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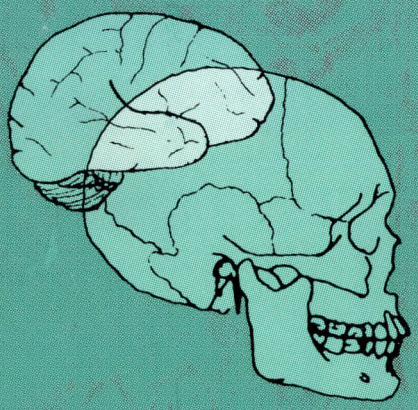

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Research report

PRODUCTION OF PURE PRIMARY RAT CEREBRAL ENDOTHELIAL CELL CULTURE: A COMPARISON OF DIFFERENT METHODS

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Summary: To study the blood-brain barrier *in vitro* pure cerebral endothelial cell cultures, without contaminating cells have to be obtained. Most other cell types besides endothelial cells can be pericytes, a few astrocytes, some smooth muscle cells, fibroblasts and meningeal cells. Careful removal of large vessels and meninges during the dissection and the optimal duration of enzymic digestions can reduce the ratio of contaminant cells. In order to further increase the purity of the culture endothelial cells can be subcloned, however, this is not useful for cells of every species. An alternative choice in cultures from rat is to perform a selective cytolysis by complement and monoclonal anti-Thy 1.1 antibody to eliminate pericytes and astrocytes. The presence of growth factors and the type of serum are also important for successful endothelial cell cultures. With the combination of the cytolysis of contaminating cells and the use of plasma-derived serum, the culturing of pure primary cerebral endothelial cells was successful.
Keywords: blood-brain barrier, cerebral endothelial cells, pericyte, complement killing

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INTRODUCTION

The blood-brain barrier (BBB) based in part on specialized characteristics of cerebral endothelial cells (CEC), including the occurrence of tight intercellular junctions, high electrical resistance, few pinocytotic vesicles as well as the presence of specific, polarized, and highly discriminatory membrane transport systems. These features form a selective permeability barrier that restricts the movement of most polar molecules and proteins.

The *in vitro* study of the blood-brain barrier (BBB) started in the 70s when Joó and Karnushina (1973) isolated fractions enriched in microvessels from rat cortex. This method could be used for short-term studies of the BBB. Couple of years later a new method was described for culture of CEC (Panula et al., 1978). These new model systems provided a better insight into the morphology, biochemistry and physiology of the CEC and new means to study the transport processes of nutrients and drugs across the BBB. In order to obtain CEC cultures porcine, murine (Tontsch and Bauer, 1989), bovine (Méresse et al., 1989), human (Vinters et al., 1987, Nagy and Vastag 1994) and rat brains (Bowman et al., 1981, Abbott et al., 1992) are used most frequently in different laboratories. Syngeneic co-cultures of rat cerebral endothelial cells (RCEC) and other cell types like astrocytes, smooth muscle cells, pericytes, neurons is an advantage of the rat model. For molecular biological studies rodents are widely used, this is also favours choosing rat CECs. Almost all methods use gray matter of brain and enzymic digestion step(s) for dissociation of microvessels. The capillary fragments are separated by Percoll gradient (Bowman et al., 1981) or by series of centrifugation steps (Tontsch and Bauer, 1989).

For characterisation of the primary culture of CEC it is necessary to check the purity and the expression of specific markers of endothelial cells. Cultures of RCEC can be easily characterised, since several antibodies are available for this species. The primary cultures are usually not pure, pericytes, astrocytes, fibroblasts, smooth muscle or leptomingeal cells may appear among endothelial cells. There are different methods to increase the purity of brain endothelial cell cultures like the subcloning of endothelial cell islands by microtrypsinisation as described by Méresse et al., (1989) and the complement mediated specific cytolysis (Risau et al., 1990).

The growth and differentiation of endothelial and the other contaminant cell types depend on the presence of different mitogenic factors. The endothelial cell growth factor (ECGF) (Folkman and Haudenschild, 1982) or the acidic and the 10-30 times more potent basic fibroblast growth factors (FGF) (Gospodarowicz et al., 1986) were found to be necessary for the

continuous growth and the expression of the phenotypic features of the endothelial cells *in vitro*. The quality of serum is also very important. There is a variability in the growth rate stimulating effect of different types or batches of serum. The fetal calf serum (FCS) different amount of platelet-derived growth factor (PDGF) which stimulates proliferation of contaminating cells as well. The plasma derived serum is free from PDGF.

The thymus-derived lymphocytes, the mesangial cells, the cerebral pericytes and astrocytes express the cell surface differentiation antigen Thy 1.1 while endothelial cells do not. Thy 1.1 antigen can be used to reduce the non-endothelial contamination of CECs (produce optimal purity of CEC cultures) by selectively lysing by antibody/complement treatment the pericytes and astrocytes (Risau et al, 1990).

In order to find an optimal culture protocol for RCEC in our laboratory we compared different techniques.

MATERIALS AND METHODS

Chemicals

Materials used in the experiments were the following origin: collagenase type II, bovine serum albumin (BSA), Dulbecco's modified Eagle's medium/ nutrient mixture F-12 Ham (DMEM/F-12), fetal calf serum (FCS), basic fibroblast growth factor (bFGF), heparin, monoclonal anti-mouse Thy 1.1 antibody, complement serum HLA-ABC (rabbit), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium, *Bandeiraea simplicifolia* isolectin B_4, 3,3'-diaminobenzidine (DAB), fluorescein isothiocyanate-dextran (FITC-dextran) (Sigma), collagenase-dispase (Boehringer), Percoll (Pharmacia), L-glutamine, penicillin-streptomycin (Gibco), plasma derived derum (PDS) (Advanced Protein Products LTD).

Preparation of primary cultures

Two-week-old Sprague-Dawley CFY rats of either sex were obtained from our Institute's animal house. Rats were anesthesized with ether. After thorough rinse with 70% ethanol, than with iodine in 70% ethanol, heads were cut, and placed into a sterile glass Petri dish. In the laminar flow box forebrains without the cerebellum were removed from the skulls with sterile microdissecting forceps and scissors, and collected in cold sterile phosphate

buffered saline (PBS, without calcium and magnesium, pH: 7.4). Meninges were removed on sterile filter paper (Whatman 3M) from each brain hemisphere while at the same time white matter was "peeled off" with the aid of fine curved forceps. Gray matter was carefully collected from the filter paper (meninges stuck to it) and minced to approximately 1 mm^3 pieces by sterile disposable scalpels in the incubation medium (270 U/ml collagenase, 1 mg/ml dispase, DMEM-F12 containing antibiotics) in a sterile glass Petri dish. Incubation media for enzymic digestion was always prepared freshly from lyophilised enzymes, then sterilised by filtration. Their pH was adjusted to 7.4.

The minced tissue was transferred into a centrifuge tube with the rest of the collagenase-dispase solution (total: 2 ml/brains) and triturated with a pipette (10 up and down), than incubated at 37 °C for 1.5 h in shaking waterbath. After this incubation, cold DMEM-F12 was added to the homogenate and centrifuged at 1000 *g* for 8 min. The supernatant was aspirated and 15% BSA/DMEM-F12 (2 ml/brain) was added to the homogenate, mixed well by trituration and centrifuged at 1000 *g* for 15 min. The myelin layer and the supernatant was aspirated, the pellet washed once in DMEM-F12 (700 *g* for 5 min) then further digested in waterbath for an other, 1 h in the incubation medium 1ml/brain. The cell suspension was centrifuged (700 *g* for 5 min). The pellet was suspended in 2 ml DMEM-F12 and carefully layered on a continuous 33% Percoll gradient and centrifuged at 1000 *g* for 10 min. For the gradient 10 ml Percoll, 18 ml PBS, 1 ml FCS and 1 ml 10¥ concentrated PBS were mixed, sterile filtered and centrifuged at 4 °C, 30000 *g* for 1 h.

The band of the endothelial cell clusters (clearly visible as a white-grayish layer above the red blood cells) was aspirated, washed twice in DMEM-F12 (at first 1000 *g*, 8 min, then 700 *g*, 5 min). The cells were suspended in culture medium (DMEM-F012 containing 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mg/ml gentamicin, 2 mM glutamine, 20 % heat inactivated FCS or PDS and from the second day 1 ng/ml bFGF) and were seeded onto rat tail collagen-coated 35 mm plastic dishes or Falcon cell culture inserts (pore size 4 mm, d=25 mm). Starting culture from 10 brains, we could obtain confluent primary culture of RCECs in 10 pieces of 35 mm tissue culture dish, equivalent approximately to 100 cm^2 surface area. The medium was changed on the next day, later on every third day.

Characterisation of the cultures

FVIII-related antigen (FVIII) immunohistochemistry. After a brief washing in PBS and fixing in ethanol at 4°C for 15 min, cells were treated with 1% H_2O_2 in PBS for 10 min, followed by washing in PBS. Non-specific binding sites were blocked by incubation in 3% normal goat serum in PBS at room temperature for 20 min. Anti-FVIII rabbit immunoglobulin (Dako) was used as primary antibody and biotin-labelled anti-rabbit IgG (Dako) as secondary antibody, both applied for 30 min. After a 30 min incubation with avidin-biotin-horseradish peroxidase (HRP) complex (ABC kit, Vector Lab, CA, USA), DAB was used as HRP substrate, followed by hematoxylin-eosin (HE) counterstaining.

Lectin-binding. RCECs were washed in PBS, fixed in 4% formalin and 70% ethanol in PBS for 15 min, treated with 1% H_2O_2 in PBS for 10 min, washed again in PBS, then incubated in 15 mg/ml HRP-conjugated BS-I-B_4 in 0.1% BSA-PBS for 90 min. DAB was used as HRP substrate. The preparations were counterstained by HE.

Complement killing

RBEC cultures were washed twice with serum free DMEM-F12, then incubated with monoclonal anti-mouse Thy 1.1 antibody (antibody: DMEM-F12 = 1:500) for 1 h at 37 °C. After washing twice with DMEM-F12, the cultures were incubated in the presence of rabbit complement (complement : DMEM-F12 = 1:3) for 2 h at 37 °C, which mediated the cytolysis of those cells which expressed on their surface the antigen Thy 1.1. After washing in DMEM-F12 the cells were cultured in the previously described complete medium.

RESULTS AND DISCUSSION

Isolation and characterisation of endothelial cells

The method we were using was based on two enzymic digestion steps to free microvessels from the surrounding nervous tissue and eliminate possible contaminating cells, followed by a Percoll gradient centrifugation to isolate the capillary fragments. The small vessel fragments obtained at the end of

the isolation procedure attached rapidly to collagen coated surfaces, and in 2-3 days, colonies of RCECs emerged and formed a non-overlapping continuous monolayer with some swirling patterns at the end of the first week. RCECs displayed a so-called "fibroblast-like" morphology: cell-shape is fusiform with an oval nucleus in the center, neighbouring cells tightly attached to each other in such a way that no intercellular space could be observed. The endothelial cells gave specific immunohistochemical staining with anti-FVIII antibody (Fig. 1 A, C), bound the galactose-specific BS-I-B_4 isolectin (Fig. 1 B) and showed positive histochemical staining for alkaline phosphatase enzyme.

For RCEC monolayers grown on Falcon 25 mm insert, 120 W¥cm^2 transendothelial electrical resistance was obtained. The passage of 70 kDa FITC-dextran was restricted through RCEC monolayer: 99.12 ± 8.79 mg/cm^2/h *vs.* 655.80 ± 12.37 mg/cm^2/h (n=6) in the case of cell-free filter (Deli et al., in press).

Effect of different sera and growth factors

Serum from fetal calf contains PDGF which stimulates fibroblast and smooth muscle cell proliferation in addition to promoting endothelial cell growth (Abbott et al., 1992). In FCS containing medium CECs loose the elongated, spindle-shape phenotype, the cells move away from each other (Fig. 2 A) and pericytes become the dominant cell type. Pericytes appear as large spreading cells with highly irregular edges which do not express FVIII (Fig. 1 C) (Shepro and Morel, 1993).

When the cultures were fed with bovine PDS which does not contain PDGF the endothelial cells showed healthy, uniform phase-bright appearance. RCECs were tightly packed against each other without intercellular gaps (Fig. 2 B). Less contaminating cells could be found than in those cultures which received FCS. There was also a difference in RCECs number between the cultures which were treated with different sera. In the presence of PDS we counted 962 ± 65 cells/mm^2 while in FCS containing medium the cells grew more sparsely: 807 ± 53 cells/mm^2.

From the second day on, basic FGF (1ng/ml) was added to the cultures, which greatly improved the growth rate of the endothelial cells.

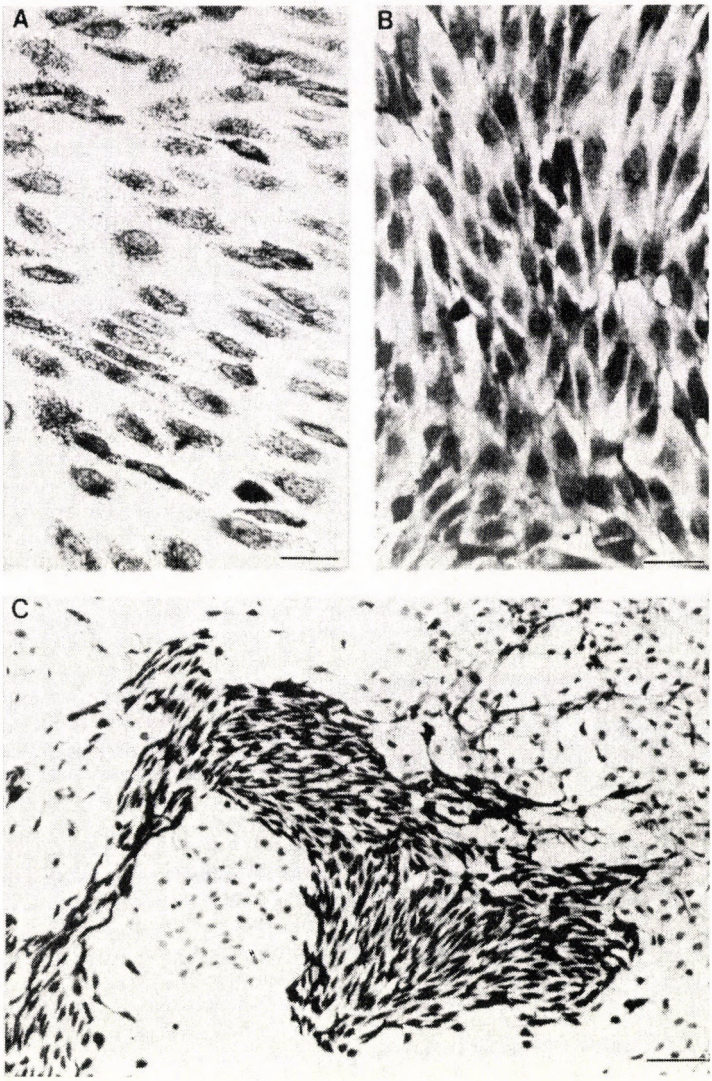

Fig. 1. (A) Antibody against FVIII related antigen gives a punctuate staining in the perinuclear zone in 5 day-old pure RCEC culture. Bar, 50 mm. (B) *Bandeiraea simplicifolia* isolectin B_4 binding gives a perinuclear homogenous staining on endothelial cells. HE counterstaining. Bar, 50 mm. (C) In a 7 day-old RCEC culture FVIII-related antigen positive endothelial cell colony is surrounded by not stained contaminating polygonal cells with processes which are probably pericytes. Bar, 250 mm

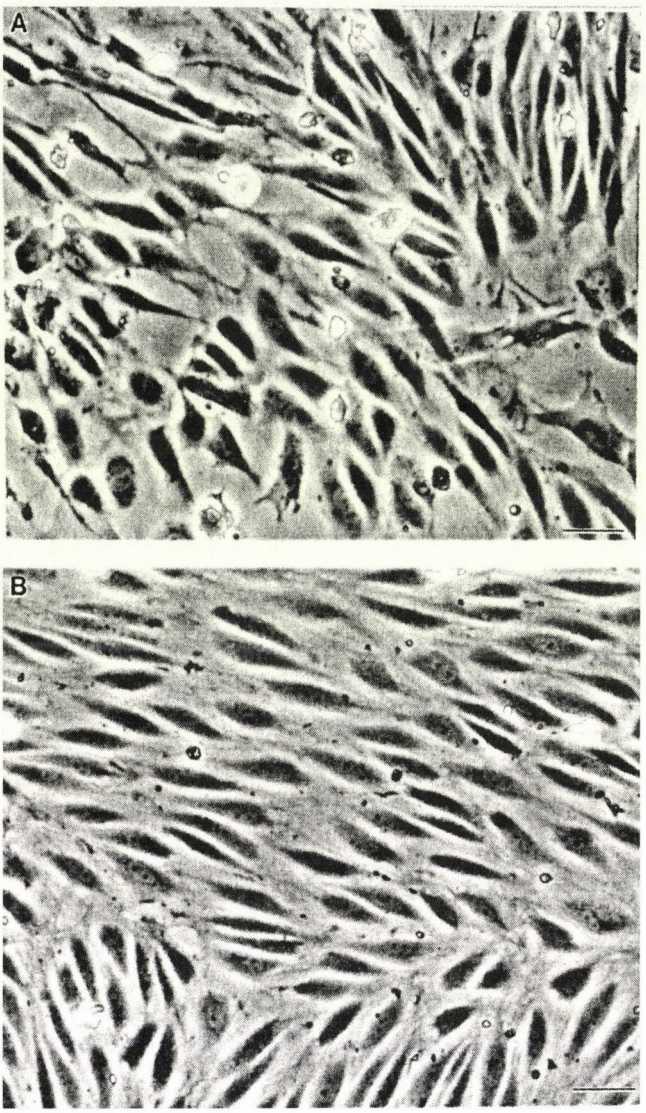

Fig. 2. (A) Primary RCEC in medium containing 20% FCS 6 days after plating. There are gaps between the elongated endothelial cells that might be caused by contaminating cells, probably pericytes. Bar, 50 mm. (B) Uniform RCEC monolayer without any other cell types at 6th day *in vitro* in medium containing 20% PDS. The cells show the fusiform, elongated morphology without intercellular gaps. Bar, 50 mm

Contaminating non-endothelial cells / Complement killing

During the production of purified RCEC cultures contamination from pericytes is the most frequent problem. Pericytes are elongated, polymorphic, multibranched periendothelial cells of mesodermal origin (similarly to the endothelium). *In vivo* they are integrated in the capillary wall and wrapped by the basement membrane of the endothelium. They are related to smooth muscle cells due to their content in contractile elements. Pericytes play a role in the proliferation and differentiation of endothelial cells, secrete vasoactive agents, release structural components of the basement membrane. The endothelium and pericytes form a morphological and functional unit (Shepro and Morel, 1993). Pericytes inhibit adrenal capillary endothelial cell proliferation *in vitro* in a contact-dependent manner and they became the dominant cell type in the culture. The activated transforming growth factor b (TGFb) is the mediator of EC growth inhibition. The co-cultures produce active form of TGFb while the separated EC and pericyte cultures secrete latent TGFb which is activated by acidic pH (Olridge et al., 1989). We also observed that pericytes tend to overgrow RCECs in long-term culture especially if the serum is not free from PDGF.

The optimal duration of enzymic digestion can keep the ratio of pericytes and astrocytes to a minimum level. If the cells are digested for a longer period or the enzyme solution is more concentrated, pericyte contamination is decreased but single endothelial cells will appear instead of capillary fragments. It turned out that single cells do not attach to the surface and do not grow well in culture, possibly because they lack 'survival factors' from their neighbours (Abbott et al., 1994). In contrast, if the basement membrane is left intact by shorter digestion time pericytes can contaminate the culture.

Some of the contaminating non-endothelial cells, pericytes and astrocytes, express Thy 1.1 antigen, whereas endothelial cells do not therefore, these cell types can be removed by selective cytolysis using anti-Thy 1.1 antibody and complement (Risau et al., 1990). We could considerably reduce the number of contaminating cells (Fig. 3) resulting in more than 95% pure RCEC cultures (Fig. 4 A). If non-endothelial cells were not removed in time, they could overgrow the RCECs in a couple of days (Fig. 4 B). It is important to find the optimal time-point for complement killing at each culture. This depends on the ratio of CECs and pericytes and the absolute number of the endothelium. If this treatment is done too early, we can kill the young and weak endothelial cells, while later we cannot remove the pericytes. We found that the optimal time is about 2-3 days after the plating of the cells.

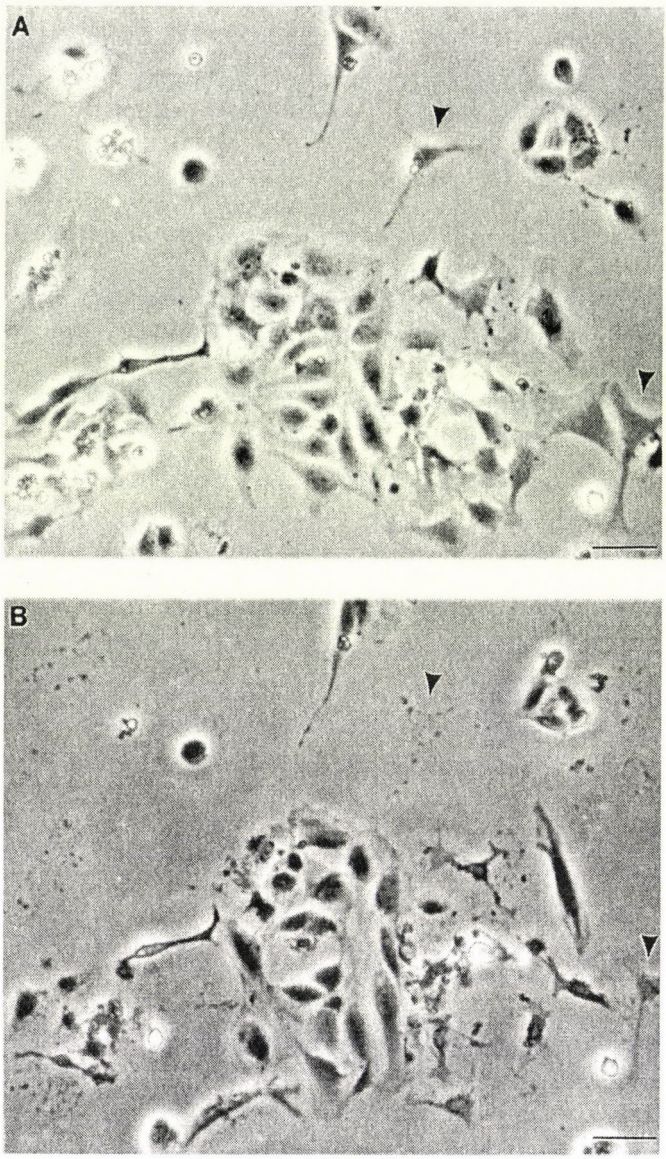

Fig. 3. Complement killing. (A) Before the cytolysis the contamining cells look as multipolar or round flat cells around or on the top of the endothelial cell clusters. (B) Immediately after the killing we can see only the shadows or the nucleus of the lysed pericytes and astrocytes, sometimes the whole cell disappears during the process (Fig. 3 B)

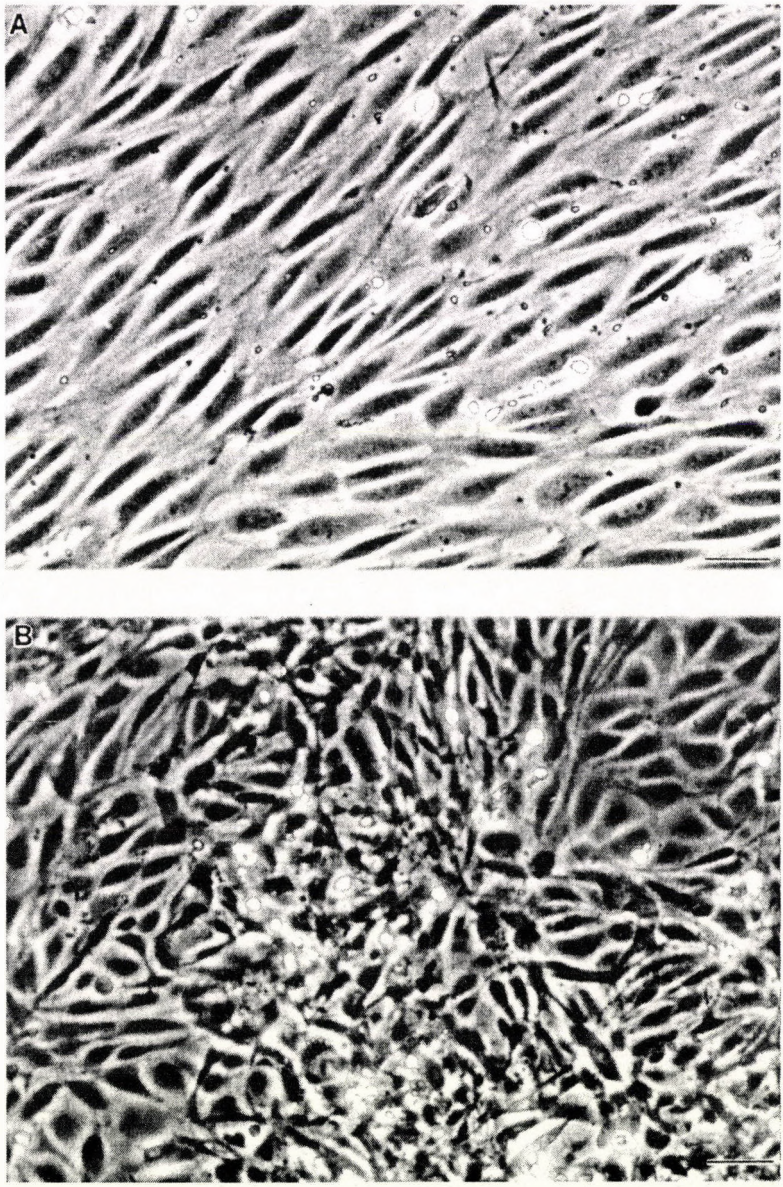

Fig. 4. (A) RCEC monolayer 4 days after the complement killing. Bar, 50 mm. (B) RCEC culture from the same preparation as A, but without any treatment at the same time-point. Contaminating cells growing on the surface of the monolayer can be observed. Bar, 50 mm

Several laboratories (Tontsch and Bauer, 1989, Dehouck et al., 1990, Nagy and Vastag, 1994) described the cloning of endothelial cells to remove the contaminating cell types and enrich the culture. Although this method is suitable for porcine, murine, bovine and human brain microvascular endothelial cells cannot be used for rat CECs. Primary RCECs cannot be subcultured because the subclones are not viable and are not able to form spontaneusly growing cell lines.

Reduction of the percentage of smooth muscle cells and fibroblasts in the cell culture can be attained by careful removal of large vessels, meninges and choroid plexus tissue during the dissection. These contaminanting cells, like pericytes, grow faster then the endothelial cells do and can overgrow the endothelium.

Angiogenesis

In some cases capillary-like structures may appear in the primary CEC cultures. Experimental evidence suggests that perivascular astrocytes play an important role in controlling the microvessel formation and growth in CNS (Laterra and Goldstein 1993). Ling and Stone (1988) described that the retinal vascularisation is coordinated by migrated astrocytes into the retina from optic nerve. The role of special cell and matrix components (collagens, laminin and fibronectin) interaction as well as some diffusible factors such as TGFb, FGF has been recently identified in modulating angiogenesis. In routine culture conditions the spindle-shaped endothelial cells form confluent monolayers in the first 7-10 days *in vitro*. When the endothelial cells differentiate into capillary-like structures they have real lumens, ramify and may develop a network in the culture dish (Robinson et al., 1990) There size, shape is similar to the capillaries seen *in vivo* and they secrete basement membrane proteins (Laterra and Goldstein, 1993). In some primary cultures of RCECs we could also observe capillary-like structures (Fig. 5 A, B).

Concluding remarks

The CECs lose the expression of differentiated functions during the culture period. Adaptation to cell culture environment might cause changes in certain properties of the cells. The tight junctions may be present but not assembled into complex structures in cell culture. On the 'filter model' - CEC monolayers grown on porous membranes - they inhibit the passage of

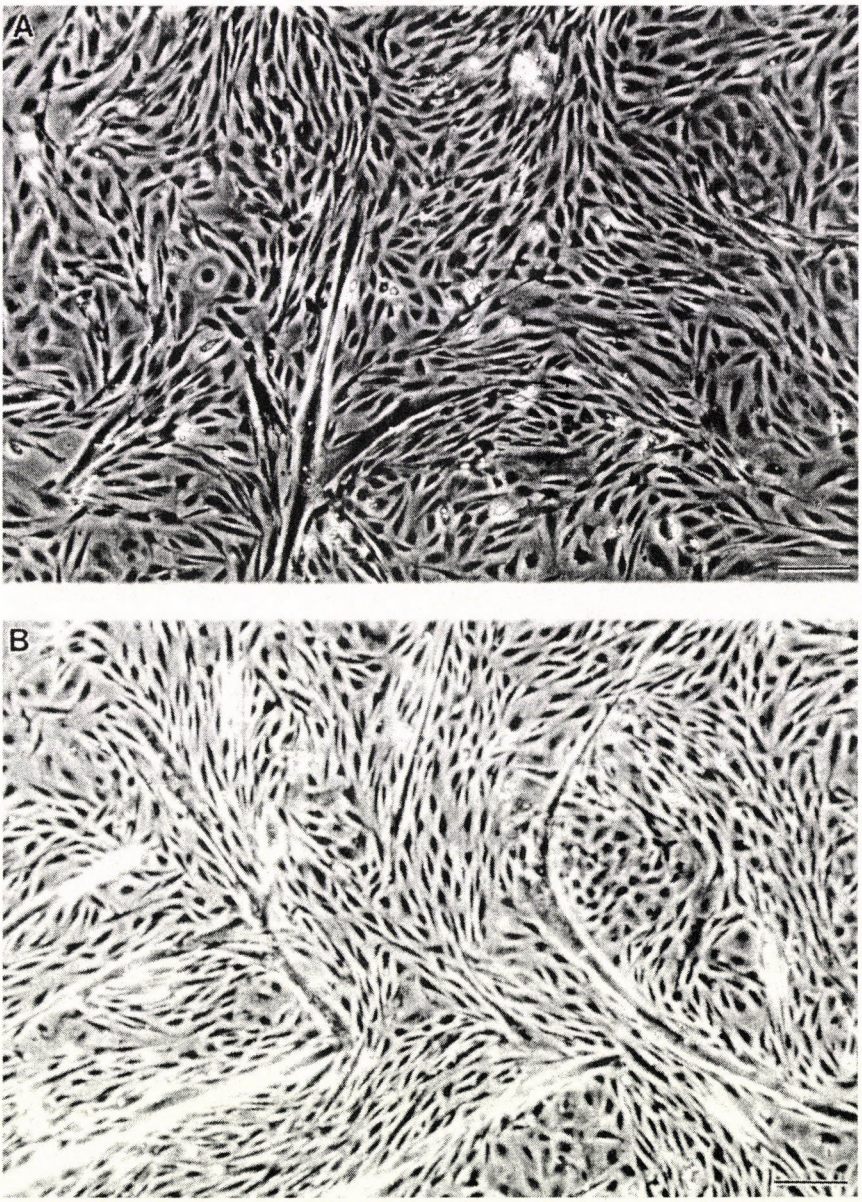

Fig. 5. (A, B) Capillary-like structures in 12 day-old primary RCEC cultures. Bar, 250 mm

proteins but do not restrict the movement of smaller molecules and ions (Bowman et al., 1983). The endocytotic capacity is elevated *in vitro* (Rupnick et al., 1988). It has been observed by Goetz et al., (1985) that g glutamyl transpeptidase and alkaline phosphatase activity may be lost with increasing time in culture. The reason of these changes might be the absence of associated cells (e.g., pericytes, astroglia, neurons). Many of the barrier features lost during culture can be reinduced by co-culture with astrocytes or by addition of astrocyte-conditioned medium (Joó, 1992) or neuronal membrane fraction (Tontsch and Bauer, 1991). Intracellular cAMP elevating drugs can further increase the tightness of the intercellular junctions and decrease the permeability of the CEC monolayers for small molecular weight substances (Rubin et al., 1991, Deli et al., 1995)

The yield of rat CECs is relatively low as compared to that obtained from bovine or porcine brain, but sufficient amounts of endothelial cells can be obtained for Western-blot or PCR techniques (Deli et al., 1993, Krizbai et al., 1995). Using the above described protocol, feeding the cells with special serum and performing the complement killing of contaminating cells, we succeded to obtain routinely the primary RCEC cultures in our laboratory that are more then 95% pure, and are good for most biochemical and molecular biological experiments

To study the structural, biochemical and physiological features of the BBB, the primary CEC culture remains one of the best objects.

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Review article

VIRAL LABELLING OF SYNAPTICALLY CONNECTED NEURONS

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Summary: A method has recently been developed to study the neuroanatomical connections in the brain by trans-synaptic tract-tracing *via* neurotropic viruses. Neurotrop viruses injected into a peripheral organ or directly into the central nervous system are transported axonally. Viruses are expressed in the infected neurons and they are transferred through synapses to reach other neurons. Many research studies illustrate by immunocytochemical detection of the viral proteins that the trans-synaptically interconnected neurons can be visualized, in addition, their neurochemical character can be identified. Thus, viruses could serve as a self-amplifying specific markers of connected neurons along hierarchial chains of functionally related circuits. Herein, we reviewed the methodology of the neuroanatomical studies obtained with a member of a-herpes viruses, the pseudorabies virus, frequently used in tracer studies in rats.
Keywords: tract-tracing, Aujeszky's disease virus, autonomic pathways, sensory pathways, immunocytochemistry, electron microscopy

Introduction

Several tract-tracing methods have been developed to investigate neural pathways in the central nervous system (CNS). One of the most recently introduced techniques is the trans-synaptic labelling by neurotrop viruses. By experimental infections of various organs with neurotrop viruses (i.e., viruses preferentially replicating in the nervous tissue) the injected virus

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progresses from the periphery to the brain with a mechanism analogous to the natural viral infection (Fig. la-c). The virus-infected neurons can be visualized with routine immunocytochemical (ICC) techniques and this method can be combined with neurochemical characterization of the neurons. Initially, neurotrop viruses were injected into the nervous system to study the mechanism of axonal transport (Kristensson et al., 1974), and capability of viruses to influence the function of neurons (Dolivo et al., 1978, for review see: Dolivo, 1980). In these classic neuroanatomical works, Sabin's postulation (1937) about the specific, trans-synaptic propagation of viruses has been confirmed. Later, this unique, circuit-specific, trans-synaptic transport of virus through CNS has been demonstrated by several investigators (Martin and Dolivo, 1983; Ugolini et al., 1987, Strack et al., 1989). Recent electron microscopic investigations have also verified the viral neuronal tract-tracing, and labelling of glial cells connected with the infected neurons (Card et al., 1993). The molecular biology of the circuitspecific infection of the CNS (Enquist, 1995), in addition, the replication and assembly of pseudorabies virus in the CNS have recently been summarized (Card, 1995). The methodology used for trans-synaptic viral mapping has been previously reviewed (Ugolini, 1995). Herein, we reviewed the methodology of the neuroanatomical observations obtained by application of the most widely used neurotrop viruses, the pseudorabies virus strains (PRV), in rats. Data obtained using other viruses and other animals are also briefly mentioned.

TRANS-SYNAPTICAL VIRAL TRACT-TRACING: METHODOLOGY

A great variety of antero- and retrograde tracer substances have been applied to study the neural pathways in the CNS. None of the classic tract-tracing techniques, such as administration of neurotoxins (tetanus toxin fragments, cholera toxin), lectins (Phaseolus Vulgaris), wheat germ agglutinin, horseradish peroxidase and fluorochromes can be used for trans-synaptic labelling. The unique property of viral trans-synaptic labelling is that the signal is amplified by replication of the virus in the neurons of the specific circuits (Fig. la-c). This property of the viral tract-tracing together with the specific, trans-synaptic passage of viruses offers a powerful neuroanatomical technique.

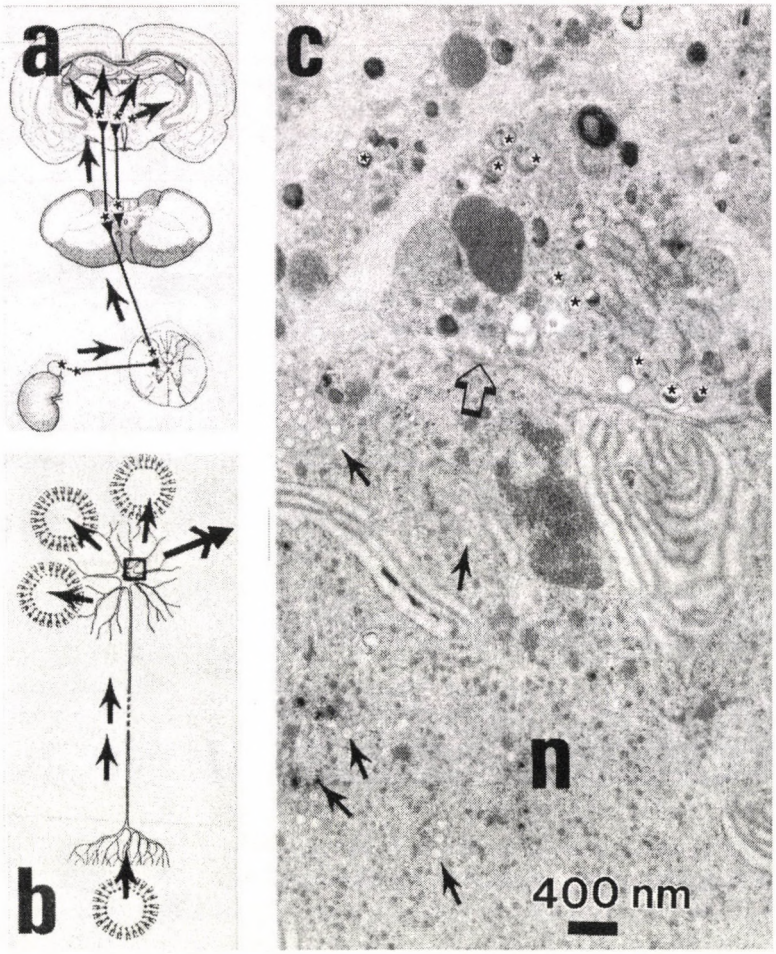


Fig. 1 a-c. Schematic diagrams and an electron micrograph of a virus-infected neuron demonstrating the principle of the viral labelling. a. The propagation of the virus at organ levels. The virus, inoculated into the adrenal gland, is transported to the autonomic ganglion, then to the intermediolateral cell column of the spinal cord, then to the brainstem and to other areas in the brain. The virus can be labelled by immunostaining. b. The propagation of virus at cellular level. The virus particles are taken up by the axon terminals then transported to the perikaryon, and, following replication, assembly and sorting, they are released. c. Replication, assembly, and sorting the viruses in an autonomic ganglion cell infected heavily with Bartha's virus. Many nucleocapsids (arrows) are in the nucleolar part (n) of the cell. The virus particles cross the nuclear membrane (empty arrows) and accumulate in the cytoplasm (asterisks) before spreading into the dendrites

Viruses used for tract-tracing studies

All families of viruses include some members that are able to invade the nervous tissue. To date, one member of a-herpes viruses serve frequently as tracers for transneuronal mapping studies: Aujeszky's disease virus (Aujeszky, 1902; taxonomic name: suid herpesvirus 1, commonly used name: pseudorabies virus [PRV]). Virulent strains of PRV are highly neurotropic and produce lytic degeneration of neurons. Naturally, PRV causes an infection in swine similar to that induced by herpes virus simplex (HSV) in human (see for review Medveczky, 1993), but many different laboratory and captive animals could be infected experimentally (Lomniczi, 1988). The several genomic variants of PRV strains used for neuroanatomical mapping primarily in rats, are as follows. (i) The virulent, wildtype PRV, most often Becker's strain (PRV-Be) is used for mapping the neuroanatomical connections of the brain. (ii) The attenuated virus, Bartha's strain of Aujeszky's disease virus was isolated in 1961 (PRV-Ba; Bartha 1961). It remains neuroinvasive but does not induce pronounced neurodegeneration. It is a well-known strain that is used in veterinary medicine as a vaccine to prevent Aujeszky's disease of swine (Lomniczi, 1985, 1990, Medveczky, 1993). (iii) The newly designed genetically engineered strains, constructed with ß-galactosidase expressing gene, could be identified with an enzyme histochemical reaction (Loewy et al., 1991, LeVatte et al., 1995; Sams et al., 1995), in addition to the immunostaining of the viral and ß-galactosidase proteins (Jansen et al., 1995a). Countries such as Canada, Great Britain and Australia have prohibited using PRV for agricultural restrictions. Thus, HSV or attenuated strains of HSV (LeVatte et al., 1995) or other virus strains are used for neuroanatomical studies in these countries.

Uptake, transport, replication and specific trans-synaptic propagation of the Auieszky's disease virus

The viruses use a strategy to invade neurons through cell surface receptors. They enter the cell by receptor-mediated endocytosis and then, delivered to the early endosomes. The virus escapes from the endosome by virtue of the special properties of one of its envelope proteins. At the acidic pH of the endosome, this protein causes the viral envelope to fuse with the endosome membrane, releasing the bare nucleocapsid into the cytosol. Nucleocapsids

containing DNA are transported to the cell nucleus (for review see: Alberts et al., 1994). The transcription, i.e., replication of viral DNA, and assembly of new capsids take place in the nucleus of the infected neurons (Figs 1c and 2a-c). The synthesis of viral gene products including the envelope proteins continues in a coordinately regulated and sequentially ordered cascade-like fashion (Roizman and Sears, 1990). The newly synthesized viruses are released by exocytosis and invade the adjacent, synaptically connected neurons (2nd-order neurons), are transported again to the perikaryon, replicate, and infect the next neuron in the hierarchy, and so on (Fig. 1a-b).

Many indirect observations support the hypothesis that viruses spread trans-synaptically. Electron microscopic investigations suggest that the progression of the viral infection of neurons is trans-synaptic (Card et al., 1993). In addition to neural infections, the glial cells which are connected with the infected neurons, absorb the viruses and became infected. However, the viral envelope proteins in glial cells are not synthesized, thus, they do not participate in the virus infection chain reaction. The degenerating neurons are finally phagocyted by microglial cells (Card et al., 1993). There is a possibility to infect the nearby neurons in a non-specific manner (Jansen et al., (1993), but in practice, virus particles have not been observed in the extracellular space (Kristensson et al., 1974; Dolivo et al., 1978; Card et al., 1993). Viruses are equally transported from subcutaneous, intraperitoneal, or intramuscular inoculations to the CNS, although, with distinct pattern, Gosztonyi et al., 1992).

PRV is approximately 150 nm large, and it consists from a nucleocapsid (an electron opaque core of DNA surrounded by an icosahedral capsid-protein) surrounded by a membrane envelope (see also Figs 1c and 2c). PRV possesses 33 proteins, 9 of them are glycosylated, forming spikes on the outer surface (for review see: Mettenleiter, 1991, 1994). The glycoproteins of PRV have been designated as gI, gII, gIII, gp63, gp50, gX, gH, gK, gL, and they are homologous to the glycoproteins found in other herpes viruses (called gE, gB, gC, gD, gI, gG and gH, gK, gL, respectively; Mettenleiter, 1991). Since 1993 the denomination of HSV and PRV proteins has been unified and names used for herpesvirus proteins is now used for designation of PRV glycoproteins, too. Viral structural proteins belonging primarily to the late genes are expressed exclusively after DNA replication had taken place. The envelope proteins mediate several steps in the virus-cell interaction, some of them are essential for replication of viruses. The cell surface receptor involved in binding of PRV to neuronal cell membrane has not been identified yet, while it has been described that one of the envelope glycoproteins, gC protein of PRV, interacts with cell membrane

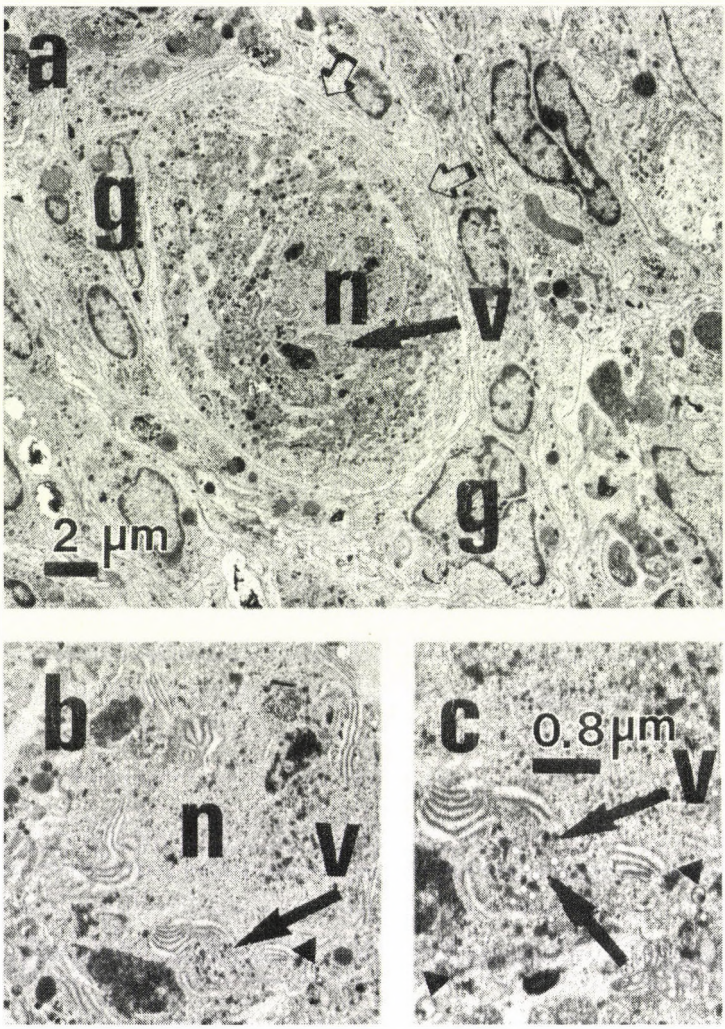


Fig. 2a-c. Electron micrographs of a part of the ipsilateral cervical superior ganglion of the rat, 4 days following Bartha's virus injection into the right extraorbital lacrimal gland of rat. The same section at different magnifications; arrows with v (virus) indicate the same virus particle in all pictures. a. The infected ganglion cell is isolated by glial cells: empty arrowheads indicate the surrounding glial processes. The nucleus (n) is disintegrated. b. The nucleus (n) at higher magnification with many virus particles at different maturation state. c. Part of the nucleus with many nucleocapsid (arrows) in close vicinity of a lamellar structure. Arrowheads (also in Fig. b) indicate mature virions. The ganglion was processed by routine electron microscopy

proteoglycans carrying heparan sulfate chains (Karger and Mettenleiter, 1996). In genetically engineered strains, used for trans-synaptic tracing, some genes of non-essential proteins have been altered to influence the pathogenicity, neural passage of the virus, and/or to introduce a marker gene to present a fast and simple identification of the virus (Loewy et al., 1991; LeVatte et al., 1995).

Application of Aujeszky's disease virus for neuronal tract-tracing

PRV has been injected into different peripheral organs of rats (Tables 1-4), into discrete parts of the CNS (Table 5) peripheral ganglia (Spencer et al., 1990; Jansen et al., 1995a,b), peripheral nerves (Li et al., 1992), muscles (Martin and Dolivo, 1983; Rouiller et al., 1989; Rotto-Percelay et al., 1992; Dobbins and Feldman, 1995) and white adipose tissue (Warren et al., 1996). PRV is taken up by the nerve terminals and subsequently transported *via* axoplasmic transport in an anterograde (e.g., by retinal ganglion cells: Card et al., 1991), and retrograde manner to the perikaryon where viral replication starts in the nucleus. Only few neuroanatomical studies have described the intrinsic brain connections by viral tract-tracing (Table 5). These observations suggest that the mechanism of intracerebral viral progression is similar to that observed in the peripheral organ infections. Namely, the virus particles are taken up by the axon terminals and glial cells participate in isolating the infected neurons. However, the viral concentration seems to influence more profoundly the number of the infected neurons (Park et al., 1996).

Animals as hosts, and symptoms following viral infection of CNS

Predominantly, rats were used for experimental studies with PRV. Rats are fairly resistant to infection, thus relatively high dose of wildtype PRV (about 10^6 plaque-forming units, PFU) and the presence of mucosal lesions are required for successful oral infection (for references see: Lomniczi 1988). Younger rats (one-day to one-week old) are more susceptible to PRV infection (Fraser and Ramachandran, 1969). Rats infected experimentally with the virulent virus strain die within 2-3 day, apparently from respiratory arrest. They develop symptoms, including pruritus, in few hours before death (Áldásy and Máté, 1969, Dolivo et al., 1978, Card et al., 1991). Administration of small dose (<1000 PFU) of attenuated strains may not

compromise the health of rats (Strack and Loewy, 1990), or, by applying higher dose, the animals may develop signs of infection later and die 5-6 days after the inoculation (Card et al, 1991). In our studies applying Bartha's strain of PRV, we observed tachypnoea, weight loss, rarely pruritus after inoculation of Bartha's virus into the extraorbital lacrimal gland or the adrenal gland. Applying 10 PFU for inoculation, these symptoms began on the third postinoculation-day, intensified on the day 4, then most of the animals of virus-injected into the lacrimal gland, became lethargic, others with intraadrenal inoculation became aggressive.

Detection of virus-infected neurons

Immunocytochemistry (ICC). The virus particles can be visualized by light microscopic ICC techniques as immunoperoxidase and immunofluorescence techniques, or by in situ hybridization histochemistry (Table 1; Sur et al., 1995) using paraffin-, cryo- or vibratome sections. Antibodies against PRV proteins for immunocytochemistry are not commercially available at present, but may be obtained from a number of laboratories. The nucleus, the first site of accumulation of viruses in the infected neuron is always stained (Figs 3a-c and 4c), however, with varying intensity. As the viral infection progresses immunostained glial cells could be seen around the infected neurons (Figs 3a and 4c). *More details are given in the Appendix* (for additional review see: Ugolini, 1995).

Jansen and co-workers have described a double-virus transneuronal labeling technique by using two different genetically engineered forms of PRV-Ba (1995a). Each strain has been constructed to express a unique marker antigen that could be identified with different antibodies in the infected host cells. Using this technique a set of the central autonomic neurons projecting to the heart and also to the adrenal gland has been identified (Jansen et al., 1995a).

Double-immunocytochemistry should be applied to phenotypic characterization of the virus-infected neurons, in addition, to neurochemical characterization synaptic connections of the infected neurons. Double or triple light microscopic immunostaining for phenotypic characterization of the virus-infected neurons have successfully been applied (Haxhiu et al., 1993; Strack et al., 1989; Papka et al., 1995; Sams et al, 1995). Furthermore, viral trans-synaptic tract-tracing can be combined with

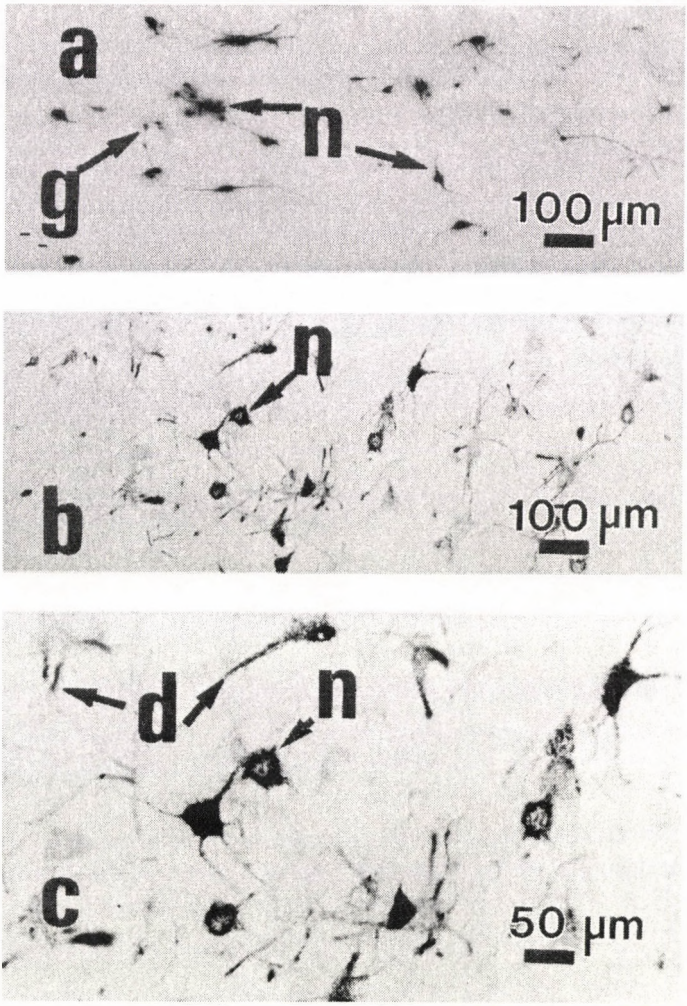


Fig. 3a-c. Immunoperoxidase labelling of different types of virus infected neurons in the CNS, 3 days following injection of Bartha's virus into the lacrimal gland of the rat. a: Small neurons (n) form a network in the lateral hypothalamic area, ipsilateral to the site of viral injection. The glial cells (g), around the infected neurons are heavily labelled, too. b and c: Larger, possibly catecholaminergic neurons (n) in the A5 area. Note the different stages of infection. i.e., some of the neurons are fully packed with viruses, some of them are lightly labelled, displaying Nissl-like staining. The dendrites (d) of some neurons are more intensely stained than the perikaryon. Vibratome sections, polyclonal antiviral antibody (code # 134) staining, ABC technique

antero- and retrograde tracing (Blessing et al., 1991; Papka et al., 1995, see also in Tables 3 and 5; Leak et al., 1996). In certain conditions colchicine treatment of the animals has also been employed to enhance the immunostaining of neurotransmitters in the neuronal perikarya (Strack et al., 1995, Lynn et al., 1996).

Enzyme histochemistry is a recent development in the methodology of viral tract-tracing. It is based the on the insertion a marker gene, LacZ gene, also called reporter gene, into the viral genome. The LacZ gene codes for the enzyme ß-galactosidase which enzyme is then expressed in the infected cells. Thus, the infected cells can be detected by a simple histochemical reaction, both at light and electron microscopic levels (Loewy et al., 1991).

Electron microscopic analysis of virus infected neurons is very sensitive method to investigate the propagation of the virus in the CNS since even a few virus particles can be readily seen. However, sporadic virus-infected neurons are difficult to find in the absence of immunolabelling. The fine structural characteristics of the different stages of viral replication have been reported (Kristensson et al., 174; Dolivo et al., 1978; Levine et al., 1994; Card et al., 1993). A systematic electron microscopic study has described the prominent role of astrocytes and microglia in the restriction of virion diffusion from the infected neurons (Card et al., 1993; see also Figs 2a-c).

Kinetic of propagation of virus in the CNS

The synthesis of viral gene products proceeds in a cascade like fashion (Roizman and Sears, 1990). The first sign of the viral infection observed by immunostaining of the viral proteins, is the neuronal nucleus, later the perikaryon then the proximal part of dendrites, finally the full extent of dendritic tree and the axon (Ugolini, 1995). There are 3 stages of infection with PRV: (i) an early stage with manifest infection of the first-order neurons, and perhaps a few second-order neurons; (ii) a middle stage, with prominent infection of second-order neurons, in addition to that of the first-order ones, and a few third-order ones; and (iii) a late stage with the prominent infection of the third- and fourth-order of neurons, in addition, the more advanced degenerative changes of the first and second-order neurons. Thus, as the viral infection progresses, more and more neurons (and also glial cells) are infected at all levels of the hierarchy. The early stage is confined to approximately 18-24 h or 42-48 h postinoculation time in the

case of wild type and attenuated PRV strains, respectively. The middle stage of infection is about 36 h or 68-74 h with wild type and attenuated PRV strains, respectively. The late stage is about 43-47 h or 90-96 h with virulent and attenuated PRV strains, respectively. The earliest detection time of virus-labelled neurons is dependent on the technique applied, electron microscopy is being the most sensitive, however, difficult to apply. The propagation time greatly varies with the different type of nerve fibers, and it is inversely correlated with the diameter and the length of the nerve fibers (Ugolini et al., 1992; Jasmin et al., 1994; Ugolini, 1995). The glial cells, including astrocytes and microglia form a barrier around the infected neurons. They are defective for synthesis of viral envelope proteins, thus mature virions here could not be developed (Card et al., 1993). The infected neurons eventually degenerate and are phagocyted by microglial cells (Card et al., 1993). The kinetic of virus infection implies that the analysis of virus infected neurons with a well-defined time course is critically important to the interpretation of the data (Card et al., 1991; Rinaman et al., 1993; Ugolini et al., 1987; Ugolini, 1995).

Modification of viral transport in the CNS

Asynchronous spreading the virulent and attenuated PRV strains from the vitreous body to the visual relay center (Card et al., 1991; 1992; Table 1), or from the cardiac ventricle to the dorsal vagal nucleus (Standish et al., 1991) has been reported. Bartha's strain induces less pronounced neuropathological changes, while dense degeneration of infected neurons could be observed in the animals infected with the virulent wild type PRV (Card et al., 1993). Recent microbiological studies with recombinant strains of PRV, confirm the previous assumption of Card and co-workers (1992), and give an explanation: the deletion of glycoprotein gE reduces the propagation of PRV in the CNS (Babic et al., 1996; Mulder et al., 1994; 1996). The attenuated strain (PRV-Ba) is negative for gE (i.e., gI) envelope protein. Thus, Bartha's strain or other, genetically engineered strains negative for gE proteins (Standish et al., 1991) seem to be an advantageous genomic variation with reduced propagation and diminished neurodegeneration properties. The reduced propagation and/or the impaired ability to induce a variety of cytokines contributing to the neuronal cell death may be responsible for the diminished neurolytic effects of viruses with the above mentioned genomic changes.

Reportedly, the administration of colchicine, a known inhibitor of axoplasmic transport, might delay the propagation of virus infection by wild type PRV up to 3 days (Dolivo, 1980). Sympathectomy or vagotomy have

been applied to influence the population of the labelled neurons (Jansen et al., 1992; Loewy and Haxhiu, 1993; Loewy et al., 1994). Transection, or ligation of the neurons of the neuraxis investigated is an evident experimental control to reveal the specificity of the method (Wesselingh et al., 1989; Li et al., 1992; Nadelhaft and Vera, 1995; Standish et al., 1995).

VIRAL TRACT-TRACING DESIGN FOR MAPPING NEURAL CIRCUITS

PRV strains have been used to study sensory pathways, even in the very first tract-tracing studies (Table 1) and for mapping the visceromotor (Table 2) in addition, the visceral neuraxis (Table 3). One endocrine organ, the adrenal gland, has been particularly extensively studied *via* viral labelling, therefore, related data are summarized separately (Table 4). We also tabulated the few intracerebral application of viral tract-tracing (Table 5).

Table 1. Viral labelling the sensory neuronal circuits

Virus	Virus dose (PFU)	Organ injected	PIT (h)	Detection of virus and neurotrans-mitters	Rat strain	Refs
PRV	10^7	Eye chamber	18 - 24, 36, 43, 47	PRV w EM PRV w IFL	n.a.	Dolivo et al., 1978
PRV-Be **PRV91** **PRV-Ba**	10^5	Eye	early late	PRV w ABC	S - D	Levine et al., 1994
PRV-Be **PRV-Ba**	10^6	Intravitreal	51 - 81 74 - 122	PRV w IPO	S - D	Card et al., 1991; Moore et al., 1995
PRV-Ba **BaLacZ**	10^2 - 10^4	Eye chamber	96	PRV w IFL and ChAT w IFL	S - D	Sams et al., 1995
PRV	n.a.	Nasal epithel	24 - 72	PRV w ABC PRV w ISH	S - D	Sur et al., 1995

Abbreviations: ABC: immunoperoxidase staining with avidin-biotin complex; AChE: acetylcholinesterase; ChAT: choline acetyl transferase; ß-GAL: ß-galactosidase enzyme; DA: dopamine; DBH: dopamine-ß-hydroxylase; EH: enzyme histochemistry; EM: electron microscopy; FG: fluorogold; 5-HT: serotonin; HSV: herpes simplex virus; HSVlacZ: HSV expressing LacZ gene; ICC: immunocytochemistry; IFL: immunofluorescence; IPO: immunoperoxidase; ISH: *in situ* hybridization; LM: light microscopy; MERGL: methionine

enkephalin Arg-Gly-Leu; na: not applicable; NMDAR: NMDA receptor; NOS: nitric oxide synthase; NP-Y: neuropeptide Y; OXY: oxytocin; PFU: plaque-forming units; PIT: post-inoculation time period; PNMT: phenylethanolamine-N-methyltransferase; PRV: pseudorabies virus; PRV-Ba: Bartha's strain of PRV; PRV-Ba-gcKA and PRV-91: genetically engineered strains of PRV; PRV-BaLacZ: PRV-Bartha with ß-galactosidase expressing gene PRV-Be: Becker's strain of PRV; S-D: Sprague-Dawley; SOM: somatostatin; SP: substance P; TH: tyrosine hydroxylase; TRH: thyrotropin releasing hormone; VIP: vasoactive intestinal peptide; w: with; WIS: Wistar

Table 2. Viral labelling of visceromotor circuits

Virus strain	Virus dose (PFU)	Organ injected	PIT (h)	Detection of virus and neurotrans-mitters	Rat strain	Refs
PRV-Ba	7×10^7	Laryngeal muscle	90	PRV w IPO	S - D	Fay et al., 1993
PRV-Ba	4×10^3	Tracheal muscle	96	PRV w ABC; PRV w IFL and 5HT w IFL and PNMT w IFL	S - D	Haxhiu et al., 1993
PRV-Ba	10^5	Pharyngeal muscle Esophageal muscle	48 - 52 57 - 62	PRV w ABC	S - D	Barett et al., 1994
PRV-Ba	10^6	Esophageal muscle	60 - 65	PRV w IFL and NMDAR w IFL, NOS w EH	S - D	Broussard et al., 1995

Abbreviations see in Table 1. Notes: * sup. cervical ggl. removed; ** with sympathectomy

Comments

With a new tract-tracing method, by viral labelling, used in combination with other neuroanatomical techniques, the synaptic architecture of the brain can be further explored. Neurotropic viruses serve as self-amplifying specific markers of synaptically connected neurons along hierarchial chains of functionally related circuits. All components of the neuraxis, including sensory, motor and visceral nerves could be infected with viruses in a hierarchial order. Glial cells have a role in isolating the infected neurons, thereby ensuing circuit specificity. As the viral infection proceeds more and more neurons and glial cells are involved at all levels of hierarchy. Therefore, analysis of viral transport in a well-defined temporal window is essential in

appropriate interpretation of the circuit related viral labelling. All the available data suggest that the use of PRV as a tracer not only enables confirmation and classification of previous reports on the neural circuits but provides further insight into the synaptology of the brain. Furthermore, in combination with the knowledge of the neural plasticity, trans-synaptic viral tracing could became the basis of gene therapy in neurological diseases (for recent review see: Kárpáti et al., 1996).

Table 3. Viral labelling of viscerosensory pathways

Virus	Virus dose (PFU)	Organ injected	PIT (h)	Detection of virus and neurotrans-mitters	Rat strain	Refs
PRV	3×10^3	Cardiac muscle	96	PRV w ABC	WIS	Ter Horst et al., 1993
PRV-Be **PRV91** **PRV-Ba**	5×10^5	Cardiac ventricle	29 - 72 51 - 78 34 - 127	PRV w ABC	WIS and R - D	Standish et al., 1995
PRV-Ba	1000	Pancreas*	96	PRV w IFL and TH w IFL, DBH w IFL, PNMT w IFL	S - D	Loewy and Haxhiu, 1993; Loewy et al., 1994
PRV-Ba	100	Kidney	72	PRV w ABC	S - D	Schramm et al., 1993
PRV-Ba	1000	Urethra	56 - 72 78 - 96	PRV w ABC TH w ABC	S	Vizzard et al., 1995
PRV-Ba	10^6	Bladder**	48 - 72 96	PRV w ABC; AChE w ICC, ChAT w ABC	S - D	Nadelhaft et al., 1992; Nadelhaft and Vera, 1995
PRV-Ba	10^4	Penis	93 - 97	PRV w ABC PRV w IFL, 5 HT w IFL, DA w IFL	S - D	Marson et al., 1993
PRV	10^4	Uterus	96	PRV, FG, ChAT w IFL; NOS w ABC	S - D	Papka et al., 1995
PRV-Ba	10^4	Clitoris	96	PRV w ABC	S - D	Marson et al., 1995

Abbreviations see in Table 1. Note: * with C8 transection; ** with transection of pelvic and hypogastric nerves

Appendix

Inoculation of viruses. Pseudorabies virus (PRV) is classified as a biohazardous agent and must be handled accordingly. In viral labelling studies multiple inoculation of small volume, i.e., 0.1-10 ml of 10 -10 plaque-

forming units (PFU) of the live, virus suspension (see also Tables 1-5) has been applied *via* a Hamilton syringe, with or without a glass micropipette attachment. Condition of storage of virus is important: PRV should be aliquoted in small volumes (200 ml) and stored at -70°C. PRV is sensitive to the fluctuations in temperature. In order to achieve maximal infectivity, high concentration and high dose of virus per injection is required. In our studies PRVBa viruses were cultured in pig kidney fibroblast (PK15), harvested in Dulbecco's modified minimum essential culture medium and stored at -70°C, at 109 plaque forming unit concentration (PFU/ml; Boldogkői et al., 1996). Before inoculation the viruses were quickly thawed to 37°C in a water bath then kept at 4°C between inoculation into the animals.

Table 4. Viral labelling for studies of the innervation of the endocrine organs (injected organ: adrenal gland)

Virus strain	Virus dose (PFU)	PIT (h)	Detection of virus and neurotrans-mitters	Species	Refs
PRV-Ba	100	96	PRV w ABC orPRV w IFL and *TH, PNMT, NPY, 5-HT, SP, VIP, TRH, SOM, MERGL w IFL	rat/S - D.	Strack et al., 1989
PRV-BaLacZ	1000	96	ß-GAL w EH (LM and EM)		Loewy et al., 1991
PRV-BaLacZ BagcKA	1000	96	PRV w IFL or ß-GAL (EH) and PNMT, TH, 5-HT, ChAT, OXY w IFL	rat/S - D	Jansen et al., 1993, 1995a
HSV-1	100	72	HSV w IPO and TH w IFL and PNMT w IFL	rat/Fisc her	Wesselingh et al., 1989
HSV-1	10^5	72 - 120	HSV w ABC or HSV w IFL	rabit	Li et al., 1992
HSV-1	1000	72	HSV w IFL	hamster	Joshi et al., 1995
HSV/HSVlacZ	<1000	72	HSV w IPO or ß-GAL w EH	hamster	LeVatte et al., 1995

Abbreviations see in Table 1. Notes: * Colchicine pretreatment for detections of all neurotransmitters by Strack et al. (1989)

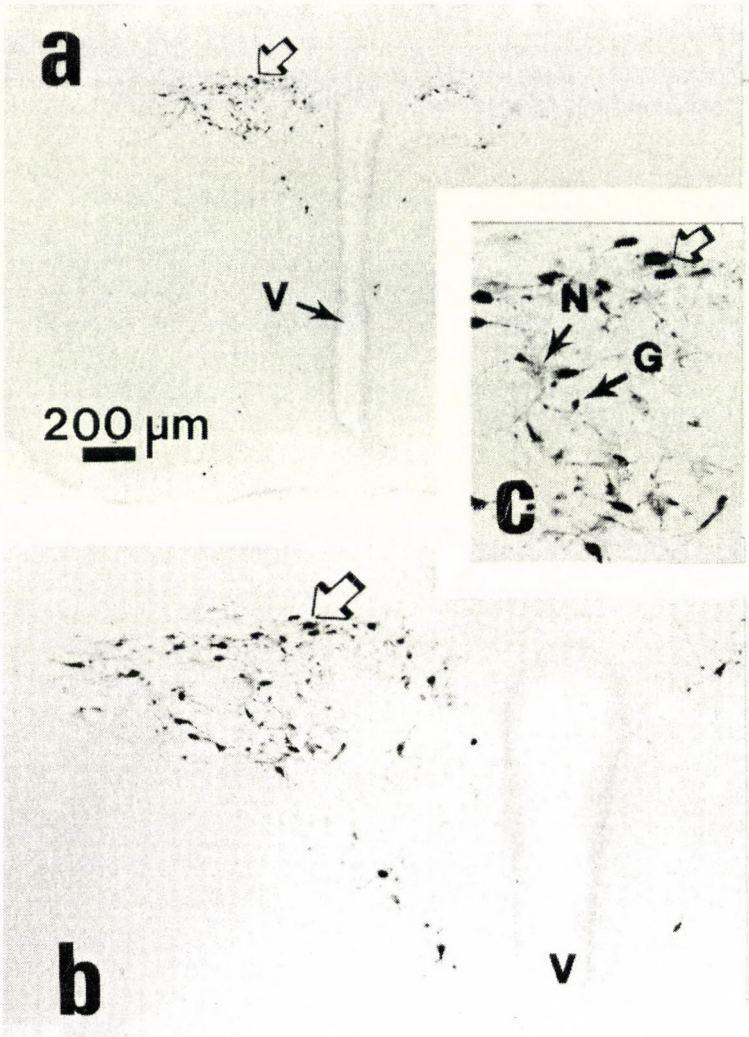
Table 5. Viral labelling of intracerebral neuronal connections

Virus strain	Virus dose (PFU)	Cerebral area injected	PIT (h)	Methods used for detecion of viruses	Rat strain	Refs
HSV	$<30^4$	Neostriatum	72 96	Electron microscopy HSV w IPO	S - D	Bak et al., 1977
HSV-1 (FMC-1)	100	Cervical vagus	72	HSV w ABC; FG w FL; HSV and 5-HT, TH, PNMT w IFL	Fischer	Blessing et al., 1991
Adenovirus-Ad. RCVßgal	25×10^6	Striatum	96	ß-galactosidase w EH	S - D	Kuo et al., 1995
PRV-Ba	1.4×10^5	Striatum	50	ICC	rat	Park et al., 1996
PRV	1.4×10^6	Hippocampus	36	ICC	S - D	Styren et al., 1996

Abbreviations see in Table 1.

Detection of virus-infected neurons: immunohistochemistry. In our experiments, Hannover-Wistar and Sprague-Dawley rats, both male and female, with 160-430 g body weight, were anesthetized with Ketamine (85 mg/kg) and Xylazine (12 mg/kg) prior to surgery. The left adrenal or the right extraorbital lacrimal gland was exposed and injected slowly with 10 PFU Bartha's strain of pseudorabies virus in 2x5 and 2x10 ml quantity, respectively. The syringe was kept *in situ* for about 5 min after each inoculation of the virus suspension. Two, 3, or 4 days following the surgery, the rats were anesthetized and following intracardial injection of 0.2-0.3 ml heparin, perfused transcardially with approximately 50 ml phosphate buffer (PB, 0.1 mol/l, pH: 7.4) followed by 250-300 ml fixative A (4% paraformaldehyde and 15% saturated picric acid in 0.1 mol/l PB at pH 7.4) for immunoprocessing of free floating cryosections; or fixative B (4% paraformaldehyde in 0.1 mol/l PB, at pH 7.4) for immunoprocessing paraffin embedded/deparaffinated sections, or fixative C (fixative B containing 0.05% glutaraldehyde) for immunoelectron microscopy on vibratome sections. The glands inoculated with the 12 virus, the autonomic or sensory ganglia, appropriate segments of the spinal cord, in addition, the brain areas identified with a brain map (Palkovits and Brownstein, 1988) were removed. The samples were fixed in the same fixative for further 1-3 hours, or kept in a diluted fixative A solutions (1:4) until sectioning (from 1 day to 1-2 months).

The injection side of tissue blocks was marked with a needle, or by incision (spinal cord), then the blocks were infiltrated with 30% saccharose in 0.1 mol/l PB, overnight and if necessary embedded in gelatine (i.e. the small superior cervical ganglion) prior to making 50 mm thick cryosections. Following thorough washing in PB (3x10 min), the sections were permeabilized with 0.5% Triton X-100 in PB for light microscopic, or permeabilized by repeated freeze-thawing in liquid nitrogen for electron microscopic (EM) immunoprocessing. After washing in PB for 3x10 min, the sections were treated with 3% hydrogen peroxide in methanol for 20 min to inhibit the endogenous peroxidase. Following washing in PB for 3x10 min, the sections were covered with 10% goat non-immune serum for 20 min, for blocking the non-specific protein blocking sites. After a brief rinsing in PB, they were incubated with the antiviral antibody (1:5000, raised in rabbit, courtesy of R.R. Miselis, see for reference Card et al., 1990) for 24-48 h. Then, the sections were washed again in PB for 3x10 min, incubated with the secondary, biotynilated antibody (1:600, anti-rabbit IgG, raised in goat, Vector Laboratories, Burlingame, CA, USA) for 1 h, washed again in for 3x10 min and incubated with ABC peroxidase complex (Vectastain Elite ABC-kit, Vector, USA) for 1 h. The immunoperoxidase reaction was developed using 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) as a chromogen and 0.003% hydrogen peroxide, in 0.05 mol/l Tris-HCl buffer, at pH 7.4. In most of the cases the DAB reaction was intensified with ammonium nickel sulphate (DAB-Ni, Figs 3a-c and 4 a-c). All reactions were performed at room temperature but the primary antibody was incubated at 4 °C. In some experiments peroxidase conjugated mouse monoclonal antibody, directed against the gII viral protein (code: 1/7, or 11/89, courtesy of Morenkov, see for references Morenkov et al., 1994) were used in combination with immunostaining of other primary antibodies against neurotransmitters, also raised in rabbit. The sections were mounted on gelatine-coated slides, and after drying, briefly rinsed in toluol then covered with depex. Each of the light microscopic immunoprocessing techniques applied have their own advantages: (i) the cryosectioning is fast; (ii) the vibratome sectioning can be easily processed for further EM study; (iii) by paraffin embedding the long-term storage of tissue before processing is possible. No appreciable differences in viral labelling by various methods could be observed. Our preliminary results have been presented (Tóth et al., 1996). Vibratome sections for EM studies were processed to epoxy resin by a routine procedure (Vizi et al., 1993).



Figs 4a-c. Light micrographs of virus-labelled neurons in the paraventricular nucleus of hypothalamus, 3 days following injection of Bartha's virus into the lacrimal gland of rat. a: The viral labelling is bilateral, with ipsilateral dominance. b and c: A network of infected neurons can be observed at higher magnification. Empty arrows show the same groups of neurons in every picture. c: Note the different stages of infection, some neurons (N) are lightly stained, many are filled with viruses, glial cells (G) around some infected neurons are labelled, too. Vibratome sections, polyclonal antiviral antibody (code # 134), ABC technique. v: 3rd ventricle

Negative and positive control experiments

Omitting the primary antibody is a commonly used negative control for immunostaining. Immunopositive areas of the brain of successfully infected animals may serve as positive controls for immunostaining, since the immunoreactivity can be preserved for a long time (in 0.1 mol/l PB containing 0.001% Na-azid). Negative control experiments include surgical procedures to ligate or transect the nerves innervating the investigated organs. Positive control experiments involve comparison of traditional tracers to PRV spreading in monosynaptic pathways (Leak et al., 1996, for review see Ugolini, 1995).

Kinetics of virus propagation, interpretation of data

The viral infection progresses both at the actual and the previous levels of the hierarchy of the infected regions. In our experiments viral immunostaining of a few first-order neurons in the autonomic (superior cervical) and sensory (Gasser's) ganglia were observed ipsilaterally to the site of the injection, at 42-44 h following inoculation of the PRV-Bartha virus into the lacrimal gland of rat. By 71-74 h postinoculation time the number of viral labelling slightly increased in ipsilateral ganglia. By 96 h postinoculation time many infected neurons in ipsilateral superior cervical ganglion was observed by EM study (see also Figs 2a-c). Second-order labelled neurons in the thoracal 1-2 segments of the spinal cord were detected 71-73 h following the virus injection. By 96 h postinoculation time many labelled cells appeared in the other upper thoracal segments, with ipsilateral dominance. Many third-order labelled neurons were observed in the paraventricular nucleus of hypothalamus at 72 h postinoculation, ipsilaterally to the site of injection (Figs 4a-c). The progress is dependent on the diameter of the fibers and the type of the neurons involved. Colchicine pretreatment, applied prior or following the virus inoculation may influence the viral labelling of neurons (Dolivo, 1980; Strack et al., 1989; Lynn et al., 1996). For exact interpretation of the viral labelling of a neuronal circuit all known synaptic connections have to be investigated at different time intervals.

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Preliminary note

STUDIES ON THE MECHANISM OF ACTION OF RGH-5002, A CENTRALLY ACTING MUSCLE RELAXANT, USING WHOLE CELL PATCH CLAMP TECHNIQUE

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RGH-5002 is a centrally acting musle relaxant compound developed by Gedeon Richter with an inhibitory action on spinal reflexes. Its chemical structure and pharmacological profile resemble that of tolperisone with slight differences. Previous electrophysiological studies suggested a presynaptic inhibitory effect (on transmitter release from primary afferents). In order to elucidate the mechanism of this action the effect of RGH-5002 on the voltage sensitive sodium channels of dorsal root ganglion (DRG) neurons were studied using whole cell patch clamp technique.

In agreement with the literature [1, 2] we found that at least two distinct sodium channel subtypes are present in the DRG cells, i.e. a tetrodotoxin sensitive (TTX-S) and a tetrodotoxin resistant (TTX-R) subpopulation, which are different not only in their pharmacological sensitivity but also in activation-inactivation kinetics and voltage dependence of steady-state current availability. RGH-5002 attenuated both the TTX-S and the TTX-R currents. It decreased slightly the maximal current measured at high negative holding potential but depression of the current was more prominent at the declining part of the steady-state inactivation curve. RGH-5002 dose-dependently (10-160 μM) shifted the normalized steady-state inactivation curve to hyperpolarized direction yielding IC50 values between 22.3 μM (TTX-S, holding: 70 mV) and 85.4 μM (TTX-R, holding: 80 mV) depending on the experiment conditions (see Figure l). The effect of RGH-5002 proved to be not use-dependent.

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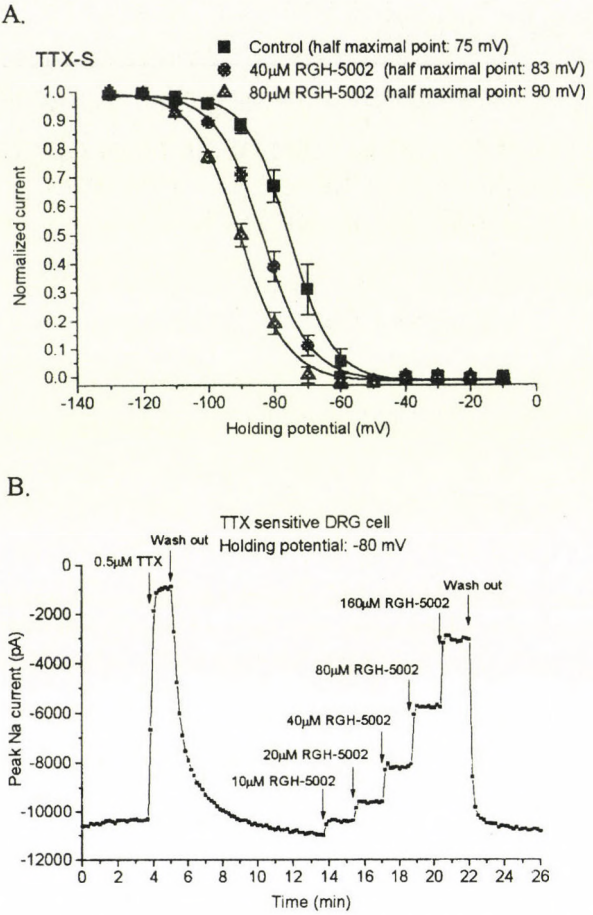

Fig. 1. A: Effect of RGH-5002 on the steady-state inactivation curve in TTX-S DRG neurons; B: Inhibitory effect of TTX and RGH-5002 on TTX-S sodium currents

In conclusion, an inhibitory effect of RGH-5002 on voltage sensitive sodium channels of primary afferentes seems to be involved in its mechanism of action. The results suggest that RGH-5002 may potentiate the physiological GABA-A mediated presynaptic inhibition.

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Preliminary note

DENERVATION SUPERSENSITIVITY: SPREADING RECEPTORS?

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Since denervation supersensitivity of striated muscles has been traditionally ascribed to the spread of nAChR on the extrajunctional surface area of the muscle fiber (Axelsson and Thesleff, 1959; Miledi, 1960), we have attempted to locate nAChR in mammalian neuromuscular junctions (NMJ) under normal conditions and after transection of the related motor nerve. For the immunohistochemical localization of nAChR, we used biotinylated α-bungarotoxin (α-BTX, Molecular Probes, Inc., USA.). It has been proved that α-BTX binds to the (α7 subunit of the nAChR (Schoepfer et al, 1990; Clarke, 1992).

Experiments were performed on 22 young adult rats (200-250 g body weight) in accordance with the European Communities Council Directive (November 24, 1986; 86/609/EEC), and the Albert Szent-Györgyi University Medical School Guidelines for Ethics in Animal Experiments. Animals were anesthetized by i.p. chloral hydrate (0.6 g/kg) and subjected to transcardial perfusion fixation with Zamboni's picric acid-formaldehyde solution, supplemented with 0.1% glutaraldehyde, preceded by a brief flush of 125 ml 0.1 M phosphate buffered saline, pH=7.4 at room temperature.

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After perfusion, the flexor digitorum brevis muscle was excised and postfixed in glutaraldehyde-free Zamboni's solution for 24 hours. For light microscopy, 30 ηm thick frozen sections were obtained; for electron microscopic pulposes, 50 ηm thick vibratome sections were used. Sections were incubated in the primary serum (I :2000) according to the free-floating technique.The immunohistochemical reaction was visualized with diamino benzidine. Specificity of the antiserum was controlled by incubation of sections in antiserum-free normal goat serum, or by pre-absorption of the antibody with αBTX prior to incubation. For electron microscopy, vibratome sections were osmicated after immunohistochemical demonstration of nAChR, applied to slides pre-treated with Liquid Release and flat-embedded in Epon. Relevant parts of the sections were excised under light microscopic control and applied to pre-polymerized Epon blocks. Ultrathin sections were obtained on an LKB ultrotome using diamond knifes, stained with uranyl acetate and lead citrate and studied on a JEOL JEM 1010 and Zeiss CEM 902 transmission electron microscopes. Transection of the sciatic nerve was performed under sterile conditions in chloral hydrate anesthesia (0.4 g/kg body weight) in the upper half of the thigh. In order to prevent regeneration, 1 cm length of the nerve was removed and the transection was repeated on the 30st and 60st day after the initial surgery.

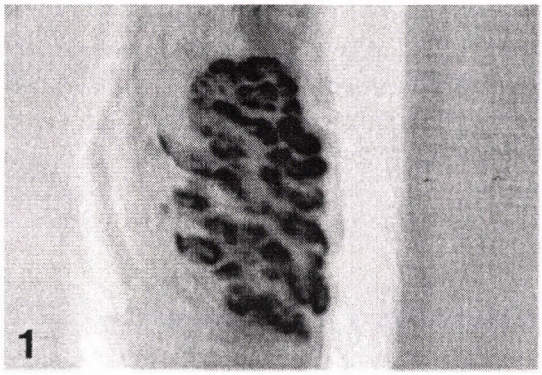
→→

Fig. 1: Localization of nACnR in the subneural apparatus of a neuromuscular junction in the flexor digitorum brevis muscle of the rat. x 1 000

Fig. 2: Shrinkage and beginning fragmentation of nAChR-immunoreactive subneural apparatuses, one month after denervation. x 1000

Fig. 3: Electron microscopic localization of nAChR in a neuromuscular junction of the flexor digitorum brevis muscle of the rat. A: axon, containing synaptic vesicles; JF: junctional folds. Arrow points at the primary post-synaptic membrane. Small arrows indicate transition between the immunoreactive 'crest' of the primary post-synaptic membrane and the non-reacting secondary post-synaptic membrane which constitutes the junctional folds.

Fig. 4: Electron microscopic localization of nAChR in a neuromuscular junction of the flexor digitorum brevis muscle of the rat, one month after denervation. dA: remnants of the degenerated axon; JF: junctional folds. Note that nearly the entire extents of the secondary post-synaptic membrane, constituting the junctional folds, are loaded with nAChR.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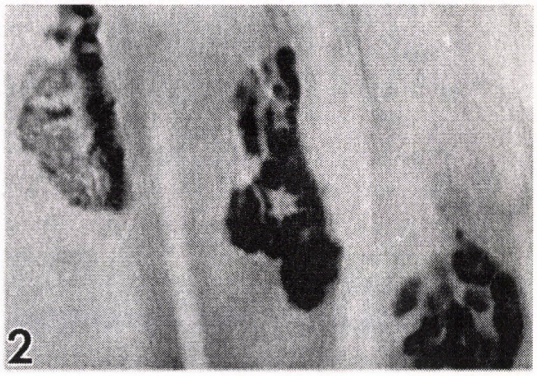
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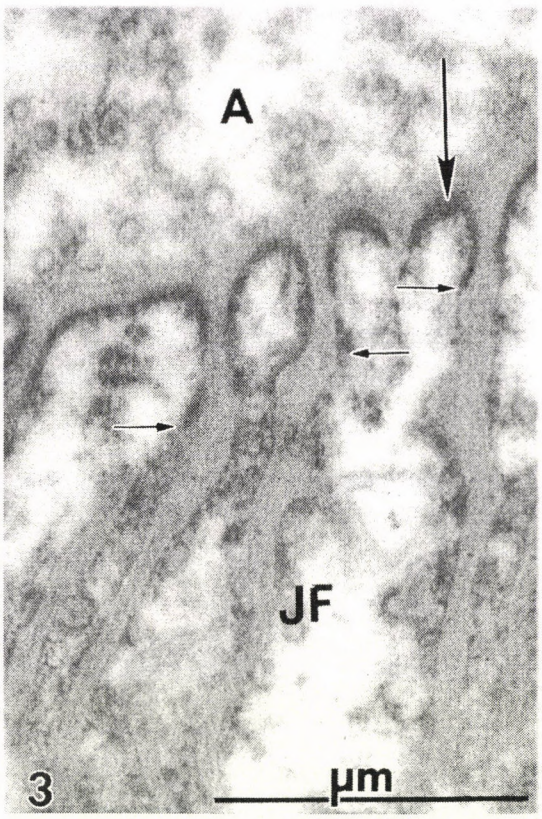
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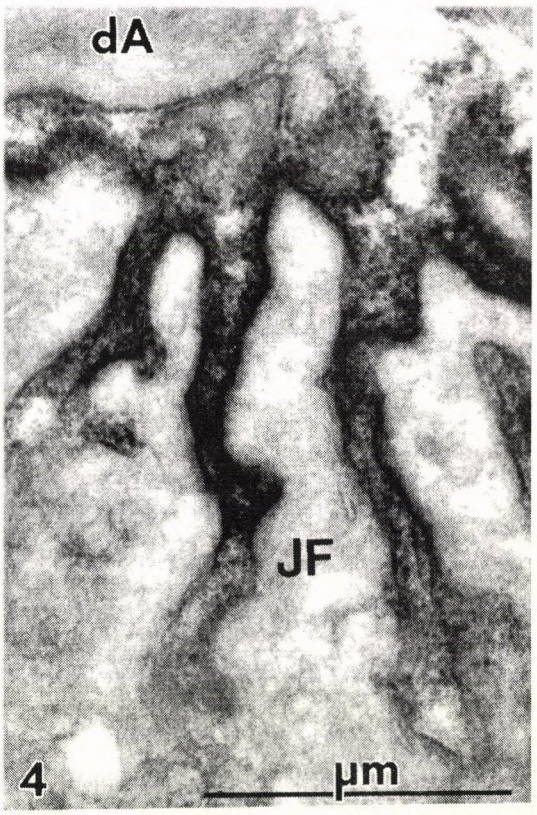
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Under the light microscope, biotinylated α-BTX visualizes structures seemingly identical with Couteaux's (1947) subneural apparatus (SNA) of the NMJ (Fig. 1.) In contrast to acetylcholinesterase specimens (Csillik, 1965) however, the outlines of the SNAs displaying nAChR immunoreactivity are completely smooth since the 'organites', the light microscopic equivalents of the junctional folds fail to react (Csillik, 1997).

At the level of electron microscopy, α-BTX outlined the primary post-synaptic membrane of the neuromuscular junction i.e. the crests of the junctional folds (Fig. 3). The secondary post-synaptic membranes, i.e. the junctional folds did not exert any reaction. The transition between the intensely reacting primary post-synaptic membrane and the immunoreaction free secondary post-synaptic membrane is clearly demarcated.

Eleven days after transection of the sciatic nerve, nAChR immunoreactivity of the post-synaptic membrane was not different from the pattern observed under normal conditions. Thirty days after transection of the sciatic nerve, the SNAs exhibiting nAChR immunoreactivity displayed signs of shrinkage (Fig. 2); at 60 and 75 days postoperatively, some of the SNAs undergo fragmentation. An important aspect of the denervated SNAs is their increased nAChR immunoreactivity. According to densitometric determinations, intensity of nAChR immunoreaction of denervated SNAs is 1.6 to 2.1 times higher than that of control samples. There was no sign of spreading of nAChR immunoreactivity over the sarcolemmal surfaces of denervated muscle fibers during the entire postoperative period.

At the level of electron microscopy, denervated SNAs are characterized by spreading of the increased nAChR immunoreactivity to secondary synaptic membranes, filling the depths of the junctional folds. Also the secondary synaptic clefts as well as what remained of the primary synaptic cleft, are filled with the end product of the immunohistochemical reaction (Fig. 4). There could not be seen any spreading of the receptors, however, on the surface of the muscle fibers.

DISCUSSION

In the present experiments, there could not be observed any sign of spreading of nAChR over the surface of denervated muscle fibers. This is in accord with the results of earlier light microscopic studies (Lentz et al, 1977). Electron microscopy proves, however, that the denervated muscle is

characterized by an entirely different, topical spreading of the receptors which is not extrajunctional, rather subjunctional, since it is directed against the depths of the junctional folds. Therefore, it appears that, at variance to the generally accepted theory, denervation supersensitivity of the muscle might be rather due to the increased amount of local nAChRs resulting in increased sensitivity of the denervated end-plate region as suggested more than fifty years ago by Kuffler (1943).

Survival of nAChRs and their increasing amount for at least 2 1/2 months after denervation, long after motor axon terminals containing calcitonin gene-related peptide (CGRP) have disappeared from the muscle, is striking. It seems to suggest that CGRP, which has been regarded to be responsible for the regulation of the biosynthesis of the α subunit of nAChR (New and Mudge, 1986; Fontaine et al, 1986) and for the increased steady-state level of nAChR-mRNA (Changeux et al, 1992), is not indispensable for the maintenance of the receptor.

ACKNOWLEDGEMENTS

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Preliminary note

IMMUNOREACTIVE GLUTAMATE IN THE PINEAL AND PARAPINEAL ORGANS OF THE LAMPREY *(LAMPETRA FLUVIATILIS)*

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In the Cyclostome lamprey, the pineal complex is composed of the pineal and parapineal organs (Fig. 1 a,b). Both are light percepting organs containing various photoreceptors (Fig. 1 c,d) and detecting circannual and circadian changes of the environmental light intensity (Collin and Meiniel, 1971; Cole and Youson, 1982; Vigh-Teichmann et al., 1983; Kuo et al., 1988; Vigh-Teichmann et al., 1989). In our previous work, we have found a presynaptic accumulation of immunoreactive glutamate in the axon terminals of the pinealocytes of various vertebrates, a result suggesting that this excitatory amino acid acts as synaptic mediator in the photoreceptor pathway of the pineal organ (Vigh et al., 1995 a, b). No data are available on the content of glutamate in the pineal complex of Cyclostomes. Therefore, in the present study we investigated the presence and localization of immunoreactive glutamate in the pineal and parapineal organs of the lamprey.

Twelve adult animals of both sexes of *Lampetra fluviatilis* were examined. The animals were kept under normal laboratory light conditions and anesthetized with MS 222 prior to fixation through the heart with 2-4% buffered glutaraldehyde. The excised pineal organs were embedded in Poly Bed 812 (Polysciences). Electron microscopic immunoreactions were performed with rabbit polyclonal antisera raised against glutamate (Sigma-Aldrich) as described earlier (Vigh et al., 1995a).

In the pineal organ photoreceptors form inner- and outer segments in the lumen of the organ. The axons of pinealocytes terminate with ribbon-type

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synapses on secondary pineal neurons. A weak glutamate immunoreaction was detected in the perikarya of pinealocytes and a stronger one in the synaptic terminals of their axonal processes. There was no considerable variation in the immunoreactivity of the photoreceptor subtypes. Except the mitochondria, perikarya of secondary pineal neurons were weakly labelled. Axons of these neurons run to the ventro-caudal end of the pineal organ. Immunoreactive glutamate was concentrated in these axons. The parapineal organ is smaller than the pineal organ and is situated ventral to the pineal end vesicle (Fig. 1 b). It is composed of a ventral and a dorsal retina. Ventrally, there are two types of photoreceptors, forming ribbon-type synapses on secondary parapineal neurons. In contrast to the pinealocytes and the ventral parapineal photoreceptors, the dorsal parapineal retina has a hormonal output.

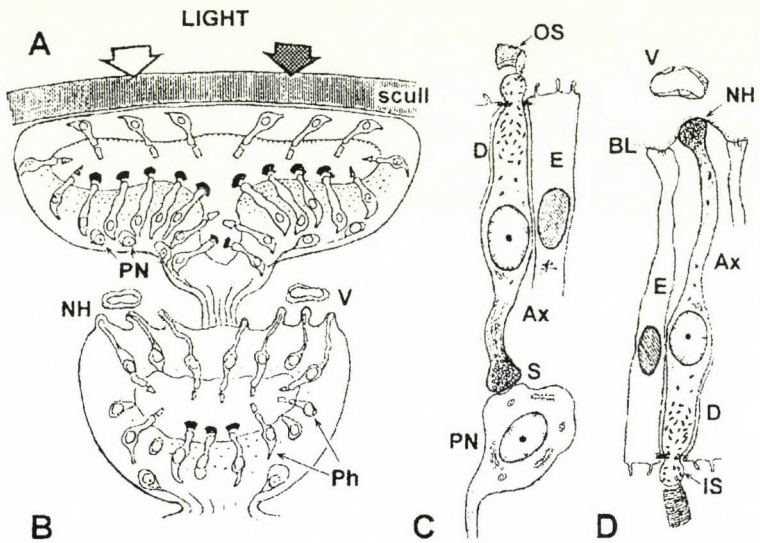

Fig. 1 Schematic drawing on the pineal (A, C) and parapineal organs (B, D) of the lamprey. Immunoreactive glutamate was found to accumulate in the axons and in axon terminals. Ax: axon, D: dendrite of the photoreceptor cells (Ph), E: ependymal cells, IS: inner segment, NH: neurohormonal terminals, OS: outer segment, PN: pineal neurons, S: synapse, V: vessels

Accordingly, the axons of the dorsal photoreceptors form neurohormonal nerve terminals in a neurohaemal layer situated between the pineal and parapineal organs. The perikarya of all parapineal photoreceptors exhibited a weak-to-medium immunoreactivity with a stronger reaction in the

mitochondria, axonal processes and in the axon-dendritic endings. Glutamate was accumulated in the neurohormonal terminals of parapinealocytes as well. Secondary parapineal neurons exhibited a medium immunoreactivity of glutamate whereas their axons were labeled intensely.

Our present data on the existence of immunoreactive glutamate in the pineal complex of the lamprey is in accord with earlier findings in othe species. We would like to stress, that glutamate immunoreactivity was the highest in the presynaptic terminals of axonal processes of photoreceptors and in the axons of secondary neurons. Consequently, it is conceivable, that this excitatory amino acid acts as synaptic mediator in the pineal photoreceptor pathway. Immunoreaction was found in neuroendocrine terminals of the parapineal photoreceptors as well. It is known, that glutamate accumulates in some neurohormonal endings, e.g. in neurosecretory terminals of the neurohypophysis (Meeker et al., 1991). Therefore, glutamate may have a role not only in the neural but also in the hormonal efferentation of the pineal complex.

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Preliminary note

COMPARATIVE CHARACTERISATION OF THE CENTRALLY ACTING MUSCLE RELAXANT RGH-5002 AND TOLPERISONE AND OF LIDOCAINE BASED ON THEIR EFFECTS ON RAT SPINAL CORD IN VITRO

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RGH-5002 is a tolperisone-type centrally acting muscle relaxant, developed by Gedeon Richter Ltd. Abundant data indicated that it had an inhibitory effect on spinal reflexes *in vivo*. The present study aimed at investigating the direct effect of the drug on the spinal cord *in vitro* in order to eliminate the influence of factors due to the complexity of in vivo systems (e.g. metabolism) and to obtain further data on the mechanism of action of the drug. For comparison, the effect of tolperisone (Mydetone, Mydocalm) and of the local anaesthetic lidocaine were also studied. The ventral root potential evoked by supramaximal dorsal root stimulation (DR-VRP) was recorded using suction electrodes for both stimulation and recording from hemisected spinal cords excised from six-day-old rats.

DR-VRP recorded in this manner consists of a compound action potential of several mV-s (probably monosynaptic reflex - MSR) followed by a prolonged depolarisation of the ventral root (see Fig. 1) which mainly reflects an electrotonic potential (EPSP). Very early (before MSR), early (after MSR), and late (poststimulus time: 80-180 ms) parts of the slow potential as well as peak-to-peak amplitude of MSR were quantitatively evaluated. All the three drugs reduced all components of the reflex potential in a dose-dependent manner. RGH-5002 was the most potent among the three drugs, being about 1.5-fold more potent than tolperisone and 2-3-fold more potent than lidocaine depending on the reflex component of consideration.

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From the fact that all the three drugs strongly diminished the EPSP including its very early part preceding MSR and that they did not possess glutamate (AMPA or NMDA) antagonist effect, it may be concluded that these drugs depressed the transmitter release from presynaptic terminals. The quantitative profile of the effects of the three drugs on the different components of the reflex suggest that the mechanism of action of lidocaine is somewhat different, whereas tolperisone and RGH-5002 are more similar to each other. Further studies are necessary to elucidate the molecular mechanism of the presynaptic inhibitory effects of these drugs.

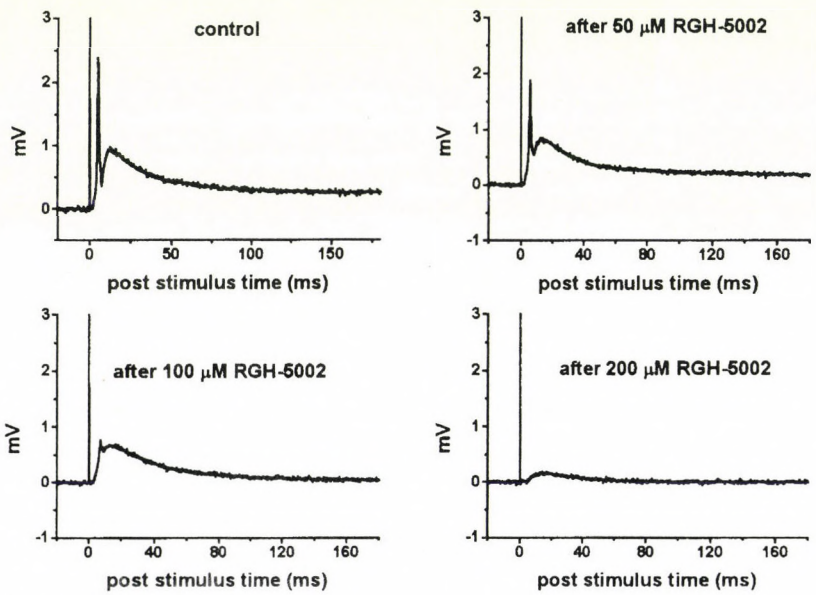

Fig. 1. Effect of RGH-5002 on DR-VRP

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Preliminary note

ACETYLCHOLINE RELEASE INDUCED IN VITRO BY BETA-AMYLOID AND ITS FRAGMENTS

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Amyloid ß-peptide (Aß) is the major component of the amyloid plaques in the brain which are characteristic of Alzheimer's disease (AD). It has been demonstrated that Aß is toxic to neurons both *in vivo* and *in vitro.* The cholinergic neurons are more sensitive than other transmitter neurons. Abe et al. (1) showed that Aß administered into the medial septum of rats decreased the ACh release from the hippocampus *in vivo,* while Pedersen et al. (2) demonstrated that ßA reduced acetylcholine (ACh) synthesis in a nontoxic way in a cell line derived from cholinergic neurons of the basal forebrain. Various lines of experimentation led to the suggestion that the cholinergic deficit in AD may be secondary to the degeneration of cholinergic nerve cells caused by Aß.

Our aim in these experiments was to establish the effects of Aß(1-42) and its fragments (Aßl-38, Aß31-35, Aß34-39, Aßl7-21) on the ACh release in tissue cultures derived from rat basal forebrain cholinergic neurons.

Neuronal cells from 16-day-old embryonic (E16) basal forebrain of rat were cultured for 4 days in vitro (4DIV) and thereafter treated with 20 µM of Aß(1-42) or one of its fragments for 3 days (DIV4+3).

The results showed that treatment of tissue cultures with synthetic Aß(1-42) or Aß(l38) for DIV4+3, reduced the ACh release by 85%; Aß(31-35) caused a 55% reduction and Aß (34-39) an 80% reduction, whereas Aß(17-21) had no such effect (Table 1).

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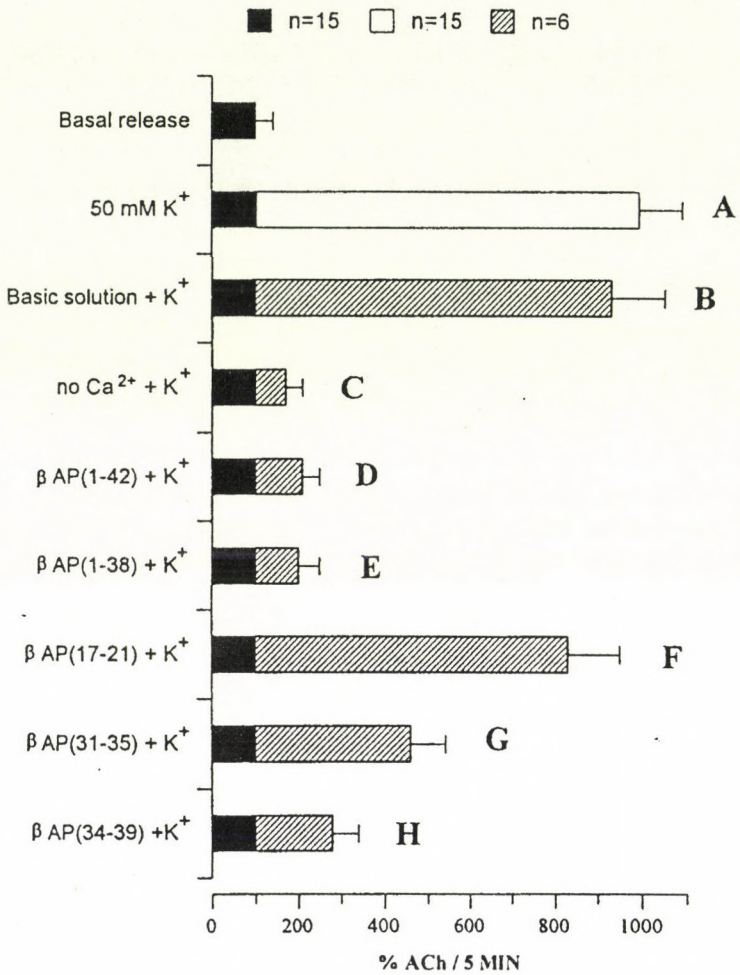
Table 1: Effects of Aß(1-42) and its fragments on the ACh content of basal forebrain neuronal cultures. Note the reduction of ACh release in the different experimental set-ups. The ACh release is expressed as %ACh/5 min/Petri dish.



The present report provides evidence that Aß and its fragments are capable of reducing the release of ACh from cholinergic neurons in *in vitro* tissue cultures perhaps by degenerating the ACh-containing (cholinergic)

neurons. It has also been demonstrated that the most toxic effect (ACh reducing effect) is exerted by those Aßs which contain lysine and methionine at positions 31 and 35. It is suggested that these aminoacids may play a significant role in the Aß-induced neuronal toxicity.

This work was supported by ETT (Grant No. T-04602/93) and MKM (Grant No. 36 and 279).

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Preliminary note

REVERSAL OF BULBECTOMY-INDUCED BEHAVIOURAL DEFICIT IN RATS

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Bilateral removal of olfactory bulbs produces a series of behavioural, neurochemical and hormonal changes in rats. The behavioural alterations, e.g. hypermotility in novel environment, deficit in passive avoidance task, show the decreasing adaptive ability of animals. The similarity of these symptoms to the human affective disorders and the results that the above mentioned alterations can be reversed by chronical treatment with antidepressants indicate that bilateral bulbectomy (OBX) can be considered as one of the animal models of human depression (1, 2).

In our previous experiments enhancement of 5-HT syndrome induced by the non-selective 5-HT_1/5-HT_2 agonist 5-MeODMT (methoxy-N-N-dimethyltryptamine) was found as a consequence of the hypersensitivity of postsynaptic 5-HT receptors in OBX rats. This enhanced 5-HT syndrome could have been reversed by pretreatment with the 5-HT reuptake blocker zimelidine (10 mg/kg s.c. for twoo weeks), and by the partial 5-HT_1A agonist buspirone (5 mg/kg s.c. for two weeks) (3).

In the present experiments the reversal by 5-MeODMT (4 mg/kg i.p.) of passive avoidance deficit and the hypermiotility was checked and it was compared to the effect of the selective 5-HT_2 agonist DOI (1-2,5-dimethoxy-4-iodo-phenyl aminopropane, 0.3 mg/kg i.p.) in OBX rats. As the data in Tables 1 and 2 show, both compounds abolished the hypermotilit, while only the non-selective 5-MeODMT was able to reverse the passive avoidance deficit.

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Table 1. Ambulatory activity of sham-operated (SO), OBX and OBX + drug-treated rats (min)

SO+sal	PBX+sal	OBX+5-MeODMT	OBX+DOI
1.59 ± 0.23	2.21 ± 0.24*	1.23 ± 0.21	1.26 ± 0.17

Table 2. Latency time (sec) of sham-operated (SO), OBX and OBX + drug-treated rats (passive avoidance, retention)

SO+sal	PBX+sal	OBX+5-MeODMT	OBX+DOI
105.7 ± 25.05	47.5 ± 22.43*	123.0 ± 24.02	69.75 ± 26.02*

* $p < 0.05$ compared to SO + sal

According to the literature there is no equivocal correlation between the hyperactivity and the learning deficit of OBX rats (2), and this is shown by our results as well. Experiments with low dose of selective 5-HT_1A and 5-HT_2 agonists suggest that 5-HT-evoked behaviour may be mediated by distinct recognition sites showing an interaction for some behaviour but not others (4). Though the role of other transmitters cannot be excluded, our results - according to which reversal of passive avoidance deficit needs the stimulation of both the 5-HT_1 and 5-HT_2 receptors, while the stimulation of the 5-HT_2 receptors seems to be enough to reverse the hypermotility - support this data.

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Preliminary note

NADPH-DIAPHORASE POSITIVE MYENTERIC NEURONS IN THE ILEUM OF GUINEA-PIG, RAT, RABBIT AND CAT

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The nitrergic elements of the ileal myenteric plexus of four mammalian species were studied by means of NADPH diaphorase histochemistry. Since previous studies assumed only minor species-specific anatomical differences, we sought similarities and differences in the nitrergic innervation pattern of the small intestine with special reference to the nerves that innervate the musculature. In rat and guinea-pig the ratio of nitrergic cells slightly exceeds 20%, in rabbit it is close to this number (16%), whereas it is lowest in cat (about 10%). The main morphometric parameters are listed in the Table below.

Parameter	1	2	3			4		5	6	7	8
			UM	MA	N	< 3	M				
rat	2247 ± 372	12 ± 4	76	16	8	not avail.		1 ± 1	377 ± 42	36 ± 8	16 ± 6
guinea-pig	2528 ± 326	14 ± 4	98	2	0	0	100	2 ± 2	392 ± 57	38 ± 11	18 ± 7
cat	917 ± 204	13 ± 5	100	0	0	not appl.		1 ± 1	443 ± 72	36 ± 10	23 ± 10
rabbit	403 ± 87	7 ± 5	45	26	29	54	46	3 ± 2	262 ± 44	29 ± 11	16 ± 6

(1) density of labelled neurons (cells/cm2±s.d.), (2) number of stained neurons in ganglia (cells±s.d.), (3) neuron type (UM: uniaxonal multidendritic, MA: multiaxonal, N: none, in %), (4) number of axons (<3 or more (M) in %), (5) dendrites > 10 μm, (6) cell surface area (μm^2±s.d.), (7) maximum length and (8) width of somata (μm±s.d.)

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The nitrergic neurons target the circular muscle layer in all investigated species, where they participate in the inhibitory motoric innervation. Apart from this, some elements of the sensory innervation of the circular musculature may derive from nitrergic neurons in rat and rabbit. The tertiary plexus is strongly supplied by NADPH diaphorase positive fibres in rat, moderately in guinea-pig and cat and not at all in rabbit. The rest of the nitrergic neurons may serve as inhibitory interneurons and control other neurons in the myenteric plexus. We conclude that the diverse physiological and pharmacological properties of the nitrergic system in different species can be connected with the anatomical heterogeneity of its elements.

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Preliminary note

NUCLEUS ACCUMBENS AND BODY WEIGHT REGULATION: NEUROCHEMICAL LESIONS

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A considerable body of evidence suggests that the mesolimbic dopamine system (MLDS) is involved in the regulation of feeding. The role of nucleus accumbens (NAC), one of the most important terminal fields of the MLDS, is considered to be crucial in translating motivational states into appropriate behavioral actions (Phillips et al., 1991). With regard to food-related behavior, dopamine (DA) depletion or blockade in the nucleus accumbens (NAC) reduce instrumental responding for food (Blackburn et al., 1989). Extracellular DA levels measured by microdialysis method in the NAC suggested that this nucleus participates in the preparatory (Phillips et al., 1991), or in the consummatory (Hernandez and Hoebel, 1988) acts of feeding behavior. There are, however, only few and contradictory data concerning the involvement of the NAC DA in homeostatic feeding (Koob et al., 1978, Salamone et al., 1991). In the present experiments, the effects of cellular damage and destructions of catecholamine as well as dopaminergic elements were compared in rats to revaluate the role of the NAC in the regulation of feeding and maintenance of body weight under physiological conditions.

For cell specific lesions. The excitotoxic drug ibotenic acid (IB) or cholinotoxic ethylcholinium mustard aziridium (AF64A) were injected into the NAC bilaterally. The latter was choosen because an important population of accumbens interneurons are considered to be cholinergic in nature, and recent findings suggested that acethylcholine (Ach) and DA interact during the initiation and completion of ingestive behavior (Hajnal et al., 1997). In

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order to differentiate the specific role of DA innervation of the NAC from the action of adjacent noradrenaline (NA) inputs, 6-hydroxi-dopamine (6-OHDA) was injected into the NAC alone or in combination with desmethylimipramine (DMI) pretreatment. DMI was used to protect NA terminals from the uptake of the neurotoxin 6-OHDA. Sham operated controls (Control) were injected with an identical volume of saline. Body weight, food and water intake were recorded before and after surgery. In order to test the motivational responsiveness to homeostatic needs (e.g. hunger and thirst), 1 h consumptions were measured after 24 h or 48 h deprivations.

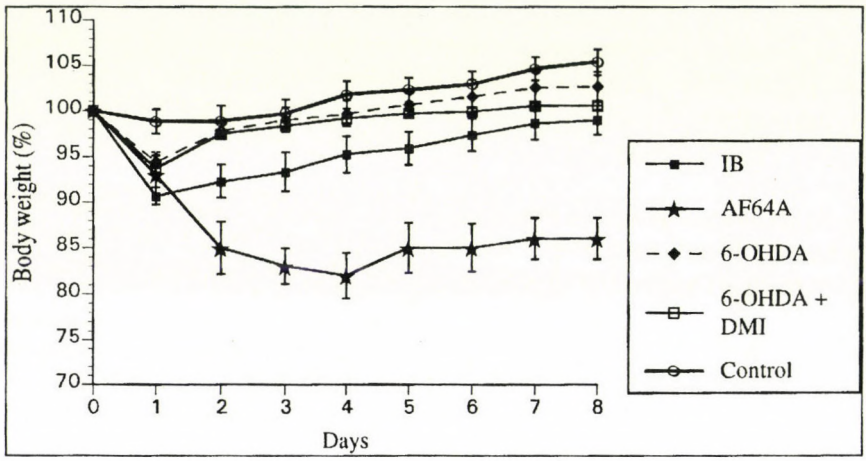

Fig. 1. Body weight changes after different neurotoxic lesions of the nucleus accumbens. Abcissa, postoperative days, ordinate, body weight in percent of initial. For abbrevation see the text

Results showed that microinjection of both cytotoxins (e.g. IB and AF64A) and 6-OHDA caused body weight decrease. However the magnitude and the dinamics of changes were different, as it is shown in Fig. 1. 6-OHDA lesions had only transient effect on body weight (1st day -5%, in comparison to controls, $p<0.01$). In contrast, the 6-OHDA+DMI group had difficulty recovering from the lesions and had significantly lower weights throughout the 3 postoperative weeks [ANOVA, F6-OHDA+DMIvsCO $(1,64)=27.4$ $p<0.001$]. The IB lesions caused more pronounced weight loss (-10%, -9%, on the 1st and 2nd day, respectively, $p<0.001$), but recovered

by the end of the 3rd postoperative week. The most pronounced body weight changes occurred in the group lesioned with AF64A. In this group, a long lasting (throughout the 22 weeks of observation) depression in the body weight was observed, with a peak on the 3rd and 4th postoperative days (-16%, -17%, respectively, $p<0.001$). The body weight decrease was accompanied by a mild hypophagia and hypodipsia in each group, except the AF64A-lesioned group, in which a minute increase in both feeding and drinking appeared. In the deprivation test, motivational deficits were found only after AF64A-lesions in response to 24 h deprivations. These deficits, however, disappeared when a longer (48 h) deprivation was applied.

In summary, our results demonstrate that (1) natural feeding and body weight regulation are impaired after lesions of both the intrinsic neurons and dopaminergic elements in the NAC, (2) the selective lesions of the MLDS terminals in the NAC cause more severe disturbances than the non-specific catecholaminergic lesions of the same area, (3) the cell specific lesions have a more pronounced effect on feeding than that of the DA-lesions of the NAC, and (4) the intrinsic cholinergic neurons of the NAC seem to be involved in the maintenance of normal body weight.

On the basis of these findings we can conclude that the MLDS has a substantial influence in the feeding-related functions of the NAC. In addition, one may suppose that the NA in the NAC may have a compensatory role in attenuating the feeding disturbances caused by the DA-lesions. Furthermore, it can be theoretized that the Ach interneurons in the NAC have a predominant role in the integration of motivational state and action.

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Preliminary note

PARTICIPATION OF NMDA AND AMPA TYPE GLUTAMATE RECEPTORS IN SPINAL SEGMENTAL REFLEX: AN IN VITRO STUDY

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In the spinal segmental reflexes the main excitatory transmitter is thought to be glutamate. In our previous in vivo study we found that the AMPA-type glutamate receptors play a key role the in early as well as in the late reflex components, since AMPA antagonists completely abolished all of them. On the other hand, NMDA antagonists produced only about 10 and 30 percent inhibition of the monosynaptic reflex and of the late components, respectively.

In the present study for comparison, the effect of AMPA and NMDA antagonists were studied in vitro as well, on isolated hemisected rat spinal cord. The ventral root potential evoked by supramaximal dorsal root stimulation (DRVRP) was recorded using suction electrodes for both stimulation and recording from spinal cords excised from six-day-old rats. NBQX and GYKI 52466 as AMPA receptor antagonists; MK-80 1, APV and CPP as NMDA receptor antagonist were the drugs used.

(DR-VRP) recorded in this manner consists of a compound action potential of several mV-s (probably monosynaptic reflex) followed by a prolonged depolarization of the ventral root which mainly reflects an electrotonic potential (EPSP). The monosynaptic component was completely abolished by AMPA antagonists. Applying a supramaximal dose the late phase of the reflex was only partially reduced by about 60-70%. NMDA

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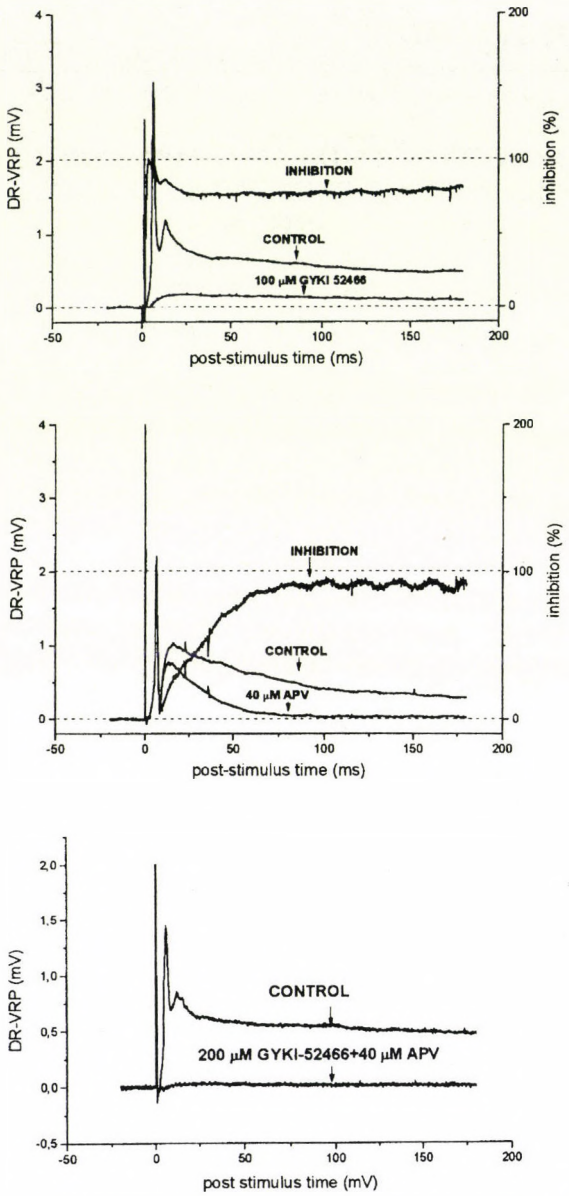

Fig. 1. Effects of the AMPA antagonist GYKI 52466 and the NMDA antagonist APV as well as of their combination on the DR-VRP. Point-to-point calculation of inhibition caused by the antagonists are also shown the above two panels as function of post stimulus time

antagonists inhibited the monosynaptic component by about 10%, and reduced the late phase by about 80%. Combination of the two types of antagonists completely abolished all reflex activities.

The results were consistent with those obtained in vivo, regarding that both types of receptors are involved in the mediation of these components and AMPA receptors play a fundamental role. However, the results concerning the relative importance of these receptors were different. Namely in vitro data suggested a greater participation for NMDA receptors in the mediation of the late reflex components than in vivo ones. We think that these apparent differences in the results may be attributed partly to differences in the techniques, most importantly in the method of recording. Namely, the in vivo recording with bipolar electrodes reflected only firing of nerve fibers while recording with suction electrodes reflects electrotonic potentials (subthreshold EPSP), too.

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Preliminary note

THE EFFECTS OF CALCITONIN-GENE RELATED PEPTIDE (CGRP) ON STRYCHNINE-INDUCED SEIZURES

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Previously we have shown that CGRP influences the pentylentetrazol (PTZ)-induced seizure (1). It is proved to be potent in decreasing of intensity and increasing of latency of PTZ-induced seizure. The aim of the present study to examine the effect of different doses of ICV administration of CGRP on strychnine-induced seizure.

Strychnine is a potent convulsant alkaloid. Its action is predominant in the spinal cord, where it competitively blocks the binding of glycine to the postsynaptic motor neurons and leads to the uncontrolled excitation of skeletal muscles. Glycine is inhibitory neurotransmitter that appears to be important in sensory processing in the spinal cord. Intrathecal administration of strychnine was reported to induce allodynia (2). Strychnine induced allodynia was shown to be mediated through the N-methyl-Daspartate (NMDA)-type glutamate receptor (3).

The experiments were carried out on adult mice. The CGRP was injected by stainless-steel Hamilton microsyringe. The needle was inserted unilaterally 1 mm to the right of the midlinepoint equidistant from both eyes and the ears and perpendicular to the plane of the skull. Peptides or vehicle (2 μl) were delivered gradually within approximately 3 sec. Mice exhibited normal behavior after the injection. The administration site was confirmed by injecting methylene blue in a few preliminary experiments, revealing that over 95% of animals were correctly injected into the ventricle. Neither insertion of the needle nor injection of the vehicle had a significant influence on the survival of the animals, neither on behavioral response or cognitive function. Thirty min after the ICV administration of CGRP, strychnine (1 mg/kg body weight) was injected subcutaneously. The mice were placed in isolated

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transparent plexiglas cages, and their behavior was observed for one hour. The intensity of the convulsions was registered according to a 6-point scale: (1) restlessness, apprehension, hyperreflexia, hyperactivity. (2) trembling in its whole body. (3) muscle stiffess in the legs and face, "jumping" on four legs. (4) 3 + turning round own axis, (5) tonic convulsions. (6) violent extensor convulsions, an arched back = opisthotonus.

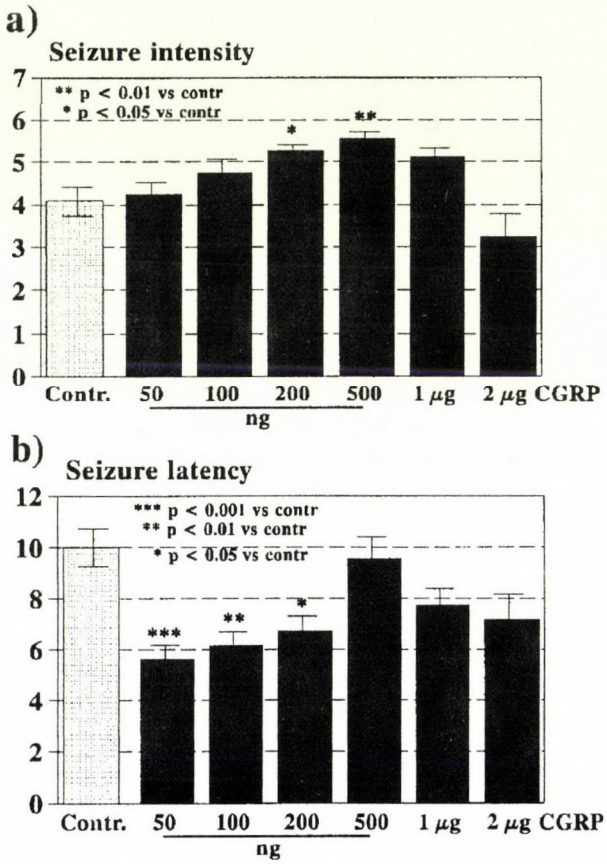

Fig. 1.

ICV injection of CGRP increased the intensity of strychnine-seizures. Dose dependency of CGRP for strychnine-seizure showed skewed bell-

shaped pattern. Maximal effect was observed with 200 ng and 500 ng (Fig l/a). The seizure latency was decreased by CGRP. The smallest doses of CGRP (50, 100, 200 ng) were most effective (Fig 1/b).

Adenosine levels increase in the cerebrospinal fluid during ischemia/hypoxia and after the seizure activity (4, 5). Adenosine can induce anticonvulsant action and analgesia (6).

We suppose that adenosin plays an important role in action of CGRP on strychnine-induced seizure.

Since the adenosine inhibits action potential-dependent release of CGRP from primary afferents in rat spinal cord and the CGRP outflow from dorsal half of the spinal cord was inhibited by selective adenosine A1 receptor agonist (7), it is possible that adenosine is consumed in prevention of hyperalgesic effect of CGRP and the intensity of strychnine-induced seizure is increased. It should be suggested, that strychnine-induced seizure threshold is decreased by lack of adenosine.

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Preliminary note

IFENPRODIL ATTENUATES THE LOSS OF PARIETAL CORTICAL PARVALBUMIN IMMUNOREACTIVITY AFTER FOCAL CEREBRAL ISCHEMIA IN THE MOUSE

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Stroke is a common life-threathening event with an abrupt onset of a focal vascular deficit. Arterial occlusion, the major source of cerebral ischemia, results in a rapid depletion of energy metabolism which in a number of ways causes intracellular Ca^{2+} overload and ultimately leads to cell death (Siesjö et al., 1985). It is believed that neuronal death in ischemia is mediated by an enhanced glutamate release and subsequent excessive activation of different voltage-dependent and neurotransmitter receptor-associated ion channels. In this regard, N-methyl-D-aspartate (NMDA) receptor overstimulation is suggested to play a critical role in the neurotoxic cascade (Goldberg et al., 1987).

Several studies have reported elevated tissue Ca^{2+} content and intracellular Ca^{2+} accumulation after cerebral ischemia which result in a multitude of disturbances in neurons (Choi, 1992). Because of their considerable Ca^{2+} buffering capacity, calcium-binding proteins (CaBP) such as parvalbumin (PV) are thought to offer endogenous protection in neurons

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against a Ca^{2+} overload (Andressen et al., 1993, Celio, 1990). PV-containing neurons were found to be resistant to global cerebral ischemia (Nitsch *et al.*, 1989), whereas Benyó et al. (1995) have recently reported a limited early loss of PV immunoreactivity (PV-ir) in hippocampal CA1 nerve cells following focal cerebral ischemia.

In the present study we set out to investigate the effects of middle cerebral artery (MCA) occlusion on PV-containing cortical interneurons in the mouse brain. As only a limited reduction of the hippocampal PV-ir neuron population was found in rat brain (Benyó et al., 1995), the effects of focal ischemia on PV expression were studied in mice. Furthermore, the putative neuroprotective action of ifenprodil, a non-competitive NMDA receptor antagonist, was also examined. Ifenprodil, by blocking the extracellular polyamine site of the NMDA receptor and thereby the Ca^{2+} entry, may prevent ischemia-induced intracellular Ca^{2+} accumulation and the subsequent generation of neurotoxic mechanisms.

Four-week old male Swiss mice (20-25 g) were used in this study. Mice were anaesthetized (chloral hydrate, 500 mg/kg of body weight, i.p.) and a burr-hole (1.8 mm) craniectomy was performed. The MCA was coagulated by bipolar diathermy. Sham-operated mice were subjected to simple exposure of the MCA. The animals received ifenprodil-tartarate (10 mg/kg, i.p.) at 5, 15, 30, 45 minutes and 3, 6, 24, 32, 48, 72, 78, 96 hours post-operation. Six days after surgery all mice were deeply anaesthetized and perfused transcardially as described previously (Harkány et al., 1995). Brains were cryoprotected overnight in 30% sucrose and 20-mm thick coronal frozen sections were cut and stained for PV according to a standard protocol (Harkány et al., 1995).

Permanent MCA occlusion elicited profound structural (necrotic) changes in the parietal cortex. An almost complete loss of PV-ir structures was observed (Fig. 1A) as compared either to sham-operated animals or to the contralateral hemisphere (Fig. 1B). Interestingly, markedly decreased PV-ir was also observed in other brain regions, e.g. in the hippocampus, thalamic nuclei, reticular formation (data not shown). It is noteworthy that the immunoreactivity of another neuronal CaBP, namely calbindin-D28k, was similarly influenced by vascular occlusion (data not shown).

Ifenprodil treatment following the ischemic insult exerts prominent neuroprotective effects on PV-ir interneurons in the parietal cortex. PV-positive cell bodies and processes scattered through all layers of the parietal cortex 6 days post-operation (data not shown). Furthermore, evidence for the beneficial drug effects was also found in all other ischemia-affected regions of the ipsilateral hemisphere (Fig. 1C).

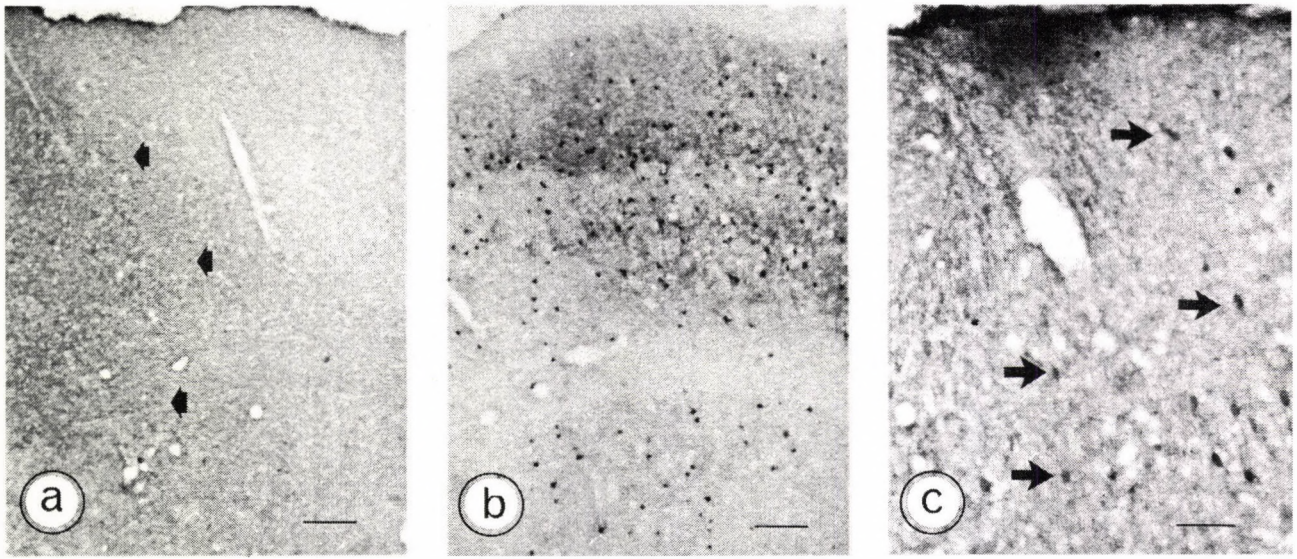

Fig. 1A-C Changes in PV-ir following focal cerebral ischemia in the mouse parietal cortex. A. depicts almost complete loss of PV-ir after middle cerebral artery occlusion (arrows indicate the most severely damaged area), while B. shows the normal distribution of cortical PV-ir structures in sham-operated animals. Note the beneficial effects of ifenprodil treatment on the survival of PV-ir interneurons (arrows) surrounding intimately the site of ischemic infarct (C.). (Scale bars: A, B = 200 mm, C = 80 mm).

Cellular consequences of cerebral ischemia involve unbalanced changes of the intracellular free Ca^{2+} concentration which are generally suggested to act as mediators of neurotoxic processes. For this reason, cells containing CaBPs (PV, calbindin-D28k) might have enhanced Ca^{2+}-buffering capacities and thereby be more resistant to Ca^{2+}-mediated excitotoxic injuries. It is worth noting that contradictory data have previously been published regarding the role and survival of PV-containing interneurons after focal ischemic insults (Benyó et al., 1995, Johansen et al., 1990, Nitsch et al., 1989). In the present study we provide evidence that occlusion of the MCA in the mouse results in a dramatic loss of PV-ir in the hemisphere ipsilateral to the occlusion. It remains to be ascertained, however, whether the reduced number of PV-ir interneurons is a result of cell death or reflects only temporary disturbances of Ca^{2+}-induced protein synthesis or increased protein degradation. Recently, Ca^{2+}-channel blockade has been shown to exert neuroprotective effects in both global and focal cerebral ischemia models (Auer et al., 1993, Benyó et al., 1995). In this regard, ifenprodil, a polyamine site NMDA receptor antagonist which is also suggested to have affinity for voltage dependent Ca^{2+}-channels and s receptors (Biton et al., 1994), considerably increased the survival of PV-ir interneurons in the parietal cortex. Therefore, the ability of ifenprodil to prevent the loss of PV-ir 6 days after MCA occlusion indicates the involvement of an enhanced Ca^{2+} entry, in part *via* NMDA receptors, in the focal cerebral ischemia-induced cytotoxic mechanisms.

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Preliminary note

LEARNING DISTURBANCES IN OFFSPRINGS OF ZIDOVUDINE (AZT) TREATED RATS

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The use of zidovudine (AZT) as a therapeutic agent in AIDS patients is limited by the fact that it causes myopathy and cardiomyopathy (Lewis et al., 1992). It has been shown in the Department of Biochemistry that similar effects develop in rats after AZT treatment and these symptoms are inherited in both sexes. These serious effects can be explained by the facts that the mitochondrial genom is especially vulnerable to AZT and other nucleotide analogues (Csere et al., in press) because of the lack of DNA repair system (Luft, 1994) and the high affinity of AZT metabolits to DNA polymerase gamma. Since the mitochondrial DNA encodes components of respiratory complexes, damage in the mitochondrial genom will cripple the oxidative energy production of the cell. This condition can be critical for cells having high energy requirements such as the skeletal and cardiac muscle cells. Deletions in the mitochondrial DNA and significant electronmicroscopic structural changes in the mitochondria with pathological electrocardiographic signs have been proven in the offsprings of AZT treated female rats. One may suppose that the central nervous system can also be affected as the neurons exhibit high energy requirement. In the present experiments the general behavior and learning capabilities of offspring of AZT treated rats (AZTO) were examined. Conventional neurological tests, open field paradigm, Morris water maze test, and in water deprived AZTO rats a water rewarded bar press learning paradigm was used in computer controlled cages (Coulbourn Instruments). In the latter situation a short light period (5 sec) was allowed for the effective bar presses to obtain water while rats

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pressing the bar in the dark period (20 sec) could not receive water reward. After completing the experiments cytochrome oxidase, NADH-dehydrogenase and NADPH-diaphorase histochemical methods were used. As conventional histology Nissl method was carried out. In several cases PCR method was also applied.

AZTO rats exhibited somewhat enhanced locomotor activity in comparison to the age-mached controls. No significant alterations have been found in orientation response latencies to different somesthetic or visual stimuli. However, the grasping strength and the climbing time in a vertically positioned grid significantly reduced in the AZTO rats. In open field AZTO rats exhibited an exploration strategy which was different from that of the controls. In Morris water maze AZTO rats could swim well and they could learn easily the place of the safety platform. When it was removed, however, their swimming strategy dramatically changed, it became chaotic and significantly differed from the controls. Significant learning deficit occurred in AZTO rats in the water rewarded bar press paradigm when the number of effective bar presses were taken into account. Although the AZTO rats pressed the bar more times than the controls, they could not press the bar in the right time.

No pathological alterations or changes have been discovered in AZTO rats when histochemical methods were used. However, by means of the PCR method it was revealed that the AZTO rats exhibited deletions in mitochondrial DNA.

Our results show that offsprings of the AZT treated rats exhibit decreased muscular strength, alterations in behavioral strategies and definite learning deficits. The existence of learning deficits observed in our experiments can not be explained by myopathic symptoms or cardiomyopathy. Our results suggest that AZT treatment in AIDS patients may induce serious disturbances of the central nervous activity in their offspring.

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Preliminary note

NOVELTY-INDUCED BEHAVIORAL DYSFUNCTIONING CORRELATES WITH THE LOSS OF CA^{2+} HOMEOSTASIS AFTER CHRONIC ETHANOL INTOXICATION

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Ethanol intoxication exerts profound effects in the brain, these being mediated in part through neuronal ion channels. L-type (dihydropyridine-sensitive) voltage-gated Ca^{2+} channels and channels gated by N-methyl-D-aspartate receptors are highly sensitive to ethanol (Gonzales and Hoffman, 1991, Gulya *et al.*, 1991). Compelling evidence indicates that ethanol intoxication induces life-long behavioral dysfunctions (Markel *et al.*, 1995) and already in low doses impairs memory processing (Melchior *et al.*, 1993); these effects are prevented by Ca^{2+}-antagonist administration. A possible molecular pathway for the neurotoxic central nervous system action of ethanol is an enhanced Ca^{2+} influx and accordingly unbalanced changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

One of the principal mechanisms underlying the maintenance of the $[Ca^{2+}]_i$ balance is the selective Ca^{2+}-binding to intracellular 'buffer' proteins (Baimbridge *et al.*, 1992). Parvalbumin (PV) and calbindin-D28k (CB-D28k) are the most prominent specific representatives of Ca^{2+}-binding proteins (CaBPs) in neuronal tissues. PV is expressed in a subpopulation of fast-firing GABAergic interneurons (Celio, 1990).

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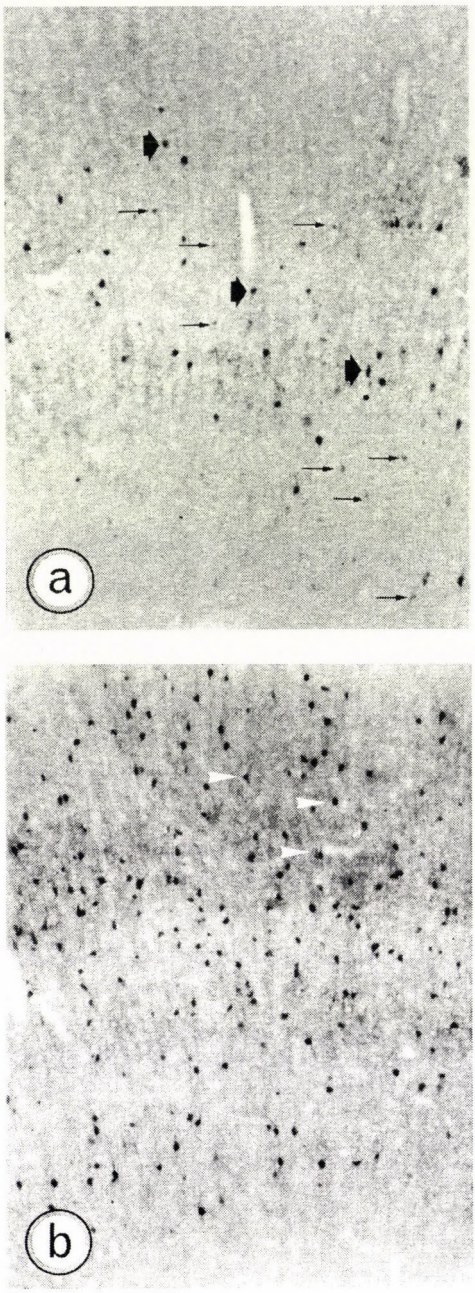
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In the present series of experiments, we investigated the possible relationship between novelty-induced alterations in spontaneous animal behavior and the expression of neuronal CaBPs, with special emphasis on PV, after 4, 7, 14 and 21 days of chronic ethanol ingestion. Brain trace element contents, and particularly those of the Ca^{2+} and Mg^{2+}, were also determined to characterize the effects of Ca^{2+}/Mg^{2+} balance on the expression of PV after 4 or 14 days of ethanol treatment.

Male Wistar rats were housed individually and fed ethanol in a liquid diet, or a control liquid diet, for 4, 7, 14 or 21 days, as previously described in detail in mice (Ritzmann and Tabakoff, 1976), with minor modifications, the regimen being adapted to rats. The group of ethanol-ingested rats was given a 7% ethanol-containing diet supplemented with vitamins, while the control animals were offered the same diet, except that ethanol was substituted by an amount of sucrose equivalent to the calories available in the ethanol. The novelty-induced activities of ethanol-fed and control animals were measured in an open-field test box as described by Nyakas *et al.* (1991). Subsequently, the rats were deeply anesthetized and perfused transcardially as described previously (Harkány *et al.*, 1995). The brains were cryoprotected overnight in 30% sucrose, and 20-mm thick coronal frozen sections were cut and stained for PV according to a standard protocol (Harkány *et al.*, 1995). Whole brain trace element contents were determined by means of atomic absorption spectrophotometry.

Analysis of the data obtained by measurement of the behavioral activities of ethanol-treated and control rats in response to a novel environment in the open-field revealed a marked impairment of explorative behavior after chronic ingestion, manifested in significantly increased start latencies, and dramatically decreased horizontal ambulation and rearing activities. Interestingly, some of the suppressant effects of ethanol on spontaneous animal behavior had disappeared after 21 days of treatment, which may reflect adaptation to and compensation of the direct effects of ethanol. Our results also indicated that the severity of these behavioral changes correlates with the degree of ethanol toxicity.

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Fig. 1. Chronic ethanol ingestion-induced changes in PV-ir structures of the rat parietal cortex. A. depicts the parietal cortex of an ethanol-fed, and B. that of a pair-fed control animal after 21 days of chronic ethanol intoxication. Note the dramatic loss of PV-ir in interneurons as a consequence of ethanol ingestion in all layers of the cortical area. Thin arrows indicate interneurons possessing a weak, though specific PV staining, while thick arrows depict densely-labeled PV-ir interneurons without stained processes. Arrowheads in B. indicate normal interneuronal PV staining patterns. Magnification ×150.

The numbers of PV-ir interneurons in frontal and parietal cortical sections and in the dorsal hippocampus were decreased significantly as early as after 4 days of ethanol treatment. The most pronounced loss of PV-ir was recorded in the parietal cortex (Fig. 1). Interestingly, the dramatically reduced PV expression was maintained up to 21 days of ethanol administration (Fig. 1). Similar changes were found in the expression of another CaBP, CB-D28k (data not shown). Furthermore, the extent of these changes correlated with the length of the treatment period and the volume of ethanol consumed. Measurement of brain Ca^{2+} and Mg^{2+} contents revealed parallel changes in the contents of these trace elements. It is noteworthy that the alterations in whole brain Ca^{2+} and Mg^{2+} contents exhibited a biphasic profile; significantly decreased Ca^{2+} and Mg^{2+} contents were recorded after 4 days of ethanol intoxication, while a dramatic hyperaccumulation was observed after 14 days of ethanol ingestion (data not shown).

In conclusion, our results demonstrate that chronic ethanol ingestion results in disturbed spontaneous animal behavior, accompanied by a deranged CaBP expression, with highly unbalanced changes in brain Ca^{2+} and Mg^{2+} contents. Taken together, our data indicate that chronic ethanol treatment influences the brain Ca^{2+} and Mg^{2+} contents, and the CaBP PV does not seem to protect against ethanol-induced neurotoxicity. Additionally, a close interaction may be suggested between the deranged neuronal functioning and behavioral dysfunctioning under open-field circumstances.

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Preliminary note

EXPRESSION OF FMRFAMIDE GENE NEUROPEPTIDES IS PARTLY DIFFERENT IN THE EMBRYONIC NERVOUS SYSTEM OF THE POND SNAIL, *LYMNAEA STAGNALIS* L.

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In the pond snail, *Lymnaea stagnalis, 5* exons (I-V) have been shown to belong to the FMRFamide gene which are responsible for the production of at least 13 different neuropeptides in the nervous system (Benjamin and Burke, 1994). Recently, a post-translational processing of the alternative neuropeptide precursor encoded by the FMRFamide gene was also demonstrated (Santama et al., 1996). Based on this molecular genetic background, the distribution of two members of the tetrapeptide group (the EFLRlamide[ILEal and the nonFMRFamide-like 22 amino acid 'SEEPLY peptide) and the post-translational, heptapeptide type acidic peptide (ACP) was investigated immunocytochemically during the embryogenesis of *Lymnaea.*

Among the three peptides investigated, ILEa-immunoreacitve (IR) elements dominated the developing central and peripheral nervous system (CNS and PNS)- The first, altogether 5, ILEa-IR cells were observed at the late trochophore stage (E30), revealing a transient character of immunostaining and located outside the future CNS. At the beginning of metamorphosis (E45), the first ILEa-IR neurons appeared within the developing central ganglia, some of them showing co-localization with either SEEPLY- or ACP-IR (Fig. 1). The number of ILEa- and SEEPLY-IR neurons, but not that of ACP-IR cells increased during metamorphosis in the cerebral, pedal, parietal and visceral ganglia, whereas the buccal and pleural ganglia contained only IR fibers in the neuropil. At hatching, the early transient cell cease to express ILE-IR At the same time, the CNS contained

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altogether 40-44 ILE-IR neurons. About 60% of them showing colocalization with SEEPLY-IR in the cerebral, parietal and visceral ganglia. On the other hand, ILE- and SEEPLY-IR neurons form distinct populations in the pedal ganglia. ACP-IR neurons (5-7) occurred exclusively in the visceral and right parietal ganglia, in all of them ILE-IR was also present.

Compared to CNS, the IR elements started to develop later, during early metamorphosis (E45) in the PNS. First a pair of ILEa-IR sensory neurons were seen in the foot, which was followed at E50 by the formation of the ILEa-IR innervation of central origin of the foot and mantle.

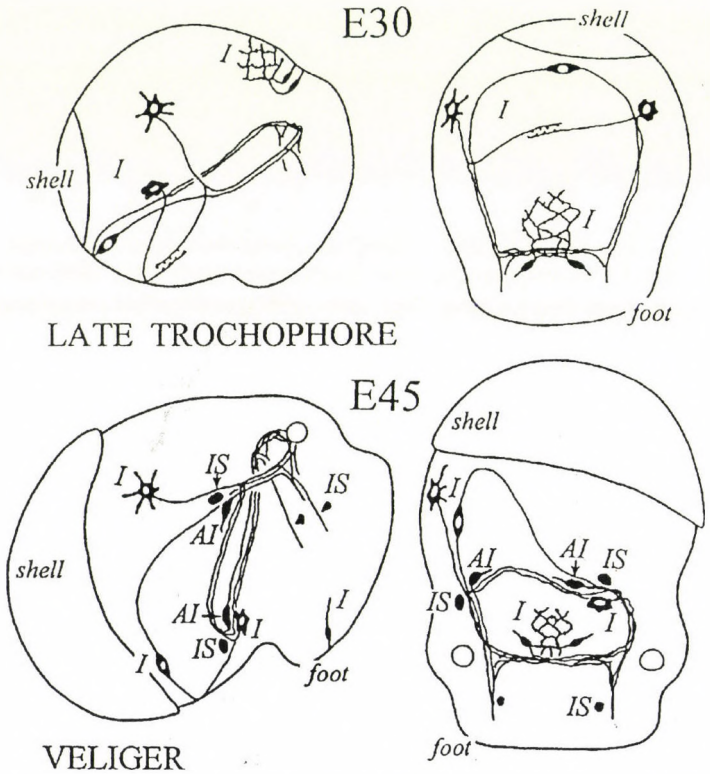

Fig. 1. Schematic representation of the distribution of ILE- (I), SEEPLY- (S) and acidic peptide- (A) immunoreactive elements in the early *Lymnaea* embryos.

However, the innervation pattern in the two peripheral organs was different; the foot received brush-like IR endings whereas the mantle was

supplied by varicose IR processes. At E60, only varicose IR elements could be found in both organs. After metamorphosis (E70) a second set of bipolar ILE-IR neurons could be demonstrated in the foot and the anterior region of mantle. Only a poor SEEPLY- and APC-IR innervation of central origin was found in the periphery by the end of metamorphosis (E80-E100).

Our findings show that the expression of the peptides encoded by the FMRFa gene is different during early and late *Lymnaea* embryogenesis. On the basis of the different temporary and cellular pattern of appearence at both the central and peripheral levels a multiple role can be attributed to them: a modulatory function in the CNS and a double, sensory and regulatory, function in the periphery. ILEa appear to be the key peptide in early central and peripheral processes, whereas SEEPLY and ACP seem to have a more specific role, first of all in central processes during late embryogenesis.

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ABSTRACTS

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EARLY MICROGLIAL ACTIVATION FOLLOWING ISCHEMIC BRAIN INJURY

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Transient arrest of the cerebral circulation leads to several metabolic changes and to neural cell death accompanied by rapid microglial reaction. In the present study we have examined the early postischemic microglial response to transient cerebral ischemic injury.

Microglial cells were detected using immunohistochemical method with monoclonal antibodies raised against the CR3 complement receptor (OX-42), the major histocompatibility complex (MHC) antigen class I (OX-18) and class II (OX-6) and leukocyte common antigen (ED- 1). Transient cerebral ischemia was brought about in two-month-old anaesthesized rats by bilateral common carotid artery occlusion for 10, 15, 20 min and 1,5 h and unilateral common carotid artery occlusion for 20 min. All the animals have been sacrificed immediatly afterwards the ischemic insult without reperfusion, except one which has been left alive for 1 h after a 20 min ischemic period.

Using the OX-42 antibody after a 10 min bilateral carotid occlusion we observed a mild microglial reaction in the hippocampus by the appearance of activated microglial cells. These cells were stained intensely and characterized by short and stout processes in contrast with the thin, long, highly branched processes of the resting or ramified cells. The intensity of the immunoreactivity was more increased after a 15 min long ischemic insult. Using Neurolucida Image Analyser program we quantified the activated cells. The number of activated microglial cells was twice greater after a 10 min, and five times greater after a 15 min ischemic period than in the intact control animal. After a 20 min of bilateral common carotid artery occlusion a widespread activity of microglial cells was apparent not only in the hippocampus, but also in the parietal cortex, in thalamic and hypothalamic areas.

Following 20 min unilateral common carotid artery ligature there was an increase in OX-42 immunostaining of activated microglial cells in the insulted hippocampus, compared with the noninsulted hemisphere. The number of activated cells in the injured hippocampus was twice higher than in the normal hippocampus.

Increased MHC I (OX-18) immunoreactivity was also observed in the hippocampus, in the parietal cortex, in thalamic and hypothalamic areas following a 1,5 h ischemic period and after 1 h postreperfusion following a 20 min bilateral ischemic injury, as well.

OX-6 and ED-1 immunostaining was observed after 1 h postreperfusion following a 20 min bilateral ischemic period.

The results show that during ischemic injury, microglial cells show an early upregulation and de novo expression of certain immunomolecules.

EFFECT OF CORTICOSTERONE AND ADRENALECTOMY ON NMDA INDUCED CELL DEATH IN RAT MAGNOCELLULAR NUCLEUS BASALIS

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Earlier studies suggest that high glucocorticoid concentrations increase the vulnerability of hippocampal neurons and consequential neurodegenerative cell death. However these experiments were almost exclusively carried out on the hippocampus while little is known of the effects of glucocorticoids on neuronal survival in other brain regions. Recently we demonstrated N-methyl-Daspartate (NMDA)-induced cell death in the Magnocellular Basal Nucleus (MBN) which could be precisely monitored by the reduction of cholinergic fibre density in the somatosensory cortex. This easy and quantifiable neurodegenerative model was used in the present study injecting NMDA into MBN of rats at different plasma corticosterone concentrations. The experiments were performed in sham-operated controls, in adrenalectomized (ADX) animals and in ADX rats supplied with 25% or 100% corticosterone pellets. The neurotoxic impact of the NMDA injection was assessed by determining loss of cholinergic fibers stained with acetylcholinesterase histochemistry in the parietal cortex. In order to evaluate the putative role of an astrocytic reaction in these experiments, glial fibrillary acidic protein (GFAP) was visualized by immunocytochemistry. Adrenalectomy significantly potentiated the NMDA-induced neurodegeneration by 50%, while chronic administration of corticosterone significantly attenuated the NMDA-neurotoxicity in a dose-dependent manner. Compared to the ADX group, 25% corticosterone application reduced the NMDA damage by 37%, whereas the 100% corticosterone pellet dimished NMDA neurotoxicity by 75%. Both adrenalectomy and corticosterone implantation enhanced the NMDA-induced GFAP immunoreactivity. The increase of GFAP immunoreactivity was most pronounced in the adrenalectomized rats supplied with the 100% corticosterone pellets. The present results provide evidence that glucocorticoids can protect cholinergic cells in the MBN against cell death. Adrenalectomy and suppletion of corticosterone potentiate astrocyte activation in the areas where the cholinergic fiber undergo degeneration. These findings indicate that corticosterone can be beneficial in neuroprotective and possibly neurodegenerative processes after brain insults involving damage to the basal forebrain cholinergic cell system.

A MODEL FOR THE INTRACORTICAL ORIGIN OF ORIENTATION PREFERENCE AND TUNING IN MACAQUE STRIATE CORTEX

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In macaque monkeys, layer IVC in striate cortex can be either divided into the two subdivisions IVCα and IVCβ which correspond to the termination zones of P and M thalamic input, or into three output subdivisions which project to different targets. Lower IVCβ projects via IVA to the blob regions in II/III, mid-IVC projects to the interblob territories of the upper layers, and upper IVCα projects to layer IVB. The output subdivisions roughly correspond to Livingstone's and Hubel's color, form, and motion pathways [1].

Although orientation selective cells are usually reported from the upper and lower layers, as well as from IVB, they are found for the first time in mid-IVC (sparse groups of orientation selective cells) and in upper IVCα (full range of selectivities). Their emergence in layer IVC coincides with the emergence of 300 ηm - 500 ηm long lateral 'sidestep' connections [2] which are sparse in mid-IVC but which are a prominent feature of upper IVCα. In upper IVCα additional longer range inhibitory connections emerge. It has been conjectured [3], that orientation selectivity may emerge in IVC and in the projection to its target and that those 'sidestep' connections may play a crucial role.

In our contribution we investigate this hypothesis for cells located in upper IVCα. Building upon a biologically detailed connectionist model of the afferent pathway [4] we add recurrent excitatory and inhibitory connections between units which correspond to cells in upper IVCα. All cortical units have circular symmetric input fields. Units, whose input fields are shifted approximately along a line in visual space, are connected by strong excitatory interactions, such that the sum of afferent inputs is largest when a grating overlaps the line of receptive fields and matches its orientation in visual space. Excitatory interactions between units corresponding to differently oriented lines fall off with their angular difference, so do inhibitory interactions, albeit less strongly. Inhibitory units have higher thresholds but steeper slopes of the contrast-response function than excitatory cells.

In contrast to previous approaches there is no afferent orientation bias included in the model. The bias is generated by the anisotropic lateral interactions and is then amplified, i.e. orientation bias and tuning are generated by the same circuitry. The model exhibits contrast invariant tuning, iso-orientation inhibition, and is in accordance with experiments blocking inhibition within one unit as well as within a whole hypercolumn. Results of a thorough exploration of parameter space as well as results related to the units' orientation and spatial frequency tuning will be reported. In contrast to models which include an afferent orientation bias [5], this model predicts non-specific PSP's in the first, transient phase of a units rsponse, similar to what has been reported, e.g., for cat striate cortex [6].

Acknowledgements

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GLIAL REACTIONS FOLLOWING STAB WOUNDS IN THE CEREBELLUM AND OPTIC TECTUM OF THE DEVELOPING AND ADULT CHICKEN BRAIN

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In the avian brain, the distribution of GFAP (glial fibrillary acidic protein) immunopositive structures is in many respects different from that found in mammals. The borderlines between the GFAP-rich and GFAP-poor areas are more distinct than in mammals and at least in one brain area, the molecular layer of the cerebellum, the glial (i.e. Bergmann-glia) cytoskeletal protein is vimentin which is characteristic only for the immature astroglia in mammals. Conversely, the granular layer of chicken cerebellum contains GFAP-immunopositive astrocytes similarly to mammals. This distribution of glial cytoskeletal elements can be observed from the first post-hatch days to adulthood. In the optic tectum, the distribution of cytoskeletal elements resembles that of the cerebellum. The deep layers, but not the superficial ones, are immunopositive against GFAP. Vimentin immunostaining reveals an intense radial glial structure in the first postnatal week. The present study investigates the glial reactions following a simple stab wound lesion to the areas characterised by specific GFAP-distribution in developing and adult domestic chickens (Gallus domesticus). Our study was complemented by similar lesions placed in the telencephalon.

The operations were performed by a sterile disposable needle, under deep anaesthesia (Equithesin, 0.25ml/l00g), on post-hatch days (P) 1, 7, 14 or 21, or in adult (at least two-month-old) animals. After a one-week survival period the animals were overdosed with ether and perfused transcardially with 4% buffered paraformaldehyde. Serial sections (100 μm thick) from the lesioned areas were cut on a vibration microtome (Vibratome), and processed for immunohistochemistry against GFAP and vimentin. The immunoreaction was developed by streptavidin-biotin horseradish peroxidase complex and the reaction product was visualised by diaminobenzidine reaction.

In the cerebellum, no trace of the lesions was found in the chicks operated on Pl, although, in the corresponding sections of the brain stem, the bottom of the wound track and the surrounding gliosis were recognized. In all older ages, the damaged area of the molecular layer was bounded by elongated, radially oriented GFAP-positive processes, which appeared to belong to Bergmann glia that acquired immunopositivity against this protein. In all other areas of the molecular layer the Bergmann-glia remained immunonegative to GFAP but immunopositive to vimentin. A demarcating meshwork of star-shaped cells (like that found usually in mammals) was only observed in the granular layer and in the white matter but not in the molecular layer. In the tectum, the Pl lesion resulted in a phenomenon similar to that observed in the cerebellum: no reactive gliosis was found in tlne GFAP-free superficial layers. From P7, however, the usual glial reaction was detectable in these layers. In the telencephalon, reactive gliosis was observed already with the Pl lesions.

In conclusion, we found an unusual form of reactive gliosis in the bird brain, in particular the molecular layer of the cerebellum. In Pl chicks, meaningful differences were observed between the glial reactivity of different areas, most probably due to the different stages of their neurohistogenetic maturation.

TESTOSTERON-NORADRENALINE INTERRELATIONS IN THE CONTROL OF AGGRESSION

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Although the interrelations between endocrine factors and the effects of pharmacological agents is in the focus of neurobiological research, we know surprisingly little about the effect of the above mentioned interrelationship on the behavior of animals. Experiments were undertaken in order to investigate whether a changed hormonal background changes the behavioral effects of pharmacological agents. The interrelationship between testosterone, noradrenaline and aggressive behavior was studied. Testosterone is among the major factors that control aggression. Testosterone affects also the function of noradrenergic system. Alpha-2 adrenoceptor blockers on their turn influence aggressive behavior. The aim of the present experiments was to investigate the effect of testosterone background on the behavioral consequences of α_2 adrenoceptor blocker treatment.

Sexually mature male rats were castrated. Half of the animals were treated with testosterone-enanthate (TE), a long-acting testosterone preparation (TE rats). Control animals were injected with vehicle (oil). After a recovery period of 1 week aggression was induced by repeated exposure of subjects to intruder male rats (30 min. during 3 consecutive days). The fourth encounter was preceded by α_2 adrenoceptor blocker (1 mg/kg idazoxan, 20 min. before the encounter). The behavior of animals was videorecorded during each aggressive encounter. Videorecordings were later analyzed.

Aggressive behavior developed more quickly, and was more intense in TE rats compared with controls. This was true for all three behavioral variables: attack frequency, threat behaviors, and dominant posture. It is worth to notice, however, that a significant aggressive

behavior developed in controls as well (though these animals were castrated, and testosterone was not replaced).

In TE rats the α_2 adrenoceptor blocker idazoxan induced a marked reduction in aggressive behavior: the frequency of attacks was lowered, while the duration of both threats and dominant behavior decreased. This results confirmed our previous results concerning the effects of α_2 adrenoceptor blockers on the aggressiveness of residents that face non-aggressive intruders.

Castrated rats reacted differently to idazoxan injections. The frequency of attacks and the frequency of threat behavior were not changed by the α_2 adrenoceptor blocker treatment. However, dominant behavior was lowered in these animals as well.

We conclude that the effects of α_2 adrenoceptor blockers on aggression depend on the presence of testosterone. Dominant behavior was changed by idazoxan irrespective to TE treatment, however, this behavior does not depend on the presence of testosterone in general.

ONTOGENY OF CALRETININ EXPRESSION IN RAT DORSAL ROOT GANGLIA

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The aim of the present work was to investigate the developmental changes in calretinin (CR) expression by means of immunohistochemistry and Western blot analysis.

Our results show that CR appears in cervical DRGs, as early as E11. At E12, most of the cells are immunopositive in ganglia observed at cervical, thoracic and lumbar level. On the following two days, CR immunoreactivity progressively disappears and CR is no more detectable at any levels between E15 and E16. From E17 till E20 the CR immunoreactivity reappears following a rostro-caudal pattern. On postnatal days 1 and 2, CR positivity is found in 12-14% of the cells, i.e. in a proportion twice as high than found in adult ganglia (5-8%). These data were confirmed by immunoblots. Neither the satellite cells nor the Schwann cells present CR immunoreactivity at any ages. According to morphometric evaluation CRimmunopositive cells belong to both «large» and «small» neuronal subpopulations.

In conclusion, our data show that calretinin is expressed very early in development and may thus have an important role during morphogenesis of dorsal root ganglion cells (S.N.F.N°3367-92).

VISUAL CONTRAST SENSITIVITY IN SCHIZOPHRENIC PATIENTS

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Schizophrenic patients treated with neuroleptics may develop a clinical picture similar to Parkinson's disease (PD), presenting a variety of extrapyramidal symptoms. Several studies

also found a considerable visual contrast sensitivity (VCS) loss in PD mainly at the middle spatial frequencies of the VCS curve. Studies measuring retinal dysfunctions in drug-induced PD, mainly VCS function, are rare. In our study static and dynamic VCS was measured in 10 schizophrenic patients and in 10 age and sex matched healthy subjects. Stimuli were vertical gratings with a sinusoidal profile. Static VCS was determined at nine spatial frequencies (SF) (between 0.5 and 14.38 cycles/degree), dynamic VCS was determined at 4 Hz temporal frequency using a forced choice staircase method. Nine of ten patients were on medication, taking different doses of haloperidol or risperidone. Static and dynamic VCS decrease was observed in the patient group at the middle spatial frequencies. The VCS loss was different in the two groups of patients taking haloperidol or risperidone. In the haloperidol group both the static and dynamic VCS decreased significantly at the middle spatial frequencies. In the risperidone group overall static and dynamic VCS loss was observed. The VCS loss depended on the doses of the drugs, and static and dynamic VCS changed in different manner in the two patient groups. Our results rise up the possibility that VCS loss observed in schizophrenic patients can be detected before the appearance of extrapyramidal symptoms. According to the data of schizophrenic patients taking risperidone, the possible serotonin-dopamine interactions in the retina and in the VCS function also need to be considered.

AUTOGRAPHIC LOCALIZATION OF DELTA OPIOID RECEPTORS IN RODENT BRAIN

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TIPP (H-Tyr-Tic-Phe-Phe-H), a synthetic, chemically stable ligand, was recently radiolabeled, resulting a radioligand highly selective and specific for the delta opioid receptors.

Cryostat sections (15 μm) from rat and mouse brains were cut and mounted onto chromealuminum/gelatin-coated glass slides. Total slide binding was determined by incubation of the slides for 120-180 min at 25°C in 50 mM Tris-HCI buffer (pH 7,4) containing 5 mg/ml $MgCl_2$, 1 μg/ml bacitracin and 1-3 nM of [^{3}H]TIPP, while nonspecific binding (12-14% of the total binding) was determined in the presence of 1 μM naltrexone. After incubation the slides were rinsed 3-Sx10 min at 4°C in 50 mM Tris buffer. While delta opioid selective ligands blocked the specific binding of [^{3}H]TIPP with high efficiency and selectivity, μ- and κ-specific and selective ligands, as well as non-opioid substances were much less effective in displacing the specific [^{3}H]TIPP binding. Our slide binding experiments determined the maximal number of binding sites (Bmax) and dissociation constant values (Kd) for rat and mouse brains as follows: rat, Bmax (fmol/mg prot.) = 15.6±1.5 and Kd (nM) = 0.463±0.15; mouse, Bmax = 23.91±0.13 and Kd = 0.85±0.12. For autoradiographic studies the sections were apposed to ^{3}H-sensitive films for 4-7 months, then developed with conventional photographic methods. Computer-assisted microdensitometry was employed to analyze the results.

This study demonstrated that [^{3}H]TIPP is a superior ligand for investigating the binding characteristics and autoradiographic distribution of delta opioid receptors.

QUANTIFICATION OF ENDOGENOUS ADENOSINE BY PRECOLUMN ENRICHMENT OF TISSUE SUPERPERFUSATE FLUID

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Adenosine is an inhibitory neuromodulator in the CNS. However, it is present at only trace levels in tissue perfusate fluid (<10 pmol.ml^{-1}) and it is rapidly deaminated by adenosine deaminase to inosine.

A sensitive and selective standard addition HPLC method was developed for the quantification of adenosine in tissue overflow fluid. The acidified and with standards admixed samples were enriched on a reversed-phase 'trap column', which was connected to the system as an extra valve. The separation was performed on a 3 µm Nucleosil-C18 analytical column, using an aqueous buffer-methanol/acetonitrile (9010(3.5/1), V/V) as the mobile phase. The aqueous buffer consisted of 10 mM potassium dihydrogen phosphate and was adjusted to pH 5.55 with potassium hydroxide. The effluent was led to an UV detector operating at 260 nm. The method was validated for the assay in tissue superfusate solution by determination of the repeatability (intra day variation), reproducibility (inter day variation) at 15 pmol.ml^{-1} added amount of adenosine 13.13±1.87, n=8; 13.41±1.49, n=18; and 30 pmol.ml^{-1} added amount of adenosine 27.74±2.26, n=8; 27.02±2.98, n=18, respectively, enrichment recovery (91.2±9.9, n=265). This direct injection method is applicable to monitoring of released endogenous adenosine in tissue overflow fluid.

EFFECTS OF AN AMPA-ANTAGONIST ON EPILEPTIC ACTIVITY EVOKED BY AMINOPYRIDINE IN IN VIVO AND IN VITRO MODELS

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Several experimental systems were developed for modelling different types of human epilepsy and investigating the effects of antiepileptic drugs. Aminopyridine (AP) administration causes a characteristic spontaneous tonic-clonic activity which resembles the ictal temporal lobe types of human epilepsy. In our parallel in vivo and in vitro experiments inhibitory effects of the specific AMPA-type excitatory amino acid receptor antagonist (GYKI 52466) were analysed on the epileptiform activity evoked by AP in the somatosensory cortex of rats. Latency of appearance, frequency and length of the spontaneous ictal events were examined in the primer and mirror epileptic foci of anaesthetised rats and in slices as well. In in vivo experiments the structure (frequency components) of each ictal periods, in slices the evoked responses were also analysed in detail.

Both in vivo and in vitro systems the first epileptic events appeared in 6 minutes after AP administration. In both experimental systems the inhibitory effect of the GYKI 52466 was almost the same, both the number and the length of the events decreased about 30 % . In slice experiments amplitude of the evoked responses was decreased also about 30 %, however length was decreased more significantly, about 65 %. In anaesthetised rats the rearrangement of frequency components of each epileptic events was striking as the effect of AMPA antagonist, the percentage of slow (1-3 Hz) frequency-component significantly decreased in primer epileptic focus and also in mirror focus.

We can conclude, that although the excitatory amino acid receptor antagonist did not fully block the seizure events in this aminopiridine-induced model, it significantly reduced the epileptic activity. This would be useful in development of combined drug delivery in medical treatment of epilepsy.

NEUROCHEMICAL ORGANISATION OF THE STOMATOGASTRIC NERVOUS SYSTEM IN OLIGOCHAETES

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The alimentary canal of oligochaetes is characterized by autonomous rhytmic movements, probably co-ordinated by intrinsic neurons located in its wall. The gut is additionally regulated by neuronal elements of extrinsic origin. The stomatogastric nervous system of earthworms consists of six paired ganglia in the wall of the pharynx and the enteric nerve plexus which is in subepithelial position. Little is known, however, about the neurochemical organization of this system. Therefore, the aim of the present study was to describe certain aspects of the immunocytochemical organization of the stomatogastric system in two prominent species of annelids, *Lumbricus terrestris* and *Eisenia fetida,* applying antibodies raised againts neuropeptides (FMRFamide [Fa], proctolin), amino acids (GABA) and monoamines (serotonin [5-HT], octopamine [OA], catecholamines [tyrosinehydroxylase, TH]), playing important roles in different synaptic and modulatory events in invertebrates.

Fa-, proctolin-, GABA-, 5-HT- and OA-immunoreactive (IR) neurons can be observed in all stomatogastric ganglia. The pharyngeal plexus contains additionally TH-IR neurons, indicative of an enteric catecholaminergic system of intrinsic origin. The number of Fa- and GABA-IR neurons is higher than that of the other IR-element. Numerous labelled fibers take their origin from intrinsic perikarya, whereas others, originating from the circumpharyngeal connectives, enter the subepithelial plexus *via* stomatogastric ganglia. All kinds of immunoreactivity investigated were detected in these fibres. OA- and GABA-IR cells were additionally revealed in the epithelial layer of the pharynx.

Our results clearly show that the neurochemical character of both the extrinsic and intrinsic nerve elements in the stomatogastric system of oligochaetes seems to be more diverse than in other invertebrates (Selverston and Moulins, 1987; Marder et al., 1995). The studied signal molecules appear to be involved in both ganglionic (integrative) and peripheral (muscular) processes.

Our observation is the first report for invertebrates on the occurrence of OA-IR neurons in the peripheral nervous system, suggesting that this important neuromodulatory substance may also have an intrinsic effector function in annelids.

The dorsal and caudal wall of the pharynx is connected with the muscle fibers of the body wall, therefore we suggest that the regulation of the body wall musculature is also performed by neuronal elements of the pharyngeal plexus. For the epithelial GABA- and OA-IR cells, a yet unknown endocrine function of these substances cannot be exluded.

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SYNTHESIS OF NEURAL NETS BASED ON SPIKE RECORDS - A MODEL STUDY

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Neuronal firing data incorporate information about their generating networks. Neural network synthesis ends with presentation of connections and parameters for units and inter-unit dynamics. It is based either on real or fictitious spike records and complementary premises. While network synthesis may be algorithmic (even automatic), the results are rarely necessary, more often sufficient, i.e. correspond to cases of possible explanations. Often incomplete interpretative networks are obtained.

In any algorithmic net synthesis an a *pnori* standardization of the steps and data are necessary. A synthesis algorithm (called state recognition method, L.E., 1980) was tested applied and developed further. It is as follows. - [1] - Replace spikes by time points. - [2] - Derive integer sequence reflect temporal densities. - [3] - Extract dynamic and statistical conclusions, from single or multiple sequences. These steps are more or less traditional. Their relation to effective net - synthesis is indirect. - [4] - Both binary and many-valued temporal logical description of sequences are possible. - [5] - Instead of (peculiar or conventional) statistical description, a non arbitrary automata synthesis is carried out. Machine outputs correspond to sequences (see in [3]) representing original spike record data. Automata may include both mono- and special multi-threshold gates. - [6] - Intuitive (informal, heuristic) steps, e.g. which aim to determine socalled essential and ignorable features of records, are necessary and represent the hardest ones.

Conclusions: (1) - The synthesis results are not unequivocal and this cannot be eliminated. Restrictions may reduce the number of alternative solutions. External information is useful to choose. Optimizations may also select from results. (2) - Goodness of fit depends strongly on the crucial step [6]. (3) - Statistical descriptions give complementary insight. (4) - Differences in explanations stem from step [6].

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EFFECT OF ACIDIC PH ON NEURONS OF THE TRIGEMINAL GANGLION

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It is well known that capsaicin acts on the B-cell population of the sensory ganglia. This effect is mediated by capsaicin receptor-gated cation channels. It has previously been proposed that protons (pH 6.0) would be the natural activators of these channels. Others have demonstrated some differences between the effects of capsaicin and acidic fluids in cultured DRG cells. The aim of the present study was to introduce a new technique to investigate acidic pH and capsaicin action upon neurons of the trigeminal ganglion isolated from young rats.

3-14 days old Wistar rat pups were used. Under ether anaesthesy the trigeminal ganglia were quickly removed and dissected in chilled oxigenated artificial cerebrospinal fluid (ACSF). The ACSF contained in mM: 124 NaCl, 3.5 KCl, 1.8 $CaCl_2$, 1.2 Mg SO_4, 1.2 KH_2PO_4, 26 $NaHCO_3$, 10 glucose. In some cases the connective tissue was digested with trypsin (4 U/mg). Ophthalmic nerve was cut and prepared for stimulation with suction electrode.

Intracellular recordings were made from over 100 cells of which 82 were suitable for further characterisation. Criteria for further analysis of the impaled cells were overshooting action potential over 50 mV and at least 5 min. duration of stable membrane potential. Input resistance of these cells fell in the range of 10 and 50 MOhm with membrane time constant around 5 ms. Time and voltage dependent rectification was strong especially in cells which fired short (<2 ms) duration of action potential (A cells). Those of over 5 ms were considered cells with C-fibers. In the majority of cases conduction velocity of the fibers were measured. Long duration action potentials came from cells with axons in the ophthalmic nerve conducting impulses with a speed below 1 m/sec.

Four cells survived testing in their membrane properties in acidic pH, 8 in capsaicin and 3 cells in both acidic pH and capsaicin. Although rectification was suppressed both by acidic pH and capsaicin, firing threshold of the cells appeared to be increased by capsaicin only. The method seems suitable for further analysis of the effects of the acidic pH and capsaicin on trigeminal ganglion cells with axonal processes.

DOPAMINE AS A LINK BETWEEN NORADRENERGIC NERVE TERMINALS AND SPLENOCYTES

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Although dopamine (DA) has been shown to be a neurotransmitter in many peripheral tissues; it is generally accepted that the spleen does not receive dopaminergic innervation.

Nevertheless, it is one of the well-established, noradrenergically innervated tissues. The existence of DA receptors on splenocytes has been shown; and functional studies suggested that they may have a role in the regulation of immunocompetence. The possible source of immunoregulatory DA in the spleen has not been validated yet. Therefore, the aim of our study was to clarify whether noradrenergic nerve endings might serve as a source of immunoregulatory DA in the spleen.

The effect of supramaximal electric field stimulation on [^{3}H] released from rat spleen strips was studied after being loaded with either [^{3}H]dopamine ([^{3}H]DA) or [^{3}H]norepinephrine ([^{3}H]NE). In some experiments, [^{3}H]DA and [^{3}H]NE stored in the tissue or released in response to electrical stimulation were separated from their tritiated metabolites using HPLC followed by radiochemical detection.

The stimulation-evoked release of [^{3}H] after loading with either isotopes was a subject to negative feedback modulation through a_2-adrenergic, D_2-dopamine and muscarinic acetylcholine receptors, and could be prevented by either calcium removal or tetrodotoxin blocking of Na^+ influx which indicated its neuronal and vesicular origin.

After the separation of radioactive metabolites by HPLC, both the tissue loaded with [^{3}H]DA and the fractions collected during its electrical stimulation contained a considerable amount of [^{3}H]NE, providing evidence that the neurons it originated from were adrenergic in function and were able to take up DA, convert it into NE, and release it as NE in response to neural activity. Tritiated DA was also released during electrical stimulation, possibly from the small synaptic vesicles (SSVs) since SSVs do not contain DBH, and the synthesis of NE from DA appears to be restricted to the LDCV population. The ratio of [^{3}H]DA and [^{3}H]NE in the spleen loaded with [^{3}H]DA was found to be dependent on both temperature and the time of loading, and could be modulated by various drugs such as desmethylimipramine, a NE uptake blocker, and fusaric acid, a dopamine b-hydroxylase inhibitor.

Our data suggest that there is a possibility for the regulated exocytosis of DA from the noradrenergic nerve endings of the spleen during sympathetic activation, that is, beside NE, DA also seems to fulfill the criteria for being a sympathetic neurotransmitter in the spleen in spite of the lack of dopaminergic innervation. Under physiological conditions, the source of DA being taken up to noradrenergic terminals could be circulating DA, especially during stress, since exposure of an organism to a variety of stressors that increase sympathetic tone is accompanied with an increase in plasma concentrations of DA. Thus, DA accumulated in noradrenergic varicose axon terminals and released into a small volume around the nerve endings would therefore be able to reach a local concentration high enough to exert an immunomodulatory effect on splenocytes. An existence of a similar mechanism for DA being collected and released in high concentrations by noradrenergic sympathetic terminals into extracellular space was suggested for the fine tuning by DA of local circulation and aldosterone synthesis in the zona glomerulosa. Our finding may reveal a new mechanism by which splenocytes can be regulated by the neuroendocrine system.

SOCIAL RECOGNITION TEST, AS A TOOL OF MEASURING SHORT-TERM MEMORY IN RATS

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The social olfactory recognition test is based on the observation that an adult male rat investigates a conspecific juvenile rat less during the second of two successive exposure, when the time interval between the encounters is relatively short. The diminished investigation is considered as recognition of the conspecific, and it is not present when a novel juvenile is encountered during the second exposure.

The test can easily be carried out using a protocol as follows: An unfamiliar juvenile rat is placed in the home cage of an adult rat for 5 min. The time spent by the adult in investigating the juvenile (close following, head-, body- and anogenital sniffing, grooming) is recorded. Following a time interval the same juvenile or a novel one is placed in the home cage of the adult rat for a second exposure period of 5-minute. When the time interval between the first and second encounter is 30 min, the adult rat investigates the novel juvenile more than the same juvenile. With a time interval of 120 min the adult rat investigates the same and novel juvenile equally, indicating that social recognition is not present any more, i.e. based on short-term memory processes.

Furthermore, the social recognition test has been shown to be sensitive to cholinomimetics. In the present experiment we studied the ability of some other treatments to facilitate short-term olfactory memory in comparison with Tacrine. The AChE inhibitor Tacrine, the nontropic agent Piracetam, the M_1 receptor agonist SR-46559A and a prolyl-endopeptidase inhibitor JTP-48 19 were administered intraperitoneally to adult rats immediately after the first exposure at doses of 3, 500, 0.5 and 5 mg/kg, respectively. Social recognition was observed after an interexposure interval of 120 min (using the same juvenile rat), while rats injected with saline did not show any social recognition. These results suggest that these drugs affect the short-term memory related processes involved in social recognition of rats.

Reference

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PANORAMA CODE: SPATIAL INFORMATION IN THE DISCHARGE OF CORTICAL NEURONS

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Dimensions of perception are represented in the mammalian brain predominantly by orderly maps containing sharply tuned neurons. Thus the location of particular stimuli might be represented by neuronal responses to stimuli from circumscribed parts of the space. There is a possibility, however, for unconventional coding of stimulus dimensions; e.g. it has been described that temporal firing code represents sound location in the feline cortex (Middlebrooks JC, Clock AE, Xu L, Green DM et al., 1994). The extremely large visual receptive fields of the neurons in the anterior ectosylvian cortex of the cat permits the testing whether or not this type of coding may also occur in the visual information processing.

We tested therefore the firing of visually responsive neurons in the cortex along the cat anterior ectosylvian sulcus (AES) as a function of stimulus location in the receptive fields. These neurons possess receptive fields that occupy most of the contralateral lower quadrant and additionally a substantial portion of both the contralateral upper quadrant and the ipsilateral hemifield: The neurons are sensitive to stimuli moving rapidly in a particular direction.

Stimulation was provided as a visual noise moving in a $8° \times 8°$ window on a screen placed 57 cm in front of the eye. The position of the window was varied pseudorandomly within the frame of a $24° \times 32°$ matrix that covered exclusively visually responsive areas. Single unit activity was recorded extracellularly in barbiturate anaesthetized, immobilized, artificially respirated cats.

A statistical analysis of neuronal firing rates showed that there was a significant difference between the responses to individually located stimulation within the receptive fields. In all receptive fields there was a best-responding area, the location of which showed no preference to any part of the receptive field. No significant difference was found between the firing rate in response to the stimulation of the best responding area and the most remote area in horizontal or vertical direction. There was a significant difference, however, between the responses to stimuli appearing on the contraletaral versus the ipsilateral side of the vertical meridian.

Our results suggest a unique activation pattern in the AES cortical visual area, in which a stimulus from nearly any location of the visual field activates a diffuse population of neurons. These active neurons may code the approximate location of the visual signal. The accurate location of the stimulation can be established by the compound activity of the neurons with similarly organized neurons in this part of the feline cortex.

INTERACTION BETWEEN A NOVEL, PUTATIVE ANXIOLYTIC DRUG, EGIS-7848, AND THE OPIOID SYSTEM

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EGIS-7848 a styryl isoquinoline compound showed strong anxiolytic activity in a series of models minimum effective dose in Vogel punished drinking test 0.001 mg/kg; in elevated plus maze test. 0.1 m/kg, in hole-board test: 0.003 mg/kg: in social interaction test: 0.1 mg/kg. The compound did not show binding to any of the following receptors: adrenenergic$_{a1}$; a2; ß, dopamine 1 and 2, serotonin 1_A, 2_A, 2_C, 1,4 benzodiazepine, 2,3-benzodiazepine and cholecystokinine. *In vitro* binding studies also revealed that EGIS-7848 did not bind the d,k,m opiate receptors, either However, some *in vitro* preliminary data indicated that an interaction may exist between the compound and the opiatergic system. The aim of the present study was to examine the nature of this potential interaction. The anxiolytic effect of EGIS-7848 was antagonized by the opiate m receptor antagonist naloxone in the elevated plus maze test. Similar effect was observed in mCPP-induced anxiety paradigm, where both naloxone and naltrexone significantly and dose-dependently antagonized the anxiolytic activity of the compound EGIS-7848 failed to significantly influence morphine-induced analgesia (a direct, m receptor mediated effect in the hot plate method. However, it dose-dependently potentiated morhpine induced catalepsy in mice (indirect effect of morphine via the dopaminergic system). The results confirm the existence of an interaction between EGIS-7848 and the opioid system. Our findings suggest that the opiatergic system might be involved in the (presently unknown) mechanism of anxiolytic action of the drug. However, on the basis of the in vitro data and the present series of experiments we suppose, that the observed interaction of EGIS-7848 with the opiatergic system is probably not the result of a direct effect on the opioid receptors.

BRLP-42 AMELIORATES DEFICIENT NERVE CONDUCTION VELOCITY AND SPINAL REFLEX INTEGRATION IN DIABETIC RATS

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Diabetic neuropathy (DN) impairs both sensory and motor functions. Small sensory fibers are affected first followed by large motor fibers, in the lower extremities of diabetic patients with polyneuropathy. DN reduces nerve conduction velocity (NCV) and disturbs central integrative (synaptic) mechanisms. Endoneurial hypoperfusion and subsequent hypoxia contribute to the aetiology of diabetic nerve dysfunction. The HSP-70 coinducer BRLP-42 (BIOREX,

Hungary), a drug under development against diabetic complications, prevented experimental retinopathy and neuropathy in previous studies.

The effects of therapeutic treatment with BRLP-42 on existing motor and sensory neuropathy in streptozotocin (STZ)-diabetic rats were examined in two experimental arrangements (I and II). Diabetic abnormalities of monosynaptic extensor- (ER) or polysynaptic flexor reflexes (FR), defined by electromyograms (EMGs), served to determine muscle motor and sensory NCVs (MNCV and SNCV, resp.) in sciatic nerve (I) or withdrawal function in response to painful skin stimulation (II).

Diabetes was induced with a single i.v. bolus (45 mg/kg) of STZ in male Wistar rats. Daily oral treatment with 20 mg/kg BRLP-42 was given after 1 month of untreated diabetes for a further month (I) or 3 weeks, followed by a 1-week washout period (II). Electrophysiological measurements were performed under anesthesia (ketamine or urethane-chloralose i.p.). ER was elicited by bipolar needle electrodes stimulating the sciatic or tibial nerve at the end of the treatment period. Latencies of the two components of averaged evoked EMGs recorded from plantar muscles and the distance between the two stimulation points were used to calculate MNCV and SNCV. FR (averaged EMG of tibial muscle) was evoked weekly by a stimulus train delivered to the plantar surface. Magnitude of FR as the subcurve area and the peak latency of the highest component were determined. Time course of BRLP-42 induced recovery of diabetic deficiency of nociceptive FR was studied.

Diabetes produced 24% and 25.8% ($p<0.05$) decreases in MNCV and SNCV, resp., compared to non-diabetic control. Administration of 20 mg/kg/day BRLP-42 over 1 month partially reversed (by 67.9% and 74.0%, resp., $p<0.01$) the motor and sensory deficits. This fact indicates that therapeutic treatment is as effective as the preventive one against peripheral neuropathy.

Magnitude of tibial FR was reduced by 42% due to 1-month diabetes. BRLP-42 treatment caused a progressive improvement, which was significant after 1 week (27.3%, $p<0.05$), almost full normalization being achieved by the end of the 3rd week (87.5%, $p<0.001$). FR declined to diabetic level after interrupting the treatment for 1 week. The peak latency was shortened by 27% with diabetes. Its maximum improvement (45% $p<0.01$) was reached within 1 week, however, it remained shorter than the onset value after withdrawal. This finding suggests that BRLP-42 can normalize the diabetic hypoalgesia, the main cause of the development of neuropathic ulcer.

It is concluded that BRLP-42 ameliorates deficient NCV and spinal reflex integration in diabetic rats. The compound is thought to act by promoting nerve survival and/or regeneration.

RHODOPSIN KNOCKOUT MOUSE AS AN ANIMAL MODEL IN THE GENE THERAPY OF RETINITIS PIGMENTOSA

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Retinitis pigmentosa (RP) is a common term of many human inherited retinopathies. These debilitating disorders collectively represent the most frequent inherited forms of human visual handicap with an estimated prevalence of approx. 1 in 3000. RP may be inherited autosomal

dominantly, autosomal recessively or in an X-linked fashion. RP involve the death of rod photoreceptor cells which ends in total blindness.

The genetic aetiology has been shown to have its origins in defects of rhodopsin and peripherin/RDS proteins. Genetic linkage studies have identified the location of loci responsible for RP in the rhodopsin gene on chromosome 3.

For the exploration of any possible gene therapy of RP, 1) we worked out a delivery system, to introduce the therapeutic gene carrying vector to the neural retina, 2) we used naked plasmid alone or with DOTAP constructed with reporter gene driven by cytomegalovirus or bovine rhodopsine promoter to investigate the possible expression of foreign genes within ocular tissues, 3) we also investigated the histology of the retina of the rhodopsin targeted mice, which might serve as an animal model of the gene therapy of Rl'

Results of gene delivery: Although the applied method of the gene delivery is effective in transfecting of the photoreceptor cells, the results are not long-lasting (2 months) and accompained by retinal degenerations.

The retina of the rhodopsin knockout mice: the targeted mouse obviously has photoreceptor cell degeneration, i.e. the absence of rhodopsin immunoreactivity, and the loss of membrane discs of the outer segment. These animals might serve as an animal model in the exploration of gene therapy of RP.

CHRONIC MORPHINE INDUCED CHANGES OF SPINAL AND SUPRASPINAL μ OPIOID RECEPTOR BINDING IN RAT CNS

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Chronic morphine administration leads to tolerance and dependence, but the molecular bases of these phenomena are poorly understood. In the present work morphine tolerance of Wistar rats was achieved by s.c. injecting increasing doses of morphine-HCl twice daily for 5 days, or administrating morphine in the drinking water for 3 weeks. Binding parameters of μ opioid receptors were measured with the specific peptide agonist [^{3}H]DAMGO. Neither the K_D nor the B_{max} values changed in crude membrane (P2) and synaptic plasmamembrane (SPM) fractions after morphine tolerance developed. In contrast, the B_{max} of [^{3}H]DAMGO binding was significantly increased after chronic morphine injection in the microsomal (MI) fractions representing intracellular binding sites.

We also examined the μ opioid binding sites in subcellular fractions of rat brain by means of affinity labeling with the chloromethyl ketone derivative of DAMGO, [^{3}H]DAMCK followed by SDS-PAGE and autoradiography or gel slicing. Specific incorporation of the label was noted into the 50kDa binding subunit to a similar extent in control and morphine treated rat brain membranes.

Functionality of the μ opioid binding sites was tested by measuring Gpp(NH)p inhibition of [^{3}H]DAMGo binding, or ligand stimulated [^{35}S]GTPγS binding in P2, SPM and MI fractions. No significant alteration was observed in the coupling of cell surface μ opioid receptors to G-proteins in the treated brains over those in untreated rats. However in MI fractions the stimulation increased after morphine treatment.

The B_{max} for [^{3}H]DAMGO binding site increased by 80% in the spinal cord P2 membranes of Wistar male rats but decreased in that of female rats after chronic morphine injection. In contrast, μ opioid binding increased in both male and female rats when morphine was administered in drinking water.

These results show that multiple molecular mechanisms accompanied morphine tolerance in rat CNS which are influenced by several factors including the route of administration, sex, cellular and subcellular localisation.

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DETECTION OF SEROTONIN 5-HT3 RECEPTOR IN HUMAN BRAIN MICROVESSEL ENDOTHELIAL CELL CULTURE

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Human brain microvessel endothelium as the structural basis of the blood brain barrier (BBB) allows the ultra-stable milieu in the central nervous system. Many vasoactive compounds interfere with the barrier properties of this endothelial cell monolayer. It is well known that 5-hydroxytryptamine (5-HT; serotonin) influences the integrity of the BBB. The 5-HT_2 receptors have been characterized on the vascular endothelium. It is reasonable to suggest, that these receptors are responsible for serotonin evoked BBB opening via an increase in vesicular transport.

On the other hand 5-HT_3 receptors have been demonstrated in different region of the brain tissue! But not in the endothelium. These 5-HT_3 receptors are the only known subtype of serotonin receptors that belongs to the family of ligand gated ion channel proteins.

In the present study we detected the expression of mRNA of 5-HT_3 receptors in human brain microvessel endothelial cell culture (HBEC). HBEC were isolated and cultured from human brain capillaries with standard procedure as we published previously, and the cells were characterized by Dil-Ac-LDL uptake and by the visualization of von Willebrand factor. RT-PCR method was applied to detect the 5-HT_3 receptor mRNA from the primary cultures.

We demonstrated, that human brain microvessel endothelial cells are capable of expressing 5-HT_3 receptor mRNA. It could be hypothesized that, 5-HT_3 receptors influence the endothelial function.

STUDY OF K-OPIOID RECEPTOR EXPRESSION BY IN SITU HYBRIDIZATION

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The K-opioid receptors have an important role in the early development of the nervous system. The aim of the present study was to determine the K_2-opioid receptor expression patern in different model systems: a/ primary neuronal cell cultures (from the forebrains of seven-day-old chick embryos), b/ granule cell cultures (from the cerebella of seven-day-old rat, c/ human cortical sections (area A4, A10).

Radioligand binding studies with non-selective antagonist (naloxon) revealed, that the expression of K_2-opioid receptors increase during the timecourse of the cultivation until the seventh day then declines. We performed an immunocytochemistry on the cell cultures and cortical sections with mAb, KA8 which is specific for K_2-opioid receptor. In accordance with the radioligand binding, the density of the immunostaining increased until the seventh day of cultivation. In the granule cell cultures, type II astocytes displayed K_2-receptor immunoreactivity. The number of the labelled cells and the density of labelling increased until the fourth day of cultivaton. In the human prefrontal cortical sections (area 10) the pyramidal-like neurons in layers II/III and V displayed primarily immunostaining.

Comparing the distribution patern of the K_2-opioid receptor immunocytochemistry and K-opioid receptor mRNA we performed in situ hybridization study applying oligonucleotide probes (sense and antisense, -36 bp to + 12 bp), on the basis of the known K_1-opioid receptor nucleotid sequence. The probes were 3'-endlabeled with digoxigenin-11-ddUTP, the specific hybrides were detected with anti-digoxigenin-AP.

We found, that in the granule cell cultures the type II astocytes, expressed K_1-opioid receptor mRNA. The number of labeled cells and the density of labelling increased until the fourth day of cultivation in agreement with the result of the immunostaining. In the human prefrontal cortex (area 10) the pyramidal-like neurons in layers II/III and V expressed primarily K_1-opioid receptor mRNA. In addition multiform neurons in layer VI were also labeled. In the motor cortrex (area) the distribution of K_1-opioid receptor mRNA expression closely resembled that of A10. Furthermore, some neurons in deeper layer II and the Betz giant pyramidal neurons were marked. K_1- and K_2-opioid receptor protein are colocalised in the studied systems either/or the K_1-probe also hybridize with the of K_2-receptor mRNA.

NUMBER AND DISTRIBUTION OF GAD_{65} mRNA-CONTAINING NEURONS IN THE RAT HIPPOCAMPAL FORMATION

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The number and distribution of GABAergic inhibitory neurons was examined in the hippocampal formation using the presently available most reliable method, the GAD mRNA in situ hybridization. Adult male Sprague-Dawley rats were used. 30 mm thick coronal sections were cut and every tenth section was processed for GAD_{65} in situ hybridization as described previously (Esclapez et al., 1993). Similar sections from other animals were stained with Cresyl Violet. In addition, serial 10 mm thick coronal sections were cut and stained with Cresyl Violet for total cell counts of principal cells.

The number of GAD-positive cells was counted at 15 levels along the septo-temporal axis. The individual sections were drawn into a computer and the location of each individual cell of that section was plotted. The total number of neurons in the different layers of the different areas (strata oriens, radiatum and lacunosum-moleculare of the CA1-CA3 areas of Ammon's horn, hilus and molecular layers of the dentate gyrus) was estimated from Cresyl Violet stained sections at the same septo-temporal level.

The number of GAD-positive cells in the rat hippocampal formation is approximately 100.000, that is much higher than previously estimated. Approximately 1/3 of the inhibitory cells is found in the dentate gyrus and 2/3 of them locates in Ammon's horn. Our data and the the data found in the literature suggest that the number of granule cells is 1 million in the dentate gyrus, and the number of pyramidal cells is 400.000. The ratio between pyramidal and GABAergic cells is close to 7:1, which is comparable to the ratio found in neocortex (4:1). In contrast, the ratio between granule cells and GABAergic neurons is larger, being around 40:1. In the Ammon's horn, GABAergic cells are distributed almost equally in the different layers, except for the stratum lacunosum-moleculare which contains only a few cells. Examination of Nissl stained sections suggests that all neurons are GABAergic in the non-principal cell layers of Ammon's horn (strata oriens, radiatum, lucidum and lacunosum moleculare). In the dentate gyrus, the majority of GABAergic cells is located in hilus and at the hilar border of the granule cell layer. There are a few cells in the molecular layer, where, except for the non-aligned granule cells, all of the neurons appear to be GABAergic. In the hilar region of the dentate gyrus approximately 50% of the neurons showed GAD-positivity (range in individual sections varied between 45-65%), confirming our previous suggestion that half of the hilar neurons are GABAergic (Seress and Ribak, 1983).

AN INTRACELLULAR STUDY OF KAINIC ACID INDUCED HYPEREXCITABILITY OF CA3 PYRAMIDAL CELLS

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In slices of adult rat hippocampus made from control animals and rats exposed to 600-rad Xray irradiation, responses were studied to kainic acid (KA) application upon the CA3 subfield. Neonatal irradiation reduced the number of granule cells in the dentate gyrus significantly, as revealed by histological procedures. Slices were cut from 4-8 week old rats, and data were discarded if irradiation failed to destroy at least 60% of granule cells in the dentate gyrus of the slice studied with electrophysiological techniques. Conventional techniques were used to maintain the slices in a recording chamber and for intracellular recordings. In 24 slices, the impaled cells were labeled with biocytin for subsequent anatomical investigation. KA (300-500 nM final concentration) were diluted fresh in the oxygenated bath fluid and delivered using a 3-way tap system. Intracellular recordings was made in the cell body layers of CA3 and dentate areas. Over 80 impalement were accepted for detailed analysis of passive properties and synaptic potentials of the cells. CA3 cells had 40-80 MOhm input resistance and 18-20 ms membrane time constant. These data fell in the range of those found in non-irradiated rats. The action potentials were larger than 60 mV. All CA3 cells in the irradiated rats discharged to MF volleys, and many cells fired more than one spike to single MF volleys. IPSPs of short (about 50 msec) duration commonly followed the initial excitatory component. KA facilitated the excitatory components of evoked postsynaptic responses. Around 40% of pyramidal cells in irradiated rats fired recurrent bursts of 4-10 action potentials on top of a large depolarizing wave after KA application but before depression of evoked responses to presynaptic volleys. Kainic acid induced bursts were more often found in cells impaled in slices cut from the temporal than from the septal part of the hippocampus. Many other impaled cells reacted with an increase of 'spontaneous' PSP activity and some depolarization to kainic acid, but no bursts were detected in them. On the other hand, all healthy CA3 pyramidal cells respond with bursting to KA application in slices from non-irradiated rats. Thus in contrast with normal CA3 pyramidal cells (Ben-Ari and Gho, J. Physiol. 404, 1988) not all pyramidal cells in irradiated rats react to KA with bursting. On the other hand our data on some pyramidal cells which do react to KA with bursting is slices from irradiated rats contradict the conclusion of Gaiarsa et al (J. Neurophysiol. 71, 1994) that neonatal irradiation prevents the epileptic action of KA in rats. We propose that partial removal of MF inputs to CA3 pyramidal cells reduces but does not prevent KA induced hyperexcitability. Quantitative measures of granule cells lost or preserved MF terminals may explain the considerable differences found among cells and slices.

EFFECT OF LONG-TERM (-) DEPRENYL TREATMENT ON THE LIFE SPAN AND COPULATORY ACTIVITY IN FEMALE RATS

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Previously it was found that longevity treatment with (-) deprenyl extended the life span in sexually inactive male rats and increased their copulatory activity (Knoll, Dalló, Yen, 1983). Experiments were conducted whether this observation could be detected in female rats as well.

A group of females was made sexually inactive by ovariectomy without hormone replacement. They were treated with physiologic saline (N=9) and (-) deprenyl in a dose of 0,25 mg/kg sc. three time a week, (N=9) from six months of age till decay. It was found that each physiologic saline treated female rat decayed within fifteen months. At the same time the (-) deprenyl treated group was still alive. (-) Deprenyl treated females started to decay slowly, after seventeen months and the last (-) deprenyl treated females decayed 43 months after the treatment.

Sexual activity was quite absent in both groups. The dissociation of the life span and copulatory activity in female rats suggests the elementary role of the ovarian steroids in the observed effect.

EXPRESSION OF OPIOID RECEPTORS IN CEREBRAL ENDOTHELIAL CELLS

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Cerebral endothelial cells (CECs) regulate the entry of solutes, macromolecules and immune cells from the blood circulation to the brain tissue in the majority of vertebrates, providing a separate compartment for the central nervous system. Monolayers of endothelial cells derived from cerebral capillaries, proved to be excellent objects for studying the blood-brain barrier (BBB) *in vitro*. Opioid receptor binding was first demonstrated in isolated cerebral microvessels by Peroutka et al. 1980, using ^{3}[H]-naloxon and DALE. Later, μ-opioid receptor agonists were shown to increase the permeability of the BBB through central histaminergic and cholinergic systems (Baba et al., 1988).

Our studies, carried out on primary rat CEC cultures, were designed to investigate the presence of opioid receptors on the BBB. Dot-like perinuclear immuno-reactivity was visualized in the cytoplasm of cultured CECs with a monoclonal antibody KA8, raised in mice against lc-opioid receptor enriched frog brain membrane fractions, recognizing κ-, but not μ- and δ-opioid receptors (Maderspach et al., 1991). In addition, low affinity and low capacity binding sites were revealed by ^{3}[H]-naloxon-binding assay performed on intact cultures, cell

suspensions and cell homogenates. By using RT-PCR with primers specific for different opioid receptors we could demonstrate the expression of κ- and δ-subtypes.
The presence of these receptors may confer responsiveness to endogenous opiates on CECs, and may participate in the regulation of BBB permeability.

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HIGH FREQUENCY CORTICAL ACTIVITY IN DEEP URETHANE ANESTHESIA IN RATS

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Frequency and amplitude of cortical EEG waves have been considered as indicators of the level of thalamo-cortical activation since the classical works of Berger describing δ, θ, α and β patterns. Recently, a new high-frequency rhythmic activity around 40 Hz receives considerable attention. While there is no general agreement about the functional implications of this pattern, it is usually associated with high levels of attention or consciousness. One important exception seems to be its occurrence during REM sleep, but this sleep stage resembles highly activated wakefulness at the thalamo-cortical level. In agreement with the correlation between EEG patterns and thalamo-cortical activity, most drugs used to induce general anesthesia cause slowing of the EEG. At deep levels of anesthesia suitable for intensive surgical manipulations, a special EEG pattern not found in undrugged animals might appear. It consists of few-second long inactive periods followed by short epochs of EEG activity and therefore it is called burst-suppression.

In experiments aimed to analyze the correlation between basal forebrain (BFA) neuronal activity and cortical EEG patterns we observed that in deep urethane anesthesia, when EEG is characterized by burst-suppression pattern, fast waves close to 40 Hz can appear during the active periods of the EEG in parallel with an activation of the wake-active neurons in the BFA. In a follow-up, we examined the occurrence and characteristics of these waves in 8 rats chronically implanted with cortical and hippocampal electrodes for other purposes and in 10 acutely prepared rats. Using appropriate filter settings (0.16-100 Hz) and high sampling rate (200 Hz), it became clear that the active EEG epochs consisted of 500-800 millisecond long shifts which were negative at the level of the pyramidal cells. On the descending part, a sharp deep-positive wave appeared in most cases, followed by a burst of more or less rhythmical waves superimposed on the long deep-negative shift. The frequency of this waves seemed to depend on the abruptness of the descending part of the shifl. The shifts usually were largest on the frontal and smallest on the occipital cortex, but they were generalized and could be observed in the hippocampus as well. Due to their generalized occurrence, the shifts were best seen in transcortical recordings between the pial surface and the pyramidal layer, and all but disappeared in bipolar recordings, when only the superimposed waves could be noticed.

The presence of the reccurring high frequency epochs indicates intense thalamocortical activation. It raises the possibility that some analysis of sensory information might happen in the cortex during these short time gaps. If true, it may offer a possible explanation for at least some of the cases when awareness is experienced by patients undergoing surgery. In addition, similar burst-suppression can be induced by cortical anoxia, thus it is also possible that in certain circumstances this pattern appears near to death in the EEG. If this is the case, then

the ability of resuscitated patients to recall events happened when they were unconscious might be accounted for by the presence of the short, highly activated periods.

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TARGET CELLS OF CORTICOTROPIN-RELEASING HORMONE-IMMUNOREACTIVE NEURONS IN THE HUMAN HYPOTHALAMUS

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Stress-induced pattern of the pituitary hormone secretion, including elevation of adrenocorticotropic hormone (ACTH) and suppression of growth hormone and gonadotropin release, is predominantly regulated by the hypothalamic corticotropin-releasing hormone (CRH) in human and other mammalian species.

It has not been identified yet whether the suppressor effects of CRH are direct actions on other hypophyseotrophic neurons and/or involve interactions with other hypothalamic interneurons. In order to elucidate this issue, the existence of appositions of CRH-immunoreactive (IR) axon varicosities to growth hormone-releasing hormone (GHRH)- and gonadotropin-releasing hormone (GnRH)-containing neurons was tested in the human hypothalamus by means of consecutive, double-labeling immunohistochemistry at light-microscopic level. In another set of experiments, we analyzed other putative target structures in the tuberoinfundibular region. Of several candidates, galanin and substance P (SP) were immunolabeled after the detection of CRH-IR fibers.

Many CRH-IR neurons of middle size were located throughout the paraventricular nucleus, while few small-sized neurons were also found in the medial aspect of the infundibular nucleus. CRF-IR axons coursed laterally from the paraventricular nucleus, then turned ventrally in the lateral hypothalamus, and curved medially toward the external zone of the median eminence. In addition to these thick coarsing fibers, many thin CRH-IR axons with numerous varicosities were observed in the infundibular nucleus, which tended to outline some perikarya and their stem dendrites. Application of subsequent immunolabeling for GHRH, galanin and SP revealed that the majority of these peptidergic cell bodies within the tuberoinfundibular region were found to be in single or multiple contacts with CRH-IR varicosities. Even some of the perikarya immunoreactive for any one of these peptides were densely studied with CRH-IR axon swellings. GnRH-IR perikarya also appeared to be innervated by CRH-IR varicosities.

The present findings supply morphological basis for multiple roles of CRH in the induction of the stress-like pattern of pituitary hormone release. Our results indicate that hypothalamic CRH-IR neurons may exert direct effects upon:

(I) GHRH-containing neurons, beside the indirect inhibitory effect which is mediated by periventricular somatostatinergic neurons; (2) Galaninergic neurons, which may act in a stimulatory manner on the release of growth hormone and prolactin; (3) SP-containing neurons, thereby CRH may contribute to various inhibitory influences on the pituitary prolactin secretion.

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PURINE AND PYRIMIDINE NUCLEOSIDES AND DEOXYNUCLEOSIDES IN THALAMIC EXTRACELLULAR SPACE

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Adenosine as a neuromodulator is known to be involved in many CNS physiological and pathophysiological processes. There are, however, several lines of investigations suggesting the involvement of other nucleosides in brain function as well. To analyse synchronously nucleosides from in vivo brain microdialysis samples, a new chromatographic method was developed. This method can detect adenosine and other purines and also so far undetectable pyrimidines, pyrimidine nucleosides, purine and pyrimidine deoxynucleosides We report the extracellular thalamic concentrations of these compounds.

To be able to monitor the concentration changes after a particular treatment of the animal, the stability of control values were measured for the whole duration of an acute microdialysis experiment. These data indicate that the concentrations do not change significantly after the first hour of probe insertion.

To examine the origin of these compounds in the extracellular space, tetrodotoxin and high potassium concentration were applied in the perfusate. Interestingly, tetrodotoxin markedly decreased the concentration of uridine, hypoxanthine, xanthine, inosine and adenosine, whereas high potassium concentration increased the level of the same compounds without changing the level of other compounds. These results suggest that the presence of uridine, hypoxanthine, xanthine, inosine and adenosine in the extracellular space is neuronal activity dependent. Adenosine is a known neuromodulator and the other purines may be its metabolites. However, in view of our results and the recently cloned pyrimidinereceptors, one could hypothesize a regulatory role of uridine as well. Whether the release or uptake of these compounds are membrane potential or released neurotransmitter dependent remains to be answered. We also achieved post mortem measurements and a rise in concentration of many of these compounds was found suggesting intracellular accumulation of many of the examined compounds.

COMPARATIVE STUDY ON INHIBITION OF SEIZURE ACTIVITY IN IN VITRO EXPERIMENTS

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Spontaneous epileptic activity develops under different circumstances in thin slices prepared from the rat neocortex. The goal of our investigations was to compare the phenomena of two different in vitro epilepsy models and the effect of the specific AMPAtype excitatory amino

acid receptor antagonist (GYKI 53784). We provoked seizure activity applying either magnesium-free Ringer solution (MFR) or 4-aminopiridyne-containing Ringer solution (APR). NMDA-type excitatory amino acid receptors are oversensitive in MFR while 4-aminopiridyne, blocking K^+ ion channels, causes membrane depolarization. Both of them enhance excitatory processes, so the opportunity of development of epileptic activity increases. In electrophysiological experiments we measured the latency of appearance frequency and the length of spontaneous events in MFR and in APR (60 mM) and the effects of GYKI 53784 (10, 20, 40 mM) application on the parameters mentioned above. We also analysed the amplitude and the length of responses evoked by white-matter stimulation and the effectiveness of AMPA antagonist.

While latency of appearance of first seizure event was longer (15.6 vs. 5.8 min) and frequency of seizures-complexes was smaller (2.1 vs. 4.7 1/min) in MFR than in APR, each of the seizures were longer and more complex in MFR. The AMPA-antagonist inhibited more effectively the length of the spontaneous seizure events in MFR. We could not detect however, significant difference in the inhibitory effect of 20 mM GYKI 53784 on the evoked responses in the two models. In both cases the threshold of evoked response increased about 30 % and the amplitude decreased about 35 % as a consequence of drug-application.

ROLE OF DIFFERENT ADRENOCEPTOR SUBTYPES IN THE SYMPATHETIC CONTROL OF THE PROXIMAL URETHRA

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Urethral smooth muscle tone is maintained by its sympathetic innervation. The effects of the latter are mediated by different subtypes of postsynaptic adrenoceptors. In humans, a_1-adrenoceptors are responsible for noradrenalineinduced contractions of the urethra. In the present paper, the effects of a_1-adrenoceptor antagonists have been studied.

In cats anaesthetized with pentobarbital-Na (35 mg/kg, i.p.) or a mixture of a-chloralose (50 mg/kg) and urethane (400 mg/kg, i.p.), urethral constriction was elicited, 1) by i.v. infusion of phenylephrine (Phe), 2) by i.v. administration of adrenaline (A), 3) by administration of the ganglionic stimulant dimethyl-4-phenylpiperazinium (DMPP) and 4) by a somatosympathetic reflex upon supramaximal electrical stimulation of the sciatic nerve. Intraurethral pressure and blood pressure were measured and recorded by means of a Hellige or a Grass polygraph.

CH-38083, an antagonist of a_{2A-B}-adrenoceptors (50 mg/kg, i.v.) elicited urethral movements, increased blood pressure and potentiated the pressor reaction due to sciatic nerve stimulation. In CH-38083 treated animals, infusion of Phe (16 mg/kg/ min) interrupted the urethral movements, potentiated urethral effect of sciatic nerve stimulation and while it failed to contract the urethra, it induced systemic hypertension.

5-methyl-urapidil, an antagonist of a_{1A-C}-adrenoceptors (50-100 mg/kg, i.v.) antagonized the Phe-induced urethral contraction and elicited hypotension.

Among other a_1-antagonists, prazosin (100 mlg/kg, i.v.) inhibited both the Phe-induced urethral constriction and hypertension, while alfuzosin (50 mg/kg, i.v.) selectively inhibited the urethral effect of Phe.

In conclusion, 1) measurement of the urethral pressure is an appropriate indicator of one of the sympathetic outputs. 2) the a_2-adrenoceptor antagonist CH38083 enhances the urethral sympathetic tone; 3) an a_1-adrenoceptor antagonist that selectively antagonizes the constrictory effect of urethral sympathetic drive without affecting systemic blood pressure could be useful for the pharmacological treatment of the symptoms of benign prostatic hypertrohpy.

CHARACTERIZATION OF VIP-IMMUNOREACTIVE NEURONS IN THE ENTORHINAL CORTEX OF THE RAT

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While much is known about the extrinsic connectivity of the entorhinal cortex (EC), there is a paucity of information available about its intrinsic connectivity, especially with regard to the synaptic organization of its inhibitory circuits. Given that the EC is reciprocally connected to both the hippocampus and neocortex, and in light of emerging electrophysiological and computer modeling findings that suggest a central role for interneurons in the control and synchronization of principal cell networks and synaptic plasticity (Traub and Wong, 1982; Whittington et al., 1995), the analysis of the synaptic organization of interneuronal circuits within the EC is particularly crucial in understanding how the hippocampus communicates with cortex.

One class of GABAergic interneuron in the hippocampus and neocortex colocalizes the neuropeptide vasoactive intestinal polypeptide (VIP). VIP-containing cells and fibers were previously shown to be distributed throughout the EC, with a preferential bias toward superficial entorhinal layers (Köhler and Chan-Palay, 1984). VIP-ir cells, therefore, can potentially regulate the activity of principal neurons in layers II and III which originate the primary cortical input to the dentate gyrus and hippocampus (Steward and Scoville, 1976). Nothing is known, however, about the synaptic organization of VIP-ir neuronal circuits in the EC.

In the present study, we used a sensitive antibody against VIP (Gulyás et al., 1990) to determine the detailed dendritic and axonal morphology, and synaptic targets of VIP-ir interneurons in the rat EC, with the aim of clarifying one class of inhibitory circuit in the EC. Sections through the EC from 5 male rats were either immunostained for VIP or double-immunostained for VIP and the neuropeptide somatostatin or one of three calcium binding proteins (parvalbumin, calbindin, calretinin). The GABAergic nature of the postsynaptic targets of VIP-ir axon terminals was determined using the postembedding immunogold staining for GABA according to the procedure of Somogyi and Hodgson (1985). VIP-ir boutons that synapsed onto GABA(-) and GABA(+) profiles were identified at the electron microscopic level, mapped and counted using a computer-aided digitizing system *(EM Wander...)*, Dr. A. I. Gulyás) linked to a Hitachi H-7100 electron microscope. Candidate VIP-PV, VIP-CB, VIP-CR, and VIP-SOM targets were identified at the light microscopic level and then re-embedded in Durcupan for correlated electron microscopic analyses.

While all EC layers received VIP-positive input, fibers and terminals distributed mainly to layer II. VIP-ir terminals frequently encircled unstained somata in layer II. VIP-ir terminals contacting the soma and proximal dendrites of other VIP-ir cells were common. Double-immunostaining revealed that VIP-ir interneurons innervate the soma or proximal dendrites of somatostatin-, parvalbumin-, and calretinin-positive cells. Although VIP-ir terminals rarely contacted calbindin-positive interneurons, numerous VIP-ir terminals were apposed to calbindin-positive neurons displaying the morphology of principal cells. Postembedding immunostaining for GABA demonstrated that postsynaptic targets of VIP-ir boutons included somata and dendrites of GABA-positive and -negative cells. In layer II, VIP-ir boutons tended to target GABA-positive somata and proximal dendrites; whereas in layer III, VIP-ir boutons tended to contact GABA-positive and -negative small caliber dendritic profiles. The laminar organization and target specificity of VIP-ir fibers and terminals allow the possibility for modulation of principal cells, as well as other interneurons that may participate in the regulation of entorhinal intrinsic circuits and EC output to the hippocampus and other structures.

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INTRODUCTION OF AMINOPYRIDINE-INDUCED CHRONIC SEIZURE MODEL TO EXPERIMENTAL EPILEPSY

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Focal seizure induced by 3-aminopyridine (3Ap) has been proven to be a useful acute experimental model because of the characteristic spontaneous electrocorticographic development of epileptiform paroxysms which resemble human epilepsy. It is a useful tool to study the manifestation and spreading of neocortical epileptiform activity as well as to

analyze abnormal intracellular events accompanying ictal discharges, both in the primary and in mirror foci of anesthetized cats or rats.

In order to analyze the effects of repeated epileptic activity and long-term treatments with drugs (like antiepileptics) on the electrographic correlates on adult rats and the intrauterin development of the nervous system we introduce the chronic 3Ap model of epilepsy. Adult rats were injected intramuscular by 3Ap solution at a dose above seizure threshold on every third day. Injections induced the development of seizure, generally evolving through the following 6 behavioral classes:

1. restless, trembling, stereotypic sniffing,
2. head nodding, face washing movements,
3. alarm reactions, trembling of the whole body,
4. forelimb contraction, rearing,
5. jumping
6. rearing with loss of balance and falling, hind limb expansion, unconsciousness.

In our present experiments female rats with chronic epileptic activity are treated with antiepileptic drugs (valproat and carbamazepin) through the drinking water during their pregnancy and the sucking period. After birth the induced mothers and the developing rats will be studied by electroProf. physiological, biochemical and immunohistochemical methods.

CATECHOLAMINERG INNERVATION OF THE LUTEINIZING HORMONE-RELEASING HORMONE-PRODUCING NEURONS IN THE HUMAN DIENCEPHALON

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Catecholamines have been proven to modulate gonadal functions via an interaction with the hypothalamic luteinizing hormone-releasing hormone-synthesizing (LHRH) neurons in rodents. LHRH release appears to be inhibited by catecholamines in the human being. In order to reveal the morphological background of this phenomenon, the distribution of LHRH neurons and tyrosine hydroxylase (TH)-immunoreactive (IR), catecholaminergic structures was mapped in diencephalic sections of four, post-mortem human brains.

First, the location of the LHRH and TH-IR neuronal elements was analyzed, then the overlapping and the interrelationship of the two different systems were examined. The LHRH-IR cell bodies were mainly present in the medial preoptic, infundibular areas and in the median eminence. The TH-IR perikarya were located in the infundibular, periventricular, paraventricular and supraoptic nuclei, while the TH-IR fibers were numerous in septal, infundibular, periventricular and lateral hypothalamic regions. The brown, diaminobenzidine labeled LHRH-containing perikarya received black, silver intensified, TH positive axon terminals in the infundibular area, while in the preoptic and caudal parts of the diencephalon only some juxtapositions were noted. Thirty-four percent of the examined LHRH-IR cells did not receive detectable catecholaminergic innervation, fifty-four percent was lightly, and twelve percent heavily innervated by TH-IR axon terminals. The TH-IR neural elements targeted mainly bipolar LHRH-IR neurons.

The present results indicate that the hormone release of diencephalic LHRH-IR neurons can be influenced by the central catecholaminerg system via direct synaptic mechanisms. The

data also support the concept that the LHRH-IR neural elements receive different percentages of catecholaminergic innervation depending on their locations.

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COMPARISON OF NEUROPROTECTIVE DRUGS IN VARIOUS MODELS OF BRAIN INJURY

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Main molecular mechanisms of neuronal cell death involve oxidative stress, excitotoxicity, disturbance in energy metabolism and intracellular ion homeostasis. The present study is an attempt to compare the effectivity of MK-801 (NMDA antagonist), GYKI-52466 (AMPA antagonist), a-tocopherol (antioxidant), vinpocetine (sodium channel blocker) and bimoclomol /BRLP-42/ (HSP-70 co-inducer) in cytotoxic hypoxia (prolongation of survival time), global cerebral ischemia (inhibition of cell death) and arachidonic acid (AA) induced vasogenic brain edema (inhibition of extravasation) models. Cytotoxic hypoxia was induced by intravenous potassium cyanide injection (10 mg/kg) in mice. Test compounds were applied *orally* 30 minutes prior to anoxia. GYKI-52466 (40 mg/kg), vinpocetine (50 mg/kg) and BRLP-42 (100 mg/kg) showed statistically significant antihypoxic effect (137%, 137% and 132% prolongation of the survival time, respectively, compared to the control, 100%), while a-tocopherol (100 mg/kg) and MK-801 (1 mg/kg) were ineffective. Antiischemic activity of four molecules was further investigated using a gerbil model of transient global cerebral ischemia. Drugs were given *intraperitoneally* 30 minutes after the termination of five minutes ischemia and the number of living pyramidal cells in hippocampus CA1 area was evaluated four days later after a Nissl staining. Vinpocetine (25 mg/kg), MK801 (3 mg/kg) and GYKI-52466 (25 mg/kg) showed similar protection against the cell damage (60-65%), while BRLP-42 (50 mg/kg) caused weaker effect (20%). In the rat brain edema test drugs were administered *intravenously* 15 minutes prior to the induction of edema by intracerebral injection of M (10 mg/rat). The effect of drugs on edema formation was characterized by Evan's blue staining of the brain slices, and evaluated by a computerised scanning method. Vinpocetine (5 mg/kg, 55.1%), GYKI-52466 (5 mg/kg, 34.2%) and a-tocopherol (20 mg/kg, 23.8%) potently inhibited the extravasation, while BRLP-42 (5 mg/kg) and MK-801 (0.3 mg/kg) were practically inactive in this system in the doses applied.

Major findings of our study are as follow: (1) Vinpocetine and GYKI-52466 proved to be effective in all the three models employed. (2) The characteristic effect of GYKI-52466 in cytotoxic hypoxia test suggest a role for AMPA receptors in the pathology of this histotoxic process. (3) Prominent activity of vinpocetine in brain edema test may result from its cerebrovascular selectivity and/or antiosmotic (sodium channel blocker) property. (4) Finally, the well-defined acute effect of BRLP-42 in the cyanide anoxia test is in line with the previously recognized protective effect of the drug against cardiac ischemia/reperfusion.

SPECIFIC ALTERATIONS IN THE AMOUNT OF HETEROTRIMERIC G-PROTEIN SUBTYPES IN MORPHINE TOLERANT RAT BRAIN MEMBRANES

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Our previous studies showed alterations of G-protein levels in subcellular fractions of rat brains after chronic morphine treatment. Rats were rendered tolerant to morphine by s.c. injections of the drug twice daily for 5 days. GTP-γ-S binding, photoaffinity and pertussis toxin labelling, as well as Westem-blotting experiments supported the observation that chronic morphine treatment increased the level of α-subunits of G-proteins by 63% resp. 30% in synaptic plasma membrane (SPM) and microsomal (MI) fraction, calculated from GTP-γ-S binding studies (1). Recent data in the literature showed that a single type of opioid receptors can couple to several G-proteins (2); and μ and δ opioid receptors interact with different sets of G-protein subtypes (3). Based on these findings it is particularly interesting to determine which G-protein subtypes are affected by repeated morphine exposure in rat brain. Western-blotting performed with specific antisera revealed significant increase in the amount of α_{s1} α_{i1}, α_{o1} in MI; increased a_s and decreased α_{o2} labeling in SPM upon chronic morphine. The level of $Gq_{/11\alpha}$, a G-protein subtype previously not shown to be coupled to brain opiate receptors, was elevated in the former, and decreased in the latter fractions.

These results demonstrate that specific changes of G-protein subtypes accompany the development of morphine tolerance.

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COMPLEMENT C5a RECEPTOR-MEDIATED APOPTOSIS IN NEUROBLASTOMA CELLS

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Activation of the complement system (C') was reported in Alzheimer's disease (AD); however, its exact role in the pathogenesis of the disease has not yet been elucidated. One of the products of C' activation is the anaphylatoxin C5a. In order to analyze its role, TGW human neuroblastoma cells were exposed to an oligomer of a C5a fragment peptide terrned PL37-MAP (amino acids: 37-53) and the effects were evaluated by electrophysiological and morphological techniques. Extracellular administration of 100 nM peptide evoked repetitive inward current pulses with an amplitude of 200-300 pA, as demonstrated by patch clamp measurements (holding potential: -70 mV. administration time: 2 minutes). The pulses were blocked by 0.2 mM cobalt-chloride revealing that the current was a calcium influx. At higher doses (0.5 nM - 1 μM), the CSa fragment peptide evoked current pulses of 600-1000 pA, followed by an irreversible, high, inward current (> 2000 pA). Calcium influx can be influenced by C5a and pertussis toxin pretreatment of the cells, respectively, suggesting that the apoptosis is associated with the CSa receptor (C5aR). DNA fragmentation, demonstrated by the TUNEL method, was accompanied by an increased c-FOS expression. The first morphological signs of apoptosis were apparent 30 minutes after the beginning of the administration. A more advanced DNA fragmentation and cell degeneration were noted after 4 hours. Patch clamp and morphological experiments were also performed in Ltk- fibroblast cells either expressing or not expressing C5a receptor. PL37-MAP induced apoptosis in Ltk- cells expressing C5aR, only, demonstrating that the apoptosis is associated with CSaR. In AD, the presence of Kunitz-type proteinase inhibitors and plaque formation indicate an impaired protease function and the possibility of an abnormal fragmentation of C5a anaphylatoxin. The present findings suggest that at least one of the C5a fragments might be involved in the genesis of AD by inducing apoptotic cell death.

A COMPUTER MODEL OF THE CORTICAL NETWORK

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A multilayered network consisting of 140 neurons was constructed, hundred of them being excitatory and forty inhibitory. The model is aimed to simulate the function of the primary

and secondary sensory areas of the mammalian cortex. Characteristic features of the model are:

1. five layers of which 1, 2, 3 represent the primary, 4 and 5 the secondary sensory areas.
2. units of the network are not neurons but synapses creating divergences and convergences specific for the given sensory modality
3. synapses are most part of Hebb type.

Without altering the basic circuitry the network is able to analyze, classify and abstract the incoming signals specifically to the requirements of the visual resp. auditory function. Conditioning, learning, and related phenomena could be simulated.

MODULATION OF THE CENTRAL HISTAMINERGIC SYSTEM BY ESTROGEN

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The hypothalamo-hypophyseal-ovarian system is regulated by feedback actions of estrogens and several diencephalic transmitter substances, including histamine. Because luteinizing hormone-releasing hormone (LHRH)-synthesising neurons do not possess a substantial amount of estrogen receptor, the elucidation of the estrogen feedback sites to other neuronal systems has been crucial. The putative estrogen reception of histaminergic neurons was analyzed in the present study by means of combined transmitter and receptor immunocytochemistry. Histamine and estrogen receptor (ER)-synthesising neurons were mapped in the hypothalami of ovariectomized, female rats.

Multipolar histamine-immunoreactive neurons were found exclusively in the five subgroups (E1-E5) of the tuberomammillary nucleus. A high density of the estrogen receptorimmunoreactive neurons was observed in the arcuate, ventromedial, dorsomedial nuclei and all subgroups of the tuberomammillary nucleus. The immunocytochemical double labelling indicated that the majority of histamine-immunoreactive neurons (over 75%) also contained ER-immunoreactivity in their cell nuclei. The ratio of double-labelled neurons was found between 65% and 85% in all subgroups.

Because a population of histamine-containing neurons projects into the septum and the preoptic region, and histamine modulates the secretion of the luteinizing hormone-releasing hormone-producing cells, the presented morphological evidence suggests that estrogen effect might be mediated to LHRH neurons through the histaminerg system.

This work was supported by OTKA grant T016354.

ISCHEMIC PRECONDITIONING PREVENTS REPERFUSION INDUCED INCREASED S-100 IMMUNOREACTIVITY OF ENTEROGLIAL CELLS IN RAT JEJUNUM

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While intestinal mucosal injury due to ischemia and reperfusion (IR) is well established, the effect of IR on enteroglial cells has not been investigated. Recently we demonstrated that ischemic preconditioning (IP) prevents ischemic reperfusion induced jejunal injury in rats. The objective of this study was to determine the effect of IR and IP (1) on immunohistochemical characteristics of jejunal enteroglial cells and (2) on the morphology of enteric neurons.

Methods: Studies were performed on anesthetized rats. Ischemia was induced by occluding the superior mesenteric artery. Fifty rats were examined from each group. In the first group the small intestine was subjected to 20 min of ischemia followed by 30 min of reperfusion (IR). In a second group of animals (IP), the same protocol was used except that the intestine was subjected to 5 min ischemia followed by 10 min period of reperfusion prior to induction of IR. Sham operated animals served as controls. After the reperfusion period, samples of jejunum were processed for histological examination. The distribution of enteroglial cells in jejunum was analysed by immunohistochemistry, using an antibody against S-100 protein (Dakopatts Z 311) described by Jensses K.R. et al. (J. Neurosci. 1983). The fine structural features of enteric neurons in different groups were studied by transmission electron microscopy.

Results: In controls, in the myenteric plexus (MP) only low level of S-100 immunoreactivity was found in the myenteric ganglia and internodal strands. In the submucous plexus (SP) moderate immunoreactivity was detected in both regions. In the IR specimens there was a profound increase in immunoreactivity in the MP in particular in the interganglionic fiber tracts. There was no apparent change in immunoreactivity of the SP. Interestingly, however, ganglionic S-100 immunoreactivity was slightly increased in the MP and in the SP after IP. In contrast, IP prevents IR induced increased immunoreactivity in the interganglionic fiber tracts in the MP. Electron microscopic studies revealed a well preserved neuronal ultrastructure both after IR and IP, although a pronounced swelling of glial processes and endothelial cells has been noticed in IR samples.

Conclusion: These results suggest that (a) enteroglial cells are significant target for the ischemic reperfusion insult; (b) this response of enteroglial cells by IR may have implications in enteric neuronal survival that occur after IR; (c) inhibition of IR induced response of enteroglial cells may play a role in IP induced neuronal protection.

BEHAVIORAL EFFECTS OF PALLIDAL BOMBESIN MICROINJECTIONS IN THE RAT

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It has been suggested that BN and BN-like peptides represent neurochemical signals evoking preabsorptive satiety. Experimental data show that intraperitoneal or intracerebroventricular (i.c.v.) applications of bombesin (BN) induce short latency transient inhibition of food intake in food deprived rats. On the other hand, i.c.v. or hypothalamic BN injections increase locomotor activity and grooming behavior and decrease sleeping and the resting periods. Furthermore, i.c.v. application of BN may cause hypothermia.

It has been shown that the globus pallidus (GP) is basically involved in the regulation of feeding and body weight. Bilateral electrolytic or neurochemical lesions of the GP cause feeding disturbances and deficits in food rewarded learning paradigms. Since this structure is rich in BN-like immunoreactive elements, one may suppose that BN microinjected into the GP can also modify feeding behavior.

In the present experiments BN was bilaterally injected into the GP of 24 h food deprived CFY male rats and feeding, body temperature and the general behavior of the animals were examined. Via stereotaxically implanted chronic guide cannulae 10 or 40 ηg BN in 1 μl were applied and solid food intake was measured in 20 min intervals. Animals were videomonitored and the time spent with feeding, drinking, resting and exploration was analysed. Stereotype behaviors in open field experiments were also studied. BN microinjections into the GP resulted in dose-dependent inhibition of feeding. After 40 μg BN administration food intake was significantly reduced during the first 40 min and it was followed by a transient increase. These effects on food intake were prevented by prior application of a BN antagonist. Videorecord analyses showed similar behavioral patterns throughout the observational period (160 min in 20 min intervals) after either BN or vehicle administrations. The deprived animals ate first, eating was followed by postingestion drinking and they spent more and more time with resting. After BN treatments eating periods decreased somewhat with the appearance of a simultaneous increase of groomings during the first 40 min, but these values were not significant when compared to those seen after vehicle injections. BN did not induce significant alterations in locomotor activity or stereotypies when rats were examined in an open field paradigm. Furthermore, no significant differences were observed in body temperature after BN or vechicle injections. Our results show that the inhibitory effect of BN on feeding can not be explained by an increase in locomotor activity, alterations in stereotype behavior or hypothermia. Instead, the BN may act in the GP as a specific satiety signal.

THE POSSIBLE ROLE OF PROTEIN PHOSPHATASE 2A IN THE SODIUM SENSITIVITY OF THE RECEPTOR BINDING OF OPIATE ANTAGONISTS NALOXONE AND NALTRINDOLE

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In the striatal membrane preparation used for receptor binding experiments considerable amount of protein phosphatase 1 and 2A activity was assayed using [^{32}P]phosphorylase as substrate. Sodium chloride in a concentration dependent manner decreased the activity of protein phosphatase 2A and increased the activity of protein phosphatase 1. Sodium chloride increased the saturation binding of naloxone and naltrindole in rat striatal membranes preincubated with ATP (50 mM) and $MgCl_2$ (5 mM). Addition of okadaic acid in a concentration of 2 nM, which is specific for the inhibition of protein phosphatase 2A, further augmented the number of binding sites of naloxone or naltrindole. The results suggest a protein phosphatase 2A dependent regulation of the binding of opiate ligands in the striatum.

LONG-TERM BEHAVIORAL AND NEUROCHEMICAL EFFECTS OF ETHANOL EXPOSURE DURING AGING

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Both neurotoxic episodes and subsequent neurotrophic drug treatments may exert short- and long-term consequences in neural functions, which may have a particular relevance to brain aging. In the present experiments the neurotoxic effects of ethanol treatment and the neurotrophic action of calcium channel blocker nimodipine were studied in aging rats under long-term experimental conditions. In the first stage of experiments ethanol was given at the beginning of aging (19 months) for 5 weeks and the behavioral effects of this treatment were tested in a spatial learning task and in a social behavioral paradigm. The ethanol-exposed rats showed inferior performances to controls in both tasks.

In the second part of experiments the alcohol treatment period was followed by a 5 week supply of food containing nimodipine (1000 ppm), and a marked recovery of functions could be observed in both behavioral tasks shortly after termination of nimodipine treatment.

In the last phase of experiments, both the effects of chronic ethanol and nimodipine treatments were further tested in the senescence age of 28 months, i.e. 6 months after termination of the treatments. At this age black-white discrimination learning and social behaviors were investigated and the following results were found: 1) discrimination learning deficit was present in the ethanol-treated rats but it continued to be antagonized by the subsequent nimodipine treatment, 2) the nimodipine-treated senescent animals displayed a more vivid social behavior and a better discrimination learning performance even against the control aged rats, indicating a slower involution in aging-related behaviors. At the conclusion of experiments some biochemical markers of serotonergic and cholinergic neurotransmissions were assayed in the hippocampus and neocortex: a) number of 5-HT_{1A} receptors (^{3}H-8-oHDPAT binding), b) choline-acetyltransferase enzyme activity (ChAT) and concentration of muscarine receptors (H-QNB binding). It was found that the 5-HT_{1A} receptor binding decreased in the ethanol-treated senescent rats in both brain structures investigated. This change in the 5-HT_{1A} receptor binding was prevented by the nimodipine treatment.

The results allow to conclude the following: 1) ethanol treatment at the beginning of aging exerts long-term dysfunction in behavioral and some neurochemical parameters, which can be reversed by chronic nimodipine treatment 2) furthermore, the chronic treatment with nimodipine improves the adaptive and social behaviors with long-lasting efficacy in rats of very old age.

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STRESS-INDUCED Fos-IMMUNOREACTIVITY IN THE PITUITARY

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Immediate-early gene c-fos and its protein product c-Fos, are widely used functional markers of activated neurons. Former studies from our laboratory revealed stress-related neuronal circuits by mapping c-Fos immunoreactive neurons within the central nervous system. To further characterize the activation of hypothalamo-pituitary-adrenal axis during acute stress, c-Fos expression in the pituitary has been studied in rats exposed to ether vapour. Animals were sacrificed at intervals ranging between 5 min and 4 hour after acute ether stress. Immunoreactive cell nuclei were revealed by immunoperoxidase reaction on 30 μm sections from the pituitaries. Under resting conditions we could not detect any specific staining for Fos. First Fos-positive cells were detected as early as 30 minutes after stress, and the maximal expression was found at 1 hour post-stress. Ether stress-induced Fos immunoreactivity was confined to cells in the anterior lobe, weak immunoreactivity was detected in the intermediate lobe but it was absent in the neural lobe. The peak of Fos protein was almost 1 hour earlier in the pituitary than in the hypothalamic paraventricular nucleus, and followed the maximum of ACTH and corticosterone secretion. Further co-localization studies are required for identification of stress-activated cell types within the pituitary.

LIGHT AND ELECTRON MICROSCOPIC MAPPING AND CONNECTIONS OF OPIOID PEPTIDE-CONTAINING NERVE ELEMENTS IN THE RODENT HIPPOCAMPUS

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The endogenous opioids exert an inhibitory effect on the principal cells of the central nervous system with the exception of the hippocampus, where an increase in the excitability was physiologically detected. This effect is supposed to be mediated indirectly, via the inhibition of GABAergic interneurons. The morphological basis of this mechanism has not been clarified yet. The aim of this study was to identify the opioidergic elements of the rodent hippocampus, to establish their synaptic target distribution and the proportion of possibly inhibitory postsynaptic targets. Combined light and electron microscopic immunocytochemistry was applied to identify leucine-enkephalin (leu-enk) and methionine-enkephalin (met-enk) in the hippocampus of rat, mouse, guinea-pig and golden hamster.

The enkephalins occurred in the hippocampus of each rodent species, in the mossy fibre system. In addition another varicose fibre system, morphologically different from the mossy fibres also showed enkephalin immunoreactivity. These nerves were present in the strata radiatum and lacunosum-moleculare of the CA1 region and at the border of CA2/CA3 region in the termination zone of the mossy fibre system. The temporal part of the CA1 region in the rat contained the most dense fibre system with enk-immunopositivity.

The enkephalinergic synaptic boutons always contained one or more strongly immunopositive dense-core vesicles, small agranular vesicles and mitochondria. Their synapses were, at least partly, type 2, symmetrical synapses. Both dendritic shafts and neuronal somata occurred among their postsynaptic targets. The morphological appearance and location of these postsynaptic elements at the border zone of strata radiatum and lacunosum-moleculare strongly support that these belong to non-principal neurons.

Our morphological results support previous physiological data concerning the disinhibitory mode of action of opioids in the hippocampus.

THE ROLE OF HYPOTHERMIA IN THE NEUROPROTECTIVE ACTIONS OF AMPA ANTAGONIST COMPOUNDS

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The neuroprotective actions of two AMPA antagonist molecules, NBQX (2,33-dihydroxy-6-nitro-7-sulfamoyl-benzol(F)qinoxaline) and GYKI-52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine) were investigated in the gerbil forebrain ischaemia model. Bilateral occlusion of the common carotid arteries of these animals produces delayed

neuronal cell death in the hippocampal CA1 region. NBQX has been shown to reduce the neuronal cell loss (1). However, the hypothermic effect of NBQX is well known and it has recently been demonstrated that postischemic hypothermia exerts protective effect on hippocampal cell loss (2). These findings raised the possibility that the neuroprotective action of AMPA antagonists may be due to their hypothermic activities.

The aim of our study was to investigate the possible correlation between hypothermia and neuroprotective effects of NBQX and GYKI-52466.

Male Mongolian gerbils (60-70 g) were subjected to global ischemia via blatetal common carotid artery occlusion for 3 minutes under ether anesthesia. The rectal temperatures of gerbils were recorded by an electric thermometer. The compounds were adrninistered intraperitoneally: GYKI-52466 4x15 mg/kg was injected 30, 45, 60 and 70 min, whereas NBQX 3x30 mg/kg was given 60, 70 and 85 min after reperfusion. The temperatures were measured before and several times after drug treatment up to 24 hours. Four days after the surgery, animals were deeply anesthetized with Nembutal (60 mg/kg i.p.) and perfused through the heart by a fixative solution. Alternate sections from the hippocampus were stained by silver impregnation. Neuronal damage in the hippocampal CA1 region was estimated with the use of a rating scale from 0 to 6.

GYKI-52466 showed significant inhibition of cell death (48.9%) in carotid occluded gerbils and markedly reduced the rectal temperatures of animals (-6.5 °C maximal effect, 8 hours of duration). NBQX exerted as potent neuroprotection (47.9%) as GYKI-52466, although its hypothermic action was lower (-2.0 °C maximal effect) with a similar duration of action.

The two AMPA antagonists showed similar neuroprotetie effect. However, the hypothermia induced by GYKI-52466 (AUC=29.6) was about 3 times higher than that evoked by NBQX (AUC=9.2) up to 8 hours after treatment. These findings indicate the lack of a direct correlation between neuroprotective and hypothermic actions of NBQX and GYKI-52466.

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EFFECTS OF MERCURY, CADMIUM AND TIN ON ACETYLCHOLINE-ACTIVATED CURRENTS IN *LYMNAEA STAGNALIS L.* NEURONES

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Using conventional two microelectrode voltage clamp and concentration clamp techniques we studied the effects of $CdCl_2$, $HgCl_2$ and $SnCl_2$ on acetylcholine (ACh), dimethyl-phenyl-piperazine (DMPP) and carbachol (CCh) activated currents on an identified neuron of the mollusc, *Lymnaea stagnalis L.* The agonists were applied with microperfusion or with exchange of the bath. Experiments were carried out on the RPeD1 neuron either in whole mount preparation or after isolation.

In the studied neuron at micromolar concentration Cd^{2+} as well as Hg^{2+} ions caused a decrease in the ACh and CCh induced Cl^- permeability of the membrane. DMPP evoked currents were also reduced by mercury, but were enhanced by low cadmium. The effects were essentially irreversible.

$SnCl_2$ caused a decrease in the acetylcholine induced current. The effect depended on the concentration and the application time of the $SnCl_2$, as well as on the membrane potential. The effective threshold concentration was 0.1 μM, and saturated at 5 μM $SnCl_2$. More expressed inhibitory effect was detected at the more negative holding potentials.

The recent observations support our earlier findings and the idea that agonist-activated channels are targets of toxic metals like cadmium, mercury and tin ions occurring in the environment. It can be considered, that the direct action at the neuronal membrane is an important event in modulation of the synaptic transmission and this should be taken in consideration to the understanding of the mechanism of metal toxicity.

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THE ROLE OF THE 'FIRST PASS' METABOLISM IN THE EFFECT OF (-) DEPRENYL

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Deprenyl (Selegiline) is a selective and irreversible inhibitor of monoamine oxidase type B (MAO B), which has been used in the treatment of Parkinsonism. Selegiline is readily absorbed from the gastrointestinal tract. It is distributed rapidly into the tissues because of its liphopfilic, slightly basic features. Deprenyl is metabolised to desmethylselegiline and methamphetamine, which can be further converted to amphetamine and corresponding p-hydroxylated metabolites. These parahydroxy derivatives are pharmacologically inactive after being conjugated with glucuronic acid. Metabolism takes place mainly in the liver and is dependent on the microsomal cytochrome P-450.

The aim of the present investigation was to compare the inhibitory potency of (-) deprenyl on MAO-B activity in male (150 g) Wistar rat brain and liver in the case of oral or subcutaneous administration of Selegiline (0.1 mg/kg; 0.25mg/kg; 0.5mg/kg). We also investigated the augmentation of inhibitory effect of its in consequence of pre-treatment with SKF 525-A (50 mg/kg IP), a well-known inhibitor of cytochrome P-450 mono-oxygenases. The mitochondrial MAO-B activity was assayed by a radiometric method (Wurtman and Axelrod) with slight modifications, using ~4C-phenylethylamine as substrate. Protein concentration was measured according to the method of Lowry et al.

The inhibitory effect of (-) deprenyl on monoamine oxidase B activity of liver and brain were significantly increased by pre-treatment of rats with SKF 525-A. The different administrations of Selegiline have also caused significant variance in the action of (-) deprenyl.

INVESTIGATIONS ON THE SYNAPTIC ORGANIZATION OF THE ANTERIOR THALAMIC NUCLEI WITH COMBINED TRACING AND IMMUNOHISTOCHEMICAL METHODS IN THE CAT

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In our previous studies we have analysed the structure of the anterior thalamic nuclei with neurodegeneration technique in the cat. Two main types of neurons were identified: a large thalamo-cortical relay neuron and a small interneuron. Three types of axon terminals (RL, RS and F_1) and presynaptic dendrites (F_2) were also differentiated. The characteristic thalamic complex synaptic arrangements i.e. *synaptic glomeruli* were observed, too. Results of these experiments suggested that the RL boutons are terminals of multiple origin.

To identify and characterize one possible source of these profiles Phaseolus-vulgaris-leucoagglutinin (PHA-L) was injected into the medial nucleus of the mamillary body. After two weeks of survival the immunostained fibers and terminals were identified in the ipsilateral anterior ventral (AV) and anterior medial (AM) thalamic nuclei. Postembedding GABA immunocytochemistry was carried out on the same sections and compared to the anterograde tracer labeling at light and electron microscopical levels. GABA-positive boutons were found in the synaptic glomeruli in synaptic contact with both the dendrites of relay neurons and with the presynaptic dendrites (F_2). The terminals of the mamillothalamic fibers were observed in synaptic connections with both GABA-positive and GABA-negative dendrites.

Our newly obtained results confirmed that at least a portion of the afferents to the AV and AM thalamic nuclei come from the mamillary body. The mamillothalamic fibers are GABA-negative, supporting the assumption that they are of excitatory function.

ATONIA RELATED REGIONS IN THE RODENT PONS AND MEDULLA

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Previous studies in the decerebrate cat have demonstrated the existence of atonia related areas in the pontine and medullary reticular formation. Stimulation of the medial medullary reticular formahon (MMRF), the ventral paralemniscal tegmental field (vFTP), the reticular tegmental (TRN) and pedunculopontine tegmental (PPN) nudei elicit muscle tone suppression which is intensity and frequency dependent. In this study, we have investigated this phenomena and its chemical basis in the decerebrate rat using electrical stimulation and microinjection techniques.

Experiments were performed on 42 male rats (170 to 510 grams). Decerebration was done at the precollicular-postmammillary level under halothane anesthesia. Multi-stranded stainless

steel bipolar electrodes were implanted in the hindlimb, forelimb and neck muscles for electromyographic (EMG) recordings. Bipolar stimulating electrodes were placed into the pons (rostrocaudally at AO.7 to PO.3, and 0.2 to 1.5 mm from the midline) and the medulla (from P2.0 to P3.3, and within 0.6 mm from the midline). Stimulation consisted of 500 msec trains of 0.2 ms cathodal rectangular pulses at 10-500 Hz and 5-500 mA. In the microinjection studies, when the electrical stimulation produced bilateral suppression of muscle tone the stimulating electrode was replaced with 26-gauge Hamilton microsyringe. Small amount (0.1 µl) of the agonist solution: kainate or NMDA was injected over a period of 30 sec. In studies in which the action of antagonist was examined antagonist injections ware performed 30 minutes after the previous antagonist injection. One minute later the agonist was reapplied. At the end of the studies current was passed through a microelectrode to identify the point of stimulation and injection.

The areas found to produce muscle tone suppression in the rostral pons were located between L0.6 and L1.2, while in the caudal pons the effective area was located between L0.4 and L0.8. This area corresponds to the nucleus reticularis pontis oralis and caudalis respectively. The effective area in the medulla was located from P2.0 to P3.3, L0.2 to L1.1 and H0.9 to H-0.9 which includes portions ofthe nucleus reticularis gigantocellularis, paragigantocellularis dorsalis, gigantocellularis alpha, and gigantocellularis ventralis. The contralateral muscle always showed more pronounced inhibition than the ipsilateral. The inhibitory effect was amplitude and frequency dependent. Microinjection of kainate to this area also caused muscle tone suppression, the effect was blocked by specific KA receptor antagonist CNQX. The effect of NMDA was opposite, muscle excitation was seen. This effect was blocked by specific NMDA receptor antagonist AP5. The contralateral muscle always showed more pronounced inhibition than the ipsilateral like in the electrical studies.

In summary, the regions in the rodent pons in which electrical stimulation causes muscle tone suppression are glutamatergic. Previous studies in our laboratory have shown that microinjection of non-NMDA glutamate agonists into the feline pontine inhibitory area (PIA) also induces muscle atonia. So the inhibitory areas found in rats correspond to the feline motor inhibitory regions and are probably implicated in muscle tone suppression in REM sleep.

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ACUTE CORTICOSTERONE EFFECTS ON AGGRESSIVE BEHAVIOR AND ON THE STIMULUS SENSITIVITY OF THE HYPOTHALAMIC ATTACK AREA

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Beyond long lasting genetic effects, new evidence shows that corticosterone may have short behavioral consequences that seem to be incompatible with the genetic way of action. These effects develop within minutes (3-10 min.) and involve various behaviors including exploration/locomotion, sexual behavior and aggression.

Previously we have demonstrated that these short effects of corticosterone are not behavior-, but rather context dependent. Corticosterone, when injected to male rats that

encountered an unknown opponent, stimulated aggression only in situations when the experimental setup determined a high level of aggressiveness. In situations when aggression was not likely to occur (e.g. when animals faced the opponent in a neutral arena), corticosterone increased exploration/locomotion, and aggressive behavior was not stimulated.

Although there is an increasing number of studies that support the assumption that corticosterone exerts rapid effects on the expression of behaviors, the phenomenon as a whole is still unknown. For instance, the site and way of action of corticosterone is rather obscure.

The presently described experiments were designed to clarify some aspects of the mechanism of action of corticosterone. The effects of the hormone were studied by using the hypothalamic aggression paradigm. The electric stimulation of the so-called hypothalamic attack area (HM) elicits immediate attack in the subjects if an opponent is present. The current threshold for attack ranges from 30 to 400 μA, and it is highly stable in time for a given individual. The threshold is subject to pharmacologic manipulation. In general, pharmacologic agents that reduce attack frequency in spontaneous aggression also increase the electric thresholds in hypothalamic stimulation (i.e. they lower the sensitivity of the attack-related hypothalamic centres).

The effects of acute corticosterone treatments were studied in adrenalectomised (ADX) animals. ADX rats were implanted with pellets containing 1. 100% cholesterol; 2. 75% cholesterol and 25% corticosterone, and 3. 25% cholesterol and 75% corticosterone. The treatments induced a basal corticosterone level of 1. < 10 nmol/l; 2. 100-150 nmol/l, and 3. 700-800 nmol/l, respectively. Acute increases in corticosterone were induced by corticosterone injections 10 min. before experimental testing (250 μg/kg)

Adrenalectomy itself (cholesterol pellet) did cause a very significant increase in thresholds (thus neuronal sensitivity to stimulation decreased). Acute corticosterone injections induced a marked decrease in attack thresholds (sensitivity increased) in both animals implanted with cholesterol and low corticosterone pellet. The high cholesterol pellet induced a significant decrease in attack thresholds (sensitivity increased), while acute injections had no effect in this case.

Results support the assumption that an acute injection of corticosterone may increase the stimulus sensitivity of neurons specifically involved in aggressive behavior. The effect develops rather quickly.

Corticosterone is intensely secreted at the beginning of a spontaneous aggressive encounter in males irrespective to social or residence status. Significant increases of blood corticosterone are reached within 5 minutes. The outbreak of attacks lasts approximately 5 minutes as well. Our results suggest that this naturally occurring corticosterone secretion may have a role in promoting aggression. Corticosterone may make the aggression-related brain centres more sensitive to stimuli. It is not known whether this is the case of other behaviors affected by corticosterone

To our surprise, chronic increases in corticosterone also increased the stimulus sensitivity of the HM. This observation supports the assumption widely accepted in psychology and sociology that chronic stresses increase aggression. However, it is in sharp contrast with laboratory experiments that show a decrease of spontaneous aggression after chronic corticosterone treatments.

Ca^{2+} CURRENTS OF FRESHLY ISOLATED COCHLEAR NUCLEUS NEURONES OF RAT

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Voltage-gated Ca^{2+} currents activated on depolarization have been studied in 24 cochlear nucleus neurones isolated enzymatically by simultaneous Collagenase and Pronase treatment. The dissociated cells were investigated by whole-cell voltage-clamp in a medium where all but Ca^{2+} currents were inhibited ($[Ca^{2+}]_e$ = 2 mM). Large cells with multiple processes were selected for the present study.

When depolarizing voltage steps from a prepulse potential of -120 mV to -90 mV were applied, a low-voltage activated (LVA) Ca^{2+} current was observed with fast activation and inactivation kinetics. Although the amplitude of this T-type component showed great variability in different cells (-200 pA to -1500 pA), its steady-state inactivation, which was determined by a double pulse protocol was similar in all neurones studied (the halfinactivation voltage and slope factor were -94±6 mV and 6±2 mV, respectively [n=5; mean ± SD]).

From a holding potential of -60 mV another current could be recorded. The activation of this component required larger depolarizations (to approximately -30 mV), it showed slow inactivation, and the peak of the current-voltage relationship was seen at about -20 mV. No further characterisation of this high-voltage activated (HVA) component was attempted.

Some of the investigated cells possessed a Ca^{2+} current which was - at least partially - activated at holding potentials of -60 mV and showed no apparent time dependent inactivation. As the steady-state inactivation of the LVA component is already complete at -60 mV it seems unlikely that this current component may be part of the LVA.

All described current components were reversibly blocked by the extracellular application of 2 mM $CoCl_2$, although the individual currents showed different sensitivities. Both T-type component and the Ca^{2+} current seen at -60mV holding potential were completely abolished by CO^{2+}, but only some 70% of the HVA current was sensitive to this blocker.

Our work indicates that the freshly isolated cochlear nucleus neurones possess various Ca^{2+} currents, but the precise identification and separation of these components require extensive further work.

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NIMODIPINE ATTENUATES DEHYDRATION-INDUCED OVEREXPRESSION OF CALCIUM-BINDING PROTEINS IN RAT NEOCORTEX AND HIPPOCAMPUS

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Dehydration is a commonly occurring physiological condition which may potentially alter the intracellular ionic environment, involving changes of the Ca^{2+} homeostasis of both neuronal and peripherial tissues. It is worth noting, however, that several human disorders are also accompanied by severe dehydration symptoms together with a highly increased plasma Ca^{2+} concentration. Ca^{2+} ions play a concentration-dependent bidirectional role in fundamental neuronal functions since alterations in the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) may induce neurite outgrowth and excitability but also neurodegeneration and cell death. One of the mechanisms underlying the maintenance of the intracellular Ca^{2+} balance is the selective binding of Ca^{2+} to cytosolic low molecular-weight Ca^{2+}-binding proteins (CaBP). Members of the EF-hand CaBP superfamily, parvalbumin (PV) and calbindin-D28k (CB-D28k), are the most prominent specific representatives of these proteins in neuronal tissues.

In the present experiments we investigated the effects of dehydration-induced intracellular Ca^{2+} accumulation on the expression of PV and CB-D28k in neocortical and hippocampal interneurons of the rat brain. Whereas PV is a selective marker for GABAergic interneurons, CB-D28k can be localized both to excitatory pyramidal and inhibitory interneuronal cell groups. To correlate the effects of dehydration on the expression of the CaBPs, we confined our analysis on the CB-D28k-positive (GABAergic) interneuron population. Moreover, nimodipine, a dihydropyridine-base L-type Ca^{2+} channel blocker was used to prevent dehydration-induced alterations in neuronal functioning.

Male young adult Wistar rats were administered chronically with nimodipine (BAY E 9736) for 5 weeks. Nimodipine was added to the daily food pellet in a concentration of 1000 ppm. Subsequently, rats were deprived of water for 4 days. Thereafter, animals were transcardially perfused and PV and CB-D28k immunocytochemistry was performed. Immunoreactive (ir) cell numbers were quantified in the frontal and parietal (somatosensory) cortical areas and hippocampus.

Four-day long dehydration resulted in significantly increased expressions of both PV and CB-D28k. Interestingly, while PV-ir most remarkably increased in the hippocampus, dehydration elicited the most striking increase in CB-D28k-ir in the parietal cortex. It is noteworthy that in the case of CB-D28k not only cell bodies but also neurites showed an enhanced immunoreactivity. Chronic pretreatment with nimodipine attenuated these dehydration-induced alterations with a high efficacy. Following a combined treatment the number of PV or CB-D28k-ir interneurons did not increase, relative to naive control animals.

In conclusion, our results indicate that dehydration (1) directly influences the intracellular Ca^{2+} homeostasis of neurons, (2) induces the overexpression of multiple CaBPs which may enhance the survival of nerve cells and contribute to the maintenance of a normal physiological function. (3) Blockade of Ca^{2+}-channels, particularly those of the L-type, by selective antagonists prevents the dehydration-induced changes in CaBP expression. The drug

effect may be indicative for the fact that selective antagonists could attenuate pathological increases in the intracellular Ca^{2+} concentration.

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MK-801 ADMINISTRATION PREVENTS THE CHOLINOTOXIC ACTION OF ß-AMYLOID IN RAT MAGNOCELLULAR NUCLEUS BASALIS: DIFFERENCES BETWEEN YOUNG AND AGED ANIMALS

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Major neuropathological features of Alzheimer's disease involve the enhanced generation and subsequent accumulation of ß-amyloid peptides (ßAP) forming neuritic plaques and cerebrovascular deposits. Compelling evidence indicates that ßAP exert neurotoxicity under both *in vitro* and *in vivo* conditions and might contribute to the cholinergic decline in the disease process. It is noteworthy that while several attempts were made to characterize ßAP neurotoxicity the fundamental molecular pathway leading to cellular toxicity is still to be ascertained.

In the present experiments we set out to investigate by means of behavioral and histochemical methods the time-course of the neurotoxicity of ßAP(25-35) and ßAP(1-42) fragments; differences in their neurotoxic potentials in young and aged animals, and neuroprotection by N-methyl-D-aspartate (NMDA) receptor antagonist MK-801.

Young adult (3 months of age, n=6 per group) and aged (28 months of age, n=4 per group) male Wistar rats were used in the experiments. ßAP(25-35) or ßAP(1-42) was injected in a concentration of 0.2 nmol/1 µl into the right nucleus basalis (MBN) of rats. Alterations in spontaneous behaviors of young animals were determined using the small open-field paradigm 3, 7 and 14 post-surgery. MK-801 was administered acutely in a dose of 5 mg/kg body weight (i.p.) 2 hours prior to ßAP(1-42) infusion and rats were sacrificed after a 14-day survival period. The neurotoxic potential of ßAP in young and aged rats was compared after 14 days post-operation. Following the appropriate survival period rats were transcardially perfused with 4% paraformaldehyde, the brains were dissected out and posffixed in the same fixative for 24 hours. Cortical acetylcholinesterase (AChE, EC 3.1.1.7) activity was visualized according to Hedreen et al. (1985) using a silver-nitrate intensification procedure. Loss of cholinergic projection fibers was quantified in the posterior somatosensory area by using a Leica Quantimet Q-600HR computerized image analysis system.

Marked deficits in explorative animal behavior (hypoactivity) of young rats as a consequence of ßAP infusion became apparent as early as 3 days post-surgery and were maintained throughout the survival periods investigated. Interestingly, MK-801 treatment antagonized the ßAP-induced hypoactivity and resulted in a significant hyperactivity which

was most obviously present in the first 10-minute block of the behavioral test. Comparison of the spontaneous behaviors of young and aged animals revealed a significantly reduced activity in aged rats, whereas ßAP infusion into the MBN of aged rats did not alter their behavioral characteristics 14 days post-injection.

In the histochemical evaluation we confined our analysis on the loss of the cortical cholinergic innervation visualized by AChE-positive fibers. Both ßAP(25-35) and ßAP(1-42) injections resulted in significantly reduced densities of cortical AChE-positive fibers originating in the MBN. Interestingly, ßAP(1-42) injection-induced neurodegeneration exceeded that of ßAP(25-35) fragment and this neurotoxicity was enhanced by an acidic milieu. Comparative analysis of our behavioral data and deficits in cortical cholinergic innervation revealed a strong correlation between impairments of explorative behavior and the extent of cortical cholinergic degeneration. While ßAP(1-42) injections into the MBN resulted in a 22.1% loss of cortical cholinergic innervation, MK-801 treatment decreased the extent of fiber reduction to 5.0%. Decreased ßAP neurotoxicity was determined in senescent rats as compared to young adult animals.

In conclusion, our studies demonstrate that unilateral MBN injections result in graded alterations in spontaneous animal behavior. The neurotoxic potentials of different ßAP fragments are dependent on their chemical properties, particularly on their tertiary structure, and an acidic milieu (pH 4.0). Our studies using the non-competitive NMDA receptor antagonist MK-801 together with the marked differences between young and aged rats indicate (1) the possible involvement of a calcium overload leading to the observed cholinergic cell death; or (2) a ligand-like binding of ßAP to specific cell-surface structures. We suggest that age-mediated down-regulation of receptors or receptor-like binding sites may account for the decreased neurotoxicity in senescent rats.

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PROLONGED ß-AMYLOID(1-42) INFUSION INDUCES BEHAVIORAL, BIOCHEMICAL AND NEUROANATOMICAL CHANGES IN FREELY MOVING RATS: IMPACT OF PEPTIDE ASSEMBLY STATE AND NEUROPROTECTION BY ZM-6, A PENTAPEPTIDE ANTAGONIST

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ß-amyloid (ßAP) neurotoxicity, *in vivo* properties of potentially amyloid-like peptide fragments and their probable contribution to cell death in brain areas subserving learning and

memory have recently become one of the major issues in Alzheimer's disease (AD) research. Earlier studies in our laboratory demonstrated that ßAP fragments exhibit *in vivo* cholinotoxicity in the magnocellular nucleus basalis (MBN) of rats. The neurotoxicity is accompanied by a calcium overload and intracellular calcium-binding 'buffer' proteins may enhance the survival of nerve cells. Furthermore, N-methyl-D-aspartate receptor antagonists, such as MK-801 or ifenprodil effectively attenuated the ßAP-induced neurotoxic cascade.

In the present series of experiments we investigated prolonged (120 min) ßAP infusion-induced changes in animal behavior throughout a 14-day survival period. Alterations in the release profile of excitatory amino acids during ßAP perfusion were also determined. To evaluate the neurotoxic potential of ßAP, thioflavine-S and acetylcholinesterase (AChE, EC 3.1.1.7) histochemical procedures were employed. Furthermore, the effects of a pentapeptide amyloid antagonist (ZM-6) which may prevent the cell-surface binding or aggregation of ßAP were also studied.

A concentric microdialysis probe, made of Travenol hollow fibers was implanted into the right MBN region of young adult male Wistar rats and secured to the skull with dental acrylic. ßAP(1-42) or ßAP(17-43) was dialysed through the probe in a concentration of 0.2 nmol/1 µl at constant flow rate (2 µ l/min) in freely moving rats. In case of neuroprotection studies, an equimolar ZM-6 solution was dialysed for 60 minutes followed by a 60-minute ßAP(1-42) perfusion. Extracellular amino acid levels were determined by HPLC analysis of ortho-phtalaldehyde (OPA) derivatives of amino acids. In parallel with ßAP perfusion home cage-like activities of freely moving rats were determined. Furthermore, open-field activity was determined after 24 hours, while passive shock-avoidance learning was tested 14 days postsurgery. Thioflavine-S histochemistry was employed to visualize the extent of ßAP(1- 42) deposition in the brain. Moreover, the loss of AChE-positive fibers invading the somatosensory cortex was determined by means of quantitative AChE histochemistry.

Prolonged ßAP administration resulted in the severe impairments of both spontaneous behaviors and learning and memory processes. Pretreatment with ZM-6 beneficially influenced these disturbances. ßAP infusion elicited a dramatic increase in Aspartate and Glutamate release, which was accompanied by that of Taurine. Interestingly, the time point of this enhanced excitatory amino acid release matched an activation in home cage-like behavior. Prolonged ßAP infusion resulted in an increased accumulation and deposition of the protein in the brain tissue, as compared to acute injections. Comparison of the loss of cortical AChE-positive fibers revealed a strong correlation between (1) the tertiary structure of ßAP fragments, (2) the extent of depositions formed and (3) the loss of cortical cholinergic innervation. ßAP(1-42) elicited a more pronounced neurodegeneration than ßAP(17-43). It is noteworthy that the latter sequence, which might be generated also in the brains of non-AD individuals, induced a significant decrease in cortical AChE-positive fiber densities, as well. ZM-6 administration prevented the loss of cholinergic fibers in the somatosensory cortex.

Administration of the full length ßAP sequence [ßAP(1-42)] elicited marked behavioral disturbances throughout the survival period. An underlying mechanism of these changes might be the extensive neurodegeneration which occurred as a consequence of the administration of the peptide.

Interestingly, ßAP neurotoxicity was mediated by an enhanced Aspartate-Glutamate co-release. The present data together with our previous results indicate that the neurotoxic potential of the peptide fragments and thus the loss of cortical AChE-positive innervation correlates with the extent of ßAP deposition in the MBN. It is noteworthy that the 'extracellular fragment' of ßAP (containing the [25-35] amino acid sequence) also exhibited a marked neurotoxicity. Application of ZM-6, a conformational pentapeptide antagonist effectively decreased the neurotoxic action of ßAP. Most remarkably, ZM-6 almost completely prevented the loss of cortical AChE-innervation. In summary, early phases of in vivo ßAP neurotoxicity involve an enhanced release of excitatory amino acids which may

directly lead to cell death. Furthermore, conformational amyloid antagonists effectively prevent the initiation of the neurotoxic cascade and might be potent tools in designing specific anti-amyloid drugs.

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MECHANISM OF SYSTEMIC ANTI-INFLAMMATORY EFFECT INDUCED BY ORTHODROMIC AND ANTIDROMIC STIMULATION OF THE CAPSAICIN SENSITIVE SENSORY NERVE ENDINGS

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Neuropeptides released by antidromic electrical or orthodromic chemical stimulation from the peripheral endings of the capsaicin-sensitive primary afferent neurons evoke local neurogenic inflammation in the innervated skin and mucosal areas. It has been established by previous studies that a neurogenic inflammation (primary reaction) inhibits the development of a subsequent neurogenic or non-neurogenic inflammation on a distant part of the body (secondary reaction). A pure non-neurogenic inflammation evoked by subplantar injection of dextran into the chronically denervated hindpaw had no anti-inflammatory activity.

The aim of the present study was to examine the neurotransmitter background of this phenomenon. In the first sets of the experiments the primary inflammation was evoked by orthodromic stimulation of the capsaicin sensitive sensory nerve endings by topical application of 1% mustard oil on the acutely denervated leg skin of the rats. Following the primary reaction, the secondary plasma protein extravasation in the contralateral paw evoked by mustard oil smearing was inhibited by 50%. Pretreatment with polyclonal somatostatin-14 antiserum (i.p.) in a dose of 0.5 ml/ rat which abolished the anti-inflammatory effect of 10 μg/kg exogenously given somatostatin or the opiate antagonist naloxone (1 mg/kg, s.c.) strongly reduced the inhibitory effect of the primary reaction. Orthodromic neurogenic inflammation (primary reaction) significantly decreased the oedema induced by subcutaneously injected dextran into the chronically denervated contralateral paw (secondary reaction) as well. Somatostatin antiserum or naloxone pretreatment diminished but did not completely eliminate this anti-inflammatory effect. Furthermore exogenous somatostatin (10 or 20 μg/kg, i.p.) significantly reduced the mustard oil-induced neurogenic inflammation, and this inhibition was prevented by pretreatment the rats with naloxone (1 mg/kg s.c.). All these results suggest a mediator role for somatostatin in the anti-inflammatory response evoked by the primary reaction. The second part of the study the primary neurogenic inflammation was induced by antidromic electrical stimulation of the sciatic nerve. Excitation with 5 Hz for 5 min or with 0.5 Hz for 60 min caused plasma extravasation in the innervated skin of the paw and also reduced the secondary antidromic neurogenic inflammation or the inflammation induced by subcutaneous injection of 1% carrageenin on the contralateral side. Stimulation with 0.1 Hz for 4 h did not have inflammatory action but did exert anti-inflammatory activity.

It is concluded that orthodromic as well as antidromic neurogenic inflammation inhibit the development of a subsequent inflammatory reaction at a distant site. Major transmitter role for somatostatin and possible participation of opioid peptides in this anti-inflammatory action is suggested. The anti-inflammatory mediator(s) is likely to be released directly from the activated sensory nerve endings and not from the inflamed tissue, as low frequency electrical stimulation induces inhibitory action without causing plasma extravasation.

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IN VITRO NEUROGENESIS FOLLOWED BY COMPUTER-CONTROLLED VIDEOMICROGRAPHY

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Our knowledge concerning the early steps in neuron formation is far from clear. The in vivo examination of changes in cell-to-cell interactions is set back by many difficulties. The use of in vitro model systems (e.g. primary cell cultures or neural cell lines) can solve some of the problems arising from the limited quantity and life span of the in vivo neural progenitors.

In our laboratory we use several p53-deficient immortalized neuroepithelial progenitor cell lines, which can be differentiated into neurons and astrocytes upon induction by retinoic acid (RA) (1). In our previous work we determined the typical stages of in vitro neuron formation by describing several morphological patterns formed by specific cell-to-cell interactions in cultures (2). We used immunostained cultures fixed at definite stages of differentiation. It is obvious, that these preparations provide only "snapshots" from the processes of in vitro neurogenesis.

Using computer-controlled video-micrography, the fate of single cells, the kinetics of cell migration or differentiation or the maturation of morphological patterns can be followed continuously, during several weeks. In the poster we present data on the use of computer-controlled video-micrography in analysing in vitro neurogenesis Video-micrographic data show that the decrease in cell number during the first 3 days of RA treatment was due to the inhibition of cell proliferation rather than to the enhancement of cell death.

We demonstrate that cell migration and the changes in the cell shape can occur within minutes. Cell groups can alter actively their morphology, without further cell divisions. Our data also show that morphological patterns are not stable and they can transform into each other during maturation. Thus, while immunostained preparations could give information only about the distribution of morphological patterns at the moment of fixation, video-microscopy can give informaton about the continuous changes during neuronal maturation.

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EVIDENCE FOR TASTE RESPONSIVENESS IN THE PREFRONTAL CORTEX OF THE RAT: ANALYSIS OF SINGLE UNIT RECORDINGS IN THE ORBITOFRONTAL CORTEX

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The orbitofrontal (ventral-lateral prefrontal, OBF) cortex is widely considered as a secondary taste area in the primate. Though morphological and functional homology of this area is also present in the rodent, the OBF is mainly claimed to be a structure of higher level olfactory information coding and taste-odor association learning, there. Also in rodents, illness-aversion deficits and disturbances in body weight regulation together with food and fluid intake are also reported. Despite the abundance of literature data on feeding related OBF functions, the specific roles of its intrinsic neurons in the information processing of gustatory stimuli are poorly understood. To investigate specific, gustatory coding characteristics of OBF neurons, extracellular single unit activity was recorded during intraoral taste stimulations.

Slightly anaesthetized CFY rats were stereotaxically fixed and single neuron activities in the OBF were recorded by glass microelectrodes. Two concentrations of five basic tastants (sweet, salty, sour, bitter and umami, respectively) and a complex one (orange juice) were individually administered into the mouth through a stimulus delivery device. Analogue data were AID converted, single cells were off line separated, and frequency histograms computed. Taste-responsive neural firing rate changes were found in the OBF of the rat. The proportion of taste responsive neurons was approx. 11%. Gustatory neurons showed distinct responsiveness to the various stimulus modalities, changing their spontaneous activity mainly to one or two taste cues. Almost all gustatory cells showed dynamic, motivation associated discharge rate

changes (habituation or sensitization) during the subsequent application of the same stimuli. Concentration-dependent responses were also recorded.

The present results, along with previous findings, indicate that OBF is intimately involved in the higher order coding of gustatory information. Complex response patterns of OBF gustatory neurons suggest the evidence for a neural level motivational control of taste associated learning (e.g. sensory-specific satiety) mechanisms.

THE NEURONAL NETWORK REGULATING THE MOVEMENT OF THE OLFACTORY ORGAN AND ITS IMMUNOCYTOCHEMICAL ANALYSIS IN THE SNAIL *HELIX POMATIA*

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Hungry snails after a few presentation of a kind of food pieces are able to find them among others from significant distances. The posterior tentacles which are the olfactory organs of the snail have characteristic spatial positions during both the appetitive food-finding and the consumatory phase of feeding behavior (Teyke 1995, Peschel et al. 1996). The olfactory organs are innervated by the olfactory nerve (on) from the tip to the base, and by the peritentacular nerves (ptns) from the base to the tip of the organ.

In this study we describe the afferent and efferent elements of the neuronal network regulating the movements of the olfactory organ by applying parallel retrograde Co and Ni labelling through nerves which innervate the organs. Furthermore, we describe the serotonin immunoreactive (5HT-ir) and FMRFamide immunoreactive (FMRFa-ir) elements of the network.

The parallel retrograde Co and Ni labelling through the ptns and the on shows that the unipolar neurons efferent to the tip and the wall of the organs are both separated and overlaped. The parallel lucifer yellow and Co labelling through the on and the ptns shows that the cerebral neurons efferent to the base of the organ, send side branches to the lucifer yellow labelled olfactory afferents, suggesting that they may have direct contacts.

Immunocytochemistry revealed that the organs are densely innervated by both 5HT-ir and FMRFa-ir elements, suggesting that numerous 5HT-ir and FMRFa-ir neurons innervate the organsPeripheral FMRFa-ir, but not 5HT-ir, cell bodies can be deteted in the wall of the organs showing that the 5HT-ir fibers exclusively, whereas FMRFa-ir fibers only partly originate from the brain.

Following the removal of the tips of the olfactory organs, the majority of 5HT-ir, but not FMRFa-ir fibers disappeared in the wall, demonstrating that the rest of 5HT-ir fibers originated from Cvl the only identifiable serotonergic neuron efferent to both ptns.

From the ventral side of the tip of the organ four thin muscle bundles originate which are anchored at the base of the organ, therefore they seem to be suitable to bend or elevate the protracted organ. They receive both 5HT-ir and FMRFa-ir fibers from both the ptns and the on.

Our observations suggest that olfactory input are capable of regulating the spatial positions of the olfactory organs which are set in by different neuronal populations efferent to the organs and by its intrinsic neurons as well. Furthermore, the precise directioning of the

olfactory organs towards the source of the proper odour suggests that snails possess a space cognition ability.

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IDENTIFICATION OF A LOW AFFINITY SEROTONIN RECEPTOR IN INSECT HAEMOCYTES

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It was previously shown that serotonin (5HT) enhances phagocytic aetivities of cockroach haemocytes through the IP_3, second messenger system. In the present study we investigated the binding properties of the radiactive labeled agonist (^{3}H5HT) and antagonists (^{3}H-LSD, ^{125}I-LSD) to demonstrate the presence of 5HT receptor in the cricket haemocyte membranes.

The haemolymph was collected from cooled crickets *(Acheta domestica)* and the haemoeytes were sedimented by centrifugation. The haemocytes were homogenized in 50 mM Tris-buffer pH 7, and centrifuged 4 times at 17,000 g for 60 minutes with intermediate resuspension. For the characterization of the kinetie and pharmacological properties of the binding, the membrane pellet was incubated with ^{3}H-5HT in the presence and absence of different pharmacons.

^{3}H-5HT was found to bind specifically to the cricket haemocyte membrane. The specifie binding was saturable and the Scatchard plot fitted the one site model. However, the 5HT binding to the haemocyte membrane has a low affinity. The K_d value was 109.4 nM and the maximal binding (B_{max}) value 2841.8 fmol/mg prot. The binding was reversible, the association was fast, whereas the dissociation was low. Investigating the affinity of a series of $5HT_1$, $5HT_2$, $5HT_3$ receptor specific pharmacons was found that only the agonists (5HT, α-methyl-5HT, 2-methyl-5HT, 1 [naphtyl]piperazine) could displace the bound ^{3}H-5HT. The antagonists LSD, mianserin, ketanserin, ICS205-930, cyproheptadine, methiothepin did not inhibit the ^{3}H-5HT binding. It was also found that neither the ^{3}H-labeled nor the ^{124}I-labeled antagonist LSD bound specifically to the haemocyte membranes.

It seems that the 5HT receptor in the insect haemocytes has anusual properties, it can be stimulated by agonists but not inhibited by antagonists.

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ETHANOL AND NMDA RECEPTOR FUNCTION

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The activity (output) of neurons depends on the balance of their excitatory and inhibitory inputs. The major inhibitory neurotransmitter in the central nervous system is GABA, and the major excitatory transmitter is glutamate. There are several subtypes of glutamate receptor, including the N-methyl-D-aspartate (NMDA) subtype.

The NMDA receptor is a heteromeric ligand-gated ion channel that consists of an NR1 subunit, which is required for activity, in combination with one or more NR2 subunits (NR2A-D), which confer pharmacological specifity to the response of the receptor. The NMDA receptor is activated by the agonist glutamate and the required co-agonist, glycine, and can be specifically inhibited by various compounds that act at the ion channel or at ligand recognition sites. When activated, the NMDA receptor-coupled ion channel is permeable to Ca^{2+}, as well as to Na^{+} and K^{+}. The NMDA receptor is believed to play key roles in learning and neuronal development. When subject to excess activation, the receptor is involved in generation of seizure activity and excitotoxicity.

Our work, and that of others, has shown that ethanol is a potent inhibitor of NMDA receptor function in a number of neuronal preparations. In cerebellar granule neurons, the mechanism of action of ethanol involves a reduction of the potency of the co-agonist, glycine. However, the mechanism of action of ethanol may vary among cells from different brain regions, possibly due to differences in NMDA receptor subunit composition within these regions. We have also investigated changes in the NMDA receptor that occur after chronic treatment of cultured cells or whole animals with ethanol. When mice are treated with ethanol in a paradigm that produces physical dependence on ethanol, there is an up-regulation of NMDA receptors in brain, as measured by ligand binding. This change may represent an adaptation to the initial inhibitory effect of ethanol, and is associated with increases in the level of proteins for several NMDA receptor subunits. However, there is no corresponding change in the level of mRNA for these receptor subunits, suggesting the possibility that chronic ethanol treatment results in post-transcriptional changes in the NMDA receptor. The time course of the change in the NMDA receptor in brains of ethanol-treated mice parallels the time course for the appearance and disappearance of ethanol withdrawal convulsions, suggesting a role for the NMDA receptor in the generation and/or expression of this withdrawal sign. This conclusion is supported by the finding that NMDA receptor antagonists can attenuate ethanol withdrawal convulsions. Furthermore, our recent studies have shown that concomitant treatment of mice with gangliosides during chronic ethanol exposure both prevents the up-regulation of the NMDA receptor, and reduces the severity of ethanol withdrawal convulsions. In addition to ethanol withdrawal signs, the NMDA receptor may play a role in generating excitotoxic cell death following chronic ethanol exposure.

When cerebellar granule neurons are exposed to ethanol in vitro for several days, there is also an up-regulation of NMDA receptor number and function. As in brain, this up-regulation is associated with changes in the level of NMDA receptor subunit proteins, as well as with an increase in the amount of a "glutamate binding protein" that is thought to be a component of another form of the NMDA receptor. As a result of the up-regulation of NMDA receptor

function, the cells become more sensitive to glutamate-induced excitotoxicity, which is mediated by the NMDA receptor. This enhanced sensitivity to glutamate-induced cell death could represent a mechanism underlying the neuronal damage that is observed in chronic alcoholics. The enhanced excitotoxicity can be prevented by treatment of the cells with various antagonists of the NMDA receptor, as well as with gangliosides. These data suggest the possibility that therapies can be developed that would both reduce the severity of ethanol withdrawal seizures/convulsions, and reduce or prevent ethanol withdrawal-induced neurotoxicity.

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STUDY OF COMPOUNDS ACTING AT POLYAMINE SITE OF NMDA RECEPTOR BY 'CORTICAL WEDGE' METHOD

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Ifenprodil and eliprodil are neuroprotective compounds and this effect is thought to be the consequence of their antagonist property at the polyamine site of N-methyl-D-aspartic acid (NMDA) receptor (1). However in some models (2, hippocampal cell culture) the neuroprotective effect of ifenprodil seemed to be not mediated at polyamine site of NMDA receptor. It has been proposed that the antagonist action at polyamine site is dependent on the brain tissue used for the study.

Even the NMDA antagonist action of ifenprodil was questioned in a study on rat cortical wedges (3).

The rat cortical wedge is a widely used in vitro method for characterisation of compounds acting on excitatory amino acid receptors. Measurement of NMDA perfusion-evoked depolarisation and/or the spontaneous epileptiform activity (SEA) appearing in magnesium-free medium are reliable methods for detection of NMDA antagonists either competitive or non-competitive (including channel blockers and glycine site antagonists).

In the present study we examined the effect of polyamine agonists spermine (SPN) and spermidine (SPD) and antagonists ifenprodil and eliprodil on wedgeshaped slices of rat brain cingulate cortex. Both the NMDA evoked depolarization response and SEA were measured, similarly to a previous study (4).

In accordance with previous studies (3), our results confirmed, that 1-300 μM ifenprodil or eliprodil did not decrease response to NMDA. Contrary to all other types of NMDA antagonists studied, ifenprodil and eliprodil up to concentration of 50 μM increased the frequency of SEA, 100 μM produced a biphasic effect, and only higher doses caused clear-cut decrease. The amplitude was unambiguously dose-dependently decreased.

The erythro diastereomer of ifenprodil was found to be more effective than threo-ifenprodil in our experiments studying SEA. Notwithstanding, Chenard et al. (5) found threo-ifenprodil more effective in an in vitro neuroprotective test.

Our results suggests that ifenprodil and eliprodil do not exert a significant effect on NMDA receptors in brain slices of rat cingulate cortex. The decrease in SEA may be caused by a

mechanism other than NMDA antagonism. Dissimilarity in relative efficacy of diastereomers as compared to other studies also supports this conclusion.

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THE DISTRIBUTION OF PUTATIVE NITRIC OXID SYNTHASE CONTAINING NEURONS IN THE CENTRAL NERVOUS SYTEM OF FOUR ISOPOD SPECIES

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It has recently been demonstrated that the central nervous system (CNS) of various invertebrate species including arthropods have nitrergic neuron populations. In this study using NADPH-diaphorase (NADPHd) histochemistry the putative nitrergic network in the CNS of four terrestrial crustacean species *(Porcellio scaber, Oniscus asellus, Armadillidium nasutum* and *Armadillidium vulgare)* has been described in wholemount preparations and consecutively serial paraffin sections.

Several NADPHd positive perikarya and neurofibres were found in all ganglia of the CNS in all investigated species. The intensity of staining varied from specimen to specimen even if they belonged to the same species and derived from the same incubation. Two distinct populations of labelled neurons could be distinguished: one is characterised by constant and strong staining while other neurons did not get stained in all samples. There were characteristic interspecific differences in the distribution of NADPHd-positive perikarya and fibres. In *P. scaber, O. asellus* and *A. nasutum* mainly the perikarya stained, while in *A. vulgare* only few labelled perikarya and a well developed, strongly stained network of fibres were found. The distribution of labelled cells showed some similarities and a few marked difference. Only a few NADPHd-positive cells occurred in the proto-, deutero- and tritocerebrum, while in the subesophageal and thoracic ganglia and especially in the terminal ganglion several labelled neurons were found. In the terminal ganglion of *P. scaber* and *A. nasutum* the ventral unpaired

median neurons and paired neurons with small diameter gave positive reaction while in *O. asellus* and *A. vulgare* several large sized paired positive cells were revealed.

Variability of staining within species and the interspecific differences in the distribution of NADPHd-positive neurons suggest the plasticity of nitrergic network during life cycle and speciation.

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ROLE OF METABOTROPIC GLUTAMATE RECEPTORS IN THE HIPPOCAMPAL RELEASE OF GABA

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The effects of glutamate agonists and their selective antagonists on the Ca^{2+}-dependent and independent releases of [^{3}H]GABA from rat coronal hippocampal slices were studied in a superfusion system. The Ca^{2+}-dependent release evoked by glutamate, kainate and N-methyl-D-aspartate (NMDA) gradually declined with time despite the continuous presence of the agonists. Quisqualate (QA) caused a sustained release within the 20-min period of stimulation. This release was enhanced in Ca^{2+}-free medium. The release evoked by QA in $^{2+}$Ca containing medium was significantly inhibited by dizocilpine and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), indicating that QA activates NMDA receptors directly or indirectly through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors. The inhibition of dizocilpine was slightly diminished and that of CNQX totally abolished in Ca^{2+}-free medium. Verapamil inhibited the QA-activated release in both Ca^{2+}-containing and Ca^{2+}-free media. The effect of QA but not that of AMPA was blocked in Ca^{2+}-free medium by L(+)-2amino-3-phosphonopropionate (L-AP3). We suggest that the sustained release of GABA is also mediated partly by activation of L-AP3-sensitive metabotropic receptors and mobilization of Ca^{2+} from intracellular stores. Such long-lasting increase in hippocampal release of GABA may be involved in long-term potentiation.

DYSTROPHIN IMMUNOREACTIVITY IN A PRIMARY SENSORY EPITHELIAL CELL. IMPLICATIONS FOR SYNAPTIC LOCALIZATION

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Dystrophin has been identified as the protein lacking or being truncated in the lethal genetic disease Duchenne muscular dystrophy (DMD), as well as in the milder Becker muscular dystrophy (BMD). The dystrophin gene contains seven independent promoters, giving rise to various dystrophin transcripts and at least seven proteins of different molecular weights, in a tissue- and cell-specific manner. The exact function of these proteins is still to be clarified. Data on their intracellular localization are also scarce, except for the full length, 427 kDa dystrophin in the neuromuscular junction. In this case localization of the protein in the postsynaptic membrane has been convincingly documented.

In primary sensory epithelial cells a peculiar synaptic specialization, the "synaptic ribbon" is found in presynaptic position but displays the features also of the postsynaptic element. By using a monoclonal antibody specific for the C-terminal region of dystrophins (Dys2, Novocastra), we were able to demonstrate immunoreactivity in the "rod" component of this structure in pinealocytes. This finding indicates that the synaptic ribbon might be a synaptic complex incorporated by the presynaptic element. This also explains why dystrophin was observed at both synaptic sides in the outer plexiform layer of the retina [Schmitz et al., Histochemistry (1993) 100, 473-479 and Ueda et al., Invest Ophtalmol Vis Sci. (1995) 36, 218-232]. A further implication of our finding is that dystrophin might be a general constituent of the postsynaptic density anchoring actin filaments and receptors to the postsynaptic membrane. This assumption is supported by the demonstration of dystrophin immunoreactivity in postsynaptic densities of hippocampal (present work), cerebral cortical and cerebellar [Lidov et al., (1990) Nature 348, 725-728] synapses.

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THE EFFECT OF CNP ON THE ACTIVATION OF THE PITUITARY-ADRENAL SYSTEM INDUCED BY DIFFERENT STRESSORS

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Earlier experiments carried out in our department established that ANP (atrial natriuretic peptide) can eliminate the ether induced activation of the pituitary-adrenal system.

In the present experiments we examined the effect of CNP, which is the most common type of the natriuretic peptides in the CNS on the actions listed below.

In order to get the activation of the pituitary-adrenal system we used the following stressors: Ether stress applied for 1-1,5 minutes, restraint for 30 minute, electrical stimulation (1mA applied on the paws for 5 sec in every 15 sec during 1 minute). Plasma corticosterone level was measured by fluorescence assay.

CNP (2 μg-20 ng), which was administered intracerebroventricularly was able to reduce in a dose dependent manner the increase of the corticosterone level caused by ether stress. Although it shows a tendency of attenuation of the activation induced by restraint or electric shock, the decrease was not statistically significant.

These data show, that CNP depending on the type of the stressors, can abolish or reduce the activation of the pituitary-adrenal system, which may indicate that this neuropeptide can block only some "stress pathways" and it is not able to diminish the response induced by the sensory system.

MOLECULAR BIOLOGY OF IONOTROP GLUTAMATE RECEPTORS. EXPRESSION OF GluRl AND GluR2 SUBUNITS IN HETEROLOGOUS MAMMALIAN CELLS

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In the last decade a new group of AMPA antagonists has been investigated in Institute for Drug Research, Budapest. These 2-3 benzodiazepine based antagonists proved to be non-competitive and might be potential drug candidates. One of their well-known representative is the compound GYKI 52466. At that moment little is known about the pharmacological significance of the different AMPA receptor subtypes and that whether each of the various AMPA receptor subtypes have GYKI 52466 binding sites. As the first step of this investigation GluRl and GluR2 receptor subtypes were cloned and expressed in mammalian cells. The final goal of these experiments is to investigate different non-competitive AMPA antagonists synthetized in Institute for Drug Research using whole cell voltage clamp method.

Cloning work started with whole-cell RNA isolated from rat brain GluRl and GluR2 cDNA were prepared using RT-PCR technique and inserted into Bluescript phagemid vector (Stratagen). Sequencing experiments revealed minor differences in comparison to published sequences. cDNAs were inserted into pcDNA3 mammalian expression vector and transfection into HEK 293 cells was carried out by calcium phosphate precipitation method. Stably expressing clones were isolated, and the presence of the proper glutamate receptor subunites was verified using either Northern blot analyses or ^{3}H-AMPA binding assay. Our results showed that the level of the expression was high enough to produce detectable binding of ^{3}H-AMPA, but, up to now, it was not possible to record AMPA-induced transmembrane currents from these cells using patch clamp technique.

EFFECTS OF CHEMICAL MODIFICATION OF ARGINYL RESIDUES ON IONOTROPIC GLUTAMATE RECEPTORS

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The possible involvement of arginyl residues in the binding of ligands to ionotropic glutamate receptors and to modulatory sites in the N-methyl-D-aspartate (NMDA) receptor was assessed in porcine cortical synaptic plasma membranes after covalent modification with phenylglyoxal (PGO). Binding of [^{3}H]glutamate to the ionotropic sites was inhibited by PGO in a dose-dependent manner. The maximal effect of PGO was observed at 20 mM. At this concentration PGO also markedly reduced the binding of [^{3}H]kainate to kainate sites and slightly inhibited the binding of (S)-5-[^{3}H] fluorowillardiine to the 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors. Twenty mM PGO almost completely diminished the binding of 3-((R}carboxypiperazin4-yl)[1,2-^{3}H]propyl-l-phosphonate ([^{3}H]CPP), a competitive antagonist of the NMDA receptor. The strychnine-insensitive binding of [^{3}H]glycine to the coactivatory sites of the NMDA receptor was likewise inhibited by PGO. The glycine- and glutamate-activated binding of [^{3}H]dizocilpine, a use-dependent channel-blocker of the NMDA receptor-ionophore, was also inhibited by treatment with PGO. In protection experiments, preincubation of the membranes with nonlabeled glutamate, kainate, AMPA, CPP and glycine prevented or reduced the inhibitory effect of PGO, only dizocilpine being without effect. Our data suggest that arginine residue(s) may be directly involved in the binding of the ionotropic glutarnate receptor ligands but not in the binding of dizocilpine.

COMPARATIVE STUDY ON REACTIVE GLIOSIS AFTER STAB WOUNDS AND PARTIAL CORTICAL ABLATIONS IN DEVELOPING RATS

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Reactive gliosis follows the different kinds of lesions in the mature central nervous system and is generally considered to be one of the most likely inhibitors of axonal regeneration. The immature central nervous system, however, shows no reactive gliosis and has fair capabilities of wound repair and axonal regrowth. The precise time course of development of glial reactivity during ontogeny, however, has not been sufficiently explored. The only systematic study by Berry et al. (Acta Neurochir. Scand. Suppl. 32:31, 1983) known to us was still not based on the immunohistochemical detection of glial fibrillary acidic protein (GFAP), which is a good marker of astroglia, especially of the reactive type. The present study investigates two model systems in developing (postnatal day -PD- I to 14) rats. In the first model we can

compare the effects of stab wounds and partial cortical ablations. The question is, whether a large and persisting tissue defect can provoke reactive gliosis at that age when a small stab wound can still be repaired without residua. In the second model stab wounds were placed in the rostral and caudal parts of the cortex. The question is, whether the rostrocaudal trend of the corpus callosum formation determines a similar spatial trend in the appearance of glial reactivity in the developing cortex. The data published in this abstract are preliminary since the last experiments are still in progress.

Lesioning by the stab wounds was performed by sterile disposable needles, percutaneously. For partial cortical ablation we opened the scalp in a straight line, the soft skull was incised by a pair of sterile scissors and folded up, a part of the cortex was removed gently by suction using a waterjet pump, and then the skull wound was closed by repositioning of the reflected piece folded up and the skin was sutured. The procedure lasted approx. 5 minutes hence an ether narcosis was enough. After operation, the pups developed normally. The postoperative periods lasted for 6 to 10 days and then the animals were perfused by 4% paraformaldehyde, the brains were embedded into agarose and serial sections were cut on a Vibratome. Floating sections were incubated with antibody against GFAP and the immunoreaction was developed according to the avidin-biotinylated horseradish peroxidase method.

In 4-day-old or older animals both the sites of partial ablation and the stab wounds were surrounded by reactive gliosis within 6 days. In the second model, the glial reactivity was observed in the full length of the cortex after P4. Our former experiments presented in the previous meeting showed that in E20 rat fetuses a deep penetrating lesion resulted in porencephalia but no reactive gliosis; and cortical (but not subcortical) stab wounds can repair without residua in P0 rats. Hence, the further experiments are focused on the period P0 to P4.

INVESTIGATIONS OF THE POSITIVE MODULATION OF AMPA RECEPTORS IN VITRO AND IN VIVO

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Positive allosteric modulators of the AMPA receptors represent an interesting group of compounds which might be used as memory enhancing agents. The aim of this study was to investigate the potentiation of AMPA receptor mediated responses by such drugs in various preparations.

The potentiation of the AMPA evoked spreading depression in chicken retina by bath application of the AMPA receptor positive modulator aniracetam (ANI), cyclothiazide (CYZ), 1-BCP and two new derivates of 1-BCP were tested in vitro. Dose dependent responses to ANI (250-1000 μM), CYZ (10-40 μM), and 1-BCP (500-1500 μM) and the lack of the effects of BDP-12 (GYKI 47195) (50-1500 μM) and GYKI 47203 (50-1000 μM) were obtained.

1-BCP (500 μM), ANI (500-1000 μM) and CYZ (20-100 μM) had no significant effects on the slope of population EPSPs and the amplitude of population spikes when evoked synaptic responses were recorded from the CA1 region of slices of rat hippocampus. Monitoring the area of EPSPs using paired pulse stimulation of the Schaffer collateral-commisural pathways 1-

BCP (500-1000 μM) and ANI (1000 μM) were found to potentiate synaptic responses and also BDP-12 (GYKI 47195) (500 μM) and GYKI 47203 (200 μM) caused some increase in the responses.

Patch clamp whole-cell recordings from acutely isolated rat cerebellar Purkinje cells were used to study the effects of 1-BCP (500 μM), ANI (500-1000 μM), CYZ (20 μM) and the two new 1-BCP derivates (200 μM, respectively) on AMPA-induced inward currents, using an electronically controlled fast drug application system. The drugs displayed similar effects to those seen in the retinal spreading depression model.

1-BCP (0.5-20 mg/kg) was tested in flexor reflex studies in cats. This positive modulator caused a dose-dependent potentiation of the miografic responses to plantar stimulation. At 5 mg/kg, or higher doses seizures were also observed.

We conclude that aniracetam, cyclothiazide and 1-BCP are active in all of the four models we used. Our results with BDP-12 in hippocampal slices are in agreement with data reported by Arai et al. (1996). BDP-12 and its congener GYKI 47203 had no significant effects on AMPA responses in our retinal and patch clamp experiments.

DEFICITS OF SALT APPETITE AFTER MICROELECTROPHORETIC KAINATE LESIONS IN THE PALLIDUM AND ORBITOFRONTAL CORTEX

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The globus pallidus (GP) and the ventral-lateral prefrontal (orbitofrontal) cortex (OBF) are known to be involved in various aspects of the central regulation of feeding. These ontogenetically, morphologically and functionally interrelated forebrain regions have also been implied in central gustatory pathways in the primate. Recent behavioral and electrophysiological findings suggest that the above structures are important taste representation areas in the rodent as well. In the present series of experiments, alterations of a specific, homeostatically determined, feeding-associated gustatory function, the salt (or sodium) appetite was investigated in kainate lesioned and control CFY rats. Kainate (or its vehiculum in the sham operations) was administered microelectrophoretically either into the ventral-medial subdivision of the GP or into the rostrocaudal middle part of the OBF. Subsequent to preoperative control tests, 12 h and 24 h intake of two concentrations (0.3 M and 0.5 M) of sodium chloride (NaCl), with or without furosemide pretreatment, was compared to water consumptions of identical intervals in a two bottle test paradigm. After repeated s.c. injections of furosemide, only a slight decrease of intake of the milder NaCl was found in both kinds of lesioned groups compared to controls. However, when preference scores [NaCl intake / total fluid intake (H_2O+NaCl)] were calculated, remarkably reduced preference, i.e., salt appetite deficit of the GP or OBF lesioned animals was clearly detectable. The present data,

along with previous findings and results of our parallel experiments, indicate that neurons of the GP and OBF are involved in complex, feeding-associated gustatory information processing.

FUNCTIONAL HETEROGENEITY OF ORBITOFRONTAL NEURONS IN THE RAT AND RHESUS MONKEY

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The orbitofrontal cortex (OBF) in the primate and its homologous area, the ventral-lateral prefrontal cortex (vlPFC=OBF) in the rodent are well known for their important roles in several processes of the central regulation of feeding. Although its importance as a secondary relay in the central taste pathway of the monkey is widely discussed, its gustatory attributes in the rat are very poorly understood. At the same time, only a few studies aimed so far to elucidate neurochemical properties of orbitofrontal cells in both species. The present experiments, therefore, were designed to provide complex characterization of feeding-associated endogenous and exogenous chemosensory attributes of OBF neurons in the rat and rhesus monkey. To do so, extracellular single neuron activity was recorded in the OBF of anesthetized rats and anesthetized or alert rhesus monkeys by means of carbon fiber multibarreled glass microelectrodes during: 1.) microelectrophoretic administration of glucose and other chemicals as well as during 2.) gustatory stimulations. Specific, feeding-related cells, both the so-called *glucose-sensitive* (GS) (whose activity is suppressed by microelectrophoretically applied glucose) and *glucose-receptor* (GR) units (whose firing rate is increased to glucose) have been identified in all kinds of preparations. These very same neurons were also likely to respond to one or more taste qualities (injected via chronically implanted intraoral tubing). Gustatory neurons in the rat (less in the monkey) OBF were found to be narrowly tuned. The majority of (both endo- and exogenously) chemosensory cells exhibited sensitivity to catecholamines and/or other neurochemicals (glutamate, GABA, Ach, interleukin 1ß, etc.) as well. The present studies provided evidence for the existence of glucose-detecting, at the same time gustatory, cells both in the primate and rodent OBF. Our results, along with previous data, indicate important associative roles of these neurons in the central feeding control.

TASTE REACTIVITY ALTERATIONS AFTER PALLIDAL AND ORBITOFRONTAL CORTICAL KAINATE MICROLESIONS IN THE RAT

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Our parallel behavioral experiments revealed alterations of taste-associated learning abilities and sodium appetite of globus pallidus (GP) and ventral-lateral prefrontal (orbitofrontal) cortex (OBF) lesioned animals. To further characterize their gustatory functions, a generally accepted technique, the taste reactivity test was performed in CFY male rats, previously subjected to bilateral microelectrophoretic kainate lesions in either the GP or OBF, and the results were compared to those obtained in sham operated control animals. Two concentrations of sucrose, sodium chloride, monosodium-L-glutamate, hydrochloric acid and quinine hydrochloride were injected via intraorally implanted polyethylene cannula both in the lesioned and control rats. Gustatory stimulation elicited behavioral changes, i.e., ingestive or aversive activity patterns were videotaped and 'off-line', frame-by-frame analyzed. Though OBF lesioned animals were somewhat more likely to be affected by tastants than those with GP damages, both groups of the kainate treated rats showed poor responsiveness to taste stimuli. Similar to the controls, there was no significant difference in the incidence of ingestive or aversive behavioral patterns in these animals. Our present findings, along with data of previous, behavioral and single unit recording experiments, suggest a major role of GP and OBF neurons in the central taste information processing. Whether the above alterations are due to a reduced taste-sensitivity in these lesioned rats remains to be further studied.

DOMAIN-RESTRICTED EXPRESSION OF TWO GAD GENES DURING MOUSE EMBRYOGENESIS

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Glutamic acid decarboxylase (GAD) is the biosynthetic enzyme for GABA the major inhibitory neurotransmitter in the CNS of vertebrates. In rodents, two forms of GAD have been characterized (GAD65 and GAD67), products of distinct genes. They show high degree of evolutionary conservation and share almost identical cellular distribution in adult brain. We have found, that the two genes are also expressed, at both mRNA and protein levels, during mouse embryonic development. This suggests GAD and GABA might play a specific developmental role. To further explore this hypothesis, we sought to examine the pattern of

expression of GAD67 and GAD65 in mouse midgestation embryos (stages E10.5-E12.5) by *in situ* hybridization using radiolabeled and digoxigenin-labeled GAD67 and GAD65 cRNAs as probes. Our results could be summarized as follows: 1) The hybridization signal in the embryonic CNS, even at early stages of neuronal differentiation (E10.5) was detected in domains with sharply defined boundaries; 2) These domains overlap with expression domains of some homeotic and paired (Pax) genes implicated in the CNS patterning; 3) GAD67 and GAD65 transcripts are colocalized almost exclusively in cells of the *subventricular* zone and rarely to single cells in the ventricular zone; 4) The intensity of hybridization signal increases with age in some sites, others, however exhibit only transient expression. 5) During stages E10.5-E12.5 GAD expression in brain structures with a laminar organization, like cortex, hippocampus, cerebellum was very low or missing. Our results support the idea for a specific role of GABA in the differentiation of the GABAergic neurons in the mouse CNS. Furthermore, the commitment to a GABAergic neuronal phenotype probably follows a specific timecourse in each GAD embryonic expression domain.

SOMATOSTATIN-CONTAINING INTERNEURONS IN THE HIPPOCAMPUS: CORRELATION OF CONNECTIVITY AND NEUROCHEMICAL PROPERTIES

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Somatostatin (SOM)-containing interneurons form a well-defined subpopulation of hippocampal interneurons. However, recent results suggest that these cells are heterogenous with regard to their neurochemical marker content. In the present study we investigated whether the SOM-immunoreactive cell population overlaps with distinct interneuron types, immunoreactive for calbindin (CB)- or calretinin (CR). Three different subtypes of SOM-immunoreactive cells have been identified on the basis of the colocalization data. The first type was located in str. oriens of the CA3 and CA1 subfields of the hippocampus. They contained CB, and constituted 32% of all SOM-containing neurons (87% of all CB-immunoreactive cells) in this layer. This type of SOM-immunoreactive neurons presumably project to the medial septum. Thus, according to the present results, SOM is also present in the majority of GABAergic neurons involved in the hippocampo-septal feedback. The second type of SOM-positive cells was located in the hilus and str. lucidum of the CA3 region, and they colocalized with CR. All spiny (but none of the aspiny) CR-containing cells were positive for SOM, while they constituted 5% of all SOM-containing neurons. The third type was negative for both calcium bindig proteins. We showed that, in the CA1 region they arborize within the hippocampus, particularly in str. lacunosum-moleculare. The postsynaptic targets of SOM-immunoreactive boutons in this layer were mostly GABA-negative, which supports the hypothesis that these cells participate in feed-back dendritic inhibition of principal cells in the entorhinal termination zone. We also conclude that the presence or absence of calcium binding proteins in specific subsets of SOM-containing interneurons is accompanied by differences in their connectivity features.

DISTRIBUTED PROCESSING OF SENSORY INFORMATION AND MODULATION BY FEEDBACK FROM MOTONEURONES IN THE FEEDING NETWORK OF *LYMNAEA STAGNALIS*

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Previous models of the organization of the neuronal circuitry underlying feeding in *Lymnaea* assumed that sensory inputs activated the system hierarchically, in the following order: modulatory neurones~central pattern generator (CPG) interneurones~motoneurones (Benjamin and Elliott, 1989). The motoneurones in this model were thought to be passive followers of the interneurones, their only role being the activation of the feeding muscles.

We investigated the validity of these assumptions in semi-intact preparations where fictive feeding can be triggered by either sensory stimuli or activation of modulatory interneurones and multiple electrophysiological recordings can be made from different types of neurones in the feeding network. We have already shown that, in contrast with the hierarchical model, chemosensory stimuli can activate the feeding system at various different levels, from modulatory to CPG interneurones, which, in turn, interact in a distributed rather than a hierarchical manner (Yeoman et al., 1995; Staras et al., 1995). We also performed new experiments in which we systematically depolarized or hyperpolarized motoneurones in the feeding system during ongoing fictive feeding in order to study their possible contribution to the generation of the rhythmic pattern. The investigation of the effect of removal of motoneurones from the circuitry by hyperpolarization was supplemented by laser photoinactivation experiments. We now demonstrate that certain types of motoneurones, which, up to now, have been thought to be passive followers of the CPG interneurones, are capable of modulating the rhythm generated by the CPG interneurones. This, in addition to the distributed rather than hierarchical nature of the effect of sensory inputs, makes the neuronal model of the feeding system much more complex than previously thought. This complexity may help explain the high level of variability and flexibility found in this system in both behavioural experiments with intact animals and electrophysiological experiments with semi-intact preparations.

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ON DIFFERENCES BETWEEN SOMATIC AND DENDRITIC INHIBITION

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We studied the specific effects of synaptic inhibition on the firing patterns of hippocampal CA3 pyramidal cells. The starting point of our simulations was Traub's bursting model (1991). Our simulations were done using the GENESIS neural modeling tool. As an experimental background to our computational study first we sought to reproduce the results of Miles et al. (1996) who examined the differences between inhibitory cells whose terminals contact pyramidal cell somata and those that form synapses with pyramidal cell dendrites.

The simulation experiments discovered many quantitative details of the effects of the location, timing, kinetics and strength of the IPSPs transferred by GABA-A synapses. The model reproduced the suppression of (i) the repetitive firing by somatic inhibition, and (ii) the slow phase of dendritic burst by dendritic inhibition as observed experimentally. Our results suggest that the differences between the shapes of somatic and dendritic IPSPs - as measured at the soma - can be explained by cable theory alone. Although there might be large differences between kinetics, the most important factor in determining the effectiveness of IPSPs seems to be (1) maximal conductance, (2) the dynamics and the state of the membrane. Especially with case of a multi-compartmental phenomena - such as complex bursting - we can expect dramatically different effects from the same IPSP arriving at functionally disjoint segments of the membrane.

Unitary inhibition was not successful in suppressing the burst phenomenon. Repetitive IPSPs were needed whose timing was critical in the submillisecond range. Somatic inhibition could not modulate the burst only delay its generation or when timed appropriately suppress it altogether. The same inhibition arriving at slightly (1 msec) different timing sometimes failed to suppress the burst.

Dendritic inhibition had a drastically different effect on the bursting event. Although it could modulate the somatic burst generation, and even change the burst into repetitive firing, it was hardly able to suppress it altogether. We have also shown an instance of paradoxical inhibition when due to dendritic IPSPs more spikes appeared at the axon IS than without it.

In addition, based on our simulations we hypothesize that the complex-burst behaves as an information unit for somatic but not for dendritic inhibition. Basket cells impinging primanly on perisomatic membrane segments of hippocampal pyramidal cells might deal with somatic bursts as units. The basic operations they can perform are suppression and delay. In contrast interneurons impinging on dendritic membrane segments could modulate somatic bursts only in

graded manner thus looking at spikes as separate units. Thus our simulations suggests that the question, whether or not bursts are units of neural information (Lisman 1997) cannot be answered by a simple 'yes-or-no' statement.

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INFORMATION PROCESSING AND REPRESENTATION IN SCHIZOPHRENIA: A COGNITIVE NEUROPSYCHIATRIC APPROACH

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Cognitive theories of schizophrenia emphasize the disorder of semantic memory when objects of reality are classified on the basis of their conceptual meaning. Research was designed to investigate perceptual (presemantic) categorization in schizophrenia: in this case the shared structural (and not semantic) features of category exemplars are represented. In graded categories (GCs) it is a summary, averaged prototypal representation, while discreet categories' (DCs) features determine category-membership in an all-or-none fashion. In the first part of the experiment, (i) subjects were presented the members of two GCs of geometrical shapes to induce initial representation. This phase was followed by (ii) the verbal definition of categories and (iii) feed-back guided learning. In the second part, instances of two DCs were presented to extract category-relevant cue-feature. Each phase was tested with presenting new exemplars. We found that (1) in the schizophrenia group the categorization performance for GCs was below the control group's performance after initial induction, (2) while the effect of verbal correction was similar. (3) Improvement after feed-back guided leaming was lower in schizophrenia, but (4) DC-performance appeared intact. These results suggest the impairment of realty-abstraction and representation on a perceptual (presemantic) level, which points to the dysfunction of modality-specific neocortical areas.

THE EFFECTS OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE ON THE ARACHIDONATE CASCADE OF RAT PLATELETS AND ON ARACHIDONIC ACID-INDUCED PLATELET AGGREGATION

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Pituitary adenylate cyclase-activating polypeptide (PACAP) has recently been shown to increase the cAMP level in platelets. An elevated cAMP level is known to inhibit platelet activation. This suggests that PACAP may inhibit the functions of platelets. The arachidonate cascade is one of the most important inter- and intracellular messenger system involved in the regulation of platelet activation. It has been reported that cAMP inhibits the activation of platelet phospholipases, which raises the possibility of a decreased release of arachidonic acid. In order to test the latter hypothesis, the effects of PACAP were studied on platelet aggregation and on the arachidonate cascade of platelets. During the examination of the arachidonate cascade, the washed platelets were labelled with ^{14}C-arachidonic acid, and the ^{14}C-eicosanoids were then separated by means of overpressure thin-layer chromatography and the radioactivity was determined in a Packard Tri-Carb 2100TR liquid scintillation analyzer. Platelet aggregation was monitored in a modified Born aggregometer. Changes in light transmission of washed platelet suspensions were continuously recorded at 620 nm. PACAP inhibited the arachidonic acid-induced platelet aggregation, without modifying the activity of phospholipases, and decreased the syntheses of TxA_2 and $PGF_{2\alpha}$ in platelets. It is concluded that, besides elevating the intracellular cAMP level, PACAP may inhibit platelet functions by attenuating the synthesis of TxA_2.

CHANGES IN NEURONAL ACTIVITY OF TRIGEMINAL BRAINSTEM NUCLEI FOLLOWING INFRAORBITAL NERVE TRANSECTION AND REGENERATION IN ADULT RAT

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Although there have been many advances in the medical treatment of nerve injuries, patiens still complain of unpleasant sensations and faulty sensory localization after suffering peripheral nerve transection.

The rat trigeminal system has become an important model for the study of these kinds of phenomena. The course of vibrissal sensory receptor denervation and its subsequent reinnervation was studied following transection of the rat infraorbital nerve. The right infraorbital nerve was exposed, transected and the stumps readjusted carefully.

Possible peripheral injury-induced changes in electrophysiological properties of neurons (response latency, receptive field) in the trigeminal nucleus principalis and subnucleus

interpolaris were evaluated. The neuronal activity in the brainstem trigeminal nuclei evoked by vibrissa stimulation reappeared between 18 and 25 days after surgery. Response latency in most of the recorded neurons was significantly longer in the operated animals than in controls. In most cases, vibrissae from rats subjected to transection exhibited enlarged receptive fields which may be considered as an evidence of misdirected axons and abnormally reinnervated receptors throughout the course of regeneration.

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PARTICIPATION OF GLUTAMATE/ASPARTATE-CONTAING FIBRES, ORIGNATING IN SUPRAMAMMILLARY NEURONS, IN THE EXCITATORY INNERVATION OF SEPTAL AND HIPPOCAMPAL AREAS OF THE RAT BRAIN. A COMBINATION OF [^{3}H]D-ASPARTATE AUTORADIOGRAPHY AND IMMUNOCYTOCHEMISTRY

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Recent studies have demonstrated that, in the lateral septum, in addition to the hippocamposeptal excitatory amino acid-containing axon terminals, a large number of other boutons establish asymmetric synaptic contacts. Also results from studies on the area- and layer-selective termination pattern of supramammillary (SUM) afferents in the rat hippocampus, have shown that SUM neurons projecting to the hippocampus contain calretinin (CR).

We suggest that SUM CR-containing neurons projecting either to the septal or the hippocampal complex contain the excitatory transmitter aspartate/glutamate. Furthermore, we theorize that these SUM neurons innervate septal cells, including septohippocampal projection neurons and that the excitatory fibers arising from the SUM CR-containing cells supply the innervation of the dentate gyrus and the CA2-CA3a subfields in the hippocampus.

Two days before killing, [^{3}H]-D-Aspartate (DA) was injected unilaterally into different areas of 1) the lateral and medial (MSDB) septum, or 2) the hippocampus. After transcardial perfusion, vibratome sections (50 μm) from the SUM area were immunostained for CR, dipped in a nuclear emulsion and stored in the dark for 4-8 weeks. The sections were developed in D19 and coverslipped for light microscopic observation.

In order to examine the relationship between cholinergic (MSDB) or calbindin (CB) containing (lateral septum) neurons and CR-immunoreactive axon terminals, correlated light and electron microscopic double immunostaining experiments were performed.

In the case of septal injections of the radiolabeled D-aspartate, a large number of autoradiographically (AR), [^{3}H]-DA labeled neurons could be observed in the SUM area, and ipsilateral hippocampus. All of the AR-labeled hippocampal cells appeared to be pyramidal neurons. Three major types of neurons could be demonstrated in the SUM: 1) neurons containing only CR, 2) a few cells which were only AR-lalebed with [3]-DA, and 3) double labeled neurons containing both aspartate/glutamate and CR. In the oro-caudal direction, the double labeled neurons were homogeneously distributed in the lateral part of the SUM area.

Results of a unilateral septal undercut demonstrated that only the extrinsic CR-axons oliginating in the SUM are aspartate/glutamatergic. These fibers which show asymmetric

synaptic contacts at the EM level, terminate on both CB and cholinergic neurons in the septum. After large injection of [^{3}H]-DA into the temporal hippocampus, radiolabeled CR-neurons were found to be in the lateral part of the SUM area, near the cross-section of the mammillothalamic tract (mt). At this type of the injection, the AR-labeled CR-positive cells were present mainly in the anterior part of the SUM region and localized on the area ventro-lateral to the mt. In conclusion, the recent study demonstrated intrinsic and extrinsic CR systems in the septal complex. The latter is an aspartate/glutamatergic system, which originates in the SUM and forms asymmetric synapses with CB neurons in the lateral septum and cholinergic cells in the MS. The connections of the latter may be essential in hippocampal type 2 theta rhythm generation.

Both the morphological and electrophysiological data are controversial with regard to the excitatory or inhibitory nature of the SUM-hippocampal neuronal connection. The morphological data that are available on the transmitter content of the hippocampal afferents that originate in the SUM nuclei are not sufficient to attribute either on inhibitory or excitatory function to this pathway. The present observations unequivocally provide evidence for the presence of an excitatory transmitter in a population of the supramammillo-hippocampal projection neurons.

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SUBSTANCE P INNERVATION OF CHOLINERGIC AND GABAergic NEURONS OF THE RAT AND MONKEY MEDIAL SEPTAL AREA. A COMPARATIVE IMMUNOHISTOCHEMICAL ANALYSIS

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Lateral septal subregions receive a very rich peptidergic innervation including substance P (SP)-containing fibers, which form pericellular baskets around calbindin (CB)-containing neurons (Szeidemann et al., 1995). Recent studies have demonstrated that SP receptor mRNA is also localized in choline acetyltransferase (ChAT) mRNA-containing neurons in the medial septum and basal forebrain cell groups of the rat, in which the SP receptor appears to be expressed selectively by cholinergic neurons (Gerfen, 1991). In an earlier investigation, SP was reported to excite neurons within the medial septum-diagonal band (MSDB) complex of the rat (Miller, 1981). However, no data is available so far on the SP innervation of medial septal neurons either in the rat or the monkey. This prompted us to examine the SP innervation of neurons localized in the rat MSDB complex in comparison to that of monkeys.

To examine the relationship between SP-immunoreactive fibers and neurochemically identified neurons in the MSDB complex of the rat and monkey, animals were perfused with either a) 1% acrolein containing 3% paraformaldehyde in 0.1 M phosphate buffer (PB), or b) 0.1% glutaraldehyde containing 4% paraformaldehyde in PB. Fifty (50) µm vibratome sections were cut from the septum. The neuronal organization ofthe MSDB was first investigated in the African green monkey *(Cercopithecus aethiops)* using single immunostaining for ChAT, parvalbumin (PA) and CB. Light microscopic double

immunostaining for SP and neurochemically identified MSDB target cells was done for both species using the avidin-biotin-peroxidase (ABC) and the peroxidase-antiperoxidase (PAP) methods. For analyzing the synaptic membrane specializations between the axon terminals and identified target elements, correlated light and electron microscopic analyses were performed on rat and monkey sections.

The most significant difference that we found in the neuronal organization between the rat and monkey MSDB complex was that no PA immunostained neurons were seen in the midline area of the monkey medial septum. Instead, a large number of CB-positive cells were distributed in this region. It is well known that the rat MSDB complex contains the majority of the septohippocampal projection neurons including the medially-located PA-containing GABAergic and the laterally distributed cholinergic cells (Kiss, J. et al., 1990). PA-immunoreactive neurons in the monkey septum were found only in the intermedio-lateral septal area (iLS), near the border between the medial and lateral septal regions. These large, multipolar, PA-positive cells were never seen in the iLS area of the rat septum. The examination of septal sections double-stained for SP and ChAT7 PA or CB revealed that only ChAT-immunopositive neurons in the rat MSDB complex and both the ChAT and CB-immunoreactive neurons in the monkey medial septum were among the targets of the SP-containing axon terminals. The correlated light and electron microscopic experiments showed that all of the SP-positive axon terminals formed asymmetric synapses with somata and dendritic shafts on ChAT-positive cells both in the rat and the monkey.

In conclusion, our results demonstrate a significant difference in the neuronal organization of the septohippocampal projection system between the African green monkey and the rat. In general, the region of the rat septum that encompasses PA-containing GABAergic septohippocampal projection neurons showed no SP contacts on PA-positive cells. In contrast, neurons in the medial area of the monkey medial septum which lack PA-immunoreactivity but contain calbindin D28k protein are extensively innervated by SP-containing fibers. Further studies are required to understand the functional significance of these findings.

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THE ARBORIZATION OF AFFERENT TERMINALS IN THE MEDIAL GENICULATE BODY

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The medial geniculate body is the last subcortical relay center of the auditory pathway where specific afferents of various order from the central nucleus of inferior colliculus articulate with the soma and dendrites of geniculocortical relay cells and local interneurons. Protrusions located in the brush-like dendritic branching points of geniculocortical relay cells are surrounded by and postsynaptic to dendritic, axonal and presynaptic dendritic profiles forming from the point of view of synaptic transmission, important complex synaptic glomeruli. From observations with Golgi impregnation and axon degeneration techniques, based mostly on indirect evidence, these profiles were considered to be of cortical origin, initial axon collaterals and processes of local neurons.

The aim of the present study was to identify the axonal and dendritic components by directly detecting their parent and target cells as well as to understand on the basis of the synaptic type, their role in impulse transmission.

In the experiments microinjections of Phaseolus vulgaris-leucoagglutinin, Biotinilated Dextran Amine and Horseradish Peroxidase were administered with iontophoresis or pressure injections with Hamilton syring into the primary auditory cortex and the central nucleus of inferior colliculus in rat and cat. Following the optimum survival time samples were taken from the ventrolateral division of medial geniculate body, processed according to immunocytochemical procedures and the sections were examined under light and electron microscope. Four surgical approaches were applied.

In the first group Horseradish Peroxidase injected into the primary auditory cortex was retrogradely transported to the fusiform relay cells arranged in onion skin-like parallel layers in the ventrolateral division of the medial geniculate body. The accumulation of label enabled us to tell the projection cells and the unlabelled short axonal local interneurons apart.

In the second group fibers originating from the primary auditory cortex were anterogradely labelled with Phaseolus or Dextran Amine and observed running in parallel with the cell layers of medial geniculate body and breaking up into multiple terminals at the end of short side branches. Observed with the electron microscope in serial sections the labelled terminals established synaptic contacts with proximal dendrites and protrusions of relay cells, as well as with dendrites, dendritic spines and presynaptic dendrites of the local interneurons. A single terminal containing round vesicles and asymmetric membrane specialization frequently articulated with dendrites of two different types of neurons.

In the third group Phaseolus or Dextran Amine injected into the central nucleus of inferior colliculus anterogradely labelled the terminals of specific afferents grouped into grape-like clusters. The labelled terminals were found presynaptic in the synapses with the perikarya and dendrites of relay cells and interneurons. Some terminals established contact with dendrites of two different types and exhibited presynaptically round vesicles with pronounced postsynaptic membrane specialization.

In the fourth group, as a combined experiment, the anterogradely labelled colliculogeniculate axon terminals were found to engage in synapses with the proximal

dendrites and perikarya of geniculocortical relay cells containing retrograde marker. The labelled terminals were articulating also with presynaptic dendrites, and were involved in synaptic triads. The unlabelled profiles containing round vesicles were considered as initial axon collaterals of relay cells, ovoid vesicles were found in the axons and pleomorphic vesicles in the presynaptic dendrites of interneurons.

The specific afferents, in addition to conveying biological important information, contribute to surround inhibition via interneurons and to recruitment via the initial axon collaterals. On the other hand the cortical fibers, by direct and indirect feedback mechanisms, may modify in both directions the synaptic transmission at the level of the medial geniculate body.

L-NAME INDUCED REDUCTION OF CEREBRAL BLOOD VOLUME CAN BE RESTORED BY OPIATE RECEPTOR BLOCKADE WITH NALTREXONE

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Involvement of L-arginine-nitric oxide system and the endogenous opioid peptide system in the regulation of regional (hemispheric) blood volume (CBV) and local hypothalamic blood flow (HBF) was investigated in Urethane anesthetized, ventilated, normoxic, normocapnic, male Sprague-Dowley rats (n=8). CBV and HBF was measured simultaneously, CBV by Tomita's photoelectric method in our modification (Sándor et al., Life Sci. 1986) and HBF by Aukland's H2-gas clearance method. Selective blockade of the NO-synthase enzyme by 30 mg/l<g i.v. administered L-NAME (Nω-nitro-L-arginine methyl ester) resulted in a significant reduction of both hemispheric CBV (from 5.25±0.72 vol % to 4.13±0.51 vol %, $p<0.01$) and local HBF from (0.89±0.09 ml/g/min to 0.60±0.07 ml/g/min, $p<0.05$) while systemic arterial pressure (MAP) increased and heart rate (HR) decreased significantly. General opiate receptor blockade by 2 mg/kg i.v. administered Naltrexone caused no change in HBF, MAP and HR values of the NOS-blocked animals. CBV, however, returned to it's steady state control value (5.4±0.72 vol %) at the same time.

These findings suggest 1) major role of L-arginine-nitric oxide system in the regulation of both hemispheric CBV and local HBF of the rat, and 2) a possible involvement of opioid peptides and/or opiate receptors in the NOSblockade induced reduction of the cerebral blood volume.

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SIGNIFICANCE OF CELL-CELL INTERACTIONS FOR CYTOTOXIC EFFECTS OF EXCITATORY AMINO ACIDS

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The aim of this study was to establish a rat primer cortical neuronal system to screen the effects of compounds having glutamate-antagonistic effects within seven days after seeding the cells out to the sterile plates.

The most common ways using primer neuronal cell cultures were unusable for that purpose. The systematic changes in the methods written in reference articles have resulted a successful screening system.

The cortical cells from 1-2 days old SPRD rat pups were separated by gently trituration and seeded out to sterile, untreated 96-well plates. The cultures were ready to screen the excitatory amino acid antagonists from the fourth day. The use of insuline-selenite-transferrin media supplement during the 24-hour incubation period with 1 mM kainate, and the leaving off of the cytozine-arabinozide treating of the cells have resulted a higher sensitivity of the system. The rate of viable cells have been established by measuring the amount of lactic-dehydrogenase enzyme (LDH) in the cultures supernatants and in the living cells sticked down to the wells bottom. Above the leaving off of the trypsinization of the cortical cells, the leaving off of the poly-L-lysine coat from the 96-well plates was the most important for the cells to answer the glutamate-agonists properly. After these modifications our rat primary cortical cultures were able to detect the effectiveness of kainate specific antagonist GYKI-52466. The microscopical studies on cultures revealed the importance of developing the cell-cell contacts during the pre-test incubation period for the most effectiveness of gutamate-agonists.

TRANSCRIPTIONAL REGULATION OF THE MOUSE GENE ENCODING THE 67-KD FORM OF GLUTAMIC ACID DECARBOXYLASE

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Gamma-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the vertebrate nervous system. The rate-limiting step in GABA biosynthesis, the decarboxylation of L-glutamic acid, is catalyzed by the enzyme glutamic acid decarboxylase (GAD). In mammals, the adult brain contains at least two forms of GAD encoded by two distinct genes. The molecular masses of the two isoforms are 65-kD and 67-kD, respectively (GAD65 and GAD67). In producing GABA, the two glutamic acid decarboxylases appear to be regulated by different mechanisms. GAD65 is regulated mainly at the level of enzyme activity through the interaction with its co-factor, while GAD67 is primarily controlled through gene transcription.

We have previously identified the exon-intron structure of mouse GAD67 gene and the transcription start sites. The transcription can initiate at three different promoters (P1, P2, P3). Hence the translation start codon is found in the second exon, the protein coded by the three different mRNAs is the same. The vast majority of the transcripts initiates from the proximal promoter (P1). This promoter lacks the common promoter elements e.g. TATA and CAAT boxes, but possesses three binding sites (GC-boxes) for the general transcription factor Spl. However, both distal promoters (P2 and P3) contain TATA and CAAT-like boxes. The promoter region of GAD67 gene is located within a large (~4kb) CpG island. In order to examine the properties of the island, we sequenced a 7kb fragment containing the 5'-region of the gene, the first two exon, the first intron, and a part of the second intron. The CpG island spans the first two exons, the first intron and ends in the second intron. The P1 promoter is contained within the CpG island, while the other two (P2 and P3) are found in a region of lower CpG content. All things considered, P1 promoter resembles that of constitively expressed housekeeping genes, while P2 and P3 share characteristics of tissue specific promoters. Based on sequence homology, several putative regulatory DNA elements have been identified at the 5'-region of GAD67 gene. It is tempting to speculate that the NF-KB consensus located 500 bp upstream from the multiple promoter region, several AP2 transcription factor binding sites, two sequence motifs resemble the canonical binding site for the egr/krox transcription factors, as well as the neuron-specific silencer element (NRSE) in the the P1 promoter region might play an important role in the tissue specific regulation of GAD67 gene.

EFFECTS OF 192-IgG-SAPORIN ON CHOLINERGIC AND GALANINERGIC NEURONS IN THE BRAIN OF RAT

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Degeneration of cholinergic neurons in the different parts of the human brain is a prominent neuropathological feature of Alzheimer's disease (AD). The galanin-containing peptide fiber system which innervates acetylcholine-containing basal forebrain neurons has been shown to hypertrophy and hyperinnervate the remaining cholinergic perikarya in AD (1). In the present study we examined, whether a similar hypertrophy and/or hyperinnervation occurs within those areas in the rat brain where cholinergic neurons are present the medial septum (MS), the vertical limb of the diagonal band nucleus (VLDBN), the horizontal limb of the diagonal band nucleus (HLDBN), and the nucleus basalis magnocellularis Meynert (nbM)].

For selective destruction of the cholinergic neurons, an immunotoxin was used (2), where a monoclonal antibody (192-IgG) to the rat nerve growth factor (NGF) receptor (p75NGFr) was armed with the ribosome inactivating protein, saporin. Wiley et al. (2) discovered that 192-IgG-saporin is highly toxic to neurons expressing p75NGFr.

Injections of cholinotoxin (4.0 µg/5 µl) (Chemicon) were administered into the left lateral ventricle and after different time intervals (3, 4, 6, 8 and 12 weeks) the animals were sacrificed. Sham-operated animals received 5 µl of sodium phosphate buffer (pH 7.4).

The brains of the animals were fixed, sectioned and the distribution and structural localization of galanin was demonstrated by immunocytochemical means. The cholinergic neurons were stained immunohistochemically, using polyclonal antibody against choline acetyltransferase. The cholinoceptive structures (i.e. acetylcholinesterase-positive structures) were demonstrated histochemically. The hypertrophy of galanin-positive structures was studied in a Nikon FXA light microscope, while the hypertrophy of peptidergic structures was demonstrated with a Leica Laborlux 'S' microscope equipped with a computer-assisted image analyser (Quantimet 500).

The results show that after 4 weeks and subsequently the 192-IgG-saporin treatment reduced the numbers of cholinergic neurons in the MS, HLDBN and nbM, and in parallel the galanin-positive nerve fibers hypertrophized in the lateral part of the septum, in the dorsal parts of the hippocampus and in the NbM. The remaining neuronal perikarya in the VLDBN, HLDBN and nbM were not hyperinnervated by galaninergic fibers. Changes in galanin staining were not detected in the different areas of the cortex.

It is suggested that, after selective degeneration of cholinergic neurons and axons, in some areas of the brain the galanin-positive fibers may hypertrophy but the reduction in the cholinergic structures is not necessarily followed by an increase in the peptidergic fibers.

The results support the suggestion that 192-IgG-saporin is a powerful tool for study of the galaninergic neuronal plasiticity in the central nervous system.

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EXPRESSION OF MOLECULES MEDIATING THE EFFECT OF GLUTAMATE IN CEREBRAL ENDOTHELIAL CELLS

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Glutamic acid has been shown to be involved in a large number of neuronal processes and is believed to be an important factor in the pathogenesis of many CNS disorders like cerebral hypoxia, neurodegenerative disease and epilepsy. Two classes of molecules play a pivotal role in the regulation of glutamate effect: glutamate receptors and glutamate transporters. Beside neurons and glia, glutamate receptors and transporters may occur on cerebral endothelial cells (CECs) as well. The aim of our study was i) to determine which glutamate receptors are expressed in CECs and to find intracellular processes which can be initiated by the activation of these receptors; ii) to check for the possible expression of glutamate transporters in CECs.

By using RT-PCR we have shown that CECs express NMDA receptors (NR1 subunit which is necessary for the formation of functional NMDA receptors - and NR2A-C subunits), AMPA receptors (GLUR1-4 subunits) and metabotropic receptors (mGLUR). NMDA receptor subunit

NR2D and the kainate receptor GLUR6 could not be detected in CECs. Exposure of the cultures to 2 mM glutamate, a well-established mediator of ischemic damage, for 30 min increased significantly the phosphorylation of CAM-PK II even after 10 and 60 min recovery time. This effect could be prevented by the NMDA blocker MK-801. Moreover, exposure to glutamate induced a raise in the intracellular Ca^{++} level as revealed by electron microscopy using the oxalate-pyroantimonate method.

We have investigated the expression of mRNAs for glutamate transporter (GLT1) and glutamate/aspartate transporter (GLAST) in primary cultures of rat CECs and we could show that both transporter types are present in these cells.

Since it has been shown that glutamate is able to open the BBB, our results suggest that endothelial glutamate receptors (especially NMDA receptors) may be directly involved in the reactions of CECs in ischemic brain oedema and the CECs may play an active role in the protection of the CNS from the icreased extracellular glutamate levels.

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NEURAL NET ARCHITECTURES AND RELATED PERFORMANCES

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(1) What is architecture ? – The concept of 'architecture' is distinguished from the traditional term of 'structure'. It encloses organizational principles in space which have specific functional relevance. Background structural conditions belong into the frame of 'structure'. As a functional term it refers to temporal organization.

(2) Organizational principles viewed as architectural features (AF) of a neural network wiring. – [1] Density of wiring – It is limited by volume constraint. Probably influences complexity of dynamics. Real CNS-s have very sparse wiring. – [2] Clusters, assemblies, modules, centres, glomeruli – With or without intrinsic sub-organization. Hypernet and hypergraph concepts are required and helpful. – [3] Layers – It is a general AF in all animal phyla. Layers are also important in subroutines of composite (artificial) neural computations. Reduction of total number of units in efficient layer systems is an unsolved problem. – [4] Crossing wires – Crossbar connections assure complete connection of neurones but with disadvantages. – [5] Asymmetry in graph theoretical sense. – Among generic (random) graph properties the asymmetry is only one. It suggests neuronal individuality and argues for fragmentary representation properties of neurons. – [6] Merged convergent or divergent projections, reshuffling, delays in forward coupling. – May support finer resolutions of overlapping (in space or time) receptive field events and may also implement (logical) auto- or cross-correlations. – [7] Independent regions, autonomous centres. Controlled multicentralized organization, called starry wiring. Both independence and co-operation are possible with sparse wiring. – [8] Strong connectedness – It is also a generic graph property and supposed to be present in various CNSs. It means e.g. the mutual functional accessibility of neurons.

Architectural features can be regarded either (1) from point of view of their generation mechanisms and (2) of their occasional functional importance instead of neutrality or even

harmfulness. If an AF is recognized, these aspects merit examination. (3) In evolution generic AF emerge automatically. Other designs need control.

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EFFECT OF THAPSIGARGIN ON THE KAINATE- AND NMDA-INDUCED Ca^{2+} SIGNALS IN CHICKEN TELENCEPHALIC NEURONS

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Recent findings indicate that a large proportion of the neurotoxic Ca^{2+} during NMDA receptor activation originates from an intracellular Ca^{2+} pool. In addition, activation of both ACh, NMDA and Glu receptors mobilized Ca^{2+} from a thapsigargin-sensitive pool in cerebellar granule cells. We have found in patch clamp experiments that elevation of $[Ca^{2+}]_i$ plays crucial role in the kainate-induced depression of potassium conductances in chicken telencephalic neurons. The aim of this study was to investigate the role of the cytoplasmic Ca^{2+} pools in the $[Ca^{2+}]_i$ elevation by EAA agonists.

The $[Ca^{2+}]_i$ was monitored in a single cell or in more chosen cells using calcium sensitive dye fura-2 with an intensified charge coupled device (ICCD) camera (Photon Technology International (PTI), USA). Pressure application of KA and NMDA from micropipettes placed at the neighborhood of a few chosen cells elicited elevation of $[Ca^{2+}]_i$ in chicken neurons. After KA-induced Ca^{2+} increase, the medium over the culture was exchanged in the microscope stage to Cd^{2+} (100 μM) containing solution. The effect of kainate seen in the normal medium was decreased in the presence of Cd^{2+}. Since Cd^{2+} could prevent the effect of KA we can conclude that the Ca^{2+} entry (at least a large part of it) via VDCCs contributed to the effect of KA to induce $[Ca^{2+}]_i$ elevation in this cell culture. The above observation was confirmed when KA was applied in Ca^{2+}-free medium. In the absence of extracellular Ca^{2+}, response to KA was not observed indicating that KA needs Ca^{2+} entry from the extracellular space.

We investigated the possible involvement of intracellular Ca^{2+} stores in the effect of KA and NMDA on the $[Ca^{2+}]_i$ responses in chicken neurons. We found that in the presence of thapsigargin the KA- and NMDA-induced $[Ca^{2+}]_i$ elevation was decreased after 15 min incubation in chicken telencephalic neurons. However, in our experiments there were also cells not responding to thapsigargin treatment. Thus, more experiments are needed to prove the unique nature of the observed phenomenon since the chicken telencephalic cell culture is very heterogeneous in cell types so it is likely that the above characteristics of KA- and NMDA-induced $[Ca^{2+}]_i$ elevation is restricted to just a few cell types.

ORGANIZATION OF HIPPOCAMPAL OUTPUT TO THE ENTORHINAL AND PERIRHINAL CORTICES IN THE RAT

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A central idea in several theories of hippocampal function is that the hippocampus plays a temporally limited role in long-term memory formation (e.g. Squire, Cohen and Nadel, 1984; Squire and Zola-Morgan, 1991; Cohen and Eichenbaum, 1993; McClelland, McNaughton and O'Reilly, 1995; Buzsáki, 1996). This role is envisaged to involve a process, sometimes called memory consolidation, by which initially fragile memories become robust and persistent, leading gradually to 'permanent' instantiation in neocortical sites. Little is known about the mechanism(s) of memory consolidation. However, one anatomical implication is that hippocampal output must have access to the neocortex, either directly or through some indirect route. While it has been known for some time that the CA1 field of the hippocampus of both rodents and primates originates direct projections to the temporal cortices around the rhinal sulcus (e.g, Swanson and Cowan, 1977; Rosene and van Hoesen, 1977), the topographical and laminar organization of this projection have not been systematically studied to date, and some apparent discrepancies exist in the literature.

In the present study we used sensitive anterograde tracing and 'unfolded' maps of the distribution of CA1 terminal fibers to determine the organization of hippocampal output to the perirhinal and entorhinal cortices in the rat, with the aim of clarifying one part of hippocampal-neocortical circuitry that may mediate memory consolidation.

Under equithesin anesthesia, iontophoretic injections of *Phaseolus vulgaris* leucoagglutinin or biotinylated dextran amine were made at various septotemporal locations along the longitudinal axis in either distal (toward the subiculum) or proximal (toward CA2) portions of CAl in male rats (n=12). To date most of the injections are focused in septal and mid-septotemporal levels. Following a survival period of 6-8 days, animals were deeply anesthetized and transcardially perfused with 0.9% saline followed by a solution containing 4% paraformaldehyde, 0.05-1% glutaraldehyde, 15% picric acid in 0.1 M PB (pH 7.3). The brains were then serially sectioned (60 μm) in the coronal plane on a vibratome, washed extensively with PB, cryoprotected overnight at 8-10°C, and finally freeze-thawed with liquid nitrogen. Injection sites and labeled fibers were visualized using standard immunocytochemical techniques in coronal sections (Freund, 1993). Adjacent series of sections were stained for Nissl, parvalbumin, or calretinin to aid in the identification of entorhinal and perirhinal boundaries. The nomenclatures of Insausti, Herrero, and Witter (in press) and Burwell, Witter and Amaral (1995) were used to define the entorhinal and perirhinal cortices, respectively. To appreciate the areal and laminar distribution of CAl projections, the density of labeled fibers in superficial layers (I-III) and deep cortical layers (V-VI) were plotted onto separate unfolded cortical maps following a modification (Suzuki and Amaral, 1996) of the method described by Van Essen and Maunsell (1980).

The regional and laminar pattern of CAl projections was related to both the septotemporal and proximodistal axes of CAl. At mid septotemporal locations, injections in distal CAl resulted in terminal fibers in layer VI of caudal portions of perirhinal cortex (areas 35 and postrhinal) and layers V, III, II, and I of caudolateral portions of entorhinal cortex (areas

DLE, DIE, and lateral parts of CE). In the entorhinal cortex, after such injections, fibers were observed to enter layers III-I only in the caudal part of the projection, whereas a relatively denser plexus was present in layer V throughout the entorhinal terminal field. At septal locations, injections in proximal CA1 resulted in terminal fibers only in layers V and III of caudomedial areas of entorhinal cortex (area CE); after such injections no terminal fibers were observed in any region of perirhinal cortex. One injection in distal CA1 at a temporal location, resulted in terminal fibers in layers V, III, II, and I of mid-rostrocaudal to caudal portions of ventral entorhinal cortex (areas DIE, VIE, and ME); no terminal fibers were observed in any region of perirhinal cortex after this temporal injection. In summary all regions of CA1 that were studied projected to the entorhinal cortex, while only the *distal* portions of approximately the *septal* one-half of CA1 projected to the perirhinal cortex. In general CA1 projections to entorhinal cortex distributed fibers to superficial layers (especially laterally) in addition to the more dense distribution in layer V, while those projections to perirhinal/postrhinal cortex were restricted to layer VI.

The present results, together with previous anatomical findings, prompt two comments about the organization of hippocampal output that will be relevant for understanding memory consolidation. First, because hippocampal output reaches neocortex indirectly through its connections with the perirhinal and entorhinal cortices (among other indirect routes), it appears that the product of hippocampal information processing requires additional synaptic mediation before permanent memory instantiation in neocortex. Second, a clue about the nature of this mediation may lie in understanding the relationship between the laminar organization of hippocampal input pathways and the laminar targets of hippocampal output. Because projections to CA1 originate from superficial layers of both the entorhinal and perirhinal cortices (Witter and Groenewegen, 1984), the finding that CA1 axons distribute to both superficial and deep layers of the entorhinal cortex on the one hand and only one deep layer of the perirhinal cortex on the other, the question arises as to whether hippocampal output has differential feedback influence on these two cortical-hippocampal pathways. A first step in answering this question lies in identifying the postsynaptic targets of CA1 axons in the entorhinal and perirhinal cortices.

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COLOCALIZATION OF SEROTONIN AND FMRFAMIDE-LIKE IMMUNOREACTIVITIES IN THE NERVOUS SYSTEM OF THE EARTHWORM, *EISENIA FETIDA*

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The aim of the present study has been to show colocalization of serotonin and FMRFamide-like immunoreactivities in the central nervous system of the earthworm, *Eisenia fetida.*

Alternate serial sections were stained with antisera raised against serotonin and FMRFamide, using the peroxidase-antiperoxidase method. The immunostained serial sections were compared on reconstructed images with the aid of NIH Image and Neurolucida programs.

Although there are a large number of serotonin and FMRFamide-like immunoreactive perikarya in the central nervous system of *Eisenia,* colocalization of these substances is comparatively infrequent. In the cerebral ganglion no cells were found that stained for both antisera. In the subesophageal and ventral cord ganglia the neurons stained for both antisera represent approximately 17-23% and 10-14% of the serotonin-, 7-11% and 9-17% of the FMRFamide-like immunopositive cells, respectively.

It is suggested that FMRFamide-related peptides may act as neuromodulators on serotoninerg neurons, influencing mainly innervation of muscles.

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THE EFFECT OF VASOACTIVE INTESTINAL POLYPEPTIDE ON MORPHINE TOLERANCE AND DEPENDENCE IN MICE

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Vasoactive intestinal polypeptide (VIP) which was first isolated from porcine small intestine is containing 28 amino acids. VIP has numerous effects on peripheral organs and the central nervous system. It has also effects on the endocrine and immune system. The effect of VIP on pain has not yet been clarified therefore, in the present study we aimed to investigate the effect of vasoactive intestinal polypeptide on pain sensitivity and on opiate tolerance and dependence in intact mice.

The analgesic effect was tested by hot plate and tail-flick methods. Change of rectal temperature, body weight and latency of stereotyped motion behavior was assessed as morphine withdrawal signs. VIP itself was analgesic when given alone either peripherally (subcutaneously, sc.) or centrally (intracerebroventricularly, icv.). Naloxone treatment abolished this analgesic effect. VIP was able to decrease the analgesic effect of morphine (sc.) and chronic tolerance to morphine. Morphine withdrawal was also diminished after VIP pretreatment.

Our findings indicate that vasoactive intestinal polypeptide may play a role in pain sensitivity. VIP is able to modify morphine tolerance and withdrawal, and that an opiate component may play a role in this effect.

ASTROGLIAL REACTIONS TO MECHANICAL INJURY, IN VIVO AND IN VITRO

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Astroglial responses to mechanical injuries were investigated in cultures of embryonic and postnatal rat forebrain cells and also, in adult rat brains. The changes in the accumulation of nestin and glial fibrillary acidic protein (GFAP) intermediary filaments were immunostained and assayed by Western blot analysis. At the sites of injury, increased nestin immunoreactivity was revealed in both in vivo and in vitro. In vivo, the accumulation of nestin-immunopositive filaments preceeded that of the GFAP-reactive material in injured cortical and striatal brain tissues. *In vitro,* increased nestin immunoreactivity was found in the edges of cells expanding into the wound, regardless of their proliferative status. The observation suggests that nestin accumulation may indicate the locomotory activity of the cells (migration or process

elongation), rather than their progenitor-like nature. As expected, progenitor cells characterized by high locomotory activity display strong nestin immunoreactivity, but not all of the nestin-positive cells are necessarily progenitors.

The validity of scratch-wounded cultures as in vitro models for post-traumal changes has also been investigated. Alterations in *in vitro* cell proliferation were followed by bromodeoxyuridine (BrdU) staining to visualise dividing cells after various post-trauma periods. Studies on the distribution of BrdU-positive cells gave some insight into the time-scale of cell production by cultivated astrocytes lining the wounded areas. The data indicated that the latency of trauma-induced cell division decreased with the in vivo age of the source material. The replacement of decayed cells seemed to be accelerated in older cultures.

Also, assays on (tumor necrosis factor) TNFα-production by cells in in injured cultures indicated certain developmental stage-dependency of the post-traumal reactions. In neuron-enriched cultures derived from embryonic brain tissue, mechanical injury resulted in enhancement of TNFα-activity in the fluid environment of the cells. On the other hand, in cultures containing mainly astrocytes derived from older brain material, the TNFα-activity decreased as a consequence of mechanical trauma.

TERMINATION PATTERN OF THE SECOND-ORDER VESTIBULOCOCHLEAR FIBERS IN THE FROG BRAINSTEM AND SPINAL CORD

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Based on labelling the branches of the VIIIth cranial nerve we have found that the primary vestibulocochlear nuclear complex have the same parts in the frog as in higher vertebrates. The Phaseolus vulgaris-leucoagglutinin (PHA-L) injections into the vestibular and cochlear nuclei revealed that the projections of the second-order vestibulocochlear fibers are also similar to that of the mammalian species. The aim of this experiment was to identify both the postsynaptic targets and the neurochemistry of axons originating from vestibulocochlear nuclear complex. In this study the vestibular terminals were analysed in the spinal cord and in the oculomotor nucleus; the cochlear terminals were studied in the principal nucleus of torus semicircularis (TP). Phaseolus vulgaris-leucoagglutinin (PHA-L) was injected either into the (LVN) or cochlear nucleus (CN), respectively. On the consecutive ultrathin sections GABA and glycin immunocytochemistry were processed.

Descending projections of the LVN can be followed as far as the lumbar segments of spinal cord, giving rise to collaterals terminating mainly in the medial part of gray matter. The PHA-L labeled terminals form exclusively symmetrical synapses on both the cell body and proximal dendrites of motoneurons. Ascending fibers from the LVN terminate in the oculomotor nucleus bilaterally, with an ipsilateral dominance. The vestibular terminals make symmetrical synapses on proximal dendrites and somata of neurons of the oculomotor nucleus. The PHA-L telminals were GABAergic both in the spinal cord and oculomotor nucleus.

The majority of the ascending second order cochlear fibers terminate in the TP. The labelled terminals form symmetrical synapses on the proximal and asymmetrical synapses on

the distal dendrites. The symmetrical synapses were positive for glycin in 10 percent, no GABA positive cochlear terminals were detected in the TP.

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METHODS FOR THE DETERMINATION OF SSAO ACTIVITY ON VARIOUS BIOLOGICAL SAMPLES

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Semicarbazide-sensitive amine oxidase (SSAO) belongs to a subgroup of amine oxidases in human sera and smooth muscle cells. SSAO is not affected by monoamine oxidase (MAO) inhibitors such as chlorgyline, pargyline or deprenyl, however, shows sensitivity to semicarbazide or other hidrazines. Its physiological role and main substrate have not been determined. A number of biogenic amines (tyramine, tryptamine, dopamine, etc.) are in vitro good substrates for the enzyme. The oxidative desamination of allylamine to acrolein or methylamine to formaldehyde may be responsible for the endothelial cytotoxicity of SSAO.

The elevation of SSAO activity was detected in diabetes, congestive heart failure and hepatic cirrhosis. The activity of the enzyme is lowered in cancer, after severe burns or in the course of glucocorticoid therapy.

Our presentation considers the possibilities of the detection of human SSAO and compares the advantages and disadvantages of fluorimetric and radiometric measurements. We report here a simple and sensitive radiometric method using ^{14}C-benzylamine as substrate for the measurement of SSAO activity in human plasma. The assay shows good linearity and reproducibility. In healthy controls the measured SSAO activity was 0.056±0.015 nmol/hour/mg protein.

EFFECTS OF CHLORALOSE ADMINISTRATION ON THE BACKGROUND AND EVOKED ACTIVITES OF POSTGANGLIONIC SYMPATHETIC NERVES IN CATS ANESTHETIZED WITH URETHANE

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In the neurophysiology of the sympathetic activity, the body of knowledge has been built up, first of all, of the results of animal experiments performed in chloralose or chloraloseurethane anesthesia (cf. Sato and Schmidt, 1973), although there have been indications (e.g. those of

Molnár et al., 1969) that chloralose might deeply influence the integration of some sympathetic reactions.

In order to get closer to the nature of chloralose-induced changes of sympathetic activity, cats were anesthetized with urethane (600-800 mg/kg, iv.) and immobilized with pipecuronium bromide (60 μg/kg, iv.). Artifical ventilation was adjusted to maintain end-tidal CO_2 at about 4%. Monophasic compound action potentials of renal and vertebral sympathetic postganglionic efferent nerve branches (innervating blood vessels of a visceral organ and skeletal muscles, respectively), ECG, arterial blood pressure, ventilation, and capnogram were continuously recorded. Somatosympathetic reflexes were elicited by repetitive stimulations (series of single impulses or short trains of stimuli [3-5 shocks at 100 Hz]) of the central stump of cut tibial or brachial nerves. Spontaneous and evoked activities of both sympathetic outputs were compared before and after chloralose administration (50 mg/kg, iv.).

The results showed that the background activity was altered by chloralose in such a way that the still synchronized discharges seemed to lose partially or totally their cardiac and respiratory (ventilatory) rhythmicity. In the evoked reactions, the inhibitory component ('silent period') became longer and more marked, giving rise thereby to more distinct reflex volleys. As for the destinations of the sympathetic nerves studied, administration of chloralose usually brought about differential effects.

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INVESTIGATION OF SPECIFICITY OF NADPH-DIAPHORASE HISTOCHEMICAL REACTION IN THE CENTRAL NERVOUS SYSTEM OF SOME INVERTEBRATE SPECIES

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Histochemical demonstration of NADPH-dependent diaphorase (NADPHd) activity is widely used as a marker for nitric oxide synthase (NOS) in the nervous system of both vertebrates and invertebrates. It is generally accepted that after short time paraformaldehyde fixation NOS containing neurons could only be stained using ß-NADPH as substrate.

In this study we have investigated the distribution of selectively stained cells in the central nervous system (CNS) of *Porcellio scaber* (Crustacea) using various experimental protocols. The effect of fixation time (40 minutes-72 hours), quality of fixative (4% paraformaldehyde,

2.5% glutaraldehyde) and substrate specifity (α-NADPH, ß-NADPH, α-NADH, ß-NADH, ß-NAD, ß-$NADP^+$, D-glucose-6-phosphate) of labelled cells were investigated. In some experiments the CNS *of Eisenia fetida* (Annelida) and *Leucocephaea maderae* (Insecta) were used as controls. We established that some neurons in the CNS of *P. scaber* could be stained after short-term paraformaldehyde fixation using α-NADPH, ß-NADPH, α-NADH and ß-NADH as substrate. After 3 hours fixation the number and distribution of labelled cells were identical in all ganglia if the incubating solution contained either ß-NADPH or ß-NADH. Markedly stained neurons were also found after incubation with α-NADPH and α-NADH though their distribution showed only partial identity with labelled cells using ß-NADPH. No specific staining occurred in the ganglia of control species. After glutaraldehyde fixation a few selectively stained neurons and prominent neuronal fibre network were revealed in all ganglia of *P. scaber* if the staining solution contained ß-NADH, while they were not found in other species. The development of reaction product could be abolished by a specific NOS inhibitor 2,6-dichlorophenol-indophenol. No selective staining was found after incubation with ßNAD^+, ß-$NADP^+$ and D-glucose-6-phosphate.

These results show that the CNS *of P. scaber* contains a specific diaphorase which is possibly identical with neuronal NOS. The fixation resistance and wider substrate specificity strongly suggest that this enzyme has various isoforms in *P. scaber*. The exact evidence of the identity of this specific diaphorase and NOS needs further examinations.

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DISTRIBUTION OF GABAERGIC NEURONS IN THE CENTRAL NERVOUS SYSTEM OF PORCELLIO SCABER

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Occurrence and distribution of g-aminobutyric acid (GABA) in the nervous system of several invertebrate species is well documented but no data are available considering the terrestrial crustacean isopods. Therefore we aimed to describe the distribution of GABA-like immunoreactive neurons in the central nervous system (CNS) of an isopod *Porcellio scaber* using immunocytochemical methods. The exact position of labelled cells was determined in whole-mount preparation and semithin sections of CNS. Small GABA positive neurons occurred in all ganglia and their distribution showed a characteristic arrangement in the CNS. A few GABAergic neurons were found in proto-, deutero- and tritocerebrum as well as in the subesophageal ganglion. They form conspicious groups in the optic and antennal (olfactory) lobe. Ratio of stained perikarya in ventral nerve cord increased rostrocaudally. Numerous GABA positive neurons were found in the 4-8th thoracic ganglia and in the terminal ganglion. Labelled cells are positioned anteroventrally and posteroventrally in all ganglia except for terminal ganglion in which a few positive neurons appear also at the dorsal side.

Orientation of immunoreactive cell populations and considerable staining throughout the neuropil areas suggest a role of GABA neurotransmission in several neuronal functions, especially sensory information processing.

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TELENCEPHALIC EFFERENTS OF THE THALAMIC NUCLEUS DORSOMEDIALIS ANTERIOR (DMA) IN THE DOMESTIC CHICKEN *(GALLUS DOMESTICUS)*

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The avian thalamic dorsomedial nuclei are thought to be equivalent to the intralaminar, midline and dorsomedial nuclei of the mammalian thalamus. The efferent connectivity of the avian dorsomedial thalamus has not been studied in detail. The telencephalic projection of the DMA of domestic chicken was investigated using the anterograde tracer Phaseolus lectin. Fibres were present in the following regions. Lobus parolfactorius with topographically oriented projections from various subdivisions of DMA. Nucleus accumbens and paleostriatum augmentatum and ventrale receiving small to modest input from DMA. The ventral DMA sent a few but profusely branching fibres to the tuberculum olfactorium. Ventral septum with a small topographically distinct ipsi- and contralateral input from DMA. Medial septum where abundant axonal plexuses ensheathed fibres and cell bodies. Weak input to the fasciculus diagonalis Brocae. Wulst, rostromedial hyperstriatum accessorium and hyperstriatum ventrale. Fibres from dorsolateral and ventrolateral DMA were more frequent in mediodorsal neostriatum and lateral to field L, respectively. Topographically oriented projections were seen in the archistriatum. A large ventrolateral DMA injection revealed extensive input to the dorsal area corticoidea lateralis. In summary, DMA projects mainly on limbic telencephalic areas. With its rich tegmental input, the region may constitute an interface between telencephalic and mesencephalic limbic centres.
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MORPHOLOGICAL AND BEHAVIORAL CHANGES AFTER TREATING THE ISOLATED SMALL INTESTINAL FISTULA WITH BENZALKONYUM-CHLORIDE IN RATS

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Benzalkonyum-chloride is a cationic detergent which has been extensively used to selectively eliminate the myenteric plexus of the small intestine without damaging the submucosal region. Treatment means to cover the serosal surface of the 2-4 cm long intestinal portion with a gauze impregnated with a 0.062% benzalkonyum-chloride (BAC) for 30 minutes. A week after the BAC administration, about 90% of the myenteric cells die while the rest of the cells show

serious degradation. The extrinsic (visceral) innervation is also affected or damaged. The external longitudinal and the inner circular muscle layers are also affected but regenerate soon afterwards. Their passive growth starts already at the 5th post-treatment day leading to hypertrophy and hyperplasy.

Our experiments had a special feature: BAC treatment was administered to an isolated small intestinal fistula. The questions raised were:

1. Is it possible to successfully treat an isolated Thiry-Vella fistula with BAC?
2. Are the morphological changes similar to those obtained by others in intact intestinal tract?
3. How does the isolated intestinal fistula behave as an isolated organ compared to those being treated whilst still in the passage?
4. How does the behaviour of the animal change after the treatment while mechanically stimulated; is there a perception of the signal?

Results show that the answer to the first question is positive: It is possible to treat the isolated intestinal fistula with BAC and the resulting changes are similar to those found in the literature with continuous intestine.

The answer to the second question is a definite none: The fistulae were histologically examined 2 or 3 months after the treatment. Cross-sections were prepared from the samples and were stained with toluidin-blue. Isolated fistulae from non-treated animals as well as pieces from the normal passage of both the treated and non-treated animals were compared to the treated loops. Altogether, 15 stained sections were made of each piece by keeping every 5th cross-section and discarding the rest of them. The studied variables were:

- the diameter of the intestine, being always smaller in any fistula than those from the passage;
- the musculature of the intestinal wall, which was no different among the treated and non-treated specimens;
- the number of neurons in the myenteric plexus area; this proved to be 20-78% of the controls in the treated fistulae.

The isolated organ experiments used the Magnus-preparation. Spontaneous activity of the treated intestine was similar to those described in the literature: long intervals of infrequent contractions were interrupted by increased tone, high amplitude and high frequency bursts. The authors who first described this phenomenon suggested that this is the rhythm of the muscles themselves manifested in the absence of the plexus.

To answer the last question, a traditional free-drinking experiment was run. The rats were deprived of water for 23 hours with food available *ad libitum.* Preceding the experiment, a rubber balloon to be filled with water was inserted into the fistula and was taped to the body. Stimulation started just prior to the 15 min. experiment during which both the fluid consumption and the behaviour was recorded by an observer. Zero, weak, strong and painful stimuli were applied determined earlier by threshold experiments. Indexing the behavior was possible because of the stereotypy observed in drinking. Characteristic elements of the satiety behaviour (drinking, grooming, orientation, food search, rest) were recorded with a 3 sec resolution, were assigned to a weight according to the expected order of occurrence, were multiplied by their respective duration and summarized for each minute. A separate aversivity index was also calculated similarly. Results show that stimulation of the treated fistula elicits much less aversivity than that in the control animals, while satiety and the intake did not differ significantly.

Our results clearly proved the success of the BAC treatment. At the same time, however, we found more neurons than expected according to the literature and have not found hyperplasy and hypertrophy either, though the movement was similar to that obtained with intact intestines. This contradiction requires some considerations. The effect of the BAC is probably due to several factors, namely:

- BAC damages and sensitizes muscle cells and neurons of the myenteric plexus;
- motility changes in the treated interval, hence - if continuous with untreated parts - gut content accumulates and distends the wall eliciting permanent pressure and restricting circulation;
- the treated area is reach in inflamed elements due to the cell damage, thus the micro-environment becomes significantly altered.

These factors act together to evoke significant cell loss in the traditional preparation. In addition, accumulation of the content also results in the thickening of the muscle layers. However, in our case accumulation was absent since there was no gut content and the fistulae had been cleaned daily; thus only the first and the last mechanisms were present. This might account for the differences.

To sum it up, we found that most of the myenteric plexus of the isolated intestinal fistula was destroyed by the BAC treatment, probably together with the majority of the extrinsic visceral efferent and afferent fibers. This, in turn, probably prevented the mechanical stimuli from effectively disturb the normal behaviour.

ON THE NATURE OF GRASPING REFLEX

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The problem. The grasping reflex is a characteristic of all apes, amongst whom it is very adaptive, but in the naked ape, the human it is almost without function. In spite of everyther the grasping reflex remained a 'living' symbol of attachment and we suppose may serve as a natural model of psychophysiological bonding in attachment where mother and infant pairs may be considered to be evolutionary dyads. Human attachment functions develoMitsányi p during the continuous interaction of this dyad. Grasping reflex as the most obvious sign of attachment behaviours, may be a tool in analysing the underlying mechanisms.

Method. Grasping reflex was elicited in 27 newborns 2.5-50 hour post partum with simultaneous measuring of accompanying heart rate changes. We examined and analyzed the psychophysiological changes during the activity of the grasping reflex in newborns.

Results. Results show, that eliciting the grasping reflex is accompanied by very significant HR deceleration (p 0.0001) and newborns become behaviourally more quiet. This deceleration clarifies the existing psychophysiological model of attachment, and the existing positive effect of the grasping reflex.

Conclusion. As potential application of this study, feed-back mechanisms are suggested to be used in treating difficult mother-infant interactions and attachment disturbances.

INVESTIGATION OF THE EFFECT OF CHLORDIMEFORM ANALOGUES ON LOCUST. *LOCUSTA MIGRATORIA MIGRATORIOIDES RF.*

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It is generally accepted that the interaction of chlordimeforms with the octopaminergic system results in toxicity in insects. At the same time, their possible effect onto other transmitter system cannot be excluded either. The present study examines the correlation between the lethal dose the feeding inhibition and the effects of chlordimeform analogues on the octopamine (OA), tyramine (TA), dopamine (DA), serotonin (5HT) and γ-aminobutyric acid (GABA) receptors.

The investigated chlordimeform analogues were the following: CDM, DCDM, DDCDM, 2-methyl-DCDM, 2-Cl-DCDM, 4-Cl-DCDM, 2,4-dimethyl-DCDM, 2,4-di-Cl-DCDM, 3,4-di-Cl-DCDM, 2-methyl-4-Br-DCDM, 2-methyl-5-Cl-DCDM, 2-Cl-4-NO2-DCDM and 2,4,6-tri-Cl-DCDM. The effects of the different analogues on the receptors were analysed by investigating the inhibition of the binding of ^{3}H-labelled ligands (^{3}H-octopamine, ^{3}H-tyramine, ^{3}H-haloperidol, ^{3}H-serotonin, ^{3}H-flunitrazepam).

It was found that the DCDM has a high affinity only for the OA receptor. DCDM has a 10 times lower affinity for the tyramine receptor than to the OA receptor, whereas its affinity for DA. 5HT, and GABA receptors was found to be very low. A close correlation was found between the lethal dose of the chlordimeform analogues and their effectiveness on the octopamine receptor. A correlation could also be demonstrated between the inhibitory effect of the analogues on the feeding behaviour and on the OA receptor. Our results clearly show that the action of chlordimeform analogues is specific on the octopamine receptor, whereas its effect on other transmitter receptors is not significant.

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EMBRYONIC DEVELOPMENT OF THE NERVOUS SYSTEM OF THE POND SNAIL, *LYMNAEA STAGNALIS* L.: QUALITATIVE ULTRASTRUCTURAL INVESTIGATIONS

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At two specific developmental stages, E21 (E35%) and E26 (E75%), of *Lymnaea* embryogenesis (lasting altogether 8 days) we have investigated i) the development of CNS in 1 μm serial semi-thin sections; ii) the ultrastructural characteristics of neuronal perikarya and neuropil; iii) the vesicule and granule content of axon profiles; and iv) the ultrastructure of interneuronal (synaptic and synaptoid) contacts. For these purposes, the embryos removed from the eggs were processed for double (GA-PFA/Os) fixation and routine EM-embedding

procedure, with the only exception of applying 0.01 M phosphate buffer for the dilution of the aldehyde fixative. According to our experience, this low buffer concentration proved to be the crutial condition to obtain a good fine structural preservation.

At stage E21, the very small embryonic cerebral and pedal ganglia contained only a few cells, and a laterally (excentrically) located neuropil. The ultrastructure of nerve cell perikarya was very simple: a large nucleus with homogeneous chromatine content was surrounded by a narrow cytoplasm which contained only few organelles, mainly rER system elements. The majority of axon profiles in the neuropil were found to be 'empty'; in some of them agranular vesicles and granules occurred and mitochondria could only be observed rarely. Varicosity-like swellings of the axons occurred occasionally. Specialized synaptic contacts were not seen at all, however, a number of axon profiles were found contacting the few perikarya along closely attached unspecialized membrane segments.

At stage E26, the entire circumoesophageal ganglion ring has already been developed. All central ganglia revealed a typical ganglionic structure with an external cell body layer and a centrally located neuropil. However, a segregation of the cell body layer and the neuropil could not be observed, as seen in late juveniles and adults, but neuronal perikarya frequently appeared intermingled with neuropil structures. Two major types of neuronal perikarya could be observed: i) those showing ultrastructural characteristics of high activity with a nucleus of rich heterochromatine content, extremely well-developed rER system, many mitochondria and free ribosomes, numerous Golgi-units and granules of different morphology; and ii) those revealing furthermore a less developed ultrastructure with large nucleus, relatively small cytoplasma containing mostly rER elements, free ribosomes and a few mitochondria. In the neuropil, the majority of axon profiles did not contain vesicles and/or granules; axon varicositites occurred rarely. Mature, specialized synaptic contacts can only be found rarely. On the other hand, membrane contacts with asymmetric clustering of vesicles but less developed pre- and postsynaptic membrane specializations, resembling developing synapses, were seen more frequently. It was striking that numerous axon profiles situated alongside the neuronal perikarya and formed close but in most case unspecialised contacts with them. An important ultrastructural feature of these late embryonic ganglia was the yet underdeveloped glia system in and the almost total lack of connective tissue (neural) sheath around the ganglia. This latter was represented only by thin muscle fibres and some collagen fibers, whereas glia elements were absent.

We suggest that i) corresponding to the relatively low (motion, feeding, respiration) activity and little behavioural repertoire of embryonic life of *Lymnaea,* a very limited number of synapses seem to be 'required' for integrative processes; ii) in the absence of ganglionic sheath the neuronal perikarya may be exposed freely to external influences be it of hormonal or trophic nature; iii) development of nerve cells can also be regulated in a modulatory way from the direction of the neuropil by the numerous unspecialized contacts with axon profiles.

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CONTRALATERAL CORTICAL PROJECTION TO THE MEDIODORSAL THALAMIC NUCLEUS: ORIGIN AND SYNAPTIC ORGANIZATION IN THE RAT

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The origin and termination of the contralateral corticothalamic projections to the mediodorsal nucleus (MD) were investigated in adult rats with double retrograde fluorescent tracing and the anterograde transport of biotin-dextran-amine (BDA). After injection of fast blue, diamidino yellow or nuclear yellow in the MD of one side, neurons were retrogradely labeled in the contralateral prefrontal cortex (PFC) in the same areas in which ipsilateral labeling was also observed, i.e. orbital, agranular insular, anterior cingulate, prelimbic and infralimbic areas. The laminar distribution and morphological features of labeled neurons were similar in the PFC of both sides. In the orbital and agranular insular areas, 15% of labeled corticothalamic cells were of contralateral origin, while the proportion was lower (3%) in the anterior cingulate cortex. Up to 70% of the contralateral cortical neurons were double labeled by bilateral MD injections. Unilateral BDA injection in the orbitofrontal cortex resulted in bilateral anterograde labeling of corticothalamic afferents in MD, with a mirror distribution and a higher density in the ipsilateral side. At the electron microscopic level, BDA-labeled boutons in the contralateral MD were identified as small terminals and were found to establish asymmetric synaptic contacts and to contain densely packed round vesicles. In the ipsilateral MD, in addition to such labeled small terminals, large corticothalamic labeled boutons were observed. The diameter of postsynaptic dendrites contacted by labeled small axon terminals was significantly larger in the ipsilateral MD than contralaterally.

These findings demonstrate that contralateral corticothalamic projections to MD derive mainly from the orbital and agranular insular areas and are formed mostly by collaterals of the ipsilateral afferents. Our observations also point out differences in the synaptic organization of prefrontal fibers innervating the ipsilateral and contralateral MD.

REINNERVATION OF DENERVATED HINDLIMB MUSCLES BY AXONS OF GRAFTED MOTONEURONES VIA THE REIMPLANTED L4 VENTRAL ROOT

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Embryonic spinal cord grafts placed into the lumbar region of an adult spinal cord survive, differentiate but neurones from such grafts are not able to extend their axons into denervated ventral roots. However, they can be made to reinnervate a skeletal muscles via a peripheral nerve bridge. We attempted to guide out axons of grafted motoneurones via the reimplanted L4 ventral root in order to reinnervate denervated hindlimb muscles. Solid pieces of embryonic spinal cords prelabelled with 5-bromodeoxyuridine were grafted into the L5 spinal segment, the L4 ventral root was pulled out and reimplanted close to the graft. Three to six months later fluorescent dyes were applied both to the L4 ventral ramus and hindlimb muscles and retrogradely labelled neurones were traced. A considerable number of labelled neurones had grown axons into the ventral root and 75% of these reached the hindlimb muscles originally supplied by L4 ventral root axons. Many of the retrogradely labelled neurones were of embryonic origin and also calcitonin gene-related protein (CGRP) immunoreactive. Intracellular labelling of these retrogradely labelled cells with biocytin showed that approximately 30% of them exhibited the features of differentiated motoneuronelike cells with an extensive dendritic tree. Stimulation of the L4 ventral ramus elicited strong contractions in the reinnervated Tibialis Anterior and Extensor Digitorum muscles (37% op/ctrl). In the absence of embryonic grafts the reimplantation of the L4 ventral root resulted in poor reinnervation. The immunohistochemical analysis of descending 5-HT and dopaminergic fibres showed that these axons did not penetrate the grafted tissue and therefore could not induce proper suprainnervation of the graft. However, abundant CGRP immunoreactive fibre networks could be seen throughout the graft.

These results show that grafted embryonic motoneurones are able to reinnervate remote denervated skeletal muscles via reimplanted ventral roots.

OCCUPANCY OF POSTSYNAPTIC $GABA_A$ RECEPTORS IS DETERMINED BY RECEPTOR NUMBER

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Neurotransmission is quantal at the neuromuscular junction where the amplitude distribution of miniature end-plate currents can be fitted by a single Gaussian and the size of the miniature

events is equal to the quantal size of the evoked responses. In contrast, in most of the GABAergic synapses in the brain the amplitude distribution of the miniature (tetrodotoxin resistant) GABAA receptor mediated postsynaptic currents (mPSCs) is skewed toward larger values and the distribution of the evoked responses does not show equidistant peaks. To study the mechanisms underlying the variability in the amplitude of $GABA_A$ receptor mediated mPSCs and the receptor occupancy at synapses on cerebellar stellate cells, we combined whole-cell patch-clamp recording and quantitative immunogold localisation of synaptic $GABA_A$ receptors.

The amplitude distribution of mPSCs was bimodal or skewed, having a 70-fold difference between the smallest and largest events. A benzodiazepine agonist (flurazepam), which increases the channel open probability (P_o), prolonged the decay of all mPSCs but increased the amplitude only of those larger than 1.5-1.75 pS. Thus for these 'large' events, the PO of the synaptic $GABA_A$ receptors is smaller than the maximum open probability (P_{omax}), resulting in a receptor occupancy of smaller than 1. However, for the 'small' (<1.5-1.75 pS) events the amplitude and therefore the P_o could not be increased by flurazepam, indicating a receptor occupancy of 1. Peak-scaled non-stationary fluctuation analysis revealed a conductance of 28pS for a synaptic $GABA_A$ receptor channel, thus the 1.5-1.75 pS events correspond to the opening of approximately 55-65 channels. It is concluded that synaptically released GABA saturates up to 55-65 postsynaptic $GABA_A$ receptors, but occupies only a fraction of them if more than 65 receptors are present in the postsynaptic membrane.

The number of immunoparticles for the α1, ß2/3 and g2 subunits of the GABAA receptor varied remarkably at symmetrical synapses on interneurons in the molecular layer. Although, some of the synapses contained over 140 immunoparticles for the α1 subunit, the majority had only 20-40 particles, resulting in a skewed distribution of synapses according to their receptor content. The mean number of particles per synapse was 55.6 with a coefficient of variation (C.V.) of 82.6%. Similar large variability in the number of receptor immunoparticles was also seen between synapses on individual cells. A positive, linear correlation was found between the number of particles and the size of the synapses and therefore the synaptic area could be used as a measure of receptor content. The distribution of synapses with regard to their size was also skewed toward larger values (mean=0.152 μm^2; C.V.=65.6%), having a 20-fold difference between the smallest and the largest synapse.

We conclude that the variation in the number of GABAA receptors between synapses parallels the variability in the quantal size of the postsynaptic responses and that on a single cell there may be two functionally distinct types of GABAergic synapses.

SELECTIVE DECLINE OF S-HT_{1A} RECEPTORS IN RAT CORTEX, HIPPOCAMPUS AND CHOLINERGIC BASAL FOREBRAIN NUCLEI DURING AGING

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The effect of aging on 5-HT_{1A} receptor expression in several forebrain areas associated with the basal forebrain cholinergic system, was investigated in rats of 3-, 24- and 30-months old by receptor autoradiography and biochemical binding assay using [^{3}H]8-OH-DPAT as a ligand. Autoradiographic measurements demonstrated a marked region-specific decline of ligand binding in: (i) the basal forebrain cholinergic cell groups, i.e. the medial septum, diagonal band nuclei and magnocellular nucleus basalis, (ii) the frontal and parietal neocortex and (iii) the dentate gyrus of the hippocampus. No change or only a slight decrease of the $5HT_{1A}$ receptor density was found in other areas investigated: the CA1 and CA3 sectors of hippocampus, the cingular and perirhinal cerebral cortex and the lateral septum. The autoradiographic findings were substantiated by the biochemical binding assay, which revealed a comparable loss of 5-HT_{1A} receptor in the hippocampus and neocortex at the age of 30 months.

The results clearly show that with increasing age the decrement of 5-HT_{1A} receptor expression in the rat forebrain is remarkably region-selective and particularly affects the cholinergic cellgroups that innervate cortex and hippocampus. This phenomenon appears to be especially significant in relation to the neuronal substrates underlying the age-related alterations of mood and cognition.

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EFFECTS OF THE GABA TRANSPORT INHIBITORS NIPECOTIC ACID AND THPO ON EXTRACELLULAR AMINO ACID LEVELS IN RATS

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It is established that compounds capable of inhibiting GABA transport and in particular astroglial uptake can act as anticonvulsive agents. One of the first recognised potent and specific inhibitors of high affinity GABA uptake is nipecotic acid which inhibits neuronal and glial uptake equipotently, while THPO, a compound structurally related to nipecotic acid, preferentially inhibits glial GABA uptake. As the extracellular GABA concentration in the brain may related to the efficiency of the high affinity GABA transporters and may be a

determining factor in the generation of seizures, in our aim was to study the effect of the two GABA uptake inhibitors on extracellular level of GABA. To further investigate whether treatment of rats with these compounds may affect other amino acids metabolically or functionally linked to GABA, the extracellular levels of aspartate, glutamate, glycine, serine, threonine were also monitored. We implanted microdialysis probes into the ventrolateral thalamus of rats and collected samples for amino acid analysis. In order to investigate the effect of elevated extracellular GABA level alone on amino acid levels 3 mM GABA was applied through the microdialysis probe. To study the effect of the two uptake inhibitors on amino acid levels, 5 mM THPO alone and together with 3 mM GABA or 0.5 mM nipecotic acid plus 3 mM GABA were infused through the microdialysis probe. Administration of 5 mM THPO alone significantly increased the extracellular GABA concentration. We found a different action of THPO and nipecotic acid on the steady state increase in the extracellular GABA concentration produced by infusion of GABA. Infusion of GABA led to a 500% increase in extracellular GABA and simultaneous application of 5 mM THPO further increased the GABA concentration reaching the level of 1200% 90 min. after the start of the infusion of THPO together with GABA. These findings suggest that glial cells may be quantitatively important for removal of GABA when the carriers are working at maximal activity. It underlines the notion previously put forward that selective inhibition of astrocytic GABA uptake is more efficient than inhibition of both neuronal and glial uptake when an anticonvulstant action of GABA transport blocker is wanted. The concentration of aspartate and glutamate were only elevated after application of GABA plus THPO. The levels of glycine, glutamine, serine and threonine were elevated when GABA was infused together with nipecotic acid or THPO, the effect being most pronounced in the latter case. When GABA or THPO was administered alone the levels of these four amino acids were less increased. The possibility of disinhibition might explain the elevated levels of excitatory amino acid transmitters, and the increased extracellular level of asparate and glutamate may, in turn lead to release of other amino acids due to depolarization and reversal of carriers. Since the effect was almost the same when GABA level was elevated by GABA itself or by GABA in the presence of nipecotic acid, a direct action of THPO is unlikely. The results indicate that glial GABA uptake inhibitors may constitute and interesting group of drugs by which the efficiency of GABAergic neurotransmission may be enhanced leading to higher thresholds for seizure activity.

DECLINE IN CORTICAL SEROTONERGIC AND NORADRENERGIC ACTIVITY AFTER ß-AMYLOID-INDUCED LESIONS IN THE RAT MAGNOCELLULAR NUCLEUS BASALIS

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One of the major protein constituents of neuritic plaques in the brains of patients with Alzheimer's disease (AD) is the ß-amyloid peptide (ßAP) which consists of 39-42 amino acid residues in mature form. Ample evidence has recently accumulated regarding the *in vivo* neurotoxic properties of ßAP. It is noteworthy, however, that while several attempts were made to demonstrate a close correlation between cholinergic denervation and accumulation of pathological hallmarks, such as ßAP deposition, in AD; relatively little is known about the alterations of other neurotransmitter systems.

According to one of the hypotheses on the cellular patho-mechanism of ßAP action, the peptides interact with specific cell-membrane components (receptors) or form cation-selective membrane-spanning channels and generate a rapid inward Ca^{2+} current which might directly be an initiator of long-lasting cellular disturbances. Another component of the neurotoxic pathway is the generation of free radicals, particularly that of reactive oxygen intermediates (ROS). It is well established that the aforementioned mechanisms may interact and potentiate each others effects.

Whereas fundamentals of the above hypothesis are primarily based on *in vitro* data, in the present study we aimed at investigating (1) the interaction between altered animal behaviors and decline in cortical cholinergic activity, (2) effects of ßAP infusion and subsequent cholinergic denervation on the action of distinct cortical neurotransmitter systems, namely the serotonergic (5-HTergic) and noradrenergic (NAergic) systems, and (3) changes in cortical nitric oxide synthase (NOS) activity. Activity changes of NOS might indicate the presence of ongoing inflammatory or even neurodegenerative processes. Furthermore, inducible NOS-generated nitrogen oxide may form highly reactive and diffusible intermediates with ROS.

Young adult Wistar rats were used in the experiments. For standard behavioral and histochemical experiments ßAP(1-42) was unilaterally injected into the magnocellular nucleus basalis (MBN) in a concentration of 0.2 nmol/µl. Thereafter, spontaneous behaviors in the small open-field paradigm and the loss of cortical cholinergic innervation (by means of quantitative acetylcholinesterase (AChE) histochemistry) were determined. Moreover, ßAP(1-42)- or ßAP(25-35)-induced (0.4 nmol/2 µl) changes in cortical 5-HT, 5-hydroxyindoleacetic acid (5HIAA) and NA contents as well as NOS activity were biochemically measured. A 7-day survival period was used in all experiments. In neuroprotection studies the noncompetitive polyamine-site-binding NMDA receptor antagonist, ifenprodil tartrate (10 mg/kg body weight) was administered intraperitoneally 24 hours after ßAP infusion and repeated every other day up to 7 days post-surgery.

In accordance with earlier studies in our laboratory ßAP(1-42) injections induced marked decreases in explorative (rearing) behavior which were accompanied by a 16.79 + 2.8% loss of

cortical AChE-positive projection fibers. Interestingly, cortical 5-HT, 5-HIM and NA levels were found biochemically to be dramatically decreased as a consequence of ßAP(1-42) infusion, whereas ßAP(2535) induced the decline of these neurotransmitters to a lesser extent. In parallel with the cortical decline of 5-HT and NA an 8-fold induction of NOS activity was determined 7 days after ßAP(1-42) injections, which was effectively antagonized by if enprodil.

The present data indicate that cortical cholinergic denervation processes induced by ßAP directly affect (1) explorative behavior and (2) the activity of other neurotransmitter systems even after a short survival period, and these alterations closely resemble the neurotransmitter changes observed in AD. (3) Neurodegeneration of cholinergic ascending projections to the cerebral cortex is accompanied by a dramatic NOS induction. Enhancement of the enzyme activity supports the involvement of ROS-mediated pathways in the neurotoxic cascade and a direct intracortical interaction between neurotransmitter systems. Furthermore, NOS induction may also reflect local inflammatory reactions. (4) Blockade of NMDA receptors within 24 hours after ßAP infusion might prevent a persistent intracellular Ca^{2+} overload and thus the initiation of the neurodegenerative changes. (5) Peptide conformation, the degree of peptide aggregation is an important factor which determines the neurotoxic potential of ßAP.

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ADAPTATION AND OTHER DYNAMICAL EFFECTS ON THE NEURAL SIGNAL TRANSFER

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In the light of the latest results concerning the dynamics of synaptic transmission (Markram et al., 1996) and spike frequency adaptation, the question how the signals are transferred between neurons and what are the meaningful signals in the neural information processing, has to be reconsidered. We constructed simple models of these phenomena and computed qualitatively the neural transfer properties. In these models we examined only the transmission of two parameters of the neurons' membrane current: the mean and the standard deviation. In the cerebral cortex there is usually significant convergence between neurons and so, if the inputs follow the Central Limit Theorem, these two parameters can fully describe the summed input of the cell. From these transfer properties we can conclude, that the big observed coefficient of variation (Softky and Koch, 1993) can be produced, at least partly, by these mechanisms. The low firing rate and spike frequency adaptation do not allow us to use simple firing rate code for longer period of time simulation of real neurons, as it is assumed in the majority of regular artificial neural network models (ANN) (Gerstner et al., 1992). The measured effects of long-term potentiation on the dynamics of synaptic transmission show, that the transfer of membrane currents mean does not change appropriately. Namely, its

modification can not be considered as synaptic weight change. Our result shows, that there could be an additional parameter, the standard deviation of the synaptic currents, which can provide the same sort of transfer properties as the average firing rate in typical ANN models. The formation and operation of this type of 'code' is discussed.

IN VITRO STUDY OF THE ROLE OF CALCIUM BINDING PROTEINS IN AMYLOID TOXICITY

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ß-amyloid protein (ßAP), which accumulates in the senile plaques in the brain of subjects with Alzheimer's disease, is cytotoxic to neurons. We recently showed that ßAP1-42 (Kása et al., 1993) and various of its fragments (Pákáski et al. in press) damaged the GABAergic and AChE-positive neurons in rat basal forebrain cultures. Although the mechanism by which ßAP exerts its neurotoxicity is not yet understood, several observations have focused attention on the increase in the intracellular free calcium content induced by exposure to ßAP (Mattson et al., 1992, 1993). This has led to the suggestion that the presence of calcium binding proteins might be related to the relatively high resistance of certain neuronal populations to amyloid toxicity. Cells immunoreactive for GABA are also immunoreactive for various calcium binding proteins: parvalbumin (PV), calretinin (CR) or calbindin D28. A neuronal culture obtained from embryonic basal forebrain was used earlier to study the toxic effect of ßAP. Since this culture system contains a number of GABAergic cells, it is a potentially important tool in studies of agents protecting against amyloid toxicity.

In the present study, dissociated cells from E16 rat basal forebrain were cultured after being plated onto ply-L-lysine-coated coverslips. The cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Four days after plating, the cultures were grown in the presence of 20 µmol ßAP1-42, ßAP31-35, ßAP34-39 or ßAP33-35. Control cultures were incubated with 10% fbs/DMEM containing the solvent of the ßAPs. One to 3 days after ßAP treatment, the cultures were fixed for PV or CR immunocytochemistry. In some cases, the cultures were double-labeled for GABA and PV.

Addition of 20 µmol ßAP1-42, ßAP31-35, ßAP34-39 or ßAP33-35 to the culture medium for 1-3 days did not alter the morphology of PV- or CR-positive neurons. Double staining of cultures for GABA/PV revealed intact cells in control cultures. In cultures exposed to ßAPs, there were morphological differences between the PV-negative and PV-positive neurons. Most GABA-positive/PV-negative neurons exhibited the morphological signs of neuronal death: initially, their processes underwent fragmentation; later, the processes disappeared and the perikarya became round. In contrast with these cells, GABA-positive/PV-positive neurons seemed to be healthy, with long, intact neurites. The morphological features of cultures treated with different ßAPs were similar, except for those incubated with ßAP33-35, which had no toxic effects on GABA-positive/PV-negative cells.

These results suggest that PV or CR may have a protective role in neurotoxicity induced by ßAPs. Since PV and CR may be involved in the maintenance of intracellular calcium homeostasis, our data support the earlier hypothesis that the mechanism of ßAP toxicity occurs via an increased intracellular free calcium level.

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RETINAL OSCILLATIONS EVOKED BY RHYTMIC IMPULSE SERIES

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In the representation and distribution of the different parts of visual information the synchronous or asynchronous activity of the certain cell populations play important role which can be detected both on the level of unit activity and field potentials. That is why the examination of oscillatoric behavior of different parts of the visual system and the temporal correlation between the synchronous activity of these parts became so important. The relation between the retinal representation of the information and the retinal evoked electrophysiological responses, as well as the connection between the evoked electrical activity measured on the retina and visual cortex are relatively unkown. We had got three questions: 1) wheater there could be a periodic behavior in the frequency range of visual cortical oscillations on the level of the retina, 2) how do the retinal field potential changes represent the impulse series showing different temporal patterns and 3) how do these retinal signs correlate with the visual cortical activity.

Spontaneous or evoked oscillatoric behavior cannot be detected either on the isolated tissue slices or on the isolated retina and it is impossible to explore the retinal unit activity on freely moving rats. So light emitting diodes for stimulation with flash and stainless steel electrodes were implanted around the eyeball and above the visual cortex (Proc. Natl. Acad. Sci. USA, 1994, 91: 5153) for recording the retinal and visual cortical activity. The retina was stimulated by impulse series between the frequency of 10 and 30 Hz in freely moving rats. We found that the frequency of the retinal evoked responses follow precisely the frequency of the impulse series. We also measured a visual cortical activity belonged to a constant frequency

range (25-30 Hz) evoked by any of the impulse series we used. Frequency preferred stages were found in time according to the first stimulus both in the intensity of the retinal evoked responses and the oscillatoric behavior followed the end of the stimulation. The power spectral intensity of the responses and oscillations evoked by impulse series above the range of 25 Hz was larger, generated at the same time as the C-wave of the electroretinogram, than it was measured at the time of the other retinogram components. The opposite pattern was found, when we used smaller frequency to stimulate the retina.

From our examination we conclude that the synchroning occurrences which were known on thalamic, thalamo-cortical, intra- and inter-cortical levels so far and which can form the basis of the information processing are able to develop in simpler retinal neural networks. The retinal oscillations in these experiments do not correlate with the visual cortical oscillatoric activity regarding the coding of the different impulse series. Previously we had examined the recovery occurrences of certain retinogram components but the data we obtained do not explain the existing of the frequency preferring stages. There can be some unknown mechanisms in the retinogram genesis, which are responsible for the features of the oscillatoric activities mentioned above.

EFFECT OF ACUTE LEAD INTOXICATION ON IN VIVO HIPPOCAMPAL POPULATION SPIKES OF RATS

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Lead is a toxic heavy metal being nearly ubiquitous in our environment, first of all from road traffic and industrial origin. Lead causes a variety of toxic symptoms some of which concern the nervous system. The adverse effect of chronic lead exposure on mental development of children has been described by several authors. Impaired IQ, different behavioral difficulties as well as characteristic EEG and auditory evoked potential alterations in schoolchildren after several years of lead exposure were observed. Even in acute cases, functional and morphological alterations corresponding to a lead-induced organic brain lesion were seen. In animal models, postnatal lead treatment induced EEG disorders and learning disability in young rats. Lead-exposed rhesus monkeys had a diminished discriminative auditory performance. The above clearly show the importance of lead exposure which, in turn, poses the need for experimental toxicological investigations. These can provide the basis for future noninvasive electrophysiological methods, to be used for early detection of harmful effects of lead. The use of these, in turn, needs to be justified by more direct experimental studies. The aim of the present work, done on acutely treated rats, was to investigate the lead-induced changes in the hippocampal population spike.

Male Wistar rats (250 to 350 g) were used. In urethane anaesthesia (1000mg/kg i.p.), the animal's head was fixed in a stereotaxic frame and the left hemisphere was exposed. Wounds of the skin, muscles and skull were sprayed with lidocaine. The dura was removed and the cortical surface was covered with warm paraffine oil. Hippocampal population spikes were recorded with low (3 - 7 Mn) resistance glass microelectrodes from the CA1 zone. For stimulation, a bipolar electrode was placed at the perforant path; the electrical stimuli were square shaped (1 - 4 mA, 0.05 msec) with 10 sec period time. Twenty evoked population spikes were averaged

and stored, followed by a 10 min interval. This stimulation sequence (20 stimuli, 10 min break) was chosen to avoid plastic modifications and was held throughout the whole experiment. For tetanization, 50 Hz trains, applied in 3 x 1 sec with 1 sec intervals, were used. The signals from CA1 were amplified (1000×) and fed to a TL-1 interface. Storage and analysis of the signals was performed using appropriate features of the pCLAMP software. The hippocampal population spikes were characterized by the usual parameters: amplitude, latency and duration. Most of the population spikes recorded were monophasic and were followed by a slower field potential. Lead, 1500 mg/kg i.p., was administered after three 10-min control records. Amplitude was the only parameter of the population spikes being significantly influenced by lead. We saw a gradual decrease of the amplitude which could already be observed at 3 - 5 min following lead administration and became more and more expressed up to ca. 1 hour. At the same time, the temporal parameters remained practically the same. On high-frequency tetanization, the hippocampal population spikes undergo a typical amplitude increase called long-term potentiation. In our experiments, 3 × 1 sec 50 Hz tetani induced a ca. 1.3× increase of the amplitude which remained for over an hour.

When such a potentiation was already established, it was, in numerous cases, not influenced by lead treatment at all. The amplitude was not reduced, in a few experiments even a further growth was seen. In other experiments, the potentiated and then lead-treated population spike showed an amplitude reduction up to the control after ca. 30 min. Here, a further potentiation was possible but no longer so effective as had been before lead treatment. Already lead-intoxicated, reduced population spikes showed practically no potentiation on tetanization. Heavy metal intoxications are usually treated with a chelator. We thus decided to study the effects of EDTA in combination with lead. It was found that 100 mg/kg EDTA could prevent the lead-induced reduction of the population spike amplitude only if it was given before lead. When, however, lead had been given first and its effect had been established, EDTA treatment could not reverse it.

Our results clearly show that lead-induced neurotoxicity can be detected on the hippocampal population spikes. As these represent a well-described phenomenon, further studies here can help to reveal the details of the mechanism of lead neurotoxicity. Hippocampus is known to play a central role in features of memory and behaviour. The alterations found by us may thus have a connection with abnormalities of higher nervous functions often described in lead intoxication.

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THE EFFECT OF NATRIURETIC PEPTIDES ON RAT THERMOREGULATION

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The natriuretic peptide family consists of endogenous peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). These peptide hormones and their receptors are equally present in peripheral organs and in the central nervous system. It is likely that natriuretic peptides play a role in the control of body fluid and circulatory homeostasis. In the past few years the possibility has arisen that these peptides

may act as neuromodulators/neurotransmitters within the CNS. The aim of the investigations was to assess whether natriuretic peptides play a role in thermoregulation.

Peptides were administered into the lateral brain ventricle of the rat. Rectal temperature was measured by an electronic thermometer in the 0^{th}, 30^{th}, 60^{th} and 90^{th} minute of the experiment. Each member of the natriuretic peptide family was able to significantly increase the body temperature of the rats in the 30^{th} and 60^{th} minute of the experiments (more in the 60^{th} than in the 30^{th} min.) in a dose dependent manner. Algopyrin (a NSAID containing noraminophenazonum, given intramuscularly) was able to diminish the hyperthermic effect of each natriuretic peptide.

Our findings underline the probable significance of natriuretic peptides in regulatory processes in the central nervous system by demonstrating their effect on rat thermoregulation.

PROPRIOSPINAL AFFERENT AND EFFERENT CONNECTIONS OF THE LATERAL AND MEDIAL SUBDIVISIONS OF THE DORSAL HORN (LAMINAE I-IV) IN THE RAT SPINAL CORD

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A large body of the experimental evidence has been accumulated in recent years indicating that the medial and lateral subdivisions of the superficial dorsal horn of the spinal cord show many distinct features concerning primary afferent inputs, synaptic and neurochemical properties of interneurons as well as the numbers of neurons with axons projecting to various supraspinal brain centers in mammals. A substantial functional link between the two sides of the dorsal gray matter at the level of the lumbar spinal cord has also been demonstrated. Here we demonstrate that, in addition to these disparities, the medial and lateral subdivisions of laminae I-IV of the dorsal horn appear to be distinct entities also on the basis of their propriospinal afferent and efferent connections. After injecting *Phaseolus vulgaris* leucoagglutinin and biotinylated dextran amine into various areas of the dorsal horn (laminae I-IV) at the level of the lumbar spinal cord, we found that a substantial number of propriospinal fibres travel one-two segments rostrally or caudally and terminate in areas of the dorsal horn that are identical to that in which their cells of origin are located, forming rostro-caudally oriented cellular laminae in which the neurons are highly interconnected. These cellular laminae both in the medial and lateral subdivisions of the superficial dorsal horn receive strong inputs from laminae V-VI. It has also been revealed that the medial subdivision of laminae I-IV projects extensively to the lateral subdivision, but terminals were only occasionally found in the medial dorsal horn after injecting the tracers into the lateral part of laminae I-IV. We found a substantial direct commissural connection between the lateral subdivisions of laminae I-IV on the two sides of the lumbar spinal cord. The lateral aspect of laminae I-IV gave rise also to fibres that terminated in the ipsi- and contralateral ventral horns or projected to supraspinal brain centers. Injecting the tracers into the medial dorsal horn, however no commissural fibres were revealed, and terminals in the ventral horn as well as fibres projecting to higher brain centres were found only in a limited number. The findings

indicate that the medial and lateral subdivisions of the superficial spinal dorsal horn of the rats may play different roles in various aspects of sensory information processing.

The study was supported by the Howard Hughes Medical Institute and the Hungarian National Science Fund; OTKA.

ROLE OF NEUROPEPTIDE Y AND NORADRENALINE IN SYMPATHETIC REGULATION OF MICROCIRCULATION OF THE SKIN

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Microcirculatory changes evoked by electrical stimulation of sympathetic efferent fibres of the saphenous nerve were measured by laser-Doppler flowmetry in the rat's skin. After perineural capsaicin (2%) pretreatment excitation of the peripheral stump of the cut saphenous nerve produced a reduction in microcirculation (vasoconstriction) followed by a minimal late enhancement. This late vasodilatation was further reduced by resiniferatoxin (RTX; 1 μg/kg i.V.)7 and vasoconstriction was prevented by guanethidine (8 mg/kg i.v.) indicating the involvement of sensory and sympathetic fibres in the respective responses. The vasoconstrictor response was examined after elimination of antidromic vasodilatation by the combined capsaicin-RTX pretreatment. α-adrenoceptor antagonists (1 mg/kg phentolamine, 0.5 mg/kg prazosin and 1 mg/kg GYKI-12743 i.v.) inhibited but did not abolish blood flow reduction in response to 3 Hz stimulation. Only GYKI-12743 caused significant inhibition at 10 Hz excitation. No inhibitory effect was observed with propranolol (10 μg/kg i.v.) in any case. A functional neuropeptide Y (NPY) antagonist, a-trinositol (D-myo-inositol-1,2,6-trisphosphate, PP56; 50 mg/kg i.v.) markedly diminished the vasoconstrictor response remaining after treatments with the α-adrenoceptor blockers. Diminution was more pronounced at 10 Hz frequency stimulation. Since 3 Hz corresponds to an average, and 10 Hz approaches the maximal firing rate of the sympathetic efferents, these results emphasize important role of NPY in regulation of cutaneous microcirculation by sympathetic fibres under physiological conditions, particularly throughout increased sympathetic activity.

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SPECIFIC AFFERENTS TO GROWTH HORMONE-RELEASING HORMONE-SYNTHESIZING NEURONS IN THE INFUNDIBULAR AREA OF THE HUMAN DIENCEPHALON

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Growth hormone-releasing hormone (GH-RH) is one of the main hypothalamic factors regulating growth hormone (GH) production of the anterior pituitary gland. In order to reveal the chemical nature of the specific afferent systems to GH-RH-synthesizing neurons, double labelling immunocytochemical studies were performed in the adult human hypothalamus. We have analyzed the putative relationship of catecholaminergic, neuropeptide-Y (NPY) and methionine enkephalin (met-ENK) containing axons with GHRH-producing neurons.

In accordance with previous reports, the majority of GH-RH immunoreactive neurons was distributed in the hypothalamic infundibular nucleus. The neurons were densely innervated by tyrosine hydroxylase (TH)-immunoreactive (IR), catecholaminergic axons. Infundibular neurons also received a substantial NPY- and met-ENK-IR innervation. By means of dual antigen detection, a rich innervation of GH-RH-neurons by catecholaminergic axons was revealed. The TH-immunopositive fibers established multiple contacts with dendrites and perikarya of GH-RH cells. Similar juxtapositions were noted among NPY-IR, met-ENK-IR axons and GH-RH-synthesizing neurons. In addition to that, GH-RH-IR processes also innervated GH-RH-neurons.

These morphological findings indicate that the hormone production of hypophysiotrophic GH-RH-synthesizing neurons is influenced by the central, catecholaminergic, NPY- and ENK-containing systems. The connections observed among GH-RH neurons indicate the existence of an ultra-short loop feedback regulatory mechanism.

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THE LOCALIZATION, MORPHOLOGY AND NEUROCHEMICAL CHARACTERIZATION OF LAST-ORDER PREMOTOR INTERNEURONS IN THE LUMBAR SPINAL CORD OF RATS

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There is strong evidence that neural circuits underlying certain motor behaviors are located in the spinal cord. Such local central pattern generators are thought to coordinate the activity of motoneurons through specific sets of premotor interneurons, some of which (last-order interneurons) establish monosynaptic contacts with motoneurons. In the experiments presented here we intended to identify, localize, morphologically and neurochemically characterize interneurons that can likely be regarded as last-order premotor interneurons.

After small iontophoretic injections of neurobiotin and biotinylated dextran amine into the lateral and medial motor columns as well as the adjacent white matter at L1-L2 and L3-L6 segments, retrogradely labeled spinal interneurons were investigated in the lumbar spinal cord of rats. A substantial number of spinal interneurons showed a homogeneous intense staining that labeled both the somata and a considerable part of the dendritic arbor. On the basis of the size and shape of the cell bodies and the dendritic arborization patterns, small and large pyramidal-like cells, multipolar and small fusiform neurons could be identified both in the lower and upper segments of the lumbar spinal cord. Regardless of the locations of the injection site, labeled interneurons were revealed in laminae V-VIII along a 3-4 segment long section of the spinal gray matter. Although most of the stained cells were confined to laminae V-VIII in all cases, the distribution of neurons within the confines of this area varied according to the site of injection. In order to estimate the proportion of inhibitory and excitatory interneurons within the total population of labeled premotor interneurons, GABA and glycine immunoreactivities of stained perikarya were investigated by postembedding immunocytochemical methods. In cases in which the tracer was delivered into the lateral motor column and the adjacent white matter at the level of L4 segment, 40% and 25% of the total number of labeled cells showed immunoreactivity for GABA and/or glycine respectively.

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ACETYLCHOLINESTERASE EXPRESSION IN RAT THALAMUS

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Acetylcholinesterase (AChE) appears critical to both development and the function of the nervous system. It may play a role in the development and the synaptogenesis of nerve cells. However, regulation of AChE during ontogenesis remains poorly understood, We used a reverse transcriptase-polymerase chain reaction assay, standardized with reference to "house keeping" cyclophilin mRNA, to investigate transient expression of AChE in newborn rat thalamus. Relative densities of DNA bands were quantitated by computerized video densitometry of ethidium bromide stained agarose gels. AChE enzyme activity was also measured biochemically and histochemically. Analysis focused on a non-cholinergic but AChE rich area, namely the medial geniculate nucleus (MGN), where transient increases and decreases of AChE expression during postnatal development are especially obvious (Robertson and Yu, 1993, Brimijoin and Hammond, 1996). Whole thalamus was studied at 1, 3, 6, 9, 12, 16, and 22 days of age, and in adult rats; MGN was examined at 8 and 23 days. Levels of AChE mRNA in the thalamus rose steadily for about nine days and then decreased. The MGN showed high levels of AChE mRNA and enzyme activity at day 8 but at day 23 both measures of AChE expression were 55% lower ($p < 0.001$). This result may reflect reduced message stability. In vivo experiments were performed to assess that possibility by determining the decay of mRNA levels after treatment with actinomycin D and adenosine analog 5, 6-dichloro-1-ß-

ribofuranosyl-benzimidazol. In conclusion, our data support the view that AChE up-regulation during neural morphogenesis may be an important feature of brain development.

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CHROMATOGRAPHIC ANALYSIS OF PURINE AND PYRIMIDINE NUCLEOSIDES AND DEOXYNUCLEOSIDES FROM IN VIVO BRAIN MICRODIALYSIS SAMPLES

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The in vivo brain microdialysis technique has become an accepted way of monitoring the extracellular space of neuronal tissue in situ. Dialysis samples are relatively clean and can easily be analysed by HPLC, because the samples can be injected directly without a cleanup procedure. The measurement of release of neurotransmitters, like catecholamines and amino acids, is an established technique. The analysis of purines and purine nucleosides from in vivo brain microdialysis samples has also become a widespread technique during the last decade and helped us to understand the role of adenosine and other purines in physiological and pathological brain processes like blood supply and hypoxia. There are, however, several lines of investigations suggesting the involvement of other nucleosides in brain function as well. Uridine and uracil nucleotides might have receptors distinct from the purin-receptors, certain deoxynucleosides can induce neuronal apoptosis.

The investigation of the role of nucleosides in neuronal function by microdialysis was hampered by the limitation of available chromatographic procedures because no more than 3 to 5 compounds were possible to be analysed. To solve this problem here we describe a chromatographic method to measure about 20 of these compounds from in vivo brain microdialysis samples. We used LKB Pharmacia SMART gradient chromatographic system with UV detection at 254 nm. Injection type chromatographic pumps, minimised dead spaces and cooled small bore C18 reversed phase columns enabled us to measure the so far undetectable compounds. The cooling of column is an unusual solution in the reverse phase analysis of small molecules, but according to our experience, it improved the selectivity of the separation of the hydrophilic molecules with small retention times and also decreased the bend broadening effect of diffusion. The identification and purity analysis was performed by the retention times, addition of known substrates to the samples, detection at two different wavelengths and also with electrospray MS-MS analysis of collected fractions of chromatographic peaks.

CHARACTERISTICS OF INCORPORATION OF 3-H B-AMYLOID PEPTIDE IN THE NEOCORTEX OF THE CAT

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High concentrations of B-amyloid peptides (BAP) have neurotoxic effects on neurones that might play a role in their pathogenic actions in Alzheimer disease. Incorporation of the labelled 3-H BAP 31-3S fragment with 11.2 μCi activity/25 mm^2 surface of the motor cortex of anaesthetized cat was investigated during control conditions, thalamic VL and pyramidal tract stimulations. Following 1 hour incorporation the cortex was excised and processed for light (LM) and electronmicroscopic (EM) autoradiography.

Incorporation of BAP induced intensive labelling of cells in all layers of the motor cortex. Excessive swelling, rounded cell contours and vacuoles in the cytoplasm and nuclei were observed in LM preparations. All types of cells (pyramidal, non-pyramidal and glia cells), mostly the postsynaptic structures of neurons and about 1/3 of the countered synapses were found to be labelled at EM level. Signs of cellular degeneration were disclosed in all preparations including chromatic condensation, swelling of nuclear envelopes, vacuoles and swelling of mitochondria and in endoplasmic compartments, dispersion of polyribosomes, disintregration of dentritic infrastructure etc.

The early signs of nuclear degeneration and excessive swelling indicates participation of apoptosis-like processes in the patogenic effect of amyloid peptides.

CHARACTERIZATION OF OSPHRADIAL SYSTEM AND ITS CHANGES UNDER THE INFLUENCE OF HEAVY METALS IN *LYMNAEA STAGNALIS L.* (GASTROPODA)

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Osphradium is a sensory organ of Molluscs living in aquatic biotop specialized for sensing mechano-, chemo- and osmotic stimuli of environment. Osphradium consists of the sensory epithelium and a ganglia connected to the CNS with the osphradial nerve. However, the physiological knowledge in regard its functioning is rather poor.

In the present investigations the osphradial system was studied in the *Lymnaea stagnalis* L. (Gastropoda, Pulmonata). It was shown that application of L-glutamate, L-aspartate, NaCl or urea to the surface of osphradium generates impulses transferred to the neurons of CNS. It has been proved that identified neuron taking part in the regulation of respiration (e.g. neurons of J-, H- and A-clusters, neurons of VV2 , RPeD1 and VD4) receive synaptic inputs from the osphradium. These inputs can be either excitatory or inhibitory. The acute treatment of the

osphradium or the chronic treatment of the whole animal with heavy metals modify the information transfer from the periphery to the CNS in a way corresponding to the substances applied to the osphradium.

More often the activity pattern of the central neurons became asynchronous or burst-type following heavy metal treatment and the neurons started to fire in an independent way.

It was found that osphradium is sensing all the changes in the environment and sends adequate information into the CNS resulting the modulation of vital physiological functions, in this case of respiration.

The model we studied can be used for investigating the origin and transfer of sensory information, as well as for studying the formation and reorganization of neural networks.

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FLASH-INDUCED RESPONSES OF THE RETINA AND THE CORTEX IN DIFFERENT BACKGROUND LIGHT AND STIMULUS INTENSITIES: DOES THE B-WAVE REFLECT STIMULUS INTENSITY?

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The B-wave of the retinogram is the only signal in the central nervous system which is sufficiently established to be of glial origin, however, its relation to the incoming stimulus is still a question of debate. The main point is whether the B-wave area does or does not reflect the stimulus parameters as strictly as it is known about the responses of the neuronal elements of the retina. It is an important problem because the spatial buffering current in the retina is the best model actually available for studying glial functions in the CNS.

In the present study we used freely moving rats implanted with LEDs; eyeball-, retrobulbar and visual cortex electrodes. A small LED (3 mm) served for the application of different background lights independent from the head position of the rats, a high emission LED (5 mm, bright LED) served for stimulation. We recorded intensity series adjusting the flash duration from 10 μs to 400 ms and we recorded responses to 1 ms flashes at 6 different stimulus intensities. Simultaneously, cortical evoked responses were recorded to compare the flash induced responses of the cortex and retina.

Our results show that the stimulus intensity is reflected in the retinogram in a surprisingly small range of intensities, than it saturates. The intensity dependence of the oscillatory potentials sitting on the uprising leg of the B-wave, changes with the stimulus intensity in a wide range of flash duration. At increasing background lights, the B-wave areas decreased with light intensity very sensitively as well as the oscillatory potentials.

Cortical responses were also sensitive to background light, and although the pattern of change in the intensity series to flash durations from 10 μs to 400 ms was similar to that of the B-waves, they showed a much smaller decrease than B-waves when the flash duration was becoming shorter.

On the basis of our findings we assume that B-wave is more sensitive to background light than to stimulus duration so it rather reflects the actual state of light adaptation than the neuronally released quantity of ions to flash. It is interesting to note that cortical responses

changed quite similarly to B-waves in intensity series but the pattern of change of the oscillatory potentials was significantly different.

EFFECT OF LOW TEMPERATURE ON THE NICOTINIC RECEPTOR AGONIST PRODUCED SEROTONIN RELEASE IN RAT HIPPOCAMPUS

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Dual effect of 1,1-dimethyl-4-phenylpiperazinium (DMPP) and lobeline has been observed in the study of mechanism of serotonin release induced by nicotinic receptors stimulation in rat hippocampus. Mecamylamine, a specific nicotinic antagonist, failed to influence lobeline-produced increase in 5-HT release but the effect of DMPP was antagonized. It has also been found that the DMPP-produced 5-HT release was extracellular Ca^{2+}-independent since Ca^{2+}-channel blockers had no effect and DMPP was also active in Ca^{2+}-free medium, challenging the conventional view of high Ca^{2+}-permeability of nicotinic Acethyl-choline receptor (nAChR). These resultes suggest that DMPP and lobeline act not exclusively on the nicotinic receptors. The goal of the present study was to investigate the possible role of other mechanisms such as carrier uptake in the nicotinic receptor activation by DMPP and lobeline.

In our experiment we attempted to find explanation that the Ca^{2+}-independent DMPP-produced 5-HT release assuming that at 7 °C when the membrane carrier proteins are inhibited in a Ca^{2+}-insensitive manner can play a role in the nicotinic receptors-produced release. At 7 °C, in general, the carrier, located in the membrane of the terminal, takes back the transmitter at physiological ion concentrations, inhibit of this carrier results in enhance of level of the transmitter. However, the direction of uptake process may turn out and results in release of transmitter. Hypothermia induced inhibition of release by lobeline while the DMPP-evoked 5-HT release was rather potentiated.

These data shown, that the nicotin agonists (DMPP and lobeline) act using different mechanism: lobeline releases 5-HT from the cytoplasm by the reversal of carrier uptake while DMPP produced release of 5-HT mediated via nAChRs in rat hippocampus.

COMPUTATIONAL MODEL OF A DIRECTION SENSITIVE MOTION DETECTOR NEURAL NETWORK

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A neural net was constructed simulating a retinal network being capable of direction sensitive motion detection. The computer program that simulates the network was written in Assembly programming language.

The network contains ten input neurons taking place in a straight line, they coordinate two output neurons and forty inhibitory cells.

If the input pattern is stationary, neither output neuron shows firing activity. However, if a dot moves consistently to either direction in the receptive field, one of the output cells - depending on the direction of the movement - fires, and the network gives information about the movement and its direction within the receptive field. It can distinguish the input patterns of different velocities: if the movement of a dot is too slow or too fast, the output cells remain silent.

This behavior shows correspondence with experimental results dealing with motion perception in amphibian retina.

LOCALIZATION OF NADPH-DIAPHORASE ACTIVITY DURING EMBRYOGENESIS IN THE POND SNAIL, *LYMNAEA STAGNALIS L.*

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Applying NADPH-diaphorase histochemistry, the distribution and development of the nitric oxide-synthase system (NOS) was investigated during ontogenesis in the pond snail, *Lymnaea stagnalis L.* For the early embryonic development, from stage E15 (E15%) to stage E21 (E35%), the appearance of a pair of large NADPH-diaphorase positive structures was characteristic, resembling the protonephridia. These structures formed two projections, one running to the future site of the developing eyes and the other projecting to the site of the future pedal ganglia. In a later stage of embryogenesis (metamorphosis, E50%-E75%), the shape of these characteristic structures completely disappeared although the staining of the remaining individual cells was not altered. No positive NADPH-diaphorase staining of neuronal perikarya could be observed in the developing ganglia until late embryogenesis (E27-28). At the same time, numerous positively stained sensory and epithelial cells were found in

the periphery, such as mantle edge, lips, pharynx, oesophagus and certain gland cells. The afferent axon processes of these elements formed tracts in the periphery which then could be followed to and in the developing central ganglia. In late embryonic stages, from E75% to hatching (E100%) and in the CNS of juvenile (postembryonic) snails, a small number of NADPH-diaphorase positive neurons were found in the cerebral and pedal ganglia. Additionally, a stained fiber tract in the neuropils was observed running around in the ganglionic ring. Some non-neuronal structures as the liver revealed also positive staining at late stages of embryonic development.

Based on our findings, the presence of nitric oxide (NO) in the developing nervous system of *Lymnaea* is suggested. NO seems to appear first in the peripheral nervous system and it is supposed that through the centrally projecting NOergic sensory elements it may participate in the formation of neuronal networks of central ganglia during the late developmental stages of *Lymnaea.* On the other hand, NADPHdiaphorase staining can also be considered as indicative of additional cell activity during embryogenesis.

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MOSAICS OF LARGE GANGLION CELLS IN THE *XENOPUS* RETINA

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To reveal their natural types, retrogradely labelled ganglion cells of the aquatic mesobatrachian frog *Xenopus laevis* were studied in retinal flatmounts. Cells with large somata and thick proximal dendrites were found to constitute three types, each with its own dendritic stratification pattern and mosaic. Following a scheme recently used for teleosts, these types were named αa, αab and αC. Cells of the αa mosaic (~0.4% of all ganglion cells) had very large somata and very large trees branching diffusely within sublamina *a* most sclerad sublamina of the inner plexiform layer. Their sparsely branched distal dendrites achieved consistent coverage by crossing those of their neighbours. Displaced and orthotopic cells belonged to the same mosaic, as did cells with symmetric and asymmetric trees. Cells of the αab mosaic (~1.2%) had large somata, smaller trees appearing bistratified at low magnification, and profusely branching dendrites. Their distal dendrites arborized diffusely through sublamina *b* and the vitread part of *a,* tessellating with their neighbours. All were in orthotopic position; most of them had symmetric dendritic tree. Cells constituting the αc mosaic (~0.5%) had large perikarya and very large, sparsely branching, flat, and overlapping trees in sublamina *c* (the most vitread). All were orthotopic; some of them seemed asymmetric. Nearest-neighbour analyses and spatial correlograms confirmed that each mosaic was regular and independent, that displaced and orthotopic cells belonged to the same mosaic, and that spacings were reduced in juvenile frogs. Mosaic statistics for α cells were compared with types previously defined by size and symmetry in *Xenopus.*

SUBCELLULAR LOCALIZATION OF CEREBRAL CALCINEURIN AND AKAP79 IN CORTEX

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Protein phosphorylation plays an important role in signal transduction and long-term changes in neuronal excitability. The phosphorylation state of the proteins is regulated by diverse protein kinases and protein phosphatases. In neurons, cAMPdependent-protein kinase A (PKA), protein kinase C (PKC) and the Ca^{2+}/calmodulin dependent phosphatase 2B (calcineurin) are attached to a scaffold protein, A kinase anchor protein (AKAP), thought to anchor these three enzymes to postsynaptic densities. Positioning the kinases and phosphatases near their substrates may ensure the spatial specificity for the regulatory action. Therefore, the cellular and the subcellular localization of calcineurin and AKAP was investigated in human and rat cerebral cortical tissue with a preembedding immunogold histochemical method. In human cortex, AKAP was found to be present at the postsynaptic site near, but not attached to the postsynaptic density. The presence of calcineurin in different kinds of neurons was examined with the mirror technique. We found no calcineurin immunoreactivity in rat hippocampal GABAergic interneurons. The absence of calcineurin from interneurons should profoundly alter the negative feedback exerted by calcium entry onto the NMDA (n-methyl d-aspartate) subtype of glutamate-gated ion channels. Patch clamp recordings performed in the presence of FK506, a specific calcineurin inhibitor, confirmed differences in the regulation of NMDA channel openings between interneurons and principal cells. By promoting the phosphorylation state of the channels, inhibition of calcineurin prolonged NMDA channel openings in CAl pyramidal and granule cells, but in the absence of calcineurin, it had no effect in interneurons. These results are consistent with the presence or absence of calcineurin, most likely anchored to the postsynaptic membrane by AKAP, playing a pivotal role in the regulation of glutamatergic transmission through controlling the phosphorylation state of NMDA channels.

STUDY ON THE CO-RELEASE OF ENDOGENOUS ATP AND [^{3}H]NORADRENALINE FROM RAT HYPOTHALAMIC SLICES

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The release of endogenous ATP, measured by the luciferin-luciferase assay, and of [^{3}H]noradrenaline from the in vitro superfused rat hypothalamic slices were studied. ATP and [^{3}H]noradrenaline were released simultaneously during resting conditions and in response to

low and high frequency field electrical stimulation; the release of both substances were frequency dependent between 2 Hz and 16 Hz.

The stimulation-induced release of ATP and [^{3}H]noradrenaline was diminished by 83% under Ca^{2+}-free conditions. Tetrodotoxin inhibited the majority of the evoked release of both ATP and [^{3}H]noradrenaline, however, it was less effective in reducing the release of [^{3}H]noradrenaline, than that of ATP.

Bilateral stereotaxic injection of 6-hyroxydopamine to the ventral part of the ventral noradrenergic bundle, originating from the A1 cell group in the brain stem, resulted in a 55% reduction of endogenous noradrenaline content of the hypothalamic slices, and the tritium uptake and the stimulation-evoked release of [^{3}H]noradrenaline was also markedly reduced. While the basal release of ATP was not affected, the evoked release was diminished by 72% by this treatment.

Perfusion of the slices with noradrenaline (100 μM) initiated rapid and continuous tritium release; on the other hand, it did not release any ATP.

In contrast, bolus administration of (-)nicotine and DMPP evoked parallel release of ATP and [^{3}H]noradrenaline which was inhibited by the nicotinic receptor antagonist mecamylamine; 6-hydroxydopamine lesion of the ventral part of the ventral noradrenergic bundle did not affect the nicotine-evoked ATP and [^{3}H]noradrenaline release.

While CH 38083, a non subtype-selective α_2-antagonist and BRL44408, the subtype-selective α_{2AD} antagonist augmented the evoked release of [^{3}H]noradrenaline, ARC239, a selective α_{2BC} antagonist was without effect. In contrast, neither of the α_2-antagonists significantly affected the evoked-release of ATP.

In summary, we report here that endogenous ATP and [^{3}H]noradrenaline are co-released stimulation-dependently from superfused rat hypothalamic slices. A significant part of the release of both compounds is derived from the nerve terminals, originating from the A1 catecholaminergic cell group of brainstem nuclei. Unlike that from the peripheral sympathetic transmission, NA and α_1-adrenoceptor agonists were unable to promote the release of ATP. Conversely, parallel ATP and NA release could be induced by nicotine receptor activation, but this release does not originate from the same nerve endings, but rather from cell bodies / or nerve terminals of median eminence. The release of [^{3}H]noradrenaline is inhibited by endogenous NA via α_{2AD} subtype of adrenoreceptors, while the release of ATP is not subject to this autoinhibitory modulation. In conclusion, our results support the view that ATP is involved in the neurotransmission in the hypothalamus, but the sources of the released ATP and NA seem to be not identical under different stimulatory and modulatory conditions.

EFFECT OF INHIBITORS ON PROLYL ENDOPEPTIDASE ISOLATED FROM DIFFERENT SPECIES

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Prolyl endopeptidase (PEP) [EC 3.4.21.26] is a serine protease cleaving small peptides on the carboxyl side of proline, with the exeption of P-Pro bonds. PEP is widely distributed in

mamals and can be purified from various organs, including brain.[1,2] The enzyme is found both in cytosolic and membrane bound forms.[3] Prolyl endopeptidase has been shown to cleave several naturally occurring peptides: oxytocin, vasopressin analogues, angiotensin, bradykynin, substance P, neurotensin, lutenising hormone-releasing hormone and thyrotropin releasing hormone.[4] Through the breakdown of neuropeptides playing important role in memory and learning processes, PEP has a key function in keeping the peptide balance in the brain. A potent inhibitor of the enzyme could have a therapeutic value for treating progressive memory deficits and cognitive dysfunction related to aging and neurodegenerative diseases of central nervous system. The goal of our investigation was to compare PEP inhibitors of different structures in the presence of various mammalian brain prolyl endopeptidases. Known PEP inhibitors (JTP 4819, SUAM-1221)[5,6] were tested on pig, rat and human PEP enzymes and the inhibitors have shown species-pendent inhibitory activity.

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EGIS-6979 A NOVEL 5-HT_{2A} RECEPTOR ANTAGONIST DRUG AS A PUTATIVE ANXIOLYTIC AGENT

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EGIS-6979 was synthesized for the serotonin (5 HT) project in EGIS Pharmaceuticals Ltd. According to *in vitro* receptor binding studies this molecule has selective affinity to the 5-HT_{2A} receptor subtype of the 5-HT receptor family and also shows high affinity to sigma receptor. It does not have considerable effect on adrenergic, dopaminergic, CCK and 1,4-benzodiazepine receptors.

In *in vivo* experiments EGIS-6979 effectively antagonizes the 5-HT_{2A} receptor-mediated (2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI)-induced head twitch reaction in mice (ID_{50}=2.5 m/kg), as well as in rats (ID_{50}=10.9 mg/kg). The compound exhibits potent activity in three models of anxiety: (1) it significantly increases the time spent on and the number of entries to the open arm in the elevated plus maze test in Wistar rats, (2) effectively antagonizes marble-burying behavior in C57Black mice and (3) also remarkably inhibits the stress-induced hyperthermia in DBA/2 mice.

On the basis of these findings EGIS-6979 might be a new putative anxiolytic agent, which presumably exerts its activity through 5-HT_{2A} receptors, however, the possibility that sigma receptors may also contribute to the anxiolytic effect of the compound cannot be excluded. Further investigations are required to understand the precise mechanism of action of the compound.

ELEVATION OF ENDOTHELIAL ACID PHOSPHATASE ACTIVITY IN HISTAMINE AND ISCHAEMIA INDUCED VASOGENIC BRAIN OEDEMA

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Cerebral ischaemia results in increased blood-brain barrier (BBB) permeability and significant changes in endothelial enzyme activities. Recent *in vitro* and *in vivo* studies indicated that different isoforms of acid phosphatase (APase) enzyme may play a role in the regulation of transendothelial transport properties. The aims of the present study were to investigate (1) the changes in APase activity concomitantly in isolated cortical microvessels (CMV) and in the whole cortical tissue; and (2) vasogenic brain oedema formation in cerebral cortex during pathological conditions. Two animal models were used in the present study. In the first part of study, different doses of histamine (0, 10^{-6}, 5×10^{-6}, 10^{-5}, 5×10^{-5}, 10^{-4} mol, respectively; n=8 in each) was injected in 1.0 ml isotonic saline into the internal carotid artery of anesthesized newborn pigs. In a model of global cerebral ischaemia, common carotid arteries of Sprague-Dawley CFY female rats were occluded on both sides and the animals were subjected to cerebral ischaemia of different duration (1, 2, 4, 8 and 16 h; n=12 in each). Cortical tissue were taken from each animal and CMV were isolated. Total acid phosphatase activity was measured both in CMV and cortical tissue by the rate of hydrolysis of *p*-nitrophenyl-phosphate. The changes in the activity of different APase isoforms were determined. BBB permeability changes were characterized by the extravasation of sodium fluorescein and Evan's-blue-albumin into the cortical tissue. Statistical analysis was performed using either one-way analysis of variance (ANOVA) or Kruskal-Wallis ANOVA on ranks followed by the multiple comparisons by the Student-Newman-Keuls method. Histamine did not change total and tartrate-resistant acid phosphatase activity in porcine brain tissue, but a dose-dependent increase was measured in isolated CMV. During global ischaemia total APase activity was significantly increased in CMV 2 h after the occlusion compared to the values measured in other groups. In the same time, both lysosomal and cytosolic types of enzyme were activated. Lysosomal APase activity was significantly elevated in cortical tissue 1 h after the occlusion, while both activities were decreased in cerebral cortex 4 and 16 h after the induction of ischaemia. As soon as 1 h after intracarotid injection of histamine, a dose-dependent BBB opening was found, but only higher doses resulted in significant ($P < 0.05$) increase in permeability both for SF and albumin. In rats, BBB permeability for SF was increased all through the ischaemic period, while EBA transport was elevated more than 2 h after the carotid occlusion. We conclude that (1) both histamine and cerebral ischaemia resulted in changes of activity of APase isoforms in CMV and cortical tissue; (2) there is a dysregulation between APase activity changes in cerebral endothelial cells and in whole cortical tissue which mainly contains neurons and glial elements; (3) elevated endothelial APase activity may have a role in the increase of BBB permeability during pathological conditions.

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THE EFFECT OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE ON ANALGESIA, MORPHINE TOLERANCE AND WITHDRAWAL IN MICE

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Pituitary adenylate cyclase-activating polypeptide (PACAP) has been detected in a number of areas in the spinal cord and medulla, where can participate in a variety of autonomic and sensory functions. The role of PACAP however, on pain sensitivity has not been fully established in various species.

The aim of the present study was to investigate the effect of PACAP on pain sensitivity upon intracerebroventricular (icv) peptide administration and to determine the action of the polypeptide on tolerance to and dependence on morphine, including naloxone-precipitated withdrawal signs in mice.

Acute administration of the polypeptide (62.5-1000 ng/animal icv) did not alter nociceptive threshold of the tail-flick latency at various time-intervals, but decreased the antinociceptive effect of a challenge dose of morphine. Repeated peptide injection significantly blunted the analgesic response of morphine. Development of chronic morphine tolerance, induced by pellet implantation was not affected by PACAP. Naloxone precipitated withdrawal signs (e.g. hypothermia and decrease in body weight) were altered by peptide treatment however, the onset of stereotype jumps upon morphine antagonist were not altered.

The results indicate that PACAP may participate in pain transmission in mice.

EFFECTS OF CGP 35348, A SELECTIVE $GABA_B$ RECEPTOR ANTAGONIST ON EPILEPTIC ACTIVITY OF ADULT AND YOUNG RATS

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CGP 35348 is a widely used drug, as a specific blocker of $GABA_B$ receptors, however most of the work is concentrating on the different area of the hippocampus. In our present work we investigated the effects of inhibition of $GABA_B$ receptors on the development and maintenance of cortical ictal epileptiform activity, induced by 3-aminopyridine (3-Ap) in adult and young (from 9 to 24 days old) anesthetized rats. In one group, the somatosensory cortex of the animals was pretreated by CGP 35348 solution, before induction of the primary focus by local application of 3-Ap crystal, in order to study the $GABA_B$ blocking effects on

the initiation of the epileptiform activity. In the other group, the animals were treated on the primary focus by CGP 35348 in the stage of ongoing epileptic activity, to analyze the changes in the maintenance of epileptiform activity.

Our results show a facilitation in both the induction and maintenance of electrocorticographic epileptiform activity of the CGP 35348 treated animals in comparison to the control ones. This was indicated by a reduction in the latency of the first ictal period, increase in the frequency and amplitude of epileptiform discharges, ancl an overall increase of ictal activity on the account of interictal period. However, these changes were more significant in the maintenance of epileptiform activity, suggesting, that: more $GABA_B$ receptors are in active condition in an already functioning epileptic focus, displaying some anticonvulsive effects. It is suggested, CGP 35348 blocks presynaptic $GABA_B$ receptors, which controls excitatory transmitter release. For further conclusions, additional experiments are needed.

CALCIUM BINDING PROTEINS IN AVIAN HIPPOCAMPUS UNDER NORMAL AND ISCHEMIC CONDITIONS

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Calcium binding protein containing neurones are abundant in the hippocampus of vertebrates. Such neurones contain parvalbumin (PV), calbindin (CB) or calretinin (CR) and they belong to morphologically and functionally different groups. The presence of calcium binding proteins has been verified in birds although their distribution and functional relations are less well studied in the avian hippocampus. In the present study we report the distribution of PV, CB and CR immunoreactive structures in the hippocampi of domestic chicks. These neuronal groups have been found to be particularly vulnerable to brain hypoxia in mammals, therefore we complemented our observations by comparing normal and ischemic experimental animals.

Cerebral ischemia was elicited by 15 min bilateral occlusion of the internal carotid arteries of day-old domestic chicks, and cell loss was verified by the Gallyas method. Following a 10-day recovery period, both ischemic, sham-operated and normal birds were perfused under terminal anaesthesia and the brains were processed for immunohistochemistry using monoclonal antibodies against PV and CB7 and a polyclonal antiserum against CR (SWant). Microscopical observations were assisted with computerised image analysis.

In normal and sham-operated birds, PV was present in multipolar neurones of diverse morphology, forming three major groups: (i) small and densely packed perikarya in the tip of the ventral subdivision, (ii) large cells embedded in a strongly immunoreactive fibrous neuropil in a subpial region arching from rostral to dorsal and caudal, equivalent of the dorsomedial subdivision, (iii) large multipolar neurones and diffusely stained neuropil in the dorsolateral subdivision, partially overlapping the substance P field. Between groups (ii) and (iii) a laminar region was impoverished in cellular and neuropil immunoreactivity. CB-containing cells were more uniformly distributed, without marked nodes of density. The site of group (ii) reactive for PV was devoid of CB immunostaining. However, the tissue band appearing poorly reactive for PV was filled with intensely stained CB-positive neuropil. CR reactive neurons were smaller in size and evenly distributed throughout the hippocampus. Following ischemia, marked

alterations were noted only in PV reactive neurones: a reduction in PV immunostaining in Group (i), shrinkage of PV reactive neuropil in group (ii) with a shift in size distribution of perikarya.

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DEPENDENCY OF EVOKED POTENTIALS ON THE BURST-SUPPRESSION PATTERN OF THE EEG IN DEEP URETHANE ANESTHESIA IN RATS

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In rats deeply anesthetized with urethane a special EEG pattern called burst-suppression can be observed. It consists of long (few seconds) deep-positive flat periods and shorter (about 500-800 ms) deep-negative shifts. During the shifts, high frequency waves or oscillations appear. As the EEG pattern is closely related to the level of thalamo-cortical activation, it can be supposed that these short epochs indicate a higher level of activation in which sensory inputs can reach the cortex more easily. Burst-suppression pattern can be induced by different anesthetics, not only by urethane, thus the question arises whether this phenomenon is related to the occasionally occurring awareness during surgical anesthesia.

To compare the effect of sensory stimulation during the flat periods and the shifts the following experiments were carried out: In urethane anesthetized rats (1.2 g/kg) monopolar recording electrodes constructed of teflon insulated stainless steel wires (diameter 250 μ) were placed into the frontal cortex, the representation of the hind limb on the primary somatosensory area, as well as into the primary auditory and visual cortices. The tip of the electrodes was in the pyramidal layer. To examine cortical responses, stimulating electrodes were placed on the nervus ischiadicus, while visual and acoustic stimuli were generated by a LED and a piezoelectric device, respectively. Stimuli were given at every 5 second. Evoked potentials were assigned into one of two groups according to their arrival during flat periods or shifts off-line.

All stimuli which arrived during a flat period induced shifts in all cortical areas. No differences were seen in the amplitude or length of spontaneous and evoked shifts; high frequency waves or oscillations were also present in the evoked ones. There was no apparent difference between the three sensory modalities in their effectiveness to induce shifts. The shifts in a given cortical area did not depend on the type of the stimulus except that the appropriate stimulus induced evoked potential as well. High frequency oscillation at 40 Hz was most prominent after visual stimuli and on the contralateral visual cortex; it was visible in the averaged evoked potentials too.

In general, evoked potentials recorded during the deep-negative shifts contained more early components than those recorded during flat periods. The latency of the evoked potential peaks were longer and their amplitude smaller in flat periods than in shifts. This tendency was especially true for the late components. The differences were best seen in the auditory responses, while the visual evoked potentials were nearly identical. This phenomenon is in conformity with the fact, that the burst-suppression pattern was the less observable in the EEG recorded from the visual cortex.

We can conclude, that sensory inputs can reach the cortex more easily during the deep-negative shifts compared to the flat periods. However, the stimuli themselves also induced shifts, thus the later components of the evoked potentials were generated in similar

background in both cases. Therefore, it would be interesting to see if more prominent differences can be obtained with weaker stimuli.

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ELECTRICAL ACTIVITY OF YOUNG RATS BORN TO MOTHERS WITH CHRONIC EPILEPTIC ACTIVITY AND VALPROATE TREATMENT

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In clinical practice the therapy of pregnant epileptic women is a big challenge because of the potential harmful effects of antiepileptic drugs to the fetus.

In order to model this situation 10-10 rats were treated with 600 mg/body weight kg valproate (VPA) per os (1. group) or 300 mg/body weight kg VPA (2. group), respectively. In the 2nd group epileptic seizures were induced by 3-aminopyridine (3-Ap) injected i.m. in an 25 mg/body weight kg dose on every other day. Basic and epileptic electrocorticographic activities of offspring (10-24 day-old) were examined. Epileptic activity was induced by local application of 3-Ap crystal to the somatosensory cortex of anaesthetized rats. Features (latency, duration, frequency) of focal and secondary generalized periodic ictal activity were analyzed during one hour recording time.

Our preliminary results show moderated changes in the cortical seizure activity of the offspring of VPA-treated mothers comparing to the control group. According to our data, there are significant differences between the cortical epileptic activity of groups 1 and 2. A few members of group 2 exhibited unusual short (5-7 sec) and frequent (33-36/hour) ictal periods, when they became 30-33 day-old. In order to make further conclusions additional experiments are needed.

COMPUTER ANALYSIS OF FIRING PATTERN OF *LYMNAEA* NEURONS

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Widely used quantitative methods for characterization of neuronal spike patterns are based on the time of action potentials and interspike data. Most of the calculated parameters (e.g. first- and higher order interspike intervals, interspike-histograms, mutual intervals) characterize the static properties of the pattern. The local firing frequency, however, is a time-dependent dynamic measure of the single neuronal activity. Conventional spike-counting method usually do not allow to observe slight modulation of firing activity in preparations with frequencies

less than 10 Hz. Convolution of the discrete spike train with a continuous and symmetric function (Gauss-function here), however, results a 'smooth' and continuous spike density function (SDF) as a proper measure of the local firing frequency. The spike density function is a sensitive character of the neuronal activity and can be used as the initial function for further analysis (Fourier-spectra, auto- and cross-correlation, return map, phase-trajectories, etc.).

Two computer programs have been developed for recording and evaluating spike trains and firing patterns of *Lymnaea* neurons. Recording of the firing pattern is based on the accurate measurement of the interspike-intervals during the experiment. In our experiments the discharge patterns of up to 4 neurons have been simultaneously recorded by the computer program supporting multichannel AD-converter. Analysis of the time series has been performed by the off-line evaluation program calculating first- and higher order distributions of the interspike-intervals, spike density functions, Fourier-spectra and correlation. The neuronal firing patterns have been characterized by the methods of nonlinear dynamics (return maps, phase trajectories), too.

These computer aided recording and analysis techniques have been in the studies of effects of neuromodulators and environmental toxic substances. Also the modulation of activity of postsynaptic neurons have been examined when presynaptic central pattern generator neurons have been stimulated by intracellular electrodes (current clamp and dynamic clamp). The experimental and computational methods, as well as the quantitative properties of the firing patterns of neurons in the *Lymnaea* respiratory network will be presented.

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DORSAL ROOT GANGLION CELLS EXPRESSING THE NK1 RECEPTOR IN YOUNG RATS

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The expression of neurokinin-1 receptors was studied in the fourth lumbar dorsal root ganglia of young rats using immunohistochemical and electrophysiological techniques. Use of the specific immunoserum raised against the C-terminal fragment of rat neurokinin-1 receptor revealed immunoreactivity in 32±1.5% of dorsal root ganglion neurones. The majority of these immunostained neurones belonged to the medium diameter cell range. A double immunohistochemical reaction with antibodies for substance P and neurokinin-1 receptor showed a complete absence of double-labelled cells in the ganglia excluding the possibility of auto-activation in ganglion neurones.

Superfusion of substance P (1~1M) to an *in vitro* preparation of fourth lumbar dorsal root ganglion induced a reversible long-lasting depolarisation measured by extracellular suction electrodes attached to the dorsal roots. This response to substance P was partially antagonised by the selective neurokinin-1 receptor antagonist RP 67580 (1 μM). Intracellular recordings distinguished between Aα/ß-, Aδ- and C-sub-types of ganglion neurones. Superfusion of substance P (1 μM) evoked excitatory depolarising responses only in Aδ-type neurones.

These results strongly suggest the localisation of the neurokinin-1 receptors on medium sized sensory ganglion neurones and the selective excitation of Aδ-type neurones in these ganglia by substance P. Our results also demonstrate possible physiological importance of peripheral neurokinin-1 receptors located on non-substance P-producing dorsal root ganglion neurones.

INTERACTION BETWEEN POSITIVE AND NEGATIVE MODULATORS OF THE AMPA RECEPTOR

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It is not clear whether the allosteric site through which GYKI 52466 and its analogs exert their negative modulatory action on AMPA receptors is the same which is responsible for the inhibition of desensitization by the positive modulators cyclothiazide (CYZ), aniracetam (ANI), or 1-BCP. In our present study the nature of the interaction between these two types of compounds was investigated in various experimental models.

In the retinal spreading depression test, where agonist-induced responses were dose-dependently blocked by GYKI 53784, and potentiated by the positive modulators, we found that CYZ, at the concentration range of 20-160 μM shifted the dose-response curve of GYKI 53784 to the right. 1-BCP (1000 μM) had similar, but weaker action.

The interaction of ANI and GYKI 52466 was investigated in hippocampal slices. GYKI 52466 dose-dependently inhibited the Schaffer/commissural fibers stimulation-evoked field potentials in CA1. Its IC50 was not influenced by the coperfusion of 500 or 1000 μM ANI. Further, the fact that the negative modulator 2,3-benzodiazepines and the positive modulator ANI, CYZ, or 1-BCP affected different components of the synaptic response suggests a distinct site of action.

Measurement of AMPA evoked whole-cell currents from acutely isolated cerebellar Purkinje cells is a straighforward way of studying AMPA receptor modulation. A rapid, electronically controlled drug application system was developed, which greatly helped pharmacological work. In this model, ANI (500 μM) had no influence on the current inhibitory effect of GYKI 53784, IC50s were 0.31 and 0.27 μM in the absence and in the presence of ANI, respectively.

In chloralose anesthetized cats, myographically recorded flexor reflexes were reduced in a dose-dependent way by 2,3-benzodiazepines, while 1-BCP caused a huge potentiation of the responses, and seizures at higher doses (>10 mg/kg i.v.). The quantitative evaluation of the interaction was rather difficult because both compounds had a short-lasting effect. It was obvious that 1-BCP caused marked reflex potentiation even when reflex responses were blocked by GYKI 53784 almost completely, but the effect of GYKI 53784 seemed to be weaker after 1-BCP.

In summary, the results of the retinal spreading depression test suggest a competitive interaction between positive and negative AMPA receptor modulators. The interpretation of the experimental data in this model, just as in the flexor reflex model, however, is difficult, and also the test systems are rather complex. Our results in the hippocampal field potential

and whole-cell current experiments do not support the existence of a common site of action for the two classes of compounds.

IN VITRO RADIOLIGAND BINDING STUDIES ON VOLTAGE-DEPENDENT SODIUM-CHANNELS IN RAT BRAIN SYNAPTOSOMAL FRACTION

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Voltage-dependent sodium-channels (Na-channels) are multisubunit protein complexes in the membranes of the neurons and muscle cells and play fundamental role in the generation of electric stimuli. The function of Na-channel can adopt at least three distinct conformational states: the resting, activated and inactivated states. Understanding the molecular basis for voltage-dependent activation, rapid inactivation and ion-conductance is a major goal of the current research. On the voltage-dependent Na-channel several neurotoxin binding sites have been identified. Batrachotoxin (BTX), a specific activator for these channels is a widely accepted probe for the demonstration of the activated channel-state. Lifarizine, a diphenylpiperazine analogue displaces BTX binding on its sites with a moderate affinity, indicating an interaction with the Na-channel. ^{3}H-Lifarizine binds specifically to the activated channel but has higher affinity for the channel in its inactivated state. Patch clamp measurements, however, showed that Lifarizine blocking Na-current (K_I=0.2μM at -70 mV holding potential) can effectively inhibit the L-type and T-type calcium currents as well (K_I values 0.8 μM and 2.8 μM, respectively). In order to examine the selective ^{3}H-Lifarizine binding to inactivated Na-channels, direct saturation analyses have been performed in the presence of ligands to L-type Ca-channels Nitrendipine and Diltiazem. In synaptosomal fraction, depolarized with 130 mM KCl, saturable and high affinity binding of ^{3}H-Lifarizine was observed (K_D= 57 nM, B_{max}= 7.1 pmol/mg protein). Changing the binding buffer for 50 mM Tris-HCl resulted in a two fold increase in the apparent affinity and also some slight alteration in the estimated density of the binding sites (K_D=26.5 $\pm$ 2.6 nM, B_{max}= 5.7 $\pm$ 0.6 pmol/mg protein). In a concentration range of 0.001 to 0.3 μM, only a minor component (< 20 %) of the ^{3}H-Lifarizine binding was sensitive for Nitrendipine and Diltiazem regardless to the nature of the depolarization. ^{3}H-BTX and ^{3}H-Lifarizine radioligand binding assays used in this study in order to detect activated and inactivated channel states may provide a useful tool for studies on voltage-dependent Na-channel.

VIRAL LABELLING OF LACRIMAL GLAND-SPECIFIC NEURONAL PATHWAYS

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The precise knowledge about the neuroanatomical basis of the secretory regulation of the lacrimal gland and the neurochemical characteristics of the neurons involved is not complete. We used trans-synaptic viral labelling for mapping the nervous system nuclei containing preganglionic cells involved in the innervation of the extraorbital lacrimal gland of rat (Sprague-Dawley, both genders). Ten µl (lOE6 PFU) of Bartha's strain of pseudorabies, e.g., Aujeszky's disease virus was injected into the gland, and after 20 h (2 rats), 45 h (2 rats) 72 h (3 rats) and 90 h (2 rats) the brain, the thoracic spinal cord, the Gasserian and superior cervical ganglia were removed and processed for routine, light microscopic immunocytochemical procedure (ABC technique). Monoclonal antiviral antibody (code number I/7, courtesy of O.S. Morenkov) or polyclonal antiviral antibody (code number 134, courtesy of R.R. Miselis) were used for the detection of viral proteins in the infected neurons.

Following 20 h of inoculation of the virus, labelled neurons were not detected in the brain, spinal cord, or ganglia. A few virus-labelled neurons were observed in the ipsilateral ponto-medullary salivatory area by 45 h postinoculation time. The number of labelled neurons of this area increased progressively by 72 h, and other neurons involved in the innervation of this area became labelled, too. The label became numerous and bilateral by 90 h. The labelled neurons in the ventrolateral ponto-medullary area formed a compact nucleus located lateral to the facial motor nucleus, in the vicinity of the A5 noradrenergic cell group. The neurons of this area were not readily separated from the neurons of the A5 cell group, therefore, double immunocytochemistry for detection of vws protein and tyrosine hydroxylase (TH) immunoreactivity simultaneously were performed. The majority of virus-labelled neurons of this area did not contain TH. They represent parasympathetic preganglionic neurons projecting to the lacrimal gland, and correspond to the superior salivatory nucleus described in the human brain. These neurons possibly form a subdivision of the so-called lacrimo-nasopalatine nucleus, which contain all the preganglionic neurons projecting to the sphenopalatine ganglion. A few neurons of the ipsilateral superior cervical ganglion (SCG) labelled following 45 hours of virus inoculation represent the sympathetic pathway. By 72 h after inoculation of the virus into the lacrimal gland their number increased in the ganglion and the neurons of the intermediolateral cell column of the thoracic spinal cord segments 1-2, (ipsilateral) projecting to the SCG became infected. The number of labelled neurons in the spinal cord increased progressively and the label became numerous and bilateral by 90 h, as well as, in other brain nuclei projecting to the spinal cord. The sensory afferent fibers originating partly from the lacrimal gland, partly from the surrounding connective tissue capsule, became also infected, as revealed by a few virus-labelled neurons in the ipsilateral Gasserian ganglion 45 h after the injection. Their number increased by 72 h, and their projection site, the nucleus of the spinale trigeminal tract became bilaterally infected by 90 h.

In conclusion, by injecting Bartha's strain of the Aujeszky's disease virus into the extraorbital lacrimal gland, the sympathetic, parasympathetic and sensory neurons projecting to or from the gland became progressively labelled. By the aid of this trans-synaptic labelling technique, a preganglionic parasympathetic cell group, equivalent to the human superior salivatory nucleus has been first localized in the brainstem of the rat.

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FINE TOPOGRAPHY OF THE HYPOTHALAMIC PARAVENTRICULAR AXONS TO THE MEDIAN EMINENCE

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The hypothalamic paraventricular nucleus (PVN) has a pivotal role in the control of the pituitary function. The paraventricular projections may reach the median eminence-pituitary stalk-pituitary gland through three different pathways: 1) through the lateral hypothalamus, 2) through the anterior hypothalamic nucleus and 3) periventricularly. The axons of the magnocellullar, oxytocin- and vasopressin-containing cells of the PVN are called the paraventriculo-hypophyseal (hypotalamo-pituitary) tract. These fibers sweep round the fornix, reach the lateral retrochiasmatic area and pass the internal zone of the median eminence on way to the posterior lobe of the pituitary (route #1). Neurons in the the parvocellullar subdivisions of the PVN project to the external zone of the median eminence and secret their neurohormones into the portal plexus which supplies the anterior lobe of the pituitary. In order to map the exact topography of this parvocellullar efferents toward the external zone of the median eminence we injected an anterograde tracer, *Phaseolus vulgaris* leucoagglutinin iontophoretically, into the different parvocellullar subdivisions of the PVN. The dorsal and lateral-posterior subnuclei do not give rise axons to the median eminence, these cells have efferents to the brainstem autonomic centers and the spinal cord. The axons of the anterior and the medial parvocellullar subdivisions leave the PVN lateralwards, parallel to the magnocellullar axons, arching over the fornix (route #1) or just medial to it (route #2) and terminate in the external zone of the median eminence, ipsilaterally. The periventricular subdivision of the PVN, however, has a unique pattern of projection. Axons leaving the PVN run mainly along the third ventricle (route #3) and terminate on the both sides of the median eminence. Besides the median eminence, periventricular fibers form a dense neuronal network in the ipsilateral arcuate nucleus without any significant projections to the other side of the hypothalamus. The possible neurochemical character of these periventricular axons is discussed.

KAPPA-OPIOID RECEPTOR EXPRESSION IN RAT ASTROCYTES AND OLIGODENDROCYTES IS REGULATED BY CYCLIC AMP AND GROWTH FACTORS

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In the CNS, the expression of opioid receptors is not restricted to neurons. According to latest in vitro data rat astroglial and human microglial cells express kappa opioid-receptor (KOR) mRNA. We confirm these observations by immunohistochemical methods in rat cerebellar cultures and by using RT-PCR and southern hybridization methods in rat glial cell cultures (Gaveriaux 1995). We have observed that KOR mRNA was expressed in matured and precursor astrocytes as well as in matured oligodendrocytes and in their precursor cells. Furthermore we showed the existence of this mRNA in preparations obtained from microglial cells. Expression was the highest in the astrocyte precursor cells and decreased with differentiation. No such changes were observed during maturation of oligodendrocytes.

Very few data are available for the regulation of KOR expression by extracellular signals. Therefore, we examined the effect of three growth factors known to be present in adult brain. Matured astroglial and oligodendroglial cell cultures were treated for two days with bFGF (10 ng/ml), or PDGF-BB (10 ng/ml), or LIF (10 ng/ml). We tested the effect of dbcAMP (lmM) as well - which is a possible secondary messenger in the opiod signal transduction pathway. It was found that both in astrocytes and in oligodendrocytes KOR mRNA expression decreased dramatically (90% decrease) under the effect of bdcAMP compared to the control. In astrocytes PDGF-BB and bFGF resulted in a decrease in the mRNA level while LIF treatment increased the KOR mRNA expression. In oligodendrocytes PDGF-BB and LIF treatement increased the level of the KOR mRNA while results with bFGF were not clear. Microglial cell cultures were treated only with LPS (0.1 μg/ml) - the most used activator of macrophages. After 24 hours incubation with LPS a 50% decrease was observed in the amount of KOR mRNA compared to the control.

These results bring new evidence on expression of opioid receptor mRNA in the glial cells of rat CNS. The regulation of this expression under the effect of extracellular signals suggests that opioids take part in the dynamic processes in glial cells possibly related to glial-neuron communication.

Abbrevations:
CNS.- central nervous system, RT-PCR - reverse transcriptase polimerase chain reaction, bFGF - basic fibroblast growth factor, PDGF-BB - platelet-derived growth factor, LIF - leukemia inhibitory factor, dbcAMP - dibutiril cyclic AMP, LPS - lipopolysaccharides.

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GENOME-SIZE STABILITY AND NEURAL DIFFERENTIATION OF NEUROECTODERMAL CELL LINES IMMORTALIZED BY pS3-DEFFICIENCY

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Spontaneously proliferating cells were isolated and cloned from brain cell cultures prepared from young (E9) embryos of mice lacking functional p53 genes. Genome-size stability of two cell-lines (NE-4C, NE-7C2) were examined through 400-900 cell cycles by counting the chromosome numbers of different passages. The chromosome number kept decreasing during the maintenance of the two cell lines. Proliferating activity, the duplication time and the developmental potencial were apparently not influenced by the loss of the chromosomes.

Treatment with retinoic acid (RA) resulted in the appearance of neurons and astrocytes in an amount of 30-40 percent of NE-7C2 cells. Neurons were characterized by their typical morphology (e.g. neurite-like processes and cell shape) and neurofilament immunoreactivity. Astrocytes were identified by GFAP immunoreactivity. During in vitro maturation, neurons displayed marked synaptophysin immunopositivity in the cytoplasm.

The importance of direct cell-to-cell interactions during RA induced neurogenesis was examined by disrupting the connections between the cells. Our data show that in the first 72 hours of RA treatment, direct interactions play a key role in establishing neural phenotype.

MODULATION OF THE CENTRAL FEEDING CONTROL AFTER PALLIDAL ADMINISTRATION OF IL-1β IN THE RAT

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The globus pallidus (GP) is intimately involved in the maintenance of body weight, metabolic control as well as in the regulation of various aspects (quantity, quality, sensorimotor integration, etc.) of food and fluid intake. During inflammatory processes, feeding is known to be altered, and the cytokines are those primary mediators which are supposed to possess causal role in the behavioral modifications. Among these immunoreactive substances interleukin-lbeta (IL-lß) has the strongest effect, and plays the most broadly discussed roles. The present experiments were designed to elucidate the effect of direct pallidal application of IL-lß m the regulation of feeding behavior. Short- and long-term food intakes, water intakes and body temperatures were measured after bilateral intrapallidal microinjections of IL-lß (with or without paracetamol /PAR/ pretreatment), and the effects were compared with those found in vehiculum-treated control animals and intraperitoneally (i.p.) injected IL-lß, IL-lß+PAR or control rats. Conditioned taste aversion (CTA) inducing capacity of the centrally or

peripherally administered cytokine was also tested. Not only the peripheral (i.p.) but the direct GP microinjection of IL-lß also remarkably reduced short-term food intake. In the long-term food intake, however, there was no significant difference among groups. Water intakes showed only slight variations. A preference-like enhanced saccharin consumption in the CTA tests revealed that anorexigenic effect of pallidal IL-1ß administration could not be due to a learned aversive mechanism. IL-lß microinjections into the GP were also followed by a significant increase in body temperature. This IL-lß induced hyperthermia, but not the anorexigenic effect, was attenuated by paracetamol pretreatment. These data, along with previous findings, indicate that the GP is one of the potential routes through which cytokines exert their direct modulatory effect on the central regulation of feeding. The underlying mechanisms of pallidal IL-lß effects are yet to be defined.

RESPONSES OF FOREBRAIN NEURONS TO IL-1β IN THE RAT AND RHESUS MONKEY

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Alterations of feeding have been reported to be induced by lateral hypothyalamic or, recently, by pallidal microinjections of an immunomodulator, the interleukin-lbeta (IL-I~). On the basis of these findings, a direct neuromodulatory role of the cytokine is supposed. To investigate this hypothesis, single-neuron recording studies with simultaneous microelectrophoretic drug applications were designed. In the present experiments, action potentials in the globus pallidus (GP) or orbitofrontal cortex (OBF; a key structure of the central feeding control, functionally and morphologically interrelated with the GP) of anesthetized rats and anesthetized or alert rhesus monkeys were extracellularly recorded by means of carbon fiber multibarreled glass microelectrodes during microelectrophoretic administration of a) IL-1ß, b) glucose, and c) "classical" neurotransmitters, such as norepinephrine, dopamine, glutamate, GABA and acetylcholine. Both in the GP and OBF (in all kinds of preparations), IL-lß-responsive neurons were found. These same cells, practically with no exception, were also sensitive to microelectrophoretically applied glucose, i.e., proved to be the elements of the previously discovered, hierarchically organized glucose-monitoring neural network (GMNW). The other chemicals elicited differential discharge rate changes of the neurons tested. Our results provided evidence for that IL-I ß can directly modulate neuronal activity in the GP and OBF. Furthermore, it is also suggested that cytokine effects are exerted via forebrain cells of the GMNW to contribute to the central regulation of feeding.

PHARMACOLOGICAL STUDIES OF THE PHARYNGEAL RETRACTOR MUSCLE OF THE GARDEN SNAIL *HELIX ASPERSA*

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The pharyngeal retractor muscle (PRM) of *Helix aspersa,* running from the columellar part of the shell to the buccal mass, is part of the muscular system responsible for withdrawing the animal into the shell. This elongated, 3-4 cm long muscle is easy to handle, and may provide a useful model to study the mechanisms of neuromodulation of the muscular contraction.

A series of putative neurotransmitter/neuromodulator substances were tested on the contraction (single twitches) evoked by direct electrical stimulation of the PRM. Glutamate applied to the bath evoked direct effect (fast contraction) of the muscle suggesting its direct role in the neuromuscular transmission. Other substances did not evoke fast contraction but modulated the parameters of the electrically evoked twitch responses. Dopamine decreased both the amplitude and the half relaxation time of the twitches, while octopamine only increased the amplitude but did not affect the half relaxation time.

More detailed studies were carried out comparing the serotonin effect to a neuromodulatory tetrapeptide FMRF-amide. Both substances increased the amplitude of the contractions and decreased the half relaxation time. FMRF-amide additionally evoked a slow but clearly visible contraction suggesting a possible catch mechanism involved in the regulaton of PRM activity.

Immunocytochemical studies visualized dense network of both serotonin- and FMRFamide immunoreactive fibers all along the muscle, arising from from the suboesophageal ganglia through the paired pharyngeal retractor nerve. The highly varicose fibers containing either serotonin or FMRF-amide dispalyed different patterns suggesting separate location of each of the modulatory substances.

Our results indicate that the relatively simple-task (fast contraction) of the pharyngeal retractor muscle of *Helix* is controlled by many substances, possibly by different mechanisms. To understand the functional significance of this complex regulation further studies are required both on isolated muscle and semiintact (muscle - central nervous system) preparations.

This work was supported by a Travel Grant from the Wellcome Trust to A.V. and the Hungarian Scientific Research Fund (OTKA) grant No. 016015 to K.E.

NUCLEAR [^{3}H]-NALOXONE BINDING IN RAT HYPOTHALAMUS: CHANGES DURING SEXUAL DIFFERENTIATION

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Nuclear [^{3}H]-naloxone binding in rat hypothalamus was demonstrated in our previous study. The observed binding parameters of these binding sites were sensitive to oestrogen and changed characteristically during puberty.

The aim of present investigation was to study the changes of nuclear [^{3}H]-naloxone binding in the hypothalamus during the period of sexual differentiation.

CFY rats aged 1-14 days were used (the day of birth is referred to as day 1). On the third day of life, group of male pups was surgically gonadectomized, female rat pups were treated intraperitoneally with 1 mg/rat of testosterone propionate dissolved in sesame oil. The animals were killed by decapitation. [^{3}H]-naloxone binding was assessed by in vitro saturation analyses in the oestrogen sensitive hypothalamic regions (preoptic area, anterior hypothalamus, medio-basal hypothalamus, eminentia mediana). For controls, tissue left in the cerebrum after removal of the hypothalamus was used.

High affinity (K_d: 0.5 - 2 nM) and low capacity of [3H]-naloxone binding sites were detected in the nuclear fraction of all examined hypothalamus, nevertheless, the shapes of the saturation curves reflecting the cooperativity in the bindillg were sex and age dependent. During the first three days of life, the Scatchard plots of the saturation curves of [^{3}H]-naloxone binding from both male and female rat hypothalamus were curvilinear, and the Hill coefficients were 1.89±0.3 and 1.93±0.2, respectively. Later, in females, the binding capacity continuously increased during the first two weeks of life, but no specific changes in the binding affinities were detected, the K_d values varied between 0.5-2 nM. From the third day of the life, the Scatchard analysis of the saturation data was linear, the Hill coefficient was ~ 1. Similar data were obtained from male rats castrated on the day 3, hut the curvilinear Scatchard plots turned to linear forms at only the end of the first week. In the males and as well in the testosterone treated females the binding capacities of the nuclear [^{3}H]-naloxone binding were lower than in the intact females at corresponding age, no changes in the binding affinities were found, but the Hill coefficients in every examined group were > 2, suggesting positive cooperativity in the binding.

The above-mentioned changes in the nuclear [^{3}H]-naloxone binding of the cerebrum could not be found.

The results suggest the contribution of the nuclear [^{3}H]-naloxone binding and its developmental changes to the sexual differentiation of the rat hypothalamus.

This work was supported by the National Science Research Fund (OTKA T 016316).

EFFECTS OF CO-ACTIVATION OF BASAL FOREBRAIN AND VENTROLATERAL THALAMIC NUCLEUS ON EVOKED POTENTIALS IN THE MOTOR CORTEX OF CATS

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Previous data indicates that the cholinergic afferents of cortical structures originate mostly from the basal forebrain (BF) and might play a role in learning processes. Before detailed cellular studies we surveyed the motor cortex if changes could be induced in neocortical excitability in general, or more specifically in identified synaptic pathways following activation of the BF.

Motor cortical surface potentials evoked by stimulation of thalamic VL nucleus were recorded before, under and after having paired them with electrical stimulation of the basal forebrain, in chronic awake and acutely anaesthetized cats. The aim was to examine the effect of BF activation on the excitability of the motor cortex. Two pulses at 100 Hz were applied to the BF, with 0-100 ms before paired VL stimuli in every third second, 500 times in each training period. After training of this type long-term enhancement of evoked potentials was not observed. Autocorrelation analysis was performed separately on the amplitudes of evoked potentials of the 20 min control period, the cca. 25 min training and the 20-40 min. after-training period on waking cat .

It was found that a very slow amplitude oscillation (T: 1-5 min) occurred in the control period, which disappeared during training and reappeared with altered frequency (even slower) in the after-training period. This phenomenon was most prominent when the time difference between BF and VL activation was 40 ms.

Our data indicates that changes both in excitability of motor cortex and in identified synaptic pathways could be induced following simulation of the BF cholinergic system.

This study was supported by OTKA T/7 16408 and by the Neurobiology PhD program of József Attila University of Sciences, Szeged.

DISTRIBUTION AND COLOCALISATION WITH OTHER NEUROCHEMICAL MARKERS OF CALRETININ IN FROG AND RABBIT RETINA

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Most of the nerve cells have been shown to contain one or more members of the EF-hand calcium-binding protein family. The role of them in nerve cells is very important in regulating calcium homeostasis. Furthermore, they may also serve as specific neurochemical markers of

neuron populations in the central and peripheral nervous system. Previous studies carried out on the retina of frog and rabbit investigated parvalbumin- and 28 kDa calcium-binding protein immunoreactivity in these structure. In the present study we aimed at examining calretinin-immunoreactive structures in the retina of *Rana esculenta* and rabbit, in order to identify specific cell populations containing this protein on the basis of their morphology and colocalisation with other neurochemical markers. In the frog retina numerous bipolar cells and a small group of amacrine cells were observed to contain calretinin in the inner nuclear layer. Some displaced amacrines and ganglion cells also show calretinin immunoreactivity in the ganglion cell layer. Calretinin was observed to colocalise with GABA in some amacrines and bipolar cells in the inner nuclear layer and in displaced amacrines in the ganglion cell layer. In rabbit retina some cones, ganglion cells, and numerous amacrines express calretinin. In our investigations calretinin could be colocalised with parvalbumin and GABA in some amacrine populations but not with calbindin or tyrosinhydroxilase. The AII amacrine cells contained both parvalbumin and calretinin. Further unidentified amacrine population showed either calretinin or GABA immunopositivity. There are some calretinin-positive displaced amacrines and ganglion cells in the ganglion cell layer too. On this basis calretinin can be used as neurochemical marker to identify neuron subpopulations in the retina.

This study was supported by an OTKA grant (F 16040).

A NEW GRAPHICAL REPRESENTATION OF THE ATTENUATION OF IMPULSES PROPAGATED ALONG THE SPINAL MOTONEURON DENDRITES IN THE FROG AND ITS RELATION TO THE DENDRITIC GEOMETRY

WOLF, E. and LÁZÁR, L.

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The dendritic trees of three brachial and three lumbar motoneurons of the frog spinal cord were intracellularly stained and 3-dimensionally reconstructed. By using the reconstructed geometry of dendrites a high-fidelity passive cable model of dendritic impulse propagation was set up to compute the voltage attenuations of Postsynaptic Potentials between various dendritic locations and the soma. In order to visualise the electrotonic architecture of dendrites the method of Morphoelectrotonic transform (A. Zador et al., J. Neurosci. (1995) 15 (3) 1669-1682) was adopted and used. In this graphical representation the whole branching pattern of the dendrites was taken into consideration thereby improving the classical approach, which neglects the significant boundary effects emerging from the finite length and branching of dendrites. The dendritic trees were morphoelectrotonically transformed with assuming various inhomogenities in the resistance of the soma-dendrite membrane. The new graphical representation could reflect the functional structure of dendrites that was different from the original geometrical structure and depended on the extent of membrane inhomogenities. To quantify the functional structure of dendrites relative to their morphological appearance, hundreds of dendritic points (synapses) were sampled on each dendritic tree and the percentages of their functional relocalisations (following the Morphoelectrotonic transform) were tabulated.

These tables or matrices of functional redistributions that we have introduced may help to find the missing link between the form and function of dendrites.

This work was supported by the Hungarian National Research Fund OTKA T020257.

THE ROLE OF DIFFERENT ALPHA-2 ADRENOCEPTOR SUBTYPES IN THE REGULATION OF ACTH SECRETION DURING ETHER STRESS IN THE RAT

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The effect of different alpha-2 adrenoceptor subtype agonists and antagonists on plasma ACTH induced by ether stress was examined in unanaesthetized, unrestrained male Wistar rats. Ether inhalation induces a significant increase in plasma ACTH level with a peak in 7 min. Central noradrenergic neuronal activity is thought to be a major mediator to hypothalamic CRH and pituitary ACTH release. It has been assumed that postsynaptic alpha-2 adrenoceptors are inhibitory to the secretion of corticotrop releasing hormone (CRH). Yohimbine and some other alpha-2 antagonists may stimulate the secretion of CRH via blockade of postsynaptic alpha-2 adrenoceptors.

The central (i.c.v.) injections of the alpha-2 receptor agonist clonidine (1-10-30 mg/ rat) or the alpha-2A subtype specific agonist oxymethazoline (1-10-30 mg/ rat) failed to influence the basal ACTH levels. The ether stress-induced ACTH increase was inhibited by the i.c.v. administration of clonidine in a dose dependent manner. I.c.v. oxymethazoline or the peripherally administered alpha1-agonist methoxamine (250 mg/ kg i.v.) failed to inhibit the ether stress-induced increase. Pretreatment with the alpha-1/alpha-2B,C-antagonist prazosine (i.p. 0.5 mg/kg) prevented the effect of clonidine on the ether stress, while the alpha-1A,C,D-antagonist WB-4101 (at a dose that has been found to cause a significant inhibition of the clonidine and oxymethazoline-induced growth hormone secretion in the rat) was unable to counteract the inhibitory effect of clonidine. Prazosine alone had no effect on the ether induced plasma ACTH elevation. These results suggest that noradrenalin in the central nervous system may inhibit the stress-induced hypothalamo-pituitary-axis activation via alpha-2B,C-adrenoceptor subtypes and prazosine antagonised the effect of clonidine on these receptors.

EFFECTS OF STIMULATION OF CALLOSAL AND THALAMIC AFFERENTS ON MOTOR CORTICAL EVOKED POTENTIALS IN RAT BRAIN SLICES

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Our aim was to study the role of corpus callosum (CC) in changes of synaptic efficacy in the motor cortex. Interactions between the stimulated callosal and thalamic afferent fibers coming from ventrolateral nucleus (VL) were examined on motor cortex potentials in complex rat brain slices included callosal and thalamic structures, because the most specific inputs of the

motor cortex originated from the VL and from the other hemisphere. Conditions for induction of enhancement of evoked potentials were investigated.

Results: 1. Motor cortical potentials evoked by stimulation of CC had a delay of about 17 ms in relation to potentials evoked by VL stimulation (cross-correlation analysis). 2. Tetanic stimulation (100 Hz) to VL fibers enhanced the evoked potentials and this effect lasted for about 1-1.5 hour. Tetanic stimulation to CC enhanced the evoked potentials induced both by CC and VL fibers (ns). 3. Coactivation of CC and VL fibers (with 10, 20, 30 ms interstimulus interval) made a short term enhancement of VL evoked potentials.

This study was supported by OTKA T/7 16408 and by the Neurobiology PhD program of Albert Szent-Györgyi University of Medical Sciences, Szeged.

MORPHOLOGICAL INVESTIGATION OF THE NEUROTOXIC EFFECTS OF PARAQUAT IN SOME GANGLIA OF VENTRAL NERVE CORD OF *EISENIA FETIDA*

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The free radicals are considered to be important mediators of neuron damages in the central nervous system however mechanism of their action is not well understood. Therefore we have examined the in vivo effect of a well known free radical generator, paraquat (1,1'-dimethyl-4,4'-bipyridium dichlorid) in the ventral nerve cord ganglia of *E. fetida.*

Paraquat caused a dose- and exposition time-dependent neuron lesion at the ventromedial and ventrolateral region of ganglia. However several cells had a high resistance against paraquat toxication, although a marked loss of Nissl substance and vacuolisation of cytoplasm were also observed in their perikarya. Ultrastructurally these vesicular/vacuolar structures were identified as degenerated mitochondria, endoplasmic reticulum cisternae and Golgi stacks. In some animals damage of the giant axon fibres (swelling and demyelinization) was also detected. Occurrence of mitotic cells in damaged ganglia strongly suggests that paraquat by disturbing metabolic pathways initiates neuron proliferation in *E. fetida.*

These results suggest that the ventral nerve cord of *E. fetida* could serve as a simple model of neurotoxicological investigations.

This study was supported by OTKA No. T 016838.

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Preliminary notes (continued)

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Balázs, R. and Richter, D. (1973) Effects of hormones on the biochemical maturation of the brain. In: Biochemistry of the Developing Brain (ed. Himwich, W.) Vol. 1, pp. 254-304. M. Dekker, New York.

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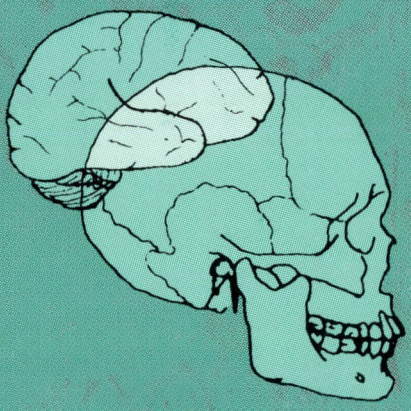

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Research report

DIFFERENTIAL EXPRESSION OF THE *K*- 2 OPIOID RECEPTOR PROTEIN UNDER THE EFFECT OF OPIOID AGONISTS IN EMBRYONIC CHICK NEURONS *IN VITRO*

CSERPÁN, E., TRYOEN-TÓTH, P., BAJENARU, L.,
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Summary: Neuronal cells cultured from 7-day-old chick embryos and differentiated under the chronic effect of opioid drugs were studied for kappa-opioid receptor expression. Plasma-membrane integrated receptors were measured by radioligand ([^{3}H]-naloxone 1 nM, [^{3}H]-ethylketocyclazocine, 4 nM) binding to intact neurons. These data were compared to the results of k-opioid receptor immunostaining (mAb KA8, Maderspach et al., 1991). The chronic presence of kappa-selective opioid agonist dynorphin$_{1\text{-}13}$ (10^{-6}-10^{-7} M) or bremazocine (10^{-7}-10^{-8} M) between cultivation days 2-4 resulted in the significant (80%) down-regulation of the membrane integrated binding sites in concentration dependent fasion. Antagonists naloxone (10^{-5} M) and norbinaltorphimine (10^{-8} M) alone were ineffective, however, they essentially balanced the changes caused by the agonists. Mu ligand morphine was without effect while kappa-1 selective U 50 488 H caused moderate decrese only at high concentrations. Interestingly, mAb KAB also caused down-regulation indicating that it has some agonist character. The down-regulation became measurable in short time (58% within 24 hours) and persisted during the three-day presence of the agonists. Kappa-receptor immunostaining of the neurons was not significantly influenced by the agonists. We suppose that the chronic opioid treatment may influence the intracellular traffic of the receptor protein.

Keywords: opioid receptor, down-regulation, immunocytochemistry, neuron, differentiation, *in vitro*

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INTRODUCTION

Opioid peptides and receptors are present already in the early stages of ontogeny and are expressed in both neurons and glial cells in the avian, mammalian and human brain (Belcheva et al., 1994; Csillag et al., 1990; Hendrickson and Lin, 1980; Maderspach and Solomonia, 1988; Maderspach et al., 1995; Rius et al., 1991; Zagon et al., 1991). As an important part of the intracellular signalling system they are reyulatory in early development and growth (Basheer et al., 1992; Belcheva et al., 1992; Gorodinsky et al., 1995; Maderspach and Nemeth, 1993; Stiene-Martin et al., 1991; Tempel et al., 1995) as well as adult function and plasticity (Beitner-Johnson et al., 1993; Giaccino et al., 1996; Matus-Leibovich et al., 1985).

Existence of multiple opioid receptor types (μ, ∂, k) *is* well documented (Goldstein, 1987; Goldstein and Naidu, 1989; Mansour et al., 1995; Martin et al., 1976; Reisine and Bell, 1993) and was encouraged also by the recent molecular cloning results. μ-, ∂- and k- opioid receptor genes are encoding different though highly homologous proteins which have seven transmembrane regions and a G protein binding epitope (Pan et al., 1995; Simonin et al., 1995; Uhl et al.. 1994; Wang et al., 1994). The molecular background of the pharmacological (subtype) heterogeneity, however, still remained hidden (Mollereau et al., 1994; Pan et al., 1995; Rossi et al., 1995; Unterwald et al., 1991; Wang et al., 1994; Wollemann et al., 1993).

Data are highly contradictory concerning the changes in opioid receptor expression caused by the chronic effect of opioid agonists in the developing or adult nervous system. Down-regulation, preceded by desensitization and uncoupling from G proteins, was described in cells of nervous system origin *in vitro* (Ericsson et al., 1993; Tang et al., 1995; Zadina et al., 1995) and in early stages of ontogeny *in vivo* (Basheer et al., 1992; Belcheva et al., 1992; Tempel, 1991; Tempel et al., 1995). In matured animals when treated *in vivo* with opioid drugs, instead of down-regulation, often an up-regulation or no effect was demonstrated (Bhargava et al., 1991; Zadina et al., 1995). Down-regulation of opioid receptors was described only in discrete areas of the brain (Tao et al., 1993; Trujillo and Akil, 1991). The apparent contradictions between *in vivo* and *in vitro* data may arise, among others, from the differences in the complexity of the two systems, the lack of several factors *in vitro*, the differences in the developmental stages and the local concentration of the drugs. However, in the *in vitro* system, measurements

on homogenous population of intact cells under controlled environment is possible.

Hereby we try to reconcile the apparent contradictions using a primary chick neuronal cell culture model. We are analysing the changes in opioid receptor expression during the chronic effect of agonists and antagonists by two independent methods. Radioligand binding to intact cells measures plasma-membrane integrated receptors, while immunocytochemistry (applying our kappa-2 opioid-receptor-specific monoclonal antibody, mAb KA8, Maderspach et al., 1991) labels also intracellularly localized receptor-protein (Maderspach et al., 1995; Schmidt et al., 1994).

MATERIALS AND METHODS

Cell culture

Primary neuronal cultures were established from 7-day-old embryonic chick forebrains according to the procedure described earlier (Maderspach and Solomonia, 1988). Dissociated cell suspensions were seeded to poly-L-lysine-coated Falcon plastic Petri dishes (6 cm dia) in an average density of 6 $\times 10^8$ cells/cm^2 and were cultivated in Ealge's MEM + 20 % FCS (Gibco) + antibiotics in a standard incubator.

Chronic drug treatment

The applied drugs and the final concentrations were as follows:

Agonists: morphine HCl (Alkaloida, Hungary). 10^{-6} M; bremazocine HCl (Sterling Winthrop Res. Inst., Rennselaer, NY), 10^{-8} M; dynorphin$_{1-13}$ (Bachem Feinchemikalien, Switzerland), 10^{-6}, 10^{-7} M; U-50 488 H (Upjohn, USA), 10^{-6} M. Monoclonal antibody KA819 was used in ascitic fluid form diluted to 1:1000 and 1:10 000.

Antagonists: naloxone HCl (ENDO Labs., USA), 10^{-5} M; nor-binaltorphimine (gift of P. Portoghese), 10^{-7} M, 10^{-8} M.

The drugs were dissolved in Hank's balanced salt solution (BSS) under sterile conditions and were added alone or in combinations into the medium of the cultures in a final volume of 50 µl. Controls received Hank's BSS. First treatment occurred 24 hours after the establishment of the cultures, and it was repeated daily until the fourth cultivation day (for dynorphin$_{1-13}$ in every 12 hours) without medium change (see also Maderspach and Németh, 1993).

Radioligand binding

Equilibrium binding experiments were made and analysed as formerly (Maderspach and Fajszi, 1982; Maderspach et al., 1991). The following kinetic parameters were established for [^{3}H]-naloxone (3.1 TBq/mmol) binding: B_{max} = 4-9 fmol/10^6 cells, Kd = 0.63 nM, and for [^{3}H]ethylketocyclazocine (EKC, 0.74 TBq/mmol, New England Nuclear) binding: B_{max} = 6 fmol/10^6 cells, Kd = nM and cooperativity index = 2. The change caused by the chronic opioid treatment in the binding capacity was measured here at about 80% receptor occupancy according to the procedure described earlier (Maderspach and Solomonia, 1988). Briefly, cultures of proper ages were carefully washed which Hanks BSS (3×15 ml) in order to remove drugs and preincubated in 1 ml serum-free grown medium at 37°C for 30 min. Equilibrium binding was measured by adding 1 nM [^{3}H]-naloxone or 4 nM [^{3}H]-EKC into the medium of intact cultures for 4 min at 37 °C. Termination was made by washing in 4 °C physiological saline (3x25 ml). Bound radioactivity was removed by icecold methanol and measured by liquid scintillation method. Non-specific binding was estimated in the simultaneous presence of 10 μM unlabelled naloxone HCl or 40 μM EKC respectively. Calculated specific binding values, plotted in Figures, are averages of two-three determinations measured in duplicate.

Heterologous displacement curves were established at 0.8 nM [^{3}H]-naloxone concentration (60% partial receptor occupancy) under equilibrium circumstances. Non-labeled bremazocine or norbinaltorphimine were given to the cultures simultaneously with the labelled naloxone in concentrations of 10^{-12}-10^{-5} M, and incubated for 4 min. Determination of specific binding values occurred as given above.

Immunocytochemistry

Immunochemical staining was performed according to the procedure given earlier (Maderspach and Németh, 1993). Briefly, cultures were rapidly fixed in icecold methanol and preincubated in blocking buffer containing 0.5% BSA (Serva). Murine monoclonal antibody KA8 (Maderspach et al., 1991) for specific labelling of kappa-opioid receptors was applied as primary antibody (ascitic fluid 1:1000). Antimouse IgG, conjugated with peroxidase (BioRad), served as a secondary antibody and immunoreactivity was visualized by substrates 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2 (Sigma). Results were evaluated by light microscopy.

RESULTS

Kinetical studies

The results of the heterologous displacement experiments are presented in Fig. 1. Both the opioid agonist bremazocine (it is partially selective for kappa-type opioid receptors) and the kappa-selective opioid antagonist norbinaltrophimine provoked a biphasic curve with a maximum at about 5×10^{-11} M cold ligand concentration. Equilibrium binding of [^{3}H]-naloxone to chick neurons shows apparent positive cooperativity kinetics as was described earlier. The value of receptor occupancy at 0.8 nM concentration is only partial, about 60%. Under such circumstances a relatively low concentration of heterologous ligand, if it could effectively interact with the binding sites causes an increase in the apparent affinity reflected in a characteristic peak (above 100 %) in the radioligand specific binding; as a result of ligand interaction more binding sites became open for ligands (Maderspach and Fajszi, 1982; Maderspach and Solomonia, 1988). In the present experiment the antagonist norbinaltorphimine and the agonist bremazocine proved to be very effective in increasing the specific radioligand binding supporting that the studied binding site is of kappa-opioid character.

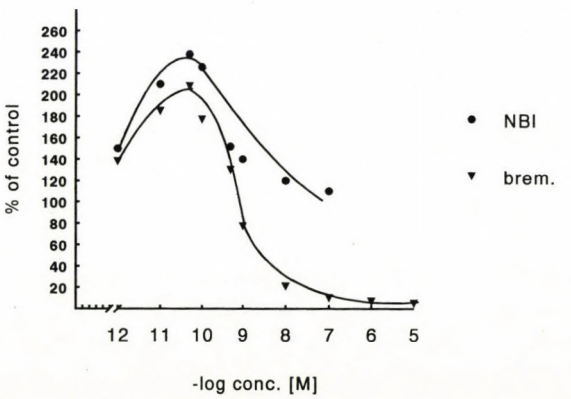

Fig. 1. Competition of bremazocine (-●-) and norbinaltorphimine (-▲-) for the [^{3}H]-naloxone binding sites. Radioligand (0.8 nM) was given to chick neurons at the 4th cultivation day alone or together with increasing concentrations of cold ligand and incubated for 4 min. Non-specific binding was measured in the simultaneous presence of 1 μM naloxone. Control (100%) value was measured without cold ligands under equilibrium circumstances and corresponds to 3.1 fmol/10^6 cells (see also Maderspach and Solomonia, 1988). Results of a representative experiment

Specific radioligand binding under the effect of chronic opioid treatment
Specific opioid receptor equilibrium binding of the cultured chick neurons was measured with the non-selective radioligand [^{3}H]-naloxone and in some experiments also with [^{3}H]-EKC, which has some selectivity to kappa-opioid receptors (Martin et al., 1976). The results with the two radioligands were not essentially different.

Figure 2 shows the naloxone specific binding values in the function of the length of the opioid treatment. Bremazocine, given in 10^{-7} M, decreased the specific naloxone binding to 42% already within a 24 hours treatment period. Further decrease (to 21%) was measured after 48 hours (three days-old cultures after 2 days treatment) while this tendency was not continued later and the specific binding does not decreased by the further presence of the drug (20%). Bremazocine in smaller (10^{-8} M) concentration was much less effective, the decrease in specific naloxone binding does not exceeded 30%, and the tendency with the length of the treatment was not so clear. The kappa-1 selective ligand U 50 488 H, even in higher, 10^{-6} M, concentration caused only a slight decline within 24 hours (15%) which amounted to 30% until the end of the three days treatment period.

Figure 3 summarizes the specific [^{3}H]-naloxone and [^{3}H]-EKC binding values of 4-day-old chick neuronal cultures after three days agonist treatment. According to these data dynorphine$_{1\text{-}13}$ proved to be similarly potent in the down-regulation of the opioid receptors as bremazocine and the effect was concentration-dependent (83% and 59% decrease at 10^{-6} and 10^{-7} M, respectively). Morphine, the μ-ligand, given in 10^{-6} M, caused only a slight decrease in the receptor binding (13% and 16% was measured by [^{3}H]-EKC and [^{3}H]-naloxone, respectively), similarly to that of U 50 488H.

The chronic treatment of chick neurons with the mAb KA8 also provoked down-regulation, however the results are not always coinciding.

The down-regulation caused by the agonist bremazocine was partially restored by the non-selective antagonist naloxone, if given in 10^{-5} M or by the kappa antagonist norbinaltorphimine, if given in 10^{-8} M simultaneously with the agonist (Fig. 4). The antagonists itself had no effect or caused a small increase in the receptor-binding (see Fig. 3).

Immunocytochemistry of kappa-opioid receptors
Kappa-opioid receptors were visualized by the mAb KA8 at the fourth cultivation day. Figure 5 selective compares the immunoreactivity of control (Fig. 5A) cultures and those of grown in the three-day presence of 10^{-7} M

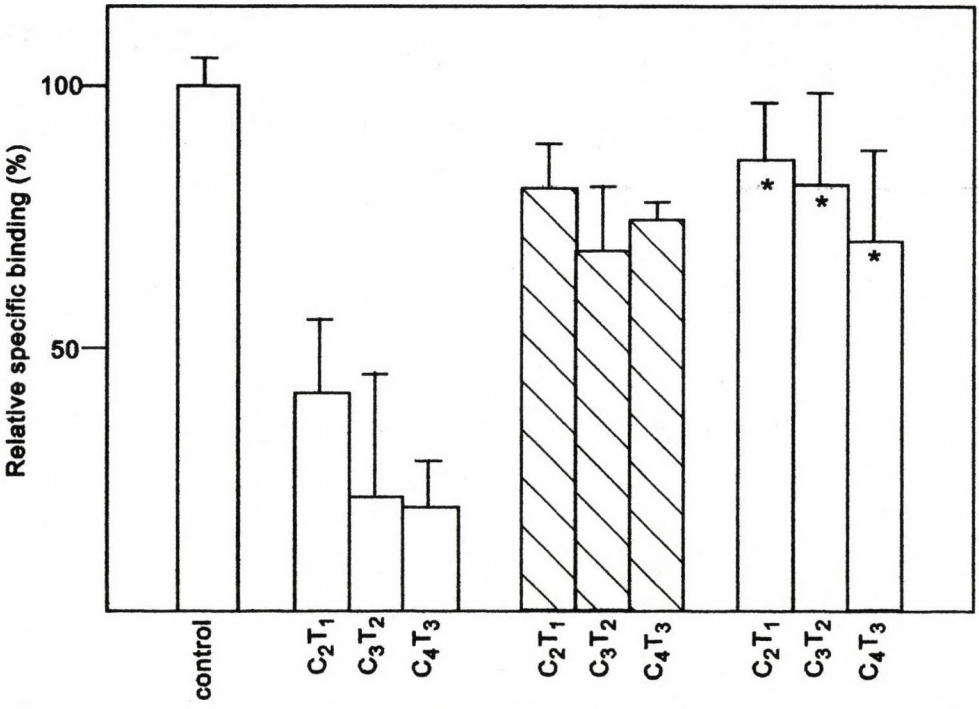

Fig. 2. Specific [^{3}H]-naloxone binding of chick neuronal cultures under the effect of bremazocine, 10^{-7} M (white columns), 10^{-8} M (striped columns) or U 50 488 H, 10^{-6} M (columns with label), as a function of the cultivation (index of C) and treatment (index of T) days. Drugs were given to the growth medium in 50 µl BBS once a day, control cultures received vehiculum. Equilibrium binding was measured at 1 nM [^{3}H]naloxone, 37 °C, 4 min. Control (untreated) value corresponds to 4.9 fmol/10^6 cells. Average of three determinations measured in duplicate

bremazocine (Fig. 5B), 10^{-5} M naloxone (Fig. 5C) as well as bremazocine and naloxone together (Fig. 5D). The neurons after four-days cultivation are morphologycally differentiated. In the presence of bremazocine, as we described earlier (Maderspach and Németh, 1993), the differentiation is promoted, the cell body is more developed, the processes are more arborized than in the control cultures. The kappa-opioid receptor-like immunoreactivity manifests as 'patchy' grains in the cell body, plasma-membrane and processes (Fig. 5A). The characteristic differences in the intensity and arrangement of the immunoreactivity between the individual cells is also visible, furthermore, some immunonegative cells are present.

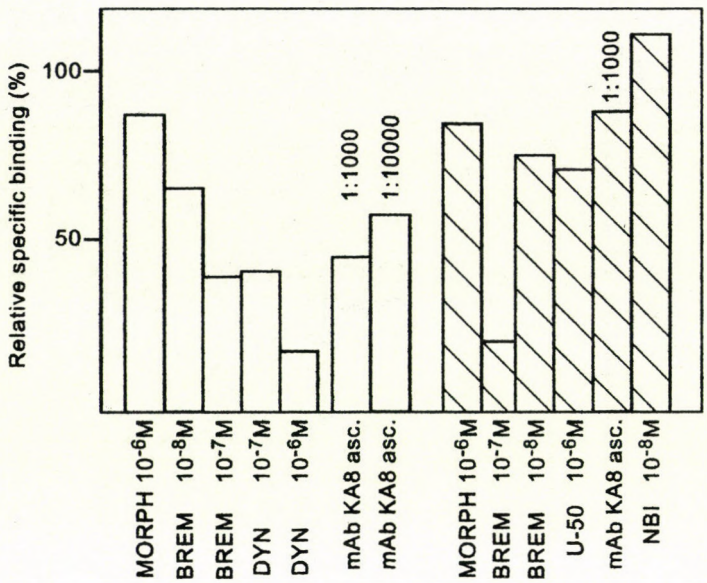

Fig. 3. Specific [^{3}H]-naloxone (1 nM, white columns) and [^{3}H]-EKC (4 nM, striped columns) equilibrium binding of chick neuronal cultures after three-day chronic treatment with different opioid agonists and norbinaltorphimine. mAb KA8 was added as ascitic fluid diluted according to the indicated value. Dynorphin$_{1\text{-}13}$ was added to the cultures in every 12 hours while the other ligands once a day. 100% corresponds to the control values of Fig. 2. Average of three determinations measured in duplicate

The immunostaining was not significantly influenced by the chronic opioid treatment. Agonist bremazocine somewhat decreased its intensity (56% immunopositive cells instead of the control 64%, Fig. 5A and B) while a slight increase in immunoreactivity was visible in the chronic presence of naloxone, the ratio of the immunopositive cells increased to 80% (Fig. 5C). The result was simular if both naloxone and bremazocine have been present in the culture medium during the treatment period (80% immunoreactive cells, Fig. 5D).

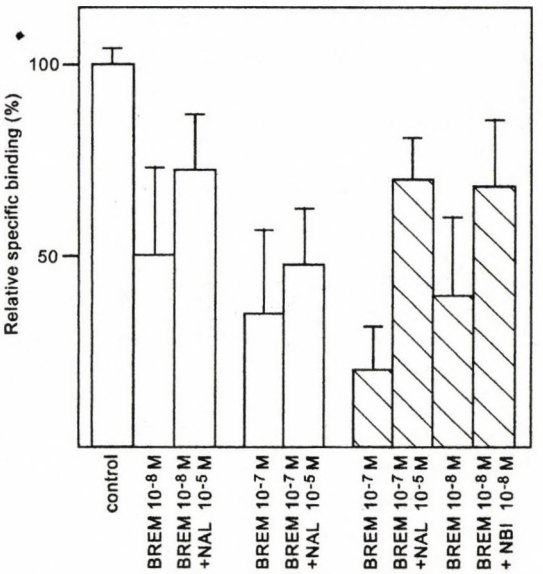

Fig. 4. Specific [^{3}H]-naloxone (1 nM, white columns) and [^{3}H]-EKC (4 nM, striped columns) binding of chick neuronal cultures after three-day chronic treatment with bremazocine (10^{-7}, 10^{-8} M) alone or in combinations with naloxone (10^{-5} M) or norbinaltorphimine (10^{-8} M). Control value is the same as in Fig. 2. Average of three determinations measured in duplicate

DISCUSSION

Earlier results from this laboratory showed, that neurons deriving from 7-day-old chick embryo forebrains and differentiating *in vitro* express opioid receptors and the majority of these receptors proved to be pharmacologically of *k*-type (Maderspach and Solomonia, 1988). Furthermore, the immunoreactivity to the monoclonal antibody KA8 which was raised against a frog *k*-2 opioid receptor preparation as antigen supports the possibility that they are of *k*-2 subtype (Maderspach et al., 1991; 1995). Data here further support this classification. *k*-selective bremazocine and norbinaltorphimine resulted in biphasic curves in the heterologous displacement experiments with maxima at about 5×10^{-11} M concentration. The high efficacy of these ligands in the interaction with the opioid binding sites indicates that these receptors are primarily of *k*-type and also shows the existence of the apparent positive cooperativity in the binding kinetics as we published earlier (Maderspach and Fajszi, 1982; Maderspach and Solomonia, 1988).

Formerly we also presented that early *k*-opioid agonist signals promote morphological differentiation and neurofilament 200 expression in chick neurons (Maderspach and Németh, 1993). A suggesstion emerged that opioid receptor expression itself could be regulated by these signals. In the experiments here neurons were chronically treated with opioid agonists and antagonists during the *in vitro* differentiation and opioid receptor expression was followed at protein level.

Amphipatic radioligands like naloxone or EKC under conditions, as in our binding experiments (nM concentration range, 4 min incubation time), does not pass plasma-membrane of intact cells. Therefore the results of radioligand binding measurements represent only those receptors which are open for external ligands. The monoclonal antibody KA8, if inoculated onto cells after methanol fixation, can reach and recognize intracellularly localized receptor protein (Maderspach et al., 1995; Schmidt et al., 1994) as well.

According to the radioligand binding results here, the *k*-type opioid agonists are able to down-regulate the plasma-membrane integrated binding sites. The endogeneous peptide ligand dynorphin$_{1\text{-}13}$ and bremazocine proved to be similarly effective. The antagonists balanced this down-regulation, showing that the effect was primarily opioid-receptor-mediated. Moreover, neither morphine (μ ligand), nor U 50 488 H (*k*-1 ligand) caused valuable decrease in the naloxone or EKC specific binding within a three-day chronic treatment period. This indicates that both μ and *k*-1 receptors are present on these cells only in small proportions. Nevertheless, ineffectivity of morphine could be caused by the low efficacy of this drug in μ-receptor down-regulation contrary to the endogeneous μ peptide ligands (Arden et al., 1995; Zadina et al., 1995). ∂-opioid receptors have not been studied in this work because its ratio on these cells in this early stage of differentiation is negligible (De Vries et al., 1990; Kornblum et al., 1987).

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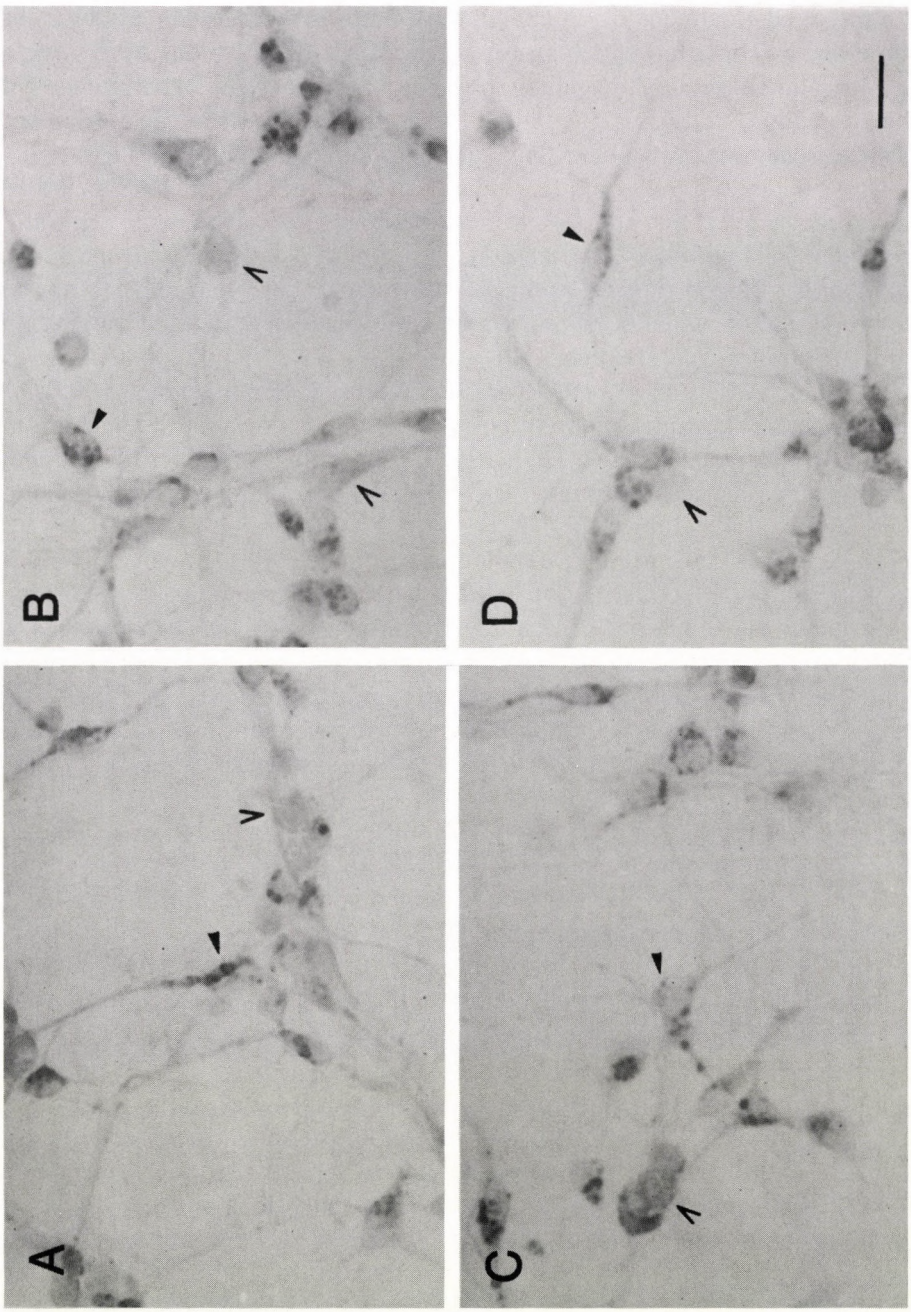
Fig. 5. Kappa-opioid receptor immunostaining of chick neuronal cells after 4-day cultivation applying the monoclonal antibody KA8 (Maderspach et al., 1991). A: untreated control cultures. B-D: cultures treated for three days with B: 10^{-7} M bremazocine, C: 10^{-7} M norbinaltorphimine and D: 10^{-7} M bremazocine and 10^{-7} M norbinaltorphimine simultaneously. Peroxidase labelled secondary antibody and diaminobenzidine + H_2O_2 substrates were used for visualization. Black arrowheads mark some immunopositive while open arrowheads some immunonegative neurons. Bar represents 10 μ


B
D
A
C

Prolonged treatment of chick neurons with mAb KA8 can provoke also some down-regulation which suggests that this antibody could have some *k*-agonist character on these chick neurons. Displacement experiments with this antibody on rat brain membrane fractions in the presence and absence of Na ions (data not shown here) have also supported this possibility.

Down-regulation of opioid binding sites from the plasma-membrane occurred in relatively short time. The loss in the receptor concentration exceeded 50% within 24 hours and it was slightly increased with the further treatments. This is in accordance with former data that have described down-regulation (Zadina et al., 1993) and functional desensitization in 3 hours (Fujimoto and Holmes, 1990) and recent data showing internalization in 4 hours (Arden et al., 1995) or 30 minutes (Sternini et al., 1996). An increase in receptor phosphorylation is lying very possibly in the background of these changes (Arden et al., 1995). The prolonged agonist treatment persisted this process during the 3-day period preventing dephosphorylation and functional recycling.

The agonists causing down-regulation of binding sites, however, promote the differentiation of the neurons (Maderspach and Németh, 1993). This is expressing the function of kappa-opioid signals in the early stages of ontogeny (De Vries et al., 1990; Gorodinsky et al., 1995; Hendrickson and Lin, 1980) which could be, however, transient. Some data are presenting that in mammalian species *k*-opioid receptor concentration is declining after birth (Fujimoto and Holmes, 1990; Rius et al, 1991) and with ageing (Maggi et al., 1989).

The down-regulation denoted by radioligand binding does not reflected in the kappa-receptor immunoreactivity of the neurons. Neither the intensity nor the character of the staining changed significantly after three days exposure to the agonist bremazocine.

The above results support the suggestion that chronic opioid treatment in cultured neurons caused translocation of the receptor protein from plasma-membrane into intracellular compartments. This could not be visualized, however, because of the high abundancy of the intracellular immunoreactivity. This is supported by our earlier electron microscopical studies for the subcellular localization of the kappa-opioid receptor protein in human (Schmidt et al., 1994) and in young rat and chick brain (Maderspach et al., 1995). We presented immunoreactivity of ribosomes, endoplasmic reticulum membranes, neurotubules and areas in close vicinity to plasma-membrane. These are in context with the intracellular traffic of the receptor

protein from place of synthesis to functioning sites and way of recycling and degradation.

The lack of visible changes in the immunoreactivity after chronic agonist treatment here do not exclude however, that agonist signals could modulate the steps of intracellular traffic or kappa-opioid receptor gene expression. Studies are in progress to analyse these possibilities.

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Research report

SINGLE LOW DOSE OF MPTP DECREASES EXTRACELLULAR LEVELS OF NORADRENALINE AND MONOAMINE METABOLITES IN THE VENTROBASAL THALAMUS OF THE RATS

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Summary: A single low dose of the neurotoxin: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) results paradoxical sleep deprivation and reduction in food intake without any detectable motor deficiencies. In the present study we monitored the *in vivo* extracellular levels of monoamines and their metabolites following intraperitoneal (i.p.) administration of a single dose of MPTP (5 mg/kg). Microdialysates were collected from the ventrobasal thalamic nucleus (VB) of Halothane anesthetized rat. We found a significant decrease in noradrenaline (NA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) levels and a significant increase in 3,4-dihydroxyphenylalanine (DOPA) concentration whereas amino acid levels were unchanged throughout the 4-hour long perfusion. We found no significant difference in the *post mortem* release of NA and DOPA between the control and MPTP treated animals, suggesting that the intracellular NA pool were maintaned. The above findings support the idea that the neurochemical mechanism of rapidly developing and transient behavioral changes induced by MPTP may be an immediate decrease in monoaminergic transmission and metabolism following MPTP injection.

Keywords: MPTP - Rat - Thalamus - *In vivo* dialysis - Monoamines - Amino acids

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INTRODUCTION

Chronic treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces Parkinson-like motor symptoms in humans and laboratory animals [Davis et al., 1979, Langston, 1985, Schneider et al., 1986, Kopin, 1987] resulting in severe loss of the dopaminergic [Chiueh et al., 1984, Ricaurte et al., 1986, Schneider et al., 1986, Herkenham et al., 1991] and, to smaller extent, a destruction of other monoaminergic neurons as well [Forno et al., 1986, Namura et al., 1987, Brooks et al., 1988]. Despite the definite motor deficiencies observed after chronic MPTP treatment, a single, low dose of MPTP does not result in marked alteration of motor performance. However it was shown to produce other behaviorally detectable changes minutes after injection in cats, such as paradoxical sleep (PS) deprivation [Pungor et al., 1990, Pungor et al., 1993], vegetative symptoms (hypersalivation, pupillary dilatation, diarrhoea) and a decrease in food intake [Schneider et al., 1986, Hajnal et al., 1991]. Application of a singe low dose of MPTP intraperitoneally (i.p.) resulted in total PS deprivation [Pungor et al., 1993] in our previous study on cats and it was confirmed in rats as well [Lelkes et al., 1992]. Rats are relatively resistant against the Parkinsonian effects induced by systemically administered MPTP [Riachi et al., 1991]. So rats are good models for studying those short-latency behavioral effects of MPTP that are presumably induced by the modulation of monoaminergic systems. Concentrations of monoamines and monoamine metabolites change following intracerebral MPTP and MPP^+ administration in the striatum of rat [Ozaki et al., 1987, Sirinathsinghji et al., 1988, Miyake and Chiueh, 1989, Caliguri and Johannessen, 1991, Santiago et al., 1991] but no data exists about other structures.

The somatosensory system is involved in the modulation of sleep [Koella, 1981, Steriade and Llinas, 1988] and other behavioral events, including those which are modified by MPTP [Miller, 1981, Jacquin and Zeigler, 1983]. The somatosensory thalamus receives monoaminergic inputs from the noradrenergic and serotonergic cell groups in rats [Koella, 1981 Jones, 1985, Steriade and Llinas, 1988]. Thus we implanted the microdialysis probes into the ventrobasal nucleus (IVB). We investigated the short-term effects of MPTP on monoamine metabolism in the time epoch in which the non-Parkinsonian type behavioral effects of MPTP were observed. It is known that MPP^+ causes cellular oxidative stress [Desole et al., 1995, Sriram et al., 1995] that induces elevated glutamate and taurine release [Globus et al.,

1988, Sandberg et al., 1986]. To assess the influence of oxidative stress on monoamine levels the extracellular amino acid concentrations were measured as well. MPTP could also modify the monoamine synthesis and destruction intracellularly which may contribute to its short-coming behavioral effects. As it is known, monoamines and amino acids are released from the intracellular space *post mortem,* so after an overdose of Halothane we also collected samples and estimated the changes in their intracellular pools.

MATERIALS AND METHODS

Microdialysis

All animal treating are in accordance with the recommendations of Guidelines on the Use of Living Animals in Scientific Investigations. Rats (350-400 gr, n=14) were anesthetized in 1% Halothane-air mixture and placed into a stereotaxic frame. Body temperature was measured and controlled with a heating lamp. The concentration of Halothane was kept between 0.5% and 0.8% during the collection of samples *in vivo.* Microdialysis probes were implanted bilaterally into the ventrobasal complex of the thalamus (P: 1.5 mm, L: 2,5 mm, V: -7 mm) according to the atlas of Pellegrino and Cushman [Pellegrino and Cushman, 1967]. The position of the probes was checked using Nissl stained coronal sections.

The probes were made of Travenol hollow fibers (cut-off 5000 D) with an active surface of 3 mm length and 0.2 mm diameter. The probes were perfused with artificial cerebrospinal fluid solution microbially filtered and made of amino acid free water containing K^+ 3 mM, Na^+ 144 mM, Mg^{++} 2.1 mM, Ca^+ 2 mM, Cl^- 155 mM at pH 7. The perfusion rate was 1 µl/min which allowed about 20-30% equilibration between the extracellular space and the perfusion solution [Juhász et al., 1989]. We started the perfusion right after implantation, but sample collection started one hour later in order to wait for the recovery of the exposed tissue compartment. Thirty µl samples were collected every 30 minutes, from which 20 µl was used for monoamine, and 10 µl for amino acid analysis by HPLC. The monoamine measurements were done immediately after the sample collection to avoid sample decomposition. Samples for amino acid analysis were frozen immadiately and stored (-20 °C). MPTP (5 mg/kg, RBI) was dissolved in sterile physiological saline and injected i.p. to 10 rats after collecting two control samples (60 minutes). Physiological saline (2 ml) was injected i.p. to

4 control rats. The duration of dialysis experiment was 240 minutes following MPTP or saline injection. Two *post mortem* samples were collected at 30 and 60 minutes after the death of the animal overdosed with 4% Halothane.

Since diffusion through the extracellular space is a complex phenomena, the real extracellular concentration cannot be determined accurately from dialysis samples [Benveniste and Huttemeier, 1990, Shimizu et al., 1993]. The considerable permeability dispersion of dialysis probes and the animal to animal differences in the extracellular monoamine concentrations can make high deviation and mask the biological changes. Therefore measured values of monoamines and amino acids were normalised to the average concentration of the control samples collected for an hour previous to MPTP application.

The results are given as mean ±SEM and compared by two-talied Student's t-test. The changes were considered non-significant when p value was greater than 0.05.

High performance liquid chromatography (HPLC) analysis

Monoamines were measured in isocratic system supplied with mobile phase by LKB Pharmacia 2150, double head pump. The mobile phase was 0.1 M phosphate buffer, pH 3.75 containing 1 mM EDTA and 1 mM octane-sulphonic acid. The flow rate was 1.2 ml/min. Separation of monoamines was performed on Beckman, UltraSpher, C18 reversed phase column (75×4.6 mm). Monoamines were detected by amperometric detector, on glassy carbon working electrode (Triem Electronics, Hungary) at 700 mV detection potential. Concentrations of monoamines were estimated manually on the basis of peak heights using external standards. The detection limit was 1 pg/20 µl for noradrenaline (NA). Injection volume was 20 µl.

Amino acids were measured using precolumn derivatization technique with orthophthalaldehyde and applying fluorescent detection (excitation filter 305-395 nm, emission filter 430-470 nm) in an LKB Pharmacia AminoSys Chromatography System using two component gradient elution. Derivatization and injection was computer controlled. The mobile phase A was 50 mM phosphate buffer, pH 7.2, containing 0.018% tetraethylammonium and 0.3% tetrahydrofuran; mobile phase B was 70% acetonitrile dissolved in buffer A, pH 7.2. Separation of amino acids was performed on Chrompack, MicroSpher, C18 reversed phase columns (100×4.6 mm). Concentrations of amino acids were calculated by PE Nelson

HPLC analysis software using external standards. The detection limit was 10-20 pg amino acid in 10 µl sample. Injection volume was 10 µl.

RESULTS

None of the measured compounds changed significantly after 240 minutes of perfusion in saline injected rats (control animals, n=4). Average *in vivo* concentrations of monoamines and metabolites were: DOPA: 345±22 pg, NA: 10.2±3.1 pg, DOPAC: 23.7±5.3 pg, and 5-HIAA: 389±66 pg in 20 µl dialysate. The average *in vivo* amino acid concentrations of the dialysates were as follows: aspartate: 0.53±0.1 µM, glutamate: 2.42±0.4 µM, asparagine: 0.45±0.1 µM serine: 9.2±3 µM, glycine: 7.4±3 µM, threonine: 5.3±2 µM, alanine: 14±4 µM, taurine: 10±5 µM arginine: 8.4±3 µM.

In vivo measurement of monoamines and their metabolites

We measured 3,4-dihydroxyphenylalanine (DOPA), noradrenaline (NA), 3,4-dihydroxy-phenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) in the thalamus of rats, other monoamines and their metabolites were under detection limit. The effect of MPTP injection on monoamine concentrations is summarized in Fig. 1. MPTP (5 mg/kg i.p.) decreased the levels of NA (54±12%) and DOPAC (56±18%) 30 minutes after MPTP injection ($p<0.01$). The decrease of 5-HIAA was slower, its level was 100±4% 30 minutes after MPTP injection. The decrease started 60 minutes after the treatment (83±6%, $p<0.01$). DOPA increased in the extracellular space after the injection of MPTP, but in the 30 minutes sample it was still at the same concentration as the control: 100±5%. The increase in DOPA started 60 minutes after MPTP treatment (110±5%) and increased permanently during the 240 minutes experimental sessions and finally reached 210±22% ($p<0.01$). So at the end of the *in vivo* perfusion the average levels were as follows: NA: 20±14%, DOPAC: 8±8%, DOPA: 210±22%, 5-HIAA: 61±9%. In some cases NA and DOPAC were not detectable. A temporary drop in body temperature (2-3 °C) was observed immediately after the injection of MPTP but it was compensated by heating.

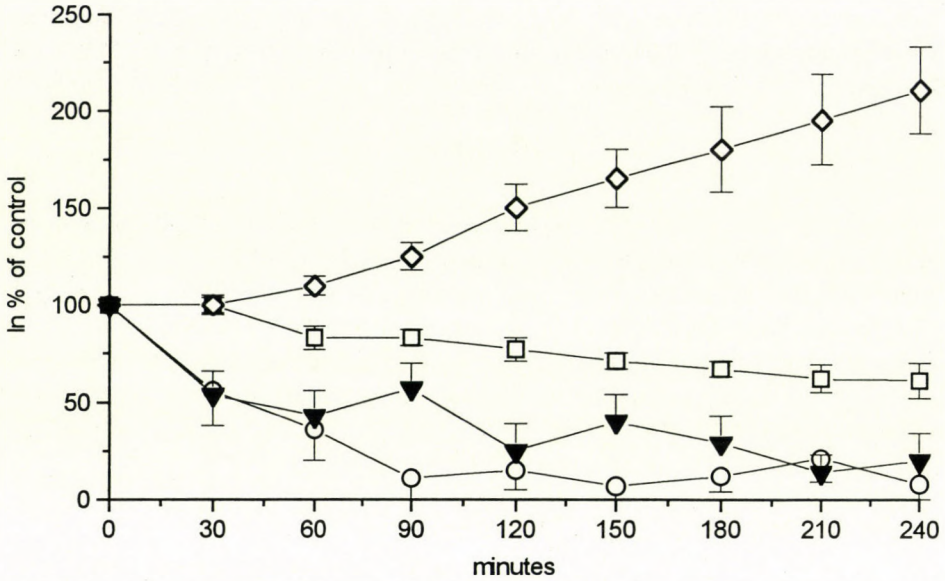

Fig. 1. Changes in the extracellular concentrations of NA, DOPAC, 5-HIAA and DOPA in the thalamus of anesthetized rat after injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropiridine (MPTP; 5 m/kg) i.p. *by in vivo* microdialysis technique. Data points were calculated as the percentage of control values. Control samples were collected for one hour previous to the drug application, and the average of control measurements were concidered 100%
Symbols: □ 5-HIAA; O DOPAC; ✧ DOPA; ▼ NA
Error bars symbolise S.E.M. (n=10)

Post mortem measurement of monoamines and their metabolites

We found no significant difference for any of the measured compounds, not even for NA between the MPTP injected and the control rats in *post mortem* samples collected 30 and 60 minutes after the death of the animal (Table 1).

However, the *post mortem* extracellular level of NA was significantly higher than the *in vivo* NA concentration ($p<0.01$). Interestingly, serotonin never appeared in the samples, DA was detected several times but not in all rats. The concentration of DOPA increased enormously and always exceeded the upper limit of detection. The metabolite, MHPG appeared in the *post mortem* samples. Overdosage of rats by 4% Halothane resulted in the further decrease of 5-HIM (Table 1). *Post mortem*, the concentration of DOPAC was always under the detection limit (Fig. 2).

Table 1. Mean values of *post mortem* extracellular NA and 5-HIAA concentration in MPTP treated (n=10) and saline injected control rats (n=4). DOPAC was often under the lowest detection limit and DOPA exceeded the upper detection limit in the *post* mortem samples. PM1 and PM2 indicate respectively the *post* mortem samples collected 30 and 60 minutes after the death of the animal. The values were calculated as the percentage of control values with Student's t-test (mean±S.E.M.). Control samples were collected for one hour previous to the drug application, and the average of control measurements were considered as 100%

	PM1 control	PM2 control	PM1 MPTP	PM2 MPTP
NA	216±48%	188±53%	264±24%	235±27%
5-HIAA	44±13%	20±3%	35±12%	17±8%

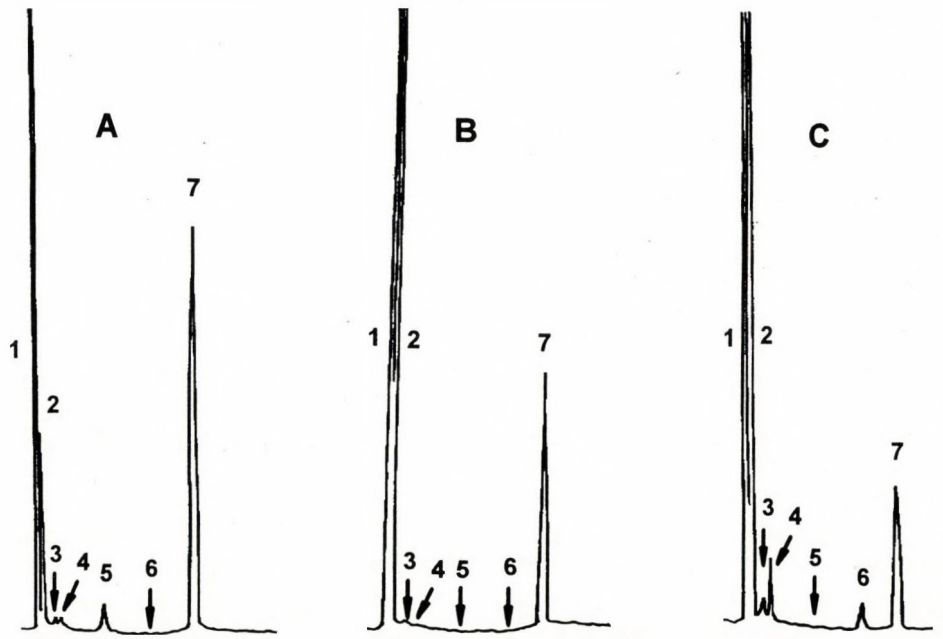

Fig. 2. Chromatograms of *in vivo* and *post mortem* microdialysates from the thalamus of an MPTP treated rat by the use HPLC analysis with electrochemical detection. The first chromatogram (A) is from a pretreatment *in vivo* sample. The second (B) that shows the increase of DOPA and decrease of 5-HIAA concentration with the disappearance of DOPAC and NA is from the final sample-collected 240 minutes after the MPTP injection. The third one (C) is the second *post mortem* sample of this rat, indicating the increase of NA and DOPA, the appearance of dopamine and MHPG, while DOPAC still remained under the detection limit

The numbers mean: 1 - front; 2 - DOPA; 3 - NA; 4 - MHPG; 5 - DA; 6 DOPAC; 7 - 5-HIAA

In vivo measurement of amino acids

The amino acids, aspartate, glutamate, asparagine, serine, glycine, threonine, alanine and taurine were regularly observed in the dialysates from the thalamus of the rat. There was no significant change in the concentration of neurotransmitter and non-neurotransmitter amino acids after MPTP injection (Table 2).

Table 2. Amino acid concentrations in the thalamus of anesthetized rat after MPTP (5 mg/kg i.p.) injection obtained by *in vivo* microdialysis technique. HPLC analysis with fluorescent detection showed no significant changes throughout the 240 min long *in vivo* perfusion. The mean values (±S.E.M. calculated with Student's t-test, n=10) of samples that were collected 240 minutes after MPTP injection are shown as the percentage of the control values. Control samples were collected for one hour previous to the drug application, and the average of control measurements were considered as 100%

Aspartate	76±27%	Glycine	130±63%
Glutamate	113±59%	Threonine	114±24%
Asparagine	109±13%	Alanine	118±20%
Serine	105±37%	Taurine	83±30%

DISCUSSION

In the present study we found an MPTP induced rapid decrease of extracellular NA level in the thalamus suggesting that the immediate effect of i.p. MPTP injection could be the alteration of thalamic noradrenergic transmission. The high amount of NA released *post mortem* and the fact that no significant difference was found between the *post mortem* monoamine concentration of MPTP and saline injected animals reveal that the intracellular pool of NA was maintained despite of the dramatic decrease in the extracellular concentration of NA after MPTP injection. This finding suggests that the decrease of extracellular NA level was not the direct result of a critical decrease in the intracellular release pool so it is possible that MPTP could modify the mechanism of NA release or uptake. Bleeding or damage of the blood brain barrier can not also be the origin of the *post mortem* release of NA, since in our *post mortem* samples we could not detect adrenaline or serotonin that are present in blood in far higher concentrations than NA [Lentner, 1984]. We believe that the noradrenergic terminals may be the origin of the *post mortem* release of DA [Sved, 1990] as the rat VB does not receive considerable amount of dopaminergic input [Jones, 1985].

Extracellular level of DOPAC and 5-HIAA also decreased after MPTP injection. There are evidences supporting the idea that the changes in the extracellular concentrations of monoamines reflect the synaptic overflow, whereas that of the metabolites are mainly related to the monoamine turnover [Ungerstedt, 1984, Vulto et al., 1986, Wolf et al., 1987]. Previously, decreased monoamine metabolite levels were reported after intracerebral application of MPTP or MPP^+ in the striatum of the rat [Ozaki et al., 1987, Sirinathsinghji et al., 1988, Miyake and Chiueh, 1989, Caliguri and Johannessen, 1991]. One of the possible explanations of the observed decreases in monoamine metabolite levels is a weak inhibition of intracellular and extracellular MAO by MPP^+ [Ozaki et al., 1987, Hall et al., 1992, Lin et al., 1995]. In the rat brain the metabolite of MPTP, MPP^+ is mainly produced in astrocytes [Ransom et al., 1987] and it can induce lipid peroxidation [Wu et al., 1994], inhibit chytochrome enzimes [Sriram et al., 1995] thus generate oxidative stress inside the cells [Grella and Mielle, 1995, Desole et al., 1995]. As it is demonstrated, the release of amino acids from neurons and glial cells is very sensitive to metabolic events, like energy production of the cell, glycogenesis, intracellular pH, etc. They all modify the calcium independent release of amino acids [Sandberg et al., 1986, Globus et al., 1988]. The fact that we failed to detect changes in amino acids in the thalamic extracellular space in MPTP treated rats suggests that either MPTP alone or the lower concentration of MPP+ than causes oxidative stress reaction could modify the monoamie turnover.

The increase of DOPA after MPTP injection and its further elevation *post mortem* indicate that the catecholamine synthesis might be interrupted by MPTP which is reflected in the accumulation of the catecholamine precursor DOPA. More probably, the increased DOPA level is related to the decreased extracellular NA concentration observed. We observed higher concentration of DOPA than extracellular levels of DOPA measured in the striatum [Westerink et al., 1990], but the monoaminergic innervation of the thalamus and the striatum of the rat is quite different [Paxinos, 1995] so it is acceptable that the extracellular monoamin and monoamine metabolite levels not match.

As it can be expected, the concentrations of the oxidative monoamine metabolites DOPAC and 5-HIAA continued to decrease *post mortem* whereas the extracellular concentration of the oxidative metabolite of NA, the MHPG increased. This suggests that one degradation pathway of NA that produces

MHPG was not affected as seriously as the other oxidative pathways, and the metabolite accumulated inside the cells.

In the thalamus monoaminergic transmitters have a key role in the fine tuning of the state of thalamic cells [McCormick, 1989], and recent experiments showed that a certain level of noradrenergic activity is required for PS generation [Mastrangelo et al., 1994]. In addition thalamus is claimed to play an important role in modifying the sleep-wake cycle [Steriade and Llinas, 1988]. Thus the decreased monoaminergic transmission and turnover observed in the thalamus may be one important component the previously detected PS deprivatory effect of a single low dose of i.p applied MPTP [Pungor et al., 1993].

In conclusion, it can be assumed that the altered thalamic noradrenergic transmission and monoamine turnover could be an early effect of one single dose of MPTP and might be related to the development of immadiete behavioral effects of MPTP.

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Preliminary note

DIFFERENTIABLE BOOLEAN DYNAMICS IS POSSIBLE AND USEFUL FOR STUDYING NEURAL NETWORK BEHAVIOUR

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Autonomous and evoked behaviour of neural nets and units expressed in terms of spiking and other electrical activity is regarded as important source of information about neural functions. Dynamic principles may influence performances of neural systems in any neural structure and in all animal species. Neural signals, especially spikes have properties which do not only permit but make the discrete mathematical approach necessary. This is often more adequate than the continuous description. However, the powerful tools of calculus, which are applicable to differentiable systems and were so successful in physics seem to be lost because of discreteness.

The authors recognized [2, 3] that recent extensions of mathematical instruments applied to differentiable systems are suitable also in discrete world, thus applicable for Boolean functions e.g. for threshold gates (TG) [7], or formal neural nets (FNN) [6]. Application of 'differential calculus in discrete world' requires a deeper analysis than that available in the literature. Therefore, this presentation is methodical and a preparatory one. We point to the main directions, and indicate some applications. It is unavoidable to deal with the mathematical tool itself, otherwise the power of applications cannot be explored at all.

(1) - Differentiability at the first glance appears to be a non-sense as applied to *'Boolean manifold'* because finitness and lack of continuity in conventional sense. Nevertheless, it is still possible, but the way is not direct. Partial derivatives with special properties have been defined in the 70s or so

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[8] and references therein. Here, we extend these concepts and introduce a broader Boolean differential-geometric calculus: (1) differential 1 - forms and k - forms (adapting definitions of Arnold), (2) gradients, (3) tangent bundle, (4) Jacobians, (5) temporal differentiation. In physical context and for discrete systems other similar considerations frequently arise. In the Boolean 'differential' world a crucial role is played by the XOR (truth-) function and finite field GF(2) in a non-trivial manner.

The listed notions mostly represent well-calculable quantities and functions. At the same time *'anomalous phenomena'* did arise e.g. with respect of Leibniz rule. Surprisingly enough, all truth-functions are differentiable both partially and for iteration by discrete time parameter too. Therefore, the underlying continuity and metric concepts need additional analysis.

Measure- and integration theoretical frames are also constructable and necessary. This offers newer, generalized, differential notions. Discrete, Boolean differential equations appear to be possible.

(2) - For the notion of *'continuity'* a survey of possible *topological spaces* - as well as various natural *metrics is* necessary and was initiated [2]. These categories can be chosen to harmonize with the concept of derivation. The continuity for discrete objects can be recaptured through various topologies.

Boolean cubes are metrizable topological lattices. But continuity is definable with numerous - non-metrized or occasionally not metrizable topologies.

(3) - A further background is offered by the *logic* and *threshold logic* [7]. Thus effect of derivation on *external* and *internal negations, dual function formation* were tested because self-duality plays an important role in synthesising autonomous invertible TG-s and FNN-s. Threshold functions offer another possibility of derivation, because they are defined through a special set of additional parameters, which has biological interpretation. However, threshold function set is a vanishing subset of the Boolean one and its special derivation does not apply to any switching function. This problem is avoided if networks and their derivation rules or the original Boolean derivation is used which apply both to threshold and other truth functions.

(4) - The concept of *antiduality* [4] proved to be a good frame to construct a special differential calculus which fulfils the axiomatic condition of Leibniz rule. This rule caused difficulties, because the literature is contradictory: (1) it is demanded as an axiom for differential operation but at the same time (2) other differential concepts defined for Boolean functions

appear to be adequate and *do not* satisfy the Leibniz rule. Consequently, at least two different differential operation seems possible. This interesting situation merits attention and is resolved in [3].

(5) - Further generalization of differential calculus towards k-valued logic, thus networks consisting of multistate neuronal units, seems possible.

(6) - The 'final' aims of the above research lines are applications of the new methods and theoretical apparatus mainly in Boolean control (neural) network circuits, especially further exploration of cyclic modes emerging both in autonomous or non-autonomous TG-nets including stability analysis.

One natural application is detection of *motion* and its parameters, as well as *spatial contrasts.* Until now XOR-s, see e.g. in [5] and the defined derivations were not directly applied in neurobiological theories, since a single XOR needs 3 (+ 2) threshold gates. That is why recognition of motion has been simulated by simple sets of suitable feed-forward networks or by mathematical apparatus not showing explicitly such network structures. The crucial question is as follows: *what is the smallest biological unit* capable of generating XOR behaviour. Is it a real neuron, or its part or an assembly of some neurons?

The previous sophisticated theories are desired to be extended also to facilitate digital dynamic and stability analysis not only for discrete models of neural nets but also for *'differentiable discrete automata'* offering further applications.

Remark: Part of this writing was first presented at the Annual Meeting of MITT in 1996, Balatonfüred.

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ABSTRACTS

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NEURAL PLASTICITY AND ANXIETY IN ANIMALS - IMPLICATIONS FOR UNDERSTANDING AND TREATING AFFECTIVE DISORDER FOLLOWING TRAUMATIC STRESS IN HUMANS

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Lasting changes in anxiety-like behavior (ALB) may be produced in rodents and felines. Brief, non-injurious, exposure of rats to cats (predator stress) lastingly increases rodent ALB in the elevated plus-maze. In this paper the impact of predator stress on behavior, neuroendocrine and CNS neuropeptide function are reviewed. ALB increases appear as early as 30 min after predator exposure and last 3 weeks or more. Path analysis shows a relationship between cat and rat defensive behavior and degree of increase in ALB one week later. Predator stress increases plasma corticosterone one week after the stress. Shortly after predator exposure, stress neuropeptides are elevated throughout limbic and brain stem circuits implicated in rodent ALB. Lasting changes in ALB following predator stress are dependent on NMDA and CCKB receptors. It is proposed that behavioral changes following predator stress may model anxiety associated with PTSD.

INTERACTION BETWEEN MORPHINE AND RISPERIDONE IN THE ACTIVE AVOIDANCE RESPONSE OF MALE MICE

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An interaction between dopaminergic and opioid systems have been previously observed in the active avoidance paradigm. In this experiment we attempt to extend our knowledge about this interaction studying the effects of the coadministration of morphine (12.6 mg/kg) and the recently developed antipsychotic risperidone (0.1 mg/kg) in male Balb/c mice that underwent 5 conditioning sessions in a two-way shuttle-box. Risperidone decreased avoidances and locomotor activity (crossings) and increased nonresponses. Conversely, morphine increased avoidances and crossings and decreased escapes. The coadministration of morphine and risperidone decreased avoidances and increased escapes, nonresponses and crossings. The results support the previous suggestion that the effects of morphine on avoidance the paradigm could be mediated by the dopaminergic system.

THE DETAILED PATTERN OF LOCOMOTOR ACTIVITY AFTER DAMPHETAMINE, COCAINE AND CAFFEINE TREATMENT IN THE RAT

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During the last decades, the behavioural effects induced by psychostimulants have been the focus of many experimental studies. Locomotor activity has been considered as a reliable and valid behavioural measurement for the animal studies. Moreover, the necessity of the direct, detailed, and continuous behavioural observation and recording (automatic or not) has been pointed out in order to extract important findings regarding the animals' behavioural pattern.

Our method tried to match:

→ the automated recording of locomotion;
→ the continuous observation and recording of the locomotor pattern;
→ a multifactorial analysis (factor analysis) for the obtained data.

The locomotor pattern seen after four doses of d-amphetamine, cocaine and caffeine was studied. All of these substances induced a behavioural activity mainly characterized by increased locomotion and a general stimulatory behavioural response. However, the whole behavioural profile derived from the animal recording and the subsequent factor analysis revealed different behavioural patterns among the three drugs.

The continuous recording of animal behaviour together with the multifactorial analysis provides the possibility for a reliable and global approach of the locomotor activity. Thus, the detailed study of the animal behaviour could really support studies related to the mechanism of action of psychostimulants, the drug abuse as well as the neurobiological state of CNS diseases.

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ETHOLOGICAL APPROACHES TO THE BIOLOGY OF PAIN

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There are few acute pain tests involving mechanical stimuli and these do not involve objects important in the evolutionary history of animals. Spines are among the most common physical defenses protecting plants and animals against consumption or predation and thus constitute natural, mechanical, pain stimuli. The present study examined the reaction to contact with such spines in the laboratory rat, utilizing a floor board with protruding spikes, located in a novel alley. All subjects showed voluntary contact with the spines, followed by avoidance, risk assessment (orientation and stretch attend/stretch approach), and freezing. Females showed reliably more risk assessment and tended to show less freezing than did males. We examined these behaviors in subjects given one of 4 doses of morphine (0.75, 1.5 and 3.0 mg/kg), finding dose-related effects on defensive behaviors. The lowest dose increased duration of contacts with the spines and crossings of the spiny object, while the highest dose attenuated all active behaviors. These results suggest the utility of procedures using discrete and salient stimuli that are painful to touch, in a testing situation involving minimal handling stress, as naturalistic models for the study of pain.

IMIPRAMINE EFFECTS IN THE FORCED-SWIMMING: GENDER DIFFERENCES

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Although women seem more prone to develop depression than man and have higher prevalence of this disorder, female rats do not show more immobility in the forced swimming test. Also, preclinical antidepressant trials use only males and the effects on females are not investigated. This study evaluates the effects of imipramine (IMI) in female rats using an ethopharmacological approach. Adult male and female rats were tested with the forced-swimming procedure: they were put in cold water for 15 min. and were retested in 24 hours. Three doses of IMI 0; 2.5; 5; and 10 mg/kg) were administered i.p. before the retest. Behaviors diplayed during the procedure were videotape-recorded. Female rats in diverse hormonal states as normal estral cycle, diestrus, or ovariectomy (OVX), treated or not with estrogen presented the same frequencies and duration of head-shake, swimming and immobility as male rats. A dose-dependent effect of IMI was seen in male rats, with a 38% decrease in immobility duration due to increased climbing in the walls; decrease in head-shakes was also observed. No anti-immobility and decrease in head-shake effect of IMI occurred in females, regardless their hormonal status. Moreover, in the OVX+estrogen group immobility increased 50% after IMI. In conclusion, in spite of an apparent behavioral similarity between sexes in the forced-swimming test, females are resistant to the effects of an antidepressant drug.

NEUROCHEMICAL AND HORMONAL CHANGES INDUCED BY CHRONIC MILD UNPREDICTABLE STRESS IN MALE RAT

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Recently the effects of acute or chronic stress on the hormonal function and the neurochemical procedure have been the focus of many experimental studies.

The aim of the present study was to investigate the effects of chronic mild stress on the dopaminergic or serotonergic system and the relation of thyroid function to stress. In addition the above experimental design was employed in two different adult male rat strains i.e. Sprague-Dawley and Wistar.

The chronic mild stress procedure lasted for six weeks. Stressed animals received a number of weekly-cycled mild stressors such as food and water deprivation, stroboscopic light, soiled cages, grouped housing, tilt cages and white noise. In parallel control group was housed under normal controlled conditions.

At the end of the experiment rats were decapitated, blood samples were collected while the brains were removed rapidly and the following brain areas were dissected: hypothalamus, hippocampus, striatum and prefrontal cortex. In addition thymus and adrenal weight (absolute and relative) were measured in both rat groups.

DA, DOPAC HVA, 5-HT and 5-HIAA were measured using HPLC with ED. Moreover DA and 5-HT turnover rate were estimated. Following sera extraction, T3 and T4 levels were assayed using in-house human T4 and T3 RIA modified assays.

The present results showed a decrease in the cortical 5-HT turnover rate in the stressed Sprague-Dawley animals compared to the control group. Moreover T3 and T4 levels were increased in stressed animals compared to their control only in Sprague-Dawley rats. Furthermore thymus weight was decreased in stressed rats in both strains.

The above results suggest a differentiation, between Wistar and Sprague-Dawley rats, regarding the thyroid function as well as the neurochemical effects induced by the chronic mild stress but the thymus response seems to be the same in both rats' strains.

BEHAVIOURAL AND NEUROENDOCRINE DEVIATIONS IN ADULT RATS AS LATE SEQUELS OF PERINATAL DRUG TREATMENT

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Drug treatment during late gestation and perinatal period may interfere with the gene controlled, time and space determined programme of ontogenetic processes, initiate disturbances of cytodifferentiation and receptor formation in the brain, endocrine and immune systems resulting in irreparable deviations of adult behaviour and immunoendocrine functions. Diazepam (D) is frequently prescribed for its tranquilizing and myorelaxing action by obstetricians and neonatologists to treat acute risk condition. With the aim to evaluate the functional teratogenic risk of D when given in various phases of ontogeny, animal model study was performed using D administration (5 or 10 mg/kg s.c.) in neonatal Wistar rats, either on postnatal days PD 4-5 (N=38) or on PD 9-10 (N=24), simulating thus clinical D treatment in the 6th-8th gestational month in human fetus/preterm neonate and in the full-term newborn, respectively. Comprehensive behavioral and neuro-immuno-endocrine follow-up till the age of 6 month revealed that D application on PD:4-5 caused retardation of somatic development, memory deficit and reproduction defects, whereas D applied on PD:9-10 induced significant decrease of immunoreactivity and enhancement of seizure susceptibility.

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THE MOUSE DEFENSE TEST BATTERY: WILD-LAB MOUSE COMPARISONS AND DRUG EFFECTS

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The value of a Mouse Defense Test Battery (MDTB) for analysis of pharmacological effects on defensive behaviors in laboratory mice depends in part on the correspondence of the behaviors shown by laboratory mice to those of wild congeners. Wild type (WT) mice in the MDTB showed excellent overall agreement with Swiss-Webster (SW) patterns. However, WT mice showed increased flight speed when chased by a predator (anesthetized rat), and more jump escapes. They showed each component of a risk assessment pattern (stops, head orientation, reversals) to the rat, but with reliably reduced frequency of the first two of these. They made more upright, vocalization, bite, and jump attacks to forced contact with the predator. These quantitative differences were considerably less than those seen when comparing wild and laboratory rats, and suggest that the SW mouse can be used as a valid model for pharmacological studies.

Using this model, we have examined the effects of a variety of benzodiazepines (BZ). Classic BZ agonists reduced risk assessment and defensive attack but did not alter flight at nonsedative doses. In contrast, antipanic compounds (imipramine, fluoxetine, alprazolam, moclobemide, phenelzine) given on a chronic basis, reduced flight, as well as defensive attack. These results coupled with previous studies in wild and lab rats document the usefulness of natural antipredator behaviors for preclinical ethopharmacological studies of defense-related psychopathology.

AUTOMATION OF BEHAVIORAL TESTS USING DIGITAL IMAGING

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During the past decade, video tracking technology has evolved dramatically. Early systems, based on analog video and hard-wired electronics, were only able to track a single animal under very stringent light conditions and in highly artificial environments. Modern systems, based on video frame grabbers and flexible software, can track multiple animals simultaneously against a variety of complex backgrounds. In 1993, we introduced EthoVision as a general-purpose video tracking, motion analysis and behavior recognition system. This is used to automate behavioral tests, in both academic research and preclinical drug testing in pharmaceutical industry. So far, EthoVision has mainly been used to measure changes in behavior of animals using continuous path-related parameters. Examples are the use of "total distance moved" as a measure for exploratory behavior or activity and the use of "latency time to entry in a predefined target zone" as a parameter for search efficiency. As such, it can automate a wide range of locomotory tests and memory tasks in the open field, Morris water maze, plus maze, radial maze, etc. Animals can be observed singly, in pairs or in groups. By partially painting animals in the color of the background or by applying color markers, multiple freely interacting animals can be identified. This allows prolonged automated observation of socially housed animals in semi-natural environments.

Here we report on our research into new methods of behavior recognition. Two different approaches are used. The first approach is based on the classical method of path analysis. Complex behavioral patterns are characterized using statistical analysis methods on continuous parameters (object position, velocity, etc.). Using this approach, parameters have been developed that allow an objective quantification of social behaviors. The second approach is based on shape analysis. By analyzing the continuously varying shape of the animal, behaviors that are not directly related to the locomotion of the animal can be measured. Examples are the detection of rearing, based on changes in the surface area and roundness of the animal, and recognition of specific body parts (e.g. tail and nose of the rat) for distinction between social and agonistic interactions. The overall objective of this research is the development of a system which can quantify behavior at a high level of accuracy and detect subtle behavioral effects of pharmacological treatments. The autonomous operation of the system will allow for a high degree of automation of ethopharmacological tests.

SPATIAL ACQUISITION AND RETENTION IN AGED RATS: NATURAL VARIABILITY AND EFFECTS OF ACETYL-L-CARNITINE ARGININE AMIDE TREATMENT

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The behavioural performance heterogeneity observed in young rats is increased by the aging process and this variability is related to different degrees of learning impairement. The present experiment examined the effects of aging and Acetyl-L-carnitine arginine amide (ST 857) in Fisher 344 rats on retention in the Morris water maze. The natural variability of aged rat performances was used to evaluate the effect of (ST 857) on aged-related spatial memory impairement. Rats 24 or 3 months old were trained in the maze and the acquisition was used by a cluster analysis to assign each old rat to one of tree classes: Good Performers (GP) Intermediate Performers (IP) and Poor Performers (PP). The three classes were equally subdivided into control and ST 857-treated rats. Old rats displayed heterogenouus performance and a spatial discrimination deficit. ST 857 enhanced the retention in the old rat, this effect was significant especially in the IP rats.

ENHANCEMENT OF THE SOCIAL TRANSMISSION OF FOOD PREFERENCES IN GERBILS BY THE ANXIOLYTIC CHLORDIAZEPOXIDE

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An 'observer' gerbil (*Meriones unguiculatus*), quickly (30 min) acquires a preference for a novel food from a 'demonstrator' conspecifics. Previous Familiarity (raised in the same litter or reproductive partners) and/or Kinship (siblings, even if raised in a different litter) between demonstrator and observer are necessary for this social transmission to occur. Exposure to an unfamiliar and unrelated gerbil does not affect individual diet preference. Likewise, prolonged (8 h) exposure to a novel food does not lead to acquisition of a food preference. This absence of social learning could be associated with aggression-related anxiety in the territorial gerbils. Similarly, lack of individual learning could be due to food neophobia-related anxiety. Oral administration of chlordiazepoxide (CDP: 2.5, 5, 10 mg/Kg), allowed the social transmission of food preferences between unfamiliar and unrelated gerbils in a gender-related manner. Administration of CDP did not, however, result in any individual learning of food preference. These results indicate that social transmission of food preferences in gerbils provides a useful ethopharmacological model for examining the roles of anxiety and associated neuromodulatory mechanisms in social learning.

'TOUGH' ADOLESCENTS ARE MORE LIKELY TO MISUSE DRUGS

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The entire year 9 (12-13 years old; n = 235) of a school completed 2 questionnaires about social status and drug use. The social status questionnaire employed the sociometric technique of peer nomination to measure social rank. The most commonly used drugs were alcohol (44% of all subjects), tobacco (22%) and cannabis (12%). Very few subjects used drugs such as heroin, cocaine, LSD and MDMA (only 7% of all subjects for all other drugs combined). Specific drug knowledge questions correlated well with self-reported drug use suggesting that it is a valid measure. It was observed that adolescents percieved as 'tough' are more likely to misuse drugs ($P < 0.0005$) and those who engage in drug misuse are socially admired by their peers ($P < 0.0005$). The majority of subjects reported that behaviours characteristic of aggression were characteristic of 'toughness'. These data suggest that contrary to animal studies, 'toughness' or aggression in adolescents is a potential risk factor in the initiation of drug abuse.

ULTRASOUNDS DURING SEXUAL ENCOUNTERS IN MICE

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Ultrasounds are emitted by mice in different contexts. In adulthood, mice emit ultrasounds during the first minutes of interaction with a potential sexual partner. The male seems mainly responsable for these vocalizations. Data from the literature suggest that these calls represent a measure of male's sexual motivation. NMRI outbred albino mice were tested for ultrasonic emission during the first three minutes of sexual encounters. Ultrasounds (70 + 5 KHz) were counted by the use of a bat detector (QMC Instruments). The number of ultrasonic calls did not correlate with measures of sexual motivation (frequencies and latencies to mount and ejaculate), but an inverse relationship between aggressiveness and ultrasound emission was observed. Pharmacological treatments affecting ultrasounds in pups (benzodiazepines and opioids) also modulate ultrasound emission in male during sexual encounters. These data do not support causal and functional implications of ultrasonic communication proposed by the literature, but, rather, they suggest that ultrasounds may represent an affiliative signal.

8-OH-DPAT IN DIFFERENT AREAS OF BRAIN HAVE EITHER ANXIOGENIC OR ANXIOLYTIC EFFECTS IN FEMALE RATS

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The present research aimed to evaluate the role of 5-HT1A receptors on anxiety in the virgin female tested in the elevated plus maze. 8-OH-DPAT were administered locally into the median raphe nucleus (N=83), medial septal nucleus (N=91), corticomedial amygdaloid nucleus (N=84), and dorsal periaqueductal gray (N=78) in different doses. The mean frequency of the behaviors were compared among the groups by an ANOVA and subsequently by Newman-Keuls test ($p<0.05$). The results showed that the 5-HT1A receptor agonist 8-OH-DPAT, in median raphe nucleus (2.0 µg/0.2 µl) had an anxiolytic effect. However, in medial septal nucleus, dorsal periaqueductal gray and corticomedial amygdaloid nucleus 8-OH-DPAT (0.5 µg/0.2 µl) increased anxiety.

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ANIMAL MODELS AND ETHOLOGICAL STRATEGIES FOR EARLY DRUG-TESTING IN HUMANS

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Views on animal 'models' of psychiatric disease range from outright negation on the grounds that human minds are unique, to the more parsimonious view that some components of animals' behaviour e.g. learning, attention, aggression or sociality, are altered in the human condition and therefore relevant. Paradoxically, however, the onus for obtaining experimental justification of each position is only demanded from holders of the latter view. Ethopharmacology, being an evolutionarly-based discipline, subscribes to the latter view. But, given the nature of early human testing, how can ethopharmacologists obtain the necessary evidence to support their claims?

The early phase of drug-testing in humans (Phase I) is focussed upon tolerability studies i.e. dose finding and side-effects. This work is performed upon healthy volunteers and only later are drugs given to patients. Even if a drug has an unconventional mode of action it is forced into a conventional mode of testing. Unfortunately, except for a Yes/No decison with respect to the indication, early clinical studies offer little opportunity of testing the validity of the original preclinical hypothesis on which the drug was originally conceived.

Human volunteers are not generally regarded as suitable for providing clues about a new drug's efficacy in a clinical indication. Nevertheless, their use is analogous to the successful preclinical use of 'healthy' animals providing their behaviour is biased in some way. We will describe a novel situation; the Ethological Challenge Interview (ECI) which is designed to bias the behaviour of healthy subjects in a manner similar to the animal situation. The situation can be used for drug studies and can be combined with endocrine and biochemical studies if required.

CHANGES IN DOPAMINE AND SEROTONIN DURING ETHANOL DRINKING AND AGGRESSION: A MICRODIALYSIS STUDY

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How is aminergic activity in n. accumbens (NAC) and prefrontal cortex (PFC) related to the initiation and consequences of alcohol heightened aggression in resident rats attacking an intruder? Resident rats were trained to drink a 10% ethanol solution immediately preceding an intruder confrontation. In NAC, no major changes in DA or 5HT were observed during aggression, but DA levels were increased after ethanol drinking. In PFC, DA increased and 5HT decreased during aggression, and these changes were at tenuated after ethanol drinking. No clear correlation existed between the changes in DA and 5HT and the level of aggression. Whether individuals that show heightened aggression after ethanol differ in DA and 5HT baseline levels, or differ in phasic DA or 5HT changes after ethanol or aggression is currently being investigated.

INTERMALE AGGRESSION HAS PHYSIOLOGICAL CONSEQUENCES IN NIH/S MICE

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Intermale aggression is a major problem in adult mice. Strain, stocking density and familiarity of mice caged together are known determinants of aggressive behaviour. Wether aggressive behaviour has impact on research results has not focused much attention. In this study, NIH/S male mice were housed in groups of four for five weeks. The number of wounds on the back of the animals was recorded weekly. The effects of wounds on weight gain and on weights of different organs were evaluated. According to the wounds, fighting occurred in two thirds of cages. In cage groups with fighting, the weights of brown and epididymal adipose tissue as well as testes decreased. Furthermore, these animals had decreased weight gain and enlarged spleens. The final body weights, weights of adrenals and serum corticosterone levels were unchanged. In conclusion, the intermale aggression in experimental groups can have effects on mice physical parameters. If this determinant is ignored, the variation of results is bound to increase and likelihood of false negatives can increase.

INTERINDIVIDUAL VARIABILITY IN MALE MICE AGGRESSIVE BEHAVIOR: DIFFERENTIAL EFFECTS OF CHLORDIAZEPOXIDE

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Recently, considerable interest has been expressed in the role that social factors have as determinants for benzodiazepine (BDZ) action in modulating social behavior in animals. We carried out a series of experiments in Swiss male mice to investigate the effects of chlordiazepoxide (CDP; 5.0, 10.0 and 20.0 mg/kg) on aggressive behavior of individuals of varying social experience (aggressive and defeated; experiment 1) and social status (dominants and subordinates; experiment 2). In experiment 1 CDP (10.0 and 20.0 mg/kg) reduced attack towards aggressive but not defeated males. However, only in aggressive animals low doses of CDP increased aggressive behavior. In experiment 2 preliminary data showed differential effects of CDP on males of different social status. Baseline behavioral differences cannot account for the differential effects of CDP. The pro-aggressive effects of benzodiazepine appear related to the experiencial background and aggressive characteristics of animals. This result could be of particular interest to understand individual variations in response to BDZ treatment especially for those clinical studies which are aimed to predict and avoid possible side-effects of BDZ on behavior. Our findings suggest that an understanding of drug effects on behavior demands consideration of the biological variability in phenotype in an evolutionary perspective.

STRESS AND STRUCTURAL PLASTICITY OF THE BRAIN

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Plastic changes in the brain are considered to accompany learning an memory as well as neuropathological processes. In this context, structural changes in the hippocampal formation are of great interest because this brain area plays a crucial role in cognitive functions and has pronounced regulatory influences on the activity of the hypothalamic-pituitary-adrenocortical (HPA) axis which is thought to be the predominant stress system. Changes of circulating adrenal steroid levels by stress exposure have been shown to affect hippocampal physiology as well as cognitive functions. Most preclinical studies relating to stress and hippocampal morphology were derived from studies in rodents. To better understand the effects of stressful psychological situations in a nonrodent mammalian species, we used the psychosocial stress paradigm in male tree shrews (*Tupaia belangeri*). Because neuronal physiology depends upon neuronal structure, we determined the morphological reactions of hippocampal neurons to psychosocial stress. In this presentation, stress-induced effects on both neuronal morphology and neurogenesis in the adult hippocampal formation are discussed with particular emphasis on the underlying mechanisms and their functional relevance.

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SUBORDINATION STRESS IN THE TREE SHREW: RESTORATIVE EFFECTS OF CLOMIPRAMINE

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Male tree shrews (*Tupaia belangeri*) represent a suitable model to study the bio-behavioral consequences of psychosocial stress. When living in visual contact with a male conspecific by which it has been defeated, the subordinate tree shrew shows dramatic behavioral and neuroendocrine changes. Since the overall pattern of these stress-induced changes resembles depression-like symptoms we analyzed the effects of long-term antidepressant treatment on bio-behavioral parameters in subordinate tree shrews. Using clomipramine for 4 weeks, a clear and time-dependent restorative effect on marking and grooming behavior, locomotor activity, risk assessment, and both urinary cortisol and norepinephrine excretion was observed. It thus appears that the clomipramine treatment counteracts the behavioral and endocrine effects of subordination stress and the time course of recovery corresponds closely to that observed when treating depressed patients in the clinic. This result is an essential requirement for animal models designed to elucidate the mechanisms underlying therapeutic actions of antidepressants.

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ANTICONVULSANT AND CALCIUM CHANNEL BLOCKING ACTIVITY OF *LAVANDULA STOECHAS* EXTRACT

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This study was carried out to validate the folkloric use of Lavandula stoechas flowers (LS) in epilepsy. Swiss albino mice were divided into 3 groups of 5 each. Group 1 served as control. Group 2 received pentylenetetrazole (PTZ; 70 mg/kg s.c.) and group 3 was treated with plant extract (i.p.) followed 1 h by PTZ and animals were then observed during 30 min for seizure and 24 h for lethality. Calcium channel blocking (CCB) activity was tested on isolated rabbit jejunum using a standard method. The methanolic extract of LS flowers (400-600 mg/kg) delayed significantly the onset of seizure from 4 to 8 min and it caused 60% protection against the lethal effect of PTZ. In rabbit jejunum, it caused a dose-dependent (0.1-3 mg/kg) inhibition of spontaneous contractions. LS also inhibited K+ (50 mM)-induced contractions at the same doses suggestive of CCB actions. Since CCBs exhibit antiepileptic action, the protective effect of LS against PTZ-induced lethality and seizures may be mediated through a CCB action.

THE ELEVATED T-MAZE AS AN EXPERIMENTAL MODEL OF ANXIETY

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Anxiety disorders are heterogeneous and existing animal models do not address to specific types of anxiety. The elevated T-maze consists of three elevated arms: one enclosed and two open. Inhibitory avoidance – representing learned fear – is measured by recording the time taken to leave the enclosed arm in three consecutive trials. Unconditioned fear is evaluated by recording the time to escape from the open arm. Restraining the animals at the end of the enclosed arm for 30 s did not change the first (baseline) withdrawal latency, indicating that aversion for the hands of the experimenter is not a key motivation for this response. In addition, rats trained in a T-maze with the three arms enclosed did not show the usual increase in withdrawal latency along the three consecutive trials of the avoidance task. These results indicate that open arm experience, but not handling stress is the main cause for inhibitory avoidance learning. The two tasks were selectively affected by anxiolytic and anxiogenic drugs, and were unchanged by psychomotor stimulants and one neuroleptic. These results support the original assumption that the two tasks relate to different types of anxiety.

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DIGGING BEHAVIOUR OF MICE IN THE LABORATORY: A POSSIBLE MODEL OF OBSESSIVE-COMPULSIVE DISORDER?

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When exposed to a proper ground (e.g. thick layer of bedding material in the experimental chamber) laboratory mice display intense digging activity. The behaviour does not habituate over repeated daily exposures for five days, though during a 60 min trial its intensity decreases with time. In a two compartment choice paradigm animals prefer the side where they can perform digging. The intensity of digging depends on the depth of the bedding material layer, properties of the ground (natural soil elicits more intense response than sawdust does), age of the animal (aged animals dig more than younger ones). Digging activity appears to be a compulsive behaviour elicited by the presence of a diggable ground. Serotonin specific reuptake inhibitors which are effective drugs in human obsessive-compulsive disorder (OCD) inhibit the digging response. The results raise the possibility that digging behaviour may be used as an animal model of OCD.

THE EFFECT OF SOCIAL EXPERIENCE ON THE BEHAVIORAL EFFECTIVENESS OF ANXIOLYTIC DRUGS

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Social stress is often claimed to be among the eliciting factors of anxiety disorders. On the other hand both laboratory animals and humans submitted to anxiolytic treatments are subjects of social experience. The literature is scarce in studies dealing with the impact of chronic social experience on anxiety measures, while studies that would link anxiolytic drug action to social experience are totally lacking. The aim of the present study is to investigate the interrelationships between subchronic social experience, anxiolytic drug treatment, and anxiety measures.

Six social experiences have been induced in male Wistar rats: 1. group housing; 2. male-female pair housing; 3. isolation; 4. daily winning in aggressive encounters; 5. daily losing aggressive encounters; 6. continuous cohabitation with a dominant male. The duration of the experience was 5 days. Anxiety has been tested in the elevated plus maze. The following order of anxiety scores has been shown: winning < male-female pairs = group housing < isolation = losing < continuous cohabitation with a dominant male. The effect of chlordiazepoxide and buspirone is currently tested in relation with experiential background. Preliminary data show that social experience may affect drug action.

TESTOSTERONE-NORADRENALINE INTERACTIONS IN THE CONTROL OF AGGRESSION

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Behavior is controlled by a multitude of external and internal factors, that are studied usually separately. However, these factors work integratedly in the living animal, therefore understanding behavior requires their integrated study. Endicrinologic and neurobiologic investigations are particularly suitable for integration, since the experimental methodologies of the two areas are rather similar, and there is ample evidence for mutual interdependence between the two categories of phenomena.

The ontogenetic development and the function of the noradrenergic system is deeply influenced by testosterone, and both noradrenergic system and testosterone are involved in the control of aggression. The aim of our study was to investigate the effects of testosterone background on the behavioral effectiveness of noradrenergic treatments. Castrated male rats have been treated with the long-acting testosterone preparation testosterone enanthate (TE) or vehicle. One week later subjects were exposed to an intruder for 30 minutes on three consecutive days. On the forth day rats have been injected with the alpha-2 adrenoceptor blocker idazoxan or vehicle. Twenty minutes later subjects were presented with an aggressive or a nonaggressive intruder. TE treatment resulted in a more rapid and stronger expression of aggressive behavior compared with vehicle treated castrates. Idazoxan showed the effects described earlier in TE treated rats, while it caused no significant changes in the behavior of rats that did not undergo testosterone replacement. It has been concluded that the effect of the alpha-2 adrenoceptor blocker idazoxan is highly dependent on the presence of testosterone. This interrelationship may be a reflection of a more general dependence of the behavioral role of the noradrenergic system on testosterone background.

NATURALISTIC MODELS OF CHRONIC STRESS IN THE RAT

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Chronic stress is widely regarded as important in the etiology of a range of defense-related psychopathologies. A subordination model of chronic stress for rats in a visible burrow system (VBS) produces behaviors isomorphic with many of the target symptoms of depression, including reductions in sexual, social, and consummatory behaviors; changes in sleep/activity cycles; increased voluntary alcohol consumption; and a deceleration of movement. Some of these behavioral changes are exacerbated in a subset of subordinates that show disruption of the hypothalamic-pituitary-adrenal axis response to acute stress, and brain neurotransmitter system effects can differentiate grouped animals from controls, subordinates from dominants, and stress nonresponsive subordinates from stress responsive subordinates. Daily administration of fluoxetine reduces some behavioral and biochemical effects of subordination. However, drug administration to subordinates may change their interactions with dominants, producing confounded effects. Newer chronic stress models avoiding this problem involve chronic exposure of subjects to a predator (cat), a procedure that also permits analysis of differential stress effects on male and female animals.

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THE EXPERIENTIAL BASIS OF 'TRIAL 2' ANXIETY IN THE MURINE ELEVATED PLUS-MAZE

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Behavioural and pharmacological evidence suggests that a single prior undrugged experience of the elevated plus-maze qualitatively alters future anxiety responses in this test (e.g. Rodgers et al., 1992). This has led to the hypothesis that 'Trial 2' anxiety may actually represent a phobic-response to the open arms/spaces of the plus-maze (e.g. File & Zangrossi, 1993). In agreement with this proposal, entrapment of rats within an open arm on Trial 1 abolishes the anxiolytic response to chlordiazepoxide (CDP), whereas similar exposure to a closed arm does not (File et al., 1990). In view of potentially important species differences in behavioural and pharmacological response to the plus-maze, the present experiments employed ethological methods to investigate which experiential parameters on initial exposure determined the development of Trial 2 anxiety in male Swiss Webster mice. Limiting Trial 1 exposure to either an open or a closed arm produced a significant but short-lasting reduction in Trial 2 anxiety but, in direct contrast to previous findings in rats, only closed arm experience was sufficient to render mice unresponsive to CDP on Trial 2. These data suggest that, following a limited Trial 1 experience (open or closed), mice show a rapid avoidance of the open arms when subsequently given free Trial 2 exploration. Furthermore, the experiential determinant of Trial 2 anxiety in these subjects appears to be exposure to a closed arm, which fits awkwardly with an 'open-space phobia' hypothesis of this state. The remaining possibility of an interaction between the Trial 1 'drug state' and its experiential parameters awaits futher clarification.

OPEN FIELD LOCOMOTION IN MICE - RELATIONSHIPS TO BEHAVIORAL EVENTS - A FACTORANALYTIC APPROACH

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Individual endogenous dispositions and environmental conditions are important factors modifying central nervous processes, as well as effects of psychotropic drugs. In our investigations mice characterized as high active (HAM) and low active (LAM) were used to study the influence of individual type related drug response and environmental reactivity. To induce the latter, two different housing conditions (group housing or isolation) and housing durations (1 day; 1 week; 3, 6, 12, 18 weeks) were used. Apomorphine and diazepam were given by acute administration. The spontaneous open field locomotor activity and neurochemical measures of drug treated and control mice were analysed.

The results of our study revealed relations between 'activity' and striatal dopamine transmission on the one side as between 'exploration' and cortical aminergic transmission and as between 'irritation' and serotonergic transmission on the other side. The dopaminergic drug primarily influenced the individual behavioral drive. The benzodiazepine exerted its effects in dependence on external circumstances and changed behavioural organization with consequences for strategy and emotionality. Independent on the fact that further variables should improve a common factor analysis of behavioral and neurochemical data the model seemed suitable to describe components of a complex behavior, their neurochemical correlates and specific drug response directions.

FIGHTING DOES NOT CORRELATE WITH AGGRESSIVENESS IN RESIDENT-INTRUDER TEST IN NIH/S MICE

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In behavioural studies it is essential to know determinants of animal behaviour. In mice, status of animal in group and its interactions with cage mates may be such determinants. In this study, the aggressiveness of male NIH/S mice, housed 4/cage, was studied with individual resident - intruder test, with group intruder test (intruder placed into the same cage with whole group), and with following the numbers of wounds in the animals. In intruder test prior to group forming, the frequency or duration of attacks toward a strange intruder or the latency time for first attack did not predict the number or severity of wounds of the animals in a new group. After housing 3 weeks in the same group, the mice with severe wounds showed increased frequency and duration of attacks against intruder, and the latency for first attack was shortened. Moreover, mice with several wounds showed more agonistic behaviour against intruder in the group. Due to large variation, the changes were not, however, statistically significant. The behaviour of animals housed in groups without fighting did not differ from that of animals with fighting. The data indicated that resident-intruder test was not sensitive to the aggressiveness in group.

EFFECTS OF FOOD PROVISIONING ON BEHAVIOUR OF COMMENSAL BABOONS (*PAPIO HAMADRYAS*) IN THE WESTERN REGION OF SAUDI ARABIA

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The effects of artificial feeding by humans on the behaviour of hamadyas baboons in the western region of Saudi Arabia were examined. Grooming was the dominant behaviour in these baboons, especially in adults throughout all seasons. Play was the dominant activity of juviniles less than 4 years of age. Provisioning locations and periods changed all kinds of behaviour.

LACK OF BIO-BEHAVIORAL CHANGES DUE TO GR-ANTAGONIST ORG34116 TREATMENT IN STRESSED TREE SHREWS

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Increased glucocorticoid concentrations play an important role in the course of major depression. Uncertain is which role the glucocorticoids play: Are they a cause for depression or is the hyperactivity of the HPA-axis a consequence of some other processes during depression? To see if we can manipulate the HPA-axis activity to counteract the stress symptoms induced by chronic psychosocial stress in male tree shrews, we treated the animals with the glucocorticoid receptor (GR) antagonist Org34116. Oral administration of the GR antagonist Org34116 to the stressed tree shrews does not have an effect on the urinary concentration of cortisol and catecholamines, nor does it affect behavioral parameters. It may be concluded that Org34116 does not seem to have an effect on the endocrinological and behavioral parameters measured in this experiment.

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EFFECTS OF RAT OR NOVEL ENVIRONMENT CONTACT ON EXPLORATION AND ATTACK BEHAVIOR BY MICE

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The effects of brief daily exposure of mice to an amicable rat (Group E), a clean empty rat cat (Group CC) or home cage experience only (Group C) on two measures of exploration as well as intruder attack were examined. Relative to Group C, novel object exploration was strongly suppressed in Group CC but significantly increased in Group E. Holeboard exploration and intruder attack, in contrast, were somewhat increased in both E and CC groups. Since electric shock, prior defeat experience and cat or novel odor exposure all inhibit intermale attack, the facilitation seen in Groups E and CC was quite unexpected and will require further study. The results also suggest that differing methods of fear induction may have more test-specific effects on exploratory behavior.

BEHAVIORAL INDIVIDUALITY AND SOME OTHER IMPORTANT CONCEPTS IN ETHOPHARMACOLOGY

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Application of some ethological concepts in the study of behavioral effects of drugs, such as observation of natural behavioral acts and postures and measurement of behavior as a whole had been discussed previously at Lisek (Krsiak, Neurosci.Biobehav.Rev. 15:439-445,1991). Another important feature of ethological approach is great attention paid to behavior of individual subjects. Mean results can sometimes obscure consistent behavioral changes occurring in individual subjects. For example, only a slight non-significant increase was found in mean scores of aggression after alprazolam discontinuation in mice given the drug 2 mg/kg/day for 8 days. However, analysis of individual data revealed that mice with low basal level of aggression showed the highest increase in aggression while subjects with high baseline of aggression were less aggressive during alprazolam withdrawal. In testing drug effects during social interaction, it is important to respect indirect drug effects, that is, effects produced by behavioral changes in untreated animals interacting with a drugged animal. This principle is still sometimes neglected although it had been formulated long ago (Miczek and Krsiak, Adv. Behav. Pharmacol. Vol. 2, pp. 87-162, 1979).

SHORT-TERM HYPOTHALAMIC ENDOCRINE FEEDBACK ON BRAIN MECHANISMS INVOLVED IN THE POST-APPRAISAL, EXECUTION PHASES OF AGGRESSION: A NEW MECHANISM?

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Hess' original reports on the elicitation of attack behaviour by activation of a specific part of the hypothalamus of the cat, had a lasting impact on the conceptualization of brain mechanisms in behaviour, in ethology, psychology and the neurosciences. Yet almost 70 years after Hess' first publication, the functions of the activated brain mechanisms are only partly understood. Neuroanatomy, ethology and pharmacology of the response suggest that only the post-appraisal, executive, or consummatory phase of the aggressive repertoire is elicited: the damgaing act of fighting itself. However, the specific function of the hypothalamus in such attack-releasing brain mechanisms is not yet clear. We present data suggesting that the hypothalamus subserves the integration of mechanisms involved in the regulation of the stress response and the mechanisms involved in aggression. Plasma corticosterone levels increase immediately in both intruder and resident rat in a territorial setting. Blocking the release of ACTH inhibits such territorial fighting, while corticosterone produces a fast facilitating effect in rats without adrenals. The increase in plasma corticosterone is probably not just a consequence of the stressor of the confrontation. Stimulation of the hypothalamic attack area in rats, in the absence of an opponent, at the stimulation intensity that would have elicited attacks in the presence of an opponent, immediately increases plasma ACTH and corticosterone to high stress levels. Stimulation of the attack release mechanisms via the hypothalamus is apparently sufficient to activate the HPA-axis without the need for an opponent as external stressor. In addition, mimicking part of the stress response by injecting corticosterone in rats without adrenals, produces an immediate, short-lasting facilitation of hypothalamic attacks. Apparently central mechanisms involved in the release of attacks generate changes in plasma hormones which immediately facilitate their own activity, generating a fast feed-forward effect. These findings may help explain why, once engaged, fighting often is self-perpetuating, and also help explain why stressors often contribute to the escalation of violence.

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NEUROCHEMICAL AND BEHAVIORAL ASPECTS OF REPEATED POSITIVE FIGHTING EXPERIENCE IN MICE

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The influence of repeated positive fighting experience in daily intermale aggressive confrontations on behavior and brain serotonergic and dopaminergic systems was studied in male mice of different strains. The sensory contact technique (Kudryavtseva, 1991*) used in such experiments enhances the aggressiveness very strong and allows the aggressive type of behavior (winners) to be formed. It has been demonstrated changes in many items of individual and social behavior in winners. Some data prove development of pathological aggressiveness in male mice. Chronic experience of aggression totally activates the brain dopaminergic systems through DOPAC formation. The changes in brain tryptophan hydroxylase activity, 5-HT and 5-HIAA levels and 5-HT_{1A} and 5-HT_{2A} receptors sensitivity have been recorded in winners. The expression of changes depends on genetically defined peculiarities of the nervous system and the duration of intermale agonistic confrontations.

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*Aggress.Behav.,1991:17:5:285.

FORMATION OF AGGRESSIVE AND SUBMISSIVE TYPES OF SOCIAL BEHAVIOR IN MALE MICE UNDER INFLUENCE OF REPEATED EXPERIENCE OF AGONISTIC INTERACTIONS IN SENSORY CONTACT CONDITIONS

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To order to generate aggressive and submissive behaviors in mice the sensory contact technique was used. Pairs of males were placed into experimental cages separated into halves by a perforated transparent partition permitting animals to see, hear and perceive the smell of neighbor, whilst preventing them from physical contact. Every day the partition was removed for 10 min, leading to agonistic interactions between males. Repeated experience of social victories or defeats in daily agonistic confrontations led to formation of alternative types of social behavior - i.e. animals with positive (winners) or negative (losers) social experiences. These animals are characterized by numerous differences in neurochemistry, physiology and behaviors. Film shows the changes in social behavior of winners and losers as a reaction to conspecific in different periods of agonistic interactions - during 1st, 10th and 20th tests. Winners demonstrate strong aggressiveness under influence of sensory contact conditions. Long experience of defeats in daily social confrontations and permanent living with aggressive males in the common cage under distant sensory contact conditions leads to development of depressive-like state, accompanying by common behavioral deficit in different situations, motor retardation, indifference, slowness, rarity of movements, passive defense during agonistic confrontation instead active defense which prevailed in first tests in losers. It is supposed, that sensory contact technique will be useful for study the neurophysiological consequences of chronic social conflicts.

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PECULIAR NOVELTY SEEKING AND D-AMPHETAMINE SENSITIZATION PATTERNS IN PERIADOLESCENT MICE

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Both rats and mice have a natural drive to seek for the experience of novelty, which has been associated, along with most amphetamine (AMPH) effects, with the activation of the meso-limbic dopaminergic system in the CNS. Also, periadolescent subjects are reported to be affected differentially by catecholaminergic agents when compared with adult animals. This study assessed levels of novelty seeking as well as AMPH sensitization patterns in Periadolescent (33-43 days) and Adult (> 70 days) mice. Subjects were IP injected for three days (treatment history: SAL, AMPH 2 or AMPH 10 mg/kg once/day) in the presence of a familiar environment. Fourty-eight h after last drug injection, mice were challenged with either SAL (to evaluate conditioning) or a standard AMPH dose (2 mg/kg, to assess sensitization) and placed in the familiar and pretreatment-paired environment. Much higher novelty seeking than Adults was found in Periadolescent mice when they were given free access to a novel environment. Interestingly, an AMPH challenge strongly increased levels of novelty preference in Adults, whereas it had a quite opposite effect in Periadolescents. A treatment history of high AMPH dosage (10 mg/kg) was associated to a marked sensitization of the locomotor response in Periadolescent mice, whereas they failed to show the AMPH-induced stereotyped behavioural syndrome that was typical of Adults. Animals of both ages treated repeatedly with the high AMPH dosage were associated to a significant reduction in body weight and to elevated plasma corticosterone levels. In conclusion, a reliable and useful experimental paradigm has been developed to investigate the issue of vulnerability to a variety of habit-forming agents or emotional experiences whose positive reinforcing properties may rely on a common neurobiological mechanism.

EARLY WEANING AND CHANGES IN SOCIAL VARIABLES DURING PRE-PUBERTY AFFECT COCAINE REINFORCING PROPERTIES IN ADULT MICE

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The aim of this study was to determine in mice if manipulation of weaning time and of social variables during pre-puberty would account for the individual features of behaviour that are correlated with the abuse of drugs. This issue was addressed by searching for differences in cocaine effects in the adult offspring of both sexes from outbred Swiss CD-1 mouse litters. On postnatal day 15, litters were split in two halves, with one half litter weaned (Precocious weaning, PW) while the other remained with the dam (Regular weaning, RW). At the same time, pups in each half litter were assigned to either a unisex or a mixed-sex rearing condition. Since postnatal day 25 to adulthood (> 70 postnatal days, time of testing), all mice were re-housed according to both sex and weaning. Both adult PW male mice and unisex-reared animals of both sexes were in general more active than corresponding controls. In a place conditioning schedule, aimed at assessing the reinforcing properties of cocaine HCl (two drug pairings; 0, 1, or 5 mg/kg, IP), mixed-sex mice of both sexes were readily conditioned by the drug. Time of weaning determined the direction of the conditioned performance. In fact, whereas RW mice showed in this specific paradigm a reduction of time spent in the drug-paired area, subjects weaned precociously developed a quite clear conditioned place preference in a dose-dependent fashion. Interestingly, irrespective of their weaning history, cocaine failed to induce conditioning in animals which underwent sexual segregation during development. These differential cocaine effects i) suggest alterations in the function of mesolimbic and nigrostriatal DA systems linked to subtle changes in social stimulation during development, and ii) may be associated with an acquired predisposition to exhibit an individual responsivity to drugs of abuse.

EFFECTS OF SCH 23390 AND RACLOPRIDE ON ACQUISITION AND PERFORMANCE OF A CONDITIONED AVOIDANCE RESPONSE IN MALE MICE

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SCH 23390 and raclopride are dopaminergic antagonists with different affinities for DA receptors: SCH 23390 is selective for D1 while raclopride is selective for D2 receptors. In this experiment we attempt to find out possible differences in the effects of SCH 23390 (0.05 and 0.1 mg/kg) and raclopride (0.1 and 0.5 mg/kg) on a two-way avoidance task in male Balb/c mice. Each subject underwent 5 conditioning sessions, called pretraining (1), acquisition (2, 3, 4) and performance (5) which differed in the treatment received by the animals. SCH had no effects on acquisition but decreased avoidance and locomotor activity (crossings) in performance phase at 0.1 mg/kg. However, raclopride decreased avoidance and crossings in the acquisition at high dose but had no effect on performance phase. The results suggest that D1 and D2 dopamine receptors could be implicated in different processes of the avoidance task.

LONG-TERM EFFECTS OF REPEATED POSTNATAL MILD STRESS IN MICE AND GENDER DIFFERENCES: REVERSAL WITH NALOXONE

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In our previous work we demonstrated that daily subcutaneous (sc) injections with saline, throughout the first three weeks of postnatal life, induce long-term body weight gain and analgesic threshold alterations in adult mice. The anti-opiate naloxone antagonised both effects. Thus they were suggested to be mediated by opioid system functioning. The litter composition is known to influence the development of the opioid system, that in mice takes place during the first three weeks of postnatal life. Then, it could interfere with the opioid mediated effects of early stress.

In this study we compare the long-term effects on body weight gain and tail flick latency of postnatally saline treated or undisturbed controls, breeded in either only males or bisexual litters. We demonstrate that in male mice, the litter composition can affect the above variables. Particularly, male mice breeded in bisexual litters show lower and later alterations in body weight and analgesic threshold, compared to same treated males breeded in unisexual litters.

Gender differences were also observed. Females breeded in bisexual litters, show no effect on analgesic threshold and lower body weight gain percentage compared to the male same treated littermates.

All the observed effects result antagonised by the active but not by the inactive isomer of naloxone. The above results suggest that sexual hormones can interfere with the long-term opioid-mediated effects of early chronic mild stress.

DRUGS OF ABUSE AND SOCIAL STRESS: COMMON NEUROADAPTATIVE MECHANISMS

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Social conflict comprises salient behavioral events that engender significant well-known immediate consequences for autonomic, endocrine and neurobiological activity. When an intruder confronts a threatening and attacking resident briefly, a sequence of defensive and flight reactions, and eventually submissive postures and signals are displayed, many homeostatic functions are constrained, and affective distress is expressed. Intense, unpredictable and uncontrollable social stress engenders naltrexone-reversible analgesia, opiate tolerance and withdrawal (Miczek et al. 1982, 1986). Even brief, non-injurious social stress results (1) physiologically, in marked elevation of pituitary-adrenal, cardiovascular and thermoregulatory activity in the intruder while being threatened (Tornatzky and Miczek 1994, 1995). The significance of brief social confrontations becomes apparent also in biological rhythm studies that reveal how recurrent aggressive episodes can become 'Zeitgeber' for the hyperthermic and tachycardic activation in anticipation of a confrontation (Tornatzky et al. in press). (2) When exposed repeatedly to confrontations with an aggressive opponent, vocal expressions of affective distress are emitted in anticipation and in reaction to threats; (3) Neurochemically, upon confronting a threatening resident rat, dopamine, DOPAC and HVA extracellular concentrations are immediately increased in nucleus accumbens and prefrontal cortex, but not in lateral and dorsal striatum. The increased DA concentration in prefrontal cortex persists for ca. 1 hour, while the intruder is threatened. Increased dopamine activity in the basal ganglia has often been associated with motor activa-tion; yet, increases in dopamine in accumbens and prefrontal cortex in the socially stressed intruder rat are synchronous with behavioral 'restraint', i.e. display of crouch postures and orients toward the threatening opponent (Tidey and Miczek 1996). Within one hour of the social confrontation, the immediate early gene cFOS is expressed in periaqueductal grey area, dorsal raphe n., and locus coeruleus (Nikulina et al. 1995). Significantly, long-term neuroadaption is initiated as evidenced by long-lasting tolerance to opiates and sensitization to dopaminergic challenges. A single aggressive episode also triggers a cascade of long-term neuroadaptive changes beginning with immediate early gene expression. Eventually, cocaine self-administration is initiated more rapidly and maintained at higher rates (Miczek and Mutschler 1996, Tidey and Miczek 1997). Tolerance to opioid analgesia, behavioral sensitization to psychomotor stimulants, and increased self-administration illustrate the pharmacological significance of a single salient social defeat stress.

ADVANCES IN DIGITAL VIDEO TECHNOLOGY: PERSPECTIVES FOR ETHOPHARMACOLOGICAL RESEARCH

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The use of computers for the collection and analysis of behavioral data is now widespread. A desktop, notebook or handheld computer, equipped with an event recording program, allows easy entry and accurate timing of behavioral events and eliminates data transcription. One can key in the data while watching the animal(s). However, *live* observation and scoring is not always possible or desirable, e.g. when behaviors occur too rapidly (e.g. agonistic behaviors in rodents) or if too much happens at once (e.g. social interactions in a colony of primates). In such cases, one typically videotapes the process and codes the behavior from tape, with the VCR playback speed adapted to the complexity of the behavior observed. Accurate timing of videotaped events, however, requires synchronization between what is visible on the tape and the computer timer. Through the integration of event recording software with video time code and multimedia hardware, fast and convenient coding, annotating and reviewing of video tapes becomes possible. During preparation of a tape recording, a time code generator adds an invisible time code to each video frame. During data collection, a time code reader retrieves the time code from the tape, allowing frame-accurate event timing independent of VCR playback speed. The VCR can be controlled by the computer, allowing software-controlled jog, shuttle and search functions. For optimal visual feedback during coding, one can display the video image in a window on the computer screen. Marked video episodes can be copied to an *edit decision list* for easy creation of highlight tapes.

The disadvantage of video tapes is that they allow only linear access. As a result, search operations can be associated with considerable tape (re)wind times. Furthermore, tape quality deteriorates with repeated playback. These problems are solved by digital storage media, such as CD-ROM and Digital Video Disk, which will gradually replace analog video tapes. Digital video allows immediate access to any video frame as well as multi-point playback (e.g. from a video server in an institutional network). Video files can also be copied without loss of quality. This offers exciting opportunities for the ethologist. Research results can thus be illustrated with video images and clips (digital movies) of selected behavioral patterns into multimedia reports. These can be distributed on CD-ROM or published on the Internet or an organization's intranet. Some examples of this new technique will be demonstrated during the presentation.

COGNITIVE ABILITIES IN TREE SHREWS: EFFECTS OF STRESSFUL EXPERIENCE

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Stress is thought to be involved in a variety of pathophysiological events. We are interested whether learning and memory might be affected by psychosocial stress. Most experimental studies in rodents employed noxious stimuli or physical restraint. In addition, cognitive tests conducted in rodents also imply several potentially confounding and uncontrollable factors such as anxiety and food deprivation. To better understand the effects of a psychological type of stress on cognitive function, we are investigating the cognitive abilities in adult male tree shrews (*Tupaia belangeri*) under stressful and non-stressful conditions and developed a paradigm with a modified hole-board. This allows the examination of the cognitive performance in the animals' home cages and - during the stress period - in the visual presence of the dominant animal (stressor). Results indicate that psychosocial stress in male tree shrews leads to various modulations of learning and memory abilities which may be due to elevated glucocorticoid hormones as they occur under stressful conditions.

THERAPY FOR ANIMALS? ENCOURAGEMENT OF EXPLORATION AS A MODEL OF RECOVERY FROM DEPRESSION

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Learned helplessness consists of prolonged exposure to unpredictable, uncontrollable, and negative events. Thus, an 'outcome independency' of the animal behaviours is established, which lead to passivity. Behavioral procedures aimed at the reversal of helplessness have received little attention. How could animals be induced to regain and express their sense of control? Two procedures have already been proposed. Helpless dogs have been treated by dragging them away from shock, while helpless rhesus monkeys have been presented with a fear stimulus which triggered escape behaviour. Both treatments reduced helplessness. In cognitive therapy for depression in humans patients are encouraged to develop and systematically test hypotheses about the outcome of their behavior. By having the patients systematically monitor the consequences of their actions, generalized apperception of the world as aloof, rejecting or unresponsive (the cognitive component of helplessness) makes way for more realistic hypotheses allowing for the initiation of new activities. In rodents, it has been argued that exploration can be understood as a process of active hypothesis formation and testing by the animal. Helpless animals could be said to have given up on this active testing. A possible road to recovery from helplessness might therefore consist of drawing the animal into exploratory activity by: moving known objects to new loci within the cage, or by removing and re-introducing them; the attention of the rats should be directed towards these objects by changing their odours and moving them; objects should be introduced that are liable or not to manipulation, by this inducing learning about difference between manipulable and unmanipulable objects; animal should be allowed exploration of novel chambers. A key tenet of the state of learned helplessness is inappropriate passivity. Hence, a test for reversal could involve the frequency and persistency of exploration after introduction of a new test stimulus, both of which should be increased relative to control group or baseline levels. If it would be possible to reverse helplessness and passivity reliably in this way, an animal model of recovery from depression might be at stake. Subsequently, anti-depressants might be administred and tested for their potential to enhance the process of exploration and/or hypothesis formation.

AN EVOLUTIONARY APPROACH TO BEHAVIORAL PHARMACOLOGY: UNDERSTANDING PROXIMAL AND ULTIMATE MECHANISMS OF DIFFERENT FORMS OF AGGRESSION IN MICE

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In the present study we examined the effects of GABAergic and Serotonergic drugs on defensive and offensive forms of intraspecific aggression. Fluprazine (a serotonergic anti-aggresive drug) and chlordiazepoxide (CDP; a benzodiazepine anxiolytic) were used to examine underlying mechanisms of maternal aggression towards intruder of differing sex. Fluprazine showed more potent inhibitory effect on attack directed towards female (offensive attack) than towards male (defensive attack) intruders. This may reflect the diverse function of attack in that males pose a much greater threat to the pups than do female. In aggressive naive dams (NAD) CDP decreased defensive forms of attack (towards males). In aggressive experienced dams (AD) CDP had opposite effects on defensive forms of attack (i.e. increased attack towards the male). In both NAD and AD conditions, CDP modified the attack strategy of lactating females against the male, switching it from a defensive to an offensive pattern. These results suggest that the effects of CDP on maternal aggression depend not only on the drug but also the object of attack, and hence the function of attack and the prior experience of the attacker. Fluprazine was also used to investigate the relationship between intrasexual attack and infanticide in both sexes in wild and Swiss mice. Results showed that, in both strains, fluprazine inhibited intrasexual and infanticidal attack in both sexes. Thus, motivational and neural substrates underlying the two forms of aggression appear to be related to each other, and similarly modulated in both sexes. This suggests a common neurobiology of competitive forms of aggression. These data suggest that ethopharmacology can be used not only to understand proximal mechanisms of behavior but also the related function of different forms of aggression. This evolutionary approach to behavioral pharmacology may allow a better understanding of neurochemical mechanisms of behavior in relation to its biological significance.

PK/PD MODELLING OF THE ANXIOLYTIC EFFECT OF 5-HT AGONISTS BY MEANS OF FEAR-INDUCED ULTRASONIC VOCALIZATIONS

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A detailed understanding of the concentration-effect relationship of anxiolytic drugs is essential to develop a rational dosing regimen. Thus far, the methods applied in the pharmacological screening of anxiolytic and antidepressant drugs do not provide information on the time course of the effect. Within the scope of pharmacokinetic-pharmacodynamic (PK/PD) modelling we have developed a behavioural model of fear-induced ultrasonic vocalizations to characterize the concentration-effect profile of 5-HT agonists.

According to a cross-over design, the pharmacodynamics of buspirone was determined on basis of quantitative monitoring of ultrasound emissions in the 20-30 kHz frequency band, both after intravenous (5 mg/kg) and oral (10 mg/kg) administration (Groups 1 and 2). Physiological fluctuation of the response was investigated in a control group, which received 5% glucose solution (Group 3). Distress calls were induced by random electric stimulation during a 10 min period using a computer-controlled shock generator and scrambler. Only animals vocalizing more than 100 calls/10 min were selected for the actual experiment. Indwelling cannulas were implanted in the aorta artery and jugular vein for drug administration and blood sampling, respectively.

On the experiment days, the anxiolytic effect was assessed before and at four different intervals after drug administration. A wash-out period of 4 days was observed before the second treatment. Recordings were carried out using a condenser microphone system consisting of a measuring amplifier and a programmable dual filter. The ultrasound emissions were subjected to analysis and quantified as calls by the software program REGUS2.0®. From these data the number of calls (calls/10 min) was calculated and used as measure of drug effect.

The ultrasonic vocalizations were immediately suppressed upon administration of buspirone and gradually returned to baseline levels, as plasma concentration decreased. The sigmoidal-E_{max} model was used to describe the concentration-anxiolytic effect relationship. Interestingly, we could incorporate physiological fluctuation of the parameter in the model and account for changes observed in baseline values.

Our results show that the inhibition of fear-induced ultrasonic vocalizations constitutes a reliable, objective measure for the time course of the anxiolytic effect. Furthermore, this approach demonstrates the feasibility of using quantitative behavioural analysis in combination with integrated PK/PD modelling to characterize drug effect and potency.

CHARACTERIZATION OF SEIZURE PATTERNS IN KINDLING AND CORTICAL STIMULATION MODELS OF EPILEPSY

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Evaluation of the anticonvulsant effect of antiepileptic drugs is based on behavioural seizure models. We have applied an ethological technique to characterize electrically induced-seizures using an ethogram with encodings for actions and postures that are not in the natural repertoire of the species. This approach seems to allow the detection of drug effects or drug-drug interactions that would not be detectable otherwise. The aim of this study was to provide a detailed quantitative and qualitative behavioural description of the ictal and post-ictal components in two experimental models of epilepsy: kindling and cortical stimulation. Seizure activity was induced in two groups of 6 Wistar-derived rats according to each model, as previously described in the literature. Ictal and post-ictal behaviors were recorded on VHS tape and analysed according to an ethogram consisting of the following components: whisker movements, eye-closure, myoclonic jerk, facial gasping, forelimb clonus, forelimb tonus, hindlimb tonus, immobility and chewing. Intensity (total duration), incidence (frequency) and sequence patterns of the behavioural components during stimulation and seizure activity were scored and encoded from video record to floppy disk using the video time-frame encoder/decoder CAMERA®. It appears that both models are in many respects qualitatively similar. However, the models differ quantitatively. Cortical stimulation is characterized by shorter and less severe seizure activity than kindling. This comparison of the ictal and post-ictal behavioural components suggests that observational measures can be applied in experimental settings to assess differences in potency and mechanisms of action of antiepileptic drugs. Furthermore, an additional natural ethogram allows one to detect the impact of the methods on animal distress and welfare.

EFFECTS OF DOPAMINERGIC ANTAGONISTS ON MORPHINE WITHDRAWAL INDUCED-AGGRESSION IN MALE MICE

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The effects of the dopaminergic antagonists haloperidol (0.1 mg/kg), SCH 23390 (0.5 mg/kg) and raclopride has been examined in mice dependent on morphine (2.5 mg/kg) during 15 days. In the first experiment, the dopaminergic antagonists were administered after 48 hours without morphine. In the second, the drugs were administered 10 min after a naloxone injection and in the third the neuroleptics were administered 20 min before the naloxone. Dependent male mice were confronted in a neutral area with anosmic conspecifics and aggression was evaluated by estimation of times allocated to 11 behavioural categories, the tests were carried out 30 min after the neuroleptic administration. The results show that in all the situations the neuroleptics were antiaggressive, but SCH 23390 and haloperidol showed a stronger effect in the naloxone-induced withdrawal compared with the first experiment, impairing motor activity in the three experiments. Raclopride was similarly antiaggressive in all experiments without affecting motor behaviour.

SEROTONERGIC ALTERATIONS DURING SOCIAL ISOLATION OF MICE

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Previous studies have shown, that especially aggressive behavior of individually housed mice was affected in the dependence on isolation time and endogenous dispositions. The aim of this study was to determine pre- and postsynaptic serotonergic alterations in the time course (1 day-18 weeks) of social isolation and in dependence on the activity state (high and low active mice, HAM and LAM). Individual housing of mice was associated with a reduction of serotonin metabolism in dependence on isolation time and brain structure. Whereas a transient decrease in the striatum and the cortex was detected (3-6 weeks), in the hippocampus a reduction of serotonin metabolism was found after long-term isolation (18 weeks). Serotonin metabolism of HAM was more affected by social isolation compared to LAM. Isolation induced up-regulation of cortical 5-HT2A receptors was measured only in HAM. Densities of postsynaptic 5-HT1A receptors in the hippocampus were not different between grouped and isolated mice, but a transient down-regulation of 5-HT1A receptors in the raphe region were found in isolated mice after 3 and 6 weeks. These results are discussed in terms of interactions between serotonergic alterations and isolation induced aggression.

ANXIOLYTIC POTENTIAL OF 5-HT_{1A} RECEPTOR ANTAGONISTS

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Buspirone and related 5-HT_{1A} receptor partial agonists are believed to reduce anxiety by decreasing impulse flow in 5-HT projections to the forebrain. However, debate continues as to whether such effects are mediated via agonist activity at somatodendritic 5-HT_{1A} autoreceptors, antagonist activity at postsynaptic 5-HT_{1A} receptors, or both. In the present experimental series, we have used highly sensitive ethological techniques to examine the effects of a range of 5-HT_{1A} receptor ligands on the behaviour of male Swiss-Webster mice in the elevated plus-maze test of anxiety. Results show that whereas full agonists (e.g. R(+)8-OH-DPAT, LY293284) produce non-specific behavioral suppression and partial agonists (e.g. S(-)8-OH-DPAT, buspirone, LY315712) exert weak anxiolytic-like effects only, potent and anxioselective profiles are consistently seen with 5-HT_{1A} receptor antagonists (e.g. (S)-WAY 100135, WAY 100635, p-MPPI, (-) pindolol, pindobind-5-HT_{1A}, SDZ 216-525, & LY297996). Importantly, control studies rule out the potential involvement of α_1, $ß_1$ and $ß_2$ antagonism in these response profiles. Data will be discussed in relation to dose-response considerations, and inconsistencies in the literature on 5-HT receptor ligands in animal models of anxiety.

INTERACTION OF DIFFERENT DOPAMINERGIC ANTAGONISTS AND MORPHINE IN AGONISTIC BEHAVIOUR OF MALE MICE

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In a previous study we have shown that morphine counteracts the antiaggressive effects of haloperidol but increases its impairing effects on motor activity. To clarify the role of the different dopaminergic receptors we have studied the effects of morphine plus raclopride (D2 antagonists), or clozapine (D4 and D2 antagonists), or risperidone (D2, 5-HT2 and alfa 2 antagonists) on aggressive and motor behaviour, using the paradigm of isolation-induced aggression. Isolated male mice were confronted in a neutral area with anosmic conspecifics 30 min after the injection of both compounds, and aggression was evaluated by estimation of times allocated to 11 behavioural categories. The results show that the dopaminergic antagonists were antiaggressive and that this effect was not influenced by morphine. The mu agonists did not show antiaggressive effects at this dose. However, when added to clozapine (that by itself did not produce changes) an impairing effect on motor activity was observed. Morphine also increased the risperidone-induced impairment of motor activity.

IMAGE-PROCESSING METHODS FOR AUTOMATED CLASSIFICATION OF RAT BEHAVIOUR

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The use of automated systems to register behaviour in ethopharmacological and toxicological research has been spurred by the necessity to increase observational validity and reproducibility. Widespread use of automated systems has increased in last couple of years due to the diminishing costs of computing hardware and the availability of customised software. A large number of standard tests, like the open-field and Morris maze are currently conducted using image-processing hard- and software. These methods classify behaviour using information such as location, movement and changes in size of an object. The behaviours thus classified include approach, avoidance, fleeing, freezing and rearing. A larger number of behaviours can be automatically classified if the entire posture of a rat is considered. Therefore we attempted to apply methods developed in the fields of pattern-recognition and image-processing to behaviour classification in ethopharmacology.

Video images of 20 Wistar rats placed on a black plate were digitised at a rate of 5 images per second during a period of 10 minutes per rat. Two video-camera's were placed above and in front of the plate. A number of parameters describing the shape of a rat were calculated from these images. These include the centre of gravity, the position of the nose and tailbase, body-axis length and orientation and invariant high-order moments. The behaviours on the video tapes were classified by 4 experienced observers. Using feature selection techniques the best parameters to classify the behaviours were determined and used as input for a standard back-propagation neural network and a self organising map (Kohonen) network. The output of the back propagation network was compared to the classification of the observers.

EFFECTS OF DIAZEPAM, CITALOPRAM AND METHADONE ON PCP-INDUCED STEREOTYPED BEHAVIOUR AND SOCIAL ISOLATION IN THE RAT SOCIAL INTERACTION TEST

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Phencyclidine (PCP) can induce a model psychosis in humans that mimics the positive and negative symptoms of schizophrenia. In rats PCP induces stereotyped behaviour and social isolation, and recent findings have shown that antipsychotic drugs inhibit the stereotyped behaviours and have distinct effect on social isolation. However, only antipsychotic drugs have been tested in this model, and it is therefore necessary to extend these findings with studies of other classes of componds to evaluate the predictive validity of this model. In the present study the effects of the anxiolytic drug diazepam (0.005 to 5.0 mg/kg), the antidepressant citalopram (0.3 to 4.0 mg/kg) and the analgesic opioid agonist methadone (0.13 to 2.0) on PCP-induced behaviours were determined in the social interaction test. The drugs were administered daily for 3 days in combination with 2.0 mg/kg of PCP, and the experiments demonstrated that diazepam, citalopram, and methadone did not alleviate the behavioral effects of PCP. So far only antipsychotic drugs have been shown to alleviate the PCP-induced behaviours, suggesting that this animal model of schizophrenia may have predictive validity.

TESTOSTERONE-SEROTONIN INTERACTIONS IN AGGRESSION

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Our understanding of the neurochemical and neuroendocrine systems regulating the display of offensive intermale aggression has progressed substantially over the past twenty years. A compelling argument now can be made that serotonin, via its action at $5HT_{1A}$(1A) and/or $5HT_{1B}$(1B) receptor sites, modulates the display of intermale aggressive behavior and that its effects serve to decrease behavioral expression. At the same time, male-typical aggression is testosterone-dependent and studies of genetic effects, metabolic function, and steroid receptor binding have demonstrated that neuroendocrine regulation can occur via independent androgen-sensitive or estrogen-sensitive pathways. Our recent efforts have been directed toward characterizing the interaction between these systems, with a primary focus on hormonal modulation of serotonin function at $5HT_{1A}$ and $5HT_{1B}$ receptor sites. These investigation have shown that the androgenic and estrogenic metabolites of testosterone differentially modulate the ability of 1A and 1B agonists to decrease offensive aggression and that there are partially distinct neuroanatomical substrates mediating these effects. Western analyses of hormonal effects on $5HT_{1A}$ and $5HT_{1B}$ receptor populations in brain regions linked to the expression of aggression are in progress. The combined findings should contribute to the development of an integrative neurobiology of offensive intermale aggression.

IMPORTANCE OF GENDER FOR THE DISPLAY OF SOCIAL IMPAIRMENT IN PAIRBOND DISRUPTED GERBILS

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Gerbils (*Meriones unguicalatus*) form stable male/female pairbonds. Disruption of these has been shown to produce profound effects including alterations in patterns of sleep and impairment of subsequent social behaviour (Starkey & Hendrie (1996) J Psychopharm 10:120, Starkey et al., (1996) J Psychopharm 10:103). This manipulation has been proposed as a new model of clinical depression. In keeping with this, marked sex differences have been noted. In social interactions with other males, pairbond disruption has been shown to reduce initiation and reciprocation of social contact. Males also show decreased offense. These effects were not seen in interactions with females. By contrast, pairbond disrupted females show decreased reciprocation of social contact regardless of the sex of their social partner. Therefore, the effects of pairbond disruption are only apparent in males when interacting with other males, whilst the social behaviour of females is impaired in the presence of gerbils of either sex. As such, it may be concluded that the impact of pairbond disruption on social behaviour is global in females but situation specific in males.

THE ELEVATED T-MAZE CAN DETECT BOTH ANXIOLYTIC AND ANTIPANIC-LIKE EFFECTS OF CHRONIC IMIPRAMINE IN THE RAT

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The elevated T-maze consists of three elevated arms: one enclosed and two open. In this test, inhibitory avoidance of the open arms – representing learned fear – has been related to generalized anxiety and the unconditioned escape from one of the open arms to panic. In the present study, the effects of chronic imipramine (daily 5, 10 and 15 mg/kg; 21 days; ip) were measured in male Wistar rats previously exposed for 30 min to one of the open arms of the T-maze 24 h before the test. This pre-exposure shortens the first escape latency. Imipramine impaired the acquisition of inhibitory avoidance, representing an anxiolytic effect. In contrast, escape latency was prolonged by the drug, indicating an antipanic effect. These results correlate with the reported clinical anxiolytic and antipanic effects of chronic imipramine.

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EFFECTS OF LOBELINE ON SPATIAL LEARNING IN AGED MICE

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Spatial tasks performed by rodents are useful to evaluate interventions directed to improve age-related cognitive deficits. In the present study effects of lobeline on spatial memory were evaluated in 20 adult C57BL/6 male mice (6 months-old) and 14 aged mice (20 months-old). Mice received a daily SC injection of either lobeline (3.5 mg/kg) or saline vehicle for 10 days. Spatial learning was assessed during the last 5 days of drug treatment in a water maze (1 m diameter and 30 cm high) adapted for mice. Latency to escape to a submerged platform (6x6 cm) was measured by the experimenter. Results showed that aged animals learned the task more slowly than adult ones. No significant effect of drug treatment was observed either in adult or in aged mice. These results are discussed in relationship with the use of nicotinic agonists in the treatment of cognitive deficits associated with neurodegenerative disorders.

DIFFERENTIAL EFFECTS OF PREVIOUS EXPERIENCE IN A SPATIAL TASK IN NMRI AND C57BL MICE

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Effects of previous experience on the performance of spatial tasks has been demonstrated in rats. The aim of the present study was to compare effects of previous training in the water maze in two strains of mice (20 NMRI and 20 C57BL/6 male mice). Spatial learning of the animals was assessed in a water maze adapted for mice (1 m diameter and 30 cm high). The training took place at 2 months of age and consisted of 3 trials during 5 daily sessions. Four months later, mice were retested in the same task and their performance was compared with 'naive' animals of the same age. Latencies to escape to the platform were measured. The ANOVA showed significant differences in the factors Group ($p < .001$), Day ($p < .001$) and Trial ($p < .01$) and in the interaction Group × Day × Trial ($p < .05$). Post-hoc analyses showed that previous training in the water maze improved spatial learning in NMRI but not in the C57BL/6 mice. Implications of these results for future studies including pharmacological manipulations are discussed.

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First page. This should contain the title of the paper, the authors' names (first names with initials) and affiliations, **abstract (not exceeding 150 words),** a maximum of 6 keywords and a running title not exceeding 50 characters (including spaces) and the full address of the author to whom reprint requests should be sent.

Text pages. These should include Introduction, Materials and Methods, Results, Discussion (followed by Conclusions and Acknowledgements). **Illustrations** (tables, diagrams and half tones) should be submitted on separate sheets. Their explanations, however, should be inserted into the appropriate sections. **Use the same character size and line spacing for explanations as for the text!** Above the explanation the place for the illustration should be left empty.

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Shepherd, G. M. (1988) Neurobiology. 2nd edition. Oxford Univ. Press, New York and Oxford.

Balázs, R. and Richter, D. (1973) Effects of hormones on the biochemical maturation of the brain. In: Biochemistry of the Developing Brain (ed. Himwich, W.) Vol. 1, pp. 254-304. M. Dekker, New York.

These should be cited in the text by the names of the authors and the year of publication, e.g. [Balázs and Richter, 1973; Shepherd, 1988; Butt et al., 1990].

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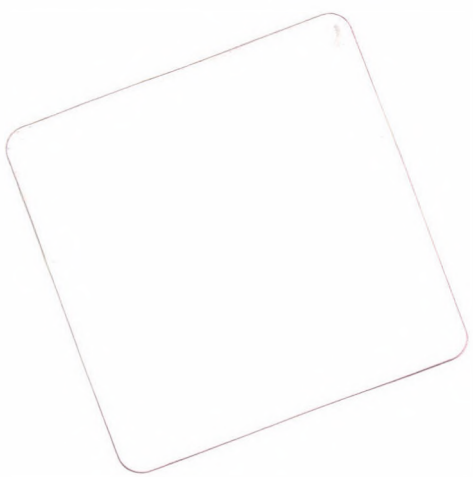

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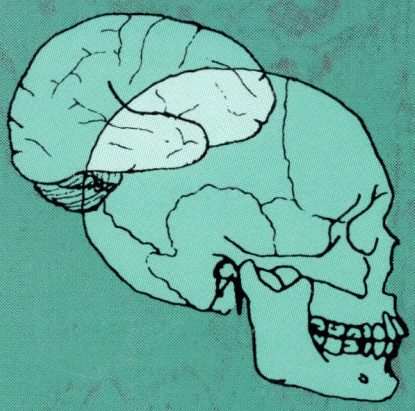

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Neurobiology

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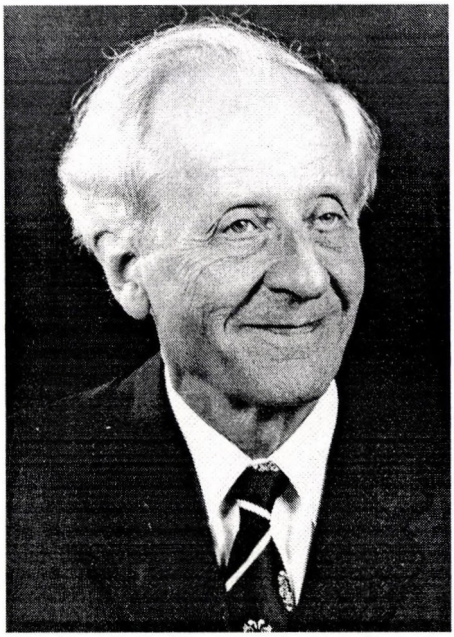
NEUROENDOCRINOLOGY: MY 'HOBBY'

BÉLA HALÁSZ



Being the son of a school master of a small village, very early in my life, I got in contact with various animals like horses, cows, pigs, dogs, cats, pigeons etc. and decided to become a veterinary. I kept this decision till the age of eighteen, when my brother, who studied geography and history at the university, got ill, and his sickness was gradually progressing. He suffered from tuberculosis at the time when the antituberculotic drugs were not yet discovered. It was an extremely hard time for me to realize that there was no real help for him. I became interested in medical sciences, changed my mind, and entered the Medical University in Pécs, which is a charming town in the southern part of Hungary. I was very lucky; at the time of my medical studies this University was one of the best ones, if not the best of the country in medical education. The anatomy course completed with histology

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and embryology, taking four semesters, was delivered by Professor János Szentágothai, who was an outstanding lecturer. His lectures and his great personality deeply impressed me from the very beginning, and eventually determined my career. Together with a very bright friend of mine I entered this department as a second-year medical student. At that time (1949) the whole staff of the department consisted of very few (4-5) doctors only, all of them very young, less than thirty, except the head of the department, who was "already" thirty seven. The teaching duties of the department were extremely heavy: gross anatomy, histology and embryology including neuroanatomy had to be taught. The students were divided into small groups, and for practical classes each group had to have its group-leader teacher of the department. Each group worked in a separate room within the section unit. Due to the shortage of teaching staff, students who passed the final examine of anatomy with an excellent mark and belonged to the so-called scientific student circle of the department became involved in teaching as group leaders of gross anatomy in the section room and in the histology laboratory. For a student, teaching was a highly stimulating and exciting job. I also enjoyed it a great deal but, at that time, I did not think of teaching anatomy during my whole life. The reason for this was the fact that with progressing in my medical studies I became more and more interested in internal medicine, so I decided to specialize in this field. I have to add that at the time of my medical academic years the medical faculties were separated both from the universities and the Ministry of Education, and became independent universities belonging to the Ministry of Health. Simultaneously, major reforms were introduced in medical education. Very often, the changes lasted merely for a very short time, one or two years, and were followed by further reforms. In general, one can say that reforms in medical education have not stopped ever since. In our country, like in several others, we live under permanent reforms and I am personally not convinced at all that reforms per se always significantly improve our education. Naturally, certain reforms are needed from time to time, but I do not think that the efficiency of our education primarily depends on the system. The effect of our teaching work is largely determined by us, how we are performing it, whether we are doing it professionally with devotion and enthusiasm, or just as a duty without any interest. A good teacher is usually able to develop a lively contact with his/her students, who realize within a short time what kind of a teacher they have, and their further behaviour largely depends on this. My excellent Master, Professor Szentágothai, from whom I learned a great deal about teaching, considered teaching should be above all our activities. He expressed it in the following way: "my dear friend, you should know teaching is holy, it is a sacred affair".

The reforms in medical education introduced during my studies were fairly fundamental. One year before graduation we could choose a special field of medicine, and spent the whole year at one clinic. I chose internal medicine, and worked 12 months at one of such departments. I enjoyed this work and was promised to get a position at the clinic after graduation. On the very last evening before submitting my application, I went to Professor Szentágothai's office to inform him about my plans. Our discussion ended up with changing my original plan. I decided to stay and go on working in the Anatomy Department. Since that time, in 1954, I have never regretted this decision.

My research activity started when entering the Anatomy Department as a second-year medical student. At that time one of the main topics studied in the Department was neuroendocrinology, but it was the very early age of the discipline, when its basic principles were not really established. I started to investigate the control of the adrenal cortex for the simple reason that the person who studied this organ earlier left our Department.

From the very beginning, I was interested in the structural and functional aspects, at first of the control of the adrenal cortex, and later on of the control of the hypothalamo-hypophysial-target endocrine gland system. At that time the methods available for us were, unfortunately, very simple, I should say rather indirect and timeconsuming. For several years one of the main indicators we used to detect functional changes was nuclear size. We determined the size of the cell nuclei of the zona fasciculata of the adrenal cortex, and later on the size of nuclei of neurons of different hypothalamic cell groups. Looking back from about four decades, it appears that the results we (i.e., the neuroendocrine group of the Department) obtained by these very simple approaches are consistent with the findings of more sophisticated techniques applied later. I am just referring to the nuclear size changes observed after removal of several various glands or following several other interventions. The aim of such investigations was to acquire knowledge about the hypothalamic localization of the control of endocrine glands. At the light of the information available at that time showing that electrical stimulation or lesion of certain hypothalamic region caused alteration in gonadal, thyroidal or adrenocortical function, we assumed that there would be well-defined regions within the hypothalamus in which the size of the nuclei of neurons would change after the interventions applied. It turned out, however, that this was not the case. We found nuclear size changes in several hypothalamic cell groups after an intervention. Of course, this is not surprising nowadays, already knowing the complexity of the hypothalamic structures involved in the control of the pituitary and the target endocrine glands.

From the second half of the nineteen-fifties on, several neuroendocrinologists focused their interest on the hypophysiotrophic substances postulated by Harris and Green. Some of them studied the effect of hypothalamic extracts on the anterior pituitary, while others, including myself, tried another approach, i.e., to implant anterior pituitary tissue into various regions of the hypothalamus to see which area was able to maintain the structure and function of the implanted pituitary. At this time Nikitovitch-Winer and Everett had already reported that retransplanting the anterior pituitary from the kidney under the median eminence restored the structure and function of the anterior pituitary. The purpose of the intrahypothalamic pituitary implantation was to try to find out which regions of the hypothalamus were capable of maintaining the pituitary structure and function. My intrahypothalamic pituitary implantation studies were preceeded by Béla Flerkó's intrahypothalamic ovanan implantations providing the first experimental evidence for the existence of sex-steroid sensitive neurons within the hypothalamus. As these investigations were performed in the same department they greatly stimulated me to implant pituitary tissue into the brain. When reporting at an international meeting, on our first observations with intrahypothalamic pituitary implants, I realized that other investigators (Flament-Durand, Knigge) had performed similar studies and obtained nearly the same results. I learned through this experience that certain problems and the ways how to approach them arise at a given time and therefore, almost identical experiments may be performed in different laboratories. Priority is not always priority in the true sense, but may also depend on seemingly unimportant factors. Since that time several publications have convinced me that very often this is the case indeed and explains the competition why researchers do their best to publish as fast as possible. Nevertheless, nobody should forget about the importance of fair play in this competition. My personal experiences in this regard are all very positive although some of my friends abroad have witnessed that this is not always the case. Too bad.

At the beginning of the nineteen-sixties the neuroendocrine research group of the Anatomy Department in Pécs accepted the proposition of Professor Szentágothai as to writing a monograph on the hypothalamic control of the anterior pituitary, which came out in 1962. At that time only one monograph existed, written by Geoffrey Harris and entitled "Neural Control of the Pituitary Gland" published in 1955. Our monograph significantly contributed to the recognition of our neuroendocrine research group, although the neuroendocrine studies made in the Department, particularly those performed by Béla Flerkó were already well known.

All those who have made experimental investigations trying to get an answer to a certain question know it well that even if the results are clear

and the answer unequivocal, the observations always raise further questions. This was also the case with the findings of our intrahypothalamic pituitary implantations. The question which came up was the following: What is the functional capacity of the hypothalamic region capable of maintaining pituitary structure and function per se, i.e., in the absence of neural input. Therefore, together with Dr. Lajos Pupp, we constructed a special small bayonete-shaped knife which could be fixed on the electrode holder of a stereotaxic instrument. The knife could be lowered into the brain through a hole drilled on the top of the skull, and through the interhemispheric fissure, to the base of the brain. By combining the turning of the knife with the movements of the electrode holder, we were able to cut around completely the medial basal hypothalamus. Thus, we could interrupt all neural connections of this hypothalamic region, but keeping the connections between the disconnected hypothalamic area and the pituitary gland intact. We were very fortunate that the area we wanted to deafferent was the medial basal hypothalamus and not another region, because the blood vessels supplying this hypothalamic region reach the area from below, and therefore, the knife cut did not seriously alter the blood supply of the isolated structures. The first results we got were quite unexpected. The disconnected medial basal hypothalamus was able to maintain, to some extent, the basal secretion of pituitary trophic hormones (there was no severe atrophy of the target endocrine glands), but the function of the anterior pituitary was far from normal, indicating that neural input to the deafferented area is essential.

In 1963, Professor Szentágothai moved to Budapest to take over chairmanship of the Anatomy Department at the Medical University in the capital of the country, and Béla Flerkó became Head of the Anatomy Department in Pécs. Thanks to Béla Flerkó, who had already had good personal contacts with leading neuroendocrine scientists of several countries including researchers in the Department of Anatomy and Brain Research Institute University of California, Los Angeles, and thanks to Professors Charles H. Sawyer's and Roger A. Gorski's support, I received a Ford Foundation Fellowship in 1964. At that time it was impossible to talce your family too, because the leaders of the communist regime were afraid you would not return. Thus, I left the country alone, with five Dollars in my pocket. (Due to the rigorous restrictions it was not possible to get more Dollars, to exchange more money.)

From scientific point of view, the twelve-month period spent in Los Angeles was a most stimulating and fruitful time for me. I was very fortunate going to Los Angeles shortly after our technique for hypothalamic deafferentation had been worked out. This gave me the opportunity to carry out a number of investigations in animals bearing various hypothalamic knife

cuts, and testing LH, FSH, ACTH, TSH and prolactin secretion. The real advantage for me was that I could do these studies with leading scientists who used the most advanced techniques given in the field. The one year spent in Los Angeles was a determining period of my life, that I will never forget. My hosts, Professors Charles H. Sawyer and Roger A. Gorski did their best to make my time most prosperous, fruitful, unforgettable and enjoyable. During my stay I had the chance to meet and listen to very distinguished, internationally well-known and acknowledged scientists. Several of my international connections with outstanding neuroendocrinologists date back to this time. Beside Americans, the researchers in my neighbourhood were from Japan, South America, Mexico, Italy and France, clearly indicating the international character. For me this was a very nice experience. I highly appreciated the extremely friendly and helpful atmosphere. Being fully aware that Christmas Eve is not an easy time for those spending this day abroad, alone, far from their family, Professor and Mrs. Sawyer invited me and some other foreigners working in Sawyer's laboratory to their house for this Eve. This was an unforgettable gesture and we were most grateful to them.

On my way back from the States I visited several outstanding neuroendocrinologists and their laboratories in Western Europe, among others Geoffrey Harris's one at the Maudsley Hospital, which I found somewhat different from what I had expected.

As already pointed out the one year spent at UCLA was a most important period of my life. After having returned, I was several times invited to international meetings, had the chance to become a member of international scientific organizations. In the nineteen-sixties the neuroendocrine group of the Pécs Anatomy Department was an internationally known and recognized research team, primarily due to the very significant scientific contributions of Professors János Szentagothai and Béla Flerkó. A great number of really distinguished neuroendocrinologists visited us during those years. Besides our neuroendocrine team in the Anatomy Department, there was a very strong neuroendocrine research group headed by Professors Kálmán Lissák and Elemér Endrőczi working in the Department of Physiology. The personal contacts between these two groups were very good.

Nineteen-seventy-one was a very important year in my life, I moved from Pécs to Budapest, having been appointed as Chairman of the 2nd Department of Anatomy, Histology and Embryology. It was very hard to make a decision to change the working place and the city to live at the same time. I was greatly satisfied with my situation in the Pécs Anatomy Department: there I had excellent possibilities of work provided by Béla Flerkó; the human atmosphere in the Department was most friendly, and last but not least Pécs

is a very charming town with lively cultural life. I was hesitating till the very last moment about withdrawing my application. When submiffing it I was quite sure I would not be selected for the chairmanship. As far as I remember six persons applied. My friends persuaded me to do so. That I did not withdraw my application was due to my friends' encouragement. More than twenty-five years have passed since then. I have to confess that I did not regret my movement from Pécs to Budapest. Moreover, I should say it was a real challenge for me to take over the chairmanship. From the very beginning I had full support of my associates. The equipment of the department was according to Hungarian standards of that time, fairly good for morphological investigations, but real neuroendocrine research work was not going on, the conditions for such studies had to be established. Scientific research requires primary personal interest, which means an intrinsic drive is indispensable. Therefore, I did not force anybody to join my research group. Those who had already worked in a field continued their studies. My neuroendocrine team was gradually built up by young doctors who graduated from the Medical University shortly before. Each member of the group started to work in a slightly different field, one dealing with the neural control of LH and FSH secretion, another with prolactin, others with growth hormone, the ontogenesis of the hypothalamo-anterior pituitary system, again others with hypothalamic morphology, etc. This, of course, did not mean that the members of the group did everything separately, a number of joint investigations were performed. The advantage of each member having some special field became evident later, when he or she developed a known researcher. It was clear whose contribution was what, and everybody could continue his/her work in more or less his/her own field. To work with them, I should say to serve them, was indeed a special pleasure and I found it to be very stimulating. Of course, as time passed, the knowledge about the special fields of my associates increased very rapidly to a level when I had to listen to them. I was always delighted when this happened.

During the years I worked in various international scientific organizations, attended several meetings abroad and could welcome a number of very distinguished scientists from many different countries to Hungary. It was always good to learn that the contributions of the Hungarian neuroendocrinologists were acknowledged, and the work was supported by several researchers and international organizations. I am most grateful for their support, assistance and friendship.

Looking back upon the history of neuroendocrinology, I think we can say without self-conceit that neuroendocrinology has made a very significant progress in the last three-four decades. It has become a well-known discipline, and fertilized several fields of neuroscience. Nowadays, it is very

difficult to define its borderlines. It has become evident that the neuroendocrine system is closely linked with other systems, first of all with the immune system. I am extremely pleased that I had the chance to witness this fantastic development.

I think, in general, it can be said that scientific researchers working in a field are participating in the construction of a never-ready building. Looking at neuroendocrinology, the foundations have already been laid down, the bottom pillars, the frame (at least part of it) as well as important walls of the building are completed. I feel it a special gift for me to see how this work has been going on at international level. I am very happy that this building is already visible and what is even more heartening me is that presently several young talented scientists are working on this building. Wish them great success.

Finally, I would like to express my heartful thanks and utmost gratitude to my distinguished friends and colleagues for their valuable contribution to this special issue of Neurobiology. I am also extremely grateful to the Editorial Board of the journal for this issue. I feel greatly honoured. Let me also take this opportunity to sincerely thank my successor for all he has done to make me feel home. I am also indebted to the Hungarian Academy of Sciences for its continuous aid and for providing also further support to our neuroendocrine research group.

Neurobiology; 5 (3) pp. 337-346 (1997)

Research report

OESTROGEN-RECEPTIVE NEURONS IN THE FOREBRAIN OF THE RAT; THEIR ROLE IN THE INDIRECT, NEURO-HORMONAL, NEGATIVE AND POSITIVE OESTROGEN FEEDBACK[1]

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Constant vaginal oestrus with polyfollicular ovaries after peoptic-anterior hypothalamic lesions

After bilateral electrolytic lesions placed in the preoptic-anterior hypothalamic area constant vaginal oestrus with polyfollicular ovaries was first described by Dey (1941). The guinea-pigs bearing this type of hypothalamic lesions had constantly open vaginae, very large uteri, several had cystic hyperplasia of the endometrial glands, and ovaries with a normal number of large follicles, but no corpora lutea. The glandular cystic hyperplasia produced by continuous oestrogen action on the endometrium is well recognized in human pathology. On the other hand, the uterine changes in these animals were similar to those produced by Nelson (1937) and Barks and Overholser (1938) by longtime administration of oestrogens. It was, consequently, an obvious inference drawn by Dey (1941) that continuous secretion rather than secretion of excessive amounts of oestrogens might have been the cause of changes observed in the uterus of the lesioned animals.

Dey's results with preoptic-anterior hypothalamic lesions have been entirely confirmed in rats by Hillarp (1949), Greer (1953), Alloiteau (1954), Flerkó (1954) and others. Also in rabbits, the bilateral preoptic-anterior hypothalamic lesions resulted in a histological alteration of the endometrium of the uterine horns that resembled the glandular cystic hyperplasia of the human endometrium. In cross sections of the uterine horn of the intact rabbit (Fig. 1a), the glands of the endometrium were hardly visible, but in the lesioned animal (Fig. 1b) large, enormously dilated, cystic endometrial glands filled with secretory material were present (Flerkó, 1953).

[1] *Dedicated to Professor Béla Halász for his 70th birthday.*

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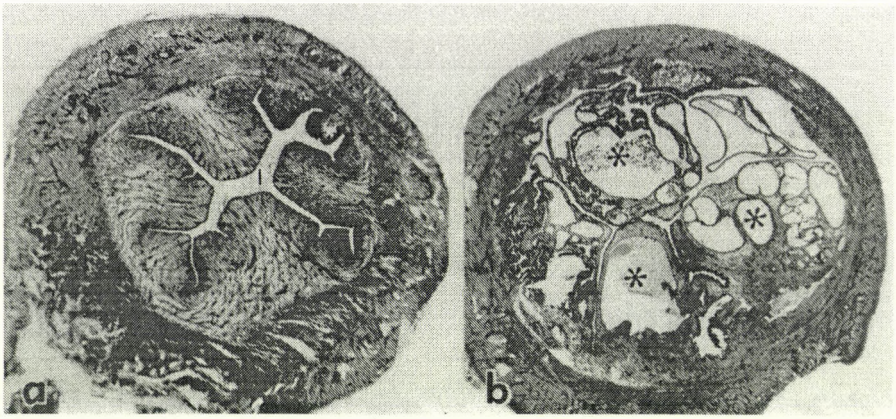

Fig. 1. Conventional histological preparations of the uterine horn obtained from intact (a) and bilaterally preoptic area-anterior hypothalamus lesioned (b) rabbits. Note the glandular cystic hyperplasia (*) in the latter group, in comparison with the endometrial glands (arrows) surrounding the lumen of the (I) in the intact rabbit. a ×30, b ×30

Oestrogen-sensitive neurons in the preoptic-anterior hypothalamic area and their role in the negative oestrogen feedback

A series of experiments was undertaken to establish why the continuous oestrogen action, which elicited the histological alteration of the uterus of the lesioned animal, failed to inhibit the secretion of gonadotrophic hormones and, as a consequence, that of oestrogenes themselves in the lesioned animals (Flerkó, 1957a; Flerkó and Illei, 1957; Flerkó and Bárdos, 1960). The results led us to the conclusion that oestrogen-sensitive neurons exist in the preoptic-anterior hypothalamic area of the brain and, at the least partly, this system responds to increasing levels of oestrogens in the blood by inhibiting gonadotrophic hormone secretion. The discovery that bilateral preoptic-anterior hypothalamic lesions in rats prevented the gonadotrophic hormone secretion-inhibiting effect of daily treatment with 1 mg oestradiol (Flerkó, 1957b) provided further evidence for this hypothesis. Flerkó and Szentágothai (1957) implanted small fragments of ovarian tissue into the preoptic-anterior hypothalamic area (Fig. 2a). Control rats carried either autotransplants of liver tissue in the same area or ovarian grafts in the posterior hypothalamus (Fig. 2c) or the anterior lobe of the pituitary. The

histological evaluation of the grafts revealed growing follicles and well-preserved interstitial glands (Fig. 2b, d) presumably secreting small amounts of oestrogens. The results of these experiments indicated, that minute amounts of oestrogen secreted by the small ovarian grafts in the preoptic-anterior hypothalamic area inhibited gonadotrophic hormone secretion. The lack of influence of ovarian grafts on gonadotrophic hormone secretion in animals bearing the implants in the posterior hypothalamus indicated that the effect of oestrogen was locus dependent. Interestingly, the released small amounts of oestrogens from ovarian grafts implanted into the anterior pituitary were not sufficient to inhibit gonadotrophic hormone secretion via a direct action upon the pituitary cells. These results furnished the first indirect experimental evidence for the existence of hormone-sensitive nerve cells in the brain through which the so-called indirect, or neuro-hormonal, long-loop feedback controls the secretion of anterior pituitary hormones. During the past four decades, several lines of experimental data have emerged to support the view that there are oestrogen-receptive neurons in the brain, through which negative as well as positive feedback effects of oestrogens are mediated on the pituitary gonadotrophin secretion.

The role of oestrogen-sensitive neurons in the positive oestrogen feedback

Merkel and Nelson (1940) assumed that the preovulatory high output of oestrogens produces, by direct action on the pituitary, the ovulatory luteinizing hormone (LH) surge, i.e., the very quick release of a large amount of LH that elicits ovulation and luteinization. Sawyer, Everett and Markee (1949) were the first to demonstrate the presence of a neural factor in the mechanism by which oestrogen induced the ovulatory LH surge in the rat. The structure of the ovaries of the so-called androgen-sterilized rats, i.e., the rats which received a single injection of testosterone in the first ten days of life, is completely similar to those of rats having bilateral preoptic-anterior hypothalamic lesions and also these animals become acyclic, anovulatory and sterile after treatment (Barraclough and Gorski, 1961). We assumed that the cause of the absence of ovulation in the hypothalamus-lesioned rats might also be the destruction of oestrogen-sensitve neurons in the brain, which neurons are indispensable for the ovulation-inducing positive, or stimulating, oestrogen feedback on the anterior pituitary gonadotrophin-producing cells. Hence, we hypothesized that in the pathomechanism of androgen-sterilization

might be an important factor the decreased oestrogen-sensitivity of hypothalamic oestrogen receptive neurons.

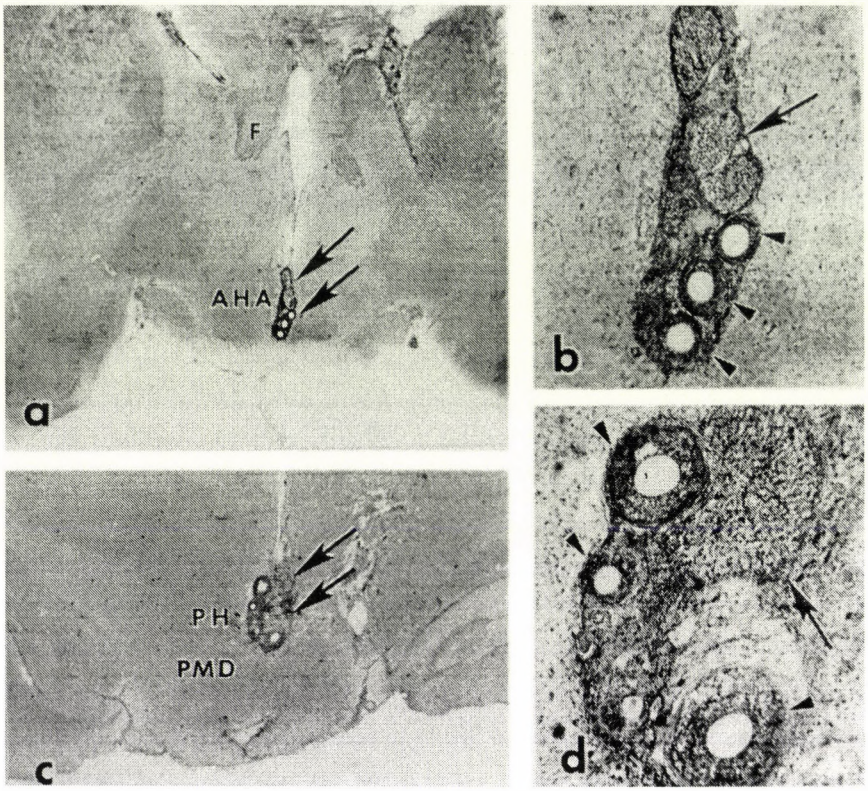

Fig. 2. Grafts of ovarian tissue (arrows) implanted into the preoptic-anterior hypothalamic area (AHA) (a) and posterior hypothalamus (PH) (c) at the level of the dorsal premammillary nucleus (PMD), respectively. The high power photographs of the implants (b, d) demonstrate growing follicles (arrowheads) and interstitial glands (arrows). F: fornix. a ×10, b ×50, c ×10, d ×50

Harris and Levine (1965) and van Rees and Gans (1966) were the first to report that sensitivity of the uterus to oestrogen was reduced in the androgen-sterilized rat. Petrusz and Flerkó (1968) observed that also the reactivity of pituitaries to oestrogen is diminished in rats injected 1.25 mg testosterone at the age of 2 days. These results suggest that responsiveness of the so-called oestrogen-reactive tissues to oestradiol is diminished by the early postnatal

androgen action. If so, it could be anticipated that not only the non-neural but also the neural oestrogen-sensitive elements could be similarly affected by the early postnatal androgen action. Experimental findings of Petrusz and Flerkó (1965) appeared to support this assumption.

The neuro-hormonal negative and positive feedback mechanisms are indispensable for the maintenance of the cyclic release of gonadotrophic hormones. It is close at hand to assume, therefore, that the loss of these feedback mechanisms by the early postnatal androgen action may account for the establishment of the non-cyclic pattern of gonadotrophin secretion in the male. In this train of thoughts, androgen-induced desensitization of the sex steroid-sensitive neural structures might play a role in the development in male direction of the hypothalamus.

A similar desensitizing effect of androgen on the sex steroid-sensitive hypothalamic structures was demonstrated also in the female rat. Compensatory ovarian hypertrophy in hemispayed rats is significantly inhibited by 0.1 mg oestradiol/day administered for a period of one month. The inhibitory effect of the same dose of oestradiol was less marked and not significant in the hemispayed rats that have received a single injection of 1.0 mg testosterone phenyl propionate one day after birth. The results suggest a desensitizing action of early postnatal androgen action on the sex-steroid sensitive hypothalamic neurons instrumental in the negative feedback action of sexual hormones (Petrusz and Nagy, 1967).

In an experiment of Flerkó and Mess (1968), the oestradiol-binding capacity of the uterus and pituitary of androgen-sterilized rats was less than that of the intact controls. Oestradiol-binding capacity not only of the anterior pituitary and uterus but also the anterior and middle hypothalamus, measured with tritiated oestradiol, was found to be significantly reduced by a single dose of 1.25 mg testosterone phenyl propionate administered two days after birth when compared to controls of approximately equal weight without early postnatal androgen treatment (Flerkó et al., 1971). This suggests that early postnatal androgen action presumably blocks the oestrogen receptor sites assumed by Jensen and Jacobsohn (1962) and interferes with the oestradiol uptake and/or retention of the neural and non-neural oestrogen-sensitive structures. Kato and Villee already in 1967 demonstrated a characteristic pattern of uptake and retention of tritiated oestradiol by the anterior hypothalamus, similar to that shown by the uterus and vagina (Jensen and Jacobsohn, 1962). It is close at hand to assume, therefore, that early postnatal androgen action destroys the specific trapping mechanism in the sex steroid-sensitive hypothalamic neurones, which retain the oestrogens in an

unconverted form and, in this way, serve in initiating neuro-hormonal feedbacks controlling the cyclic output of gonadotrophic hormones.

It was in 1962, that Halász et al. described the so-called hypophysiotrophic area (HTA) of the hypothalamus and demonstrated by various experiments (Halász, 1972) that the HTA is capable of maintaining the basic or tonic secretion of the various troph hormones of the anterior pituitary. If the capacity of the HTA for independent function is considered on one hand, and the insufficiency of the area for preserving normal trophic hormone secretion of the adenohypophysis on the other, it may be concluded, as proposed by Barraclough and Gorski (1961) and by Flerkó (1962) with respect to gonadotrophins in the female rat, that two levels exist in the hypothalamic control of the anterior pituitary in general (Halász, 1972).

One level (the so-called lower level) would be represented by the hypophysiotrophic area. It might be assumed that this area acts directly on the anterior pituitary by its hypophysiotrophic and/or releasing factors (Halász, 1972). The intention to learn the localization of the neurons that produce and transport the releasing and inhibiting factors to the capillary loops penetrating the median eminence and the neural stalk, induced the immunocytochemical investigations by Sétáló et al. (1975) for the localization of the luteinizing hormone-releasing hormone- (LH-RH-) neurons which experimental work was the overture of a long series of investigations concerning hypothalamic releasing and inhibiting hormones in the Pécs Anatomy Department in collaboration with the laboratory of A. V. Schally of the Tulane University, New Orleans, Louisiana.

The nervous structures outside the hypophysiotrophic area can be considered the second or higher level. If it is taken into account that the nerve endings around the capillary loops penetrating the median eminence belong to nerve cells of the hypophysiotrophic area, it seems very probable that nervous elements outside the area do not directly affect the anterior pituitary, but act through the HTA neurons by regulating the synthesis and release of the inhibiting and releasing hormones and factors (Halász, 1972).

With respect to the gonadotrophic hormones, the lower level of this control mechanism involves the tonic discharge of follicle stimulating hormone and LH in sufficient quantity to maintain follicle development and oestrogen secretion, but this level is not able to initiate the ovulatory and compensatory surge of the above hormones. Barraclough and Gorski (1961) localized this tonic mechanism in the ventromedial-arcuate nuclear region of the hypothalamus. According to our results, summarized by Halász (1972) and Flerkó (1972), this mechanism is located in the HTA which includes also the

arcuate nuclei and parts of the ventromedial nuclei. Of fundamental importance in maintenance of the female type, i.e., cyclic, release of gonadotrophic hormones and ovulation is, therefore, the higher level, the so-called cycle mechanism, encompassing all the brain structures that can modify the activity of the LH-RH- (and perhaps also the doubtful separate FSH-releasing factor-) producing neurons. Neurons belonging to the cycle mechanism appear to be concentrated in the preoptic-anterior hypothalamic, septal, amygdalar, olfactory and parolfactory areas as well as in the limbic system and midbrain reticular formation and rhinencephalic structures (Flerkó, 1972).

Localization of oestradoil-concentrating and oestrogen receptor-containing neurons in the brain by means of steroid autoradiography and immunocytochemistry

The first anatomical evidence for oestrogen-concentrating neurons within the brain was provided in the mid-sixties. This was achieved by autoradiographic detection of in vivo administered ^{3}H-labelled oestrogen after its retention from the circulation and gradual accumulation by neurons (Michael, 1965; Pfaff, 1968; Stumpf, 1968). The first studies focused on the endocrine hypothalamus where the medial preoptic area and the arcuate-ventromedial complex appeared to show the highest level of accumulation. Later, the entire central nervous system was mapped for oestradiol-concentrating neurons (Pfaff and Keiner, 1973; Stumpf et al., 1975). In addition to the hypothalamus, prominent uptake occurred in structures of the limbic system, the bed nucleus of the stria terminalis, the hippocampus and the medial and cortical amygdaloid nuclei. From the endocrine point of view, the oestradiol-concentrating cells in the preoptic-anterior hypothalamic area and the ventromedial-arcuate nuclei are particularly interesting since these regions take part in the regulation of gonadotrophin secretion by indirect feedback mechanisms (Flerkó 1962, 1972).

Cintra et al. (1986) and Liposits et al. (1990) reported that the most intense oestrogen receptor-immunoreactivity was found in the medial and periventricular preoptic areas, anterior parvocellular unit of the paraventicular nuclei and preoptic and lateral compartments of the bed nucleus of the stria terminalis. These results furnished the first direct evidence for the presence of oestrogen-sensitive - now we can say oestrogen-concentrating or oestrogen

receptor-containing - neurons in the hypothalamus as it was assumed and supported by indirect evidence (Flerkó, 1962) four decades ago.

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Research report

FINE-TUNING CONTROL OF TESTICULAR FUNCTIONS

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Summary: The effect of testicular administration of peptides synthesized in the testis (somatostatin, oxytocin) or peptide antagonists (opioid receptor antagonists naloxone, nalmefene, anti-corticotrop hormone-releasing hormone antiserum, ß-endorphin antiserum), partial denervation of the testis, and combination of local treatment with peptides/peptide antagonists and denervation was studied on testicular steroidogenesis in immature hemicastrated rats. The observations indicate that ß-endorphin, corticotrop horrnone-releasing hormone, oxytocin, and somatostatin exert a stimulatory, whereas enkephalin has an inhibitory action on steroidogenesis. Surgical (vasectomy) or pharmacological (local injection of 6-hydroxydoparnine) denervation suppresses testosterone secretion. Following partial denervation of the testis the effect of naloxone or oxytocin on steroidogenesis observed in fully innervated gonad is not present or the effect is paradoxical. These results indicate that steroidogenesis is fine-tuned by local peptide actions, and neural inputs. Data further suggest an interaction between local peptide action and neural control.

Key words: testis, peptides, innervation, steroidogenesis, local control

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This paper is dedicated to Dr.Béla Halász on the occasion of his 70th birthday

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INTRODUCTION

The major regulatory component of testicular functions is the hypothalamohypophysial system. In the last two decades data have been accumulated on the finetune control of the peripheral endocrine glands in general, and the testis, in particular. The minor regulatory components include: 1) local regulatory actions exerted by locally produced substances, 2) regulatory actions exerted by nerves innervating the organ, and 3) interaction between the above-mentioned two regulatory factors, i.e., modulation of local peptide effects by neural elements.

Several biologically active substances are synthesized in the testis, such as gonadal hormones/peptides (testosterone, inhibin, androgen-binding protein /ABP/, anti-Müllerian factor, etc.), 'hypophysiotrophic' hormones (gonadotrop hormonereleasing hormone /GnRH/, corticotrop hormone-releasing hormone /CRF/), growth hormone-releasing hormone /GHRH/, somatostatin), opioid peptides (the proopiomelanocortin-derived opioid peptide ß-endorphin, enkephalin, dynorphin), neurohypophysial hormones (vasopressin, oxytocin), 'neuropeptides' (vasoactive intestinal polypeptide, calcitonin gene-related peptide, substance P, etc.), and growth factors (Saez, 1994; Skinner, 1991).

Our *in vivo* studies summarized in the paper, provide data on the physiological role of testicular opioid peptides, CRF, somatostatin and oxytocin in the local control of testicular somatic cells.

Besides local regulatory actions of testicular peptides, testicular functions are also modulated by nerves to and from the testis. The testis obtains dual innervation by nerves from branches of the autonomic nervous system and, in addition, possesses a sensory afferent component. Fibers converge to the testis along two major pathways, the superior and inferior spermatic nerves. The superior spermatic nerve, the major contributor of testicular innervation runs alongside the testicular artery, whereas the inferior spermatic nerve accompanies the vas deferens. Testicular nerves contain monoaminergic and acetylcholinesterase-containing fibers as well as nerve fibers displaying immunoreactivity for different neuropeptides (Setchell et al., 1994).

The aim of these studies was to reveal the role of testicular sympathetic neural elements and the role of the inferior testicular nerve in the control of testicular functions. For this reason pharmacological sympathectomy of the testis (intratesticular injection of the neurotoxin 6-hydroxydopamin /6-OHDA/) and vasectomy (that also includes the transection of the inferior spermatic nerve) were performed.

The third issue of local control concerned the interaction between local peptide effects and testicular innervation. In these studies partial denervation

of the testis was followed by local administration of those substances whose synthesis and local regulatory action had already been demonstrated.

MATERIAL AND METHODS

Male CFY/Sprague-Dawley or Wistar rats were used in the experiments. The majority of studies reported here were performed in immature hemicastrated rats. In immature animals the local regulatory actions are also entirely exerted by somatic cells compared to adults where spermatogenic cells could also have some regulatory action. The hemicastrate model was chosen, since changes induced by local administration of peptides or antagonists were in several cases in hemicastrates more pronounced than those in animals with two testes in situ.

Local control

Different doses of peptides known to be produced and present in the testis (enkephalin, somatostatin, oxytocin), opioid receptor antagonists (naloxone, nalmefene), or antisera generated against locally produced peptides (anti-ß-endorphin antiserum, anti-CRF antiserum) were administered intratesticularly. Control animals were injected with physiological saline (in the case of peptide or receptor antagonist treatment) and with normal rabbit serum (in the case of antiserum administration). In additional groups the peptides, antagonists, antisera were injected systemically to exclude the action of these substances through the general circulation (classical endocrine action).

Neural control

In the denervation studies the following interventions were performed: 1) pharmacological sympathectomy: intratesticular administration of the neurotoxin 6OHDA, 2) intratesticular treatment with 5,6-dihydroxytryptamine (5,6-DHT) in order to destroy serotoninergic neural elements, and 3) vasectomy: removal a minimum of 2 min of piece of the ductus deferens together with the inferior spermatic nerve.

Interaction between local control and innervation

In these experiments partial denervation of the testis (local treatment with 6OHDA, 5,6-DHT or vasectomy) was performed prior to local administration of naloxone, enkephalin analogue or oxytocin.

The rate of testicular growth or compensatory testicular hypertrophy was recorded, and in some cases serum and epididymal ABP concentration was

measured by RIA (Gunsalus et al., 1978) to investigate the effects of interventions on Sertoli cell functions. Serum testosterone concentration and testosterone secretion *in vitro* of the testis were determined by RIA (Csernus, 1982), and used as an index of Leydig cell function. Testosterone secretion is known to show alterations during ontogenesis. The differences in steroidogenesis of control animals in different experiments are due to the different age of animals used.

RESULTS

Local control

Opioid peptides

Effects of opioid receptor antagonists

Our early studies indicated that in neonatal rats local injection (1 mg/testis) of naloxone stimulates testicular growth and enhances the extent of compensatory testicular hypertrophy (Gerendai et al., 1983) 5 days posttreatment. Further studies using another opiate receptor antagonist, nalmefene have also indicated that in neonatal (Gerendai et al., 1986), but not in adult (Gerendai et al., 1984) rats intratesticularly administered nalmefene facilitates compensatory testicular growth in a dose-dependent manner, and increases the serum concentration of ABP, a Sertoli cell product secreted into the vascular compartment before puberty (Gunsalus et al., 1980).

Both in neonatal (Fig. 1a) and adult rats intratesticular administration of naloxone and nalmefene significantly reduced basal testosterone secretion *in vitro.*

Furthermore, in hemicastrated opioid antagonist-treated adults, the response of incubated testes to human chorionic gonadotropin (hCG) was lower than that in testes from intact or hemicastrated controls.

Effect of anti-ß-endorphin antiserum

Local administration of anti-ß-endorphin antiserum to neonatal hemicastrated rats resulted in an effect on steroidogenesis similar to that obtained following treatment with the opiate receptor antagonist, i.e., the antiserum suppressed basal testosterone secretion *in vitro* (Fig. 1b) (Gerendai, 1991).

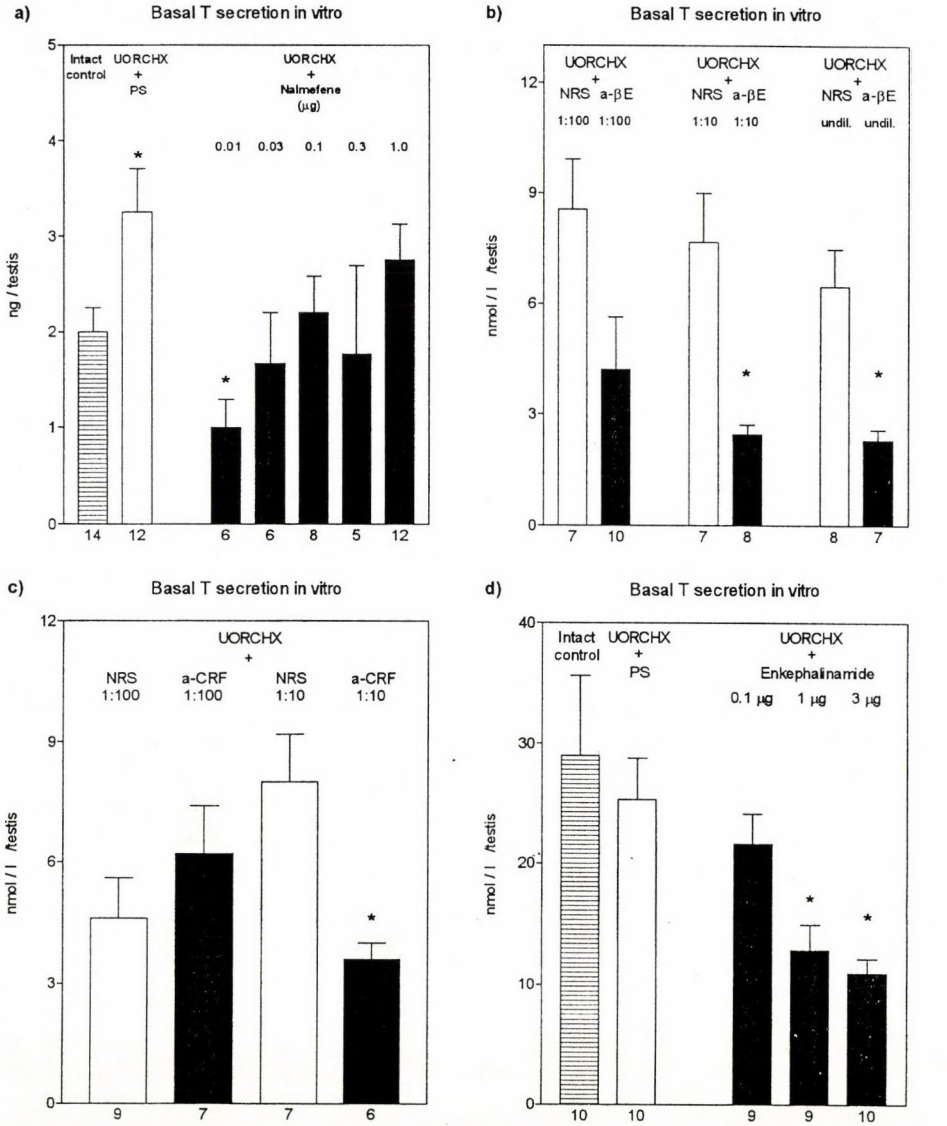

Fig. 1. The effect of nalmefene (a), anti-beta-endorphin antiserum (a-ßE) (b), anti-CRF antiserum (c) and enkephalinamide (d) on basal testosterone (T) secretion *in vitro*. Five-day-old rats were injected intratesticularly (on the right side), and immediately after the left testis was removed. Animals were sacrificed 5 days (a, b, and c) or 2 h (d) posttreatment. UORCHX: unilateral orchidectomy; PS: physiological saline; NRS: normal rabbit serum. The number below the bar designates the number of animals. The asterisks indicate significant difference from the hemicastrated vehicle-treated group

Effect of enkephalinamide
In neonatal hemicastrated rats testicular administration of a superactive enkephalin analogue (D-Met2, Pro5)-enkephalinamide (Bajusz et al., 1977) suppressed steroid secretion in a dose-dependent manner (Fig.1d). Time-course studies have indicated that the effect is a short-term one (maximal action 120 min posttreatment). Local injection of naloxone prior to the treatment with1the opioid analogue prevented the opioid-induced decrease in steroidogenesis.

'Hypophysiotrophic' peptides

Effect of anti-CRF antiserum
Two microliters of anti-CRF antiserurn (dilution 1:10 or 1:100) were injected locally to immature rats with two testes or to hemicastrates. In hemicastrates the treatment resulted in a significant decrease in serum testosterone concentration and basal testosterone secretion *in vitro* (Fig. 1 c). Unilateral testicular injection of anti-CRF in rats with two testes induced a significant drop in serum testosterone level with no change in basal testosterone production.

Effect of somatostatin
Local injection of somatostatin in adult rats with two testes *in situ* decreased serum testosterone concentration and basal testosterone secretion *in vitro*. Similar treatment in immatures had no effect on the parameters. In immature hemicastrates, however, administration of the peptide induced a significant rise both in basal testosterone secretion *in vitro* and serum testosterone level (Fig. 2c, d). In adult hemicastrates the peptide did not influence testicular functions (Gerendai et al., 1996).

'Neurohypophysial ' peptide

Effect of oxytocin
Immature rats were injected intratesticularly with oxytocin on one side, then the contralateral gonad was removed. In each age group studied local treatment with oxytocin resulted in a significant increase in steroidogenesis and/or serum testosterone concentration (Fig. 2a, b) (Gerendai and Csernus, 1995).

Neural control

Effect of 6-OHDA
In neonatal rats with two testes unilateral administration of the neurotoxin in large dose (333 μg/testis) induced severe atrophy of the treated gonad, and resulted in a significant weight gain of the contralateral gland, whereas in hemicastrates the extent of compensatory hypertrophy of the treated testis was significantly reduced. If the dose of 6-OHDA was remarkably smaller (33 ,ug/testis), in hemicastrates an enhanced compensatory testicular hypertrophy occurred (Gerendai et al., 1984).

Effect of vasectomy
In neonatal animals S days after unilateral vasectomy ipsilateral to surgery, testicular weight increased, and basal testosterone secretion *in vitro* decreased. Ten days postvasectomy the changes were opposite, and affected both testes (Gerendai et al., 1986).

Interaction between local peptide action and innervation

Effect of naloxone following pharmacological sympathectomy
Neonatal rats were injected intratesticularly with 6-OHDA, and immediately after, with naloxone. As described previously, either naloxone or 6-OHDA alone reduced basal testosterone secretion *in vitro.* However, the testes underwent combined treatment exhibited testosterone production similar to that observed in controls (Fig. 3a) (Gerendai et al., 1989).

Effect of naloxone or enkephalinamide following vasectomy
Both vasectomy and naloxone administration induced suppressed steroidogenesis. If the two interventions were combined, basal testosterone secretion *in vitro* did not show further decrease, but resulted in steroid production which significantly exceeded that observed in intact gonads. When vasectomy was performed just prior to enkephalinamide treatment, the suppressive effect of the peptide was not present (Fig. 3b) (Gerendai et al., 1992).

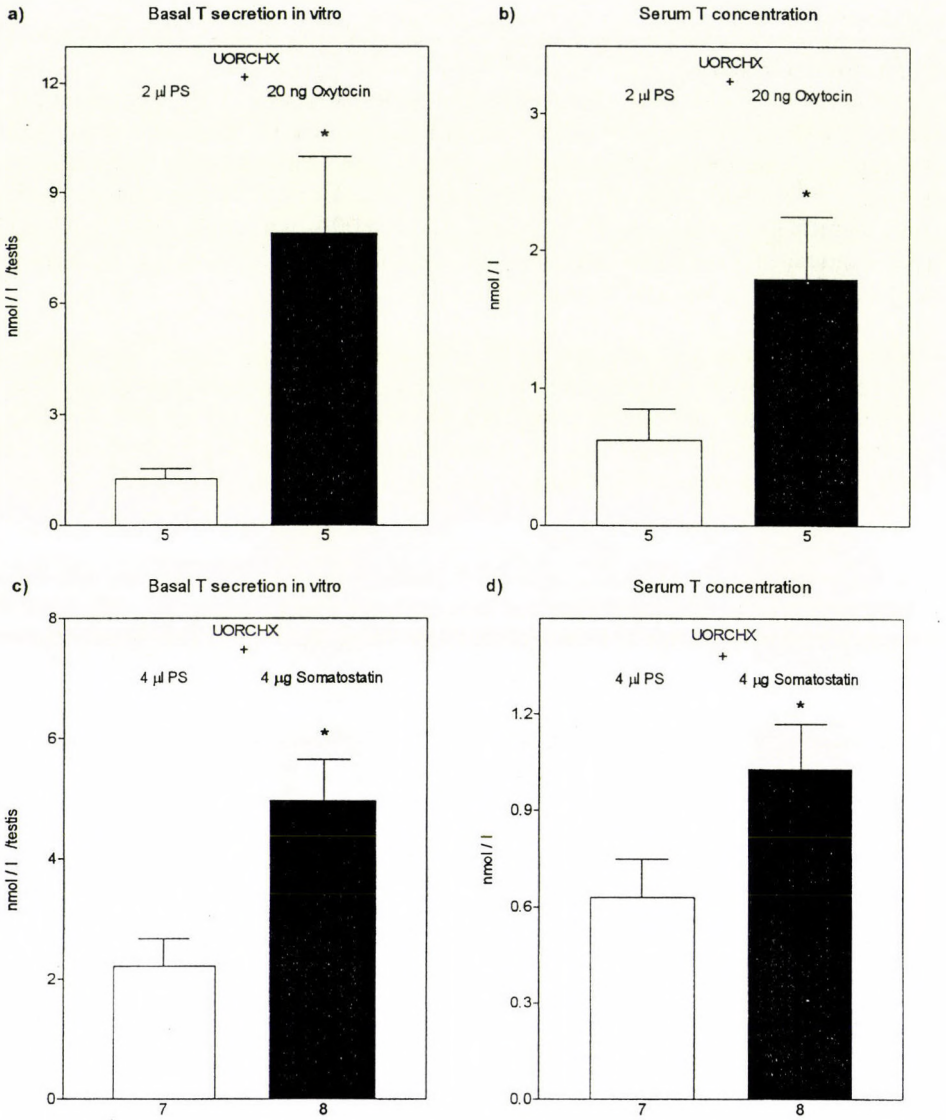

Fig. 2.The effect of oxytocin (a, b) and somatostatin (c, d) on basal testosterone (T) secretion *in vitro* and on serum T concentration. a, b: five-day-old rats were injected intratesticularly (on the right side) with oxytocin and immediately after the left testis was removed. Animals were sacrificed 7 days posttreatment; c, d: somatostatin treatment and hemicastration was performed in 18-day-old rats, and the animals were sacrificed 1 day later. UORCHX: unilateral orchidectomy; PS: physiological saline. The number below the bar designates the number of animals. The asterisks indicate significant difference from the hemicastrated PS-treated group

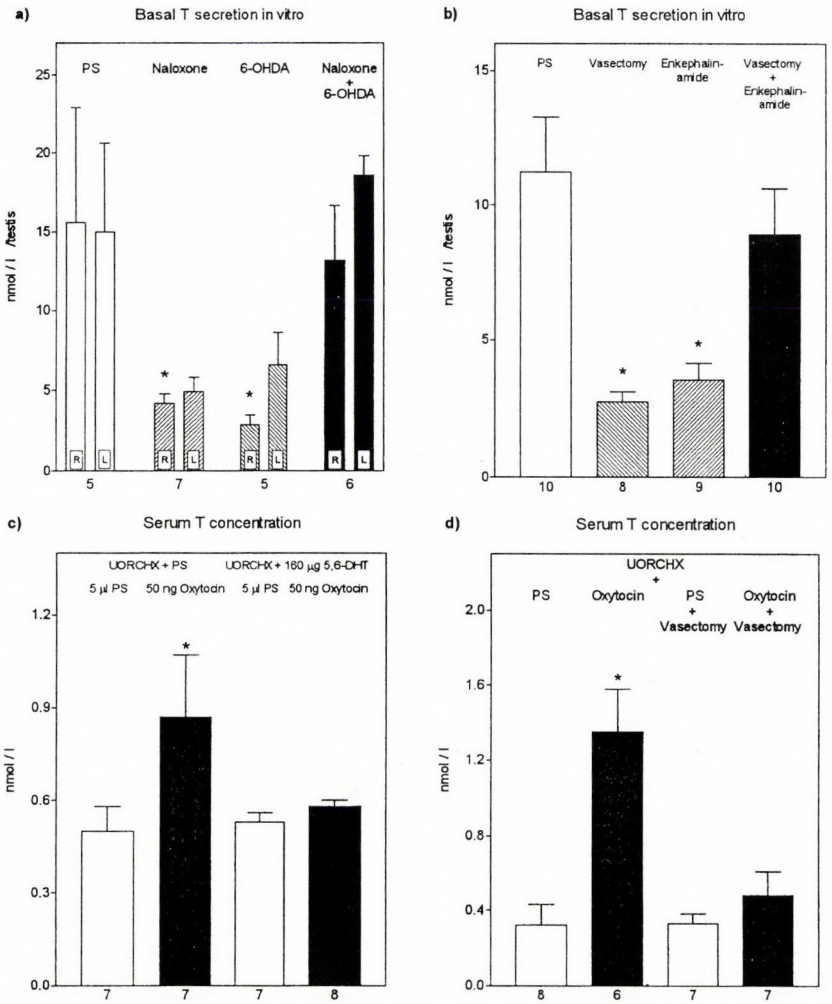

Fig. 3. Effect of intratesticular injection of naloxone, enkephalinamide or oxytocin combined with pharmacological or surgical denervation of the testis on basal testosterone (T) secretion or on serum T concentration. a: Effect of naloxone treatment of the right testis and/or 330 µg of 6-hydroxydopamine (6-OHDA) in 5-day-old rats (survival time: 5 days). b: Enkephalinamide treatment of the right testis plus contralateral hemicastration and/or vasectomy in 5-day-old rats (survival time: 2 h). c: Effect of unilateral intratesticular injection of oxytocin and/or 5,6-dihydroxytryptamine (5,6-DHT) (9-day-old rats were treated first with 5,6-DHT, 5 days later with oxytocin, animals were sacrificed 1 day after the second treatment). Effect of oxytocin treatment and/or vasectomy in hemicastrated rats (treatment, vasectomy and hemicastration were performed in 9-day-old rats, which were sacrificed 1 day later). R: right testis; L: left testis; UORCHX: unilateral orchidectomy; PS: physiological saline. The number below the bar designates the number of animals. The asterisks indicate significant difference from the PS-treated group

Effect of oxytocin treatment following 5,6-DHT administration or vasectomy

Both 5,6-DHT treatment and vasectomy prevented the oxytocin-induced rise in serum testosterone concentration (Fig. 3c, d) (Gerendai et al., 1996).

DISCUSSION

The results summarized above (Table 1) indicate that several biologically active substances, mainly peptide hormones, besides their production and action in other bodily systems, are synthesized in the testis, and exert a local regulatory action also in this organ.

Table 1. Site of production, receptor localization, effect, and presumable mechanism of action of different testicular peptides on steroidogenesis in immature hemicastrated rats

Peptide	*Site of peptide synthesis*	*Loacalization of receptor*	*Effect*	*Presumable mechanism*
Oxytocin	Leydig cell	Leydig cell	Stimulation	Autocrine
CRF	Leydig cell	Leydig cell	Stimulation	Autocrine
ß-endorfin	Leydig cell	Sertoli cell	Stimulation	Paracrine/ introcrine
Enkephalin	Spermatogenic cell	Sertoli cell	Inhibition	Paracrine
Somatostatin	Not known	Spermatogenic cell	Stimulation	?

Perhaps the prototype for those peptides first identified in another tissue and later found to be localized in the gonads are the POMC-derived peptides, including ACTH, MSH and ß-endorphin. ACTH- and ß-endorphin-like material in the testis is localized to Leydig cells (Tsong et al., 1982), whereas opiate receptors have been found in Sertoli cells, but not in Leydig cells (Fabbri et al.,1985). Therefore, the stimulatory effect observed following administration of opiate antagonists on testicular growth, compensatory testicular hypertrophy, and ABP secretion (Gerendai et al.,1986) as well as the finding that ß-endorphin inhibits Sertoli cell proliferation (Orth, 1986) indicate that the locally produced opioid peptide ß-endorphin has a suppressive effect on Sertoli cell functions, presumably by a paracrine mechanism. Opioid receptor antagonists and ß-endorphin antiserum had been reported to reduce steroidogenesis both *in vivo* and *in vitro* (Bardin et al.,

1987). These observations suggest that in long-term studies ß-endorphin may facilitate testosterone secretion. The lack of opioid receptor on Leydig cells seems to exclude an autocrine action of this peptide on steroidogenesis, therefore it is postulated that ß-endorphin exerts its effect on Leydig cells through a Sertoli cell product or by an introcrine mechanism.

Interestingly enough, another opioid peptide enkephalin synthesized in spermatogenic cells (Kilpatrick and Rosenthal, 1986) has a short-term suppressive effect on steroidogenesis. These observations suggest that different opioid peptides synthesized in different cell types of the testis exert differential effects on Leydig cells.

Both CRF (Yoon et al., 1989) and oxytocin (Guldenaar and Pickering,1985) immunoreactivity as well as their receptors (Ulisse et al., 1989; Bathgate and Sernia, 1994) have been reported to be present in Leydig cells. Our results indicate a positive regulatory action of these hormones on steroid secretion, and due to the localization of their receptors on Leydig cells, data suggest that their action on steroidogenesis is exerted by an autocrine mechanism.

No data are available at present on cellular localization of somatostatin in the testis, therefore no conclusion can be drawn on its mechanism of action related to local control of steroidogenesis.

In sum, all these results indicate that testicular steroidogenesis and Sertoli cell activity is under multiple regulatory action exerted by several substances produced in the organ.

Our studies concerning partial denervation of the testis are consistent with the observations which indicate that testicular nerves, besides their vasomotor function, exert a regulatory action on testicular functions. Interruption of the superior spermatic nerve has been reported to induce a significant decrease in testicular serotonin content (Campos et al., 1990), attenuation of hCG-stimulated *in vitro* androgen secretion, and reduction in the number of testicular gonadotropin receptors (Campos et al., 1993). In accordance with our results obtained in vasectomized immature rats are the observations of Frankel et al. (1984) who demonstrated that even in hypophysectomized animals hemivasectomy can prevent the hemicastration-induced testicular response.

The results of our experiments in which we performed local treatment of testes which had previously been denervated, indicate that the effect of naloxone or oxytocin on steroidogenesis observed in the fully innervated gonad is not present after denervation, or partial denervation results in a paradoxical effect. Interestingly enough, it appears that the different types of denervation applied modified the local peptide effect in different ways,

therefore, it can be supposed that the action of locally produced peptides is modulated by multiple neural control.

The mechanism of action of partial denervation of the testis on local peptide effect is not known. It could be supposed, however, that the interaction between the two different types of fine regulatory processes (local peptide action, neural control) might provide an additional, peripheral 'neuro-local endocrine' fine-tuning mechanism in the control of testicular functions.

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Research report

TOPOGRAPHIC LOCALIZATION OF CALRETININ, CALBINDIN, VIP, SUBSTANCE P, CCK AND METABOTROPIC GLUTAMATE RECEPTOR IMMUNOREACTIVE NEURONS IN THE SUPRAMAMMILLARY AND RELATED AREAS OF THE RAT

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Summary. A detailed description of the localization of neurons containing various neuropeptides in the supramammillary complex (SUM) is provided. Further, the neurochemical character of supramammillohippocampal and supramammilloseptal projecting neurons was investigated. The following experiments were performed: (a) immunocytochemistry for each of the eight different neuropeptides investigated, in animals pretreated or not with colchicine, and perfused in fixative containing or lacking acrolein; (b) a thorough mapping study of the localization of immunolabelled neurons at three rostrocaudal levels; (c) double-tracing retrograde labelling for two-directional neuronal projections combined with immunocytochemistry, to study neurochemical character of the projecting neurons. The observations are: (1) each type of immunolabelled elements, such as calretinin, calbindin, VIP, substance P, CCK and metabotropic glutamate receptor 1a immunopositive neurons has a characteristic localization; (2) no parvalbumin- and enkephalin-containing neurons are present in the SUM; and (3) a small population of calretinin-containing and a small number of calretinin-negative supramammillohippocampal neurons located in the lateral area also project to the medial septum-diagonal band region of the septal complex.

Keywords: supramammillary, localization, retrograde labelling, projections, neuropeptides

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INTRODUCTION

The supramammillary area (SUM) is located in the caudal region of the hypothalamus, dorsal to and overlying both the lateral and medial mammillary nuclei. It is bounded caudally by the posterior and lateral hypothalamic areas and dorsally by the periaqueductal gray matter. SUM is subdivided by the mammillothalamic tract into medial and lateral subregions, while the supramammillary decussation divides the rostrocaudally extending nucleus into dorsal and ventral areas (Paxinos and Watson, 1986; Geeraedts et al., 1990). The medial part contains densely packed small to medium sized cells, while in the lateral subregions less densely packed medium to large sized cells are primarily accumulated (Swanson, 1982).

SUM projections to the hippocampus are relatively well documented. Fibres originated from supramammillary neurons were found to terminate heavily in the hippocampal formation, specifically within the dentate granule and the supragranular molecular layers. In addition, a dense SUM fibre band was also demonstrated in strata oriens and pyramidale of the CA2/CA3a region (Wyss et al., 1979; Veazey et al., 1982; Dent et al., 1983; Haglund et al., 1984; Vértes, 1992). Recently it was reported that the targets of the SUM projection in the hippocampal formation are principal cells both in the dentate gyrus and in the CA2/CA3 subfields (Maglóczky et al., 1994).

The SUM sends fibres to numerous other brain structures too, including the cingulate, parietal, temporal, occipital and entorhinal cortices, the amygdala, the basal forebrain nuclei, thalamic nuclei, hypothalamus, midbrain central gray and the septum (Segal and Laudis, 1974; Pasquier and Reinoso-Suarez, 1976, 1987; Amaral and Cowan, 1980; Vértes, 1981, 1988, 1992; Swanson, 1982; Haglund et al., 1984; Stanfield and Cowan, 1984; Saper, 1985; Ino et al., 1988). Most recent findings (Borhegyi et al., 1997) indicate that targets of the SUM projection in the medial septum-diagonal band of Broca complex are cholinergic and GABAergic septohippocampal projection neurons, which make asymmetric synapses with the SUM axonal terminals.

On the other hand, the SUM receives afferent fibres from widespread brain regions including the infralimbic cortex, the nucleus of the diagonal band of Broca, the preoptic nuclei, the septal region, the nucleus centralis superior, the ventral tegmental and the laterodorsal tegmental nuclei (Sesack et al., 1989; Hurley et al, 1991; Swanson, 1976; Swanson and Cowan, 1979; Hayakawa et al., 1993).

On the basis of its efferent and afferent connections, the SUM was suggested to serve as a relay station in the generation of the theta rhythm of the hippocampus and the gating of information flow through the hippocampal formation (Vértes, 1992).

In previous mapping studies (Jacobowitz and Winsky, 1991; Resibois and Rogers, 1992) calretinin (CR), a calcium-binding protein, was found to be localized in a large number of neurons in the SUM. These CR-containing cells which are known to include a large number of supramammillohippocampal (Maglóczky et al., 1994; Kiss and Szeiffert, 1995) and supramammilloseptal (Leranth and Kiss, 1996) neurons, did not show any characteristic distribution throughout the SUM area. In addition, the presence of dopaminergic (Dahlström and Fuxe, 1964), substance P (SP)-containing (Ljungdahl et al., 1978), vasoactive intestinal peptide (VIP)-containing (Haglund et al., 1984; Seroogy et al., 1988; Baek et al., 1988), and cholecytokinin (CCK) immunoreactive (Haglund et al., 1984; Hökfelt et al., 1987) neurons in the SUM and related brain areas has been demonstrated.

In the present study a detailed description of the localization and cytoarchitectonic organization of neurons containing various transmitters or neuropeptides such as calbindin D28k (CaBP), VIP, SP, CCK and one of the metabotropic glutamate receptors throughout the entire rostrocaudal extent of the supramammillary region is provided and the observations are presented in comparison with the distribution of CR-containing neurons. Further, retrograde labeling was used to determine the neurochemical character of neurons that project to the septum and hippocampus.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 250-300 g were used in the present study. Eight rats, after equitensin (Chlornembutal, 0.3 ml/100g) anaesthesia, were perfused through the heart, first with 50 ml of saline (0.9% of sodium chloride), then with 400 ml of fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde and 0.2% picric acid in phosphate buffer (PB, 0.1 M pH 7.4) and, finally with 200 ml of the same fixative containing no glutaraldehyde. In a different experimental group the perfusion fixation was carried out by using a fixative containing 1% accrolein, 3% paraformaldehyde and 0.2% picric acid to achieve a more rapid fixation of neuropeptides. The brains were removed from the skull and immersed in a 2% paraformaldehyde solution for overnight at 4°C. Blocks of tissue

containing the caudal part of the hypothalamus were dissected and coronal sections were cut at 50μm with a vibratome. Sections were then equilibrated with 30% sucrose in PB and freeze-thawed in liquid nitrogen.

Immunocytochemistry

The brain sections were treated sequentially with 0.1% hydrogen peroxide in phosphate buffered saline (PBS) for 20 min to block endogeneous peroxidase activity and to enhance penetration of immunoreagents, then after extensive washing with 0.2% sodium borohydride in PB for 30 min to inactivate aldehyde groups.

To localize various neuropeptides in neurons of the SUM, antisera against CR (1:5000, Rogers, 1989), parvalbumin (PV, 1:4000, Celio, 1986), VIP (1:10,000, Gulyás et al., 1990), CCK (1:4000, Gulyás et al., 1990), SP (1:5000, Léránth et al., 1992), leu-enkephalin (LeuEnk, 1:15,000, a gift from T.J.Görcs) and, to study the appearance of metabotropic glutamate receptor (mGluR) proteins in SUM neurons, a monoclonal antibody, specific for the intracellular C-terminal region of mGluR1a (1:5, Kiss et al., 1996) was used.

Free-floating sections preincubated (as given above) previously, were first incubated either with 10% normal goat serum (NGS) or with normal horse serum (MHS) diluted in PB. Sections preincubated with NGS were processed further using CR, VIP, CCK, SP or LeuEnk antisera, while sections preincubated with NHS were immunoreacted with the monoclonal mouse antibodies to PV, CaBP and mGluR1a. Briefly, alternate sections were treated with rabbit polyclonal antisera to CR, VIP, CCK, SP or LeuEnk and mouse monoclonal antibodies against PV, CaBP or mGluR1a for two days at 4°C. As the second layer a sheep anti-rabbit Ig-biotin $F(ab)_2$ fragment (Boehringer-Mannheim, 1:200) to the polyclonal and biotinylated horse anti-mouse IgG (Vector, 1:200) to the monoclonal antibodies were used overnight at 4°C. The third layer contained avidin-biotinylated-peroxidase complex (ABC Elite, Vector, 1:400, for 3 h). After washing the immunoperoxidase reaction was developed using 3,3'-diaminobenzidine (DAB) as a substrate, intensified with ammonium nickel sulphate. Sections were then mounted and dried onto gelatine-coated glass slides and coverslipped for light microscopy.

Double-tracing labeling for divergent retrograde axonal transport combined with CR-immunocytochemistry

Two different retrograde tracers were microinjected by pressure into the dorsal hippocampus and the border zone of the medial septum-intermediate lateral septal area, the target areas of neurons located in the supramammillary region.

In this tracing study the following combination of the retrograde tracers was applied:

(a) gold-conjugated wheat germ agglutinin (WGA-Go) was used to visualize supramammillary neurons projecting to the hippocampus. This tracer was injected unilaterally into the dorsal hippocampus at stereotaxic coordinates according to Paxinos and Watson (1986).

(b) at the same time the rat received horseradish peroxidase (HRP, Sigma, apoHRP) injection into the border zone between the medial and intermediolateral septal area under stereotaxic conditions to label neurons in the supramammillary region that project to the septum. Two days after the administration the animals were perfused through the heart, first with saline and then with fixative containing 0.1% glutaraldehyde, 4% paraformaldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (PB, pH 7.4). On vibratome sections (50 μm) the transported WGA-Go was visualized using the silver intensification technique (IntenseTM Silver Enhancement kit, Amersham). Sections containing the SUM region were then processed for immunocytochemistry to label the transported HRP, using rabbit anti-serum to HRP (Sigma) and DAB staining. In this double retrograde labelled experiments the black granule-like silver-gold deposit (SG) of the transported WGA-Go could be easily distinguished from the yellowish-brown reaction product of HRP immunoreaction in the same cells. In a group of sections, CR-immunohistochemistry by DAB staining was combined with the silver intensified WGA-Go retrograde labelling.

RESULTS

Distribution of neurons immunoreactive for various neuropeptides

In general, a large number of cells located in the SUM are heavily immunostained for any one of the peptides (CR, CaBP, SP, CCK, VIP, mGluR1a) studied in these experiments. The DAB reaction product is

distributed diffusely throughout the perikaryal cytoplasm. In the case of mGluR1a, the most conspicuous characteristic of the immunostained cells was the diffuse reaction product distributed uniformly both in the somatic and dendritic cytoplasm. Proximal and distal dendrites of all types of neurons labelled by different antibodies are also extensively stained. By staining for mGluR1a, very fine discrete immunoreactive puncta as reaction product could also be seen occasionally in perikarya and cytoplasm of the proximal dendrites of immunolabelled neurons. The strongest mGluR1a-immunoreactivity of the cells was confined to the somatic and dendritic membranes, showing occasionally granule-like labelling, too. The fine, distal dendritic processes of these neurons contained regularly the granular type of the reaction product.

The dendrites of neurons immunostained with whichever antiserum we used extend irregularly without showing any particular orientation, and form an irregular network.

Localization of CR-containing neurons. Neurons immunopositive for CR appear to form a continuous mass of cells starting rostrally at the posterior hypothalamic nucleus, extending caudally as far posteriorly as the interfascicular nucleus and the ventral tegmental area of Tsai (Figs 1 and 7). At any of the rostrocaudal levels of the SUM, no characteristic arrangement of the CR-positive cells could be recognized. The number of the CR-labelled cells at the most caudal region was lower, in comparison with that of rostral levels. In the anterior region of the SUM, there is a dense accumulation of cells in the lateral parts of the nucleus, near to and surrounding the mammillothalamic tract.

Localization of SP-immunolabelled neurons. Without colchicine pretreatment, SP-immunolabelled cells were not found in the entire rostrocaudal extent of the SUM. After incubation of sections from colchicine-pretreated rats, perfused with a fixative-containing paraformaldehyde and low concentration of glutaraldehyde in an antiserum to SP, a relatively high number of cells was immunolabelled. Under these fixation conditions, there was usually a faint staining in the immunolabelled neurons.

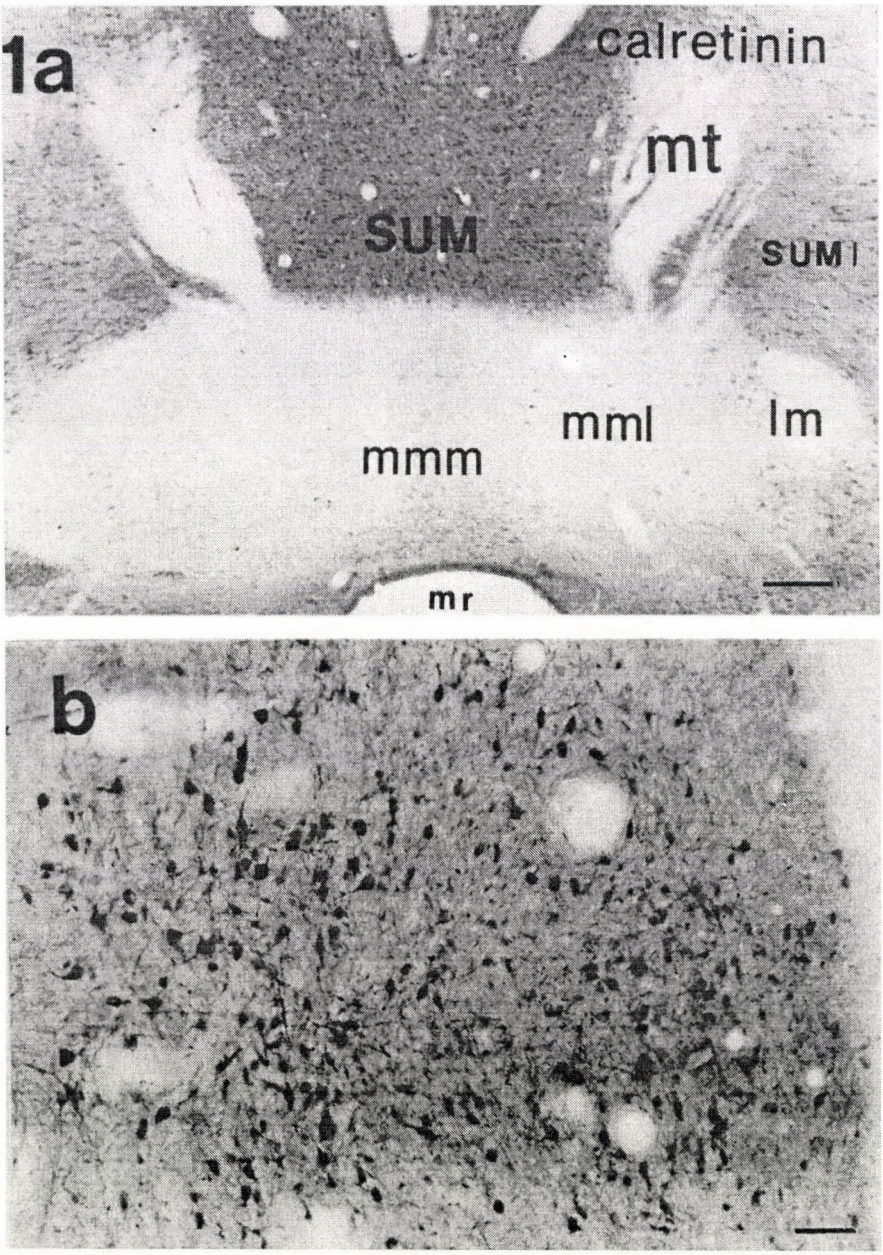

Fig. 1. Photomicrographs illustrating the localization of calretinin immunoreactive neurons in the SUM at lower (a) and higher power (b). Abbreviations: lm = lateral mammillary; mml = lateral part of the medial mammillary; mmm = medial part of the medial mammillary; mr = mammillary recess; mt = mammillothalamic tract; SUM = supramammillary complex; SUMl = lateral part of the SUM. Bars: a: 100 μm; b: 20 μm

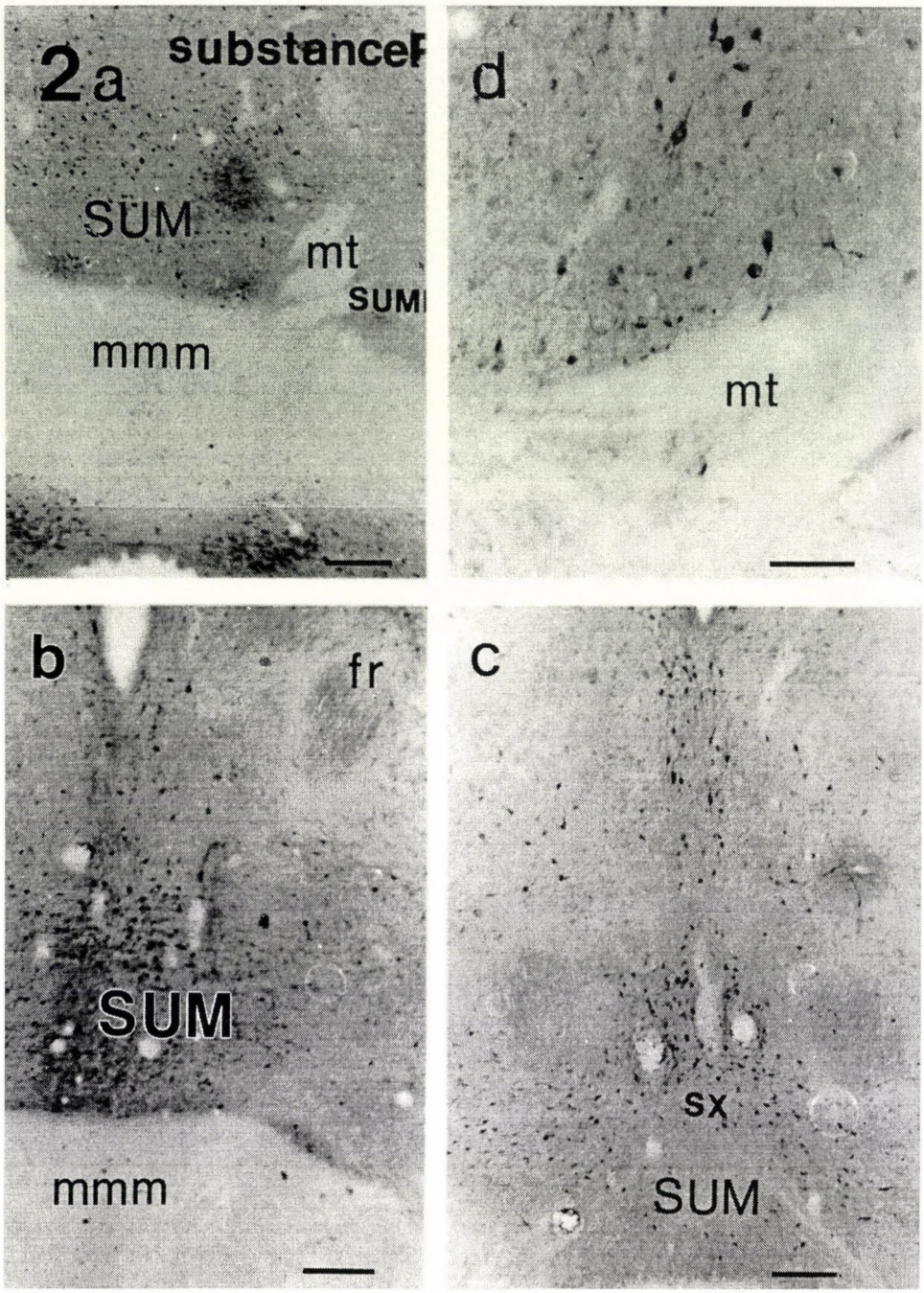

Fig. 2. Photomicrographs demonstrating the localization of substance P immunopositive neurons in the SUM at lower power at three different levels (a,b,c) and at higher magnification (d). fr = fasciculus retroflexus; sx = supramammillary decussation; for other abbreviations see Fig. 1. Bars: a,b,c: 100 μm; d = 20 μm

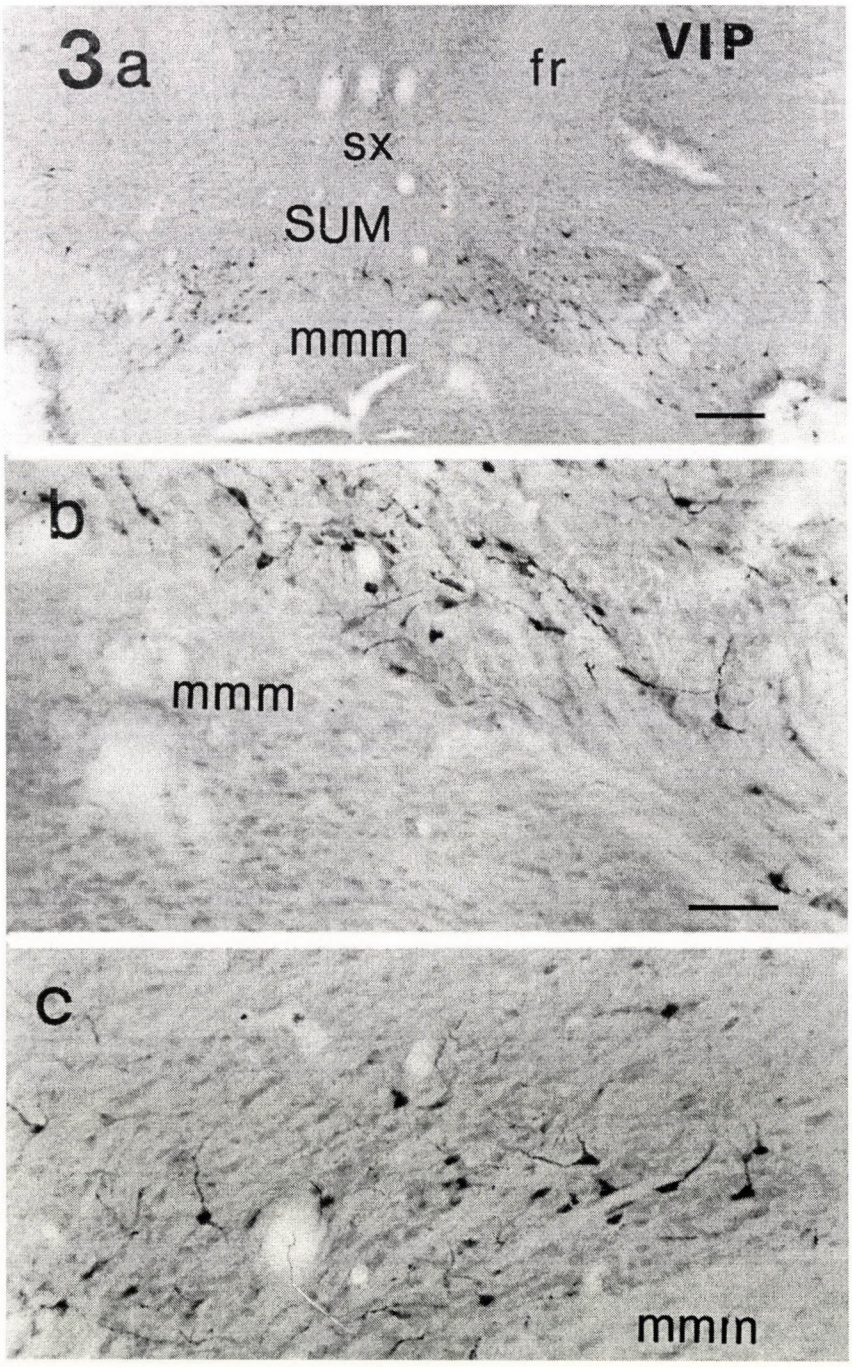

Fig. 3. Photomicrographs showing the localization of VIP positive neurons at lower (a) and higher power (b,c). For abbreviations see Figs 1 and 2. Bars: a: 100 μm; b,c: 20 μm

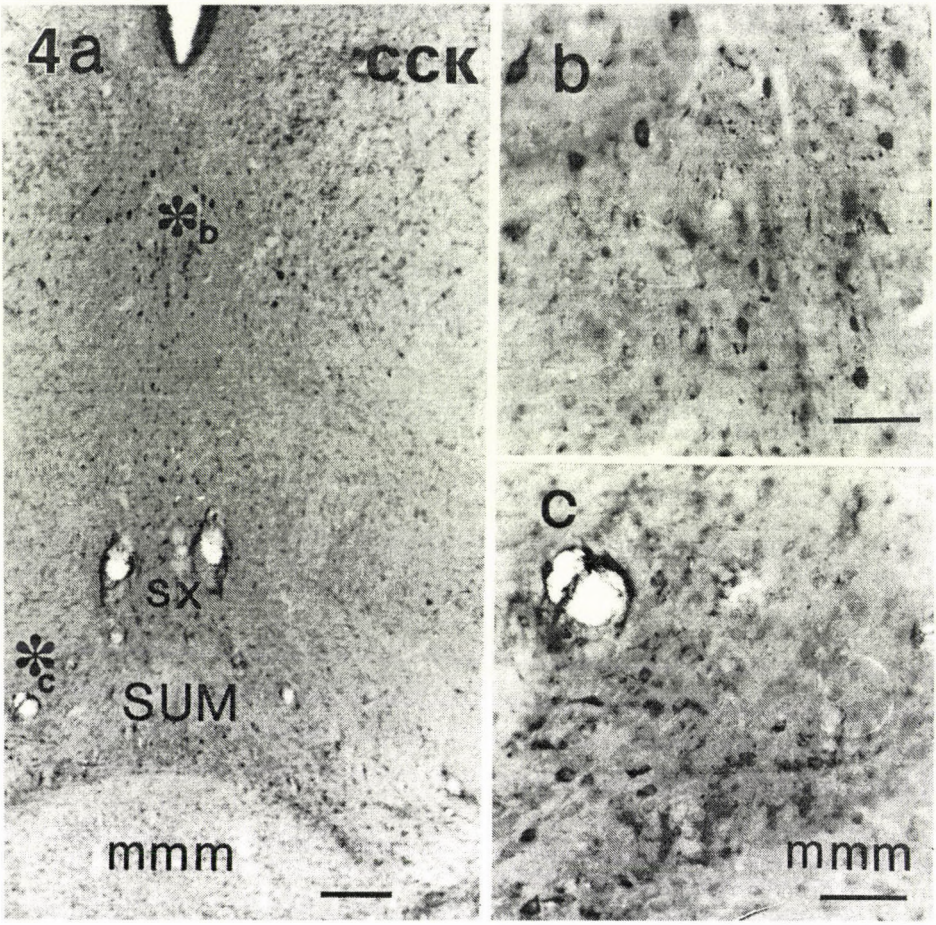

Fig. 4. Photomicrographs demonstrating the localization of CCK immunoreactive neurons at a caudal level of the SUM at lower (a) and higher power (b,c). *b is enlarged in b, and *c in c. For abbreviations see Figs 1 and 2. Bars: a: 100 μm; b,c: 20 μm

Similarly, the analysis of the SUM sections of colchicine-pretreated animals, however, perfused with a fixative-containing paraformaldehyde and acrolein indicated that a large number of cells is immunoreactive for SP (Fig. 2). The immunolabelled neurons were much more extensively stained under these fixation conditions in comparison with those using a fixative containing no acrolein. However, no significant difference in the number of the immunolabelled neurons on sections fixed with or without acrolein, could be seen. Immunoreactive SP-positive neurons were found throughout the SUM, although the majority was located in the rostral region of the nucleus (Fig. 8). The majority of the SP-labelled neurons was localized in more medial aspects of the SUM at all rostrocaudal levels examined, relative to the distribution of the CR-containing neurons. The relative occurrence of the SP-containing neurons in the lateral parts of the nucleus was low. Less number of labelled cells were seen near the mammillothalamic tract, in comparison with that of neurons immunoreactive for CR. Even though, the evaluation of the appearance of SP-labelled fibers was not in the focus of the present study, it has to be noted that a very high density of SP-positive fibers and axonal terminals was observed in the entire SUM region.

Localization of VIP-immunoreactive neurons. Medium number of immunostained cells were seen in the SUM complex, in accordance with earlier report (Seroogy et al., 1988). However, a significant difference in the occurrence of immunostained neurons was found between the rostral and the more caudal areas of the SUM complex (Fig. 9). Only very few scattered cells occurred in the most rostral region. Higher density of the immunoreactive cells could be seen at the intermediate level, where a definite group of VIP-stained neurons was located, occupying mainly the medial area of the nucleus immediately above and in the SUM decussation. No laterally extending neurons were found at this level. At most caudal level immediately anterior to the interfascicular nucleus, a large number of VIP-immunopositive neurons is localized in the SUM following the border of the medial mammillary nuclei and the SUM (Fig. 3). At this region the immunostained cells extend ventro-laterally towards the lateral mammillary nuclei, however, no VIP-containing cells could be found in these laterally located mammillary nuclei.

Localization of CCK-immunoreactive neurons. In the sections from animals perfused either with paraformaldehyde or with the fixative containing acrolein, cells in any areas of the SUM complex were stained less extensively in comparison with VIP-immunoreactive neurons (Fig. 4). The localization of these CCK-immunopositive cells was similar to the distribution of the

VIP-containing neurons (Fig. 10). However, in the rostral part of the SUM complex, where only scattered VIP-positive cells were observed in the alternate VIP-stained sections, a well-defined group of CCK-containing neurons is localized immediately dorsal to the SUM decussation (Fig. 4a and b). The neurons of this dorsally located group could be observed in the entire rostrocaudal extent of the SUM complex, and the number of the cells in this group increased from rostral to caudal.

Localization of cells containing calcium-binding proteins other than CR. No PV-immunoreactive neurons were found in any parts of the entire extent of the SUM complex. On the contrary, CaBP immunoreactivity could be observed in large number of cells in all rostrocaudal parts of the SUM. By quantitative evaluation there is a slight increase in the number of labelled cells extending from rostral to the most caudal areas. Dorso-ventrally, CaBP-stained cells occurred throughout the SUM and the location of the majority was restricted to the medial aspects of the complex (Fig. 11). No laterally extending CaBP-positive cells were present in significant number. The highest density of the immunolabelled neurons was located ventral to the supramammillary decussation, near the area of the medial mammillary nuclei. It has to be noted that both the medial and the lateral parts of the medial mammillary nucleus also contain large number of immunolabelled cells, and a high density of immunostained fibers can be observed both in the mammillothalamic tract and fasciculus retroflexus.

Localization of mGluR1a immunolabelled neurons. Using the monoclonal anti-mGluR1a antiserum, the immunostaining showed extensive labelling of neuronal processes and cells in restricted areas of the SUM (Fig. 5). The strongest immunoreactivity was confined to the somatic and dendritic membranes, occasionally showing fine granular labelling, too. The mGluR1a-immunolabelled neuronal elements were almost exclusively located in the lateral parts of the SUM (Fig. 12), surrounding completely the cross-section of the mammillothalamic tract (Fig. 5b). No, or only a few scattered cells and low density of processes were observed the medial aspect of the SUM complex. Like in our earlier studies (Kiss et al., 1996), a large number of mGluR1a immunopositive neurons and a dense immunorective network of dendritic processes could be seen in the mammillary and the tuberomammillary nuclei.

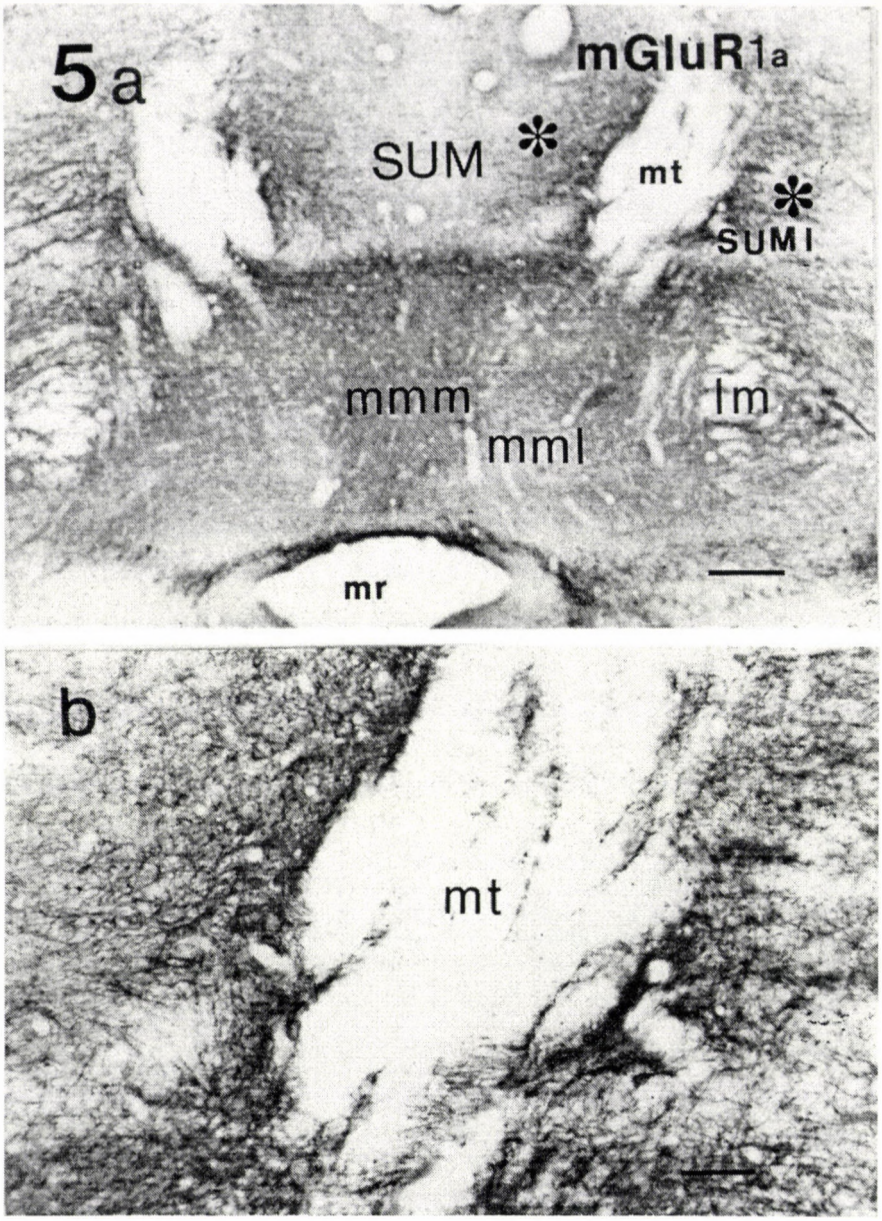

Fig. 5. Photomicrographs demonstrating the localization of mGluR1a immunopositive elements at lower (a) and higher power (b). The area labelled by asterisks in a is enlarged in b. For abbreviations see Figs 1 and 2. Bars: a: 100 μm; b: 20 μm

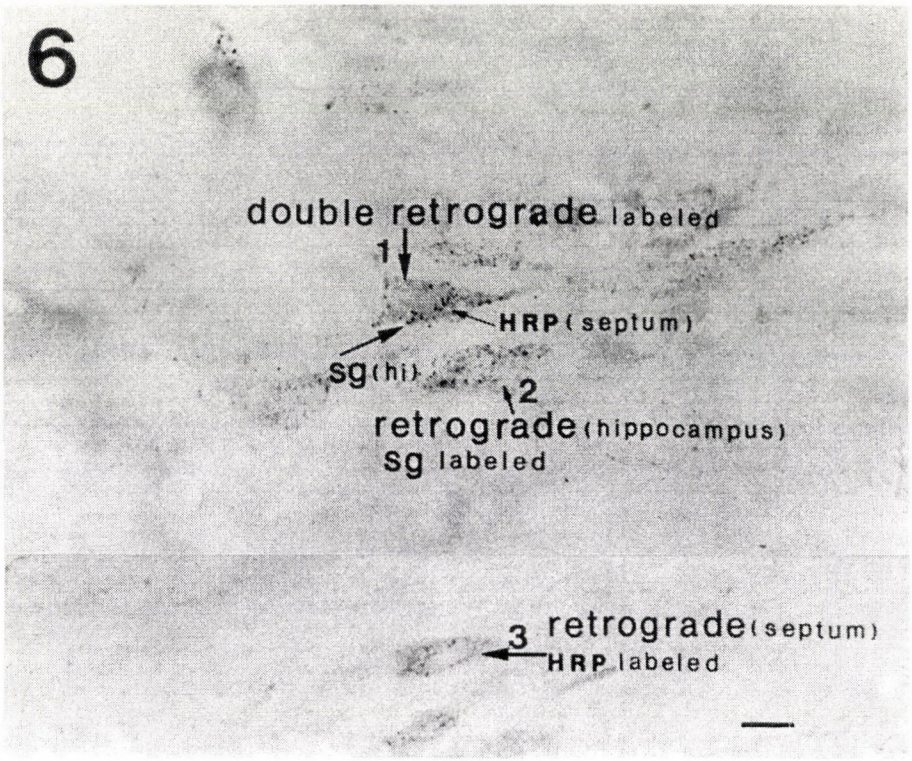

Fig. 6. This microphoto (originally in colour) demonstrates single and double retrogradely labelled neurons from the medial septum-diagonal band (MS) and from the dorsal hippocampus (hi) in the SUM. The silver-gold (sg) reaction product transported from the hi appeared as black granules in the cells, the immunoreaction product of the HRP transported from the MS was stained with DAB and seen as yellow-coloured spots in the neuronal cytoplasm in the SUM. In a double retrogradely labelled neuron (1) both the black-coloured sg granules (long arrow) and the yellow-stained spots (thin arrow) were visible. In a neuron labelled from the hi (2) only sg granules (short thin arrow) were accumulated; the neuron labelled from the septum (3) contained only DAB-stained spots. Bar: 10 μm

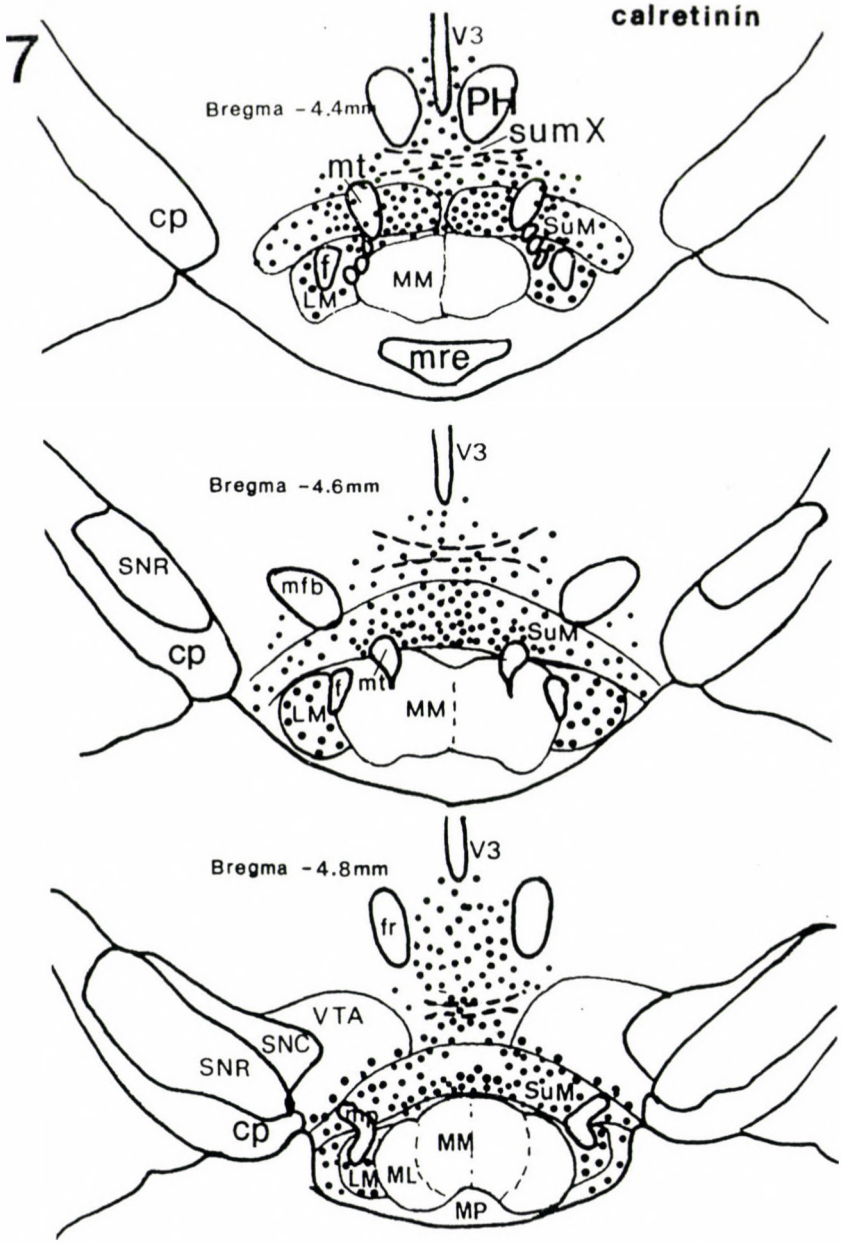

Fig. 7. Distribution of calretinin immunoreactive neurons in the SUM at three different rostrocaudal levels according to the Paxinos and Watson atlas (1986). Coordinates are: 4.20; 4.50 and 5.00 mm posterior to the bregma

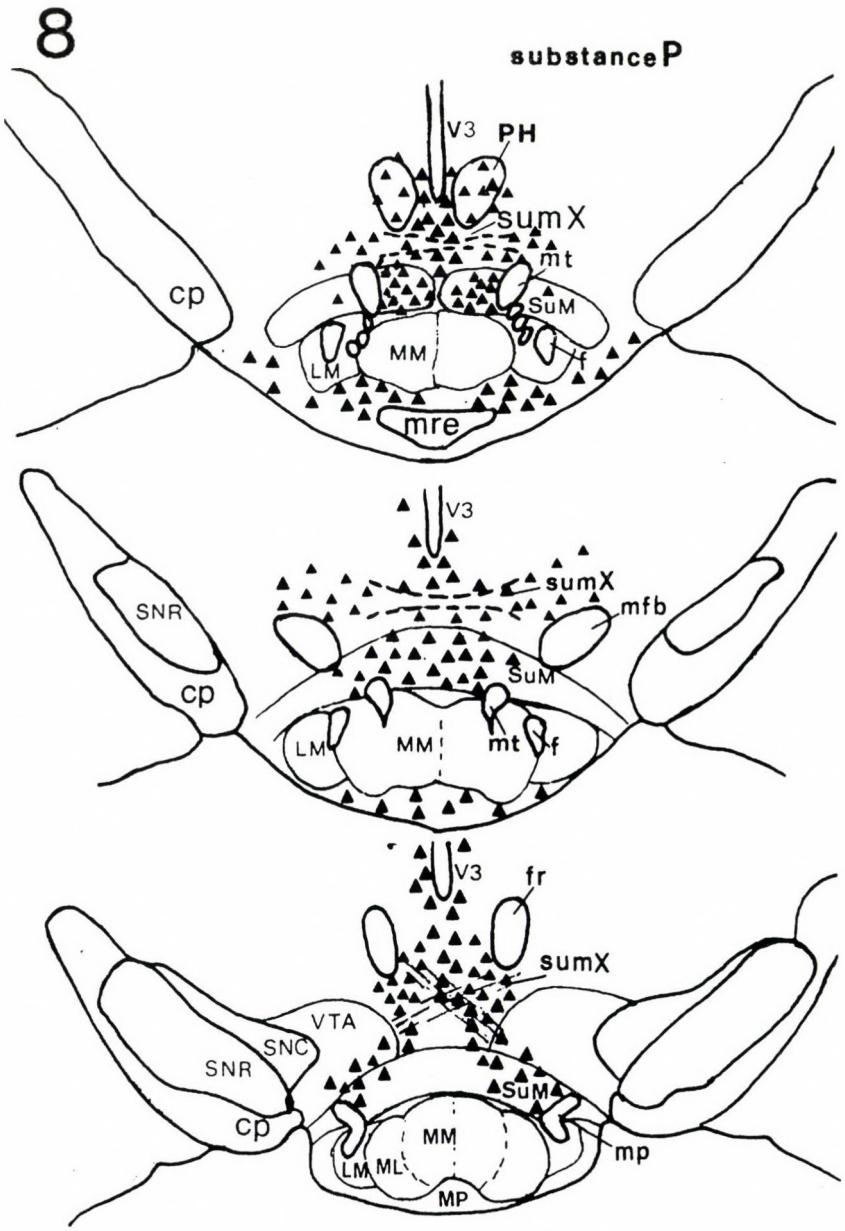

Fig. 8. Distribution of substance P immunoreactive neurons in the SUM at three different rostrocaudal levels according to the Paxinos and Watson atlas (1986). Coordinates are: 4.20; 4.50 and 5.00 mm posterior to the bregma

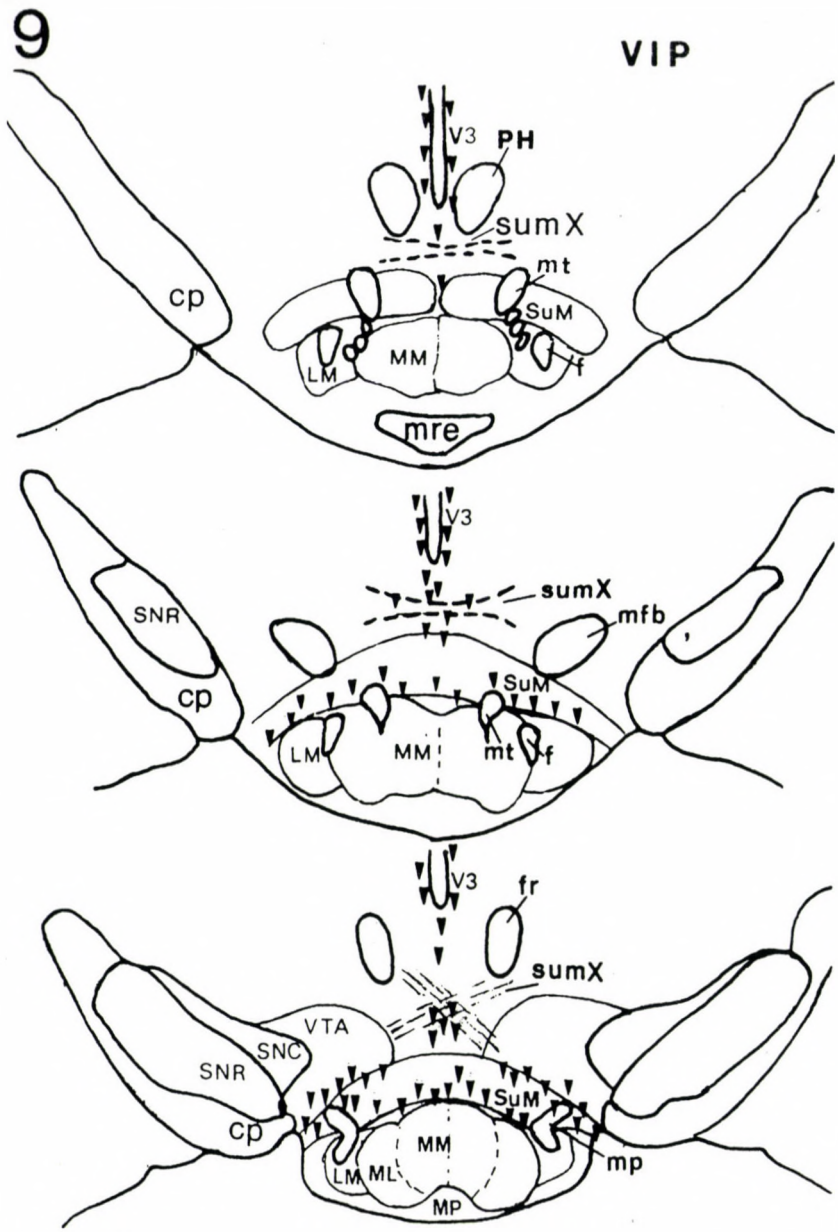

Fig. 9. Distribution of VIP immunoreactive neurons in the SUM at three different rostrocaudal levels according to the Paxinos and Watson atlas (1986). Coordinates are: 4.20; 4.50 and 5.00 mm posterior to the bregma

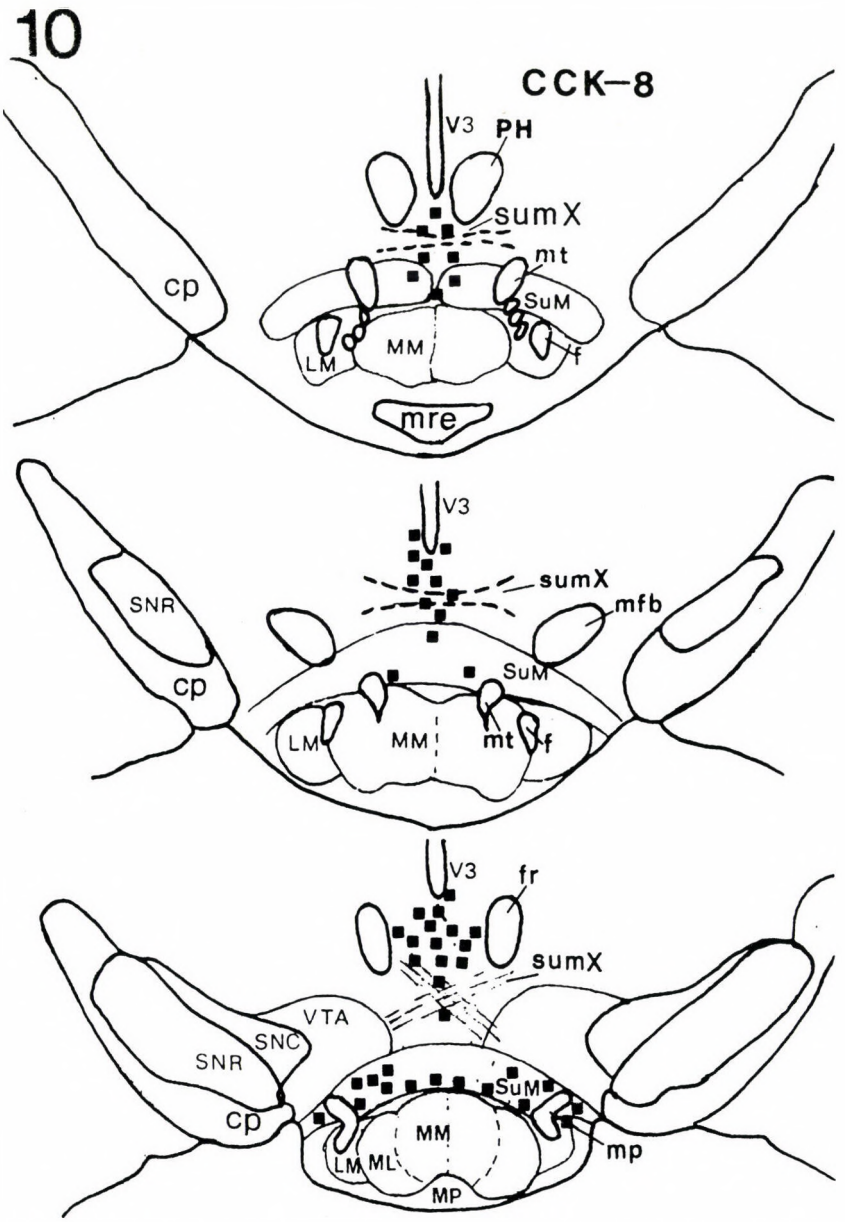

Fig. 10. Distribution of CCK immunoreactive neurons in the SUM at three different rostrocaudal levels according to the Paxinos and Watson atlas (1986). Coordinates are: 4.20; 4.50 and 5.00 mm posterior to the bregma

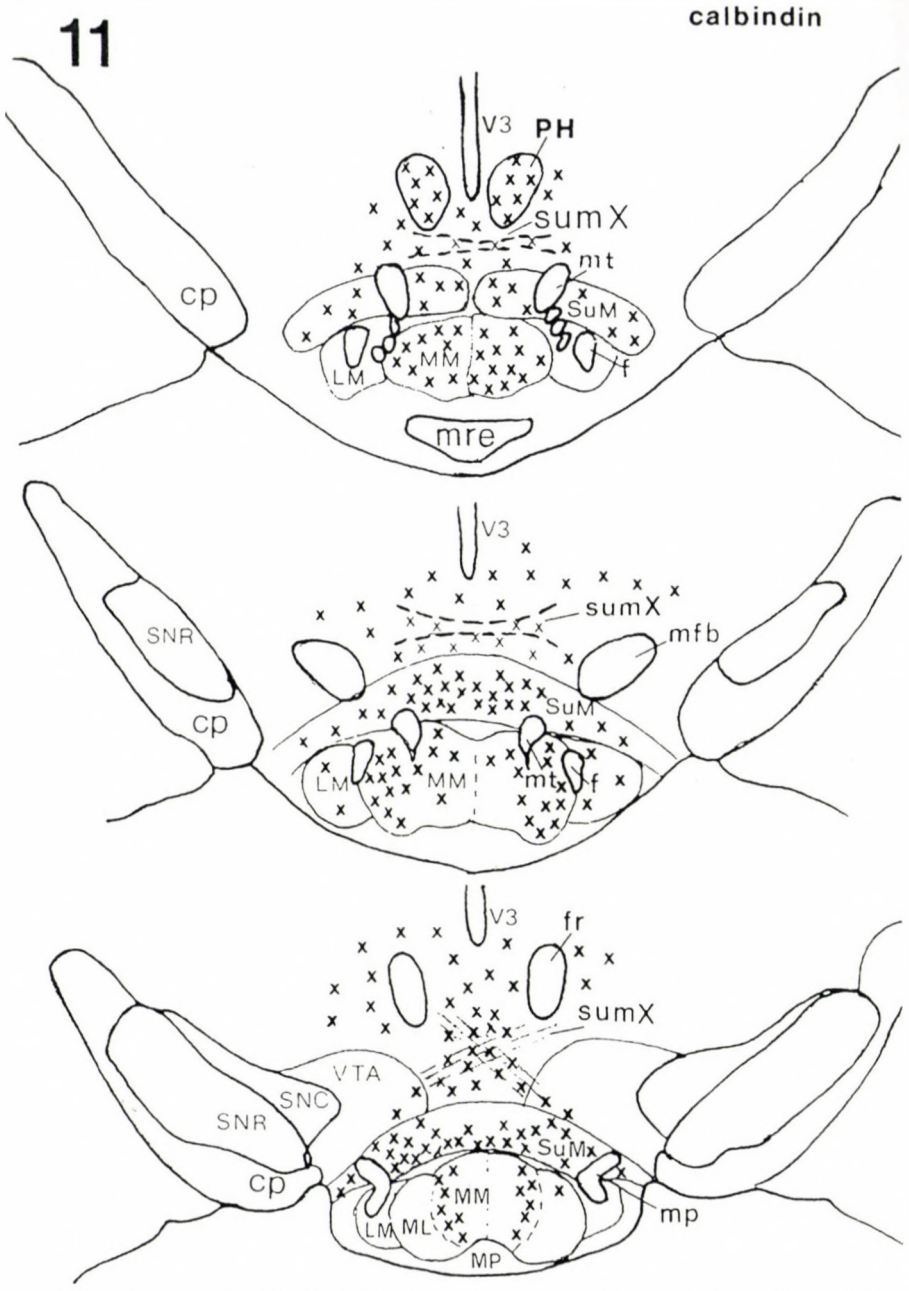

Fig. 11. Distribution of calbindin immunoreactive neurons in the SUM at three different rostrocaudal levels according to the Paxinos and Watson atlas (1986). Coordinates are: 4.20; 4.50 and 5.00 mm posterior to the bregma

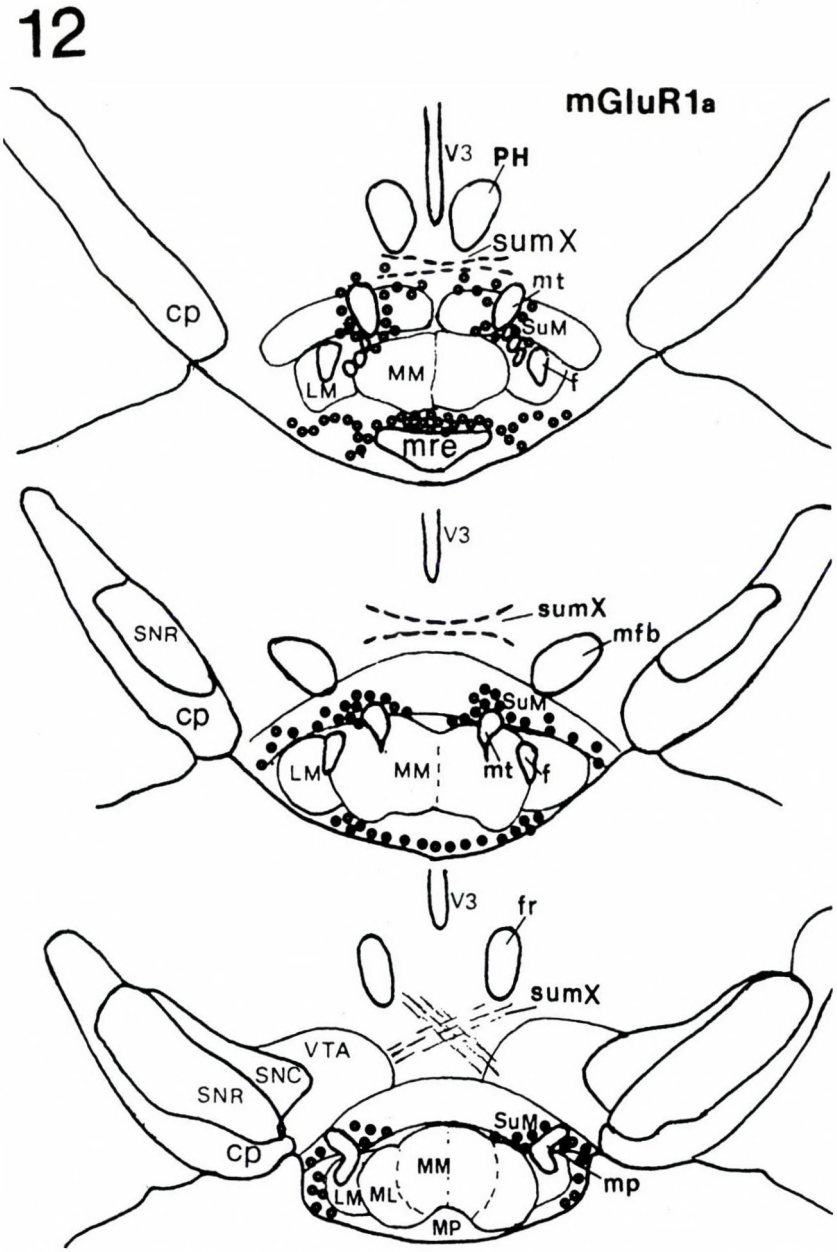

Fig. 12. Distribution of mGluR1a-immunoreactive neurons in the SUM at three different rostrocaudal levels according to the Paxinos and Watson atlas (1986). Coordinates are: 4.20; 4.50 and 5.00 mm posterior to the bregma

No enkephalin immunolabelled neuronal elements were found in any part of the SUM, perfused the animals with fixative either containing or not acrolein.

Distribution of neurons in the SUM projecting to the medial septum-diagonal band of Broca complex and to the hippocampus

Double-retrograde tracing experiments in which gold-conjugated wheatgerm agglutinin was injected into the dorsal hippocampus and apo-HRP was administered into the medial septum-diagonal band complex, resulted in three types of retrogradely labelled cells in the SUM, visualizing the gold-conjugated transported material with silver-intensification and the retrogradely transported HRP by DAB-staining, in the same sections. Large number of single-labelled cells containing the silver-gold deposit (sg) was distributed in the lateral area, and a significantly smaller number of those was scattered in the medial aspect of the SUM. By using the combination of WGA-Go-silver intensification and CR-DAB-immunocytochemistry on the same sections of a separated group, the majority of the silver-gold labelled cells were CR-immunopositive. The ventral tegmental area, the tuberomammillary and the premammillary nuclei also contained a small number of silver-gold labelled neurons. The second type of retrogradely labelled cells contained the yellowish-brown reaction product (stained with anti-HRP, -ABC, -DAB) of the retrogradely transported apo-HRP. This type of cells was distributed in the SUM, localized not only in the lateral parts but also in the medial aspects of the complex. The observations were particularly focused on the third type of labelled neurons. These cells contained both the black coloured, granule-like silver-gold deposit and the yellowish-brown DAB reaction product (Fig. 6). These double-retrograde labelled neurons, which project both to the hippocampus and the medial septum-diagonal band complex are localized in a restricted area of the lateral part of the SUM, surrounding more or less the cross-section of the mammillothalamic tract. Even though, a small number of both type of single-retrograde labelled cells was found on the side contralateral to the injection, double-retrograde labelled neurons could only be seen ipsilaterally to the injections.

DISCUSSION

The present study was undertaken to characterize in details the neurotransmitter content of neurons localized in the SUM complex and related structures (tuberomammillary, premammillary regions), the distribution of cells containing various neuropeptides and some projections of the SUM nucleus of the rat. The major conclusions of these studies are: 1) the distribution of the CR-containing neurons follows a more or less diffuse pattern in the SUM without particular accumulation in any parts of the nucleus; 2) a low proportion of the CR-immunopositive neurons in the lateral part of the SUM is double retrogradely labelled after injections of different retrograde tracers into the septum and the dorsal hippocampus indicating that these neurons besides innervating hippocampal target cells also give rise projections to the medial septum-diagonal band area; 3) a number of neurons localized in the SUM complex and the premammillary (PM) and tuberomammillary (TM) areas contain SP immunoreactivity, occupying in the SUM principally the dorso- and ventromedial parts of it. SP-immunopositive cells are present in much lower number in the lateral SUM; 4) VIP and CCK-immunoreactive neurons occur in very low number relative to the CR-positive cells, mainly in the more caudal region of the SUM, distributing in a restricted area of the dorsal part of the nucleus; 5) intense immunolabelling for 1a type of metabotropic glutamate receptor (mGluR1a) occurs in perikarya and dendrites of the lateral parts of the SUM. A characteristic distribution of these mGluR1a-immunoreactive elements is present immediately near, and surrounding the mammilothalamic tract.

The SUM is suggested to be a relay station interconnecting various limbic structures with hypothalamic and numerous other brain areas. The SUM has a considerable effect on hippocampal activity. Hippocampal theta rhythm is influenced by SUM stimulation (Segal, 1979; Mizumori et al., 1989; Carre and Harley, 1991), however, only if the medial septum (MS) is intact (Kirk and McNaughton, 1991). SUM projections to the hippocampus are relatively well documented (Wyss et al., 1979; Vértes, 1992). SUM fibres were found to terminate heavily in the hippocampal formation, specifically within the granule cell layer and the supragranular molecular layer of the dentate gyrus, and in the strata oriens and pyramidale of the CA2-CA3a region (Dent et al., 1983; Haglund et al., 1984; Vértes, 1992). It is also known that SUM fibres terminate densely in the medial septum-diagonal band of Broca complex (Vértes, 1992), and that a significant part of the SUM-medial septum-diagonal

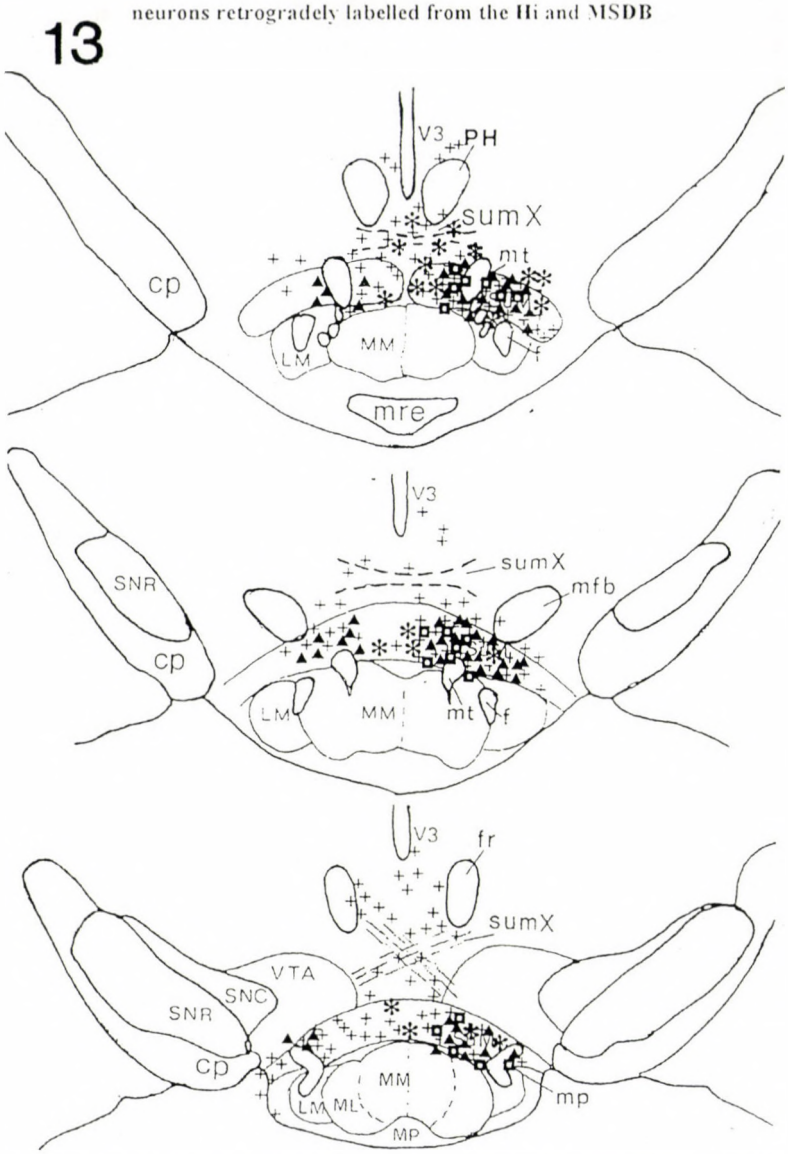

Fig. 13. Distribution of retrogradely labelled neurons from the medial septum-diagonal band and from the hippocampus. + = single labelling from the septum; ▲ = single labelling from the hippocampus; = double labelling from both the medial septum and the hippocampus; * = ^{3}H-D-aspartate autoradiographic labelling from the medial septum (Léránth and Kiss, 1996). Coordinates are: 4.20; 4.50 and 5.00 mm posterior to the bregma, according to the atlas of Paxinos and Watson (1986)

band complex fibres contains the excitatory transmitter glutamate/aspartate (Léránth and Kiss, 1996). In general, however, little information has been given to date about the possible transmitters of the fibres originated in the supramammillary complex. The calcium binding protein, calretinin, is contained both in supramammillohippocampal (Maglóczky et al., 1994) and supramammilloseptal (Kiss and Szeiffert, 1995; Léránth and Kiss, 1996) projections in the rat. It was demonstrated in previous studies, that the largest mass of the neurons in the SUM contains CR immunoreactivity (Jacobowitz and Winsky, 1991; Résibois and Rogers, 1992). Our present observations indicate clearly that the great majority of the neurons located in the entire rostrocaudal extent of the supramammillary complex contain CR. The localization of these labelled neurons shows an evenly diffuse distribution both in the medial and the lateral parts of the SUM. A small proportion of the CR-containing neurons could be identified as to be labelled by radioactive D-aspartate after retrograde transport from the septum (Leranth and Kiss, 1996) and the hippocampus (Kiss et al, 1997). These laterally localized CR-neurons seem to contain the excitatory transmitter glutamate or aspartate. In a separate experiment, after injection of HRP into the septum and WGA-gold into the dorsal hippocampus, we found double-retrogradely labelled cells which were immunolabelled also for CR in the SUM, showing that these neurons innervating hippocampal target cells also give rise projections to the medial septum-diagonal band area. These findings, taking together with recent data (Maglóczky et al., 1994) showing that hippocampal postsynaptic targets of the supramammillary projection are principal neurons, suggest that the influence of SUM neurons on the frequency of hippocampal theta rhythm may be exerted by direct modulatory effect through the medial septum-diagonal band elements. SP was also reported to be present in SUM neurons (Gall and Selawski, 1984; Ino et al., 1988) of the guinea pig, cat and monkey.

In our material, sections from animals which were not pretreated with colchicine, and were perfused with a fixative containing paraformaldehyde and low concentration of glutaraldehyde, SP immunoreactive neurons could not be found in the SUM. However, after colchicine pretreatment and by using a fixative containing acrolein and paraformaldehyde, a large number of SP-immunoreactive neurons appeared in the entire rostrocaudal extent of the SUM complex, and showed a characteristic topographic localization. The SP-containing neurons present in SUM of other species have been shown to project to the hippocampus in the guinea pig (Gall and Selawski, 1984), but not in the rat (Haglund et al., 1984). Whether there are SP-positive

supramammilloseptal projections which innervate the medial septum-diagonal band neurons in the rat, remains to be determined. The present findings indicate that a large number of retrogradely labelled neurons appears both in the medial and lateral SUM areas after retrograde tracer injection into the medial septum-diagonal band area of the septum. A higher proportion of these retrogradely labelled neurons is CR-immunopositive, however, there is a significant number of CR-negative cells that also contain the retrograde labelling. In view of the different pattern of distribution of CR- and SP-positive cell populations, SP-containing neurons in the SUM might be candidates of such projections to the medial septum-diagonal band complex.

Previously, it has been shown that CCK and VIP neurons in the SUM project to the hippocampus (Haglund et al., 1984) and to the lateral nuclei of the septum. The mapping data of the present study revealed that CCK and VIP immunoreactive neurons, although present in very low number at all rostrocaudal levels in the SUM, are in nearly similar or almost identical overlapping distributions, except the most rostral region of the SUM complex where only scattered CCK and VIP neurons occur. A growing body of evidence suggests that excitatory amino acids and their receptors may play a critical role in the regulation of hypothalamic functions. By means of an antibody against metabotropic glutamate receptor (mGluR1a), we found a well-defined immunolabelling of several hypothalamic cell groups including the nuclei of the tuberomammillary and the mammillary complex (Kiss et al., 1996). In a recent paper, van den Pol and associates (1995) have reported that neurons immunoreactive for mGluR1a and mGluR5 are present in the caudal hypothalamus including the mammillary body and the tuberomammillary nuclei. In our comprehensive mapping study the localization of these immunolabelled neurons had characteristic pattern.

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Research report

EFFECT OF THE LACK OF LIGHT IMPULSES ON THE HYPOTHALAMIC PACAP AND C-FOS IMMUNOREACTIVITIES IN RATS

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Summary. Pituitary adenylate cyclase activating polypeptide (PACAP) is a member of the secretin family. It is widely distributed in the central and peripheral nervous systems. The highest concentration of PACAP was found in the hypothalamus. In the present work it has been studied whether PACAP is involved in the mediation of photic stimuli to the anterior pituitary gland. We have examined the effect of the lack of light impulses on the hypothalamic PACAP and C-fos immunoreactivities. In adult rats 10 days after the removal of the eyes (surgical enucleation) and in those received monosodium glutamate treatment neonatally (chemical enucleation). The PACAP immunostaining enhanced in the hypothalamic magnocellular nuclei and in the extemal zone of the median eminence. C-fos immunoreactivity also enhanced in a few hypothalamic nuclei 2 hours after the surgical enucleation indicating that the lack of light impulses activated hypothalamic neurons which, in turn, might stimulate the release of PACAP into the portal circulation. It has been concluded that PACAP may be involved in photoendocrine regulations.
Keywords: PACAP, C-fos, hypothalamus, removal of the eyes, immunohistochemistry

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INTRODUCTION

Pituitary adenylate cyclase activating polypeptide (PACAP) was isolated from ovine hypothalami based on its ability to stimulate adenylate cyclase in anterior pituitary cell cultures (Miyata et al., 1989). According to its amino acid sequence PACAP is a member of the secretin family. PACAP is present in two bioactive amidated forms: PACAP38 and 27 composed of 38 or 27 residues, respectively. PACAP shows a considerable sequence homology with the vasoactive intestinal polypeptide (VIP). PACAP, as VIP, is widely distributed in the central and peripheral nervous systems of rats. Both peptides were demonstrated in the hypothalamus as well.

PACAP immunoreactivity (IR) in the hypothalamus

In intact adult (3-4 month old) Sprague-Dawley rats PACAP immunoreactive (ir) cells cannot be demonstrated by immunohistochemical techniques. Colchicine treatment had to be used for successful staining (Köves et al., 1991). Two days after 50-100 μg of intracerebroventricular (icv) colchicine injection PACAP IR was mainly observed in the supraoptico-paraventriculohypophyseal magnocellular system (Köves et al., 1991; Kivipelto et al., 1992; Piggins et al., 1996). Contraversial data are also available. Hannibal et al. (1995) using a monoclonal antibody demonstrated PACAP ir cells mainly in the parvocellular subdivision of the paraventricular nucleus (PV). In our material after a long-lasting colchicine treatment (6 days) PACAP ir cells were also observed in parvocellular cell groups as well, not in the PV but in the arcuate and posterior hypothalamic regions (Köves et al., 1994). PACAP ir fibers in the hypothalamus were observed in the median eminence (ME), mainly in its internal zone (Köves et al., 1991) and in the suprachiasmatic nucleus (SCH) (Köves et al., 1996a).

VIP IR in the hypothalamus

In intact rats VIP ir cells are always seen in the ventrolateral part of the SCH (Loren et al., 1979; Sims et al., 1980). However, after colchicine treatment they occupy the whole extent of this nucleus and a few VIP cells appear in the PV, anterior comissural nucleus (ACN) and in the medial preoptic area as

well (Köves et al., 1991). A well-definied fiber bundle leaves the SCH ascending to the subparaventricular zone (Saper et al., 1979).

The biological clock

It is well known that the SCH is the biological clock (see: Klein et al., 1991). This nucleus has a mandatory role in the regulation of the hormonal rhythms which show circadian, cyclic and annual changes. The major neurotransmitter of the SCH cells is VIP. Beside VIP, another member of the secretin family, gastrin releasing peptide (GRP) IR was also demonstrated in the same cells (van den Pol and Görcs, 1976). VIP ir cells receive afferents from various structures. The major inputs come from the retina, lateral geniculate nucleus (LGN) and the raphe nuclei. The retinohypothalamic pathway was demonstrated first by Moore and Lenn (1972) and Hendrickson et al. (1972). This fibers use glutamate as neurotransmitter (Liou et al., 1986). In our material we have observed PACAP ir fibers in the SCH. It was supposed that these fibers might derive from the retina (Köves et al., 1996a). Neuropeptide Y (NPY) containing fibers derive from the intergeniculate leaflets of LGN (Card and Moore, 1989). Serotoninergic fibers derive from pontine raphe nuclei (Hisano et al., 1987).

We have supposed that PACAP, which is structurally related to VIP and GRP, may also be involved in the regulation of hormonal rhythms. In the present experiment it was studied how the lack of the light impulses influenced the hypothalamic PACAP and C-fos IR-s. The appearance of C-fos IR was accepted as a marker for activated cells.

MATERIALS AND METHODS

Animals

Sprague Dawley male rats were kept in a light (lights on at 6:00 h and off at 18:00 h) and temperature controlled vivarium (22±2 °C). In *Group* I and *II* adult (3-4-month old) male rats were surgically enucleated. Both eyes were removed under general anaesthesia using Hexobarbital (10 mg/100 g body weight). In *Group III* the animals were treated by monosodium glutamate (MSG) according to the following protocol (Antoni et al., 1982): newborn male rats were injected with 4g/kg body weight of MSG (Reanal, Budapest,

Hungary) subcutaneously every other day for the first 10 days of life. Litter mate controls received 0.5g/kg NaCl in 10% (w/v) solution. MSG is a toxic agent. As it was demonstrated previously (Kizer et al., 1978, Olney, 1969) after this treatment the optic nerves were very thin because nearly all retinal ganglion cells were degenerated. This intervention was called 'chemical enucleation'.

Immunohistochemistry

Group I. Five animals were sacrificed 10 days after the surgical enucleation with 4% paraformaldehyde perfusion. The brains were removed and postfixed for overnight. Fifty μm thick sections were cut on vibratome or 20 μm thick sections were cut on cryotome. The sections were stained for PACAP IR according to the directions of Vectastain ABC KIT (Vector, Burlingham, USA). The primary antiserum was raised in rabbit against PACAP27 and previously characterized (Köves et al., 1990). Its working dilution was 1:6,000. To demonstrate the specificity of the immunostaining 1 ml PACAP antiserum at working dilution was preabsorbed with an excess amount (20 μg) of PACAP27 24 hours before use.

Group II. Five animals were sacrificed two hours after the surgical enucleation. The sections of the brain were similarly prepared as in *Group I.* Then they were stained for C-fos immunoreactivity.

The C-fos antiserum was raised by immunizing rabbits against synthetic 4-17 sequence of the Fos protein linked to human serumalbumin by means of glutaraldehyde or 3-maleimidobenzoic acid N-hydroxysuccinimide ester. Specific anti-4-17 Fos immunoglobulins were purified from the rabbit antiserum by 4-17 Fos affinity column chromatography. The column was prepared with 417 peptide coupled to 2-fluoro-1-methyl pyridinium toluene-4-sulphonate (FMP)-activated cellufine cellulose (Chisso Corp., Japan), loaded with the antiserum and eluted with 0.2 M glycine buffer, pH 2.45. The pH of the collected fractions (1 ml) was raised to 7.5 with 1 M TRIS and the absorbance at 280 nm was measured. Peak fractions were pooled and then dialyzed overnight against 0.01 M phosphate buffer, pH 7.4.

The specificity of the Fos immunostaining was evaluated by overnight preabsorption of the diluted antiserum with the synthetic 4-17 peptide (10-M).

Group III. Four adult male rats treated with MSG in neonatal age were perfused. The brains were similarly treated, sectioned and stained as in *Group I.*

RESULTS

Effect of surgical enucleation on the hypothalamic PACAP IR

Ten days after the removal of the eyes (*Group I*) PACAP IR tremendously increased in the hypothalamic magnocellular nuclei compared to age matched control male rats. PACAP ir material accumulated in the ACN (not shown), at different levels of the magnocellular subdivision of the PV (Fig. 1a-c and e-f) and in a part of the SO (Fig. 1d). In the rostral part of the PV PACAP ir cells were positioned in front of the parvocellular subdivision of the PV. 1 mm behind this level PACAP ir cells formed two groups: a smaller dorsal and a larger ventral groups (Fig. 1a) lying above and below the parvocellular subdivision of the PV. Further backwards PACAP ir cells formed a thin layer surrounding the parvocellular cells (Fig. 1b). In the most posterior part of the PV, above the ME, PACAP ir cells were located horizontally in the dorsal part of the PV (Fig. 1c). PACAP ir cells were also observed in the periventricular nucleus (Fig. 2a) and in accessory magnocellular cell groups which were usually located along vessels (Fig. 2b-d). In all above-mentioned nuclei rough, beaded fibers appeared between the PACAP ir perikarya (Fig. 1d-f and Fig. 2). Similarly rough fibers were never seen in intact rats. In the ME PACAP ir fibers appeared in the external zone (EZ) around the portal capillaries beside the characteristic localization in the internal zone (IZ) (Fig. 3a and b).

Effect of chemical enucleation (MSG treatment) on the hypothalamic PACAP IR

In adult animals which were neonatally treated with MSG *(Group III)* PACAP ir cells were also observed in all the magnocellular cell groups. The localization of PACAP ir cells in the ACN, PV and SO was basically similar to that observed in animals bearing surgical enucleation. From the IZ of the ME rough PACAP ir fibers migrated into the EZ (Fig. 3c). The major part of the retinal ganglion cells were degenerated (not shown).

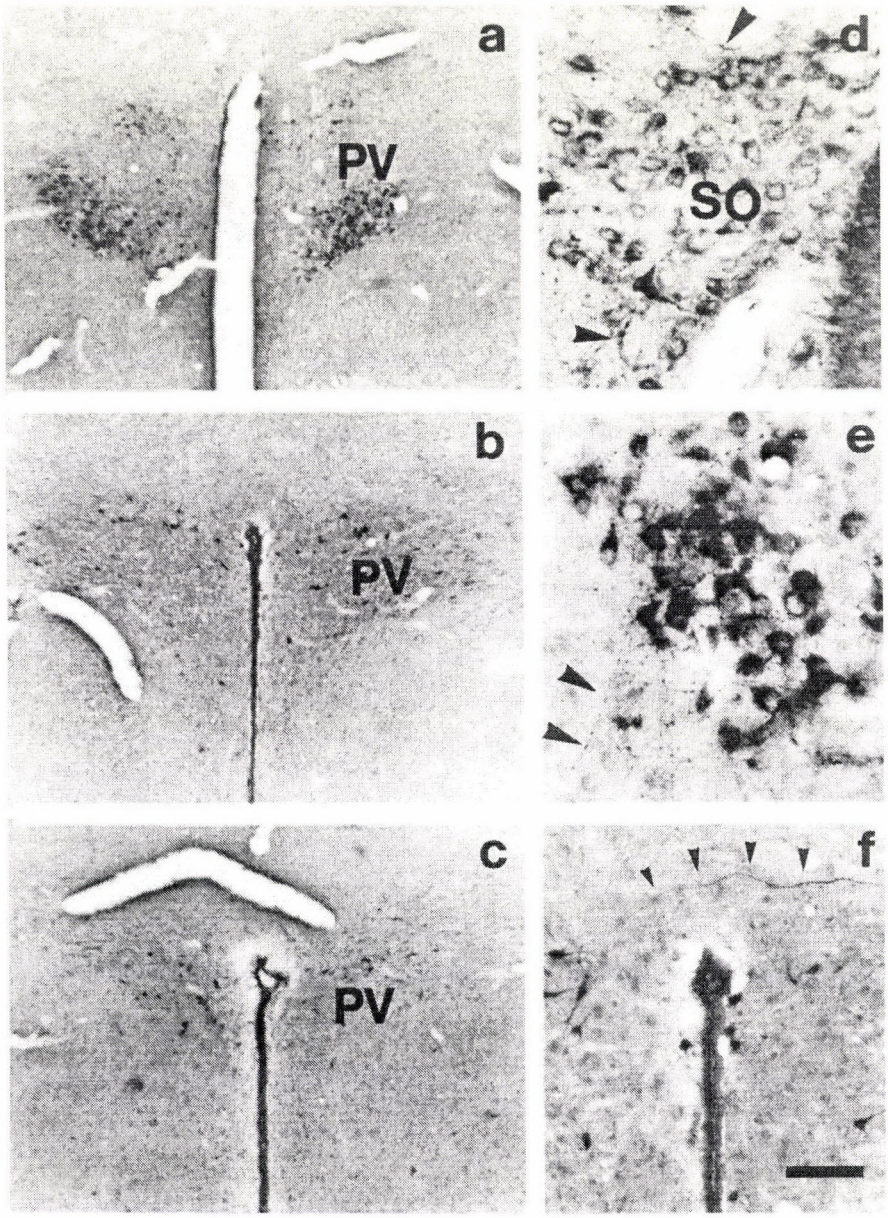

Fig. 1. PACAP IR in the paraventricular nucleus in three succeeding rostrocaudal levels *(a-c)* and in the SO *(d); e* and *f* are high power details of the PV. In *a* PACAP ir cells form 2 groups above and below the parvicellular subdivision of the PV. In *b* PACAP ir cells form a thin layer surrounding the parvicellular portion. In *c* PACAP ir cells form a horizontal row in the dorsal part of the PV. In *d* PACAP ir cells and beaded fibers (indicated by arrowheads) are seen in the SO. In *e* prominent PACAP fibers leave the PV lateralwards indicated by arrowheads. In *f* a PACAP ir fiber indicated by arrowheads crosses the midline at the same antero-posterior level of PV as in *c*. SO = supraoptic nucleus, PV = paraventricular nucleus. Scale bar: 250 μm in *a-c*, 50 μm in *d* and *e*, and 100 μm in *i*

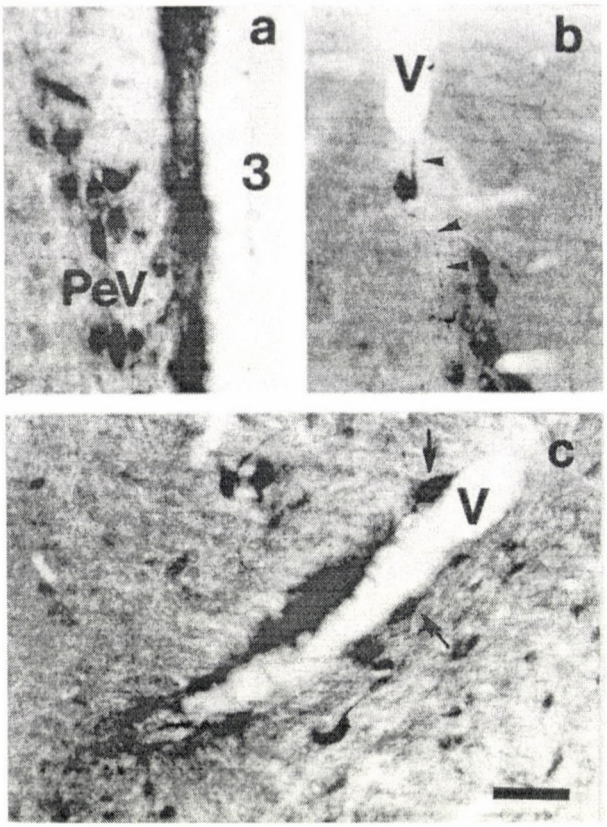

Fig. 2. PACAP ir cells in the anterior portion of the periventricular nucleus *(a)* and in accessory magnocellular nuclei surrounding blood vessels *(b* and *c).* In *b* a neuron sends processes to the wall of vessels indicated by arrowheads. In *c* PACAP ir cells are elongated and positioned parallel to a vessel indicated by arrows. PeV = periventricular nucleus, V = vessel, 3 = the third ventricle. Scale bar is 50 μm

Effect of surgical enucleation on the hypothalamic C-fos IR

In the hypothalamus 2 hours after the removal of the eyes C-fos ir cells were observed in the ventromedial part of the SCH (Fig. 4a), in the dorsal part of the PV (Fig. 4c), and in an area dorsal to the SO (not shown). In these nuclei of intact rats only a few cells exhibited C-fos IR (Fig. 4b and d).

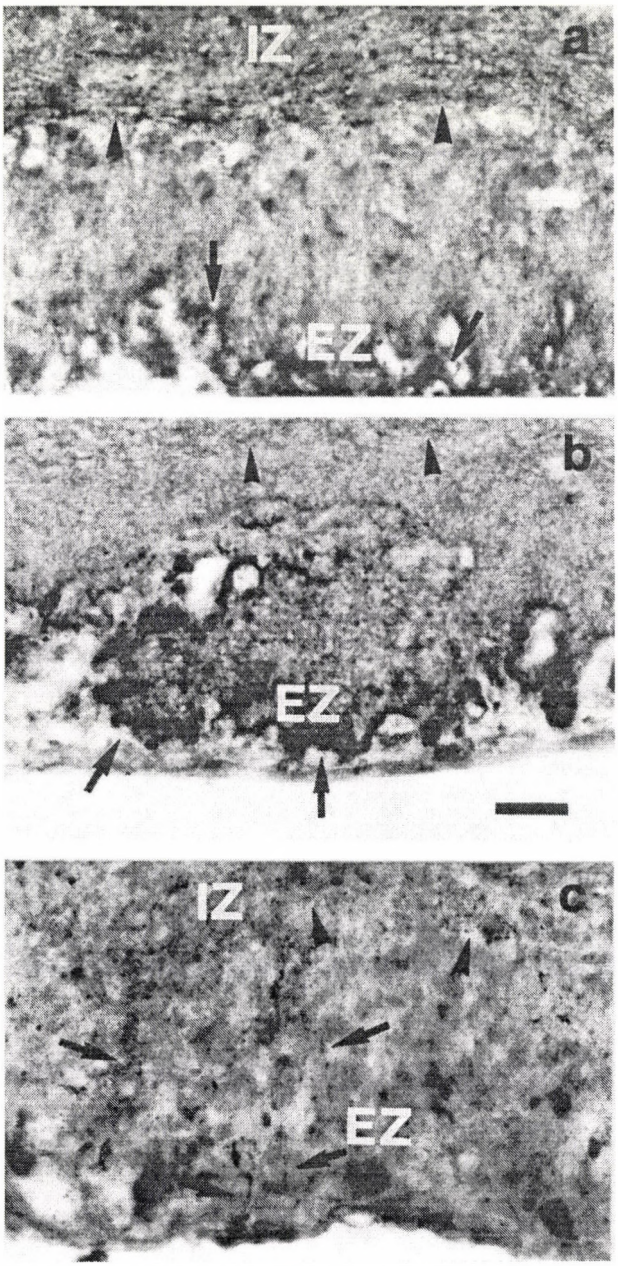

Fig. 3. PACAP ir fibers in the median eminence of a surgically enucleated *(a* and *b)* and a MSG-treated rat (c). In *a* and *b* a dense PACAP ir terminal plexus is seen in the EZ indicated by arrows besides the characteristic localization in the IZ indicated by arrowheads. In c rough PACAP ir fibers are seen in the IZ indicated by arrowheads and rough, beaded fibers penetrate downwards in the EZ indicated by arrows. EZ = external zone, IZ = internal zone. Scale bar is 50 μm

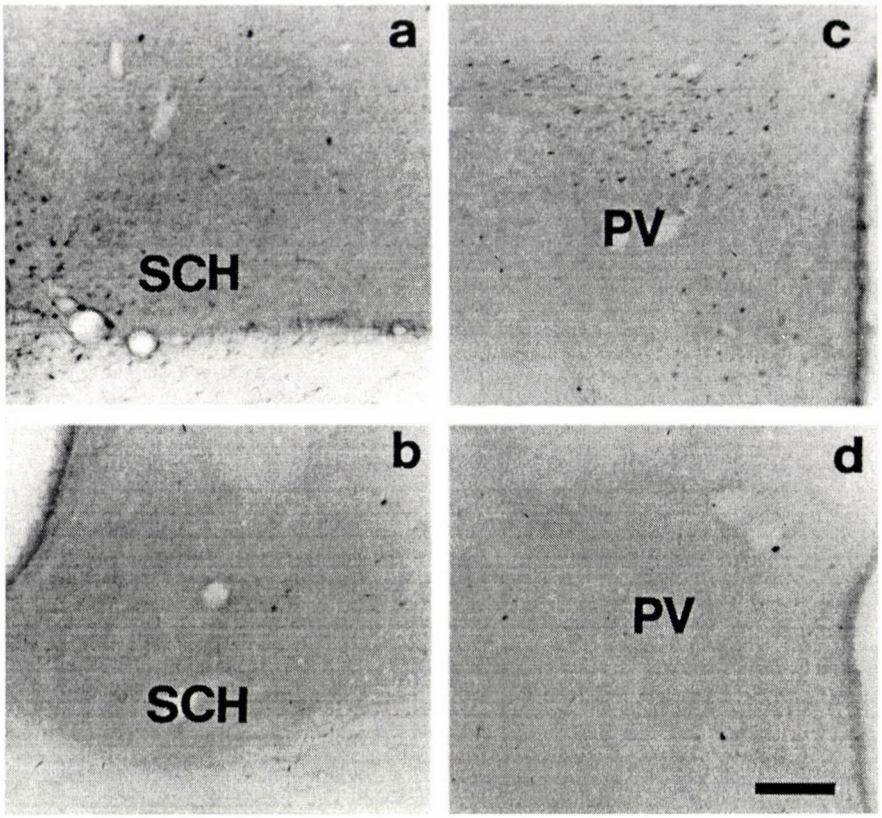

Fig. 4. C-fos ir cells in the ventromedial part of the SCH (*a*) and in the dorsal and periventricular parts of the middle level of the PV (*c*) of a rat bearing surgical enucleation. Only a few C-fos ir cells are seen in the SCH *(b)* and PV (*d*) in an intact rat. Scale bar is 100 μm

DISCUSSION

Our results clearly show that the lack of the light impulses (due to a surgical or chemical enucleation) influences the activity of special groups of the hypothalamic neurons either causing the appearance of C-fos IR or the accumulation of PACAP ir material. In our other studies (Lakatos et al., unpublished data) we have demonstrated that there are groups of PACAP

neurons in the ventral magnocellular part and in the periventricular region of the PV of rats which are hypophysiotrophic. In intact colchicine treated male rats only these neurons send their axons or axon collaterals to the portal capillaries where they may release PACAP into the portal circulation (Dow et al., 1994). It is possible that there is an interaction between the activated neurons, which exhibit C-fos IR after enucleation, and those which produce PACAP. The hypophysiotrophic PACAP neurons may release more PACAP in the portal circulation than in intact rats. We have also demonstrated that ten days after the enucleation the PACAP content measured by radioimmunoassay (RIA) in the PV and in the ME is twice than in intact rats (unpublished data).

Many data are available in the literature that light impulses activate neurons in the ventrolateral part of the SCH of rats (Rea, 1989) which is the terminal field of the retinohypothalamic (Moore and Lenn, 1972; Hendrickson et al., 1972) and geniculohypothalamic pathways (Card and Moore, 1989). In our model we have studied the effect of the lack of light impulses and we have found activated neurons in a different localization that is C-fos ir neurons were observed in the ventromedial part of the SCH, in the dorsal and periventricular parts of the PV and in an area dorsal to the SO. It seems that the light and the lack of the light impulses influence a different population of the hypothalamic neurons. Although Teclemariam-Mesbach et al. (1995) has demonstrated in golden hamsters that not 2 but 3 hours after the beginning of the activity period during the subjective night light impulses (15 min, 100 lux) induced the appearance of the C-fos IR in the SCH in similar localization to that what we have found in enucleated animals with 2 hour survival time. In our immunohistochemical and RIA studies enucleation enhanced the PACAP IR in the magnocellular hypothalamic nuclei and in the ME. These data well correlate with those demonstrated by Takahashi et al. (1989). In their experiment constant darkness enhanced the VIP IR in the hypothalamus. It was also demonstrated that the mRNA level of VIP fluctuated during the photoperiods. During the dark period the level is higher than during the light period (Stopa et al., 1988; Albers et al., 1987).

The regulatory role of PACAP in the neuroendocrine mechanism is well established. The effect of PACAP was studied in *in vitro* and *in vivo* systems.

In *in vitro* studies PACAP stimulated the adenylate cyclase activity in static anterior pituitary cell culture (Miyata et al., 1989), in corticotrop and melanotrop cell cultures. In the latter experiment the adenylate cyclase activation was associated with hormone release (Koch and Lutz-Bucher,

1992). In a gonadotrop cell line PACAP potentiated the effect of LHRH on LH release (Culler and Patschall, 1991). Studied by a reverse hemolytic plaque assay PACAP stimulated GH but inhibited the prolactin release (Nagy et al., 1993). In a superfusion system PACAP moderately enhanced the GH, PRL, LH, and ACTH release but did not influence the FSH and TSH ones (Miyata et al., 1989).

In *in vivo* studies the effect of PACAP seems to depend on the route of administration, on the species and the sex of the animals used. Intravenous (iv) administration of PACAP enhanced the plasma prolactin level in lactating rats four hours after separating from their pups. In this refracter stage PACAP was the only factor which stimulated the prolactin release (Nagy et al., 1993). In our previous experiment iv administration of PACAP in rats did not influence the ovulation administered before the critical period of the proestrous stage or was not able to prevent the blocking effect of Nembutal; however, icv injection blocked the expected ovulation (Köves et al., 1996b). In male rats not a single injection, but an intraarterial infusion of PACAP elevated the plasma LH levels (Leonhardt et al., 1992). In ovariectomized ewes icv administration of PACAP decreased the LH amplitudo and pulse frequency (Sawanjagoren and Curlewis, 1994).

Our results support the view that besides the other members of the secretin family, such as VIP and GRP, PACAP is also involved in the regulation of the hormonal rhythms. It is possible that PACAP is one of the factors which mediates the effect of photoperiodic inputs to the endocrine hypothalamus.

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Research report

LESIONING OF THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS INHIBITS ETHER-INDUCED ACTH BUT NOT PROLACTIN RELEASE

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Summary: Stress mediators, CRF-41 and vasopressin known to be synthesized in, and released from the parvocellular neurosecretory neurons of the hypothalamic paraventricular nucleus (PVN) are essential to release adrenocorticotropin (ACTH) in response to stress. In addition, suckling-induced prolactin (PRL) release also depends on the integrity of the PVN. In the present study, ether stress-induced adrenocorticotrop hormone (ACTH) and prolactin (PRL) release was studied 2, 5 and 42 days after placing lesions in the hypothalamic paraventricular nucleus (PVN) of male rats. Ether-induced ACTH secretion was strongly inhibited 2 and 5 days after lesions whereas 6 weeks later the lesion induced inhibition was fading. In contrast, PVN lesion failed to inhibit ether-induced PRL release at any time studied. The results suggest that contrary to previous suggestions the peptidergic neurons essential for stress-induced PRL release are outside the PVN.

Keywords: Prolactin, ACTH, Prolactin releasing factor, PRF

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INTRODUCTION

The hypothalamic paraventricular nucleus (PVN) is believed to integrate visceral and endocrine functions (19, 22) and to mediate a number of hormonal responses, such as adrenalectomy or stress-induced ACTH release (5, 10), cold-induced TRH release (3) and suckling-induced prolactin (PRL) release (8).

The PVN contains a number of peptides such as thyrotropin releasing hormone (TRH) (9), vasoactive intestinal polypeptide (VIP) (12), polypeptide histidine isoleucine-27 (PHI) (4), oxytocin and arginine vasopressin, all endowed with PRL releasing activity under specific experimental conditions (7, 17); for review see (19). Most, if not all of these peptides are also found in neural projections to the stalk-median eminence (SME) or the neural lobe, they are present in the hypophysial portal circulation and thus are possible candidates for mediators of stress-induced PRL release.

In male rats the stress-induced release of PRL (16) is triggered by activation of PRFs (19, 20) probably originating in the anterior hypothalamus (6), although the role of posterior pituitary in providing PIFs and PRFs has also been suggested (15). A role in stress-induced PRL release for the peptidergic neurones in the PVN and their projections to the SME received support from the findings showing that PRL release induced by ether, immobilization and 5-hydroxy-l-tryptophane was blocked by electrolytic lesions of the PVN.

Since inhibition of ether-induced ACTH release has been shown (11, 12) to fade four weeks after placing PVN lesions, ether-stress induced PRL release has been studied at various times after lesioning. The results question the importance of the PVN in stress-induced PRL release.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 220-300 g were purchased form LATI (Gödöllő, Hungary) and were acclimatized for at least 1 week to experimental rooms with controlled temperature (24±1 °C) and light (on 6:00-19:00), and were given pelleted rat food and tap water ad lib.

Paraventricular lesions were performed as described earlier (10, 11) with a rotating knife destroying an inverted cone of tissue centred over the PVN. Briefly: the rats were anesthetized with pentobarbitone (40 mg/kg b.w.) then placed into stereotaxic frame. The skull was adjusted to flat position with the nose 11° down. The knife has been lowered to the base of the brain and rotated in 360°. For sham-operation the knife was lowered, but not rotated.

Cannulation and sampling. Indwelling silastic venous cannula were placed into the right atrium in pentobarbital anesthesia. When collecting blood 2 days after PVN lesioning, the cannula was placed 2 days before the stereotaxic lesioning. When sampling was 5 days, or 6 weeks after PVN lesions, the venous cannulation was performed 2 to 3 days before sampling. After cannulation the rats were housed in individual cages. At least 3 hours before the first blood sampling an extension cannula was attached to the indwelling cannula and brought outside the cage for sampling without disturbing the rat. Blood was withdrawn before, 3, 15, 30 and 60 min after exposure to ether. The rat was placed in a closed jar with saturated ether vapours until anaesthetized (about 1 min) and then anaesthesia was maintained up to 3 min with a nose cone with ether-soaked cotton wool. Blood was collected on 20% EDTA in plastic tubes kept in an ice-bath, centrifuged at 4°C, and the plasma stored at -20°C until hormone measurements.

Radioimmunoassays. Plasma PRL concentration was measured using material kindly supplied by the NIADDK Rat Pituitary Hormone Distribution Program. Assay characteristics were: sensitivity: 2 ng/ml, intra-assay variation 9%, inter-assay variation: 15%.

ACTH was measured from unextracted plasma samples by direct radioimmunoassay developed in our laboratory: the ACTH antibody (#8514) directed against the midportion of h-ACTH1-39 molecule, was raised in rabbit. Intra- and interassay coefficients were 4.7 and 7% respectively.

Statistical analysis. Plasma ACTH and PRL levels are known to have log-normal distribution, therefore hormonal measurements were analysed after transformation to logarythms. One and two-way analysis of variance for repeated measures were performed with CRUNCH statistical software (CRUNCH Software Co. Oakland CA, USA).

Histological examination. The lesion has been checked at the end of each experiment. The brains were fixed in formaline: picric acid 1:3 fixative then embedded in paraffin. 1 out 5 series of 10 mm sections have been stained for neurosecretory material according to Brinkman and Bock (1). Only rats with complete destruction of the PVN were included.

RESULTS

Paraventricular nucleus lesion. On histological examination the lesioned group all of parvo- or magnocellular portion of the PVN was eliminated. The lesion also damaged the anterior hypothalamic nucleus. Anteriorly, the knife reached the ventral part of the anterior commissure, destroyed the posterior

parts of the median preoptic nucleus, the median preoptic area and the periventricular hypothalamic nucleus. Lateral the lesion extended to the fornix. Behind the PVN, part of zona incerta, anterior tip of the dorsomedial- and the dorsal part of ventromedial nucleus as well as the dorsal hypothalamic area were sometimes also destroyed.

Plasma ACTH. In sham-operated controls, plasma ACTH responses to ether exposure was of similar magnitude at 2 or 5 days or 6 weeks after the operation. There was significant blockade of the rise of plasma ACTH 2 days after PVN lesion, at 5 days after the operation a small, but significant rise was observed, and at 6 weeks after PVN lesioning a substantial rise was observed and only at 15 min after the beginning of ether exposure was a significant difference from the control (Fig. 1a, b, c).

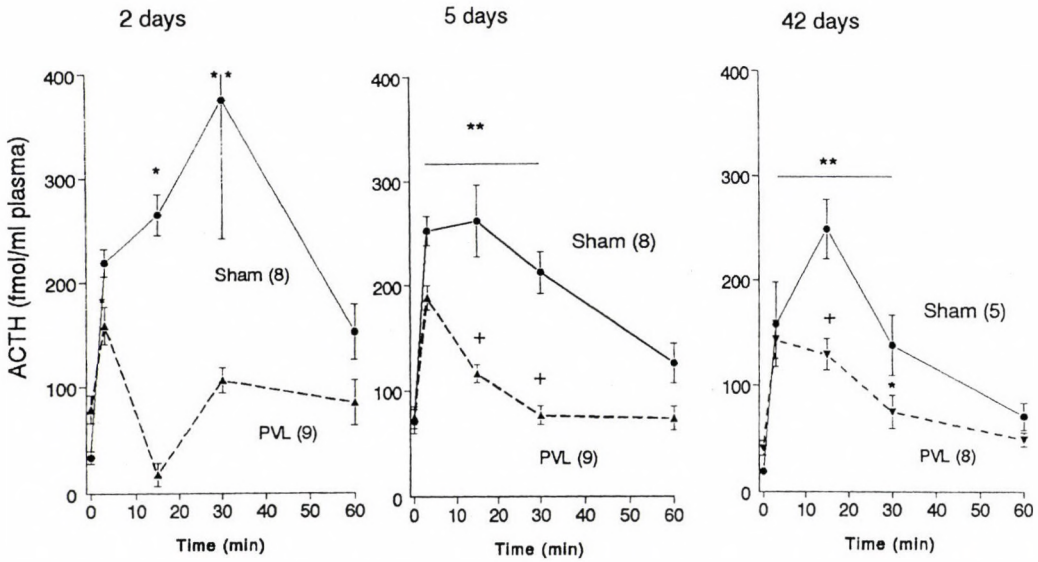

Fig. 1. The effect of 3 min ether exposure on plasma ACTH levels in sham-operated and paraventricular lesioned (PVL) rats. a. 2 days, b. 5 days, c. 6 weeks after the operation.
* $p<0.05$, ** $p<0.01$ compared to 0 time point

Plasma PRL. In contrast to stress-induced ACTH release, the ether induced PRL release (Fig. 2a, b, c) was rather small at 2 and 5 days after sham-operation and the small response was not depressed by the PVN lesion. 6 weeks after PVN lesion placement, there was a tendency to

decrease only at 30 min after ether exposure, but this was not statistically significant. At this time point the scatter of the sham-operated group was very large and the high values contributed to the sustained PRL level at this time point in the sham-operated group. Because of this anomaly, we performed another experiment at 6 weeks postoperative time interval, where the PRL level at the 30 min time point was already smaller than at 15 min, and there was not even a tendency to decreased PRL response to ether in the PVN lesioned group.

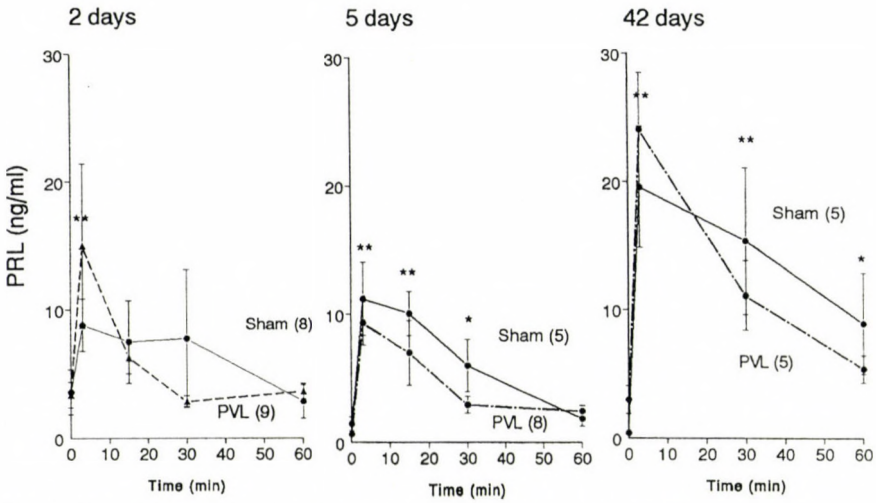

Fig. 2. The effect of 3 min ether exposure on plasma PRL levels in sham-operated and paraventricular lesioned (PVL) rats. a. 2 days, b. 5 days, c. 6 weeks after the operation.
* $p<0.05$, ** $p<0.01$ compared to 0 time point

DISCUSSION

Dissociation of the ether stress-induced ACTH and PRL secretion in paraventricular nucleus-lesioned male rats was found in this study: PVN lesions produced by a rotating knife did not result in an inhibition of ether stress-induced PRL release, while the expected inhibition of plasma ACTH release was observed.

We have repeatedly found no change in PRL responses in rats with surgical PVN lesions in a number of experiments performed at different

periods over a year; thus it seems to be a reproducible experimental result. The differences between the present findings and those of Minamitani et al. (13) might be due to any or a combination of the several technical details which are different in the two studies. We have used a rotating stereotaxic knife (5) which causes a mechanical, ischemic lesion while Minamitani et al. (13) used electrolytic lesions which may leave metal deposits around the lesioned area and thus may influence the function of the neural tissue surviving around the lesion. The postoperative interval in their study was about 2 weeks and the rats were given replacement doses of thyroid hormones to normalize thyroid hormone supply which is known to decrease following PVN lesioning. However, lack of thyroid hormones is expected to decrease function at the pituitary level, i.e. GH and PRL synthesis and thus may cause inhibition rather than lack of inhibition as observed in our present study. It seems unlikely that the postoperative time is the crucial difference since the survival times employed in the present study bracket those in the previous study. Since none of these factors seem to explain satisfactorily the different findings; we suggest that the difference ought to be due to small details in lesion morphology outside the PVN. Both the present study and that of Minamitani et al. used rats with complete removal of the PVN, however, the hypothalamic regions partially lesioned must have been different in the conflicting studies. Further studies are required to pinpoint the source of the disagreement.

Although the PVN itself is not indispensable for stress-induced PRL release, transection of other structures in the anterior hypothalamus with anterior hypothalamic deafferentation block stress-induced PRL release (6). Further studies with smaller lesions may be necessary to map these cells in the hypothalamus of the rat.

Two recent publications reported inhibition of serotonin-induced PRL release after PVN lesions (2, 17); however, only PRL release due to 5HT2C agonist was inhibited and 5 HT1A agonist injection elicited significant PRL release in PVN lesioned animals. Taken together with the inhibition of suckling-induced PRL release in the PVN lesioned rats (8) these reports also point to the complex nature of the control of PRL secretion.

The present findings cast strong doubt on the hypothesis that any of the peptidergic neurons in the PVN and containing TRH, VIP, PHI of oxytocin may play an essential role in the ether stress-induced release of PRL, since these neurons were eliminated and release of PRL followed the exposure to ether vapours remained normal. This conclusion does not refer to suckling-induced release of PRL since we have re-evaluated the study of Nagy et al., (8) and the inhibition of suckling-induced PRL release was observed also in this laboratory (unpublished data).

The finding of a strong inhibition of ether induced rise of plasma ACTH at 2 and 5 days after PVN lesion placement, and a restoration of the responsiveness at 6 weeks after lesioning is consistent with previous findings from this and other laboratories (2, 5, 10, 11); and suggest that CRF-41 and AVP of PVN origin is a dominant mediator of ACTH release under normal conditions but with time after elimination of CRF/AVP neurons in the PVN other neurons and/or other factors may take over the control of ACTH release and adrenocortical function under stressful conditions (14).

In conclusion, the present result failed to support a role for a PRF of PVN origin during stress-induced PRL release and show a dissociation of stress- and suckling-induced release of PRL.

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Research report

MELATONIN SECRETION OF THE RAT PINEAL GLAND IN RESPONSE TO NOREPINEPHRINE IN DIFFERENT TYPES OF THE ANOVULATORY SYNDROME

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Summary: Earlier experimental results show the role of altered pineal function in the development of the constant estrous-anovulatory (CEA) state induced by neonatal androgen sterilization (NA) or in the spontaneously developed anovulatory syndrome in the aging rat (AG - CEA state). Since norepinephrinergic (NE) innervation of pineal gland represents the main stimulus for melatonin secretion in mammals, in the present experimental series, the reactivity of pineal tissue to NE was investigated in *in vitro* perifusion system in rats suffering from NA - CEA or AG - CEA state, using the model of pineal body deprived from neural inputs. The data indicate that the ' melatonin deficiency ' observed in the 2 types of anovulatory syndrome (NA - CEA and AG - CEA states) is due to disturbance of norepinephrinergic innervation of the pineal gland rather than to deficient secretory capacicity of pineal tissue.

Keywords: melatonin, pineal gland, aging, perinatal age, androgen sterilization, anovulatory state

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Dedicated to Professor Béla Halász for his 70th birthday.

INTRODUCTION

Experimental results show the decline in pineal melatonin secretion observed in the period of aging (Sack et al. 1986; Rosenzwaig et al. 1987). According to our earlier studies, the modification of melatonin levels was influential for the spontaneously developed constant estrous - anovulatory (AG - CEA) syndrome in aging rat (Rúzsás et al. in press).

On the other hand, the excess or deficiency of pineal hormones was also proved to modify the development of neonatal androgen sterilization (NA-CEA) syndrome (Rúzsás et al. 1986, 1996), in association with insufficient activity of monoaminergic neurons (Timiras et al. 1980; Walker 1983).

The importance of intact sympathetic innervation in the maintenance of pineal melatonin secretion was demonstrated by earlier data (Wurtman et al. 1964). The stimulatory role of noradrenergic innervation in the regulation of melatonin secretion of the pineal gland is well documented both in *in vivo* and *in vitro* experiments (Zatz 1981; Klein 1985; Klein et al. 1983). Further data show the action of noradrenergic terminals on specific adrenergic receptors located on pinealocyte's membrane in the pineal tissue (Cardinali et al. 1987).

Data related to anovulatory rats show the insufficiency of catecholaminergic neurons in the aging rat (AG - CEA rat; Walker et al. 1980). In NA - CEA rats, decreased activity of brain noradrenergic receptors was also registered (Greenberg and Weiss 1978; 1984).

On the base of these data, in the present study, perfomed in *in vitro* perifusion system, the changes were investigated in the sensitivity of pineal tissue to norepinephrine (NE) in 2 types of anovulatory sterility syndromes: in rats suffering from AG - CEA or from NA - CEA syndromes.

The use of *in vitro* cultures provides opportunity to study the structures involved in the neuroendocrine regulation under conditions of 'deafferentation'. In a long-term perifusion system, under *in vitro* circumstances, pineal gland keeps its secretory capacity in spite of deprivation of innervation, in a milieu free from hormonal and neuronal influences which cannot be excluded in *in vivo* condi tions (Mess et al. 1991). Since this model is appropriate for investigations of action of different neurotransmitters, in the present experiments, norepinephrine (NE) induced stimulation was registered on pineal glands of CEA rats in *in vitro* system, according to the method described by Rékási et al. (1991).

MATERIALS AND METHODS

Experimental animals and general conditions

Female albino rats of Wistar strain were used, kept on standard photoperiod (14:10) and in standard laboratory conditions.
Experimental series:

1. Rats suffering from NA - CEA syndrome: Neonatal androgenization was performed by a single subcutaneous injection of 250 μg testosterone propionate (TP), injected on the 3. day of postnatal life. Corresponding control groups received the solvent only, given in the same conditions. Vaginal smears were daily recorded from the time of vaginal opening and registered up to the end of the experimental period. Sacrifice of the animals was performed in the age of 11 month of life. Pineal gland was immediately removed at sacrifice and it was submitted to *in vitro* perifusion studies.
2. Rats suffering from AG - CEA syndrome: Animals over 22 months of age showing constant estrus in the vaginal smears daily recorded throughout the experimental period were selected. Sacrifice and removal of pineal gland were performed and pineals were submitted to *in vitro* investigations, similarly to the previous series. Young (11 months old) animals served as controls of the old AG - CEA rats.

In vitro perifusion system

Culture preparation from pineal glands of rats used in the experiments were prepared according to the method described by Rékási et al. (1991), Mess et al. (1991). Pools were formed by 4 pineals of the same experimental group. Explanted pineals were perfused at low rate of 0.1 ml/min at room temperature for 4 days. Medium passing through the pineal tissue was collected by a fraction collector. Kinetics of melatonin secretion in response to NE (0.1 - 10 μM) administered to the medium was registered by dose-response curves, according to the protocols established by Rékási et al. (1991). Melatonin concentration released into the medium from pineal tissue at different intervals following administration of NE was measured by radioimmunoassay according to the method elaborated by Rékási et al. (1991). Mathematical analysis of the results obtained by perifusion technique

was performed by computer programme developed by Csernus and Schally (1990).

RESULTS

1. Effect of norepinephrine (NE) on melatonin secretion of the perifused pineal tissue from NA - CEA rats: (Figures 1A and 1B)

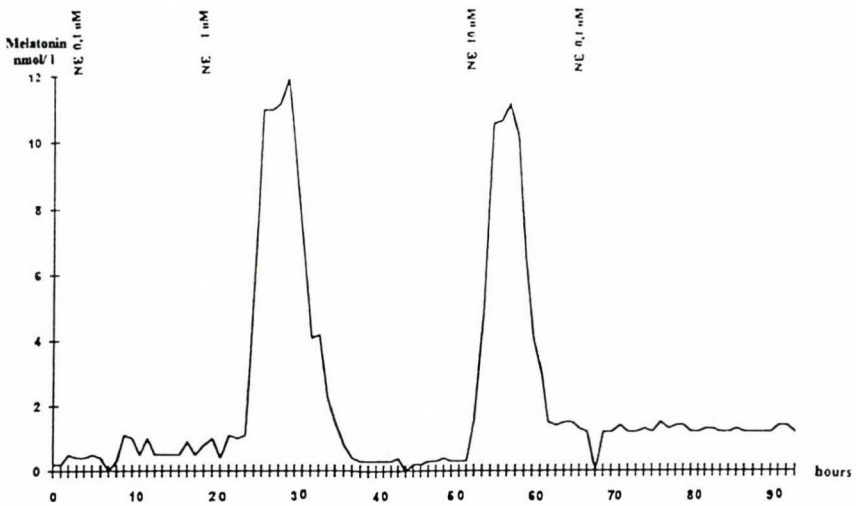

Fig. 1A. Effect of NE on melatonin release in perifusion system from pineal gland of non-androgenized (pair fed control) rats

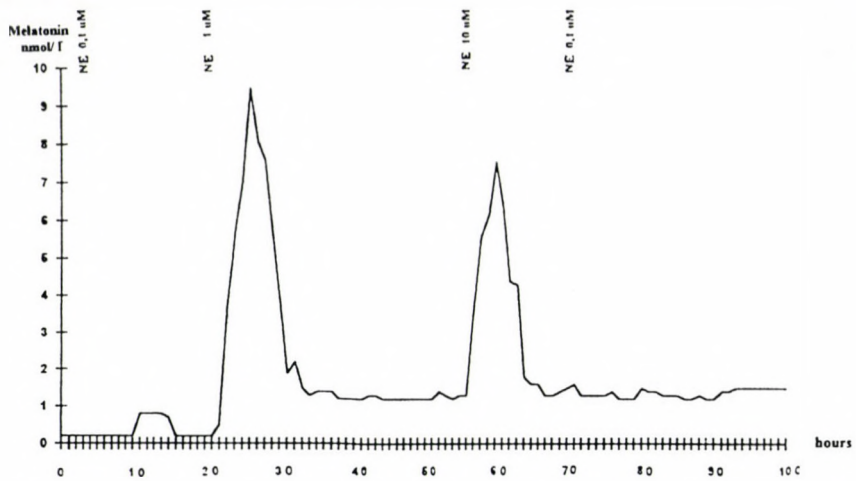

Fig. 1B. Effect of NE on melatonin release in perifusion system from pineal gland of rats suffering from NA-CEA syndrome

2. Effect of norepinephrine (NE) on melatonin secretion of the perifused tissue from AG - CEA rats: (Figures 2A and 2B)

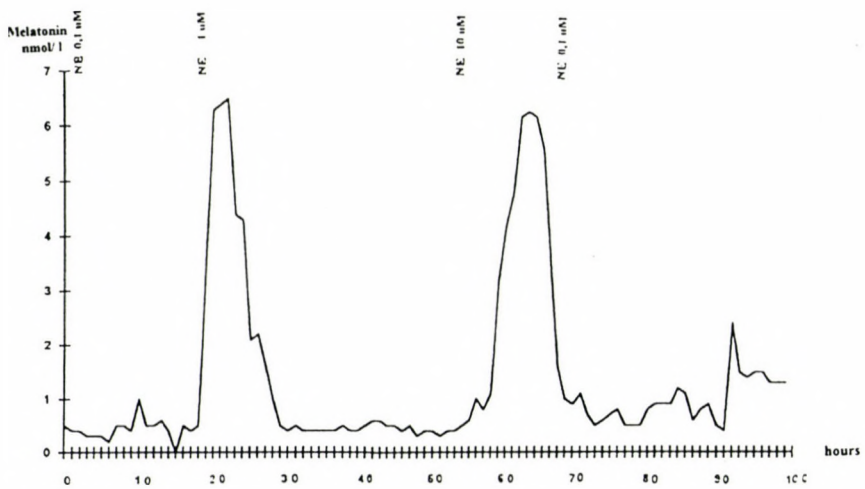

Fig. 2A. Effect of NE on melatonin release in perifusion system from pineal gland of young (11 months old) rats, controls for AG - CEA rats

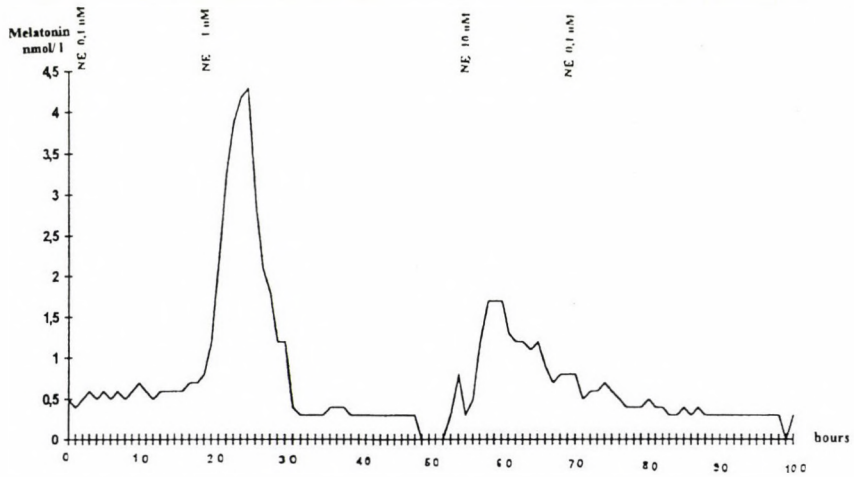

Fig. 2B. Effect of NE on melatonin release in perifusion system from pineal gland of aging (22 months old) rats suffering from AG - CEA syndrome

1. NA - CEA rats

The lowest dose of NE (0.1 μM) was equally ineffective in the control and in androgenized animals. Slightly decreased melatonin output in response to 1 μM dose of NE injection was detectable in the androgen - sterilized (NA - CEA) group. This decrease was even more pronounced at the highest dose of NE: peak value of melatonin reached 11.2 nmol / l in controls, compared to 7.6 nmol / l peak value of the NA - CEA animals. (Figs 1A and 1B) The results demonstrate that pinealocytes are able to react to NE also in androgenized (NA-CEA) state, although, with lower amplitude.

2. *AG - CEA rats*

Similarly to the NA - CEA animals, 0.1 μM dose of NE was ineffective both in aged and young animals. After 1 μM dose of NE, a moderate difference was detected between the 2 experimental groups. The decreased reactivity of pineal melatonin secretion in AG - CEA animals to 10 μM dose of NE was much more pronounced in AG - CEA animals than in the androgenized (NA -

CEA) group. (Peak value in control rats: 6.1 nmol / l; in AG - CEA animals: 1.8 nmol / l; Figs 2A and 2B).

DISCUSSION

As it is documented by data of the literature, norepinephrinergic innervation of the pineal gland represents the primary stimulus for melatonin secretion (Klein and Weller 1970). The crucial role of norepinephrinergic neural transmission in maintaining melatonin releasing capacity of the pineal gland under *in vitro* conditions is evident from experimental findings (Klein and Berg 1970). Our present data show that the responsiveness of pineal tissue to norepinephrinergic stimuli remains still preserved in both investigated types of the anovulatory syndrome, although, in minor degree.

Perifusion of the explanted pineal tissue with increasing concentrations of NE resulted in a dose dependent melatonin release also in anovulatory sterile rats. However, melatonin response to smaller dose (1 μM) of NE was reduced in both types of CEA syndrome. This reduction was even more expressed using higher dose of NE (10 μM). AG - CEA animals revealed an even more pronounced reduction in melatonin response compared to NA - CEA animals.

Our earlier data demonstrated that perinatal administration of melatonin could prevent the development of the neonatal androgen sterilization syndrome (Rúzsás et al. 1996). On the base of the present results, it seems presumable that the reduction in perinatal melatonin secretion contributing to development of NA - CEA state is not the consequence of the lesion of the synthesizing capacity of the pinealocyte itself, but it might be rather due to the lesion of norepinephrinergic nerve terminals carrying the impulses to pineal gland. Our present results suggest that the influence of pineal hormones exerted on the development of the gonadotroph hormone releasing mechanism in the perinatal age might be connected with the dysfunction of the catecholaminergic innervation of pineal gland disturbed by neonatally administered andogen hormones.

In the aging rats suffering from AG - CEA syndrome, the reduction in the amplitude of melatonin secretion was described by several authors (Iguchi et al. 1982; Sack et al. 1986). In the present studies performed on 'deafferented' pineal tissue deprived from the neurotransmitters, reactivity of pinealocytes to exogenous NE was preserved but reduced, compared to the reactivity of young animals. According to our present results, the explanation

for the reduction in the amplitude of melatonin secretion observed in aging, is not due to degeneration of pineal tissue, but it appears to be the consequence of disturbed norepinephrinergic innervation.

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Research report

CORTICOTROPIN-RELEASING HORMONE EXPRESSION IN SUPRAOPTIC NEURONS AFTER BILATERAL LESIONING OF THE PARAVENTRICULAR NUCLEUS IN RATS

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Summary: *In situ* hybridization histochemistry was used to demonstrate corticotropin releasing hormone (CRH) mRNA expression in the supraoptic nucleus of rats. Labeled cells with a range of grain densities were located mainly in the dorsal area of the nucleus. Long-term (6-weeks) lesioning of the hypothalamic paraventricular nucleus, which eliminates the major CRH pool from the hypothalamo-hypophyseal system resulted in an increased CRH mRNA density within supraoptic neurons compared to within sham-operated rats. Adrenalectomy failed to effect CRH mRNA content either in sham-operated or paraventricular-lesioned animals. CRH gene expression in supraoptic neurons of long-term paraventricular lesioned rats may exhibit a compensatory mechanism in the hypothalamus by which supraoptic neurons can take over some of the functions of the lesioned paraventricular CRH cells.
Keywords: supraoptic nucleus, adrenalectomy; paraventricular lesions; in situ hybridization histochemistry, corticotropin releasing hormone

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INTRODUCTION

The rat supraoptic nucleus consists of different cell types which express a variety of neuropeptides. The principal neuropeptides are arginine-vasopressin (AVP) and oxytocin (OX). The AVP-containing neurons are mainly situated in the ventral part of the nucleus whereas the OX-containing cells are confined to the dorsal side. The OX-containing neurons co-express cholecystokinin (Vanderhaeghen et al., 1981) and corticotropin releasing hormone - CRH (Burlet et al., 1983; Sawchenko et al., 1984). In unstressed animals, the immunostainable CRH, as well as CRH mRNA (Young, 1986, 1990) levels are relatively low in the supraoptic cells.

The major pool of CRH-containing neurons is in the parvocellular paraventricular nucleus. After bilateral lesions of the nucleus, reduced CRH content (about 10-15% of the control value) is found in the median eminence (Bruhn et al., 1984; Makara et al., 1986). Combined surgical and immunohistochemical studies indicate that CRH immunoreactive fibers in the median eminence of long-term paraventricular lesioned rats arise in the neurons of the supraoptic and perifornical nuclei (Palkovits et al., 1991). In the present study, the effect of long-term paraventricular lesions on CRH gene expression in the supraoptic neurons was investigated in rats with or without adrenalectomy by using *in situ* hybridization histochemistry.

MATERIALS AND METHODS

Adult, CFY strain male rats (250-280 g body weight) were kept under standard laboratory conditions: lights on between 6 AM and 6 PM, room temperature (23 ± 1°C), rat chow and tap water provided *ad libitum.*

Experimental protocol

The animals were divided into four groups (6 animals per group):

1) Bilateral lesions of the hypothalamic paraventricular nucleus (PVN): The animals were anesthetized with sodium pentobarbital (40 mg/kg) and the heads were fixed in a Kopf stereotaxic instrument with a 10° nose-down position. The PVN lesions were performed with a triangular-shaped rotating knife (Makara et al., 1981, 1986) with a vertical penetration in the midline, 1.8 mm caudal to the level of the bregma.

2) Sham-operated control rats: A similar surgical procedure as in group #1 was performed without lowering the knife into the hypothalamus.

3) Bilateral adrenalectomized rats: The surgery was performed *via* a dorsal approach.

4) Five weeks after PVN lesion, animals were adrenalectomized bilaterally.

Six weeks after PVN lesions, and 5 days after adrenalectomy, the rats were sacrificed by decapitation. Brains of rats from groups #1 and #4 were selected, and only the complete bilateral lesions were used for *in situ* hybridization histochemistry.

In situ *hybridization histochemistry*

After the rats were killed by decapitation, the brains were removed and frozen on dry ice. They were stored at -80 °C until 12 μm frozen sections were cut in a cryostat and thaw-mounted onto gelatin-coated slides. The sections were stored at -80 °C until warmed for processing for detection of CRH transcripts as previously described (Young, 1986, 1992). The sections were dipped into Kodak NTB3 nuclear emulsion and exposed for 2 months.

Determination of plasma ACTH and corticosterone

Plasma ACTH was measured by direct radioimmunoassay (Kovács and Makara, 1988). The rabbit antibody is directed against the midportion of h-ACTH(1-39) molecule. Intra- and interassay coefficients were 4.7 and 7.0%, respectively. Plasma corticosterone was measured by radioimmunoassay without extraction, the interference of plasma transcortin was eliminated by inactivating transcortin at low pH. The sensitivity of the assay was 0.1 pmol/tube; intra- and interassay variations were 6.4 and 23.8%, respectively.

RESULTS

Concentrations of corticosterone and ACTH in the plasma

Corticosterone (pmol/ml, mean±SEM): sham: 67.9±16.3; PVN-lesion: 45.8±12.9; ADX: 2.7±0.6; PVN-lesion+ADX: 3.5±1.1.

ACTH (fmol/ml, mean±SEM): sham: 24.1±5.5; PVN-lesion: 21.6±4.3; ADX: 698.7±160.5; PVN-lesion+ADX: 88.3±14.9.

In situ *hybridization histochemistry*

In sham-operated rats, CRH mRNA is expressed in low to moderate levels in supraoptic neurons (Fig. 1). Labelled cells are found mainly in the dorsal area of the supraoptic nucleus.

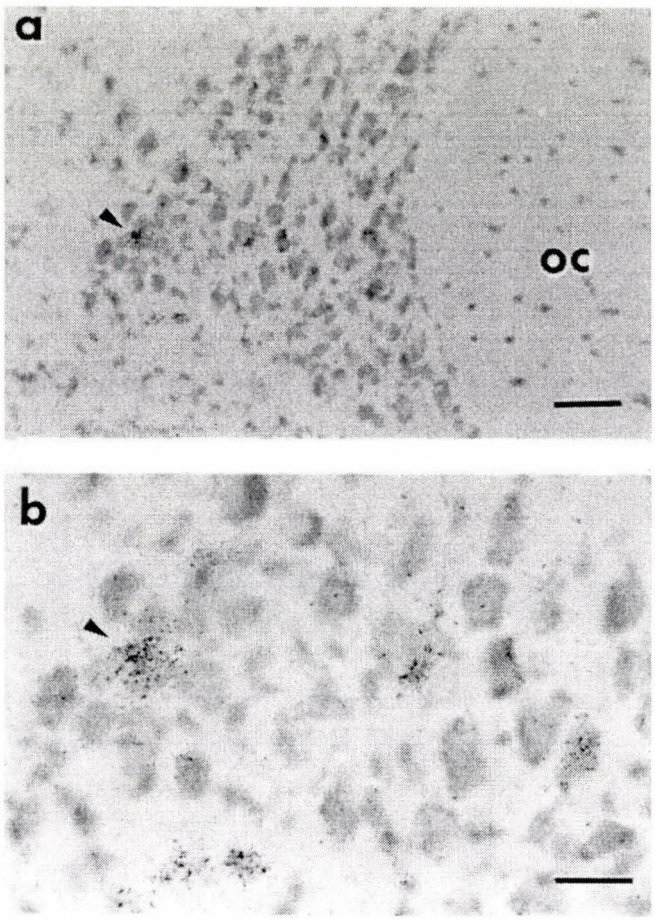

Fig. 1. a - CRH mRNA labeled neurons in the supraoptic nucleus. Fig.b shows cells from Fig.a at a higher power of magnification to demonstrate variable grain density over labeled neurons. (For topographical orientation, one of the same highly labeled cells is pointed to by arrows in Fig. a and b).
oc - optic chiasm. Bar scales: 50 μm (Fig.a) and 125 μm (Fig.b)

Adrenalectomy, which increases CRH mRNA density in the paraventricular nucleus results in minor, if any changes in supraoptic CRH gene expression (Fig. 2b).

Bilateral paraventricular lesions increase CRH mRNA density in the supraoptic nucleus. Several supraoptic cells show prominent labeling of mRNA of CRH (Fig. 2c).

In 6-weeks PVN-lesioned and 5-day adrenalectomized animals, CRH labelling in the supraoptic nucleus is similar to that in PVN-lesioned animals (Fig. 2d): cells show more intense labelling than those in sham (Fig. 2a) or adrenalectomized animals (Fig. 2b).

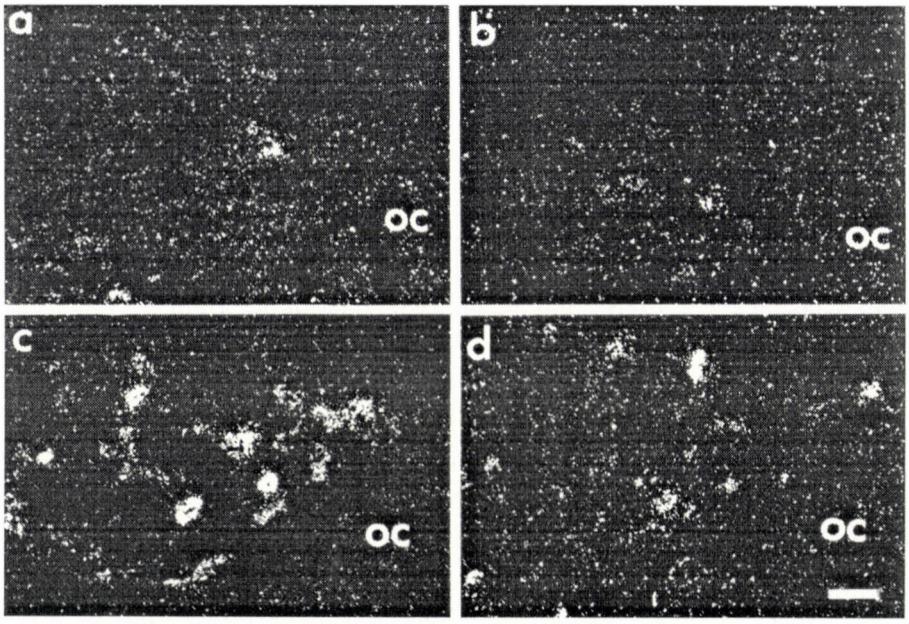

Fig. 2. Effects of 6-weeks paraventricular lesion (c), 5-days adrenalectomy (b) and their combination (d) on CRH mRNA expression in the supraoptic nucleus (a = sham-operated control). Coronal sections. oc - optic chiasm. Bar scale: 50 μm

DISCUSSION

Supraoptic neurons exhibit a relatively low level of CRH mRNA expression in intact rats. They are distributed preferentially in the dorsal and ventromedial parts of the SON in co-localization with oxytocin-containing neurons (Young, 1986, 1991). In the present study, an increased CRH gene expression is observed in these supraoptic neurons 6 weeks after bilateral lesions of the paraventricular nucleus.

CRH-immunoreactive cells are seen in the supraoptic nucleus of PVN-lesioned rats after axotomy (Palkovits et al., 1991). It was hypothetized that some of the supraoptic cells can take over - at least partly - the function of the destroyed paraventricular CRH neurons in the basic and stress-induced release of ACTH from the pituitary. Since CRH is synthesized in the supraoptic nucleus in neurons which co-express oxytocin (Vanderhaeghen et al., 1981; Burlet et al., 1983; Sawchenko et al., 1984), CRH, like oxytocin, may reach the portal circulation.

Oxytocinergic fibers enter the external layer of the median eminence (Vandesande et al., 1977) and oxytocin has been demonstrated in high concentrations in hypophyseal portal plasma (Gibbs, 1984). CRH, whose levels in the median eminence of paraventricular-lesioned animals reach 10-15% of the levels measured in intact or sham-operated rats (Bruhn et al., 1984; Makara et al., 1986), may be partly of supraoptic origin.

The low level of CRH in the pituitary portal blood in paraventricular lesioned rats may activate CRH gene expression in hypothalamic neurons, such as those in the supraoptic nucleus, which are potentially able to transport CRH into the median eminence. This hypothesis is supported by observations that after hypophysectomy, several CRH-immunoreactive neurons could be demonstrated in the supraoptic nucleus (Meister et al., 1990).

Although, the presence of the glucocorticoid receptor in the supraoptic neurons has been demonstrated by Kiss et al. (1988), supraoptic CRH cells may not serve as direct targets of a corticosterone feedback mechanism in the hypothalamo-hypophyseal system. The very low plasma corticosterone levels in adrenalectomized rats (with or without paraventricular lesions) did not elicit CRH mRNA expression in supraoptic neurons. Furthermore, we could not find any correlation between plasma ACTH levels and the density of CRH mRNA in the supraoptic nucleus.

The 'compensatory' activation of CRH neurons in the supraoptic nucleus may represent an ultra-short feedback mechanism in the hypothalamo-hypophyseal system. The depleted CRH level in the pituitary portal veins caused by the removal of the major CRH pool by paraventricular lesions may

activate all of the available hypothalamic neurons which have been enabled to express CRH and axonally are connected to the median eminence.

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Research report

TWO DECADES AROUND THE HYPOTHALAMIC MEDIAN EMINENCE

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From the mid-sixties until the mid-eighties the author worked in fruitful collaboration with Professor Béla Halász. The collaborative work was initiated by the late János Szentágothai soon following his taking over the chair of the Anatomy Department at Budapest Medical School in 1963. Although he wanted to concentrate all his time and effort to the launching of the various subcomponents of the neuroscience program in his new workplace, he still did not want to being detached completely from the hypothalamic studies in his old department in Pécs. He trusted the author who was a young assistant in Budapest to collaborate with Béla Halász who needed morphological tools to prove the distinctness of the hypophyseotrophic area (HA). This partnership was reinforced some years later after Béla Halász became the chairman of the Second Department of Anatomy (1971) and the author joined the same Department in 1972.

The center piece of the morphological studies was from the very beginning the hypothalamic median eminence (ME). Later on the histological analysis was extended towards the adjacent arcuate nucleus and some other regions in the medio-basal hypothalamus. The experimental techniques included the histological methods of the era: Golgi impregnation, fiber degeneration induced by experimental lesions, tract tracing techniques, electron microscopy and immunohistochemistry. All experiments were done on rats.

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Topically the studies could be subdivided into 5 groups: identification of the hyophyseotrophic area with the technique of the experimental degeneration, neuronal and vascular structure of the ME, studies on the hypothalamic arcuate nucleus, axon regeneration in the hypothalamus and description of the LH-RH fiber system.

Identification of the hypophyseotrophic area with the technique of the experimental degeneration

Based on pituitary implantation experiments and surgical deafferentation Halász et al. (1962) described the ventromedial portion of the hypothalamus, including mostly the arcuate and periventricular nuclei as the major if not only source of nerve fibers terminating in the superficial (palisade) zone of the ME. Complete and partial deafferentation of various parts of the hypothalamus was done using the Halász-knife and degenerating nerve terminals were searched in the palisade zone of the ME with the electron microscope (Réthelyi and Halász, 1970). The results could be summarised as follows:

i) unintentional injury of the arcuate nucleus induced massive fiber degeneration in the palisade zone;

ii) anterior deafferentation at the caudal aspect of the optic chiasma resulted in a substantial number of degenerating fragments;

iii) a parasagittal deafferentation across the ventromedial nucleus did not produce significant degeneration in the palisade zone.

It was concluded that the HA was a structural entity, i.e. the overwhelming majority of the axons terminating in the palisade zone of the ME originated from a circumscribed region of the hypothalamus. A significant difference in the delineation of the HA with respect to the origin description has not been dully emphasised in Discussion of the above mentioned paper, namely that the suprachiasmatic and preoptic areas were essential portions of the HA. From the publication of the paper the definition of the HA became equivocal, and this might be one of the reasons of the barrage of critical publications. It will be shown later in the present paper that two technical aspects, the observation of the characteristic middle portion of the ME with the characteristic capillary loops and the neglect of the observation of the palisade zone in the pituitary stalk were to be blamed for the alternate findings of some later authors.

Inspite of all of these difficulties in the interpretation of the findings this paper was frequently quoted for 15 years.

Neuronal and vascular structure of the median eminence

The ME has been known as a collection of various fiber systems arranged in an internal and external zones. These fiber bundles together with the processes of the tanycyte processes formed a three-dimensional lattice (Szentágothai, 1962). Neuronal perikarya were described around the lateral part of the ME adjacent to the arcuate nucleus.

The observation of the toluidin blue stained plastic sections helped us to recognise groups of small size neurons in the subependymal layer of the ME (Réthelyi, 1975). The neurons concentrated towards the caudal portions of the ME. Electron microscopic observations revealed axon terminals synapsing with the neurons. The original interpretation that these neurons were close relatives of the neurons in the arcuate nucleus seems to be valid. The reason why did they fail to migrate laterally together with the bulk of the arcuate neurons and their significance remained unclear.

The structure of the afferent nerve fibers to the ME was studies and the results were described in two papers. In one of them the results of Golgi specimens and experimental degeneration were outlined (Nagy and Réthelyi, 1979). It was concluded that nerve fibers approaching the ME from lateral direction could be followed along the tuberoinfundibular sulcus, sparsely in the very lateral rim of the ME. Practically no degenerated fibers occurred is the middle portion of the ME but they were distributed richly in the pituitary stalk (Fig. 1). It was learned from these experiments that the fibers severed by the lesion degenerate within few hours. These findings helped us to reconsider the interpretation of earlier results concerning the delineation of the HA.

In another study the anterograde transport of HRP stained various components of fibers in and around the ME (Réthelyi and Fockter, 1982). The most interesting findings was the description of the varicose commissural fibers in the subependymal layer that seemed to terminate in the contralateral medial basal hypothalamus. The internal zone of the ME was filled with fiber bundles of smooth contour meandering around oval areas apparently filled with the bulky tanycyte processes. The fibers of the external zone were of varicose character. Close to the internal zone they ran rostro-

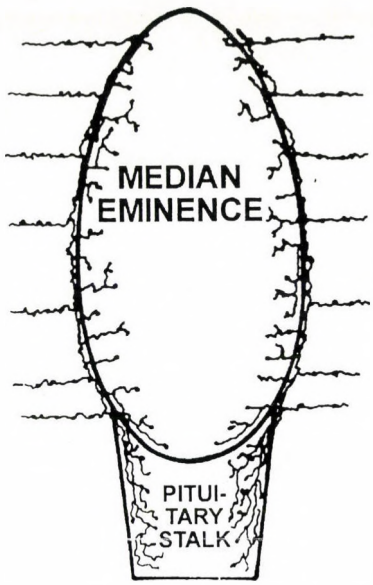

Fig. 1. Summarizing diagram indicating the arrangement of nerve fibers approaching the median eminence from lateral direction. The fine, varicose fibers formed a plexus along the margin of the median eminence (tuberoinfundibular sulcus). Some fibers proceeded more in medial direction and terminated in the palisade zone of the lateral median eminence region. Numerous fibers ran towards and terminated in the pituitary stalk. (From Nagy and Réthelyi, 1979; modified)

caudally, while more ventrally they surrounded the capillary loops in a zigzag pattern. Fig 2 shows schematically the distribution of the fibers in the ME.

The surface area of the neurovascular contact zone (i.e. the contact surface between the nerve terminals and tanycyte process versus capillary loops) along the ME and around the pituitary stalk was calculated (Réthelyi, 1979). The most interesting findings were:

i) the contact surface was larger by roughly 26% in female than in male rats;

ii) systematic local variations occurred in the relative foldedness of the neurovascular contact surface;

iii) a significant portion of the neurovascular contact surface is to be found along the pituitary stalk.

These results were discussed with respect the regular termination site of the various troph hormone containing pathways. There seems to be major differences in the contact surface available for the termination of fibers in the

palisade zone, the LH-RH pathway being in the less fortunate situation if one presumes that the extensively folded contact surface means safety in the release processes.

A large number of vacuoles were found along the tuberoinfundibular sulcus and in the posterior part of the ME (Fig 3). They easily could be misinterpreted as blood vessels along the tuberoinfundibular sulcus. Our EM studies clearly showed that the vacuoles are confined to the tanycyte processes (Bodoky et al., 1979). Their significance is to be clarified.

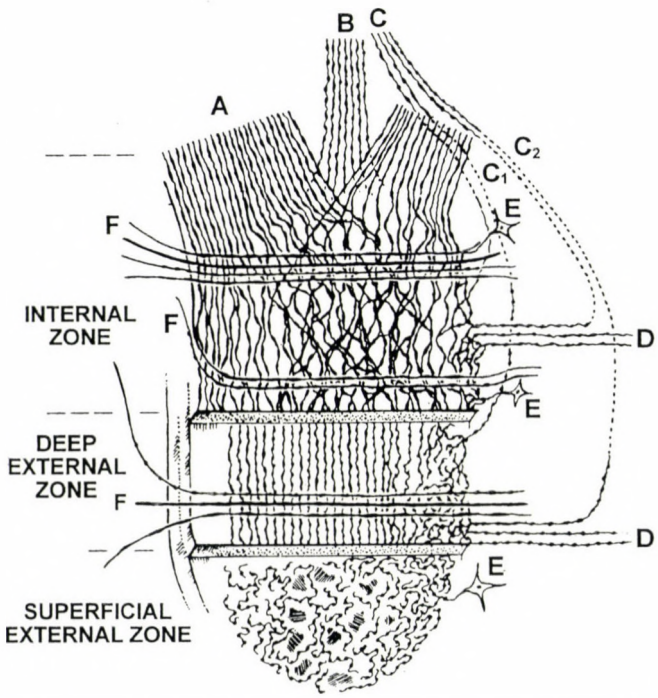

Fig. 2. Schematic diagram showing the main fiber components of the median eminence. A = fibers of the internal zone (hypothalamo-neurohypophyseal tract). B = varicose fibers in the anterior part of the hypothalamus and in the preoptic region running rostro-caudally in the external zone of the median eminence, eventually the fibers terminate in the middle region of the external zone. C = varicose fibers originating at the same site as the fibers labelled with B, but they approach the external zone from lateral direction either through the arcuate nucleus (C_1) or along the basal surface of the hypothalamus (C_2). D = varicose fibers coursing along the basal surface of the hypothalamus. E = tubero-infundibular neurons in the arcuate nucleus. Fibers labelled with C, D and E seemed to terminate in the lateral regions of the external zone. F = bundles of commissural fibers. The hatched areas in the superficial zone represent capillary loops of the portal vessels. (From Fockter and Réthelyi, 1982; modified)

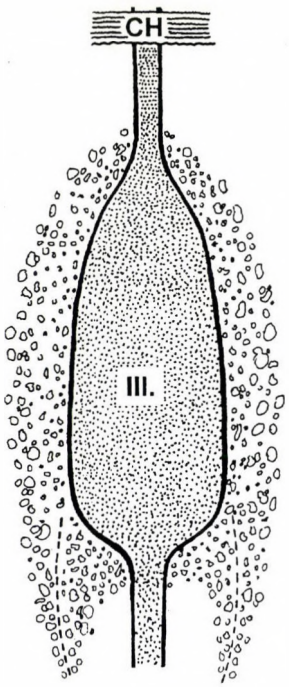

Fig. 3. Schematic drawing representing the distribution of the cisternae around the third ventricle (horizontal projection). CH = optic chasma, III. = third ventricle. (From Bodoky et al., 1979, modified)

Studies on the hypothalamic arcuate nucleus

For long time the hypothalamic arcuate nucleus was supposed to play an important role in the neuroendocrine control mechanisms. Golgi studies from our laboratory described the dendritic and axonal architecture of the arcuate neurons (Bodoky and Réthelyi, 1977, Réthelyi, 1985.)

The arcuate neurons were of two types. Medium size neurons with several dendritic branches occurred around the periphery of the nucleus whereas small size neurons with mostly two dendritic branches were found scattered in the nucleus. The dendrites of the arcuate neurons together with the arching processes of the tanycytes seemed to form an envelop around the dorsal and lateral aspects of the nucleus. The axons of the majority of the neurons converged towards the tuberoinfundibular sulcus. This characteristic course of the axons could be seen also in arcuate neurons

located in the lateral portion of the ME: they formed a loop and ran laterally. From the tuberoinfundibular sulcus the axons seemed to continue into the PS, into the postinfundibular portion of the arcuate nucleus or towards the lateral hypothalamus.

HRP microinjections into or lateral to the arcuate nucleus showed a unique pattern of diffusion of the enzyme (Réthelyi, 1984). HRP injected into the arcuate nucleus failed to diffuse laterally into the ventromedial nucleus as well as medially into the ME. Large HRP injections into the adjacent ventromedial nucleus did not penetrate into the arcuate nucleus. The delineation of the medial border of the arcuate nucleus in the first group of injection and its lateral border in the later cases was especially clear. Moreover, HRP injections reaching the lateral portion of the ME did not show any sign of diffusion towards more medial parts (Réthelyi and Fockter, 1982). It was hypothesised that the tanycyte processes around the arcuate nucleus and in the ME served as diffusional barriers. The significance of this structural peculiarities might be the division of the medial basal hypothalamus into microscopic compartments with restricted transverse communication. The characteristic course and termination patterns of the various releasing hormone containing pathway seemed to coincide with the compartmentalisation.

Axon regeneration in the hypothalamus

The multifarious application of the Halász-knive in the hypothalamus indicated the careful scrutiny of regenerating nerve fibers across the cut. In our study the course of HRP stained fine nerve fibers was traced in horizontally sectioned hypothalami (Nagy et al., 1983; Köves et al., 1986). No regeneration of nerve fibers were found in adult animals using various survival periods. Interestingly, in one rat the HRP stained fibers took a new trajectory at the caudal end of the cut line. The fibers circumvent the lesion and entered the depth of the denervated area. This unique observation may explain the functional recoveries after hypothalamic lesions.

LH-RH fiber systems

The collaboration of the researchers in three laboratories - Budapest, Pécs and Chapel Hill, NC, USA - resulted in the comprehensive description of the LH-RH fiber system (Réthelyi et al.,1981). The axons of the preoptico-

infundibular tract formed three fascicles: the median, the medial and the lateral fascicles. The termination sites of the fibers formed an elongated elliptical ring at the margin of the ME (Fig 4). Rostrally the termination area comprised the retrochiasmatic area, laterally the tuberoinfundibular sulcus and the lateral part of the ME and caudally the inframammillary region. In addition, LH-RH positive fibers were also found in the pituitary stalk. Interestingly, the surface of the hypothalamus along this ring was covered mainly by the tanycyte processes and was largely devoid of portal capillaries. Fig 5 shows the three dimensional schematic arrangement of the fascicles of the preoptico-infundibular LH-RH pathway.

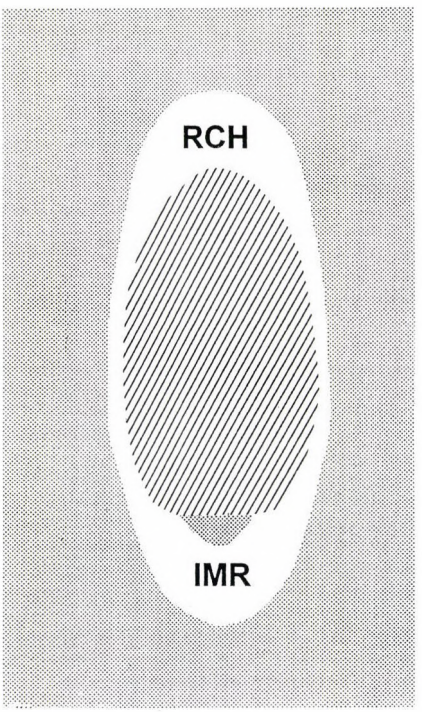

Fig. 4. Schematic drawing of the epedymal-glial covering of the basal surface of the hypothalamus. The brain surface is covered in general by a thin layer of interdigitating glial processes (stippled area). Around the median eminence this glial covering will be replaced by an oval belt of tanycyte processes (white area). The belt expands rostrally over the retrochiasmatic area (RCH) and caudally over the inframammillary region (IMR), but laterally it is restricted to the tuberoinfundibular sulcus. The belt surrounds the median eminence (hatched) and the much smaller median eminence posterior (stippled) in which tanycyte processes and nerve fiber terminals are intermixed. (Réthelyi et al., 1981; modified)

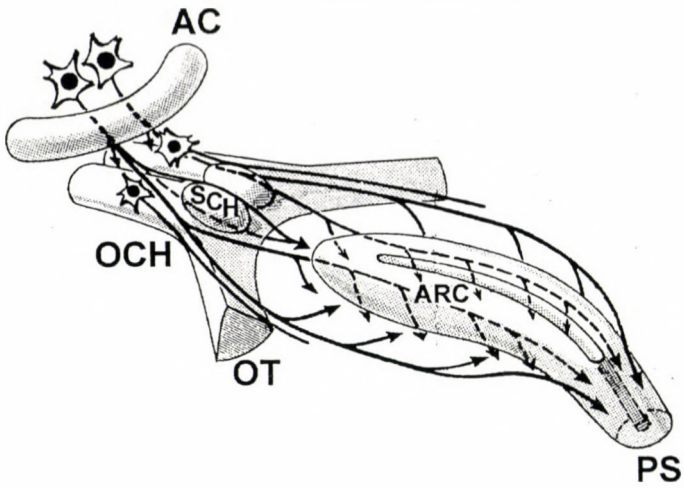

Fig. 5. Schematic diagram showing the origin, course and termination of the preoptico-infundibular LH-RH tract. Four neurons (left), two in the septal region and two others in the anterior hypothalamico-preoptic region, mark the territory where LH-RH positive perikarya were found. The fibers (preoptico-infundibular tract) take a rostro-caudal course along the midline on the dorsal and ventral surfaces of the optic chiasma (OCH), and reach the retrochiasmatic area. The remaining fibers from two laterally located bundles and divide into the medial and lateral fascicles. Fibers of the medial fascicle course along the lateral border of the suprachiasmatic nucleus (SCH), then turn medially and terminate in the retrochiasmatic area. Some fibers run further caudally within the arcuate nucleus (ARC) and seem to terminate in the lateral portion of the median eminence and in the inframammillary region. Fibers of the lateral fascicle follow the concave dorsal surface of the optic tract (OT) while coursing caudally, ventrally and lateraly. From this lateral position the fibers approach the lateral margin of the median eminence along the surface of the hypothalamus. Some fibers seem to continue into the pituitary stalk(PS). At the very rostral level LH-RH fibers seem to give rise to collateral branches to the organum vasculosum laminae terminalis (small arrows below the anterior commissure (AC). (Réthelyi et al., 1981; modified)

Conclusion

New pieces of information about the neuronal, glial and synaptic structure of the ME and the surrounding hypothalamic regions obtained with more sophisticated techniques complete now the above described 'classical' data and interpretation. Some of the old mysteries still await, however, new methods and fresh minds.

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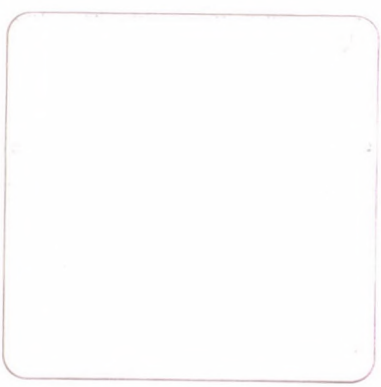

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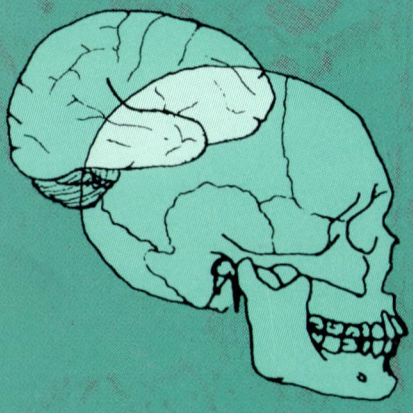

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Research report

MOTOR AND SOMATOSENSORY CONDUCTION TIME BETWEEN THE CORTEX AND THE ERB POINT IN PATIENTS SUFFERING FROM CERVICAL SPINAL STENOSIS AND TUMOUR

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Summary. Motor and sensory conduction time between the cortex and the Erb point were examined in patients with cervical cord compression. Patients were divided into two groups: the compression was caused either by cervical extramedullary tumour (9 cases), or by cervical spondylosis or herniated disc (16 cases). In response to median nerve stimulation, pathological somatosensory evoked potentials were recorded in 66% of the patients suffering from tumour and in 60% of the patients suffering from spondylosis. All of the patients disclosed pathological motor evoked potentials. On the basis of these observations it could be concluded that, in cases of cervical spinal cord compression, the involvement of the motor system could be more reliably detected than that of the sensory system with electrophysiological methods.
Key words: Motor evoked potentials; Somatosensory evoked potentials, Cervical spondylotic myelopathy, Cervical extramedullary tumour

INTRODUCTION

The external compression of the spinal cord may be caused by various diseases, most commonly by cervical spondylosis or herniated disc and occasionally by extramedullary tumours. The nerve conduction velocity is characteristically slowed down in these cases. The extent of compression can be shown with a number of different imaging techniques including myelography, CT myelography, CT and MR. In order to decide on active invasive treatment, exact information on the neural dysfunction is indispensable.

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There are two methods to obtain this information (1) the investigation of the characters of somatosensory evoked potentials (SEP) of sensory pathways, and (2) motor evoked potentials (MEP) of motor pathways. The pathological changes of both the SEP and the MEP have been described by a number of authors in cervical stenosis (Datzmann and Hamburger, 1985, Di Lazzaro et al., 1992, El-Negamy and Sedgwick, 1979, Ganes, 1980, Maertens de Noordhout et al., 1991, Masur et al., 1989, Molinor, 1992, Restucciaet al., 1994, Siilvola et al., 1981, Veilleux and Daube, 1987, Yu and Jones, 1985), whereas relatively few authors (Brunholzl, 1993, Caramia et al., 1988, Zentner and Reider, 1990) give account of pathological changes of the MEP in patients with cervical extramedullary tumour. However no reports are available on the differences of electrophysiological recordings obtained from patients with tumour and spondylosis.

In this study, we have examined the pathological changes of the motor conduction time (MCT) and the sensory conduction time (SCT) between the cortex and the Erb point in patients with cervical myelopathy caused by spondylosis, disc herniation and extramedullary tumours at the level of the 1 to 6 cervical segments.

SUBJECTS AND METHODS

Nine patients (3 men and 6 women, mean age 38 years) with cervical extramedullary tumour, and 16 patients (10 men and 6 women, mean age 51 years) with cervical spondylosis or with disc herniation, were involved in this study. The spinal cord compression was verified by neuroradiological methods including myelography, CT, myelo-CT, MR and their combinations. Clinical evidence for lesions of long tracts was present in all of the patients. The symptoms and the neuroradiological images unanimously indicated for surgical treatment.

The electrophysiological data obtained from the above patients were compared with the data of 20 control subjects (11 males, mean age 43, and 9 females, mean age 35). The SEPs were recorded with an Amplaid measuring system (EMG 15). The median nerve was stimulated at the wrist with an intensity of 12-20 mA quadrangular pulses, 0,1 ms duration at 4 Hz. The potentials were recorded from two places: from the Erb point on the side of stimulation and from the contralateral parietal tuber (P3, P4). The reference electrode was placed on the vertex (CZ). Two hundred records were averaged. Subcutaneous needle electrodes were used in the patient group, and surface electrodes in the control group. In SEPs the latencies of the negative waves were measured, which were recorded from the Erb

point (N 9) and from the sensory cortex (N 20). The SCT was calculated from the difference of these two latency values.

The MEP was elicited with a Magstim 200 (Novametrix). The instrument generated 2 Tesla at maximum (100%) stimulation intensity. The motor cortex was transcranially stimulated with 70-80% intensity, and the brachial plexus was transcutaneously stimulated with 40-50% intensity at the Erb point. MEP was recorded from the thenar muscles with surfaces electrodes during slight voluntary contraction. The latency of muscle contraction recorded from above the thenar was measured after the stimulation of the cortex and the brachial plexus. The MCT was calculated from the difference between the two latencies. The results obtained from the two groups of the patients were statistically compared to the results of the control group (paired Student's *t*-test). Both in the SEP and MEP examinations, the ground was fixed to the forearm.

From each individual latency and conduction time of the pathological groups were expressed as the percent of the cortical SCT and MCT events which exceeded the upper limit of the respective control values using the quotient:

$$\frac{A-(X+2.5\ SD)}{X+2.5\ SD} \qquad (1)$$

where A is the individual value of the latency or the conduction time in a patient, X and SD are the mean values and the standard deviations of the corresponding latencies or conduction times in the control group. These calculations allowed us to make an individual comparison of each patient with the average values of a control population.

RESULTS

The values obtained from the control group of healthy, symptomless persons, and from the two groups of patients with tumour and spondylosis, respectively, are summarized in Tables 1 and 2. In view of the rather uniform values of the different parameters, only the mean and the standard deviation are shown in the control group, whereas we find interesting to show the data of each individual person of the patient groups.

Table 1. Latencies of somatosensory evoked potentials in patients with cervical cord compression caused by tumour or spondylosis

	Case	Cortical latency (ms)		Erb point latency (ms)		SCT (ms)	
		R	L	R	L	R	L
	1	18.00	20.00	9.70	9.20	8.30	10.60
T	2	-	25.20 *	9.40	9.00		16.20 *
U	3	24.20 *	23.60 *	8.80	9.00	15.40 *	14.60 *
M	4	19.00	19.60	9.60	9.20	9.40	10.40
O	5	22.60 *	21.40	11.40	10.80	11.20 *	10.60
R	6	25.00 *	-	9.80	9.40	15.20 *	
S	7	19.60	21.00	9.40	9.20	10.20	11.80 *
	8	19.40	18.80	8.70	8.80	10.60	10.00
	9	20.80	20.50	9.50	9.60	11.30 *	10.90 *
	mean	20.1 ± 2.447 a		9.5 ± 0.723		11.8 ± 2.504 a	
	10	21.60	21.80	11.40	11.50	10.20	10.50
	11	20.40	20.20	10.40	10.00	10.00	10.20
S	12	19.20	19.80	9.80	9.80	9.40	10.00
P	13	20.60	20.00	10.80	10.40	9.80	9.60
O	14	21.60	21.20	10.60	10.50	11.00 *	10.70
N	15	22.60 *	21.60	12.00	11.40	10.60	10.20
D	16	20.20	23.20 *	10.00	10.00	10.20	13.20 *
Y	17	23.00 *	23.40 *	11.00	11.20	12.00 *	12.20 *
L	18	24.20 *	23.20 *	10.00	9.10	14.20 *	14.10 *
O	19	-	-	-	-		
S	20	22.20 *	22.00 *	11.00	11.00	11.20 *	11.00 *
I	21	no					
S	22	22.40 *	22.00 *	10.40	10.20	12.00 *	11.80 *
	23	23.00 *	22.20 *	10.40	10.40	12.60 *	11.80 *
	24	22.50 *	22.40 *	10.20	10.20	12.30 *	12.20 *
	25	20.10	20.00	10.40	10.30	9.70	9.70
	mean	21.7 ± 1.337 a		10.6 ± 0.696 b		11.1 ± 1.374 a	
n							
o	mean	18.1 ± 1.451		9.2 ± 1.139		8.9 ± 0.674	
r	mean + 2.5 SD	21.7		12.1		10.6	
m							

* = pathological value; R = right; L = left; SCT = sensory conduction time; no = not measured; - = no response; a = $P < 0.001$, b = $P < 0.01$ compared to the normal group (paired Student's t-test)

Volunteer subjects

In response to the stimulation of the median nerve, SEP appears with twice as long latency at the cortex as at the Erb point. The difference between the two values presents the Erb point-to-cortex conduction time that in the SCT, which is about 9 ms.

Motor evoked potentials require 2 ms longer time to appear whether the cortex or the Erb point is stimulated. If we consider the peripheral conduction time, which is the latency at the Erb point to the median nerve stimulation in SEP, and the muscle potential to the Erb point stimulation in MEP, we may conclude that sensory impulses propagate faster than motor ones along the median nerve. The short delay in the neuromuscular impulse transmission may partially account for the slower conduction speed in motor nerves. The difference, however, between SCT and MCT is not significant (Tables 1 and 2).

Patients with cervical tumour

Somatosensory evoked potentials could be recorded only from 15 patients. As expected, the peripheral conduction time (Erb point latencies) are comparable with normal values, the SCT is longer than in the control group. The significant difference is resulted from the increased latency proximal to the Erb point on both sides in two patients, and on one side in four other patients. Pathological SEPs were recorded in 66% of the patients with cervical tumour.

Motor evoked potentials provide more unambiguous results. The peripheral conduction time (Erb point latency) is also normal, but the latencies to cortical stimulation are significantly longer in all patients (except on the left side in patient No. 1). That is, pathological responses are recorded almost 100% of the patients examined (Table 3). Correspondingly, the MCT is significantly longer, it is almost twice as long as in the control group.

Patients with cervical spondylosis

Somatosensory evoked potentials present normal values at the Erb point but the cortical latencies are significantly longer on both side in 6 and on one side in three patients. This corresponds to 60% of the responses recorded

(Table 3). The SCT is slightly, although significantly longer than in the control group.

Table 2. Latencies of motor evoked potentials in patients with cervical cord compression caused by tumour or spondylosis

	Case	Cortical latency (ms)		Erb point latency (ms)		MCT (ms)	
		R	L	R	L	R	L
	1	23.00	29.60 *	12.20	12.20	10.80 *	17.40 *
T	2	29.50 *	25.60 *	13.60	10.80	15.90 *	14.80 *
U	3	34.20 *	29.50 *	13.80	11.70	20.40 *	17.80 *
M	4	28.60 *	-	11.90	-	16.70 *	
O	5	29.30 *	25.70 *	10.60	9.10	18.70 *	16.60 *
R	6	26.70 *	26.70 *	11.70	11.50	15.00 *	15.20 *
S	7	-	25.80 *	13.60	13.30		12.50 *
	8	27.10 *	27.80 *	9.80	10.30	17.30 *	17.50 *
	9	24.80 *	21.20	13.30	13.00	11.60 *	8.20
	mean	27.8 ± 2.661 a		11.7 ± 1.441		16.2 ± 2.458 a	
	10	49.80 *	37.50 *	15.50	14.90	34.30 *	22.60 *
	11	22.90	23.70 *	12.90	12.40	10.00	11.30 *
S	12	25.90 *	33.40 *	13.30	12.70	12.60 *	20.70 *
P	13	no					
O	14	24.50 *	24.40 *	12.00	8.70	12.50 *	15.70 *
N	15	34.20 *	28.60 *	17.40 *	15.00	16.80 *	13.60 *
D	16	29.70 *	28.80 *	15.50	12.70	14.20 *	16.10 *
Y	17	30.50 *	34.00 *	16.00 *	16.40 *	14.50 *	17.60 *
L	18	31.70 *	32.80 *	12.90	12.30	18.80 *	20.50 *
O	19	33.50 *	25.80 *	11.70	9.40	21.80 *	16.40 *
S	20	25.00 *	26.60 *	14.10	13.60	10.90 *	13.00 *
I	21	26.60 *	-	14.20	14.20	12.40 *	
S	22	25.20 *	24.90 *	14.60	14.30	10.60 *	10.60 *
	23	36.90 *	35.80 *	13.90	14.30	23.00 *	21.50 *
	24	29.50 *	27.00 *	13.20	13.10	16.30 *	16.90 *
	25	26.50 *	25.90 *	12.90	13.20	13.60 *	12.70 *
	mean	29.6 ± 6.041 a		13.6 ± 2.031 b		16.1 ± 5.511 a	
n o r m	mean	20.1 ± 1.388		11.8 ± 1.591		8.4 ± 0.838	
	mean + 2.5 SD	23.6		15.8		10.5	

* = pathological value; R = right; L = left; MCT = motor conduction time; no = not measured; - = no response; a = $P < 0.001$, b = $P < 0.01$ compared to the normal group (paired Student's t-test)

Erb point latencies of motor evoked potentials appear with slightly longer latencies than normal ones, but with exception of two patients (Nos 15, 17) the increase is not significant at the 0.05 level. The prolongation of the cortical latencies is, on the other hand, highly significant and pathological in almost 100% of the cases recorded (Table 3). In a few patients (Nos 10, 12, 17, 18, 23) the delay is almost twice (or in a single case more than twice) as long as in the control group. The individual MCTs are also significantly longer and their mean value is twice as long as in the control group.

Table 3

	Patients with tumour		
	No.	pathological EP	percentage
SEP	9	6	66%
MEP	9	9	100%
	Patients with spondylosis		
	No.	pathological EP	percentage
SEP	15	9	60%
MEP	15	15	100%

No. = number of patients; pathological EP = number of patients with abnormal evoked potentials

The comparison of sensory and motor evoked potentials

Since the peripheral conduction times are virtually normal in almost all of the patients, it is relevant to compare both the motor and the sensory cortical latencies and conduction times in the two patient groups in terms of percentage in relation to the average value of the control group. As shown in the diagrams of Fig. 1, cortical latencies of MEPs greatly increase, especially in the spondylosis group, they can be higher than 150% of the upper level of the normal range. In SEPs the latency values increase to a much less extent, they remain above the upper level of the normal range in the majority of the cases. The picture is slightly different in the cases of the conduction times in that the greater part of the SCTs are also above the level of the normal range.

These results indicate that the MEPs are much more sensitive indicators of damages caused by tumours or spondylosis than the SEPs, and the

conduction times calculated with quotient (1) is a very sensitive sign in showing any pathological alterations of evoked potentials.

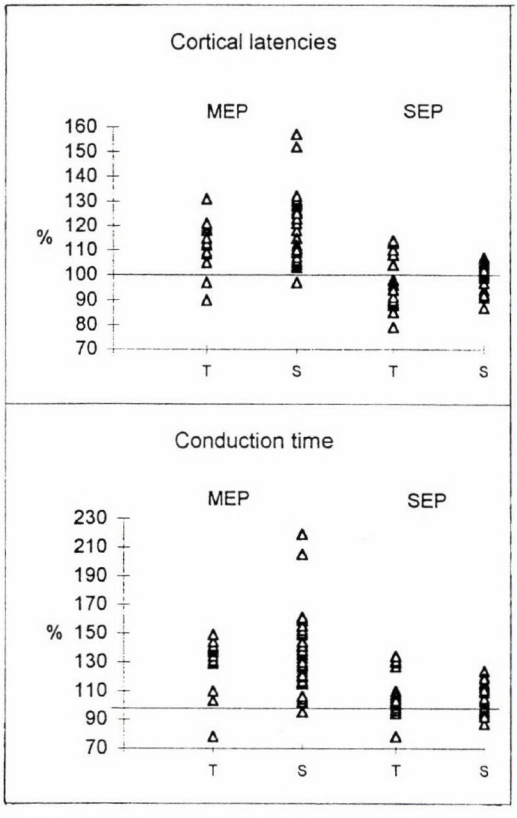

Fig. 1. Comparison of motor and somatosensory evoked potentials and conduction time in patients with cervical tumour and spondylosis. The 100% means the upper level of the normal range. Abnormal values are above the line.
T = evoked potentials in patients with cervical tumour. S = evoked potentials in patients with cervical spondylosis

DISCUSSION

The chronic, progressive external compression of the cervical spinal cord is usually caused by degenerative deformations of the spine, and occasionally by extramedullary tumours occurring at this level. Radiological imaging

methods can precisely show only the location and extent of the deformation. The functional damage to the spinal cord must be investigated with clinical and electrophysiological methods. The practicability of electrophysiological examinations in cases of myelopathies and pain syndromes caused by cervical spondylosis, or disc herniation, is well known (Datzmann and Hamburger, 1985, Di Lazzaro et al., 1992, El-Negamy Sedgwick, 1979, Ganes, 1980, Maertens de Noordhout et al., 1991, Masur et al., 1989, Molinor, 1992, Perlik and Fischer, 1985, Siilvola et al., 1981, Veilleux and Daube, 1987, Yu Jones, 1985). The most common method for the examination of sensory tracts between the levels of C1 and C6 is the SEP evoked by stimulation of the median, ulnar and tibial nerves. Veilleux and Daube (1987) examined the SEP in a case of cervical stenosis by stimulating these three nerves. The tibial nerve stimulation produced pathological values in 74%, the median nerve in 43% the ulnar nerve and in 64% of the patients examined. In this investigation a great percentage of the SEP is pathological in myelopathy, whereas this percentage is very low in cases of radicular involvement. Yu and Jones (1985) conducted SEP examinations in patients with myelopathy stimulating the median, ulnar and tibial nerves. The SEPs were pathological in 80% of patients suffering from myelopathy and brachialgy, and only in 33% of patients with pain symptoms. If patients are not separated according to clinical symptoms, the usefulness of SEP decreases (Siilvola et al., 1981).

Stimulating more than one nerve increases the diagnostic value of SEP. From the findings of Veilleux and Daube (1987) and Yu and Jones (1985) it is clear that the combination of the median, ulnar and tibial SEPs reveals the damage to the sensory tracts with a higher probability. In 80% of the patients examined, sensory damages could be shown only by this method. Cervical SEP is also an adequate technique to show disorders in patients with cervical stenosis. In El-Negamy's material (El-Negamy and Sedgwick, 1979) of 9 patients suffering from cervical spondylosis, 5 disclosed pathological results with this technique. Since efferent fibers innervating individual muscles leave the spinal cord at different levels, the MEP has a localization value in cases of motor stimulation. In compression, caused by cervical myelopathy, motor conduction time increases at the level of damage. Examining the MEP in the biceps and interosseal muscles, Maertens de Noordhout et al., (1991) regularly recorded pathological MEPs from the first interosseal muscle, whereas biceps MEPs were pathological only in 4 cases out of 8 patients with spondylosis localized at level of C2 - C4. Di Lazzaro et al., (1992) obtained similar result from the thenar muscles. The MEP of the lower limbs is also suitable for examination of the thoracic and lumbar sections of the spinal cord. Like the SEP, the MEP is

pathological only in a low percentage in cases of root symptoms without myelopathy.

Masur et al., (1989) compared the SEP and the MEP in patients with cervical stenosis. They found that the MEP recorded from the anterior tibial muscle was pathological in 17 out of 19 patients (89%), and the SEP was pathological in 16 cases (84%). Examinations of the upper limbs (Maertens de Noordhout et al., 1991) showed similar results, that is, the MEP was pathological in a greater percentage than the SEP in patients with myelopathy.

The MEP latency and motor conduction time greatly increased also in extramedullary tumours. Brunholzl (1993) and Zentner and Rieder (1990) found that MEP closely correlated with the clinical motor status. In study of Caramia et al. (1988) on the upper limbs of patient suffering from extramedullary tumour at the C 1 level, the SEP gave normal results, whereas the MEP was pathological on the affected side.

The comparison of our results with the data in the literature justifies the conclusion that MEP examinations are, in general, more sensitive and useful than SEP examinations in showing impairments of long projecting tracts, whether motor or sensory, in cases of space occupying events caused by tumours or spondylosis.

In answering of the question of why the MEP is more sensitive than the SEP in showing damages to the spinal cord in space occupying events, one may think of the multiple synaptic impulse transmission in motor commands. The mechanism of magnetic stimulation is not known to that extent that we could make a calculation about the number of synapses involved in discharging a pyramidal neuron. We do not have any idea about the involvement of the subcortical motor centers in the processing of a motor command. Finally, the motor neuron is impinged upon by a number of descending tracts, and many of them convey their commands through polysynaptic transmission. The fragility of synaptic transmission is a well-known physiological phenomenon, and it can easily be damaged by space occupying processes. We think, therefore, that it is a feasible assumption that the higher sensitivity, thus the more practicability of the MEP, in showing pathological functional changes relies on the polysynaptic nature of motor pathways.

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Short communication

MALEIC ACID IS AN ALDOSE REDUCTASE INHIBITOR

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Summary. The effects of a series of dicarboxylic acids on aldose reductase activity were investigated in a crude enzyme preparation obtained from ocular lenses of the swine. Of the compounds examined, maleic acid inhibited aldose reductase in a pronounced manner and in a concentration-dependent fashion (IC_{50} value of 83.0 $\pm$ 1.4 mM). Saturation experiments revealed non-competitive kinetics with a K_i value of 64.9 $\pm$ 1.7 mM. The present results suggest that, at higher doses, maleic acid might have some implication for the treatment of diabetic complications.
Key words: maleic acid, aldose reductase inhibition, diabetic complications

INTRODUCTION

Aldose reductase (EC 1.1.1.21.) inhibitors are known to be effective in a range of experimental models of diabetic complications, including autonomic and peripheral neuropathies (Greene et al., 1993), as well as micro- and macroangiopathies including retinopathy, nephropathy and vasculopathies (Lightman, 1993). Several of them, e.g. Sorbinil, Tolrestat, Epalrestat and Statil, have already been marketed for the treatment of one or more of the above complications (Tomlinson et al., 1992), and a number of novel inhibitors are under preclinical and clinical development. Recognized inhibitors of aldose reductase can be classified into several distinct chemical

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groups, such as sulphonylnitromethanes (Cook et al., 1995), hydantoins (Nakayama et al., 1995) and flavonoids (Varma and Kinoshita, 1976). In the present study we provide evidence that a simple organic acid, namely maleic acid inhibits aldose reductase in a concentration-dependent manner and in a non-competitive fashion.

MATERIALS AND METHODS

Chemicals

Maleic acid (cis-butenedioic acid), fumaric acid (trans-butenedioic acid) and crotonic acid (trans-2-butenoic acid) were obtained from FLUKA. Malonic acid (propanedioic acid) was obtained from LOBA. Succinic acid (butanedioic acid), oxalic acid (ethanedioic acid) and tartaric acid (D-2,3-dihydroxybutanedioic acid) were purchased from REANAL (Budapest). The phosphate buffer was from MERCK. Quercitrin, NADPH and D,L-glyceraldehyde were obtained from SIGMA. All chemicals were of analytical grade.

Enzyme preparation

Crude preparations of aldose reductase were obtained from the ocular lens of the swine, freshly dissected at the local slaughter-house. Lenses were kept on ice for less than 1 h, then homogenized, extracted with distilled water and fractionated by increasing concentrations of ammonium-sulfate according to the method of Hayman and Kinoshita (1965). The enzyme preparations were stored at -20 °C for several weeks without significant reduction in their activity.

Aldose Reductase Assay

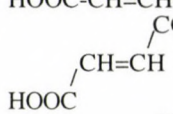
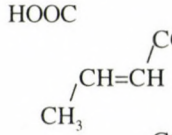
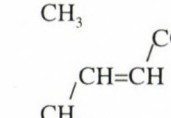
The activity of the aldose reductase was assayed spectrophotometrically by following the decrease in NADPH coenzyme adsorbance at 340 nm for 10 minutes (Hayman and Kinoshita, 1965). In brief, measurements were performed at room temperature (24 Ò 1 °C) using a JASCO V-550 spectrophotometer. The reaction mixture contained the enzyme solution and the coenzyme (NADPH, 5.5×10^{-5} M) dissolved in 92 mM phosphate buffer (pH 6.20). The reaction was started by addition of the substrate, D,L-glyceraldehyde (1 mM unless otherwise stated). In inhibition experiments, dicarboxylic acids were also present at a concentration of 5×10^{-4} M. The concentration dependency of inhibition by maleic acid was investigated in a range of 5×10^{-7} to 5×10^{-3} M. Data were corrected for the nonspecific decay of NADPH estimated in enzyme-free blank samples.

Saturation kinetics was examined at a substrate (D,L-glyceraldehyde) concentration range of 5×10^{-6} to 1×10^{-3} M, in the presence or absence of 5×10^{-5} M maleic acid. As reference compound, quercitrin was also tested. IC^{50} values were calculated by linear regression analysis. The K_M, V_{max} and K_i values were calculated from Lineweaver-Burk plots.

RESULTS AND DISCUSSION

At a concentration of 5×10^{-4} M, fumaric acid, crotonic acid, malonic acid, oxalic acid and tartaric acid caused 10% or less inhibition of aldose reductase activity, whereas maleic acid inhibited the enzyme by approximately 85% (Table 1). Another C_4 dicarboxylic acid, i.e. succinic acid, evoked a weak inhibition of about 40%.

Table 1. Effect of maleic acid and other dicarboxylic acids on aldose reducatse activity, *in vitro*

Compound	Structure	Inhibition
Maleic acid	HOOC-CH=CH-COOH	84.42 ± 0.33
Fumaric acid		8.07 ± 0.92
Crotonic acid		3.10 ± 0.28
Tartaric acid		3.85 ± 0.21
Succinic acid	HOOC-CH_2-CH_2-COOH	41.60 ± 0.56
Malonic acid	HOOC-CH_2-COOH	10.33 ± 2.00
Oxalic acid	HOOC-COOH	3.15 ± 0.07

Inhibitors were employed at a concentration 5×10^{-4} M. Data represent mean values ± SD (n = 5)

The inhibition by maleic acid proved to be concentration-dependent (Fig. 1) with an IC_{50} value of $8.30 \pm 0.14 \times 10^{-5}$ M (n = 3). For comparison, the IC_{50} value of quercitrin, a known inhibitor of aldose reductase was 4.65 ±

0.21×10^{-6} M (n = 3), indicating that maleic acid has a moderate activity to inhibit aldose reductase.

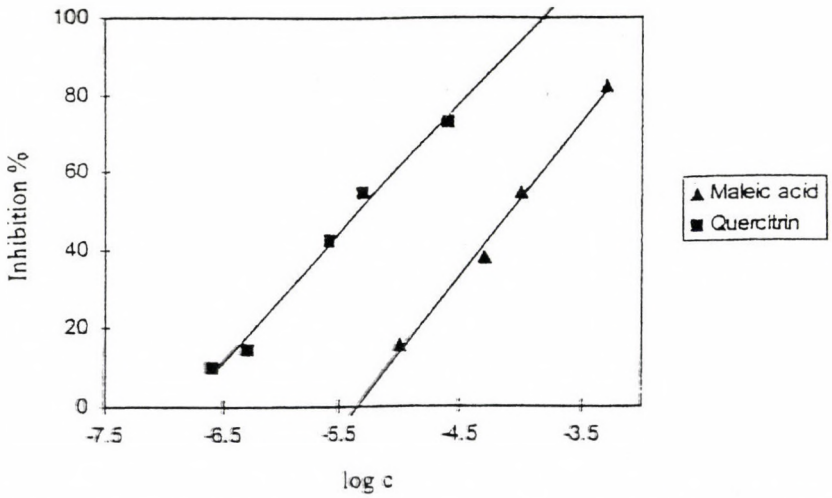


Fig. 1. Inhibition of aldose reductase activity by maleic acid. Concentration-dependent inhibition by maleic acid and quercitrin at a substrate concentration of 1 mM. Points represent mean values obtained in 3 repeated experiments

Aldose reductase activity was saturable with increasing concentrations of D,L-glyceraldehyde (Fig. 2). As revealed by Lineweaver-Burk plots, the K_M and V_{max} values (n = 3) were $3.27 \pm 0.12 \times 10^{-5}$ M and 2.54 ± 0.38 mmol/min/mg protein, respectively, which are in line with previously published data of Hayman and Kinoshita (1965).

The kinetic analysis of the inhibition by maleic acid (5×10^{-5} M) revealed a non-competitive type of inhibition (Fig. 3) characterized by a K_i value of $6.49 \pm 0.17 \times 10^{-5}$ M (n = 3).

Our results indicate that maleic acid is a moderately active, specific, non-competitive inhibitor of aldose reductase. As aldose reductase inhibitors are known to be potent in preventing diabetic organopathies, further studies are needed to elucidate whether tolerable doses of maleic acid may be effective in such experimental models.

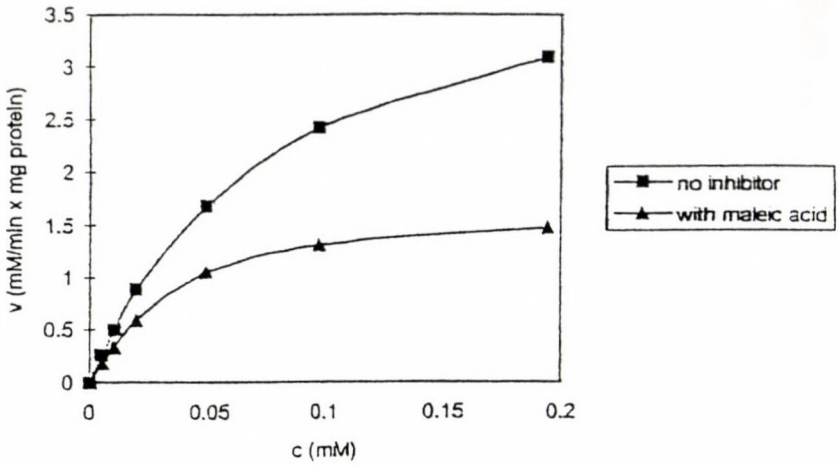


Fig. 2. Saturation of the aldose reductase by increasing substrate concentrations (5×10^{-6} to 1×10^{-3} M) in the presence and absence of maleic acid (5×10^{-5} M).
Points represent mean values obtained in one representative experiment with tiplicate samples. For K_M, K_i and V_{max} values, see text

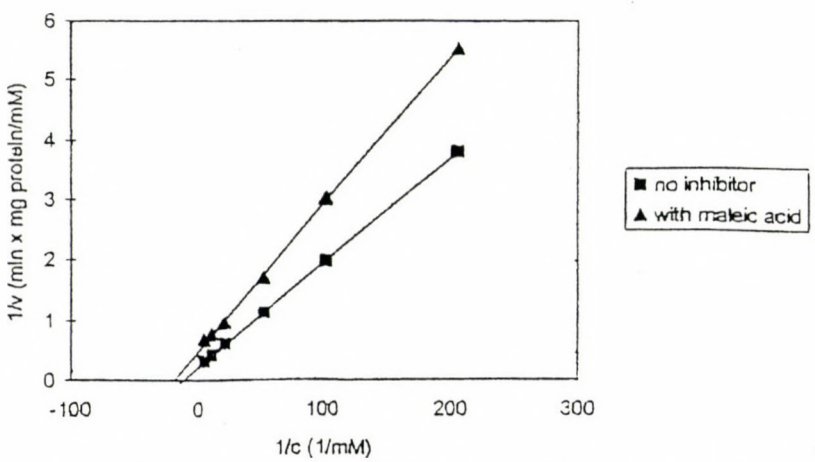


Fig. 3. Lineweaver-Burk plots showing non-competitive inhibition in the presence of 5×10^{-5} M maleic acid. Points represent mean values obtained in one representative experiment with tiplicate samples. For K_M, K_i and V_{max} values, see text

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Preliminary note

MEDIATOR SUBSTANCES OF THE PINEAL NEURONAL NETWORK OF MAMMALS

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Summary: In addition to receptor-type pinealocytes, the mammalian pineal organ contains small and large neurons and ependymal/glial cells as well. Axons of pinealocytes form synaptic ribbon-containing axo-dendritic synapses on large secondary pineal neurons and/or terminate as neurohormonal endings on the basal lamina of the vascular surface of the organ. The small pineal neurons were found to be gamma-aminobutyric acid (GABA)-immunoreactive, while large secondary neurons and pinealocytes contained immunoreactive amino acids (glutamate and aspartate). Glutamate accumulated presynaptically in pinealocytic axon terminals on large secondary neurons and in the axons of these neurons. Glutamate immunoreactive axons of pineal neurons were traced via the pineal tract to the habenular nucleus. Axons containing granular vesicles and coming from extrapineal perikarya are glutamate immunoreactive as well. Aspartate and GABA are also present in some of the myelinated axons, supposedly pinealopetal in the pineal tract.
Key words: pineal organ, pineal tract, habenular nucleus, GABA, glutamate, aspartate, immunocytochemistry, electron microscopy, mammals

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INTRODUCTION

In addition to receptor-type pinealocytes, the pineal organ of mammals also contains neurons and ependymal/glial cells. These cellular elements are similarly organized as in the submammalian photoreceptor pineal organ histologically considered to be a "folded retina" (Vígh and Vígh-Teichmann, 1988, 1992). There are two types of neuron in the mammalian pineal organ with small electron-dense and large electron-lucent perikarya, respectively. Axons of small neurons form intrapineal synaptic connections, whereas the large secondary neurons send their axons to the pineal tract. In some mammals, acetylcholinesterase, GABA, somatostatin, glutamate and aspartate were demonstrated cytochemically in the pineal neurons (Bhaskar and Joy, 1989, Lew and Lawson-Willey, 1987, Moller et al., 1993, Vígh and Vígh-Teichmann, 1988, 1992, 1993, Vígh-Teichmann and Vígh, 1992, 1994, Vígh-Teichmann et al., 1991).

To further study the synaptic mediators of the intrapineal neural network of mammals, in the present work we investigated the localization of immunoreactive GABA, glutamate and aspartate in the pineal of ten different species representing Insectivora, Rodentia and Carnivora.

MATERIALS AND METHODS

Both sexes of animals belonging to the following species were examined: 6 hedgehog (*Erinaceus roumanicus*), 16 bats (5 *Myotis blythi oxignathus*, 3 *Cynopterus sphinx*, 4 *Taphozous longimanus*, 4 *Scotophilus temmenckii*), 10 ferrets (*Putorius furo*), 12 minks (*Mustela vison*), 2 stone martins (*Martes foina*), 8 cats and 15 rats. The animals vere kept under normal laboratory light conditions and anesthetized with phenobarbital prior to fixation. The pineal including habenular and posterior commissures together with habenular nucleus were embedded in Poly Bed 821 (Polysciences, St. Goar, FRG). Sections were cut on a Reichert Ultracut S ultramicrotome. Postembedding electron microscopic immunoreaction was performed with polyclonal antisera raised in rabbits against GABA, glutamate and aspartate (Sigma Aldrich, Diesenhofen, FRG) as described earlier (Vígh-Teichmann et al., 1991, Vígh et al., 1997).

RESULTS

The pinealocytes of the mammals investigated are polarised cells with a receptory and an efferent pole. The receptory pole is marked by a sensory cilium of 9+0-type. In some species (e.g. hedgehog) the cilium is enlarged (Fig. 1a). In bats and ferrets the cilia contain vesicles resembling immature retinal photoreceptor outer segments (Fig. 1b). The efferent pole of the pinealocytes is represented by an axonal process forming two types of endings: synaptic ribbon-containing axo-dendritic synapses on large secondary pineal neurons and neurohormonal terminals on the basal lamina of the pineal vascular surface (Fig. 1c, d). Neurohormonal terminals may emerge from the surface of the organ towards vessels (Fig. 1e).

The small electron-dense pineal neurons exhibit GABA immunolabel (Fig. 2a). Axons of GABA-immunoreacting fibers form synapses on dendrites of large secondary neurons (Fig. 2b) and on profiles presumably belonging to pinealocytic processes. The pinealocytes and the large secondary neurons of all animals studied reacted with glutamate as well as with aspartate immunosera. Glutamate immunoreaction was more intense than that of aspartate. Glutamate accumulated presynaptically in the pineal axon terminals on large secondary neurons (Fig. 2c) and in the axons of these neurons. Some profiles containing granular vesicles were glutamate-immunoreactive as well (Fig. 2d). Granulated perikarya were not found in the pineal proper but were present in its habenular region. In the proximal part of the organ and in the pineal tract some myelinated fibers also showed GABA and aspartate immunoreaction (Fig. 2d, e). In serial sections, glutamate immunoreactive axons of pineal neurons were traced through the pineal tract to the habenular nucleus where accumulation of immunoreactive glutamate was observed presynaptically in axodendritic synapses (Fig. 2f).

Only slight differences were apparent between the various species examined. The number of axo-dendritic and axo-somatic terminals formed by pinealocytic processes were higher in the hedgehog, mink, ferret, cat, while numerous neurohormonal terminals were found in the bat species. The different terminals were not homogeneously distributed: axo-dendritic and axo-somatic synapses were frequently found in the proximal part, while neurohormonal terminals were predominant in the distal part of the organ. The number of glutamate immunoreactive pineal neurons was higher near the habenular and posterior commissures than elsewhere.

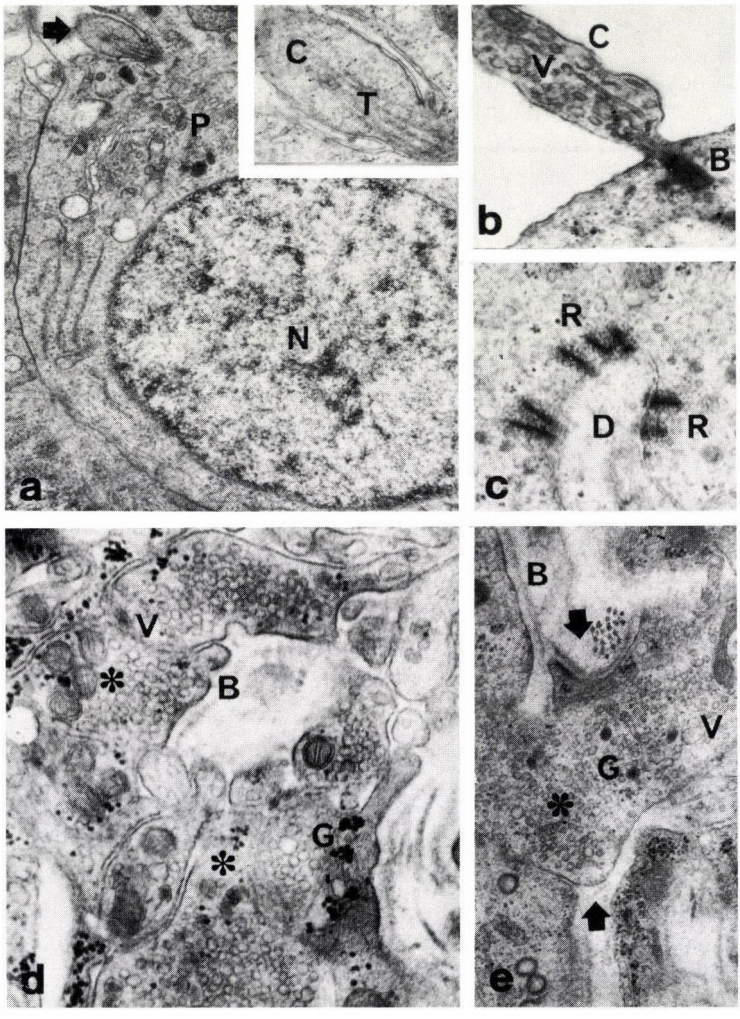

Fig. 1. Fine structure of the pineal organ of various mammals. a: Pinealocyte (P) of the hedgehog, note the fusiform cilium (arrow), N nucleus, ×9600. Inset: The fusiform cilium (C) of the pinealocyte with higher magnification, T, microtubules, ×23000. b: photoreceptor outer segment-like cilium (C) filled by vesicles (V) of a pinealocyte of the ferret (*Putorius furo*), B basal body, x21000. c: synaptic ribbons (R) of an axon terminal of a pinealocyte of the mink (*Mustela vison*), D dendrite of a large secondary pineal neuron, ×30500. d: neurohormonal nerve telminals (asterisks) on the vascular surface of the pineal of the bat (*Tadmozous longimanus*), B basal lamina, G glycogen granules, V synaptic vesicles, ×30200. e: neurohormonal terminal (asterisk), leaving the surface of the pineal nervous tissue (arrows) of the fruit eating bat (*Cynopterus sphinx*), B basal lamina, G granular vesicles, V synaptic vesicles, ×23600

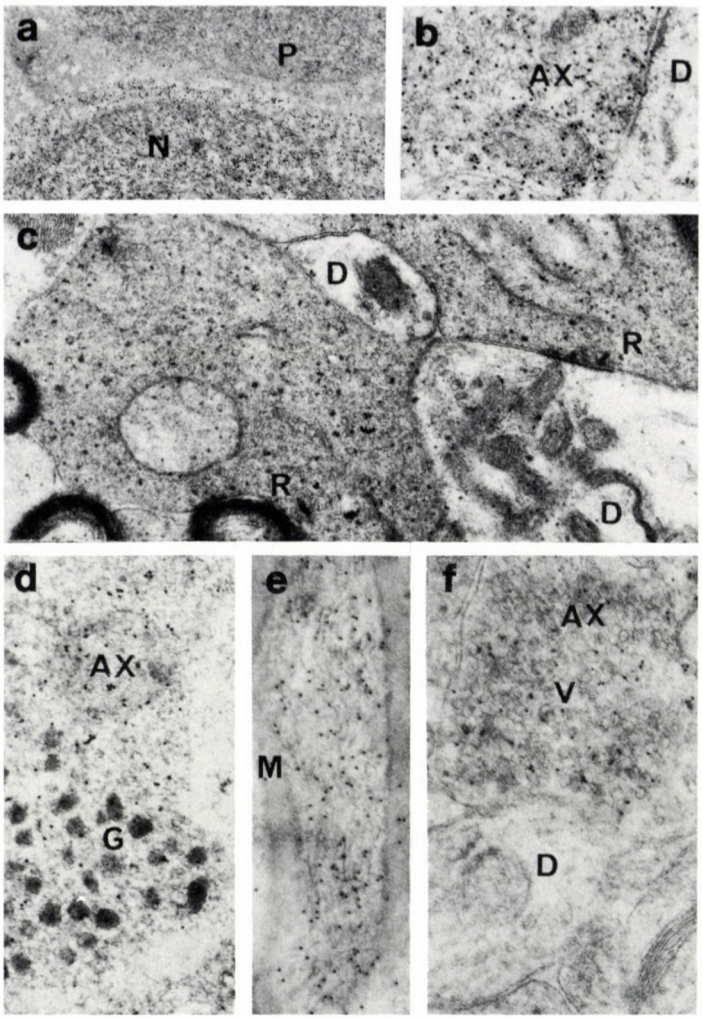

Fig. 2. GABA, glutamate and aspartate immunoreactivity (black dots of immunogold particles) of cells and nerve fibers of the pineal organ of mammals. a: GABA-immunoreactive small neuron (N), and immunonegative pinealocyte (P) of the cat, ×8400. b: GABA-immunoreactive axon terminal (AX) on a dendrite (D) of the large pineal neuron of the cat, ×l2000. c: glutamate-immunoreaction of ribbon-type (R) axon terminals of pinealocytes of the cat. D dendrites of large, secondary pineal neurons. T glial microtubules, V synaptic vesicles, ×12600. d: glutamate-immunoreactive fiber containing granular vesicles (G) in the pineal organ of the fruit eating bat (*Cynopterus sphinx*), F glial microfibrills, M myelinated fiber, x18800. e: aspartate-immunoreaction of a myelinated fiber (M) of the bat (*Tadmozous longimanus*), ×18500. f: Glutamate-immunoreaction of an axon terminal (ax) among pineal tract fibers on a dendrite (D) of the median habenular nucleus of the ferret (*Putorius furo*), V synaptic vesicles, ×20000

DISCUSSION

Using immunocytochemistry, GABA was demonstrated in the embryonic pineal cells of the quail (Araki et al., 1994). According to Brandstatter and Hermann (1996), GABA enhances the light response of ganglion cells in the trout pineal organ. Concerning mammals, Krstic and Nicolas (1992) described the localization of glutamin synthetase in glial cells of the rat pineal and its supposed role in the metabolism of glutamate and GABA. Our findings on the GABA immunoreactivity of small pineal neurons in the mammalian pineal indicates - in accordance with the aforementioned data - the existence of a GABA-ergic inhibitory component of the pineal neronal network.

Earlier biochemical, autoradiographic and immunocytochemical studies revealed the localization of glutamate in the rat pineal. Most of the authors attribute a role for glutamate in the neural regulation of pineal glandular activity (Kus et al., 1993, McNulty et al., 1992, Redecker and Veh, 1994). In the photoreceptor pineal of the frog, glutamate and aspartate were shown to exert an excitatory effect on achromatic light responses of pineal neurons (Meissl and George, 1984). Accordingly, in preceding works (Vígh et al., 1995a,b,c), we found a presynaptic accumulation of immunoreactive glutamate and aspartate in the pinealocytic synapses on intrapineal neurons and in the axons of these neurons in fishes, amphibians, reptiles and birds. These results showed that excitatory amino acids may act as synaptic mediators in intrapineal and efferent pathways of the submammalian pineal (Vígh et al., 1995a,b). Similar findings were obtained in the ferret, cat and human, suggesting that excitatory amino acids may have a similar role in mammals including human (Vígh et al., 1995c). The present results derived from ten different mammals confirm this earlier supposition. In the pineal of various insectivorous, rodent and carnivorous representatives of mammals, the localization of immunoreactive glutamate and aspartate corresponds to that of the submammalian pineal. The gradual accumulation of immunoreactivity in the axonal processes and presynaptic elements of pinealocytes and large secondary pineal neurons corroborate the view that excitatory amino acids - first of all glutamate - act as neurotransmitters in the intrapineal and efferent pineal pathway of mammals (Fig. 3). The glutamatergic granulated axons may originate from perikarya of the habenular region. Also the large myelinated axons exhibiting strong aspartate immunolabel presumably originate from outside of the organ.

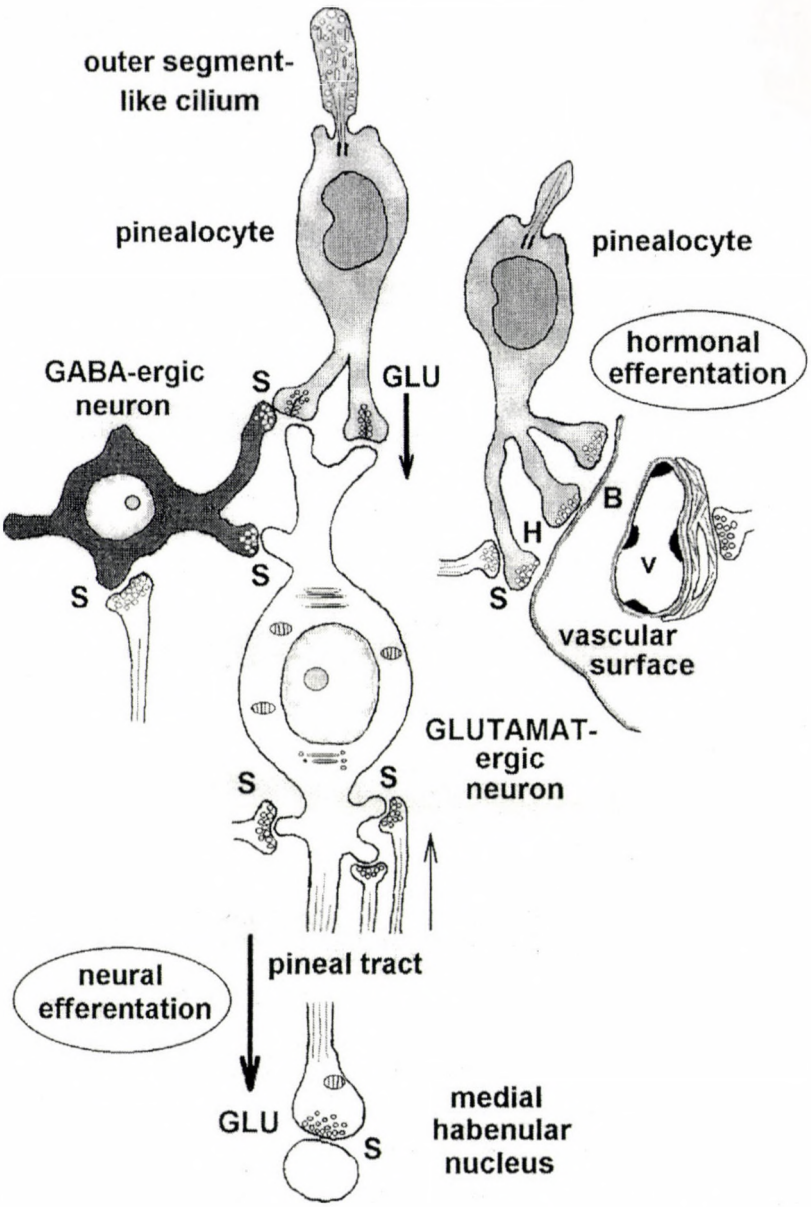

Fig. 3. Scheme on the intrapineal neural network of mammals. B basal lamina of the vascular surface of the pineal nervous tissue, GLU glutamate, H neurohormonal nerve terminals of pinealocytic axons, S synapses, V vessel

Further studies are necessary to elucidate the role of other neural elements such as those that show substance P, somatostatin and/or FMRF-amide-immunoreaction in the pineal neural network.

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Preliminary note

EFFECT OF CATECHOLAMINERGIC LESIONS OF MEDIAL PREFRONTAL CORTEX ON REGULATION OF BODY WEIGHT AND GLUCOSE PREFERENCE

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The prefrontal cortex (PFC) is involved in a variety of higher psychic functions, such as cognition and emotion. Moreover, the role of the PFC has also been considered in the ethiology of schizophrenia and depression (Weinberger, 1993). It is also known that lesions of the mesocorticolimbic dopaminergic system (MCLDS) - that has abundant projections to the PFC - induce similar impairments in the functional processes from motivation to action (Scheel-Krüger and Willner, 1991). Although previous studies revealed that the limbic terminal fields of the MCLDS (e.g. hypothalamus, globus pallidus, nucleus accumbens) have important role in the regulation of feeding behavior (Ungerstedt, 1971; Lénárd, 1977; Hernandez and Hoebel, 1988; Lénárd et al., 1988), there are only few data (Hernandez and Hoebel, 1990; Mogensen and Divac, 1993) concerning the specific role of the mesocortical areas. Therefore, the aim of the present experiments was to determine whether specific neurochemical lesions of the catecholaminergic terminals in the infralimbic area of the PFC impair feeding and body weight.

In 36 adult CFY male rats, 6-hydroxydopamine (6-OHDA, 6-OHDA-HCl, Sigma Chemical Co., 7.5 μg in 1 μl) was injected bilaterally into the infralimbic area of the PFC. In order to increase the selectivity of the neurotoxin (to avoid the destruction of noradrenaline terminals), 6-OHDA was applied in 18 rats after desmethylimipramine (DMI, GEIGY) pretreatment. Additional 18 rats were used as vehicle-treated sham-operated controls. Anesthesia, the preparation and application of 6-OHDA solution and DMI premedication were similar as published previously (Lénárd, 1977; Lénárd et al., 1988). Daily food and water intake or consumption of

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glucose solution (see below) and body weight were measured for 12 postoperative weeks. It was also examined whether the lesioned animals exhibit regulatory deficits to different physiological challenges (e.g. 24 h food and water deprivation). These challenges were accompanied by open field tests for scoring the spontaneous motor activity and stereotype behaviours.

In order to demonstrate possible changes in the feeding specific motivational state, glucose preference were tested in a two-bottle free choice paradigm. Consumptions of 5% glucose solution vs water or 10% glucose solution vs water were measured for 60 days from the 24th postoperative day on. At the end of experiments plasma glucose and hormone levels (insulin, tyroxine, trijodothyronine) were determined. Data were analyzed by ANOVA followed by post-hoc t-tests when group effects justified (Excel 4.0 for Windows).

Results showed that body weight of the 6-OHDA+DMI-treated rats decreased during the first postinjection days and animals regained their initial weight on the 7th postoperative day. In contrast, significant difference between the body weight of 6-OHDA and sham-operated control rats was observed only after the 6th day. Thereafter, the body weights of both lesioned groups remained lower than those of controls. Significant reduction in food intake was observed only in the 6-OHDA+DMI group in the early postoperative period. No significant differences were observed in water intakes between groups. In responses to deprivation tests, motivational deficits did not occur in the lesioned animals. Open-field tests, however, revealed an increased motor activity as well as an increased number of stereotypy in both lesioned groups. In two-bottle free choice tests, when both water and glucose solutions were available, animals in each group preferred to drink glucose (Fig. 1). However, the 6-OHDA+DMI rats exhibited a significantly enhanced intake of the 5% glucose solution (Fig. 1A.) when it was compared to the glucose consumption of the 6-OHDA treated or sham-operated control rats ($p<0.01$). In the 6-OHDA+DMI animals this enhanced glucose preference was also present for 10% glucose solution ($p<0.01$, Fig. 1B.) and it persisted at an extremely high level throughout the experiment (during 60 days). The plasma glucose and hormone levels did not show significant differences among groups.

In conclusion, the catecholaminergic lesions of the infralimbic PFC resulted in disturbances in the regulation of feeding and body weight. Results demonstrated different symptoms after either the selective lesions of dopaminergic terminals (6-OHDA+DMI rats), or neurochemical destructions of both dopaminergic and noradrenalinergic elements (6-OHDA animals) of

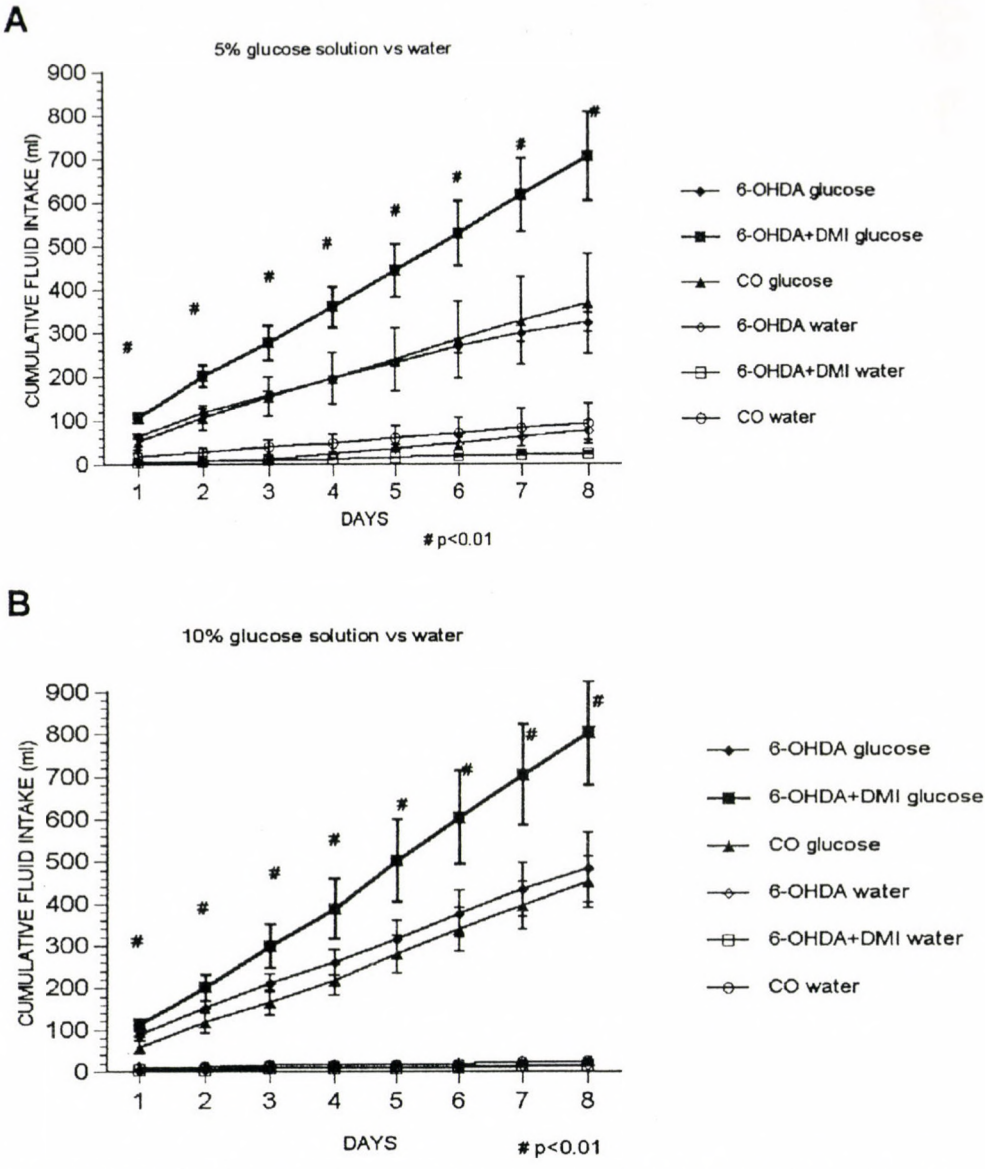

Fig. 1. Changes in glucose preference after catecholaminergic lesions of the infralimbic PFC. Cumulative intakes of 5% (A) or 10% (B) glucose solution (filled symbols) or water (opened symbols) measured during 8 consecutive days in the lesioned (6-OHDA, n=9; 6-OHDA+DMI, n=9) and control (CO, n=9) animals

the infralimbic PFC. These differences were especially pronounced in the higher glucose consumption of the dopamine lesioned animals.

Acknowledgements. This work was supported by the Hungarian National Research Fund (OTKA T022127; to A.H., OTKA C-012 to L.L.), the Ministry of Welfare of Hungary (ETT T-04731/93 to L.L.) and by the Hungarian Academy of Sciences.

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Preliminary note

ROLE OF ACCUMBENS CHOLINERGIC INTERNEURONS IN THE MAINTENANCE OF BODY WEIGHT

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The nucleus accumbens (NAcc) has been considered as a neural substrate involved in psychostimulant reward and incentive motivation. Experimental data also indicate that the NAcc plays an important role in the regulation of feeding-behavior (for reviews see: Hoebel et al., 1992; Leibowitz and Hoebel, in press). These accumbental functions have usually been studied and considered along with the role of mesolimbic dopamine input arising from the ventral tegmental area. Unlike investigations of interactions between dopamine and acetylcholine (ACh) systems in the dorsal striatum, the cholinergic regulation in the NAcc has received relatively little attention. Recently, it has been revealed that feeding exhibits a definite effect on NAcc ACh release, showing an increase during both natural (hunger-induced) feeding (Mark et al., 1992) and during feeding induced by norepinephrine microinjections in the hypothalamic paraventricular nucleus of satiated rats (Hajnal et al., 1997). To date, however, lesion studies have not demonstrated a behavioral role of cholinergic elements of the NAcc. Therefore, the present study was designed to determine whether selective lesions of ACh interneurons impair feeding and body weight regulation.

In 12 male CFY rats, AF64A (1 nmol / 1 µl) was injected bilaterally into the NAcc. Additional 12 rats were used as sham-operated controls injected with an identical volume of vehicle. The preparation of the aziridium analog of the toxin from precursor ACh mustard hydrochloride (AF64A, Research Biochemicals Inc., U.S.A.) followed the description of Moos et al. (1989). For surgery, subjects were anesthetized with ketamine (Calypsol, Richter Gedeon, 40 mg/kg, i.p.). A silica glass injector (37 µm diameter ID; Polymicro Tech.) with a 26-gauge stainless steel guide tube aimed at the

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posterior medial NAcc was stereotaxically implanted as follows: A 10.2 mm, L 1.2 mm, V 9.0 mm, with reference to the interaural line, mid-sagittal sinus, and the level of skull, respectively. Food and water consumption and body weight were measured daily during 7 postoperative weeks. In order to test the motivational state of the animals and their responsiveness to physiological challenges, 24 and 48 h food-, and water-deprivation tests were conducted during the 2nd postoperative week. These challenges were accompanied by neurological tests for additional effects of the lesion according to the standard procedures of our laboratory (Lénárd et al., 1982; Sándor et al., 1992). The body weight data were expressed in percent of the preoperative values. Daily food and water consumptions were normalized to the actual body weight of the animal. Data were analyzed by analyses of variance (ANOVAs, two-factor with repeated measures) followed by post-hoc *t*-tests when group effects justified (Excel 4.0 for Windows).

After bilateral cholinotoxic lesions of the NAcc, a body weight decrease (-18%) was observed (Fig. 1). From the 2nd postoperative day on a significant weight difference was found between the AF64A-lesioned and sham-operated groups that persisted for the whole 5-week period of comparison (ANOVA, $p<0.001$, $n=12$). Within the early postoperative period

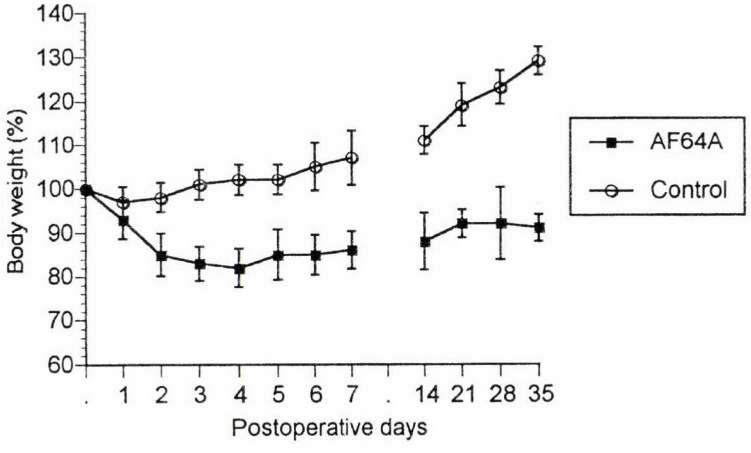

Fig. 1. Body weight changes after bilateral microinjection of AF64A into the NAcc within a postoperative 5-week period of time. Mean body weight is expressed in percent of initial weights (%). Error bars show values of SEM. Zero day on the abscissa indicates the day of operation. Control: sham-operated control group (n=12); AF64A: lesioned group (n=12). For statistics and more explanation see the text

body weight gain was not accompanied by significant alterations of feeding or drinking in comparison to controls. Later, however, the normalized food and the water intake proved to be higher in lesioned animals than in controls (ANOVA, $p<0.01$, between the 21th and 35th p.o. days, *t*-test on the 35th p.o. day: $p<0.01$, Fig. 2). A significantly decreased feeding response (72.3 ± 8.5 %, Mean ± SEM) after 24-h food deprivation was found in the AF64A group ($p<0.01$, not shown). In contrast, this difference disappeared after 48 h food deprivation. In the water deprivation tests, no significant differences were found. In the neurological tests the animals did not show motor imparments or sensory deficits.

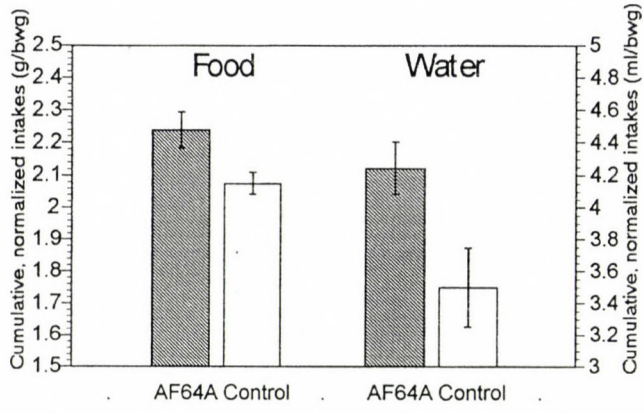

Fig. 2. Mean food and water consumption after bilateral microinjection of AF64A into the NAcc. Data of the daily food and water intake were normalized to the actual body weight (g/bwg) and counted in cumulative manner. Columns represent only the cumulative values of the groups' means on the 35th postoperative day (*t*-tests: $p<0.01$). Control: sham-operated control group (n=12); AF64A: lesioned group (n=12). For more explanation see the text

These findings demonstrate that selective lesions of the ACh interneurons in the NAcc cause feeding but not sensory-motor disturbances as reflected by 1) a lasting depression of the body weight, 2) an increase in food and water intake, 3) a motivational deficit found after a mild food deprivation test. These data suggest that besides the dopamine inputs the cholinergic interneurons of the NAcc have an impact on the maintenance of body weight. In this regard, it has recently been suggested that ACh of the NAcc has a unique role in stopping ingestive behavior. This function of ACh was suggested by the discovery that artificial elevation of ACh in the NAcc with

local neostigmine was sufficient to interrupt feeding or to induce a conditioned taste aversion (Mark et al., 1992). Conversely, a decrease in the ACh functions may be presumed to lead to an increase of feeding. Indeed, the data reported here showed an increase in food and water intake after cholinotoxic lesions of the NAcc. It is worth noting, however, that an inconsistency was found between the food intake and body weight gain of the AF64A-lesioned rats suggesting the additional possibility of an impaired metabolism. For this reason, and in order to describe the symptoms reported here in its wider context, further behavioral and metabolic studies are required.

Acknowledgements. This work was supported by the Hungarian National Research Fund (OTKA F-012978 to A.H., OTKA E-012296, OTKA C-012 to L.L.) and the Ministry of Public's Welfare of Hungary (ETT T-04731/93 to L.L.) and by the Hungarian Academy of Sciences.

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Preliminary note

GLIAL REACTIONS TO LESIONS IN NEURAL TRANSPLANTS: A PRELIMINARY STUDY

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The graft/host border is a site of lesion in both the grafted tissue as in the host, some observations (Zhou et al., 1989 and ours, unpublished) still suggest that the demarcating reactive gliosis is very weak, occasionally missing on the graft side of the border. The astrocytes of the transplants seem to be less responsive to the lesion by which the grafted tissue was isolated from the donor brain. It should be noted that the central nervous tissue can be transplanted in embryonic stage when reactive gliosis does not develop, at least in the rat cortex (for refs see Fisher and Gage, 1993, Ajtai et al., 1997). The solid transplants, however, survive for weeks or months, so it could be expected that, after the maturation of the grafted tissue, the reactive gliosis develops on the graft side of the graft/host border. The question is, whether the graft astrocytes lose their responsiveness only to the surface injury of the grafted tissue or also to other lesions.

The aim of this study was to investigate the reaction of the astroglia in the transplanted brain tissue i) to the host/graft border, ii) to a stab wound in the time, when the astrocytes in the transplant are supposed to be mature.

In the adult recipient rats a 3 × 3 mm piece of the left parietal bone was removed, the dura was opened and a pit was formed in the cortex under gentle suction by a water-jet pump. A piece of embryonic (E15) rat cortex was placed into the pit. To facilitate the re-operation, the skull was covered with thin plastic or aluminium foil pieces instead of the bone piece removed. During operations, deep ketamine-xylazine narcosis was applied.

After a four-week survival period (i.e. when the maturity of the grafts was supposed to correspond to a 20-day postnatal age (Fisher and Gage, 1993), the animals were re-operated and the transplants, if visible, were stabbed by a thin sterile needle. After another 7-day postoperative period,

the animals were perfused transcardially with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4) and postfixed overnight in the same fixative. The transplants and the surrounding host tissue were dissected out, embedded into agarose, cut into serial Vibratome sections and parallel sections were processed for immunohistochemistry against glial fibrillary acidic protein (GFAP). Mouse monoclonal antibodies (Boehringer, Mannheim) were used as primary antibodies and then the sections were incubated with biotinylated rabbit anti-mouse immunoglobulin followed by streptavidin-biotinylated horseradish peroxidase complex. The immunocomplex was visualized by the diaminobenzidine reaction. In some experiments, after the post-transplation period the animals were sacrificed and processed for GFAP-immunohistochemistry without a stab wound in the transplant.

At the present stage our results must be considered preliminary, because of the disadvantageous survival ratio, in consequence of which only in 4 cases were obtained with the survival of both the recipient animal and the re-operated transplant.

In these specimens GFAP-immunopositive astrocytes were found throughout the transplant. Actually, they appeared to be abundant in comparison to the mature rat cortex *in situ*. This phenomenon has also been reported by other authors (Björklund and Dahl, 1982; Björklund et al., 1983; Smith and Ebner, 1985; Connor és Bernstein, 1987) who attributed the phenomenon to a greater survival ratio of astroglia than of neurons and a glial reaction to the neuronal degeneration occurring in transplants. Along the graft/host borders (Fig. 1) a hypertrophy of astrocytes and a strong glial demarcation was observed in the host tissue but only a negligible gliosis affected the graft. This observation was in accordance with the findings of Zhou et al. (1989). Within the transplant, around the stab wound (Fig. 2) no demarcating gliosis developed despite the adjacent mature astrocytes.

Our results suggest that the surface formed surgically develops into a 'natural' surface at that immature stage in which brain tissues are usually transplanted. A similar phenomenon was observed by Sumi and Hager (1968) in the artificial porencephalia of newborn rats. This lack of reactivity seems to persist after the maturation of the grafted tissue. The abundance of GFAP-positive astrocytes, which is supposed to be a reaction to the neural degeneration in transplants, seems to contradict to the absence of reactivity to lesions. It appears that the anisomorph gliosis, which is provoked by penetrating lesions and the isomorph gliosis, which follows neuronal degenerations (Malhotra et al., 1993; Hajós et al., 1990; Mansour et al., 1990) may have different regulatory mechanisms.

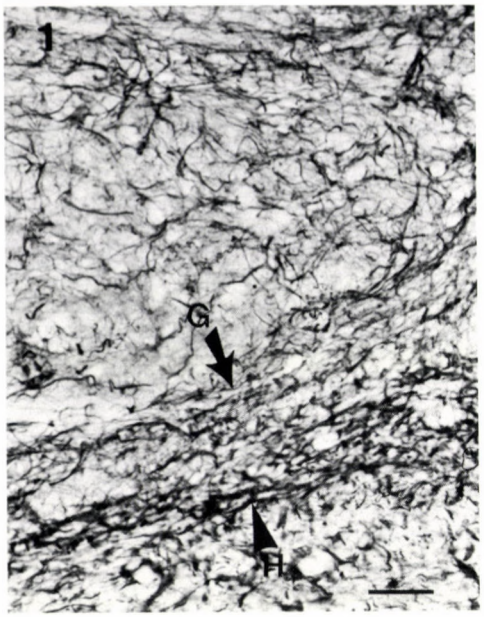

Fig. 1. Border zone of E15 cortical tissue four weeks after transplantation. Note that the reactive gliosis affects mainly the host tissue (H and arrowhead) and to a lesser extent the graft (G and arrow). Bar: 100 mm

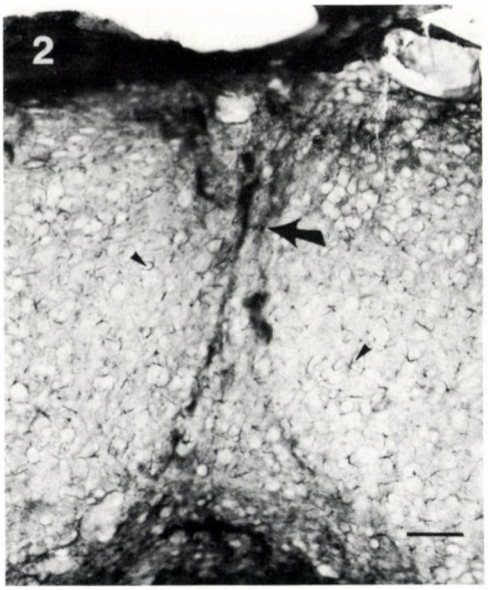

Fig. 2. Stab wound in an E15 cortical tissue, in four weeks after transplantation *plus* one week after stabbing. The trace of the lesion is conspicuous (arrow) but the glial demarcation is negligible although non-reacting astrocytes (arrowheads) are abundant near the lesion. Bar: 400 mm

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Preliminary note

DORSAL ROOT ORIGIN OF AXONAL GROWTH CONES: REGENERATIVE SYNAPTO-NEOGENESIS IN THE UPPER SPINAL DORSAL HORN OF PRIMATES

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Notorious inertness of the central nervous system in establishing viable axonal regeneration is well known since more than a century. This 'harsh decree' of the human (and in general, mammalian) central nervous system (Cajal, 1928) is still one of the major impediments which forbids reconstitution of derailed wiring after accidents, strokes and other pathological impairments. Therefore, the rare exceptions from this general rule, like regenerative proliferation in the upper dorsal horn are of major importance since these may help to decipher the structural and topochemical cues resulting in a permissive environment essential for the regenerative propensity in the mammalian central nervous system.

In a series of publications based on light- and electron microscopic enzyme- and immuno histochemical studies (Csillik and Knyihár, 1975, 1978; Knyihár-Csillik and Csillik, 1981; Knyihár-Csillik et al., 1985, 1989, 1990, 1992; Tajti et al., 1988), we have drawn attention to the peculiar axonal regeneration which ensues in the upper (superficial) spinal dorsal horn after transganglionic degenerative atrophy (TDA).

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TDA is one of the consequences of the blockade of retrograde axoplasmic transport of nerve growth factor in the ipsilateral, segmentally related peripheral sensory nerve (Csillik et al., 1985) which can be obtained, in addition to locally (perineurally) applied microtubule inhibitors, by transection or crush of the peripheral nerve. Whenever the retrograde axoplasmic transport is resumed, a process called regenerative synapto-neogenesis (regenerative proliferation, regenerative or reactive sprouting) results in restoration of the original wiring and synaptic connectivity in the upper dorsal horn, which had been destroyed formerly by TDA (Hajós et al., 1990). In this context it should be emphasized that regeneration of peripheral axons ensues earlier after nerve crush than after nerve transection, since remaining Schwann cells, constituting Büngner's bands, serve as itinerary for regenerating axonal sprouts. In contrast, peripheral axonal regeneration is retarded if transection of the nerve is accompanied by removal of a portion of the nerve. Accordingly, retrograde transport of nerve growth factor will be resumed considerably earlier after peripheral nerve crush than after nerve transection. In consequence, regenerative synapto-neogenesis, characterized by the appearence of axonal growth cones, ensues earlier after peripheral nerve crush than after nerve transection (Csillik and Knyihár-Csillik, 1981; Ferencsik et al., 1989).

While the fact of reactive sprouting and synaptogenesis has been supported by numerous studies (see Coggeshall et al., 1997), the origin of the regenerative axonal sprouts observed in the superficial dorsal horn has been repeatedly questioned (see Doubell et al., 1997). It could be argued, namely, that also local interneurons could emit reactive axons after TDA which might account for the synaptogenesis observed under such conditions. Evidently, if so, the net result of the regenerative process would not be *'restitutio ad integrum'* rather a random (and useless) luxuriant growth.

MATERIALS AND METHODS

In order to answer this equivocal question, we decided to prove dorsal root origin of the regenerative sprouts by means of experimental surgery combined with electron microscopy. Three healthy *Macaca fasciculata* monkeys (one male, two females), 8, 9.5 and 10 kg body weight, were used. The animals were sedated by Ketamine (3 mg/kg) and anesthetized with Nembutal (200 mg/kg). Care of the animals was in conformity with the guidelines controlling experiments and procedures in live animals, as described in the *Principles of Laboratory Animal Care* (NIH Publication No. 85-23, revised 1985). In one animal (M1), the left sciatic was crushed, close to the sciatic notch,

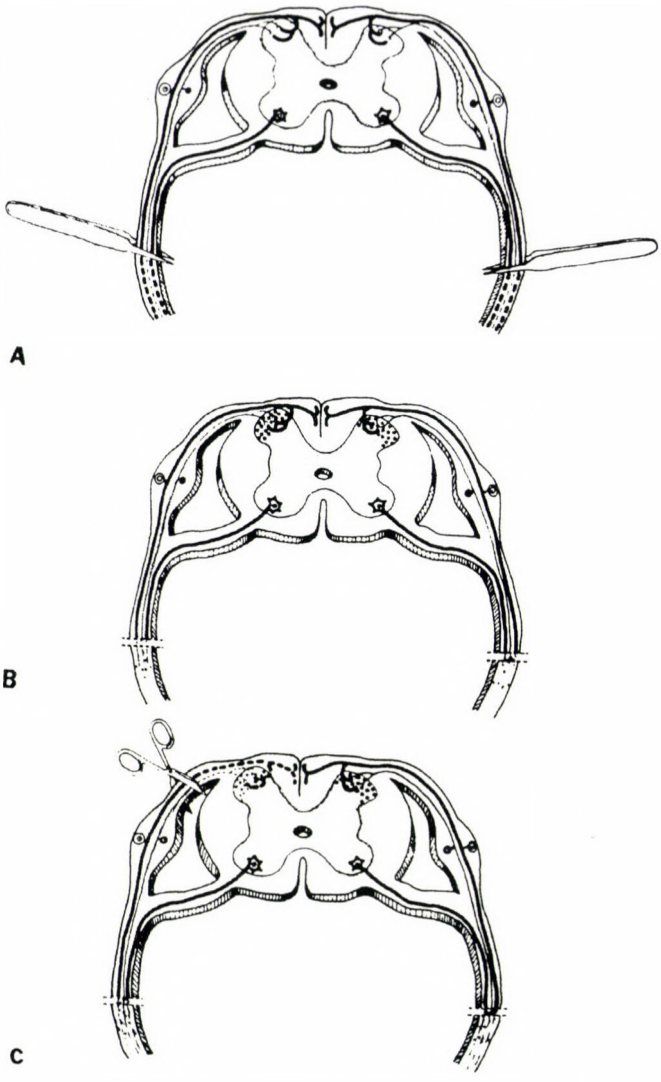

Fig. 1. Schematic drawing of the experimental set-up. A: both sciatic nerves are crushed (forceps); Wallerian degeneration ensues in both sciatics. B: in consequence of the blockade of retrograde axoplasmic transport, transganglionic degenerative atrophy ensues in both upper dorsal horns which yields for regenerative proliferation of central axonal terminals (dotted area) following regeneration of peripheral axons in the sciatic nerve. C: Transection of the dorsal root (scissor) results in Wallerian degeneration of regenerated axons and axonal gowth cones in the upper spinal cord

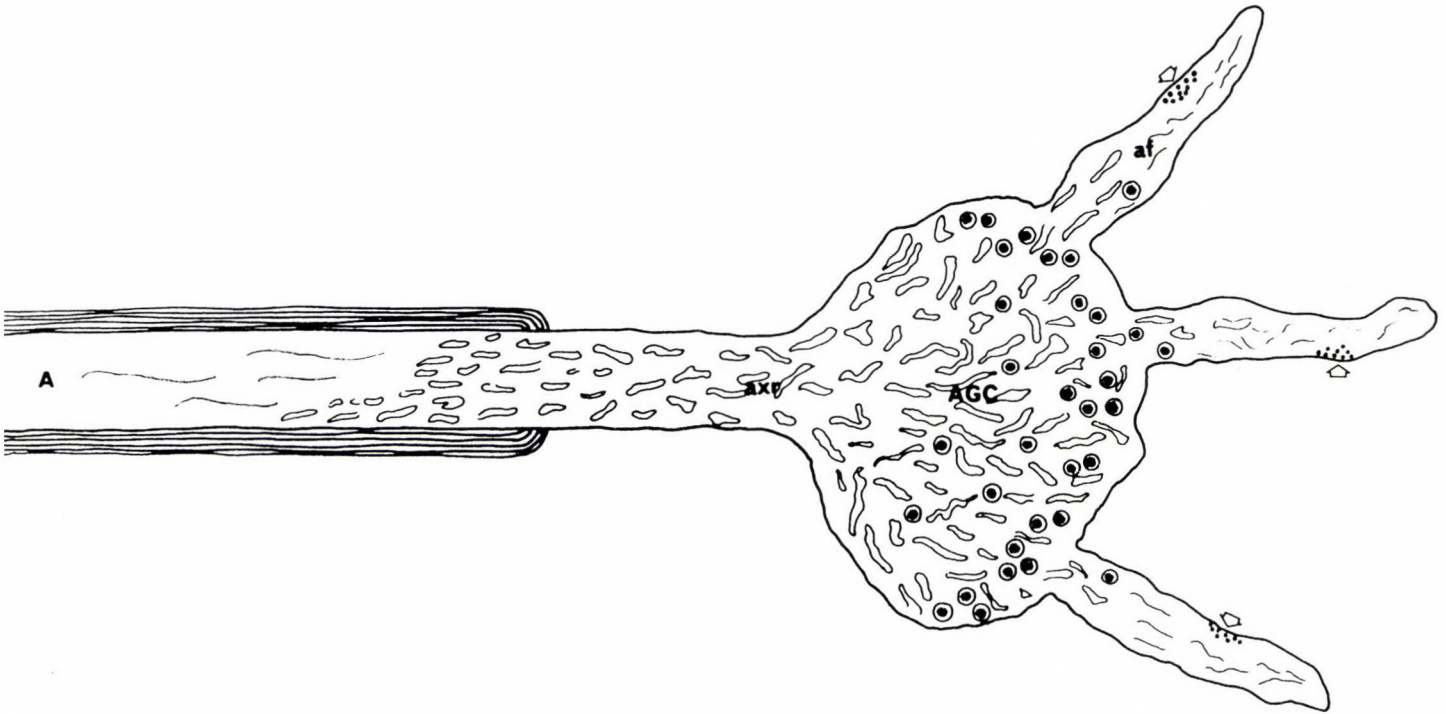

Fig. 2. Schematic drawing of an axonal growth cone (AGC) in the neuropil of the upper (superficial) spinal dorsal horn in the course of regenerative synapto-neogenesis. AGC originates from a thin myelinated axon (A) and contains axoplasmic reticulum (axr) and growth cone vesicles. Axonal filopodia and foliopodia (af) emanate from AGC and establish *de novo* synapses with local dendritic elements (hollow arrows)

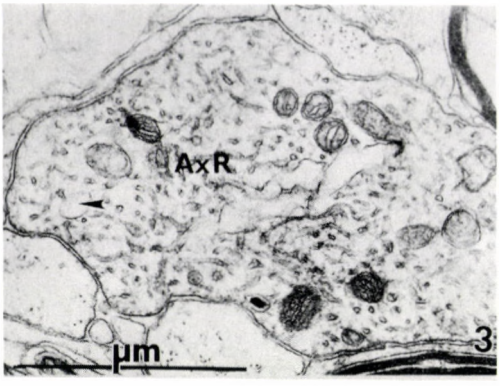

Fig. 3. Axonal growth cone in the spinal dorsal horn of the primate Macaca fascicularis, 3 months after crushing the ipsilateral, segmentally related sensory nerve (sciatic). Note growth cone vesicles (arrow) and cisterns of the axoplasmic reticulum (AxR)

while the right sciatic was transected at this site, and a 2 cm portion of the nerve was removed. One month later, an overdose of Nembutal was administered intraperitoneally, and the heart was set free in the thoracic cavity. A needle was inserted into the left ventricle, through which the vascular system was rinsed with phosphate-buffered saline (1.5 l). This was followed by transcardial fixation with Palay's formaldehyde-glutaraldehyde solution. - In another monkey (M2), the first surgery consisted of crushing both sciatic nerves, close to the sciatic notch. Three months afterwards, left lumbar roots L3, L4, L5 and L6 were transected unilaterally. Two days later, transcardial fixation was performed in anesthesia induced by an overdose of Nembutal, as described above. The third animal (M3) was treated like M2 but it was killed 3 days after rhizotomy. The lumbar spinal cords of the animals were set free and postfixed in the same fixative overnight. Blocks were cut with razor blade from the dorsal quadrants of the spinal cords and post-fixed in 1% buffered osmic acid for 24 hours. After rinsing, the blocks were embedded in Durcupan ACM and sectioned with diamond knife by means of a Reichert Ultrotome. Semi thin sections were stained for light microscopic orientation with basic toluidine blue; thin sections, silver interference colour, were stained with lead citrate and uranyl nitrate and studied by a Zeiss electron microscope.

RESULTS AND DISCUSSION

One month after the initial surgery, TDA prevailed in the lumbar dorsal horn of the first animal (M1), both at the side of transection of the sciatic nerve and contralaterally, i.e. at the side of sciatic nerve crush. In other words, transection as well as crush of the peripheral nerve induced TDA in

the segmentally related, ipsilateral upper dorsal horn. This is in accord with the results of previous studies.

Lumbar dorsal horns of the other two animals (M2 and M3) exhibited entirely different patterns. On the right side, where the sciatic nerve had been crushed three months previously, the entire extent of the substantia gelatinosa Rolandi displayed numerous axonal growth cones, structurally identical to those described in the course of similar experiments and reported in previous studies. In contrast, axonal growth cones on the left (acutely rhizotomized) side exhibited signs of Wallerian degeneration.

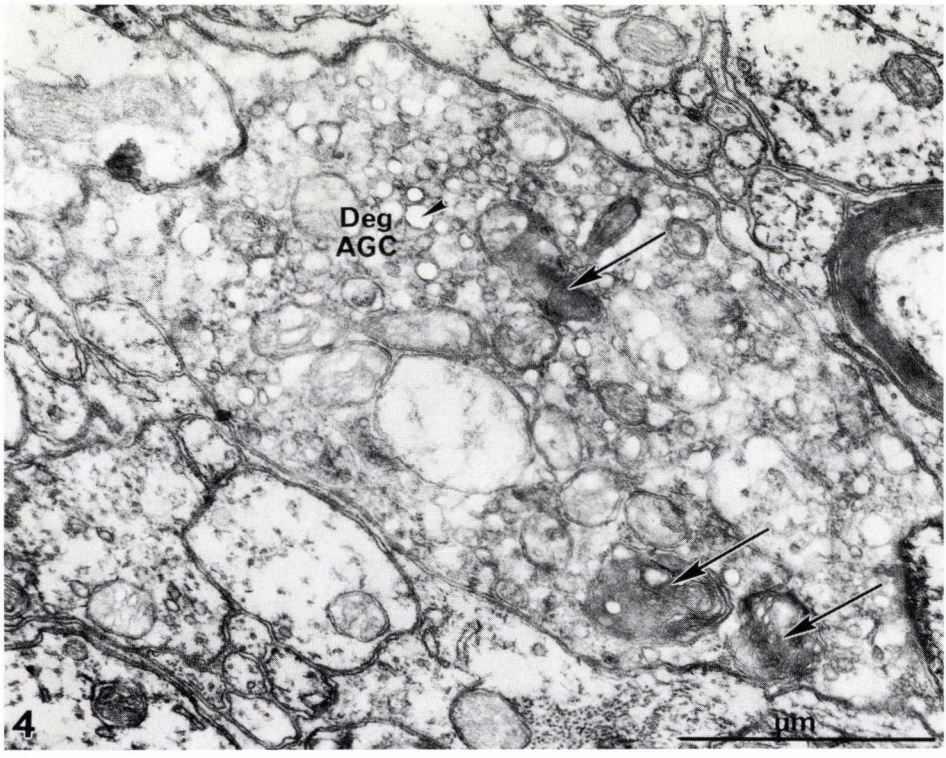

Fig. 4. Incipient Wallerian degeneration of axonal growth cone (Deg AGC), 48 hours after transection of dorsal roots L3, L4 and L5. Dorsal rhizotomy was performed 3 months after crushing the ipsilateral sciatic nerve. Arrows point at membrane whorls characteristic of incipient degeneration. Note growth cone vesicles (arrowhead)

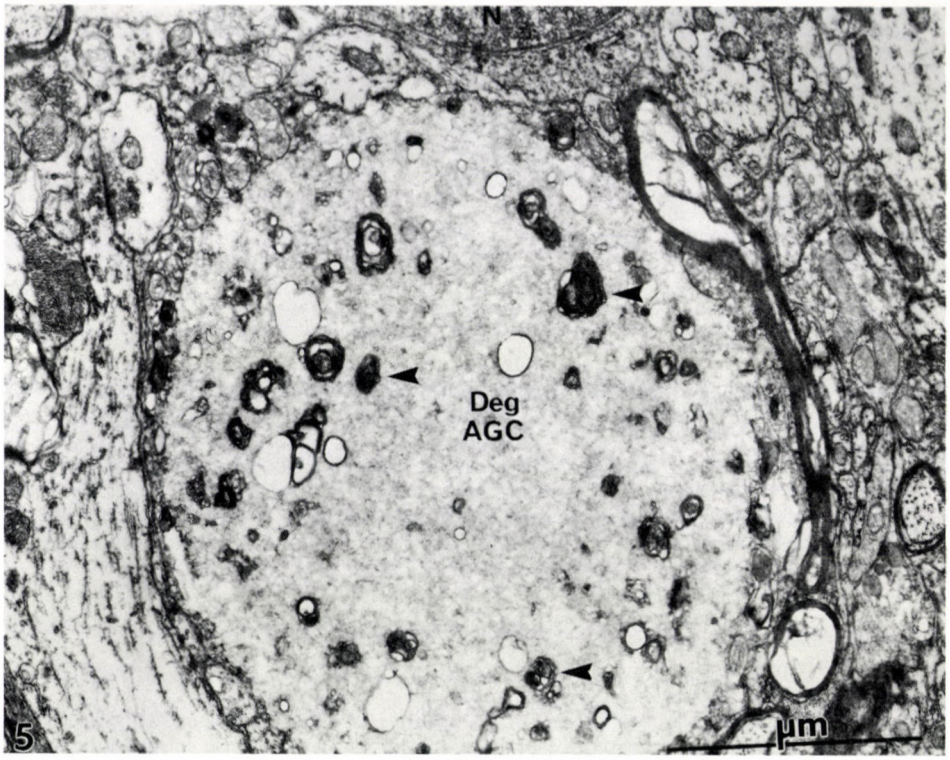

Fig. 5. Progressing degeneration of axonal growth cone (Deg AGC), 72 hours after transection of dorsal roots L3, L4 and L5. Dorsal rhizotomy was performed 3 months after crushing the ipsilateral sciatic nerve. Arrowheads point at osmiophilic degenerative debris. Note absence of growth cone vesicles. N: nucleus of a glial cell

From these experiments it is concluded that sources of regenerative synapto-neogenesis are dorsal root axons. Growth cones of dorsal root axons are operative in restoring wiring in the upper dorsal horn, resulting in a "restitutio ad integrum" after derailment of synaptic connectivity in consequence of TDA.

Acknowledgements. Our thanks are due to Dr. Attila Bezzegh for his help in experimental surgery and to Mrs. Zsuzsanna Pocsai for technical assistance.

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Preliminary note

PROJECTIONS OF THE MEDIAL AND SUPERIOR VESTIBULAR NUCLEI TO THE BRAINSTEM AND SPINAL CORD IN THE RAT

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The dorsolateral part of the rat brainstem contains the vestibular nuclei receiving primary sensory fibers from the vestibular receptors (Brodal, 1981). Physiological and morphological studies on the connections of this primary vestibular region with the second-order centers yielded a large number of contradictory results concerning the central structures involved in the vestibular function (Donevan et al., 1992; Hirai and Uchino, 1984; Rose and Neuber-Hess, 1991; Uchino and Hirai, 1984).

The lectin Phaseolus vulgaris leucoagglutinin was injected either into the superior (SVN) or the medial vestibular nucleus (MVN) in order to study their ascending and descending projections and connections with the structures of the diencephalon, brainstem and spinal cord. Injection sites were determined with the aid of the stereotactic atlas of Paxinos and Watson (1986). The following areas of termination could be discerned when the tracer was injected into the SVN: (1) In the diencephalon the ascending fibers were detected bilaterally in the caudal one-third of the thalamus. Fiber terminals were found similarly to the previous studies (Lang et al., 1979) in the contralateral ventral posteromedial nucleus of the thalamus, gustatory thalamic nucleus, posterior thalamic nuclear group and zona incerta (Fig. 1 a). (2) At the level of the mesencephalon the terminals were found bilaterally

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in the oculomotor nucleus, with an ipsilateral dominance, and in the contralateral trochlear nucleus. The nucleus of Darkschewitsch and the interstitial nucleus of medial longitudinal fasciculus involved in the coordination of eye movements were also richly supplied by the fibers originating from the SVN. Labelled terminals were also detected in the parvicellular part of the red nucleus, the anterior pretectal nucleus, the dorsal part of the medial geniculate nucleus and the deep mesencephalic nucleus. (3) At the level of the pons and medulla (Fig. 1c) the other three vestibular nuclei received fibers from the ipsilateral SVN. A small amount of commissural fibers could be followed to each of the contralateral vestibular nuclei. The sensory and motor cranial nerve nuclei received fibers on both sides. It is in good agreement with the results of physiological studies revealing connections of the vestibular nuclei with the spinal nucleus of trigeminal nerve, the motor facial and hypoglossal nuclei (Fanardjian and Sarkisian, 1988). These kind of interactions between these nuclei indicating the participation of the vestibular nuclei in the coordination of pray catching motor behavior of the frog. A large number of fibers originated from the descending part of the medial longitudinal fasciculus and terminated in the nucleus of prepositus hypoglossi and the dorsal part of the reticular formation including its dorsal paragigantocellular, intermediate and parvocellular nuclei. (4) Contrary to the previous studies the descending fibers could be followed to the spinal cord (Fig. 2a-c). The labeled fibers were detected mainly in the ipsilateral anterior funiculus of the spinal cord as far as the lumbar levels. The terminals were evenly distributed in the Rexed laminae VII, VIII, and IX, some of them were found in the laminae VI and V. A few boutons were detected in the contralateral ventral horn. Some of the descending fibers were found in the lateral and posterior funiculi.

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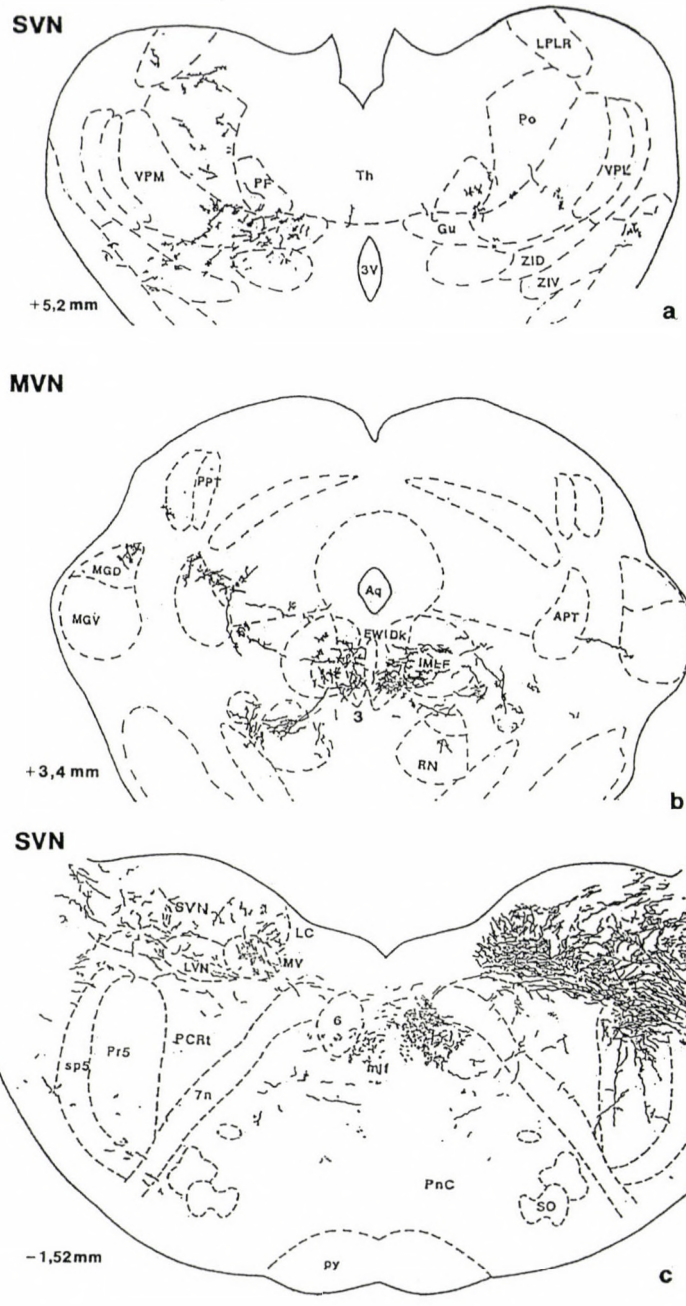
Figs. 1a-c. Cross-sections of the brainstem at the various levels showing the PHA-L labelled structures following the injection of the Phaseolus vulgaris leucoagglutinin into the superior (SVN, Fig. 1a, c) or medial (MVN, Fig. 1b) vestibular nuclei. The roman numerals at the left lower corner of each figure indicate the distance from the interaural line rostrally (+) and caudally (-). 3: oculomotor nucleus, 3V: 3rd ventricle, 6: abducens nucleus, 7n: facial nerve, APT: anterior pretectal nucleus, Aq: cerebral aqueduct, Dk: nucleus of Darkschewitsch, EW: Edinger-Westphal nucleus, Gu: gustatory thalamic nucleus IMLF: interstitial nucleus of Cajal, LC: locus ceruleus, SO: superior olive, LVN: lateral vestibular nucleus, MGD, MGV: medial geniculate nucleus dorsal and ventral part, mlf: medial longitudinal fascicle, MVN: medial vestibular nucleus, PnC: pontine reticular nucleus, Po: posterior thalamic nucleus, PrS: principal sensory nucleus of trigeminal nerve, py: pyramidal tract, RN: red nucleus, sp5: spinal trigeminal tract, SVN: superior vestibular nucleus, Th: thalamus, VPM, VPL: medial and ventral posterolateral thalamic nucleus, ZID, ZIV: zona incerta dorsal and ventral part Calibration bar is 500 μm


SVN
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PCRt
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Injection of the lectin into the medial vestibular nucleus revealed the following termination areas: (1) In the diencephalon the terminals were found in the contralateral zona incerta. (2) At the level of the mesencephalon, the terminals were less numerous than that of SVN origin and were found within the bilateral oculomotor and trochlear nuclei and in the red nucleus (Fig. 1b). (3) The pontine and medullary termination pattern was also different from the SVN origin. More intense labelling was found in the vestibular nuclei ipsi- and contralateral to the injection site. A great number of boutons was found in the ipsilateral abducens nucleus. Less intense labelling was observed in the areas of the reticular formation and in the cranial nerve nuclei except the mandibular division of the spinal nucleus of the trigeminal nerve. (4) The projection to the spinal cord was bilateral in the anterior funiculus, a large contingent of fibers were found in the contralateral lateral funiculus and a few of them in the dorsal funiculus. At the cervical level the ipsilateral termination was found almost exclusively in the central cervical nucleus.

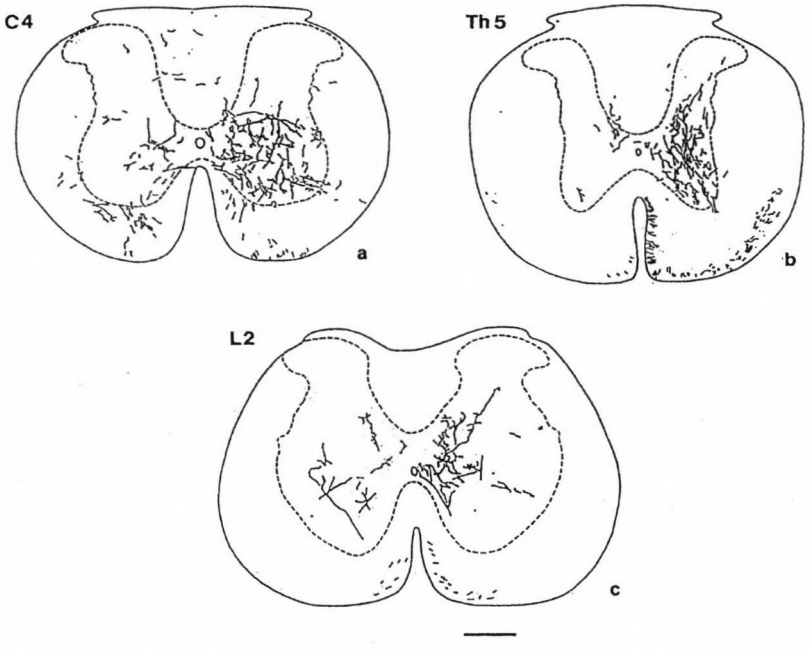

Fig. 2a-c. Cross-sections of the spinal cord at the cervical (C) thoracic (Th) and lumbar (L) levels indicating the PHA-L labelled fibers and boutons originating in the superior vestibular nucleus. Calibration bar is 500 μm

The results presented here demonstrate the differences in the termination areas of the secondary vestibular fibers originating from the superior and medial vestibular nuclei. According to the results of the present study the SVN also contribute to the formation of the vestibulospinal pathways. Our findings are in good agreement with the results of the physiological experiments that the different vestibular nuclei have different functions to maintain the balance and orientation of the body.

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Preliminary note

EFFECTS OF DIFFERENT TYPES OF ANTICHOLINESTERASE AGENTS ON IN VIVO HIPPOCAMPAL POPULATION SPIKES IN RATS

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Any anticholinesterase agent, when administered systemically, has widespread and severe physiological and/or toxicological effects. In the central nervous system, acetylcholine is mainly a modulator, responsible e.g. for the ascending control of arousal (McCormick, 1992). In the hippocampus, cholinergic input to CA1 pyramidal neurons from the medial septum (Wainer et al., 1985). Its enhancing effect of synaptic excitation has also been described (Rovira et al., 1983).

Organophosphates (OPs), used as insecticides, are irreversible AChE blockers and their effects on the central nervous activity has been widely studied. While it is obvious to attribute these effects to the AChE blockage, certain data in the literature are not consistent with this (Gralewicz et al., 1991). To see if the OPs studied by us have any non-cholinergic effects we chose the hippocampal population spike (POPSP; Anderson et al., 1971) as a phenomenon under an established cholinergic influence and compared the effect exerted on it by OPs and by physostigmine as a reference AChE blocker. This choice was supported by previous results (Papp et al., 1997).

The experiments were performed on adult male rats weighing ca. 400 g, anaesthetized with 1000 mg/kg urethane i.p. The left hemisphere was exposed and the dura removed. To elicit POPSPs, the perforant path (AP: -6, L: 4.5, V: 4 mm stereotaxic coordinates; Paxinos and Watson, 1982) was stimulated (1 mA, 0.05 ms, 0.1 Hz) via a bipolar needle. A glass recording microelectrode was put in the CA1 region (AP: 3, L: 2.5, V: 2 - 4). Every 10 minutes, a train of 20 stimuli were given and the averaged POPSP was recorded after 1000 x AC amplification in a PC using the pCLAMP sotfware. After 4 or 5 control records, the drug to be studied was given i.p. and further records were taken with the same sequence. The amplitude of the POPSP was measured afterwards and the relative change (treated/control) was calculated and plotted.

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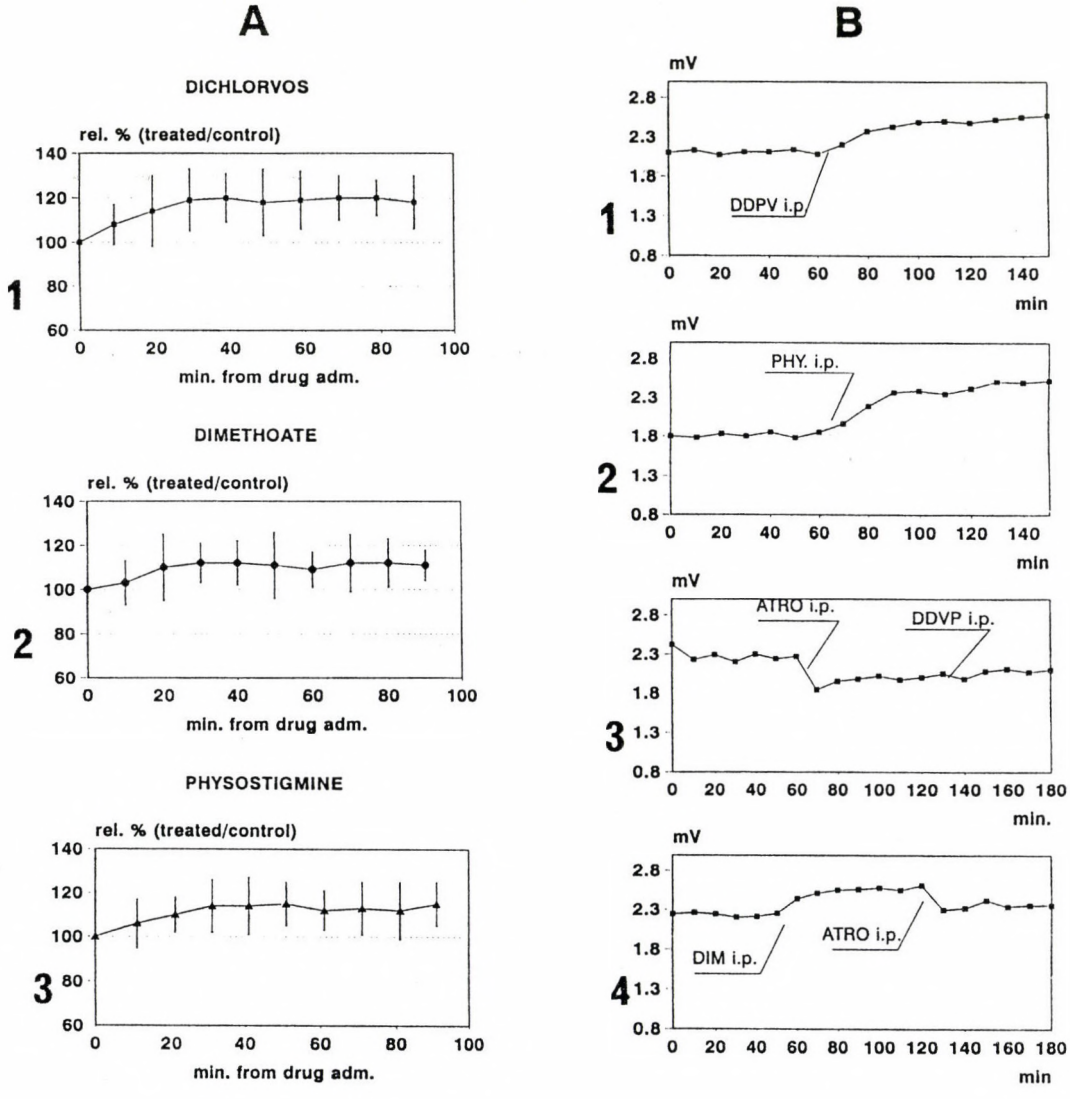

Fig. 1. A: Averaged (n = 10) relative changes of the peak-to-peak amplitude of the population spike caused by dichlorvos (1) dimethoate (2) and physostigmine (3). Error bars represent S.D. B: Time course of the POPSP amplitude in typical single experiments, illustrating the effect of dichlorvos (1), physostigmine (2), atropine and dichlorvos (3) and dimethoate and atropine (4)

The organophosphates, dimethoate (DIM) and dichlorvos (DDVP), were given in 1/5 LD50 dose (45 mg/kg DIM and 0.8 mg/kg DDVP), to keep the results comparable with previous ones (Dési et al., 1991; Nagymajtényi et al. 1994). Physostigmine (PHY) was given in 0.1 mg/kg (based on Lynch and Coon, 1972; Messamore et al., 1993). Both OPs induced an increase in the POPSP amplitude. The increase began within 10 minutes from drug application and was full-blown in ca. 30 min. There was practically no change in the latency of the POPSP peaks. The average relative amplitude change from 10-10 experiments was +19% with DDVP and +12% with DIM (Fig. 1; A/1,2 and B/1). Atropine (ATRO) in 1 mg/kg, given ca. 30 min before OP administration, induced an amplitude decrease which was not reversed by subsequent OP (Fig. 1; B/3,4). PHY as reference substance had the same effect like the OPs. The average amplitude increase of the POPSPs under PHY was +14 % (Fig. 1; A/3, B/2). The effect of atropine pretreatment was also the same as for OPs. The POPSP amplitude increase was significant with each anticholinesterase agent ($p<0.05$, Wilcoxon's signed rank test).

Our results suggest that the effect of OPs on the hippocampal POPSP in rats is a cholinergic one. Septo-hippocampal cholinergic fibres, ending mainly on the pyramidal neurons modulate neuronal activity (Benardo and Prince, 1982) thereby facilitating transmission (Krnjevic and Ropert, 1982). When, by means of the OP-induced AChE blockage, cholinergic influence is increased, a given stimulus can elicit a stronger response - as has been seen by us.

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Preliminary note

DOUBLE IMMUNOHISTOCHEMICHAL STUDY ON PARAVENTRICULAR EFFERENTS TO AUTONOMIC CENTERS

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The hypothalamic paraventricular nucleus (PVN) is well known to have an important regulatory influence on the autonomic nervous system via its direct projections to the lower brainstem autonomic centers and to the intermediolateral cell column of the spinal cord (Saper et al., 1976; Swanson and Kuypers, 1980; Sofroniew and Schrell, 1981). This pathway serves as one of the most prominent neuronal links between the neuroendocrine and autonomic regulatory centers in the brain (Swanson and Sawchenko, 1980; Swanson et al.,1980). However, the exact path of the descending fibers and their connections are uncleared.

In this study neuronal pathway descending all along the neuroaxis down to the thoracic spinal cord was followed by application of the anterograde tracer, Phaseolus vulgaris leuco-agglutinin (PHA-L) into the rat PVN (Fig. 1.1., Gerfen and Sawchenko, 1984). PHA-L was injected iontophoretically with a glass micropipette having a tip diameter of 20-40 mm according to the stereotaxic coordinates of Paxinos and Watson. In attempt to reveal the neurotransmitter character of the cells that PHA-L labelled paraventricular fibers may be in contact with, a second immunohistochemical staining was performed on some sections (Bolam, 1992). Terminal areas for PVN fibers in the brainstem, like locus coeruleus (LC), parabrachial nuclei (PB) and nucleus of the solitary tract (NTS), have already been demonstrated in rats (Luiten et al, 1985; Larsen et al., 1991). In the present study our special attention was focused on the areas

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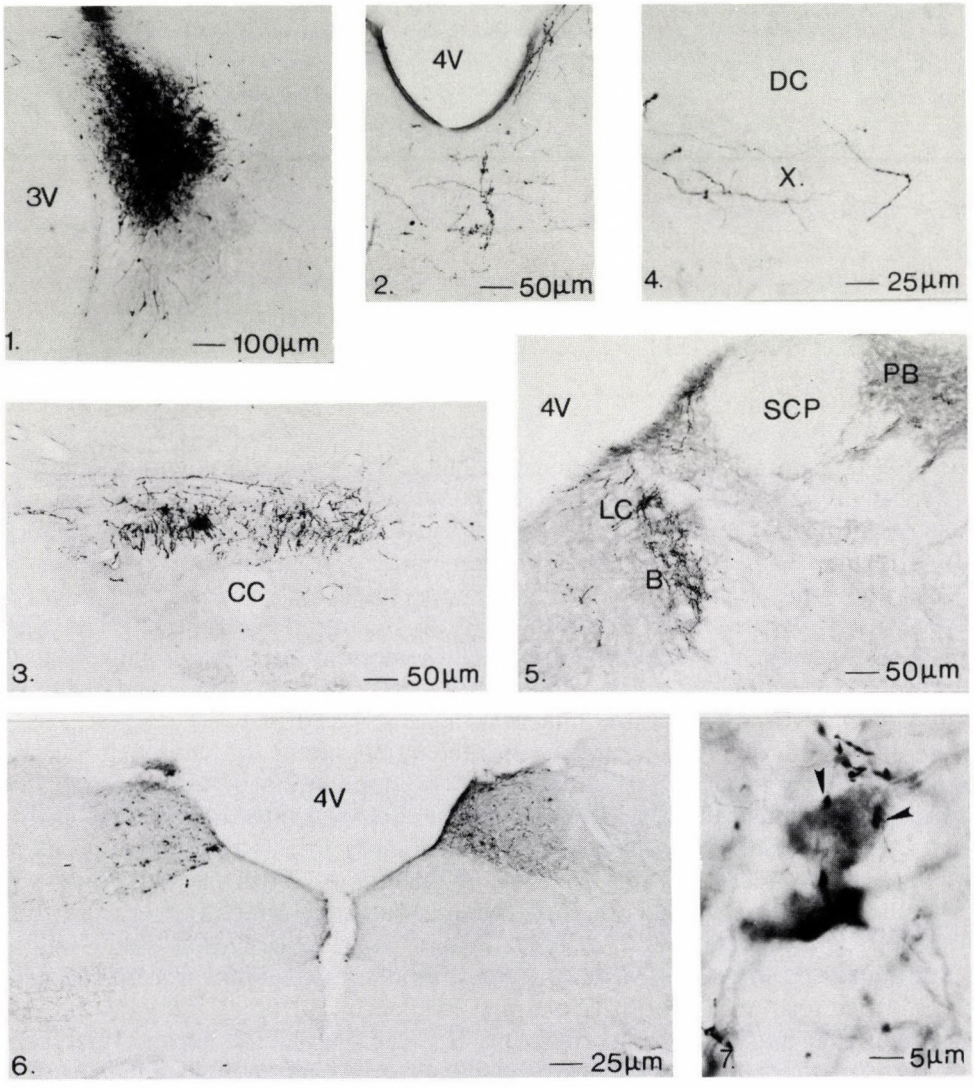

Fig. 1. 1. PHA-L injection site in the PVN. 2. Decussating fibers in the pontine tegmentum. 3. Decussating fibers in the commissural NTS. 4. Decussating fibers in the spinal cord Rexed X. 5. Ipsilateral fibers in the LC, PB and Barrington nucleus. 6. Bilateral fibers in the NTS. 7. Close proximity between a TH-ir like cell and a PHA-L-ir like fiber (arrows).
Abr.: 3V: third ventricule, 4V: fourth ventricule, CC: central canal, DC: dorsal column, X.: Rexed X., B: Barrington nucleus, SCP: superior cerebellar peduncle

where descending fibers cross-over the midline to establish bilateral inputs from the PVN. The major findings are as follows:

1. The fibers cross the midline at several levels. Decussating fibers are observed in the supramamillary, pontine tegmental (Fig. 1.2), NTS-commissural areas (Fig. 1.3.) and in the spinal cord (Fig. 1.4.), as well.
2. Ipsilateral dominance of descending fibers can be seen especially in the periaqueductal gray, LC and PB (Fig. 1.5).
3. The Barrington nucleus in the pontine tegmentum has a strong bilateral innervation.
4. There is a perpendicular path through the subcoeruleus area between the lateral tegmentum and the ventrolateral corner of the pons.
5. There is an arch between the dorsomedial and ventromedial medulla.
6. The NTS has a strong bilateral innervation as well (Fig. 1.6.), especially its commissural subdivision. It is strongly suggested from our results, that the fibers of paraventricular origin enter firstly the commissural part of the NTS, cross the midline in it and pass towards the rostral part of the NTS. Experiments are planned to verify this hypotesis by using combined approaches of experimental surgery and immunocytochemistry.

Double immunohistochemistry at the light microscopic level indicated that there was a close proximity between the PHA-L labelled fibers and galanin-ir like cells in the locus coeruleus and Barrington nucleus, tyrosine-hydroxilase (TH)-ir like cells in the NTS (Fig. 1.7) and cholinacetyltransferase (Chat)-ir like cells in the intermediolateral cell column throughout the thoracic spinal cord.

Further electron microscopic studies are needed to provide evidence for the existence of synaptic connections between the phaseolus labelled paraventricular fibers and the galanin-, TH- and Chat-ir like cells in the locus coeruleus, Barrington nucleus, NTS and intermediolateral cell column, respectively.

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