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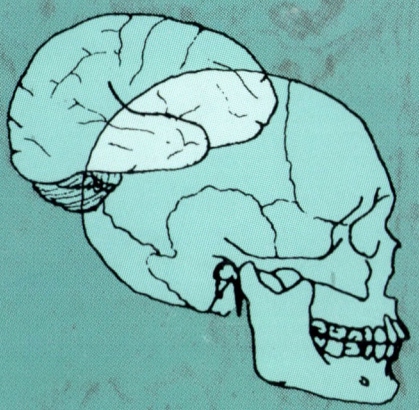

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RESEARCH REPORT

SEMICARBAZIDE-SENSITIVE AMINE OXIDASES: WIDESPREAD OCCURRENCE AND MANY NAMES AND FACES

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Introduction

In the past half century, amine oxidase activity has come to light in many different species: plants, microorganisms and animals, especially mammals. From the start the issue has been confused by the different methods used for determining the activity and by the differences in amines used as substrates. Accessability to the study of these enzymes has also been greatly hampered by the wide variation in nomenclature, which often involved the substrate used, the source of the enzyme activity, or both. Practically from the start various attempts have been made to provide a more rational approach to the study of these enzymes, based not only on substrates, but also on inhibitor profile and cofactor identity. Some excellent reviews have appeared (e.g. Buffoni, 1966; Blaschko, 1974).

The most important step was made when it was realized that there were at least two different cofactors, with concomitant inhibitor profile, present. One group of amine oxidase enzymes depended for activity on a flavin adenine dinucleotide (FAD) cofactor, and was resistant to the action of carbonyl reagents. This group included the well-known monoamine oxidases (MAO) A and B and some polyamine oxidases, and has been classified as monoamine:oxygen oxidoreductase (deaminating) EC 1.4.3.4. The other group of amine oxidase enzymes appeared to have a carbonyl-containing cofactor, of uncertain identity, as a functional unit in the cofactor. The activity of this group of enzymes was not influenced by inhibitors of the FAD entity, but instead by compounds reacting with a carbonyl group, such as semicarbazide. This latter group, now classified as amine:oxidoreductase (deaminating)

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(copper containing) EC 1.4.3.6, but often called semicarbazide-sensitive amine oxidases (SSAO) has until recently received far less attention than the FAD-dependent MAO group. In the present contribution, we will focus on this group of SSAOs, their different names and faces and their presence in nature.

SSAO: general aspects and many faces

One of the problems in the classification of SSAOs has been the large variety of substrates reported, which were often quite specific for the source of the enzyme used. Enzymes from other sources had completely different substrate patterns. However, most if not all enzymes recognised as SSAOs are able to convert (non-physiological) benzylamine to benzaldehyde, hydrogen peroxide and ammonia:

$$C_6H_5CH_2NH_2+O_2+H_2O \rightarrow C_6H_5CHO+H_2O_2+NH_3$$

For practical purposes, therefore, it seems logical to define SSAO as any enzyme which is capable of transforming benzylamine into benzaldehyde in the presence of a MAO-inhibitor, but which lacks this capability in the presence of a carbonyl reagent such as semicarbazide. The inclusion in this definition of the resistance to MAO-inhibitors is necessary, since other enzymes, notably MAO-B, are also capable of performing the same transformation. An added advantage of this definition is that most (but not all) assays developed for SSAO measurement use benzylamine as a substrate and include a MAO-inhibitor in the incubation procedure.

Some other characteristics are probably common to SSAOs, although they have not been investigated in full in all. The enzymes are glycoproteins with a molecular weight of approximately 180 000 Dalton and exist as dimers. Differences reported in the M_w could result from differences in sugar content (Falk et al., 1983). The investigated enzymes all contain copper, which sometimes is accessible to copper chelating agents, resulting in loss of activity. The carbonyl-entity containing cofactor was originally believed to be pyridoxal phosphate and later pyroloquinoline quinone (Lobenstein-Verbeek et al., 1984). In the past few years it has become clear that in most if not all SSAOs the real cofactor is a topaquinone entity, which is build-up post-translationally from a tyrosine unit in the amino-acid chain (Janes et al., 1990; Holt et al., 1998). Apart from benzylamine and other nonphysiological compounds like allylamine (Boor et al., 1990) and 3,4-dihydroxybenzylamine (Boomsma et al., 1993), natural substrates for (some) SSAOs include β-phenylethylamine,

aminoacetone, methylamine, kynuramine, histamine, serotonin, tyramine, tryptamine, dopamine, the polyamines spermine, spermidine and putrescine, mescaline and octopamine (see e.g. Lyles, 1996). At present it is still not clear whether the widely varying activity towards different amines is due to different enzymes or to differences in glycosylation. Some SSAOs are soluble, while others are membrane-bound, which may also contribute to different substrate patterns, through differences in tertiary structure attributable to surrounding conditions.

An interesting distinction between different SSAOs has been reported based on the presence or absence of stereoselectivity in the abstraction of a hydrogen from the alpha-carbon of substrates like benzylamine or tyramine in the course of the transformation reaction (Yu and Davis, 1988; Coleman et al., 1989; Scaman and Palcic, 1992).

Nomenclature

Based on the definition above, a large number of reported enzymes can now be recognised as being SSAOs (Table 1).

Table 1. Various names of enzymes and proteins belonging to the SSAO class

A	B	C	D	E	F
E. coli amine oxidase	Aminoacetone oxidase	Clorgyline-resistant amine oxidase	Plasma monoamine oxidase	Amiloride-binding protein ?	Vascular adhesion protein-1 ?
Lentil seedling amine oxidase	Benzylamine oxidase	Copper amine oxidases	Serum monoamine oxidase		
Pea seedling amine oxidase	Mescaline oxidase		Retina-specific amine oxidase		
Yeast amine oxidase	Phenylethylamine oxidase				
	Tyramine oxidase				
	Histamine oxidase				
	Histaminase				
	Methylamine oxidase				
	Diamine oxidase				
	Polyamine oxidase				
	Spermine oxidase				
	Lysyl oxidase ?				

Group A consists of amine oxidases named (only) after the species in which the activity was found. These are mainly microorganisms like *Escherichia coli*, yeast like *Hansenula polymorpha*, and plants like lentils and peas.

Group B SSAOs were named after the amines found to be deaminated: aminoacetone, benzylamine, mescaline, phenylethylamine, tyramine, histamine, methylamine, polyamines like spermine. These polyamine oxidases are a mixed group and do not all belong to the SSAO class since many if not most of them are FAD-dependent (Smith, 1985; Morgan, 1985). Lysyl oxidases catalyse the oxidative deamination of lysyl residues in proteins and are involved in the cross-linking of connective tissue; they form a distinct subclass sometimes given a separate EC denomination (EC 1.4.3.13). Diamine oxidases metabolise histamine and especially diamines like putrescine and cadaverine.

The term clorgyline-resistant amine oxidase was coined to draw special attention to the fact that the enzymes were resistant to the inhibitory action of clorgyline on the activity of MAO-B, which is likewise capable of deaminating benzylamine (Lyles and Callingham, 1975). The name copper amine oxidases is a broad term referring to the presence of copper in these amine oxidases.

Tissue or organ has also occasionally been used as a basis for naming SSAO activity. The original term of plasma (serum) monoamine oxidase has been rather confusing, because it has led to association with the FAD-dependent MAO enzymes. A novel use of such a name is the human retina-specific amine oxidase reported recently (Imamura et al., 1997).

The increasing work done on the cloning and sequencing of genes is leading to other names for proteins which may also be included in, or even may be identical to, the SSAO enzymes. Examples are the amiloride-binding protein, which is partly homologous to SSAO from pig kidney and human placenta (Novotny et al., 1994), and even more so the recently reported vascular adhesion protein-1 (Smith et al., 1998).

In many instances the names of the SSAOs incorporate more than one of the above-mentioned group names, e.g. rat kidney amiloride-binding protein and goat liver aminoacetone oxidase.

Sources of SSAO

Enzymes belonging to the SSAO group are widely present in nature. In microorganisms SSAOs have been found in e.g. *Escherichia coli* (Moënne-Loccoz et al., 1995) and *Arthrobacter globiformis* (Choi et al., 1995) and in the yeast *Hansenula polymorpha* (Cai et al., 1997).

Particularly rich sources of SSAO are seedlings of peas (*Pisum sativum*) and lentils (*Lens culinaris*) (see Hill and Mann, 1964; Smith, 1985; Medda et al.,

1996). The (poly)amine oxidases from other nonleguminous plants are probably mostly FAD-dependent amine oxidases (Smith, 1985; Morgan,1985). In animals SSAO has been found in hepatopancreas homogenates of the mollusc *Callista chione* (Nicotra et al., 1994), in chick heart (Fowler and Callingham, 1977), in amphibia in liver from the frog *Rana catesbeiana* (Kobayashi et al., 1981) and the toad *Bufo bufo* (Senatori and Nicotra, 1988), and in intestine, liver and skin of the catfish *Parasilurus asotus* (Kumazawa et al., 1989) and in the heart of the carp *Cyprinus carpio* (Yamamoto et al., 1984). Most reports however are about the abundant presence of SSAO in mammals, including man.

Mammalian organs/tissues

Many studies of SSAO have been performed in a bewildering array of organs and tissues of many mammalian species (see e.g. Lyles, 1996). High SSAO activity has been reported in vascular smooth muscle cells of a.o. rat (Wibo et al., 1980; Clarke et al., 1982; Elliott et al., 1989; Buffoni et al., 1994)], pig (Tipnis et al., 1992), sheep (Elliott et al., 1992) and dog and cow (Yu and Zuo, 1992). Studies in rat artery SSAO to be mainly located in the tunica media (Lyles and Singh, 1985. Other sources of SSAO are the (human) umbilical artery (Precious et al., 1988; Lyles et al., 1990; Lyles and Chalmers, 1992; Yu et al., 1994), dental pulp of pig (Norqvist et al., 1982) and cow (Nakano et al., 1974), liver of goat (Ray and Ray, 1987) and cow (Høgdall et al., 1998), white and brown adipose tissue of rat (Barrand and Callingham,1982 and 1984; Raimondi et al., 1991), kidney of pig (Novotny et al., 1994), heart of rat (Clarke et al., 1982), Guinea pig (Puliti et al., 1996) and cow (Høgdall et al., 1998), lung of cow (Lizcano et al., 1998) and guinea pig (Puliti et al., 1996), skin of guinea pig (Puliti et al., 1996), eye of cow (Arriba et al., 1991), rat (Cao Danh et al., 1985) and man (Imamura et al., 1997), spleen of cow (Høgdall et al., 1998), vas deferens of rat (Lizcano et al., 1991). Recent studies have shown SSAO to be present in rat adipocyte membranes and GLUT4 glucose transporters (Morris et al., 1997; Enrique-Tarancon et al., 1998).

The most extensive studies on the presence of SSAO in human and rat organs and tissues have been reported by Lewinsohn et al., 1978 and 1980. An elegant study on SSAO activity in mouse organs has recently been published (Grönvall et al., 1998).

The tissue SSAO is probably mostly membrane-bound, with a short tail inside the cell, a transmembrane segment, and the largest part with the catalytic side sticking out from the membrane.

Mammalian plasma

The plasma SSAOs are often called soluble SSAOs to discern them from the membrane-bound SSAOs of organs. Whether there is a clear-cut molecular difference between the two is not known as yet. The source of the plasma SSAOs is also not known. If the soluble SSAOs are derived from the membrane-bound SSAOs which have one transmembrane segment, one might visualise the soluble SSAOs to be formed by cutting off the outer-membrane portion. To our knowledge, there is no evidence for such a mechanism so far.

Activity of SSAO has been described in plasma of many mammalian species including pig, cow, goat, horse, rat, mouse, dog, sheep, deer, guinea pig, rabbit, and man (Eliott, 1960; McEwen and Cohen, 1963; Buffoni and Della Corte, 1972; Ray and Ray, 1983; Boomsma et al., 1993; Callingham et al., 1995; see also Blaschko et al., 1959 and Blaschko, 1962). Activity appears to be particularly high in ruminants, and much less in man and in rodents. A comparative analysis of plasma SSAO activity in different species has recently been reported (Boomsma et al., submitted).

Although all plasma SSAOs readily deaminate benzylamine, activity towards other substrates differs greatly. In order to obtain more insight into these varying properties, we have investigated a large number of compounds as possible substrates (or inhibitors) for the SSAO in the plasma of various animals. To this end, we investigated the changes in benzaldehyde formation brought on by the presence of various concentrations (1.25, 2.5, 5 and 10 mM) of more than 30 other amines in the incubation mixture, next to the usual (saturating) amount of benzylamine in our functional assay method (2.6 mM) (van Dijk et al., 1995). Results, expressed as a percentage of the formation in the absence of these other amines, are tabulated in Table 2 for the 10 mM amine concentration. Data are means of 3 experiments; standard deviations are all $<$ 10% and are not included. Blank incubations were also performed with all amines and showed no interference. For comparison, we did similar experiments in homogenates of 10-day-old seedlings of green pea and marrowfat; these data are also included in Table.

Table 2. Effects of various amines on the production of benzaldehyde from benzylamine by SSAO of different sources

Compound (10 mM)	Plasma								Seedling homogenate	
	Human	Pig	Goat	Sheep	Cow	Horse	Rabbit	Dog	Green pea	Marrowfat
Allylamine	6	52	9	11	0	19	12	8	1	1
Kynuramine	20	32	16	11	2	37	21	23	59	55
Aminoacetone	36	12	5	12	4	14	18	22	14	23
Methylamine	44	102	34	32	7	89	98	102	57	52
Ethylamine	60	95	72	75	40	56	77	49	80	62
Ethanolamine	90	74	44	44	31	59	83	82	61	60
Tryptamine	80	67	14	21	9	32	97	54	40	41
5-Methoxytryptamine	83	66	33	36	17	24	55	16	33	32
Amiloride	77	46	41	25	12	37	52	19	112	82
Phenylephrine	66	50	72	78	63	68	80	132	89	96
Histamine	105	54	38	42	27	59	102	80	14	11
Serotonin	101	65	31	38	39	51	76	53	63	71
Noradrenaline	95	83	15	16	6	19	72	38	106	116
Dopamine	101	61	31	36	22	47	62	83	60	72
Adrenaline	98	66	64	74	65	90	99	76	97	97
3,4-Dihydroxybenzylamine	103	55	14	35	14	30	43	72	87	115
Tyramine	87	91	30	34	14	58	86	31	79	71
3-Methoxytyramine	99	85	35	37	19	43	64	9	39	35
Octopamine	89	88	24	23	16	31	75	79	77	78
Spermine	81	99	29	29	11	38	102	77	37	31
Spermidine	84	96	28	32	13	83	88	70	11	9
Putrescine	98	98	83	87	83	85	93	90	6	4
5-Aminolevulinic acid	96	75	30	36	21	71	61	97	61	62
Ag mati ne	77	55	54	59	44	57	80	57	9	9
Taurine	87	100	100	99	84	86	98	96	93	87
Ornithine	104	97	98	97	89	91	103	101	86	80
Glycine	97	101	82	81	92	84	91	80	91	86
β-Alanine	101	95	90	94	101	96	95	93	96	93
Lysine	105	104	99	101	92	92	102	97	85	76
4-Aminobutyric acid	94	96	99	95	90	79	100	96	95	95
3-Aminoisobutyric acid	106	94	97	94	89	86	100	93	90	78
β-Aminopropionitril	22	49	17	18	4	71	79	95	0	0
Semicarbazide	0	0	3	6	0	1	3	2	0	0

Data (mean of 3 experiments) represent the formation of benzaldehyde from benzylamine, expressed as a percentage of the formation in the absence of the added compound. Standard variations are not included but are < 10% in all cases

β-Phenylethylamine is not reported in Table, as it gave interfering peaks at the retention time of benzaldehyde in the chromatograms, even with the highest purity of the compound commercially available. Also, aminoguanidine, an SSAO inhibitor (Yu and Zuo, 1997) is not reported, because this compound interferes with the subsequent derivatization reaction of benzaldehyde with dimedone.

Clearly, there are large differences in the influence of various amines on benzaldehyde generation. Spermine e.g. is a substrate for some, but not all plasma SSAOs. In this respect it should be mentioned that in some mammals, e.g. sheep (Elliott et al., 1992; Callingham et al., 1995), more than one kind of SSAO is present, and each could have its own preferred substrate(s). Plasma of the ruminant cow contains the most omnivorous SSAO. In all species allylamine, kynuramine and aminoacetone are excellent substrates for the plasma SSAO. Curious is the fact that methylamine and ethylamine do not appear to be substrates for pig plasma SSAO, in contrast to most other plasma SSAOs. In dog and rabbit plasma, methylamine also does not influence benzaldehyde generation, but ethylamine does. Possibly this is due to a much lower reaction rate for methylamine, as has been suggested for the SSAO from *E. coli* (Moënne-Loccoz et al., 1995). In Fig. 1 can be seen the effects of 4 different concentrations of allylamine, kynuramine, aminoacetone, methylamine and ethylamine on the conversion of benzylamine by human and pig plasma SSAO.

As expected from previous reports (Hill and Mann, 1964), the SSAO from green pea and marrowfat is prominently active against histamine, polyamines and also agmatine.

It should be kept in mind of course that such a co-substrate method has its limitations: an effect on benzaldehyde generation can be due to the compound under study being a substrate, an inhibitor, or both. A point in question is kynuramine. This amine is a substrate, which is converted into an aldehyde which spontaneously cyclises to the fluorescent product 4-hydroxyquinoline (4-HQ). In fact, a method for human plasma SSAO measurement using kynuramine as a substrate has been reported (Matsumoto et al., 1985). We have similarly used kynuramine (optimal concentration 0.15 mM; at higher concentrations substrate inhibition occurred) as a substrate in SSAO measurements, measuring the generated 4-HQ by fluorimetry (excitation 318 nm, emission 380 nm).

With goat, sheep and cow plasma V_{max} values of 14, 11 and 17 nmol 4-HQ/min/ml, respectively, were found, much lower than the V_{max} values using

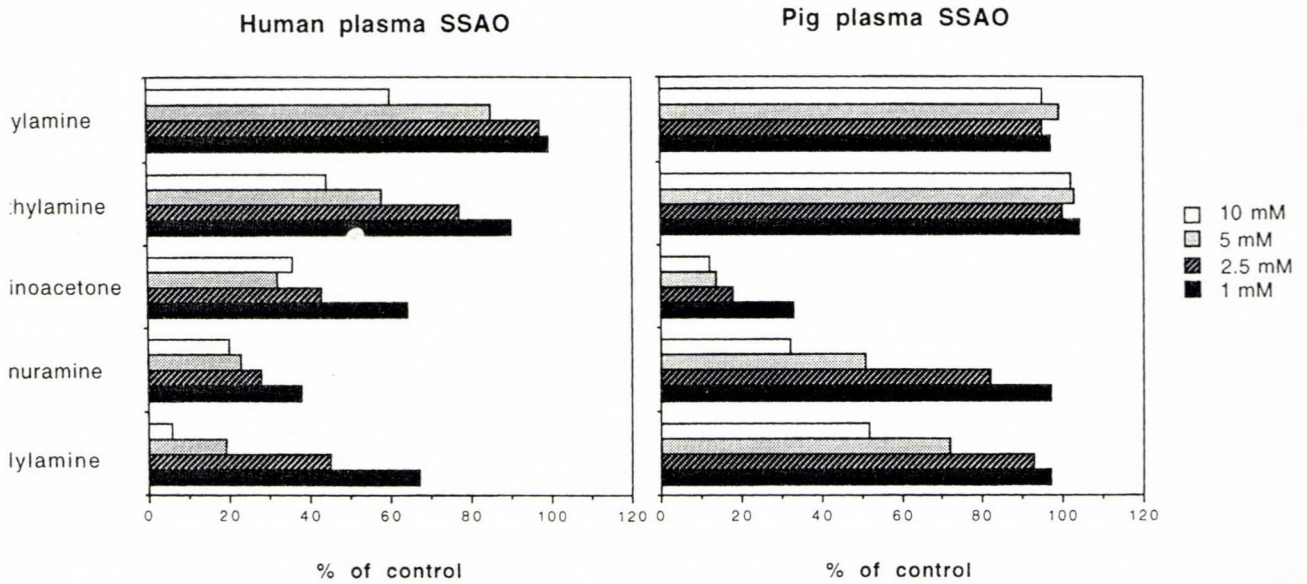

Fig. 1. Effect of 1, 2.5, 5 and 10 mM of allylamine, kynuramine, aminoacetone, methylamine and ethylamine on the production of benzaldehyde from benzylamine by SSAO from human and pig plasma.
Data are means of 3 experiments; variation coefficients all <10%

benzylamine as a substrate (respectively, 120, 75 and 59 nmol benzaldehyde/ml/min). On the other hand, K_M values with kynuramine were lower than with benzylamine (respectively, 45, 37 and 70 μM vs 283, 156 and 280 μM). These data would suggest that kynuramine binds faster to SSAO than benzylamine, but has a lower turnover rate; in practice therefore, kynuramine as a co-substrate would have an inhibitory effect on the rate of benzylamine conversion.

Since in blank studies no interferences of β-phenylethylamine and aminoguanidine were apparent in the kynuramine-substrate method, this method could be used to study any possible influence of these compounds on the SSAO activity towards kynuramine. Results demonstrate that β-phenylethylamine and aminoguanidine have an inhibitory effect on the conversion of benzylamine in cow plasma, as does aminoacetone (Fig. 2).

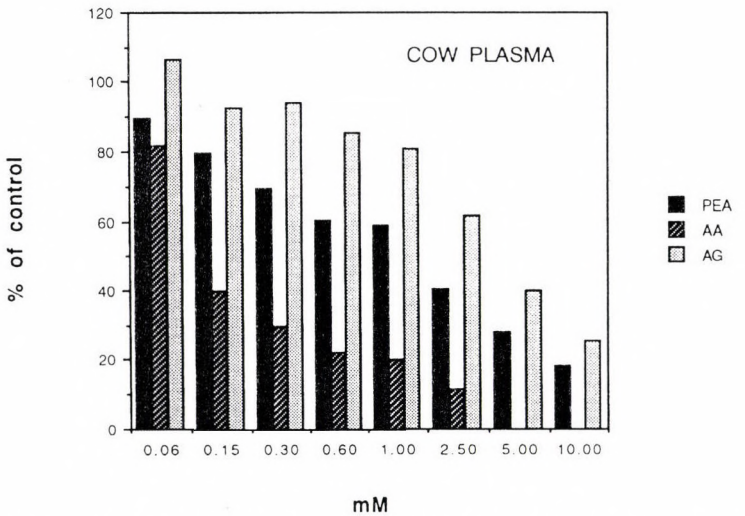

Fig. 2. Influence of various concentrations of co-substrates on the conversion of kynuramine in cow plasma
PEA: β-phenylethylamine
AA: aminoacetone
AG: aminoguanidine

In the 1960s much work has been done on the human plasma SSAO (then called serum monoamine oxidase) in relation to human pathologies. Raised levels were reported in patients with diabetes, heart failure and liver cirrhosis (McEwen and Castell, 1967; McEwen and Harrison, 1965; Nilsson et al., 1968), while lowered levels were found in patients with various cancers and with burns (Lewinsohn, 1977). Normal values were higher in young than in older subjects (Tryding et al., 1969). In recent years with newer techniques for SSAO measurement, some of these findings have been confirmed and further extended (Boomsma et al., 1995, 1997 and 1998).

Concluding remarks

Employment of new powerful techniques of gene sequencing and cloning and of amino acid sequencing may be expected to shed light on the many different aspects of SSAO in the future. The finding of the consensus sequence Asn-Tyr-Asp/Glut-Tyr for the formation of the topaquinone cofactor entity and of 3 conserved His units for copper binding in many of the SSAOs studied is already a helpful sign (Mu et al., 1994; Choi et al., 1996; Høgdall et al., 1998). Sequencing of genes and proteins may help to unravel how many separate SSAO entities there are and how differences in exon-intron programming can influence characteristics. Effects of differences in glycosylation and of surrounding conditions *in situ* should not be forgotten however.

Intriguing as all these differences between SSAOs are, the most enigmatic aspect of SSAO remains its physiological role. Based on the reactions catalysed, one could assume that its function(s) might be protection against deleterious influences of exogenous and endogenous amines, generation of hydrogen peroxide at places where this could fulfill a useful purpose, or the production of aldehydes needed for cross-linking of proteins. In different places different functions could be operative. So far, most information has been obtained on deleterious effects of such reactions (e.g. Boor and Hysmith, 1987; Yu and Zuo, 1993 and 1995). Recent work (Enrique-Tarancon et al., 1998) has for the first time shown a clear potentially positive function for SSAO in the glucose transport into adipocytes. With the increased interest in, and research on, SSAOs, it seems probable that the next decade will see the unravelling of many of the as yet enigmatic aspects intriguing class of amine oxidases. Many surprises may still be in store!

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RESEARCH REPORT

SEMICARBAZIDE-SENSITIVE AMINE OXIDASES IN HEART AND BOVINE SERUM

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In guinea pig dorsal skin the semicarbazide-sensitive amine oxidase (SSAO) is localised in fibroblasts. Fibroblasts in culture lose the ability to express this enzymatic activity with doublings, thus suggesting that the SSAO expression needs some factors which are not present in the 10% bovine serum culture medium.

Fresh bovine serum of adult animals contains two SSAO activities, one with high affinity for benzylamine and one with lower affinity. The enzyme with lower affinity for benzylamine was identified as spermine oxidase, the oxidation of [^{14}C]-benzylamine was inhibited by semicarbazide, α-aminoguanidine and B24, a specific inhibitor of benzylamine oxidase and spermine oxidase, both SSAO enzymes. The enzymatic activity of bovine serum was partially purified, the kinetic properties and sensitivity to inhibitors studied. A mathematical procedure for the analysis of the kinetics resulting from the activity of two enzymes acting on the same substrate seems to give better results than the methods previously described.

Key words: heart amine oxidase, bovine serum amine oxidase, semicarbazide-sensitive amine oxidase, fibroblasts

Abbreviations: B24 = 3,5-diethoxy-4-aminomethylpyridine, BAO = benzylamine oxidase, DAO = diamine oxidase, MAO = monoamine oxidase, SSAO = semicarbazide-sensitive amine oxidase, SAO = spermine oxidase, b-FGF = fibroblast growth factor

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INTRODUCTION

The most recent experimental results have presented new views on the possible physiological and pathophysiological role of semicarbazide-sensitive amine oxidases (E.C.1.4.3.6.) and, in particular, of the tissue-bound SSAO enzymes, that we prefer to call benzylamine oxidase (BAO).

Benzylamine is the best substrate of the tissue bound SSAO enzymes and of some plasma enzymes. The substrate specificity of the plasma enzymes differs according to species as Blaschko [1974] pointed out. A typical spermine oxidase activity (SAO) has been observed in those animals in which special and characteristic compartments for the fermentation of cellulose have evolved. These animals include all ruminants and tylopods, the hippopotamus and the hyracoidea [Blaschko, 1974].

On the other hand there is no sharp division in the substrate specificity of the plasma enzymes: benzylamine is a good substrate of both benzylamine oxidase and spermine oxidase.

The functional significance of the SSAO enzymes is still obscure.

One of the interesting properties of the plasma oxidases is that in many species their level increases during postnatal development. It is still unclear if the plasma enzymes are products of the degradation or secretion of the cells. It is known that tissue BAO is localised on plasma membranes and in cytoplasm [Callingham et al., 1995; Buffoni, 1995]. Until now we thought that the main expressing cells were smooth muscles, adipocytes and fibroblasts but recent results have shown that BAO may be expressed also by other cells, for instance, endothelial cells [Bono et al., 1998; Smith et al., 1998] where an adhesion protein in the mouse and man shows amine oxidase activity. Adhesion molecules expressed on vascular endothelial cells mediate cell-cell interaction and cell-matrix interactions that are essential for the regulation of trafficking of leukocytes across the vasculare endothelial barrier and have been shown to be critically involved in the inflammatory response. The adesion response serves as an important initial step in the host-defense response. However inappropriate attachment of leukocytes to the activated endothelial cell surface can occur by expression of one or more endothelial adhesion molecules, for instance also BAO. This may contribute to the pathogenesis of disorders such as the adult respiratory distress syndrome and ischemic-reperfusion tissue injury. An important role of BAO is its insulin-like properties. The BAO present on the plasma membranes of adipocytes seems to have a role in glucose uptake [Marti et al., 1998] because hydrogen peroxide production contributes to the *in vivo*

regulation of GLUT4 trafficking in adipose tissue [Enrique-Tarancon et al., 1998].

In mammals the other important class of SSAO enzyme is represented by diamine oxidase (DAO). This enzyme has been localised in several tissues, including thymus, intestine, kidney, seminal vesicle, placenta and pregnancy plasma. DAO is not constitutively expressed in the mammalian brain, but recent results show that it becomes detectable following focal injury [Munis et al., 1998]. A very interesting recent observation is that human vascular endothelial cells and fibroblasts express a cell-surface degradative pathway for histamine which employs a receptor for DAO [Lewis Baenziger et al., 1994].

Therefore we may state that tissue SSAO with affinity for benzylamine (BAO) and DAO may have an important role in the host defence and inflammatory responses. In agreement with these assertions we observed in the past a strong increase in BAO activity in regenerating guinea pig skin [Buffoni et al., 1993] and during the development of angiogenesis [Ziche et al., 1987] in the rabbit cornea. SSAO enzymes are copper enzymes such as lysyl oxidase, cytochrome-c-oxidase and superoxide dismutase. Copper plays a critical role in chronic neurological diseases [Multhaup et al., 1996], in cardiomyopathy [Meideros and Wildman, 1997], hypertension [Vivoli et al., 1995] and in inflammatory diseases [Milanino et al., 1993]. Copper-deficient pigs and rats exhibit a variety of cardiovascular defects including abnormalities in the connective tissue of blood vessels, ventricular aneurysms, cardiac hypertrophy, hypercholesterolemia, etc. [Meideros et al., 1997]. BAO activity in the plasma is a good marker of copper deficiency [Blaschko et al., 1965].

In two cases of Steely hair syndrome that we treated with copper-EDTA there was an increase in the serum copper content, of the caeroplasmin level and of BAO activity, but in the final stage of the illness, BAO activity was undetectable whereas caeruloplasmin and serum copper were increased by the treatment. Therefore, serum BAO activity seems a more reliable index of the clinical state, meaning that the expression of this enzyme needs not only the presence of copper but of other factors which are lacking in the final state of this disease [Papini et al., 1981].

The cardiovascular importance of copper and copper amine oxidases aroused our interest in the study of BAO activity in the heart where this enzyme may be important.

In rats BAO activity is expressed by cardiomyocytes [Pino et al., 1998]. The level of activity does not change in the heart of 3-month old spontaneously hypertensive rats which have not yet developed cardiac hypertrophy [Cerbai et

al., 1994], whereas the level of MAO A and B are significantly increased [Pino et al. 1998], in agreement with previous observations [Kumagai et al., 1974]. This increase may represent an early event in the development of hypertrophy which may be followed by a decrease. A decrease in MAO activity of platelets has been observed in stabilized human hypertension [Anselmi et al., 1976] without any change in the benzylamine oxidase activity of blood plasma [Buffoni and Sicuteri, 1980].

Lewinsohn et al. [1978] have described the presence of benzylamine oxidase activity in autoptic samples of the human heart. We had the opportunity to analyse some fresh pieces of human heart obtained during heart transplant [Pino et al., 1998] and found a semicarbazide-sensitive amine oxidase with affinity for benzylamine (BAO).

Rat and human hearts did not show any DAO activity, the histaminase activity was dependent on the presence of BAO. This was also observed in the pig heart [Buffoni et al., 1998]. The enzyme present in pig heart has been fully purified and partially characterised. It seems identical to pig plasma benzylamine oxidase [Buffoni and Blaschko, 1964]: it is formed by 2 subunits of 97 kDa linked by S-S bridges, contains 1 copper per subunit and a carbonyl cofactor the nature of which was not identified [Buffoni et al., 1998].

The effect of histamine on the heart are well known [Genovese and Spadaro, 1997]. In absence of DAO this amine is N methylated to form 1,4-methylhistamine which is a substrate of MAO B [Hough and Domino, 1979] and of some plasma oxidases [Blaschko, 1959]. The presence of BAO in the heart may contribute to the degradation of this amine. Human and pig plasma benzylamine oxidase also have histaminase activity [Buffoni and Blaschko, 1964; McEwen, 1972] which may be relevant in decreasing the level of circulating amine. Therefore, at least in human, pig and rat tissue and plasma, benzylamine oxidase activity may be important in inflammatory diseases of allergic nature, too.

In rats vascular histamine is decreased in spontaneously hypertensive animals [Holcslaw et al., 1985]. This decrease seems linked to an enhanced conversion of histamine to imidazoleacetic acid. In the mesenteric artery of the rat no diamine oxidase activity is present but a SSAO with histaminase activity and affinity for benzylamine is present [Buffoni et al., 1994]. In 7-months old spontaneously hypertensive rats an increase in BAO activity of blood vessels was observed [Del Carmen and Fuentes, 1983], which might account for the observed histamine decrease.

During these studies we had the opportunity to assay the expression of BAO by skin fibroblasts in culture and to analyse the amine oxidases which are present in bovine serum, which is used for cell culture. We present here the results obtained and a new procedure for the analysis of the kinetic of two enzymes acting on the same substrate.

MATERIALS

[^{14}C]-Benzylamine and [^{14}C]-putrescine were obtained from Amersham International, Buckinghamshire, U.K., Microgranular DEAE-cellulose (DE32) was from Whatman, Maidstone, Kent, U.K., phenol was supplied by Merck Laborchimica, Milan, Italy. Aminoguanidine hemisulphate, semicarbazide hydrochloride, bovine serum albumin, 4-aminoantipyrine, collagenase P and Dulbecco's modified Eagle's medium were obtained from Sigma Chemical Co., St. Louis, Mo., USA. Catalase, trypsin, horseradish peroxydase were from Boehringer, Mannheim, Germany. All other reagents were of analytical grade. The inhibitor 3,5-diethoxy-4-aminomethylpyridine (B24) was synthesised by Bertini et al. [1995].

METHODS

General methods

Proteins were determined according to the method of Waddell and Chapel Hill [1956] or by using the method of Lowry et al. [1951] with bovine serum albumin as the standard.

Enzyme assays

BAO activity was monitored radiochemically in a final volume of 300 µl with 83 µM [^{14}C]-benzylamine as follow: 100 µl of 67 mM-Na_2HPO_4-HCl buffer pH 7.4, 50 µl of catalase (43 U ml^{-1}), 100 µl of enzyme fraction (at different concentrations according to the activity) were preincubated with shaking at 37 C for 10 min before starting the reaction with 50 µl of 500 µM [^{14}C]-benzylamine. After 30 min the reaction was stopped with 100 µl of 3 N HCl and the benzaldehyde produced was extracted with 1 ml of ethyl acetate and counted in 10 ml of Instagel (Packard) using liquid scintillation counting. For kinetic studies the reaction was followed for 30-60 min using the following

concentrations of [^{14}C]-benzylamine 12-24-48-60-120-240-480-600-900-1200-1500-1800 μM, 50 μl in the reaction mixture. When the effects of inhibitors were studied, the inhibitors, dissolved in buffer, were preincubated with the enzyme for 30 min.

Quench correction was obtained by the channel ratio method.

The oxidation of non-radioactive substrates was studied using the method of Holt et al. [1997] in the following conditions: 1.8 ml of 67 mM Na_2HPO_4-HCl buffer pH 7.4 containing (blank) or not containing 6×10^{-4} M B24, 0.5 ml of enzyme solution, 0.3 ml of 8.2 mM 4-aminoantipyrine, 0.3 ml of 102 mM phenol, 0.1 ml of peroxidase 120 U ml^{-1}, 0.1 ml of 30 mM substrate. Hydrogen peroxide was used as standard 37.5-75-150-300 nmoles were added to the blank solution of the enzyme without substrates. 2×10^{-5} M B24 was used in the blanks in order to completely inhibit the enzymatic activity. No hydrogen peroxide was formed in the presence of B24 and each substrate.

The oxidation of [^{14}C]-putrescine was assayed as follows: 350 μl of 67 Na_2HPO_4-HCl buffer pH 7.4 and 100 μl of enzyme solution were incubated for 10 min at 37 °C, then 50 μl of 10 mM [^{14}C]-putrescine were added and the reaction was followed for 30 min at 37 °C. The reaction was stopped with 100 μl of 2 N perchloric acid and 500 μl of strong alkaline buffer. The reaction product was extracted with 2.5 ml of butyl-BPD according to [Pino et al., 1998]. For kinetic studies the following putrescine concentrations were used: 10-20-50-100-200 μM. Two milliliters of butyl-BPD were counted in a Packard scintillation counter.

Enzyme velocity analysis

Kinetic data were fitted using the method of Wilkinson [1961] with a computer program written for this purpose or by the method of Leatherbarrow [1987].

Step 1 - Experimental data were plotted on an Eadie plot to determine the values at which the action of the first enzyme was predominant, and first estimate of the kinetic constants Vm_1 and Km_1 of the first enzyme and Vm_2 and Km_2 of the second one were obtained.

Step 2 - The first estimates of the previous step were then used in a Levenberg-Marquardt method [Press et al., 1988], developed in a program of the 'Numerical Recipies in C package' or with the routine of the 'Mathematica'™ Standard Add-on package NonLinearFit, using standard deviations as weight (when available) [Wolfram,1996].

Step 3 - From the estimated values in both steps 1 and 2 of Vm_1, Km_1, Vm_2 and Km_2 values for the kinetic constant k_1, k_2, k_3, k_4 were deduced. A differential model reported in Scheme 1, with suitable initial conditions and the obtained constants were solved numerically through the NDSolve function of 'Mathematica' [Wolfram, 1996] for different values of the concentration of substrate to check the fit with the experimental values of the product obtained after 1 hour. Data are plotted for easier comparison.

$$E_1 + S \xrightarrow{k_1} ES_1 \xrightarrow{k_2} P_1$$
$$E_2 + S \xrightarrow{k_3} ES_2 \xrightarrow{k_4} P_2$$

$k_{-1}, k_{-3} = 0$

$$ds/dt = -k_1e_1(t)s(t) - k_3e_2(t)s(t)$$
$$de_1/dt = -k_1e_1(t)s(t) + k_2es_1(t)$$
$$des_1/dt = k_1s(t)e_1(t) - k_2es_1(t)$$
$$dp/dt = k_2es_1(t) + k_4es_2(t)$$
$$de_2/dt = -k_3e_2(t)s(t) + k_4es_2(t)$$
$$des_2/dt = k_3s(t)e_2(t) - k_4es_2(t)$$

$$p(0) = es_1(0) = es_2(0) = 0$$
$$s(0) = s_0\ e_1(0) = e_1^{\ 0}\ e_2(0) = e_2^{\ 0}$$

$$ds/dt = -k_1e_1(t)s(t) - k_3[e_1^{\ 0} + e_2^{\ 0} - e_1(t) - e_2(t) - es_1(t) - es_2(t)]s(t)$$
$$des_2/dt = k_3[e_1^{\ 0} + e_2^{\ 0} - e_1(t) - es_1(t) - es_2(t)]s(t) - k_4es_2(t)$$

Scheme 1

Cell culture

Guinea pigs were maintained for 1 week in our animal facility until sacrificed. This investigation conforms to the rules for the care and use of laboratory animals of the European Community (86/609/CEE).

Hair was removed from the dorsal skin and the skin was washed with soap and cleaned with alcohol before surgery. A piece of skin (about 6 cm^2, epidermal and dermal surface) was removed, washed with sterile PBS (phosphate buffer), cut in small pieces and suspended in 5-7 ml of PBS containing 2 mg ml^{-1} of collagenase P (1.7 U mg^{-1}). The suspension was kept at 37 °C for 1 h and the opalescent supernatant was decanted in a sterile centrifuge tube. Cells were obtained by centrifugation at 400 g for 10 min. The pellet was washed twice with 10 ml of sterile PBS and finally suspended in a small volume of PBS (generally 1 ml) and plated in DMEM (Dulbecco modified

Eagle medium) containing 10% fetal calf serum, 2 mM l-glutamine, 100 IU of penicillin, 100 µg of streptomycin and 0.25 µg of fungizone. A 0.1 ml suspension was plated in 3 ml of DMEM. Cells were kept at 37 °C in an incubator at 5% CO_2.

The culture medium was renewed every 4 days. Treatment with trypsin was carried out as follows: cells from each well (9.6 cm^2) were incubated for a few minutes at room temperature with 1 ml of a solution containing 4 mg of trypsin, 10 mg of glucose, 2 mg $EDTANa_2$ in 10 ml of PBS without calcium. The cell suspension was collected in a sterile centrifuge tube and the well washed twice with sterile PBS. Trypsin was neutralised with 1 ml fetal calf serum and cells were collected by centrifugation at 400 g for 10 min. The pellet was washed twice with 10 ml of PBS and cells were suspended in 1-2 ml of PBS and plated or analysed. An inverted phase-contrast microscope was used to count the cells in a Thoma chamber.

The PBS composition was: 135.2 mM NaCl, 2.64 mM KCl, 8 mM Na_2HPO_4, 1.44 mM KH_2PO_4,, 0.17 mM $CaCl_2$, 0.049 mM $MgCl_2$.

The cells were homogenised in a small volume of 10 mM Na_2HPO_4-KH_2PO_4 phosphate buffer pH 7.4 containing 0.25 M sucrose, centrifuged for 5 min at 600 g to remove unbroken cells, nuclei and debris and then centrifuged at 12,000 g for 20 min.

RESULTS

Fibroblasts and bovine serum amine oxidases

In order to study the mechanism which induced the expression of BAO, some cell cultures were carried out which showed that fibroblasts in culture lost the ability to express BAO activity, which was expressed in the primary culture (Fig. 1), this suggesting that the expression of this enzymatic activity is controlled by cytokines or growth factors which are not present in the culture medium.

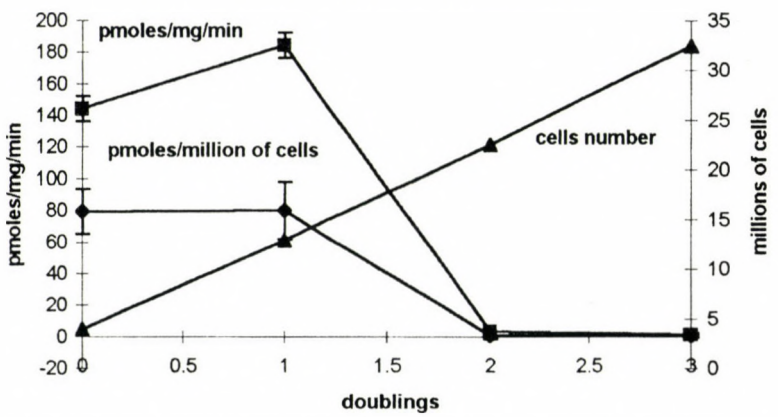
During these experiments we had the opportunity to assay the amine oxidase activity of bovine serum. The bovine serum of adult animals had BAO activity (Table 1). This serum showed two amine oxidase activities, one with high affinity for benzylamine and one with low affinity for benzylamine (Fig. 2). The enzymatic activities of bovine serum were partially purified by ammonium

Table 1. Oxidation of benzylamine by bovine serum. Mean ± s.e. 5 experiments in duplicate

[^{14}C]-Benzylamine μM	Bovine serum nmoles ml^{-1} min^{-1}
2	0.246 ± 0.015
4	0.474 ± 0.039
8	0.834 ± 0.077
10	1.121 ± 0.077
20	2.042 ± 0.140
40	3.800 ± 0.362
80	6.763 ± 0.460
100	8.169 ± 0.301
150	10.606 ± 0.275
200	13.124 ± 0.774
250	15.147 ± 1.003
300	17.154 ± 1.994
600	29.536 ± 5.381

The bovine serum was dialysed against 67 mM Na_2HPO_4-HCl buffer pH 7.4, the protein content was 22.47 ± 1.02 mg ml^{-1}. For assay method see Methods section.

Fig. 1. Benzylamine oxidase activity of guinea pig skin fibroblasts in culture

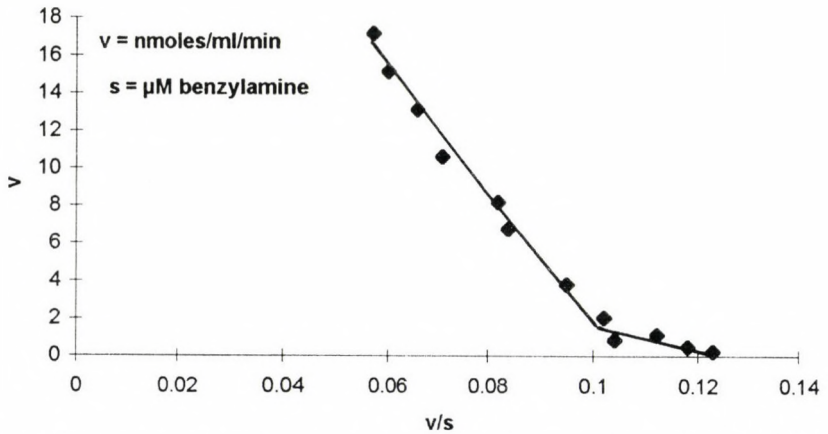


See Methods section for further information. Values are the mean ± s.e. of 4 experiments

sulphate precipitation between 0-35 and 35-55% of saturation. The active proteins were obtained between 35 and 55% of saturation. The precipitate was fully dialysed against 67 mM Na_2HPO_4-HCl pH 7.4.

The precipitate oxidised benzylamine, but the oxidation of benzylamine was strongly inhibited by 2 mM spermine (Fig. 3), and in this condition a simple

Fig. 2. Amine oxidase activities of dialysed bovine serum. Eadie plot



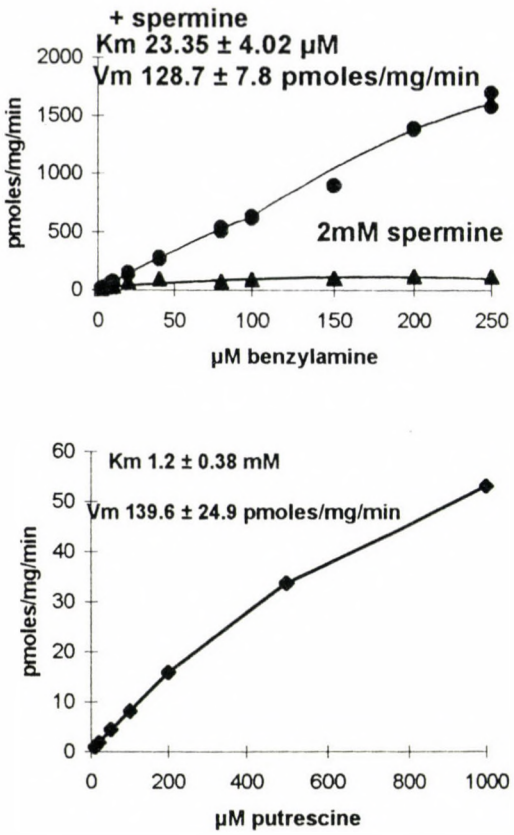
See Methods section for further information. The bovine serum was fully dialysed against 67 mM $NaHPO_4$-HCl buffer pH 7.4. Values are the mean of 5 experiments performed in duplicate

kinetic was obtained which gave the apparent Michaelis Menten constants of the high affinity BAO. Both enzymes were totally inhibited by 10^{-4} M B24 and semicarbazide and 66% inhibited by 10^{-4} M α-aminoguanidine. Therefore, bovine serum seems to contain BAO and SAO activity, both inhibited by carbonyl reagent and by B24. It is known that spermine oxidase also oxidises benzylamine and putrescine. The oxidation of putrescine gave a simple kinetic (Fig. 3) and was totally inhibited by 6×10^{-5} M B24, which does not act on DAO and by 6×10^{-5} M semicarbazide and α-aminoguanidine. Therefore this activity was due to the presence of SAO.

The ammonium sulphate precipitate fully dialysed against 10 mM Na_2HPO_4-KH_2PO_4 buffer at pH 7.0 was put on a DEAE-cellulose (2×48 cm) column equilibrated with the same buffer. Proteins were eluted by increasing the ionic strength (550 ml of 30 mM followed by 860 ml of 1000 mM Na_2HPO_4-KH_2PO_4 buffer at pH 7.0 and by 360 ml of the same buffer containing 1 M NaCl). The active fractions were pooled (Fig. 4). Each pool was assayed using [^{14}C]-benzylamine and [^{14}C]-putrescine as substrates: pool 1 was enriched by SAO, pool 2 by BAO activity and pool 3 mainly had BAO activity.

Pool 1 still contained 2 enzymatic activities, one of which was totally inhibited by 1 mM spermidine giving a simple kinetic (Figure 5). Both

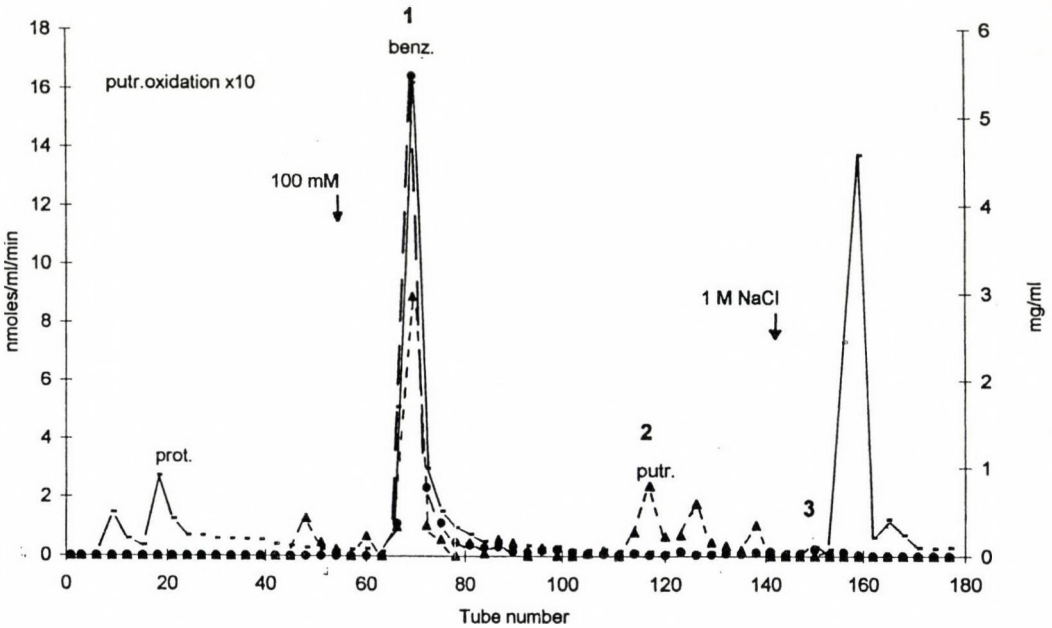
Fig. 3. Oxidation of benzylamine and putrescine by ammonium sulphate precipitate of bovine serum and effect of spermidine



The precipitate obtained between 35 and 55% of ammonium sulphate saturation was collected by centrifugation and fully dialysed against 67 mM Na_2HPO_4-HCl pH 7.4.
The activity was assayed as described in the Methods. Values are the mean of 2 experiments in duplicate. Spermine was supplemented with benzylamine

enzymes were inhibited by 5×10^{-8} M B24 (1% 44.3 ± 1.97 s.e. of 11 determinations). This pool oxidised [^{14}C]-putrescine with a simple kinetic completely inhibited by 6×10^{-7}M B24 and competitively by 1 mM spermidine (Fig. 5), a characteristic of SAO.

Fig. 4. Elution profile of the amine oxidase activity of bovine serum on DEAE cellulose column



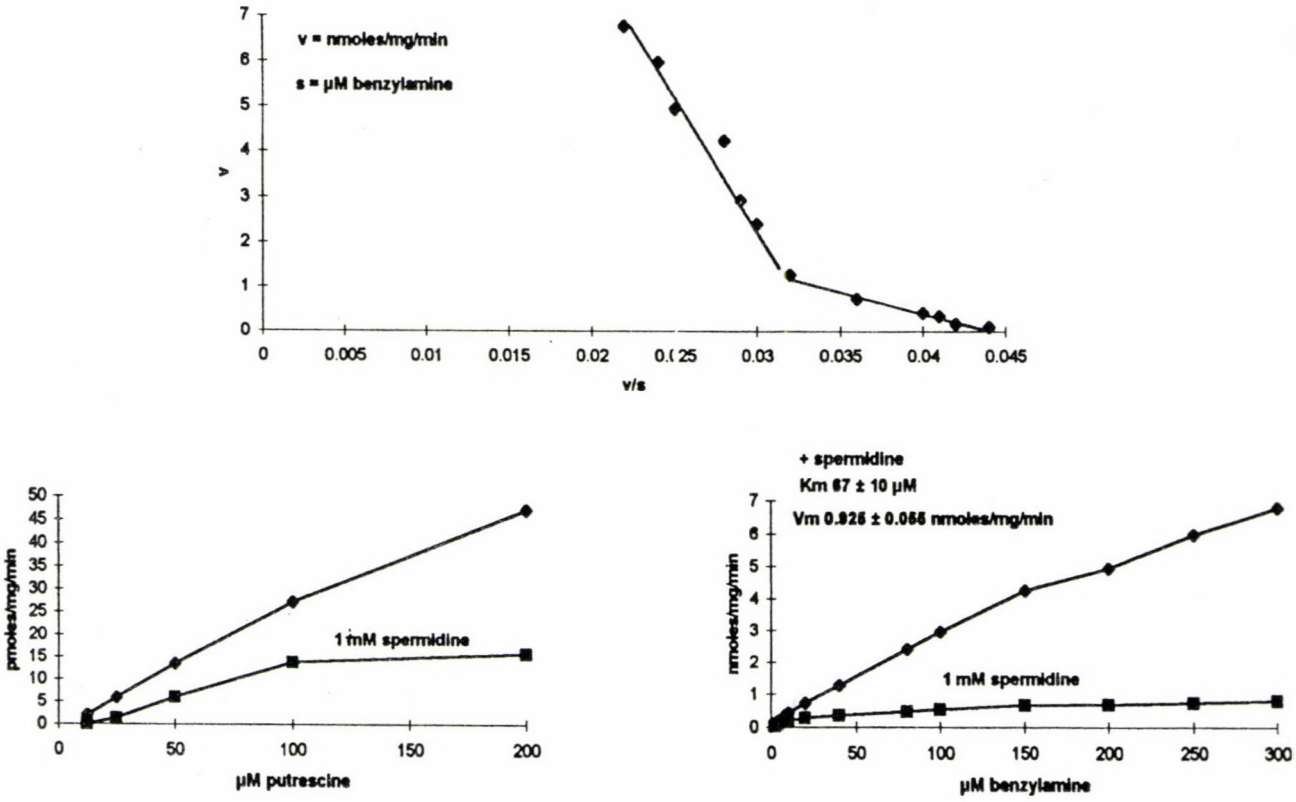
See Methods section for further information. Prot. = protein mg/ml; benz. = activity assayed using [^{14}C]-benzylamine as substrate; putr. = activity assayed using [^{14}C]-putrescine as substrate 1,2,3 indicate the position of the collected peaks. Arrows indicate buffer change

Pool 2 still contained SAO activity as was shown by its partial inhibition by 1 mM spermidine, pool 3 had mainly BAO activity (Fig. 6).

Analysis of the kinetics of benzylamine oxidation by bovine serum and by some partially purified fractions

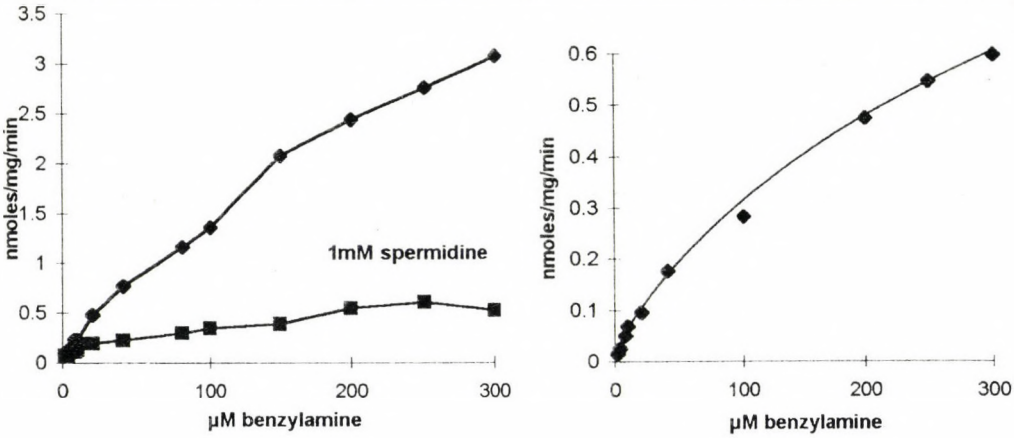
As described in the methods we tried to better approach the kinetic constants of the amine oxidases which are present in bovine serum. Figure 7 shows that the mathematical simulation of the rates gave a good fitting with the experimental results, generally better than the fitting obtained using the constants coming from the Eadie plot or by the Wilkinson method after correction of the rates of the second enzyme. Therefore the differential method seems a suitable procedure for a better approach to the apparent Michaelis-Menten constants of the two enzymes.

Fig. 5. Oxidation of benzylamine and of putrescine by pool 1 coming from DEAE cellulose column and effect of spermidine



See Methods section for further information. Values are the mean of 2 experiments run in duplicate

Fig. 6. Oxidation of benzylamine by DEAE cellulose pools 2 and 3 and effect of spermidine on pool 2



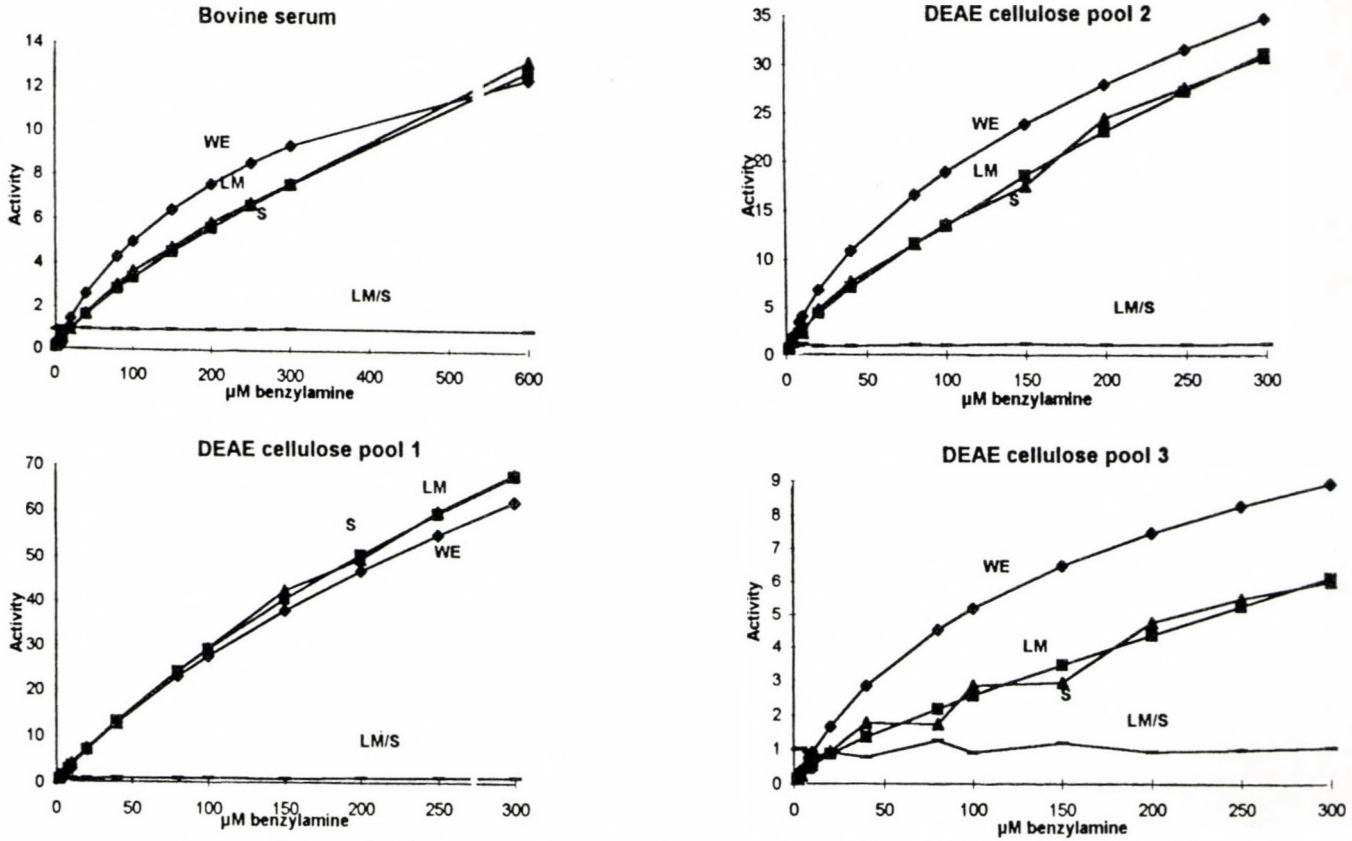
See Methods section for further information. Values are the mean of 2 determinations

The apparent Km and Vm obtained are, on the other hand, affected by the relative amount of e_1 and e_2 in the total protein content of the sample. The changes of Vm with the purification reflect changes of the e_1 and e_2 concentrations. One might deduce the change in the relative proportion of the two enzymes by the change in the ratio k_2/k_4 assuming no variation in the real constants of the two enzymes with the purification procedure.

DISCUSSION AND CONCLUSION

The first conclusion that can be deduced from the results presented in this paper is that the ability of skin fibroblasts to express BAO activity is lost when the cells are cultured. This suggests that the expression of this enzymatic activity is controlled *in vivo* by some factors which are not present in the culture medium. In agreement with this hypothesis is the stimulating effect of b-FGF on BAO (Bz.SSAO) expression in guinea pig regenerating skin [Buffoni et al., 1994 and 1995]. This may be consistent with a role of this enzyme in inflammatory reactions or more generally in conditions in which tissue is altered and histamine, cytokines and growth factors are released, as suggested in the introduction. For instance, the expression of DAO

Fig. 7. Oxidation of benzylamine by bovine serum and some purified fractions. Computer fitting of the experimental results



Activity = 10^{-10} mol min^{-1} mg^{-1} of protein. WE = constant obtained by the apparent Michaelis-Menten constants computed by the Wilkinson method or by Eadie plot. The rates of the second enzyme were corrected as described in the Methods. LM = constants obtained by Levember-Marquardt method as described in the Methods LM/S = ratio between the computed rates and the experimental rates. The amount of total enzyme was assumed to be 5×10^{-9} mol in 1 mg of protein, $e_1 = e_2$

is increased by epidermal growth factors in Caco-2-cells in culture [Daniele and Quarone, 1991].

The second conclusion is that in bovine serum of adult animals two amine oxidases are present, one which appears to be a BAO with a good affinity for benzylamine and the other a true spermine oxidase (SAO) which oxidises spermine, spermidine, benzylamine and, at much lower rate, histamine, putrescine and cadaverine. Both enzymes are inhibited by B24, semicarbazide, α-aminoguanidine. Therefore BAO activity is also present in bovine serum. The presence of BAO activity in sheep plasma has been observed by Elliott et al. [1992]. Sheep and calf are ruminant mammals, thus BAO seems to be present not only in non-ruminant but in any mammals plasma, and this indicates an important role of this enzyme. According to Blaschko [1974] SAO may have a defensive role against polyamines which are formed in the rumen. The observation that SAO activity is present in the serum of pregnant women [Gahl et al., 1982], where SAO and DAO appear to be a single enzyme protein, suggests that SAO activity is expressed in non ruminants in certain conditions in which polyamine levels are increased. SAO and DAO have a strong homology [Janes et al., 1992], seeming to differ mainly in substrate specificity, polyamines being the best substrate for SAO and diamines the best for DAO.

The observation that two amine oxidases are present in bovine serum may be considered to be in agreement with the observation of the existence of three genes for the copper amine oxidases [Hogdall et al., 1998]. Bovine plasma SAO seems to come from the liver whereas in the pig BAO is not present in liver hepatocytes [Buffoni et al., 1977]. The provenance of plasma BAO is still unknown.

A new procedure for the analysis of the kinetics deriving from two enzymes acting on the same substrate has been presented. The described procedure gives a better fitting of the experimental results than the previously suggested procedure.

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RESEARCH REPORT

ON THE PRIMARY STRUCTURE OF MEMBRANE-BOUND SEMICARBAZIDE-SENSITIVE AMINE OXIDASE (SSAO)

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Mammalian semicarbazide-sensitive amine oxidase (SSAO) activities are a diverse group of copper dependent enzymes within the classification EC 1.4.3.6. [amine:oxygen oxidoreductase (deaminating) (copper-containing)]. They include plasma amine oxidase and the membrane-bound enzyme. Although soluble plasma SSAO from several species have been successfully purified and cloned, relatively little work has been carried out on the molecular properties of the tissue-bound enzyme. At present there is not conclusive evidence that allows to conclude whether or not plasma SSAO is released from the tissue-bound enzyme. This review focuses upon recent progress made in determining the primary structure of the membrane-bound SSAO.

Key words: Semicarbazide-sensitive amine oxidase, amine metabolism, topa quinone, copper, glycoprotein

Introduction

Copper-containing amine oxidase activities are a diverse group of enzymes within the Enzyme Commission classification EC 1.4.3.6 [amine:oxygen oxidoreductase (deaminating) (copper-containing)]. They include plasma amine oxidase, tissue-bound enzyme and diamine oxidase. All of them are inhibited by semicarbazide, as a result of the presence of a carbonyl group at the cofactor site, and this is routinely used to distinguish these enzymes from

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the monoamine oxidases (amine:oxygen oxidoreductase (deaminating) (flavin containing) EC 1.4.3.4; MAO). However the substrate specifities of both types of enzyme overlap to some extent. The oxidation of aromatic and aliphatic primary amines by SSAO enzymes produces an aldehyde metabolite, hydrogen peroxide and ammonia:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

The physiological role of mammalian tissue-bound SSAO remain speculative. Although benzylamine has been shown to be the best substrate for studying SSAO activity, there is no evidence that this aromatic amine exists as an endogenously-occurring substrate. This led to some authors to search for the possible physiological substrates for SSAO. It has been reported that the aliphatic amine methylamine is deaminated by SSAO, but not by MAO, being considered as endogenous substrate for SSAO (Lyles et al., 1990). However, the ability of SSAO to convert this amine to the potentially cytotoxic formaldehyde has led to some authors to study the possible mechanisms by which SSAO could produce tissue damage (Yu and Zuo, 1993).

It is known that stress enhances excretion of adrenaline, that can be deaminated by MAO and the methylamine produced could be afterwards metabolized by SSAO generating formaldehyde, a well-known toxic agent for vascular tissues. So formaldehyde generation by SSAO in vascular tissues could be a potential risk factor for stress-related angiopathy (Yu et al., 1997). In the other hand, elevated SSAO activity has been shown in insulin-dependent diabetes mellitus patients suffering from either retinopathy or nephropathy (Boomsma et al., 1995), supporting the previous finding that raised plasma SSAO levels in rats with streptozotocin-induced diabetes mellitus could be prevented by insulin replacement therapy. In this way, it is worth to point out that SSAO colocalizes with insulin-dependent GLUT4 glucose transporter in adipocyte endosomal compartment, stimulating, via H_2O_2 production, the cellular glucose transport by promoting the GLUT4 protein translation to the plasma membrane (Enrique-Tarancón et al., 1998), Finally, Lewhinson (1984) described changes in plasma SSAO activity in fibrotic liver and patients suffering from burns, whereas a decreased membrane-bound SSAO activity has been reported in chemically-induced rat breast tumours (Lizcano et al., 1991).

Vascular adhesion protein-1 (VAP-1), a human sialoglycoprotein whose cell surface expression is induced under inflammatory conditions, is involved

in the binding of lymphocytes to endothelium (Salmi et al., 1992). Since the cDNA reported for this protein resulted 100% identical when compared to that reported for human placenta amine oxidase, the authors showed that VAP-1 possesses amine oxidase activity (Smith et al., 1998). Furthermore, Ax endothelial cells transfected with VAP-1 cDNA express VAP-1 on their cell surface and bind lymphocytes. So it seems that, at least in endothelial cells, SSAO may act as and adhesion protein.

Soluble plasma SSAOs from several species have been successfully purified and cloned. All they have been shown to contain, per mol of enzyme, 2 mol of tightly-bound copper and 2 mol of the uncommon covalently-bound organic cofactor, topa quinone (the quinone formed from the oxidation of 2,4,5-tryhydroxyphenylalanine), integrated in the polypeptide (Janes et al., 1990 and 1992).

The membrane-bound SSAO has been found in several tissues, with particularly high activity in blood vessels associated with smooth muscle cells (Lyles and Singh, 1985). However, SSAO activity has also been found in some other non-vascular cell types such as rat articular cartilage (Lyles and Bertie, 1987), adipocytes from rat white and brown fat (Barrand and Callingham, 1984), in pig dental pulp (Norqvist et al., 1981) and in different parts of the bovine eye (Fernández de Arriba et al., 1991). In spite of rapid progress on both kinetic properties and tissue distribution studies of tissue-bound SSAO, relatively little work has been carried out on the molecular properties of this enzymes and, therefore, much remains to be clarified about the relationships of tissue-bound SSAO to the soluble plasma enzyme.

At present, there is not direct evidence that allows to conclude whether or not plasma SSAO is released from the tissue-bound enzyme, and, if so, which is the mechanism of such release. In such case, plasma SSAO could be of use as an interesting clinical marker for some specific tissue dysfunctions. This review focuses upon recent progress made in determining the molecular properties of tissue-bound SSAO.

Membrane-bound SSAO from bovine lung

Our laboratory has recently succeeded on the purification of the bovine lung microsomal SSAO (Lizcano et al., 1998). The aims of this work was to obtain, for the first time, a pure preparation of tissue-SSAO that allowed molecular comparison with the plasma enzyme. The purification procedure allowed us to obtain enough pure preparation for its molecular

characterization. The enzyme behaves as an integral membrane glycoprotein as it was necessary the use of detergents during all the chromatographic steps to avoid enzyme inactivation. The pure enzyme oxidizes benzylamine, 2-phenylethylamine, methylamine and histamine, showing the highest affinity towards benzylamine. However, both methylamine and benzylamine gave similar specificity constants (kcat/Km). The enzyme does not oxidize tyramine, dopamine, tryptamine, kynuramine or polyamines

The study on the nature of the enzyme cofactor performed by the "redox-cycling" staining (Paz et al., 1991) and by enzyme derivatization with phenylhydrazine (Klinman and Mu, 1994), revealed a topa quinone-like cofactor. In this way, Holt et al. (1998) have recently demonstrated that both cow and pig aortic SSAOs contain topa quinone. So it can be concluded that both plasma and tissue-bound SSAOs have same organic cofactor.

The next question to resolve was whether or not the tissue-bound SSAO contain copper, as has been demonstrated for the plasma enzyme. KCN has been routinously used to differentiate both types of the enzyme, since 1 mM KCN irreversibly inhibits plasma SSAO by binding to copper in the active site. In contrast, crude preparations of SSAO are not affect by cyanide nor by copper chelating agents such diethyldithiocarbamate (Barrand and Callingham, 1984), suggesting that tissue enzyme might not contain copper. However, from the atomic spectrophotometry studies performed with pure bovine lung SSAO it was concluded that this protein contains 2 mol of copper/mol enzyme, as it has been previously reported for bovine plasma SSAO (Morpurgo et al., 1987). Furthermore, removal of half of the copper content caused enzyme inactivation, that was recovered when it was dialyzed against copper. These results are consistent with the involvement of copper ions in the catalytic mechanism of the tissue-bound enzyme, perhaps playing a similar role to those in plasma SSAO. Finally, with regard to cyanide, bovine lung SSAO showed and IC_{50} of 5 mM for KCN, a value 100 times higher than that reported for plasma enzyme, suggesting than this metal may be effectively shielded by the folded structure of the protein.

Despite that both tissue-bound and plasma SSAOs have same organic and non organic cofactor, rather similar kinetic behaviour, and antibodies raised against tissue-bound or plasma SSAO cross-react among them, it cannot be concluded whether they are isoenzymes or different forms of same gene product.

Primay structure of membrane-bound SSAOs

Figure 1 shows a comparison of the N-terminus sequences obtained for the bovine lung SSAO with the N-terminus sequences of membrane-bound SSAOs available. These alignments result in a more than 90% of identity when compared with rat adipocyte SSAO, human placenta SSAO, human and mouse VAP-1 (vascular adhesion protein-1) and pig aorta SSAO. In case of human retina SSAO, the identity was 60%, approximately. In fact, all these sequences are very close to each other, and all of them greatly differ from the human retina protein, that it is specifically expressed in the retina. This led to the authors to suggest and important role in retinal function for this enzyme. Figure 1 also shows the N-terminus sequence for the bovine plasma SSAO, obtained from a liver cDNA library, that clearly differs from those of the tissue-bound enzymes. Furthermore, it has a eukaryotic secretory signal sequence cleavage site between amino acids 16 and 17 since Arg 17 has been demonstrated to be the first residue of the mature protein.

Bovine lung SSAO[a]	MNQKTTLVLL	ALAVITIFAL	VCVLLAGR
Rat adipocyte SSAO[b]	MTQKTTLVLL	ALAVITIFAL	VCVLIAGR
Human placenta SSAO[c]	MNQKTILVLL	ILAVITIFAL	VCVLLVGR
Human vap-1[d]	MNQKTILVLL	ILAVITIFAL	VCVLLVGR
Mouse vap-1[e]	MTQKTTLVLL	ALAVITIFAL	VCVLLAGR
Pig aorta SSAO[f]	MNQKTTLVLL	ALAVITIFAL	V?VLLAGR
Human reitna SSAO[g]	MHLKIVLAFL	ALSLITIFAL	AYVLLTSP
Bovine plasma SSAO[h]	MFIFFILSLW	TLLVMGREEG	GVGSEEGV

Fig. 1. N-terminal sequences of purified and cloned mammalian tissue-bound SSAOs. [a]Purified bovine lung SSAO (unpublished results, Lizcano et al.). [b]Rat adipocyte SSAO (cloned, Morris et al., 1997). [c]Human placenta SSAO (cloned, Zhang and McIntire, 1996). [d]Human VAP-1 (purified and cloned, Smith et al., 1998). [e]Mouse VAP-1 (purified and cloned, Bono et al., 1998). [f]Pig aorta SSAO (purified, Holt et al., 1998). [g]Human retina SSAO (cloned, Imamura et al., 1997). [h]Bovine plasma SSAO (purified and cloned, Mu et al., 1994)

All the tissue-bound SSAO sequences contain a region of 20-22 hydrophobic amino acids between residue 5 and residue 25-27, that it might represent a transmembrane domain. Figure 2 shows the hydropathy plot for human placenta SSAO. Also are represented the six potential N-glycosilation sites, the position of the topa quinone cofactor (Tyr 471) and 3 histidines that are believed to bind the copper ion. Since previous work demonstrated that the active site is out-facing the cell (Holt and Callingham, 1984), several

authors have suggested that SSAO might be a type II membrane protein with N-terminus of the molecule located intracellularly (5-6 residues) and the C-terminus located extracellularly. Nevertheless, the use of secondary structure prediction computer programmes might result in some alternative models.

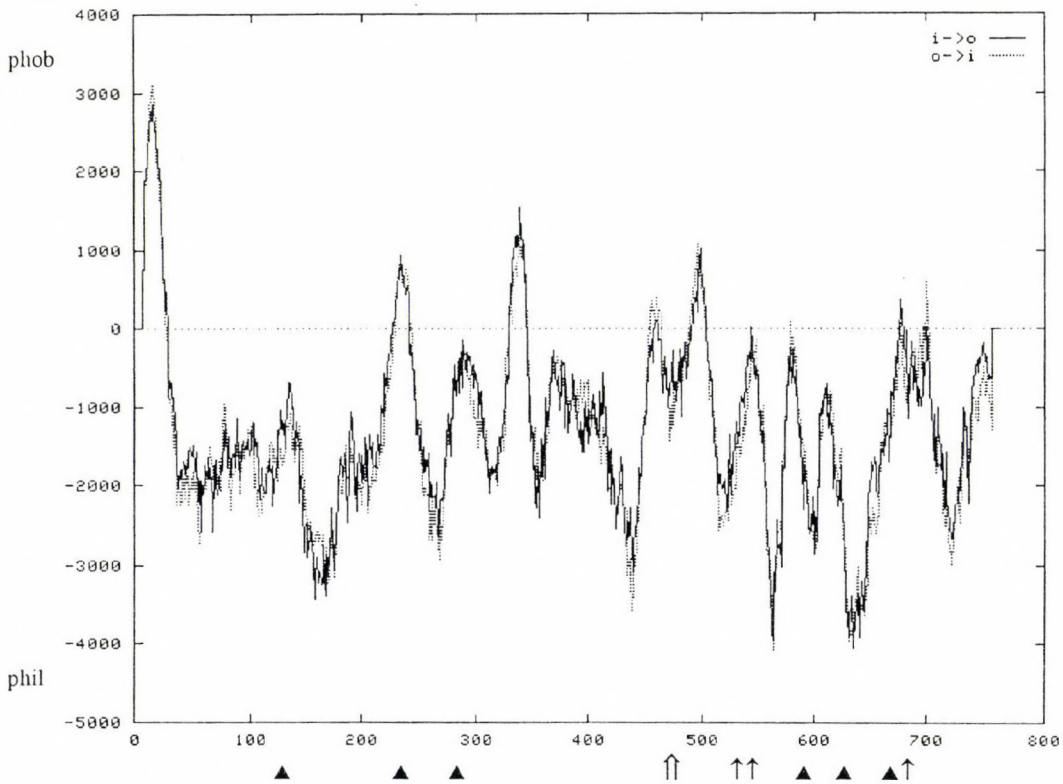

Fig. 2. Hydropathy plot for human placenta amine oxidase.
▲, Potential N-glycosilation sites (at position 137, 232, 294, 592, 618 and 666). ⇑, Topa quinone cofactor (Tyr 471). ↑, Conserved histidines (524, 526 and 689). Amino acid sequence obtained from Zhang and McIntire (1996).

Using TMpred® computer programme (Hoffmann and Stofell, 1993), a 3 transmembrane domains model for the human placenta sequence can be obtained. All 3 transmembrane regions are composed by 22-25 hydrophobic residues, resulting in 2 intracellular domains (from residue 1 to 5, and 246 →

330) and 2 extracellular regions (27 → 224 and 351 → 763). In such case Asn 294, a potential N-glycosilation site, would be located intracellularly, whereas the topa quinone cofactor and the 3 conserved histidines would be located extracellularly. Obtaining monoclonal antibodies against the polypeptide 246→330 and/or more focussed glycosilation studies on Asn 294 may be proved useful for resolving this question since it is clearly important to know how SSAO is integrated in the plasma membrane in order to dilucidate the possible mechanism by which this protein can be released from the plasma membrane.

With the present availability of sequences for membrane-bound SSAOs it can be also performed the corresponding alignments for the active site sequences (Fig. 3). All they contain the consensus sequence N<u>Y</u>DY (where underlined Y represents the tyrosine from which topa quinone derives by a post-translational modification) that has been reported for all mammalian copper amine oxidases cloned.

Human placenta SSAO[a]	MSTLL	N<u>Y</u>DYVWD	TVFMPSGA
Humam vap-1[b]	MSTLL	N<u>Y</u>DYVWD	TVFMPSGA
Mouse vap-1[c]	VSTLL	N<u>Y</u>DYIWD	MVFMPNGA
Human retina SSAO[d]	VSSVG	N<u>Y</u>DYIWD	FVLYPNGA
Bovine plasma SSAO[e]	TSTVY	N<u>Y</u>DYIWD	MVFYPNGA

Fig. 3. Active site sequences of cloned mammalian SSAOs. [a]Human placenta SSAO (Zhang and McIntire, 1996). [b]Human VAP-1 (Smith et al., 1998). [c]Mouse VAP-1 (Bono et al., 1998). [d]Human retina SSAO (Imamura et al., 1997). [e]Bovine plasma SSAO (Mu et al., 1994)

Are tissue-bound and plasma SSAOs encoded by different genes?

As discussed above the key point in current research on SSAO is to determine whether or not plasma SSAO is released from membrane-bound enzyme. When available tissue-bound SSAO sequences are compared with those for plasma the enzyme and overall 80% identity is obtained. Although it cannot be excluded that these differences might be due to a different splicing mechanism, few evidences recently appeared implying that both enzymes might be encoded by different genes.

It has been recently reported the cDNA that encodes for SSAO from bovine lung (Høgdall et al., 1998). This sequence has a 94% identity with the corresponding plasma enzyme. The use of bovine genomic DNA library led

this authors to propose the existence of at least 3 different genes encoding bovine copper amine oxidases. The first is expressed in lung, kidney, heart and spleen, and might encode for tissue-bound SSAO. A second gene is specifically expressed by the liver and might encode for the plasma enzyme (bovine plasma SSAO has been cloned using a liver cDNA library, Mu et al., 1994). A fragment of a third gene was also identified and could be related to the bovine diamine oxidase. However, the N-terminus sequence obtained in our laboratory for the bovine lung SSAO (28 aa) presented differences in half of the residues, when compared with the sequence reported by Høgdall et al. for same tissue-bound SSAO. Cloning of the protein obtained in our lab is being performed in order to elucidate whether or not they are different enzymes.

Another evidence came from a recently appeared study (Holt et al., 1998). These authors reported the active site sequence for pig aortic SSAO (Asn-Tyr-Asp-Tyr-Tyr). Asn-Tyr-Asp-Tyr is the consensus sequence to all mammalian copper amine oxidases. When this sequence was compared with that for the pig plasma enzyme, a valine at position 5, rather than tyrosine, was observed. This led to the authors to conclude that in pig, at least, plasma amine oxidase might not be derive from the blood vessel wall enzyme.

It is clear that, at the moment, there are not definitive evidences that allow us to draw conclusions. Stable expression of both plasma and tissue-bound SSAOs in mammalian cells lines hopefully will help in resolving this important question.

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RESEARCH REPORT

AGE-RELATED CHANGES OF MAO-A AND -B DISTRIBUTION IN HUMAN AND MOUSE BRAIN

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Age-related changes of MAO-A and -B were studied in human and BL/C57 mouse brain areas (substantia nigra, putamen and cerebellum). [^{3}H]Ro41 -1049 and [^{3}H]lazabemide were used as selective radioligands to image and quantify MAO-A and MAO-B respectively by enzyme autoradiography. MAO-A binding was higher in mouse, whereas MAO-B binding was higher in human. With aging, mouse MAO-A was significantly reduced between 4 and 8 weeks and remained unchanged until 19 months followed by a slight increase between 19 and 25 months. In contrast, no clear variation was observed in humans between the age of 17-93 years. In most of the structures studied a clear age-related increase in MAO-B was observed beginning in mouse brain at 4 weeks, whereas in human tissue this increase started at the age of 50-60 years. These results show marked differences in the levels and variations of mouse and human MAO-A and -B associated with aging and should be taken into account when extrapolating experimental data from mouse to human.

Key words: monamino oxidase (MAO), aging, mouse brain, human brain, autoradiography *in vitro*

INTRODUCTION

The two isoenzymes monoamino oxidase (MAO; EC 1.4.3.4) MAO-A and -B are mitochondrial enzymes which differ in their selectivity for substrates (Waldmeier, 1987) and inhibitors (Cesura and Pletscher, 1992), and in their cellular localization, and they are encoded by two separate genes (Bach et

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al., 1988). In rodent brain, 5-HT, dopamine and noradrenaline are preferred physiological substrates for MAO-A, whereas in human brain dopamine is a common substrate for both isoenzymes (Bevan Jones et al., 1972; Major et al., 1979; Riederer and Youdim, 1986; Martignoni et al., 1991). Several experiments have suggested that the cellular localization of MAO and their proposed physiological substrates are not always coincident: for example, dopaminergic neurons have no MAO-B and very little MAO-A, whereas 5-HT neurons are rich in MAO-B, as are astrocytes. This suggests that some neuronal types and astrocytes participate in the inactivation of extraneous neurotransmitters.

Variations in MAO-A and -B during ageing in rodent and human brain have been described by several authors and may reflect modifications in their main cellular location. Thus, MAO-A, which is mainly localized in adrenergic and noradrenergic neurons is believed to decrease with aging whereas MAO-B, which is mainly in astrocytes, increases due to the gliosis associated with neuronal death.

In this study we compared the results obtained with two radioligands, [^{3}H]Ro41-1049 and [^{3}H]lazabemide ([^{3}H]Ro19-6327), as selective tools for imaging and quantifying MAO-A and MAO-B in tissue sections by enzyme autoradiography *in vitro* (Saura et al., 1994 and 1997). This approach allows the comparison of age-related changes in MAO-A and MAO-B in normal mouse and human brain and may help to determine if their pattern of variations is similar in each of the species studied.

MATERIALS AND METHODS

Human tissue

Postmortem human brain was obtained through the local Bank of Neurological Tissue (School of Medicine, University of Barcelona, Barcelona, Spain) from 17 subjects with no known history of neurological or psychiatric disorder, ranging in age from 17 to 93 years. Cases with a number of senile plaques above the age threshold, assessed according to Khatchaturian's criteria (4), were not included in the study. Postmortem delay ranged from 1.5 h to 24 h and the time of storage of tissue blocks prior to experiment ranged from 3 to 29 months. No significant correlation was found between age and time post-mortem ($r=0.02$), age and tissue storage time ($r=0.25$) or post-mortem delay and tissue storage time ($r=-0.16$). At autopsy, the brains were dissected into blocks of tissue less than 3 cm thick, immediately frozen with dry ice and kept at -80 °C

until cryostat sectioning (Mahy, 1993). In the present study we used blocks of striatum (n=15), brainstem at the level of the substantia nigra (n=13) and cerebellar hemispheres (n=17).

Animals

BL/C57 male mice (Charles River España S.A.) aged 4 weeks (n=10), 9 weeks (n=7), 19 months (n=6) and 25 months (Cesura et al., 1989) were killed by decapitation under anaesthesia and their brain was quickly removed, frozen and kept at -80 °C until sectioning.

Enzyme autoradiography

Twelve µm cryostat sections were obtained from all human blocks and mouse brains, mounted on gelatinized slides, air dried and kept at -30 °C until the experiment. Sections were stored for no longer than 2 months. Enzyme autoradiography was performed essentially as previously described (Saura et al., 1992).

To reveal MAO-A, sections were incubated for 60 min at 37 °C with 8 nM [^{3}H]Ro41 -1049 (27.0 Ci/mmol) in a buffer solution (pH=7.4) containing 50 mM Tris, 120 mM NaCl, 1 mM $MgCI_2$, 5 mM KCI and 0.5 mM EGTA. Incubation was terminated by a 1min + 1min wash in cold buffer followed by a quick dip in cold distilled water. Non-specific binding was defined by coincubation with 1 µM clorgyline.

To reveal MAO-B, adjacent sections were incubated for 90 min at 22 °C in the above buffer containing 3 nM [^{3}H]lazabemide (20.2 Ci/mmol). The washing protocol was identical to that for MAO-A and coincubation with 1 µM deprenyl was used to define non-specific binding. After drying under a stream of cold air, sections were apposed to [^{3}H]sensitive film (Hyperfilm™, Amersham, Bucks, UK) for 26 days (MAO-A) or 12 days (MAO-B). Films were developed and analyzed densitometrically after calibration with plastic standards ([^{3}H]-microscales, Amersham, Bucks, UK) using a computer-assisted Image Analysis System (Interdens, Microm, Barcelona, Spain). The average brain protein content was estimated as 8%.

Statistics

Values are expressed as mean ± standard error of the mean (SEM). The effect of age on MAO-A and MAO-B was analyzed by linear regression and Pearson's linear correlation coefficient (product moment). Data were analyzed with

statistical software SPSS 6.1 (Marija Norusis/SPSS Inc., Chicago, IL) and STATGRAPHICS 6.0 (STSC Inc., Rockville, MD).

Materials

[^{3}H]Ro41-1049 and [^{3}H]lazabemide were kindly provided by Dr. J .G. Richards, Hoffmann La Roche, Basel, Switzerland. L-(-)deprenyl and clorgyline were supplied by Research Biomedical Inc. (Natick, MA). All other chemicals were of analytical grade and obtained from various sources.

RESULTS

Distribution of MAO-A and MAO-B

Distribution of MAO-A and -B revealed an heterogeneous distribution in both human and mouse tissue (see Table 1) similar to that previously reported. Differences between MAO-A and MAO-B binding were greater in human than in mouse. In all the studied areas [^{3}H]Ro41-1049 binding was lower in human, with the highest differences in the mouse binding molecular layer (603 vs. 2141) and the white matter of cerebellum (126 vs. 807). However, [^{3}H]lazabemide binding was higher in all the human areas, with the highest value and difference in mouse tissue in the molecular layer of cerebellum (1546 vs. 510).

Table 1. Distribution of MAO-A and -B in human and mouse brain

	MAO-A			MAO-B		
	human	mouse	% difference	human	mouse	% difference
Substantia nigra	1673±150 (13)	1933±120 (7)	+15	3215±148 (14)	2156±124 (8)	-34
Putamen	1325±133 (15)	1710±74 (9)	+29	2051±137 (15)	1813±50 (10)	-14
Cerebellar hemispheres						
Granular layer	1002±39 (16)	1488±81 (9)	+48	1926±162 (12)	1063±48 (10)	-46
Molecular layer	603±36 (16)	2141±99 (9)	+255	1556±145 (11)	510±24 (10)	-67
White matter	126±21 (17)	807±57 (7)	+540	1220±78 (17)	653±47 (8)	-49

Data show mean±SEM and are expressed in fmol/mg prot. for human (62.2 ± 5.0 years) and mouse (4 weeks) brain areas. Number of cases shown in brackets. % difference indicates the variation between human and mouse data. (Adapted from Saura et al., 1994 and Saura et al., 1997)

Age-related changes

In human, no significant correlation between age and MAO-A content was found in any of the structures studied (see Table 2). However in mouse, with the exception of the MAO-A in the molecular layer of the cerebellum, which was not modified, this enzyme was significantly reduced in all the areas between 4 and 9 weeks; afterwards, recovery was observed in the granubr layer; in the other areas this decrease was maintained in the 19- and 25-month-old groups.

Table 2. Effect of age on MAO-A in human amd mouse brain

	Human		Mouse			
	all ages	9 weeks (%)	19 months (%)	25 months (%)		
Substantia nigra	0.28	-27	-26	-24	**	a,b,c
Putamen	0.18	-41	-46	-33	**	a,b,c
Cerebellar hemispheres						
Granular layer	-0.16	-22	-18	-13	*	a
Molecular layer	0.16	-13	-3	0	n.s.	-
White matter	0.09	-32	-49	-47	**	a,b,c

With the exception of the left column, which shows the correlation coefficents between human ageing and MAO-A, values show the estimated change of MAO-A (in %) between 4 weeks and the 9 weeks, 19 months and 25 months mouse groups. *p<0.01, ** p<0.001. (Adapted from Saura et al., 1994 and Saura et al., 1997). a Significant differences between 4 week and 9-week-old animals, b Significant differences between 4-week- and 19-month-old animals, c Significant differences between 4 week and 25-month-old animals

Except for the substantia nigra, human MAO-B showed a significant positive correlation with age in all the structures. Analysis of the data showed that only patients older than 50-60 years presented an increase in MAO-B, and that when the MAO/B age plots were drauwn for patients older than 45 years, the strength of the correlation was enhanced (see Table 3). An age-related increase was also observed in the mouse in every brain structure studied, but was significant only in the substantia nigra and putamen in the 19- and 25-month-old groups. These increases vary significantly between the structures studied and they were greater in the 25-month-old animals: In this group MAO-B increased from 28% in the substantia nigra to 144% in the molecular layer of the cerebellum (Table 3).

Table 3. Effect of age on MAO-B in human amd mouse brain

	Human			Mouse (%)			
	age>45	% increase 60-90 years	9 weeks	19 months	25 months		
Substantia nigra	0.11	-	20	34	28	**	b,c
Putamen	0.63**	42	16	42	68	***	b,c,e
Cerebellar hemispheres							
Granular layer	0.59*	52	24	58	65	***	a,b,c,d,e
Molecular layer	0.52*	48	33	117	144	***	a,b,c,d,e,f
White matter	0.55**	50	10	14	37	**	c

With the exception of the left column, which shows the correlation coefficents between human ageing and MAO-B, values show the estimated increase of MAO-B (in %) between 60 and 90 years in humans and between 4 weeks and the 9 weeks, 19 months and 25 months mouse groups. *$p<0.05$, ** $p<0.01$, *** $p<0.001$. (Adapted from Saura et al., 1994 and Saura et al., 1997). a Significant differences between 4-week and 9-week-old animals, b Significant differences between 4-week- and 19-month-old animals, c Significant differences between 4-week- and 25-month-old animals, d Significant differences between 9-week- and 19-month-old animals, e Significant differences between 9-week- and 25-month-old animals, f Significant differences between 19-month- and 25-month-old animals.

DISCUSSION

The present study shows marked differences in the relative distribution of MAO-A and -B in human and mouse: in all the structures studied, MAO-B was more abundant in human tissue, whereas MAO-A levels were similar or higher than MAO-B levels in the 4-week-old mice. As human MAO-B remains stable before 50-60 years, and the mean age of the patients of this study was 62 years, this suggests that astrogliosis is more abundant in human than in mouse.

Another difference found is the age-related decrease of MAOA in mouse brain, which was not observed in human tissue. However, as this decrease takes place during the first weeks of life, it is difficult to compare with the human data due to the 14 to 94 years age-range of the patients included in this study. Thus, we cannot rule out a possible variation of human MAO-A during the first years of life. However, MAO-A increase has been reported in the aging rat cerebellum (Leung et al., 1981; Strolin Benedetti and Keane, 1980) and may reflect important differences of sensitivity of noradrenergic neurons to ageing between species. Ageing-associated gliosis may also contribute to the levels of

MAO-A, since glial cells seem to contain MAO-A, though at lower levels than MAOB (Konradi et al.,1988 and 1989; Westlund et al.,1988).

MAO-B increase is present throughout the lifespan of the mouse, but starts between the ages of 50-60 years in human, with the exception of substantia nigra, where it remains constant. This may help to explain the lack of reduction of MAO-A between 9 weeks and 25 months in mouse and would argue for a lack of modification in MAO-A during human ageing.

In conclusion, the present study indicates that enzyme concentrations, MAO-A: MAO-B ratios and age-related changes differ markedly between human and mouse brain. These differences should be taken into account when extrapolating experimental data from mouse to human brain.

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RESEARCH REPORT

N-METHYL(*R*)SALSOLINOL AND A NEUTRAL *N*-METHYLTRANSFERASE AS PATHOGENIC FACTORS IN PARKINSON'S DISEASE

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The pathogenesis of Parkinson's disease is still an enigma. As an endogenous MPTP-like neurotoxin, N-methyl(*R*)salsolinol was proved to induce parkinsonism in rats and apoptosis in dopaminergic neurons. It is synthesized in the human brain by two enzymes; an (*R*)salsolinol synthase and an N-methyltransferase, and accumulates in the nigro-striatum in human brains. The activity of a neutral N-methyltransferase in the striatum was found to determine the level of MPP^+-like 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion, an oxidation product of N-methyl(*R*)salsolinol in the substantia nigra. The activity of this N-methyltransferase was found to increase significantly in lymphocytes prepared from parkinsonian patients. In cerebrospinal fluid from untreated parkinsonian patients, N-methyl(*R*)-salsolinol increases significantly. These results suggest that N-methyl(*R*)salsolinol and a neutral N-methyltransferase may be endogenous factors in the pathogenesis of Parkinson's disease.

Keywords: Parkinson's disease, N-methyl(*R*)salsolinol, (*R*)salsolinol N-methyltransferase, dopamine, peripheral early markers

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INTRODUCTION

The pathogenesis of Parkinson's disease (PD) remains to be elucidated, and there has been an increasing body of evidences to indicate the involvement of neurotoxins to the cell death of dopamine neurons in the substantia nigra. In search for specific endogenous neurotoxins, 1(*R*), 2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline [*N*-methyl(*R*)salsolinol, *N*M(*R*)Sal] was found to induce parkinsonism in rats. The biochemical, pathological and behavioral features were comparable to those of an animal model of PD (Naoi et al., 1996a). As a cause of the cell death, apoptosis was detected in the dopamine neurons in the substantia nigra of parkinsonian brains (Mochizuki et al., 1996; Anglade et al., 1997) and *N*M(*R*)Sal was found to cause apoptotic cell death in human dopaminergic neuroblastoma SH-SY5Y cells (Maruyama et al., 1997a and b).

The mechanism of the selective neurotoxicity of *N*M(*R*)Sal was shown to be closely related with its biosynthesis pathway in the brain (for reviews, Naoi et al., 1997a and 1998a). Only the (*R*)enantiomer of NMSal is detected in the human brain, and the *in situ* synthesis was proved to be catalyzed by two-step enzyme reactions, as shown in Fig. 1. A precursor, (*R*)Sal, is synthesized enantio-selectively from dopamine and acetaldehyde by an (*R*)salsolinol synthase (Naoi et al., 1996b), and it is further N-methylated by an N-methyltransferase in the nigro-striatal system (Maruyama et al., 1992; Naoi et al., 1997a). The localization of the precursor dopamine and the biosynthesis enzymes seems to be responsible to the specified localization of *N*M(*R*)Sal in the nigro-striatum, because of the high activity there (Maruyama et al., 1997c). *N*M(*R*)Sal is further oxidized into MPP^+-like 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion enzymatically (Naoi et al., 1995) or non-enzymatically (Maruyama et al., 1995a) with generation of hydroxyl radical (Maruyama et al., 1995a and b). The production of reactive oxygen species (*R*OS) was found to account for apoptotic DNA damage in the dopaminergic cells (Maruyama et al., 1997a and 1997b).

To examine whether *N*M(*R*)Sal is truly involved in the pathogenesis of PD, human materials such as cerebrospinal fluid (CSF) (Maruyama et al., 1996a) human brains (Maruyama et al., 1997c, Naoi et al., 1997b) and lymphocytes (Naoi et al., 1998b) from parkinsonian patients were analyzed for the isoquinoline levels and the activity of related enzymes. The results on the altered metabolism of the dopamine-derived isoquinoline are discussed in relation to possible involvement the pathogenesis of PD as an endogenous factor.

MATERIALS AND METHODS

Analysis of NM(R)Sal in parkinsonian CSF

The CSF samples were obtained from newly diagnosed untreated parkinsonian patients (n=16) and from 29 control subjects without neurological disorders. Lumbar CSF was obtained in the morning after 9 hour rest and fast (Maruyama et al., 1996a). CSF sample was analyzed for the level of *N*M(*R*)Sal and a dopamine metabolite, homovanillic acid (HVA) by high-performance liquid chromatography (HPLC) with multi-electrochemical detectors (ECD) (CEAS, ESA, Chelmsford, MA, USA) (Maruyama et al., 1996b). The CSF from 9 out of the 16 parkinsonian patients were analyzed for the second time after an interval of 1.5 to 2 years (mean; 1.8 years). All patients were medicated and only one (Patient #6) was not treated with L-DOPA.

Analysis of (R)Sal and N*M*(R)*Sal derivatives in human brain*

Ten control human brains without neuropsychiatric disorders were obtained and stored at -80°C until analysis. Four brain regions, frontal cortex, caudate, putamen and substantia nigra were punched out and the content of dopamine, the (*R*)- and (*S*)-enantiomers of Sal and NMSal, and the isoquinolinium ion were analyzed. The enantiomeric separation of (*R*)-and (*S*)-Sal and NMSal was performed using HPLC-ECD with a cyclodextrin-bounded column as reported (Maruyama et al., 1996b) and the isoquinolinium ion was by HPLC with fluorometric detection (Naoi et al., 1995).

Determination N*-methyltransferase activity in lymphocytes and brain samples*

The activity of an N-methyltransferase which catalysis N-methylation of (*R*)Sal was analyzed in human brain samples (Naoi et al., 1997b) and lymphocytes (Naoi et al., 1998b) using (*R*)Sal as a substrate and S-adenosyl-L-methionine as a methyl donor (Naoi et al., 1997b).

Lymphocytes prepared from 56 idiopathic parkinsonian patients and 24 control patients were used for the measurement of the enzyme activity. Human lymphocyte sample was prepared from freshly taken blood by use of a lymphocyte separation medium (Flow Laboratories, Nortg Ryde, NSW, Australia). The activity of (*R*)salsolinol N-methyltransferases with the optimal pH at alkaline and neutral, and N-methyl(*R*)salsolinol oxidase was analyzed as reported (Naoi et al., 1998b) and the values were compared between the patients and control.

As another study, both lymphocytes and CSF samples were obtained from 9 newly diagnosed parkinsonian patients without treatment. The mean age of the

patients was 67 years (47-77) and the M/F ratio was 3/6. In this group, the activity of a neutral (*R*)salsolinol N-methyltransferase in the lymphocyte and the concentration of *N*M(*R*)Sal in the CSF were analyzed to compare these two parameters in each patients.

The protocols of the examination of the lymphocyte and the CSF were approved by the Ethical Committee of Iwate Medical University or Aichi Medical University, and the patients were fully informed about the risks and the benefits of the examination.

Statistics

Statistical analysis was done by Wilcoxon matched-pairs signed-ranks test or analysis of variance (ANOVA) followed by Scheffe F-test. A p value lower than 0.05 was determined as statistically significant.

RESULTS

Analysis of NM(R)Sal in the CSF from parkinsonian patients

Figure 2 shows the concentration of *N*M(*R*)Sal and HVA in untreated parkinsonian patients. The level of *N*M(*R*)Sal was found to be significantly higher and HVA was significantly lower in parkinsonian patients than control. In almost all control, except 2 out of 29, *N*M(*R*)Sal level was lower than 6 nM. On the other hand, in 12 PD patients out of 16 the level was higher than 6 nM. To estimate the biosynthesis rate of this isoquinoline from dopamine, the concentration of *N*M(*R*)Sal was compared with that of HVA, a major metabolite of dopamine. The ratio also significantly increased in PD patients compared with control ($p < 0.0002$), suggesting the increase in the biosynthesis of *N*M(*R*)Sal. On the other hand, the age and the sex of the patients were not related with the *N*M(*R*)Sal concentration in CSF.

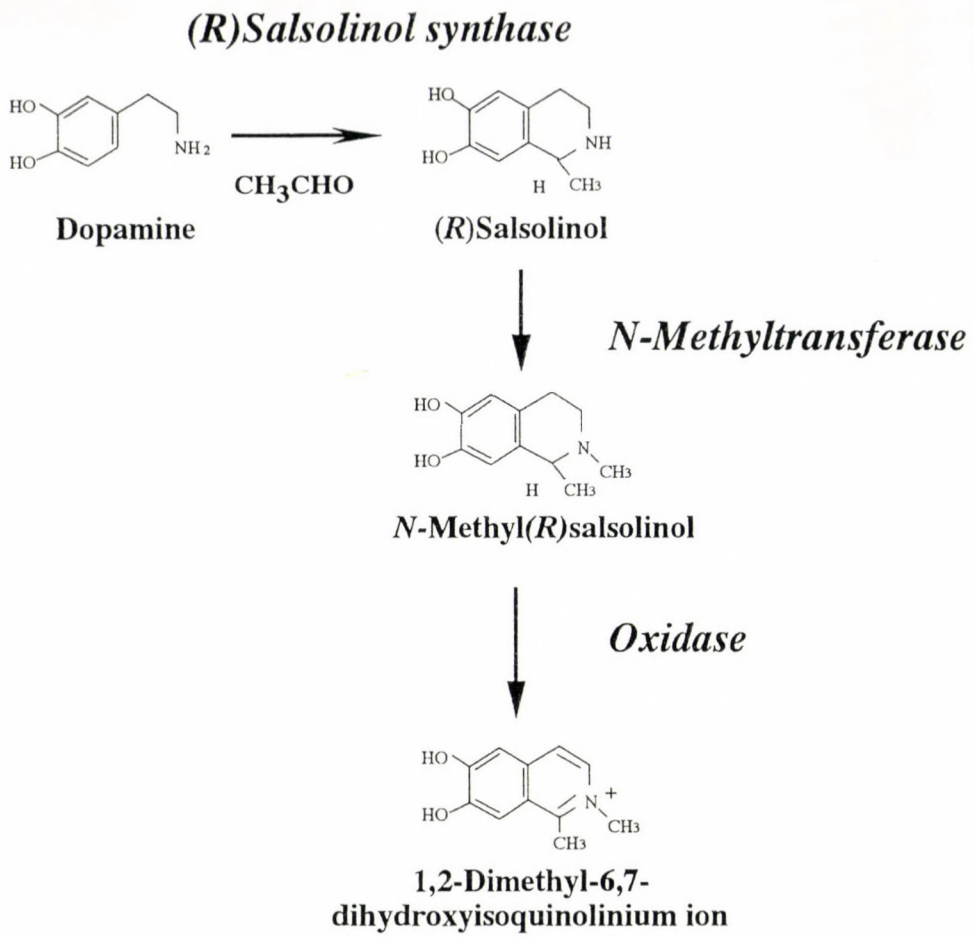


Fig. 1. The metabolic pathway of *N*-methyl(*R*)salsolinol in the human brain

Effect of the treatment and progress of the disease on the N*M(*R*)Sal concentration in CSF*

The change of *N*M(*R*)Sal level in the CSF obtained from the same parkinsonian patients before and after anti-PD medication for about 2 years was shown in Fig. 3. The level was found to decrease in 8 patients out of 9 and to increase only in one patient (#2). There was no difference in the clinical profile (age, sex, type of medication, duration of the illness, Hoehn and Yahr stage) between Patient #2 and other 8 patients. The (*R*)- and (*S*)-Sal and *N*M(*S*)Sal were under

the detectable level (< 0.1 nM). *N*M(*R*)Sal content was found to decrease significantly in the second CSF sample compared with that in the first sample from the same patient. These results may be due to the L-DOPA therapy and/or of the progress of PD, namely the loss of dopamine neurons in the brain.

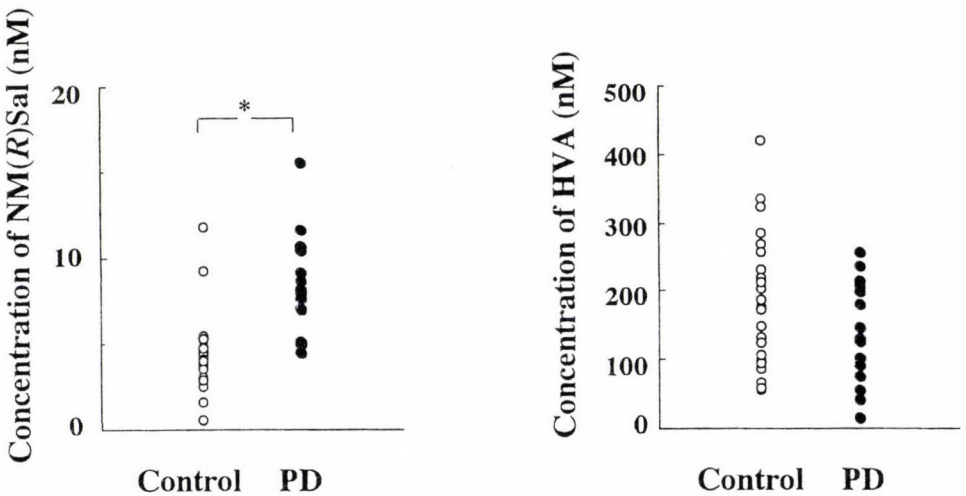


Fig. 2. The concentration of *N*M(*R*)Sal, HVA and the ratio of *N*M(*R*)Sal /HVA in the CSF from control and parkinsonian patients

Analysis of salsolinol derivatives in human brain

From control human brains (n=10), four brain regions, frontal cortex, caudate, putamen and substantia nigra, were analyzed for *N*MSal and related compounds. In human brain only the (*R*)-enantiomer of Sal and *N*MSal were detected. The distribution of (*R*)Sal, dopamine, *N*M(*R*)Sal and the isoquinolinium ion was summarized in Table 1. (*R*)Sal occurs in the brain non-selectively, whereas *N*M(*R*)Sal accumulates in the nigro-striatal system and the isoquinolinium ion is detected only in the substantia nigra. These results demonstrate that (*R*)Sal is synthesized enantio-selectively from dopamine and the high activity of a *N*-methyltransferase in dopamine neurons is responsible to the accumulation of *N*M(*R*)Sal in the nigro-striatum, as shown by use of *in vivo* microdialysis in rat brain (Maruyama et al., 1992). The accumulation of the isoquinolinium ion in the substantia nigra may be due to the binding of the

isoquinolinium ion to neuromelanin in the substantia nigra, as proved by in vitro (Naoi et al., 1994) and *in vivo* experiments (Naoi et al., 1996a).

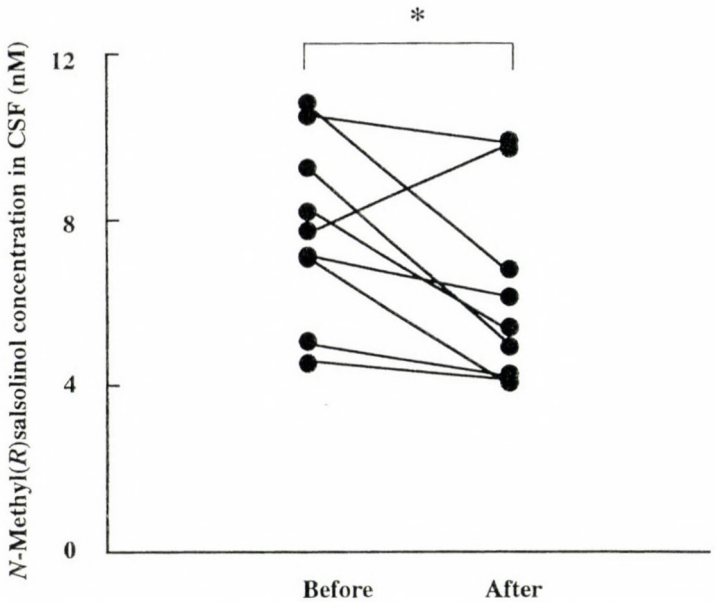


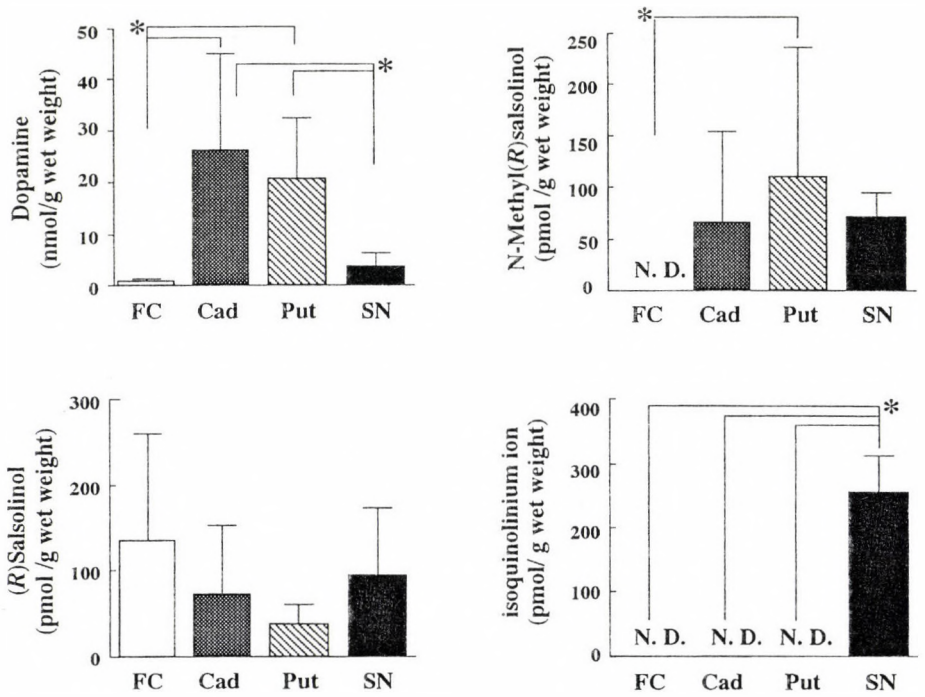
Fig. 3. The change of the concentration of *N*M(*R*)Sal in the CSF from the same patients after 2 years medication. CSF was obtained by the lumbar puncture and analyzed by HPLC-with multi-ECD

Relationship of the N*M(*R*)Sal concentration and the enzyme activity in the brain regions*

The activity of a (*R*)Sal synthase, a neutral and alkaline (*R*)Sal *N*-methyltransferase, and *N*M(*R*)Sal oxidase were analyzed in the brain sample prepared from the frontal cortex, caudate and putamen (Naoi et al., 1997b). The strong positive correlation was found between the activity of a neutral (*R*)Sal *N*-methyltransferase in the caudate-putamen and the content of the isoquinolinium ion in the substantia nigra (Fig. 4). Neither the activity of other enzymes nor that of a neutral *N*-methyltransferase in other brain regions correlated with the level of any *N*MSal derivatives. Further purification and characterization of this enzyme are now on the way.

The results suggest that *N*M(*R*)Sal synthesized in the striatum is transported by retrograde axonal flow to the substantia nigra, and oxidized there or on the

way to produce the isoquinolinium ion. As shown above, the isoquinolinium ion accumulates in the substantia nigra more markedly than the reduced isoquinoline and in other parts of the brain.



FC: Frontal Cortex
Cad: Caudate
Put: Putamen
SN: Substantia nigra

Table 1

Analysis of the enzymes related to the NM(R)*Sal metabolism in parkinsonian lymphocytes*

Lymphocytes from parkinsonian patients and control were analyzed for the activity of a neutral and an alkaline (*R*)Sal *N*-methyltransferase, and an *N*M(*R*)Sal oxidase. As summarized in Table 2, only the activity of a neutral

(*R*)Sal *N*-methyltransferase was significantly higher in parkinsonian patients than in control, whereas that of an alkaline *N*-methyltransferase and an *N*M(*R*)Sal oxidase were not significantly different in lymphocytes from parkinsonian patients and control (Naoi et al., 1998b). The activity of a (*R*)Sal synthase was not detectable in the lymphocyte sample. Considering that the activity of this enzyme in the striatum determines the level of the toxic isoquinolinium ion (Naoi et al., 1997b) in the substantia nigra, the activity may increase also in the caudate-putamen of parkinsonian brain, resulting in the increase of the neurotoxin in the substantia nigra.

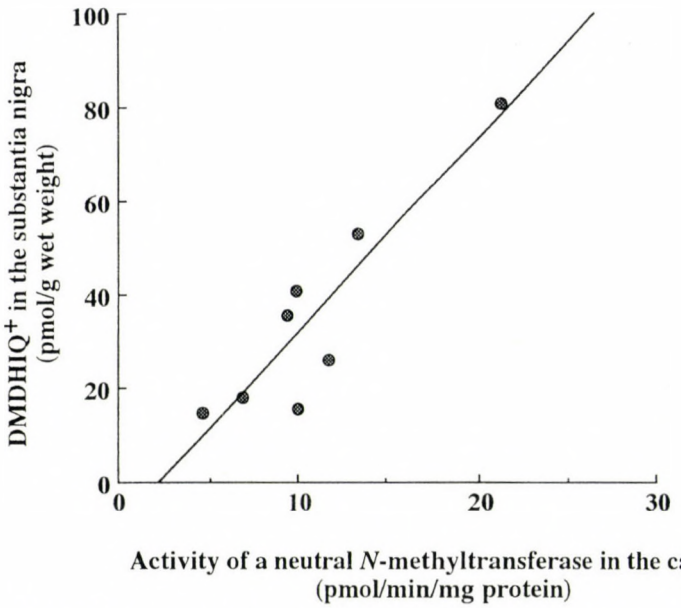


Fig. 4. Correlation of the content of the isoquinolinium ion in the substantia nigra with the activity of a neutral *N*-methyltransferase in the caudate (A) and putamen (B). $p < 0.001$ and 0.05 for caudate and putamen, respectively

Relationship of the N*M(*R*)Sal level in CSF and the activity of a neutral (R)Sal N-methyltransferase in lymphocyte prepared from the same PD patients*

It was examined whether the activity of a neutral (*R*)Sal *N*-methyltransferase in lymphocytes correlated with the concentration of *N*M(*R*)Sal in the CSF. The enzyme activity in lymphocytes and the content of *N*M(*R*)Sal and HVA in CSF

from each patients are shown in Fig. 5. Significant correlation was found between the enzyme activity and the *N*M(*R*)Sal level ($r = 0.716$, $p < 0.05$). On the other hand, there was no significant correlation between the activity of the *N*-methyltransferase and the level of HVA, nor between the level of HVA and *N*M(*R*)Sal. The results seem to present the direct relationship between the activity of this enzyme and the neurotoxin level in CSF and probably in the brain.

Table 2. Activity of neutral *N*-methyltransferase and related enzymes

	Enzyme activity in lymphocytes prepared from (pmol/min/mg protein)	
	Parkinsonian patients (n = 56)	Control (n = 24)
Neutral *N*-methyltransferase*		
	100.2 ± 81.8	18.9 ±15.0
female	111.2 ± 92.3	16.7 ± 14.4
male	84.3 ± 62.3	20.2 ± 15.6
Patients with Hoehn-Yahr stage		
II (n = 6)	40.8 ± 17.0	
III (n = 34)	121.4 ± 89.4	
IV (n = 13)	80.8 ± 55.2	
V (n = 3)	61.9 ± 99.9	
Alkaline *N*-methyltransferase*		
	41.8 ± 17.3	25.0 ± 23.0
N-Methyl(*R*)salsolinol oxidase		
	2.15 ± 2.43	1.38 ± 2.23

* The activities of neutral and alkaline (*R*)salsolinol *N*-methyltransferase were measured at pH 7.0 and 8.0, respectively.

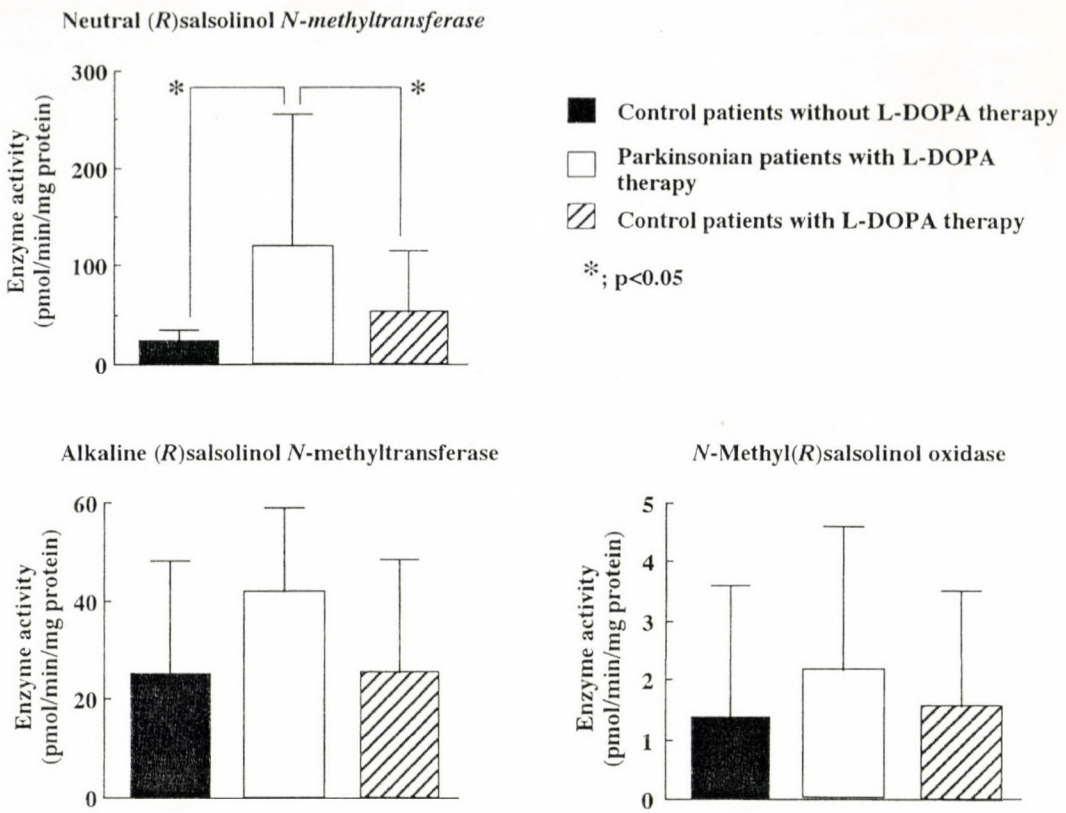


Fig. 5. The correlation of the activity of a neutral (*R*)Sal *N*-methyltransferase in the lymphocyte and the level of *N*M(*R*)Sal in CSF of newly-diagnosed and untreated parkinsonian patients. The activity of the *N*-methyltransferase and the *N*M(*R*)Sal level of each patient were compared and the good correlation was confirmed ($p < 0.05$)

DISCUSSION

An endogenous catechol isoquinoline, *N*M(*R*)Sal was proved to be dopaminergic neurotoxin by *in vivo* and *in vitro* studies. The direct mechanism of cell death is the induction of apoptosis in dopamine neurons. The neurotoxicity of *N*M(*R*)Sal is selective to dopamine neurons, as shown by selective depletion of dopamine neurons in the substantia nigra of the animal PD model after infusion in the striatum (Naoi et al., 1996a). As described here, (*R*)Sal should be *in situ* synthesized enantio-selectively and the enzyme activity of a neutral *N*-methyltransferase determines the selective accumulation of

*N*M(*R*)Sal and the oxidation product, the isoquinolinium ion in the substantia nigra. These results suggest that the enzymes related to the metabolism of this neurotoxin is closely related to the specificity and mechanism of the induction of apoptosis in dopamine neurons.

At present, the genetic marker of sporadic form of PD remains to be clarified, whereas that for familiar autosomal dominant parkinsonism was identified as the mutation of α-synuclein gene (Polymeropoulos et al., 1997). More recently, another mutation was identified in the parkin gene in autosomal recessive juvenile parkinsonism (Kitada et al., 1998). However, the relation of these mutations to the pathogenesis of the sporadic form of PD has never been confirmed.

The activity of the neutral *N*-methyltransferase is considered to be regulated by genetic and environmental factors. This enzyme seems to be novel, judging from the selective substrate specificity to (*R*)Sal, but not (*S*)Sal, the optimal pH at 7, and the distribution of the cytosol fraction of the brain. The purification and characterization of the enzyme and the isolation of its cDNA will bring us a new development to understand the pathogenesis of PD. In addition, this transferase activity in the lymphocyte may be applicable as an preclinical and peripheral maker of PD.

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RESEARCH REPORT

ANTI-APOPTOTIC FUNCTION OF L-(-)DEPRENYL (SELEGILINE) AND RELATED COMPOUNDS

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(-)Deprenyl has been proposed to be neuroprotective to dopamine neurons in the parkinsonian brains. To clarify the mechanism, the effects of (-)deprenyl and structurally related compounds on apoptosis induced by a neurotoxin, *N*-methyl(*R*)-salsolinol, and reactive oxygen species, nitric oxide and peroxynitrite, were examined in dopaminergic SH-SY5Y cells. DNA damage was quantified by the single cell gel electrophoresis (comet) assay. (-)-Deprenyl protected the cells from apoptosis in a dose-dependent way, which required pre-treatment at least for 20 min. The effect was confirmed even after washing out of (-)deprenyl, indicating that (-)-deprenyl initiates the intracellular process to antagonize the apoptotic death program. The studies on the structure-activity relationship reveal that *N*-propargyl residue with hydrophobic structure is essential for the anti-apoptotic function. These results suggest that (-)deprenyl and related compounds may be applicable as neuroprotective agents in neurodegenerative diseases.

Key words: (-)deprenyl, anti-apoptotic function, neurotoxin, peroxynitrite, nitric oxide, Parkinson's disease

INTRODUCTION

The pathological characteristic of Parkinson's disease (PD) is the selective degeneration of dopamine neurons in the pars compacta of the substantia nigra. The mechanism of the cell loss remains to be elucidated, and recently apoptosis is proposed as a death process in PD (Mochizuki et al., 1996;

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Anglade et al., 1997). Apoptosis is induced by various intra- and extracellular stimuli. We found that an endogenous dopaminergic neurotoxin, 1(*R*), 2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydro-isoquinoline [*N*-methyl(*R*)salsolinol] (for reviews; Naoi et al., 1997; 1998), induced apoptosis in human dopaminergic neuroblastoma SH-SY5Y cells (Maruyama et al., 1997a, b). More recently, peroxynitrite and nitric oxide (N0) were found to induced apoptosis in the cells, suggesting that oxidative stress may initiate the apoptotic death process (Maruyama et al., 1998). It is relevant with the findings that reactive oxygen species (ROS) may account for the cell death in PD. A product of the oxidative stress, 4-hydroxy-2-nonenal protein adduct, was found to increase in the nigral neurons of parkinsonian brains (Yoritaka et al., 1996). These results suggest that peroxynitrite and nitric oxide may be involved in the neuronal cell death in neurodegenerative diseases, such as amyotrophic lateral scrolosis (Beckman et al., 1993) and PD.

(-)Deprenyl is a well-known irreversible inhibitor of type B monoamine oxidase [monoamine, oxygen oxidoreductase (deaminating); EC 1.4.3.4., MAO-B] and is now used for the treatment of parkinsonian patients in combination with L-DOPA therapy. In parkinsonian patients treated with (-)deprenyl, slowing of the disease progression was confirmed (Parkinson's study group, 1989; Tetrud and Langston, 1989; Olanow et al., 1995; Myllylä et al., 1996). The neuroprotective or neurorescue effect of (-)deprenyl was demonstrated also by *in vitro* experiments. (-)Deprenyl protected dopamine neurons against the toxicity of another dopaminergic neurotoxin, 6-hydroxydopamine (Knoll, 1986), 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) (Tatton and Greenwood, 1991), 1-methyl-4-phenyl-pyridinium ion (MPP^+), (Mytilineou and Cohen, 1985; Wu et al., 1993), trophic withdrawal (Tatton et al., 1994), and glutamate excitotoxicity (Mytilineou et al., 1997). It also protected the cells against toxicity of a noradrenergic toxin, DSP-4 (Finnegan et al., 1990) and a cholinergic toxin, AF64A (Bronzetti et al., 1992), showing that (-)deprenyl is effective not only dopaminergic, but also other types of neurons.

In humans the metabolites of (-)deprenyl by the cytochrome P-450 system have been confirmed to be L-desmethylselegiline and L-methamphetamine (Yoshida et al., 1986; Heinonen et al., 1994). These metabolites are known to cause adverse side-effects in patients treated with (-)deprenyl (Engberg et al., 1991). However, desmethyl-selegiline was shown to exert neuroprotection against the excitotoxicity (Mytilineou et al., 1997), trophic support withdrawal (Tatton et al., 1994) and depletion of reduced glutathione (GSH)

(Mytilineou et al., 1998). Another acetylenic MAO-B inhibitor, *R*(+)-*N*-propargyl-1-aminoindan (rasagiline) (Finberg et al., 1995) is not metabolized into amphetamine, and is now in phase 3 clinical trial for treatment of PD. Rasagiline was reported to be also neuroprotective and increase the survival of dopaminergic cells in the primary culture of mesencephalic cells (Finberg et al., 1998).

The neuroprotection by (-)deprenyl does not depend on MAO-B inhibition, and the exact mechanism remains an enigma. In this paper, the effect of (-)deprenyl was studied on the DNA damage induced by an endogenous neurotoxin, *N*-methyl(*R*)salsolinol, NO and peroxynitrite in SH-SY5Y cells. NOR-4, (±)-*N*-[(E)-4-ethyl-2-[(Z)-hydroxyimino]-5-nitro-3-hexen-1-yl]-3-pyridine carboxamine, was used as a donor of N0, and as a donor of peroxynitrite, SIN-1, *N*-morpholino sydnonimine, was used, which produces NO and superoxide simultaneously to generate peroxynitrite. The DNA damage was quantitatively assessed using a single cell gel electrophoresis (comet) assay (Östling and Hohanson, 1984; Maruyama et al., 1997a). The structure-activity relationship was studied among compounds structurally related to (-)deprenyl. The anti-apoptotic function of (-)deprenyl is discussed in the relation to its possible application as neuroprotection of neurons in neuro-degenerative diseases.

MATERIALS AND METHODS

Chemicals

N-Methyl(*R*)salsolinol and the derivatives were synthesized according to Teitel et al. (1972). Cycloheximide, ethidinium bromide, (-)deprenyl and superoxide dismutase (SOD) were purchased from Sigma (St. Louis, MO); NOR-4 and SIN-1, were from Dojindo (Kumamoto, Japan); α-tocopherol, catalase, agarose (low melting-temperature), GSH and other reagents were purchased from Nacalai Tesque (Kyoto, Japan).

Single cell gel electrophoresis (comet) assay

SH-SY5Y cells were cultured with Cosmedium-001 (CosmoBio, Tokyo, Japan) supplemented by 5% Nakashibetsu precolostrum newborn calf serum (Mitsubishi Kasei, Tokyo, Japan) in atmosphere of 95% air-5% CO_2. The comet assay was performed as reported previously (Östling and Hohanson, 1984; Kasamatsu et al. 1996, Maruyama et al., 1997a). The cells were dissociated with trypsin, washed with Cosmedium supplemented with the

serum, suspended in the Krebs-Ringer solution, and incubated with various concentrations of *N*-methyl(*R*)salsolinol, NOR-4 or SIN-1 for 3 h. The cells (5×10^4 cells) were washed, suspended, and mixed with 1% low-melting agarose. The mixture (100 µl) was applied on a microscope slide and lysed in alkaline lysis buffer (10 mM Tris buffer, pH 10.0, containing 2.5 M NaCl, 100 mM EDTA, 1% sarcosine and 1% Triton X-100, and 10% dimethyl sulfoxide) for 15 h at 4 °C. The slides were equilibrated with alkaline electrophoresis buffer (300 mM NaOH containing 1 mM EDTA 2Na) and applied to electrophoresis (25 V and 300 mA for 20 min). After neutralization with 0.4 M Tris-HCl buffer, pH 7.5, DNA was stained with ethidinium bromide (20 µg/ml). Two hundred comet images were randomly selected, and the tail length (length of DNA tail from the trailing edge of nucleus) and the comet length (nucleus plus migrated DNA tail) were measured on a video camera screen connected to a fluorescence microscope.

The effects of (-)deprenyl and antioxidants on DNA damage induced by *N*-methyl(*R*)salsolinol, NOR-4 or SIN-1 were examined. The cells were incubated at 37 °C for 20 min with (-)deprenyl (20 µM in the final concentration), GSH (50 µM), catalase (0.5 mg), SOD (2000 units), or α-tocopherol (250 µM). Then, 0.5 mM *N*-methyl(*R*)salsolinol, 10 µM NOR-4 or 5 µM SIN-1 was added and incubated for further 3 h. and DNA damage was quantified by the comet assay.

To examine the effect of washing out, the cells were incubated with 20 µM of (-)deprenyl for 20 min, washed twice with phosphate-buffered saline without calcium and then re-suspended in the Krebs-Ringer solution. The cells were treated with NOR-4 (10 µM) or SIN-1 (5 µM) for another 3 h and DNA damage was quantified by the comet assay.

Statistics

The comet length and the tail length of 200 cells was measured in each experiments. To evaluate DNA damage, the cells with the comet length longer than 30 µm were classified to be positive for DNA damage. The number of DNA-damaged cells was expressed as percentages of the total, and also the tail length was compared. Statistical significance was assessed by analysis of variance (ANOVA) for the tail length. All differences with $p < 0.05$ were considered to be statistically significant.

RESULTS

The induction of apoptosis by a dopaminergic neurotoxin and protection by (-)deprenyl and anti-oxidants

After incubation with the isoquinoline, an NO- or peroxynitrite-donor, SH-SY5Y cells showed a typical comet image with migrated comet tail of fragmented DNA. The DNA damage was proved to be apoptotic, by prevention of the DNA damage by cycloheximide and actiomycin-D, and by the morphological observation with the TUNEL method (Maruyama et al., 1997a, and b).

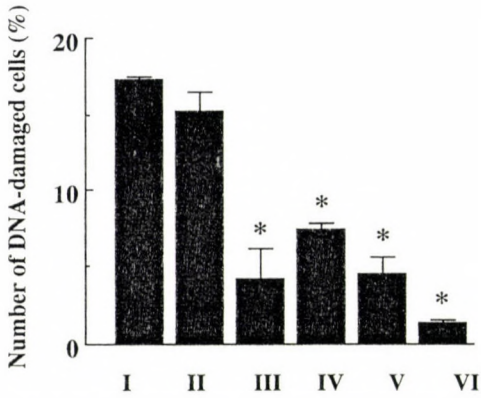

Fig. 1. The effects of (-)deprenyl and antioxidants on DNA damage induced by *N*-methyl(*R*)salsolinol. The cells were incubated with *N*-methyl(*R*)salsolinol after preincubation in medium alone (I) or with SOD (II), catalase (III), GSH (IV), (-)deprenyl (V) or semicarbazide (100 μM) (VI). Each column and a bar represent the mean ± SD of three measurements from three independent experiments. * Different significantly from the cells incubated with *N*-methyl(*R*)salsolinol alone ($p<0.05$)

As shown in Fig. 1, 20 min preincubation with 20 μM (-)deprenyl protected the cells from DNA damage. Anti-oxidative agents, catalase, GSH and semicarbazide, also prevented apoptotic DNA damage, while SOD did not. Since in SH-SY5Y cells only type A MAO is detected, the protection of (-)deprenyl did not depend on the MAO inhibition. On the other hand, clorgyline, a MAO-A selective inhibitor, did not prevent the apoptotic DNA damage. *N*-Methyl(*R*)salsolinol is oxidized by an enzyme sensitive to

semicarbazide (Naoi et al., 1995), which is relevant with the protection by semicarbazide. The oxidation, either enzymatic or non-enzymatic, of this isoquinoline produces hydroxyl radicals (Maruyama et al., 1995a, b), and he protection by catalase and GSH suggests the involvement of ROS in the apoptotic process.

Table 1. The effect of NOR-4 and SIN-1 concentration on DNA damage in SH-SY5Y cells

		Cells with DNA damage (%)	Tail length (μm)
Control cells		2	0.42 ± 3.00
Cells incubated with			
NOR-4	50 μM	40	7.26 ± 10.00*
	10 μM	33	6.73 ± 11.22*
	5 μM	23	4.49 ± 8.56*
	1 μM	7	1.31 ± 4.92
SIN-1	5 μM	36	6.77 ± 9.63*
	1 μM	16	2.92 ± 6.96*

SH-SY5Y cells were incubated with various concentrations of NOR-4 or SIN-1, then were subjected to the comet assay. The cells with the comet length greater than 30 μm were classified as positive for DNA damage, and expressed as percentages of the total. Tail length of 200 cells was expressed as mean ± SD. *; The difference from the control cells was statistically significant ($p < 0.05$) by ANOVA.

Apoptosis induced by NO and peroxynitrite and protection by (-)-deprenyl

The intracellular mechanism of anti-apoptotic function of (-)-deprenyl was studied more directly in relation to the apoptosis induced by NO and peroxynitrite, generated by NOR-4 and SIN-1. As in the case with the isoquinoline, these donors induced apoptotic DNA damage in the cells. After incubation with NOR-4 or SIN-1, the number of cells with DNA damage and also the mean value of the tail length increased significantly. Table 1 summarizes the results on the DNA damage induced by various concentration of NOR-4 or SIN-1. NOR-4 and SIN-1 induced DNA-damage in the cells in a dose-dependent way. With 10 μM of NOR-4 or 5 μM of SIN-1, about a third of the cells were positive for DNA damage.

The effects of (-)deprenyl, radical scavengers and antioxidants on the DNA damage are shown in Fig. 2. (-)Deprenyl was found to prevent apoptosis induced by NOR-4 and SIN-1 markedly. SOD, catalase, GSH and α-tocopherol reduced the number of DNA-damaged cells after treatment with NOR-4. In the cells incubated with SIN-1, SOD, GSH and α-tocopherol

reduced the DNA damage significantly, but catalase did not prevent the damage. The difference may be due to different synthesis pathway of NO and peroxynitrite. NOR-4 produces NO directly, and catalase catabolises a precursor of superoxide, hydrogen peroxide, and reduces peroxynitrite synthesis. On the other hand, SIN-1 chemically produces NO and superoxide simultaneously, which react to produce peroxynitrite, and catalase neither reduces peroxynitrite production, nor protects the cells from apoptosis. These results suggest that peroxynitrite may be a true toxic compound to initiate the apoptotic cell reactions.

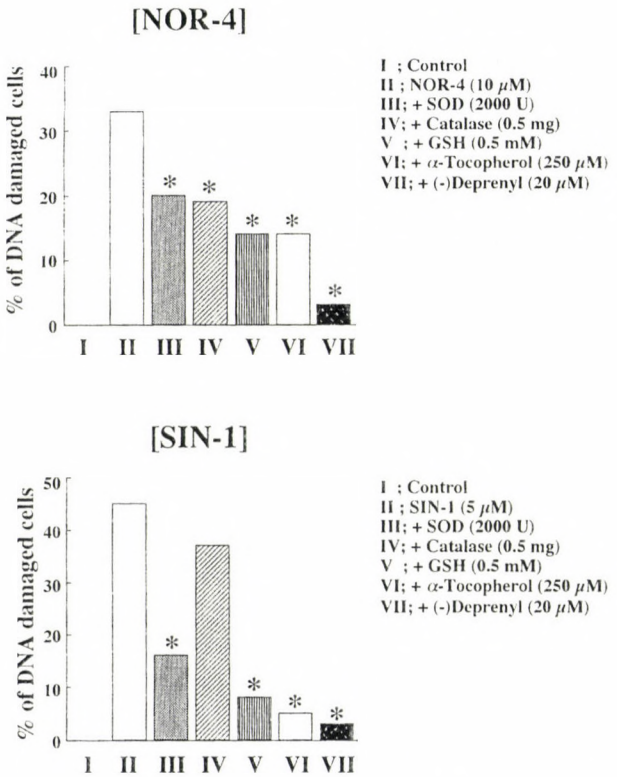

Fig. 2. Effects of (-)deprenyl and anti-oxidants on the DNA damage induced by NO and peroxynitrite. SH-SY5Y cells were treated with (-)deprenyl, SOD, catalase, GSH, or α-tocopherol and then with 10 μM NOR-4 or 5 μM SIN-1 for 3 h. DNA damage was assessed by the comet assay. The cells with the comet length greater than 30 μm were classified as positive for DNA damage. * The difference from the cells treated with NOR-4 or SIN-1 alone was statistically significant ($p < 0.05$) by ANOVA

Washing out of (-)deprenyl

The cells were incubated with (-)deprenyl and washed, and then incubated with NOR-4 or SIN-1. When compared with the cells directly treated with NOR-4 or SIN-1, pre-treatment with (-)deprenyl reduced the number of DNA damaged cells and the tail length (Table 2). This result suggest that (-)deprenyl may initiate an anti-apoptotic process in the cells.

Table 2. The effect of pre-treatment of (-)deprenyl on DNA damage induced by NOR-4 or SIN-1

	Cells with DNA damage (%)	Tail length (μm)
Control cells	1	0.41 ± 2.90
Cells incubated with		
NOR-4 (10 μM)	33	6.58 ± 10.99
+ Pre-treatment of (-)deprenyl (20 μM)	19	4.97 ± 9.71
SIN-1 (5 μM)	27	7.18 ± 12.51
+ Pre-treatment of (-)deprenyl (20 μM)	12	1.94 ± 5.63*

SH-SY5Y cells were incubated with 20 μM (-)deprenyl for 20 min and the cells were washed twice and incubated with 10 μM NOR-4 or 5 μM SIN-1 for another 3 h. The cells were applied for the comet assay. The cells with the comet length greater than 30 μm were determined as positive for DNA damage. * The difference from the cells treated with NOR-4 or SIN-1 alone was statistically significant ($p < 0.05$) by ANOVA.

Relationship of chemical structure and neuroprotection

The structure activity-relationship was studied among (-)deprenyl derivatives. The protective effects of these compounds against the apoptosis induced by peroxynitrite were studied in comparison with (-)-deprenyl. The titration studies of the protection revealed that (-)-deprenyl protected the cells at the concentration higher than 100 nM, whereas rasagiline was more potent than (-)deprenyl and effective even at 1 nM. On the other hand, (R)-aminoindan did not prevent the DNA damage. In general the (*R*)-enantiomers were more effective to protect the cells than the (*S*)-enantiomers. The chemical structure required for the protection was confirmed to be the propargyl residue with hydrophobic structure.

DISCUSSION

(-)Deprenyl was proved to protect SH-SY5Y cells from apoptosis induced by a dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol, and ROS, NO and peroxynitrite. The protection does not depend on MAO inhibition, since the cells contain only type A MAO (Maruyama et al., 1997a). The mechanism of the neuroprotection has been proposed to be associated with several intracellular mechanism. (-)Deprenyl was reported to provide neuroprotection by an anti-oxidant effect (Mytilineou, et al., 1998), enhancement of anti-oxidant enzymes, such as superoxide dismutase and catalase (Carrillo et al., 1992), inhibition of the uptake of dopamine (Zsilla et al., 1986), and preservation of membrane potential (Wadia et al., 1998).

In this paper pre-treatment of the cells with (-)deprenyl was shown to prevent the DNA damage by peroxynitrite, even after the washing out. These results suggest that anti-apoptotic system in neurons may be initiated by (-)deprenyl analogs. The neuroprotection has been shown to be associated with up-regulation of anti-apoptotic and anti-oxidant molecules (Carrillo et al., 1992: Tatton et al., 1994). (-)-Deprenyl increased the expression of Cu/Zn SOD1, Mn SOD2, glutathione peroxidase, tyrosine hydroxylase, *bcl-2*, *bcl-x* and *c-fos*, and decreased that of *bax* and *c-jun* (Tatton and Chalmers-Redman, 1996), and increase in mRNA of trophic factors; NGF (Semkova et al., 1996) and CNTF (Seniuk et al., 1994). The studies on intracellular mechanism underlying the induction of anti-apoptotic function are now under way.

The studies on the chemical structure-activity relationship confirm the neuroprotection is unique among (-)deprenyl and its metabolites. The results with rasagiline and aminoindan clearly demonstrate that a propargyl residue and an adequate hydrophobic structure are required for the neuroprotective function. However, clorgyline even with a propargyl structure could not protect the cells from apoptosis, suggesting that dichlorophenoxy group is not adequate to bind to a novel protein initiating the anti-apoptotic system.

As a conclusion, small molecules with the propargyl structure were confirmed to be transported into the brain through the blood-brain-barrier and protect neurons by inhibition of "death" signal transduction induced by endogenous and environmental factors. Such drugs may have potency to rescue the dying neurons in neurodegenerative diseases, such as PD.

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RESEARCH REPORT

MOLECULAR INTERACTION BETWEEN REVERSIBLE MAO-A INHIBITORS AND THE ENZYME

APPLICATION TO ARYLOXAZOLIDINONE, A PROTOTYPE SERIES

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Among the various chemical classes of monoamine oxidase A inhibitors, phenyloxazolidinone represent one of the major series. The purpose of this paper is to review the experimental (X-ray diffraction, NMR, electronic absorption spectroscopy, lipophilicity studies) and theoretical (quantum chemistry, molecular mechanics, molecular dynamics) studies which have led to the description of the mode of interaction between phenyloxazolidinone inhibitors and the MAO-A enzyme.
Key words: MAO-A, reversible inhibitors, phenyloxazolidinone, molecular modelling, review

Introduction

Monoamine oxidase (MAO) is an enzyme implicated in the catabolism of biogenic and xenobiotic amines. MAO exists in two isoforms, A and B, which differ in substrate and inhibitor specificity, tissue and cell distribution in addition to different immunological properties (Dosert et al., 1989; Strolin Benedetti and Dostert, 1992; Weyler et al., 1990).

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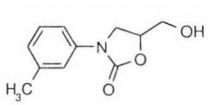
The discovery, in the 1950s of the antidepressant properties of MAO inhibitors (MAOIs) was the major finding that led to monoamine theory of depression. Earlier MAO inhibitors introduced into clinical practice for treatment of depression were abandoned due to adverse side-effects, such as hepatotoxicity, orthostatic hypotention and the so-called « cheese effect » characterized by hypertensive crises. This handicap was thought to be related to nonselective and irreversible monoamine oxidase inhibition.

Over the past years, efforts have been oriented toward the discovery of reversible and selective inhibitors of MAO-A (RIMAs) leading to a new generation of compounds and to a renewed interest in this class of drugs (Gleiter and Volz, 1996; Jegham and George, 1998).

Two compounds, toloxatone **1** and moclobemide **2** have been introduced to the market and have shown clinical efficacy in depression with good general safety (Fig. 1) (Berlin and Lecrubier, 1996).



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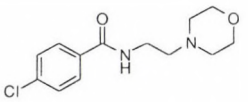

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Fig. 1. Structure of toloxatones 1 and moclobemide 2

Toloxatone **1**, the first reversible MAO-A inhibitor to be launched, belongs to the phenyloxazolidinone chemical family. Many other MAO-A inhibitors have been discovered using an oxazolidinone as key pharmacophore including cimoxatone **3** and befloxatone **4**, E-2011 **5** (Kagaya et al., 1996), and T-794 **6** (fig. 2) (Kato et al., 1997). Befloxatone **4** is the only new MAO-A inhibitor, belonging to this chemical classes which has reached an advanced stage of clinical dvelopment. It is currently in phase III clinical trials for the treatment of depression.

Beflaxotone **4**, inhibits MAO-A selectively and competitively in animals and humans (Ki = 2.5 nM). The inhibition of MAO-A by beflaxotone **4** is time-dependent and fully reversible (Curet et al., 1996).

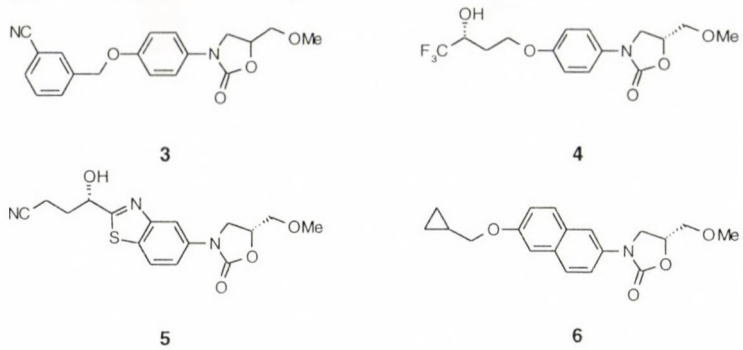


Fig. 2. Structure of cimoxatone 3, befloxatone 4, E-2011 5 and T-794 6

In order to define a model of interaction between phenyloxazolidinone derivatives and the MAO-A isoenzyme, a program has been engaged using experimental (X-ray, NMR, electronic absorption spectroscopy, lipophilicity studies) and theoretical (quantum chemistry, molecular mechanics, molecular dynamics) methods.

This paper will review the experimental and theoretical studies performed on the phenyloxazolidinones MAOIs and will describe their mode of interaction with the enzyme.

Effect of the phenyloxazolidinone moiety on MAO-A inhibition

Structural studies

Structural analysis by single crystal X-ray diffraction were carried out on several phenyloxazolidinone derivatives (Fig. 3) in order to ascertain the conformational features of this family of compounds (Moureau et al., 1992; Wouters et al., 1994a and 1994d).

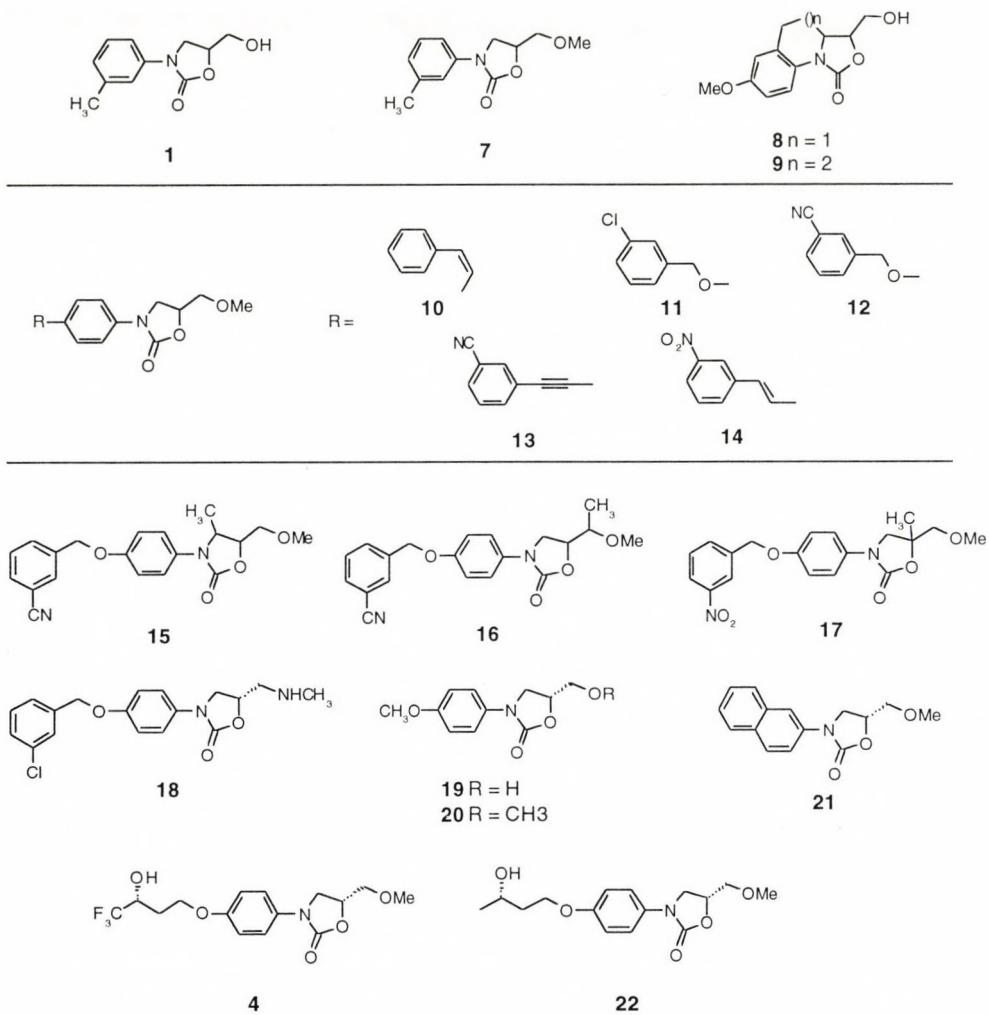


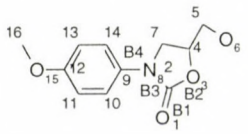
Fig. 3. Structure of the phenyloxazolidinone derivatives studied by single crystal X-ray diffraction

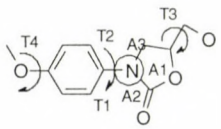
Conformation of the phenyloxazolidinone moiety

These studies have shown that the oxazolidinone ring is practically planar and the nitrogen atom of the oxazolidin-2-one ring is trigonal (the sum of the valence angles around the N atom are close to 360° for each structure) (Table 1).

Analysis of bond lengths showed that the C(2)-N(8) and C(2)-O(3) bonds are between standard single and double bond length (Allen et al., 1987), indicating electronic delocalization along N(8)-C(2)-O(3) (Table 1). The N(8)-C(9) intercyclic bond length is also intermediate between a standard C-N single and double bond.

Table 1. Bond length (Å), valence angles (°) and torsion angles (°) obtained from the CSD search with the conventional atomic numbering of phenyloxazolidinones

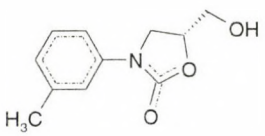




N°	Refcode	T1	T2	T3	T4	A1	A2	A3	B1	B2	B3	B4
7	BIRRAV	8.7	-172.1	63.0		111.7	126.7	121.5	1.209	1.353	1.358	1.414
10	BIRREZ	-179.6	0.2	-67.1		111.1	126.7	122.1	1.202	1.343	1.374	1.399
11	BOJKIU	-4.1	176.4	-64.2	-163.9	112.1	127.0	120.8	1.185	1.349	1.390	1.442
12	BOJKOA	-0.2	179.9	22.6	-171.3	110.9	128.1	120.9	1.213	1.345	1.360	1.414
13	BOGFAE	17.8	-167.1	-62.6		111.2	125.7	123.2	1.212	1.351	1.361	1.421
14	BUGZIM	167.7	-12.8	-65.3		111.8	125.3	122.6	1.206	1.347	1.359	1.419
14	BUGZIM	-176.2	6.4	-68.0		111.4	126.5	121.7	1.207	1.352	1.357	1.407
14	CUHYUZ	-2.4	176.8	-61.2	174.9	111.1	126.5	122.2	1.227	1.364	1.335	1.408
15	COYTUF	-136.3	46.7	-69.5	-20.3	112.4	123.8	123.5	1.208	1.356	1.354	1.426
15	COYVAN	157.7	-22.1	-72.4	175.2	112.1	125.1	121.8	1.193	1.358	1.360	1.424
16	BUHGEQ	157.6	-22.9	64.4	-8.5	111.8	125.9	122.2	1.199	1.340	1.353	1.439
17	BUGJIW	-10.1	171.3	-177.3	-179.7	111.8	125.1	122.7	1.200	1.365	1.361	1.408
19	LEBCOK	167.6	-13.9	63.2	-178.0	111.7	126.1	121.8	1.218	1.345	1.340	1.423
20	LEBDAX	-165.2	17.4	61.5	176.8	111.2	126.0	122.5	1.187	1.345	1.368	1.417
22	WIBYEL	-168.7	12.2	60.1	-3.8	112.1	125.0	122.7	1.202	1.362	1.365	1.410
	Mean								1.205	1.352	1.360	1.418
	Minimum								1.185	1.340	1.335	1.399
	Maximum								1.227	1.365	1.390	1.442

In order to improve the description of the electronic structure, *ab initio* molecular orbital calculations were undertaken on (*R*)-toloxatone **1b**. The atomic and interatomic populations calculated according to Mulliken confirmed the hypothesis of an electronic delocalization within the oxazolidinone moiety but invalidated a presumed conjugation between the

phenyl and oxazolidinone rings. From these studies, a delocalization scheme has been proposed for (*R*)-toloxatone **1b** (Fig. 4) (Moureau et al., 1992).



1b

Fig. 4. Delocalization scheme proposed for toloxatone

Despite the poor conjugation between both rings, coplanarity between the phenyl and oxazolidinone rings has been observed in the crystalline state (Table 1). Nevertheless, one exception has been observed for compound **15** (Table 1, Fig. 3) (Durant et al., 1985). This deviation from coplanarity can be explained by the steric hindrance of the methyl group on the C(7) atom. X-ray structures of bridged oxazolidinones were then analysed (Moureau et al., 1992). Only the tetrahydroxazolquinoleine **8** showed a good affinity (Fig. 3). The crystal structure of compound **8** revealed that the two-carbon bridge between the phenyl and oxazolidinone rings forces the molecule to adopt a nearly coplanar structure, the C(2)-N(8)-C(9)-C(10) torsion angle value is –12.3°. On the other hand, the three-carbon bridge structure of **9** induces a 58.3 degrees angle between both rings. Moreover, the conformational analysis on (*R*)-toloxatone **1** and 5-methoxymethyl-3-(4-methoxymethyl-phenyl)oxazolidin-2-one **20**, carried out by *ab initio* methods, revealed that the coplanarity between the oxazolidinone and the phenyl ring corresponds to an energetically stable conformation (C(2)-N(8)-C(9)-C(10) = 0 or 180°) (Moureau et al., 1992; Wouters et al., 1993). From these studies, it was proposed that phenyloxazolidinone derivatives require a coplanar conformation between the phenyl and oxazolidinone rings for MAO-A inhibition.

Crystal packing

The analysis of the forces responsible for crystal packing of phenyloxazolidinone derivatives showed that π–π interactions between the phenyl and oxazolidinone rings stabilize the structure in the solid state. Intermolecular Hydrogen bonds involving the carbonyl function and the

alkoxy chain contribute to the stability of the oxazolidinone compounds in the solid state.

Electronic properties studies

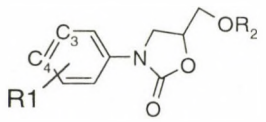
The essentially planar structure of the phenyloxazolidinone MAO-A inhibitors and their aptitude to participate in π–π interactions, as deduced from the structural studies, led us to propose the hypothesis that such compounds could exert their inhibitory activity by reversible interactions with the FAD cofactor of the MAO enzyme. Indeed, the FAD cofactor of MAO is an isoalloxazine derivative well known for its acceptor properties. In order to verify the validity of such an assumption, experimental and theoretical studies were undertaken (Moureau et al., 1994 and 1995).

Electronic absorption spectra

Electronic absorption spectroscopy was used to analyze the formation of complexes, by charge transfer, between riboflavin (a model of FAD) and several phenyloxazolidinone derivatives. The difference spectra for the riboflavin/phenyloxazolidinone solutions was characteristic of charge-transfer complex interactions. Indeed, the difference spectra revealed the existence of two absorption bands. The first band found around 445 nm is characteristic of riboflavin and the second, called the "extra-band", is characteristic of the intermolecular transition between the riboflavin and MAO-A inhibitors (Table 2).

The effect of the phenyl substitution has also been assessed on several extended heteroaromatic oxazolidinones derivatives, such as naphtyl derivative **21** (Fig. 1), using electronic absorption spectroscopy. Moreover, it has been shown that oxazolidinones derivatives having a number of alternative aromatic nuclei induce a shift of the absorption band relative to toloxatone **1** and befloxatone analogues (Table 2).

Table 2. Extra band (λ_{max}) and complexation constants (Kc) characteristic of the charge transfer complex observed in solution between riboflavin and phenyloxazolidinone derivatives



Compound	R1	R2	l (nm)	Kc(°C,M^{-1})
toloxatone **1**	3-Me	H	487	107
Befloxatone **4**	(*R*)-4-O-$(CH_2)_2$-CH(OH)-CF_3	Me	490	-
23	4-O-$(CH_2)_3$-NH_2	Me	492	40
24	(*R*)-4-O-$(CH_2)_2$-CH(NH_2)-CH_3	Me	492	28
25	(*S*)-4-O-$(CH_2)_2$-CH(NH_2)-CH_3	Me	492	32
21	(*R*)-(3,4)-Phenyl	Me	493	-

Molecular orbital calculations

Beside the charge transfer component, other bonding parameters such as electrostatic interactions and van der Waals forces contribute to the stability of the different complexes. The relative contributions of these different types of interactions between the phenyloxazolidinones and riboflavin, as well as the relative orientation of the two partners within the complex, is governed by their electronic properties. Therefore, in order to explain the experimental results obtained by electronic absorption spectroscopy and to identify the different types of interactions which contribute to the stabilization of the riboflavin/phenyloxazolidinone complexes, several electronic parameters of the studied MAO-A inhibitors were determined by *ab initio* methods (Moureau et al., 1994 and 1995). Considering the relatively large dimension of FAD (57 heavy atoms), the oxidized form of lumiflavin (Wouters et al., 1994b) was chosen as a model for FAD. A thiomethyl (SCH_3) group was added to the 8α-CH_2 in order to simulate the start of the enzyme peptide chain.

The MEP calculated around lumiflavin showed two large attractive zones induced by the carbonyl function of the heterocycle and a large repulsive zone which spreads over the isoalloxazine nucleus (Fig. 5). The MEP calculated around the phenyloxazolidinones moieties systematically showed large attractive zones centered on the carbonyl (Fig. 5). The analysis of the HOMO topology of phenyloxazolidinones and the LUMO of lumiflavin revealed that both are π-type orbitals (Fig. 6). The atomic orbital

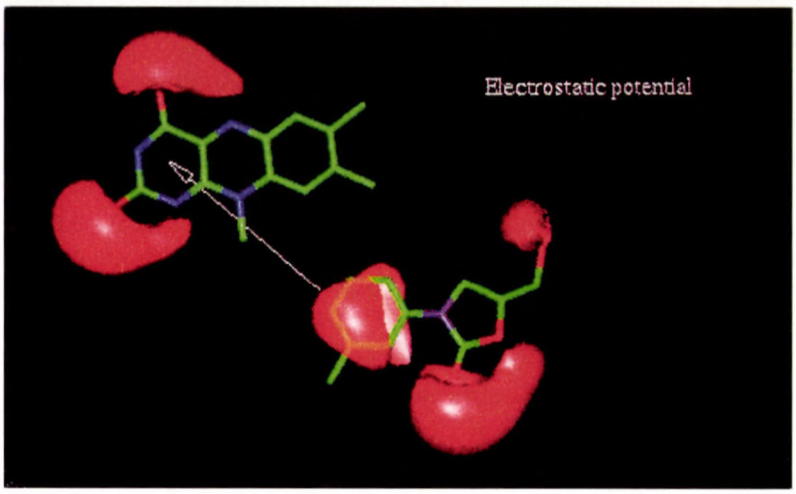


Fig. 5. Molecular Electrostatic Potential calculated around the isoalloxazine nucleus and the toloxatone 1. Attractive region (-15 kcal/mol) are displayed in red

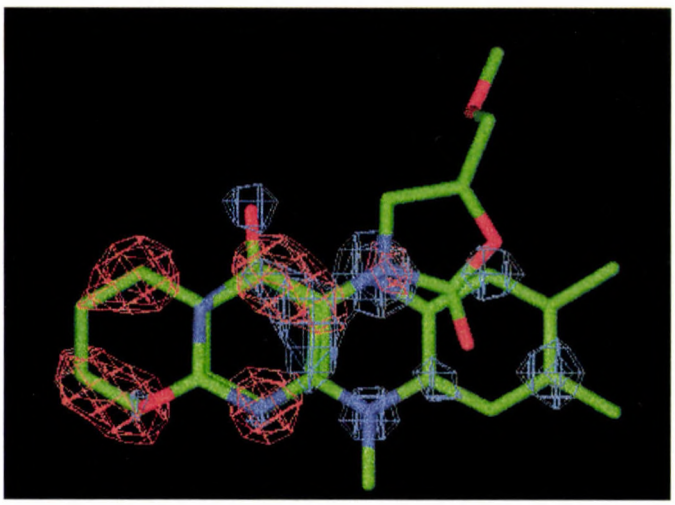


Fig. 6. Frontier orbitals calculated calculated around the isoalloxazine nucleus and the toloxatone with the deduced orientation for the two partners. The LUMO of the isoalloxazine ring is represented in blue (0.005 e^-/ Å3) and the HOMO of SL71.0068 21 in red (0.005 e^-/ Å3)

perpendicular to the molecular plane is the only one to contribute to the electron density. The electron density of the LUMO of lumiflavin is delocalized all over the isoalloxazine nucleus. Analysis of the HOMO topology of the phenyloxazolidinones has revealed the particularly high electron density of the HOMO on the phenyl ring and on the O(1) and N(8) atoms and has underlined the donor properties of this entity.

Analysis of the molecular electrostatic potential (MEP) calculated around the substituted lumiflavin and several phenyloxazolidinone MAO-A inhibitors showed a good complementarity between the attractive potential zones of the flavin moiety and the repulsive zones of several phenyloxazolidinone derivatives and *vice versa* (Fig. 5). This interaction hypothesis has been further confirmed by the analysis of the frontier orbital (fig. 6). Indeed the orientation deduced from the MEP studies allows a maximum matching between the HOMO of the donor phenyloxazolidinone, and the LUMO of the acceptor flavin moiety (Fig. 6).

Moreover, a good correlation has been found between the HOMO energy and the MAO-A affinity for the "extended" phenyloxazolidinones studied ($r^2 = 0.95$; $n = 3$). Regression analyses have been carried out between compounds having approximately the same steric hindrance. These results show that when the energy of the HOMO is less negative i.e. the better the ability of the compound to donate its electrons, the greater will be its MAO-A affinity.

Preliminary model

The combination of experimental (electronic absorption spectroscopy) and theoretical (*ab initio* calculations) methods demonstrated the existence of labile, reversible and weak interaction between the phenyloxazolidinone MAO-A inhibitors and the FAD cofactor of the enzyme. In addition, it defined the origin of the molecular association and allowed a proposition of a model for the interaction which gives an indication on the relative orientation of the two partners (Figs 6 and 7).

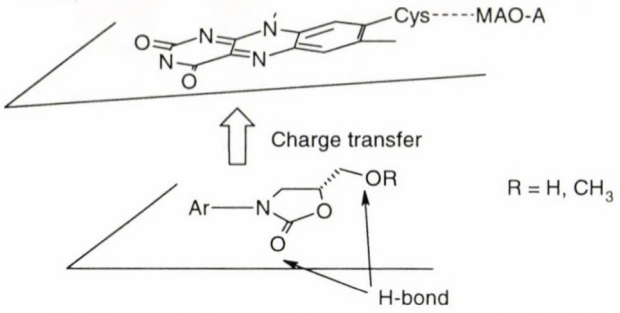


Fig. 7. Preliminary model of interaction between phenyloxazolidinones and the MAO-A enzyme

Influence of the substitution of the oxazolidinone moiety

Oxazolidinone substitution

Modulation of the hydroxymethyl, on position 5 of the oxazolidinone ring, has been undertaken in order to increase the MAO-A inhibitory potency (Fig. 8). These studies have shown that substitution of the hydroxy by a methoxy group increases activity, and that the amine function also gives active products. However, introduction of a sulfur atom leads to loss of activity (Dostert et al., 1982). The stereoselectivity of the methoxymethyl chain has also been assessed. The 5-(*R*) enantiomer always shows a greater affinity and selectivity for MAO-A than the 5-(*S*) enantiomer. The results obtained with the amino derivatives have also shown that reversible and irreversible MAO inhibitors could be obtained depending on the degree of substitution. The main trends of the structure activity relationships are given in Fig. 8.

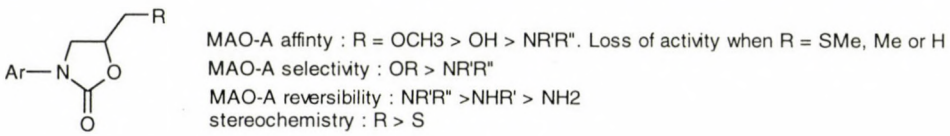


Fig. 8. SAR of phenyloxazolidinone compounds. 5-(aminomethyl)-3-aryl-2-oxazolidinones were used by Silverman and coworkers in mechanistic studies of MAO (Ding et al., 1993; Ding and Silverman, 1993a; 1993b; Silverman, 1997)

Structural studies

The conformation of the methoxymethyl chain has been studied by single crystal X-ray diffraction and by theoretical studies. The stereoselectivity and the conformational properties, taken together, have allowed us to determine critical values for the O(3)-C(4)-C(5)-O(6) torsion angle. The critical values for the O(3)-C(4)-C(5)-O(6) torsion angle are between -170 and -80 degrees. The conformations corresponding to these angles are stable in the case of the active (4*R*, 5*S*) isomer of compound **16** (Durant et al., 1982) but forbidden in the case of the inactive (4*R*,5*R*) isomer (Table 1) (Durant et al., 1984).

Phenyl substitution

Modulation of the phenyl substitution has also been undertaken. These studies have shown that substitution of position 2 leads to a loss of activity. Modifications of the methyl group in position 3 did not clearly improve the MAO-A affinity whereas the alternative alkoxy substituents introduced in position 4 led to more potent MAO-A inhibitors than toloxatone **1** (Dostert et al., 1982).

The improved MAO-A affinity obtained with the 4-alkoxyphenyl-oxazolidinones derivatives compared to the 4-methoxy group led to the syntheses of other alkoxy compounds and to the discovery of befloxatone and analogues which are some of the most potent selective and reversible MAO-A inhibitors known. Some informations about structure-affinity results among this series are summarized in Figure 9.

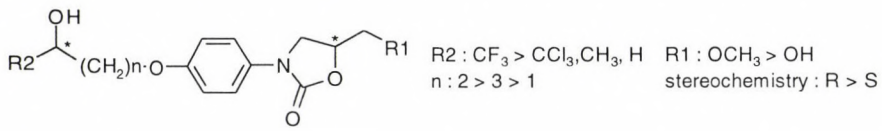


Fig. 9. Qualitative SAR of phenyloxazolidinone

Electronic properties studies

Electronic absorption spectra

Like toloxatone **1**, and other reversible MAO-A inhibitors, befloxatone **4** contains a large planar electron-rich fragment that may be engaged in a privileged interaction with the enzyme flavin cofactor. In order to understand

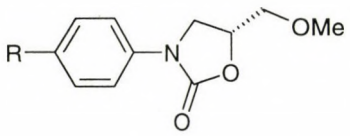
the role played by the befloxatone alkoxy side chain and other analogues on the charge transfer interaction previously postulated, electronic absorption spectroscopy studies have been undertaken (Wouters et al., 1998 and 1994c). The complexation in solution of riboflavin by befloxatone **4** and different analogues has also been clearly characterized as a 1:1 charge transfer complex identified by a new absorption band (Table 2). Moreover this study has shown that introduction of an alkoxy group on the phenyl ring induces a shift of the absorption band characteristic of the intermolecular electron transfer in the complex. Complexation constants (Kc) deduced from a Foster-Hammick-Wardley plot were also calculated (Table 2). The fact that Kc constants were in the same order of magnitude for several substituants of phenyl oxazolidinones and that they presented the same λ_{max} values, strongly suggested that only the phenyloxazolidinone part of the molecules is involved in complexation with the flavin ring. Moreover, in solution, introduction of the butoxy side chain diminishes complexation. These observations implicitly demonstrate the need of additional interaction, in the active site of MAO-A, in order to account for a better affinity of those compounds towards the enzyme.

Lipophilicity of the lateral chain

The lipophilicity of substituted phenyloxazolidinones was studied (Ooms et al., 1998), using a set of MAO inhibitors belonging to the phenyloxazolidinone chemical families. Those compounds differ in the nature of their side chain and for which log k_w (capacity factor) values could be obtained by reverse-phase high performance liquid chromatography (RP-HPLC) (Table 3). The experimental results obtained have been compared to the log P calculated by CLIP (Carrupt et al., 1997), a computational method based on the concept of the Molecular Lipophilicity Potential (MLP). This was done in order to check the reliability of the method for this type of molecule. Indeed, computational approaches are particularly interesting for this class of compound since log P values are experimentally difficult to obtain due to their poor water solubility.

It has been shown that the CLIP approach used in this study is a valuable tool for estimating log P_{calc} values for MAO inhibitors belonging to the phenyloxazolidinone chemical families. No correlation was found between lipophilicity and MAO-A inhibitory potency.

Table 3. Chemical structures of the phenyloxazolidinone derivatives, capacity factor (log k_w), calculated log P_{calc} and MAO-A inhibitory potency (K_{iA})



Compound	R	log k_w	log P_{calc}	K_{iA} (μM)
26	HO-$(CH_2)_2$-	1.00	1.13	0.080
27	HO-$(CH_2)_2$-O-	1.00	0.77	0.390
28	(R, S)-CH_3-CH(OH)-CH_2-O-	1.17	1.17	0.220
29	HO-$(CH_2)_3$-	1.30	1.27	0.0016
21	(S)-H_3C-CH(OH)-$(CH_2)_2$-O-	1.60	1.71	0.0065
4	(R)-F_3C-CH(OH)-$(CH_2)_2$-O-	2.45	2.48	0.0035
30	(S)-H_3C-CH(OCH_3)-$(CH_2)_2$-O-	2.21	2.70	0.170
31	HO-$(CH_2)_4$-O-	1.58	1.84	0.029
32	(R, S)-H_3C-CH(OH)-$(CH_2)_3$-O-	1.88	2.27	0.013
33	H_3C-C(O)-$(CH_2)_2$-O-	1.43	1.37	0.046
34	H_3C-C(CH_2)-$(CH_2)_2$-O	3.08	3.03	0.028

Molecular orbital calculations

Frontier orbital calculations performed on befloxatone **4** and its analogues also showed a good complementarity between the MEP of these MAO-A inhibitors and the isoalloxazine nucleus. The orientation deduced from the MEP allows a good overlap between the HOMO of the phenyloxazolidinones and the LUMO of the isoalloxazine ring.

Moreover, the MEP reveals that, in addition to the attractive regions already observed for toloxatone **1** and other phenyloxazolidinones derivatives, a new attractive potential well was induced by the befloxatone hydroxyl group an its analogues. From this study, an additional interaction through an H-bond between the hydroxy group and a residue of the active site was proposed (Wouters et al., 1998).

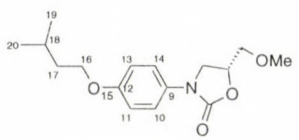
Structural studies

X-ray crystallography, NMR (2D-NOESY) and molecular modeling methods were used to characterize the conformational features of the befloxatone **4** side chain and some of its analogues (Wouters et al., 1998). X-ray structure analysis performed on several phenyloxazolidinone has allowed the characterization the spatial position of the hydroxy group.

Conformation of the side chain

In all the alkoxyphenyloxazolidinone derivatives studied by single crystal X-ray diffraction the O(15) atom is sp2 hybridized as indicated by the analysis of the bond length, the valence and torsion angles (Table 2). The lateral trifluorobutoxy chain of befloxatone **4** adopts, in the solid state, a **ttg**$^+$ conformation (Wouters et al., 1994c). The same conformation of the side chain has been observed for the racemic mixture of befloxatone **4** (*RR/SS*). An all *trans* conformation of the side chain has been observed for compound **22** (Table 4).

Table 4. Selected torsion angles of befloxatone 4 and one analogue 22



Torsion angles	4a	4b	4 (racemic RR/SS)	21
C(12)-O(15)-C(16)-C(17)	-169.2	170.7	-179.5	-172.2
O(15)-C(16)-C(17)-C(18)	68.8	67.2	64.1	-169.2
C(16)-C(17)-C(18)-O(19)	67.3	68.0	60.2	-56.7
C(16)-C(17)-C(18)-C(20)	-172.2	-174.9	-177.8	-178.4

As the X-ray structure might not correspond to the stable conformation of a molecule, conformational analysis was performed on befloxatone **4** and its analogues. Semi-empirical (AM1) and molecular dynamics (cff91) methods were used to sample the conformational space of the side chain of several substituted phenyloxazolidinones. From the affinity and the conformational properties of the studied compounds, allowed and disallowed regions could be determined.

The allowed regions correspond to extended conformations of the side chain in agreement with the X-ray diffraction results.

Crystal packing

Analysis of the crystal packing of befloxatone **4** also showed that π–π interactions between the phenyl rings stabilize the structure in the solid state

(Wouters et al., 1994c). In addition to this interaction, intermolecular hydrogen bonds between the hydroxyl group of the side chain of one molecule and the carbonyl of the oxazolidinone ring of another increased the packing stability.

Model of interaction

As a result of these studies a model has been proposed for the reversible inhibition of MAO-A by phenyloxazolidinones. The pharmacophoric elements are summarized bellow and illustrated in Fig. 10.

a) a charge transfer interaction between a planar electron rich moiety, the phenyloxazolidinone, and the isoalloxazine ring of the FAD cofactor
b) H-bond interactions involving
   -the carbonyl of the oxazolidinone ring
   -the methoxymethyl group of the oxazolidinone ring
   -the hydroxy group present on the side chain of befloxatone

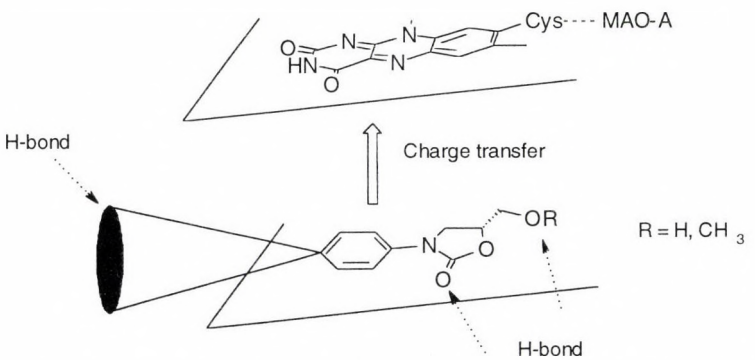


Fig. 10. Model of interaction of the phenyloxazolidinone proposed for the reversible inhibition of MAO-A

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RESEARCH REPORT

POSSIBLE DIFFERENT FATES FOR THE HYDROGEN PEROXIDE PRODUCED BY RAT WHITE ADIPOCYTE AMINE OXIDASES

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Monoamine oxidase (MAO) and benzylamine oxidases (Bz-SSAO) of rat white adipocytes, deaminating benzylamine and tyramine produce hydrogen peroxide at different cellular levels. The peroxide produced by their activity has a very short half-life in adipocyte suspension. The addition of a catalase inhibitor allows for the recovery of the intact peroxide within the first 10-min of its production. Thus, benzylamine and tyramine-dependent peroxide recovery is different, suggesting that the fate of the peroxide produced by the two amine oxidases might be different depending on the cellular site of its production.

Key words: hydrogen peroxide, amine oxidases, tyramine and benzylamine metabolism

INTRODUCTION

Hydrogen peroxide (H_2O_2) mimics several of the metabolic and growth-promoting effects of insulin and related growth factors [Little and de Haen, 1980; Ramasarma, 1982]. Most of these effects are particularly evident and have been studied in adipocytes [Mukherjee et al., 1978]. In these cells H_2O_2 was found to work synergistically with insulin [Hayes and Lockwood, 1987] and it can be produced locally in several ways. In rat white adipocytes, one source is represented by the membrane bound NADPH-oxidase activity [Krieger- Brauer and Kather, 1991] but also by other cellular enzymes like the amine oxidases, as result of amine degradation. Rat white adipocyte

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amine oxidase population is constituted of the mitochondrial amine oxidases (MAO, E.C. 1. 4.3.4), essentially MAO type A, and the semicarbazide-sensitive benzylamine oxidising activities (Bz-SSAO, E. C. 1.4.3.6.). These two classes of proteins are characterised by different subcellular localisation, cofactor and affinity toward substrates, can be distinguished by using selective inhibitors [Buffoni, 1993]. In addition to this, MAO activity in rat white adipocytes control endogenous amine concentrations and functions [Fisher et al., 1995], whereas Bz-SSAO seems to influence cell metabolism [Tarancon et al., 1998; Marti et al., 1998]. This latter effect was related to the peroxide produced extracellulary by the Bz-SSAO-dependent tyramine degradation. However, MAO represents a source of intracellular production of low amounts of peroxide or free radicals, which could also influence cell metabolism.

To study the metabolic effect of the peroxide produced by MAO in rat white adipocytes, we first investigated the amount and the fate of the peroxide produced by benzylamine, a selective substrate for Bz-SSAO, and by tyramine, a common substrate for Bz-SSAO and MAO.

MATERIALS

Collagenase type ll, pargyline and semicarbazide hydrochloride, sodium azide, benzylamine and tyramine hydrochloride was purchased from the Sigma Chemical Company (St. Louis, Mo, U. S. A.). Homovanillic acid was from Merck (Darmstadt, Germany). Horseradish peroxidase was from Boehringer (Mannehein, Germany). Male Wistar rats were from the Morini breeding colony (San Polo D'Elsa, Reggio Emilia, Italy). [^{14}C]-tyramine hydrochloride (53 mCi/mmoles) was obtained from ICN.

METHODS

Adipocytes preparation

Male Wistar rats (250-300 g) were killed by cervical dislocation and the epididymal portion of white adipose tissue was used to isolate the adipocytes. Mature adipocytes were prepared according to Rodbell [1964].

Amine oxidase activity assays

Fluorimetric measure of H_2O_2 accumulated in cell medium as a result of tyramine and benzylamine oxidation

The fluorimetric measure of hydrogen peroxide was performed according to Matsumoto et al [1982]. Fivehundred microliters (50 µl) of adipocyte suspension (4.82 + 1.29 × 105 cells/ml) in phosphate buffer containing homovanillic acid and horseradish peroxidase were preincubated at 37 °C in a humidified atmosphere. Benzylamine or tyramine were added and the H_2O_2 produced at 37 °C was measured at different times after the addition of the substrates. Two ml of 0.1 N NaOH was then added to stop the reaction. In some cases the reaction mixture contained 1 mM sodium azide (NaN_3) in order to inhibit catalase. The fluorescence of the samples was measured in a spectrofluorimeter. Results were expressed as nmoles of H_2O_2 as concentration of the peroxide (µM). Calculations were made in respect to standard curves obtained using 2, 10 and 20 µM of exogenous H_2O_2 added to adipocyte suspensions and treated as the samples.

Fluorimetric determination of residual H_2O_2 in adipocyte suspension

Using the coupled reaction between homovanillic acid and peroxidase, the peroxide is trapped, as it is being produced (total hydrogen peroxide).

Weather or not homovanillic acid and peroxidase are added after amine oxidase substrates incubation the peroxide measured represents the residual H_2O_2, coming from amine oxidation, at that time.

To measure this amount of residual peroxide, adipocyte suspensions were prepared as required for the fluorimetric method, except that homovanillic acid and peroxidase were omitted from the medium of reaction. Both reagents were added just after incubation of the substrates at different times at 37 °C in a humidified atmosphere of 5% °C 2 and 95% air. In some cases, the reaction mixtures contained 1 mM NaN_3 in order to inhibit catalase.

RESULTS

Hydrogen peroxide vanishing time in adipocyte suspension

Hydrogen peroxide (10 µM) exogenously added to cell suspensions disappeared quickly from the cell medium.

The presence of this peroxide concentration was not detectable within the first 5 min after its addition. This demonstrates that peroxide undergoes to a metabolic degradation or bioconversion. The permanence of H_2O_2 in the extracellular spaces could be increased by blocking catalase using 1 mM NaN_3, indicating that, in adipocytes, H_2O_2 is primarily degraded enzymatically by catalase. This enzyme can derive from cell rupture or be released by cells. Under these conditions, the calculation of the half-life (t 1 /2) for H_2O_2 disappearance from the medium was allowed (21.8 + 2.0 min) (Fig. 1).

Benzylamine and tyramine-dependent accumulation of H_2O_2: fluorimetric measures

The H_2O_2 produced by amine oxidase catalysis could be easily measured by the coupled reaction between peroxidase and homovanillic acid using benzylamine (10 μM) or tyramine (50 μM) as substrates.

Both substrates gave a time-dependent production of hydrogen peroxide. In particular, benzylamine (10 IlM) was maximally oxidised in 5-10 min., whereas tyramine oxidation proceeded up to 30 min (data not shown).

Residual H_2O_2 in the medium

When homovanillic acid and peroxidase were absent, the H_2O_2 found in the samples at each time tested (5, 10, 20, and 30 min) was at basal levels. Indeed for exogenous peroxide, the H_2O_2 produced by 10 μM of benzylamine disappeared from the medium in the first 5-min of incubation with cells (data not shown).

As for the exogenously added hydrogen peroxide that was also produced by benzylamine (10 μM) oxidation remained longer in the medium when catalase was inhibited (Fig. 2). Under these experimental conditions, the residual H_2O_2 recovered within the first 5 min remained close to the values measured when homovanillic acid and peroxidase were added at the beginning of benzylamine oxidation. This time is comprised of the maximum rate of benzylamine oxidation (5-10 min). After the first 5 min of reaction the peroxide recovered was almost half of that totally produced. At that time, H_2O_2 disappeared from the medium even when in the presence of NaN_3. When tyramine was used as a substrate, residual H_2O_2 in the medium was measured at different concentrations, tyramine being a substrate for both Bz-SSAO and MAO. When using only 50 μM of tyramine oxidation, the amount of residual H_2O_2 found in the medium, 10 min after amine addition was

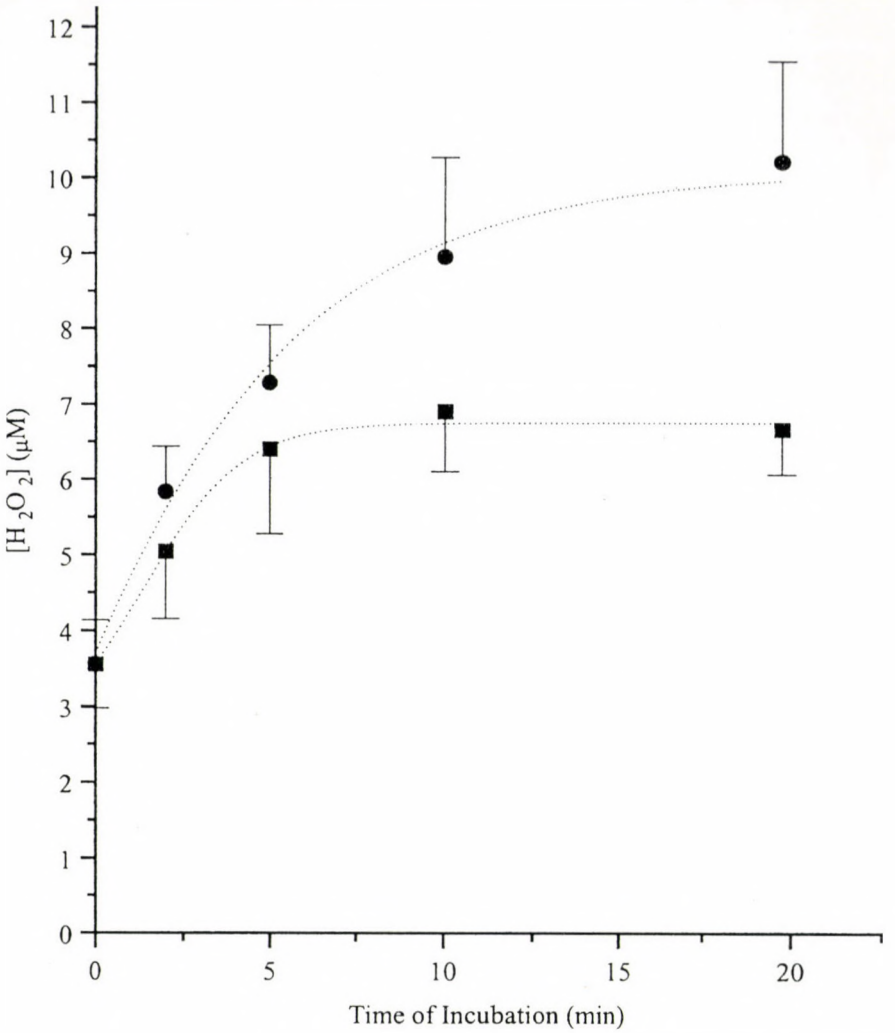

Fig. 1. Total and residual H_2O_2 in cell medium from benzylamine oxidation. Adipocyte suspensions were prepared for fluorimetric determination of H_2O_2 in solutions containing 1 mM NaN_3 . For total H_2O_2 determination (●), cells were incubated with benzylamine (10 μM) for different times at 37 °C in a humidified atmosphere. When residual peroxide was evaluated (■), cells were incubated under the same conditions as above in a medium containing NaN_3, but devoid of homovanillic acid and peroxidase. Both reagents were added at different times after the addition of tyramine and the reactions stopped soon after. Results are expressed H_2O_2 concentration (μM) and represent the mean ± s.e. of 4 different experiments run in duplicate

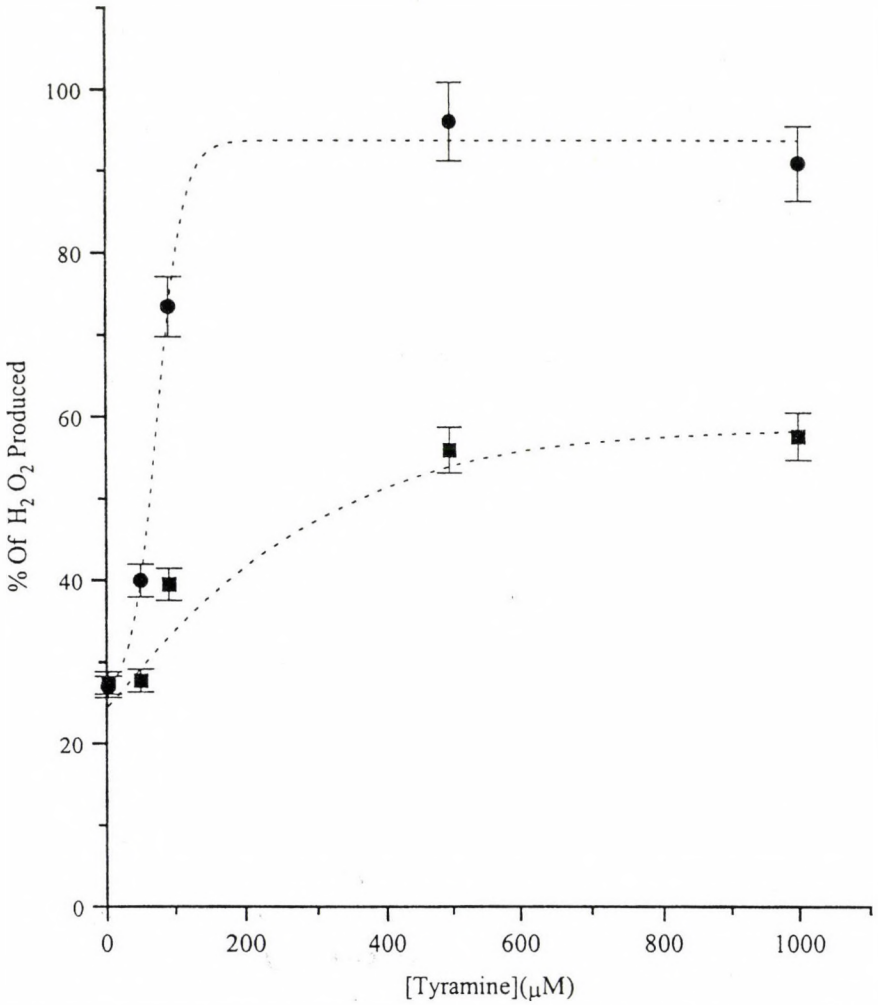

Fig. 2. Total and residual H_2O_2 in cell medium from tyramine oxidation. Adipocyte suspensions were prepared for fluorimetric determination of H_2O_2 in solutions containing 1 mM NaN_3. For total H_2O_2 determination (●), cells were incubated with different tyramine concentrations for 10 min at 37 °C in humidified atmosphere before stopping the reactions. When residual peroxide was evaluated (■), cells were incubated under the same conditions in a medium containing NaN_3, but devoid of homovanillic acid and peroxidase. Both reagents were added 10 min after the addition of tyramine and the reactions stopped soon after. Results are expressed as % of the maximum H_2O_2 production (nmoles/mg of proteins/10 min), measured when 1 mM tyramine was used. They represent the mean ± s.e. of 4 different experiments run in duplicate.

close to the total amount produced by this amine concentration. By increasing tyramine concentration, the value of residual H_2O_2 at that time was only 50% of the total, even in the presence of NaN_3 (Fig. 3).

DISCUSSION

H_2O_2 is a reactive oxygen species, which undergoes rapid bioconversion because of its short half-life in biological fluids. Many effects of the peroxide have been described in tissues or isolated cells [Jacobson et al., 1996; Watanabe et al., 1996].

Hydrogen peroxide is endogenously produced by different sources in the different cell types. Among these, rat white adipocytes possess two main systems of endogenous peroxide generation: NADPH-oxidase and amine oxidase systems (MAO and Bz-SSAO). It is already known that both NADPH and amine oxidase activities are quite highly expressed in these types of cells [Mukherjee et al., 1977; Raimondi et al., 1991]; where they both, to a different extent, produce H_2O_2.

MAO and Bz-SSAO oxidatively deaminate amines among which are benzylamine and tyramine. Oxidation of the substrates occurs in a time-dependent fashion with the subsequent production of low amounts of peroxide which, however, rapidly disappear from the medium of reaction. One of the first hypotheses verified was that this disappearance of peroxide could generate lipid peroxidation in cell membranes. However, using the method of thiobarbituric acid [Ciuffi et al., 1992], no lipid peroxidation was measured. We verified, on the contrary, that the first attack on peroxide is an enzymatic one and it is carried out by catalase; we were, in fact, able to increase the time of intact peroxide storing in the cell medium by using NaN_3 as a catalase inhibitor. Under this conditions, both exogenously or endogenously produced peroxide can be recovered within 10 min from its addition or formation.

Some differences in the recovery of peroxide were observed when exclusive substrates for Bz-SSAO or for both Bz-SSAO and MAO were used. Benzylamine and tyramine gave different time-dependent kinetic of oxidation; in particular, benzylamine oxidation occurred quickly within the first 5 min after its addition to the cells. At that time, the peroxide was totally recovered when catalase was inhibited. Tyramine, instead, degraded more slowly than benzylamine and its oxidation continued for up to 30 min.

Consequently, benzylamine produced relatively high amounts of peroxide in a few minutes, whereas tyramine did the same with a different kinetic. In this way, it is possible that benzylamine-dependent peroxide is attacked by catalase as soon as it is produced. In fact, 10 min after the addition of benzylamine, the peroxide is fully recovered when catalase is inhibited.

Peroxide, instead, was recovered in the first 10 min using tyramine, even if NaN_3 was present, however only 50% of the total produced. Since tyramine is a substrate for MAO and Bz-SSAO, this indicates that the amount of peroxide attacked by catalase represents only a portion of that totally produced. Benzylamine, however, escapes this type of degradation. This last finding could suggest that the fate of the peroxide produced by the two amine oxidases might be different, probably because of its different. These findings suggested that the fate of H_2O_2 produced by benzylamine or tyramine could be different because of its different cell compartmentalisation.

Then it can be hypothesised that tyramine oxidation, being carried on by MAO and Bz-SSAO, could produce different cell effects than those related to benzylamine.

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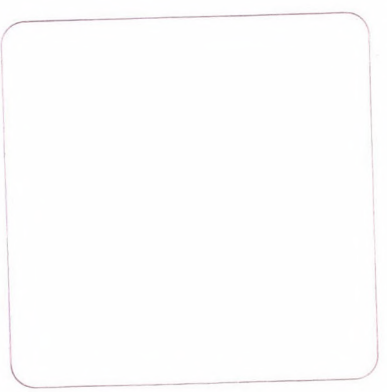

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RESEARCH REPORT

UNUSUAL PATTERN OF ß-PHENYLETHYLAMINE DEAMINATION IN THE RAT HEART

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The present study was aimed to determine type A and B MAO activities in rat heart and renal cortex homogenates and evaluate the sensitivity of deamination of ^{3}H-5-HT and ^{14}C-ß-PEA to selective MAO-A and MAO-B inhibitors, respectively Ro 41-1049 and lazabemide. Deamination of ß-PEA in the rat heart was not affected (V_{max} = 53±10 vs 42±6 nmol mg protein^{-1} h^{-1}) by lazabemide (250 nM), but was significantly reduced (V_{max} = 10±1 nmol mg protein^{-1} h^{-1}) by Ro 41-1049 (250 nM). Deamination of ß-PEA in the rat heart is a low affinity process (when compared with that in the kidney) with high K_m values (244±98 vs 18.6±5.8 µM). On the other hand, deamination of 5-HT in the rat heart and renal cortex revealed high K_m values, which were similar to those for ß-PEA in the heart. Deamination of ß-PEA (1000 µM) in the rat heart was inhibited in a concentration-dependent manner by Ro 41-1049 with a K_i value of 32 nM (22, 48; 95% confidence limits), but not by the selective MAO-B inhibitor lazabemide (up to 500 nM). Inhibition of 5-HT (1000 µM) deamination in the rat heart by Ro 41-1049 was also a concentration-dependent process with a K_i value of 21 (16, 26) nM. Deamination of 5-HT (1000 µM) in the rat renal cortex, was inhibited in a concentration-dependent manner by Ro 41-1049 with a K_i value of 12 (8, 17) nM. Deamination of ß-PEA in the renal cortex was inhibited by lazabemide with a K_i of 5 (3, 7) nM. In the rat heart, in contrast to that in the renal cortex, the specific MAO-B substrate ß-PEA is deaminated by a form of MAO which most probably corresponds to MAO-A.
Key words: MAO-A, MAO-B, rat, heart, renal cortex

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INTRODUCTION

Monoamine oxidase (MAO, amine-oxygen oxidoreductase, E.C. 1.4.3.4) is a flavin-containing enzyme bound to the mitochondrial outer-membrane which catalyses the oxidative deamination of a variety of monoamines to their corresponding aldehydes (1, 2). MAO exists in two forms, A and B, encoded by separate genes (3, 4) with the same exon-intron organization (5). They have also been separated on a biochemical basis according to their substrate specificities and sensitivity to inhibitors (6, 7). Classically, MAO-A preferentially oxidizes biogenic amines such as 5-hydroxytryptamine (5-HT), adrenaline and noradrenaline, and is inactivated by the acetylenic inhibitor clorgyline. On the other hand, MAO-B preferentially oxidizes β-phenylethylamine (ß-PEA) and benzylamine and is inactivated by the irreversible inhibitor selegiline. Dopamine, tyramine and tryptamine are considered common substrates for both forms of MAO. However substrate specificities depend on the species being considered and also on the tissue studied (2, 8).

In the rat heart there is almost exclusively MAO-A with little MAO-B present (9), while in the rat renal cortex both forms of MAO are present (10-12). As already stated, ß-PEA is generally considered as MAO-B preferential substrate (2), but in the heart it has been described also as a substrate of MAO-A (13). Whether this substrate preference just reflects differences in tissue distribution of MAOs (14) or reflects structural differences between the two isozymes is not clear. As recently shown for both MAO-A and MAO-B, a single amino acid has a vital role in determining the substrate selectivity (15).

In the present study we determined type A and B MAO activities in rat heart and renal cortex homogenates and tested the sensitivity of deamination of ^{3}H-5-HT and ^{14}C-ß-PEA to selective MAO-A and MAO-B inhibition by, respectively, Ro 41-1049 and lazabemide (Ro 19-6327). These are highly selective, mechanism-based and reversible inhibitors of MAO activities (16), and lazabemide bears remarkable structural resemblance to the MAO-B preferential substrate ß-PEA (17, 18). These mechanism-based inhibitors, being nothing more than substrates that are converted by the normal catalytic pathway in products that inactivate the enzyme, are quite useful to study the mechanisms of action of the enzyme itself (19). Their interaction with MAOs appears to be similar to that occurring with substrates, being oxidized to form tightly, but reversible, bound adducts with the active sites. Taking advantage of the type of interaction of these MAO-A and MAO-B inhibitors it was decided to examine their effects upon deamination of 5-HT and ß-PEA in the rat heart.

METHODS

Male Wistar rats (Biotério do Instituto Gulbenkian de Ciência, Oeiras, Portugal), 45- to 60-day-old and weighing 200 to 280 g were used in the experiments. Animals were kept two per cage under controlled environmental conditions (12-hour light/dark cycles and room temperature 24 °C). Food and tap water were allowed *ad libitum*. The experiments were all carried out during daylight hours.

Preparation of homogenates

In brief, rats were killed by decapitation under ether anaesthesia, the heart and kidneys removed through a midline thoraco-abdominal incision and immediately washed in ice-cold saline. The kidneys were decapsulated, cut in half and placed in ice-cold saline. The outer cortex was cut out with fine scissors and homogenised in 67 mM sodium phosphate buffer, pH 7.2, at 4 °C with a Thomas Teflon homogeneiser kept continuously on ice. As for the heart, after being rinsed with ice-cold saline, a portion of ventricle was used for the homogenisation in the same buffer conditions described for the kidney.

Assay of MAO-A and B Activities

MAO-A and MAO-B activities were determined using, respectively, [^{3}H]-5-hydroxytryptamine (^{3}H -5-HT) and [^{14}C]-ß-phenylethylamine (^{14}C-ß-PEA) as preferential substrates. The reaction mixture contained 50 µL of homogenate and 50 µL of 67 mM phosphate buffer and increasing concentrations of each substrate (^{3}H-5-HT for MAO-A and ^{14}C-ß-PEA for MAO-B). After 20 minutes of incubation at 37 °C with continuous oxygenation and shaking, the tubes were transferred to an ice-water bath and the reaction stopped by the addition of 10 µL of 3 M HCl. The deaminated products were then extracted with ethyl acetate (500 µL) and measured by liquid scintillation counting (20, 21). The MAO activity is expressed in nanomoles of substrate metabolized per mg of protein per hour of incubation (nmol mg protein^{-1} h^{-1}).

Saturation curves for deamination of ^{3}H-5-HT (50 to 2000 µM) were determined in the absence and in the presence of lazabemide (250 nM), whereas deamination of ^{14}C-ß-PEA (5 to 500 µM) was performed in the absence and the presence of both Ro 41-1049 (250 nM) and lazabemide (250 nM). Homogenates were pre-incubated for 30 min at 37 °C either with phosphate buffer or phosphate buffer plus the inhibitor, lazabemide or Ro 41-1049, and then incubated with the substrates, ^{14}C-ß-PEA or ^{3}H-5-HT, for further 20 min, in the same conditions as described above.

In another set of experiments, selective MAO-A and MAO-B inhibitors, respectively Ro 41-1049 and lazabemide were used to study the deamination of ^{3}H-5-HT (1000 μM) and ^{14}C-ß-PEA (1000 μM). Homogenates were prepared as described above and pre-incubated for 30 min at 37 °C in the presence of increasing concentrations of each inhibitor, ranging from 0.5 to 500 nM. This was also followed by an incubation with the substrates for further 20 min, in the same conditions as mentioned above.

Protein assay

The protein content in the homogenates were determined according to the method of Bradford (22), using human albumin as a standard.

Data analysis

V_{max} and K_m values for the deamination of ^{3}H-5-HT and ^{14}C-ß-PEA, as determined in saturation experiments, were calculated by non-linear regression analysis, using the GraphPad Prism statistics software package (23). For the calculation of the IC_{50}'s for lazabemide and Ro 41-1049, the parameters of the equation for one site inhibition were fitted to the experimental data (23). K_i's were calculated as defined by Cheng and Prusoff (24) for competitive inhibition. Arithmetic means are given with S.E.M. and geometric means with 95% confidence values. Statistical analysis of the saturation curves was done with a one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. A P value less than 0.05 was assumed to denote a significant difference.

Drugs

Drugs used were: ^{14}C-ß-phenylethylamine hydrochloride (50 Ci/mmol) (NEN Chemical), ^{3}H-5-hydroxytryptamine creatinine sulphate (23.6 Ci/mmol) (NEN Chemicals), Ro 41-1049 [N-(2-aminoethyl)-5-(m-fluorophenyl)-4-thiazole carboxamide hydrochloride] (RBI, Natick, USA), Ro 19-6327 (generic name lazabemide) [N-(2-aminoethyl)-5-chloro-2-pyridine carboxamide hydrochloride] (F. Hoffmann-La Roche Ltd).

RESULTS

Deamination of ß-PEA, considered a specific MAO-B substrate, in the rat heart was found to present an unusual pattern, as can be observed in Fig. 1.

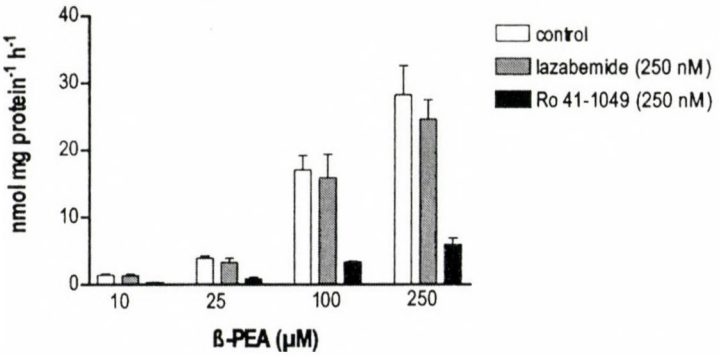


Fig. 1. Deamination of ß-PEA in rat heart homogenates in the absence (□) and the presence of lazabemide (250 nM; ▨) or Ro 41-1049 (250 nM; ■). Symbols represent means of four experiments per group and vertical lines show SEM

The saturation curve for deamination of ß-PEA in the rat heart was not affected (V_{max} = 53±10 vs 42±6 nmol mg protein^{-1} h^{-1}) by the selective MAO-B inhibitor lazabemide (250 nM), but was significantly reduced (V_{max} = 10±1 nmol mg protein^{-1} h^{-1}) by the selective MAO-A inhibitor Ro 41-1049 (250 nM). As shown in Table 1, deamination of ß-PEA in the rat heart is a low affinity process (when compared with that in the kidney) with high K_m values (244±98 vs 19±6 µM). On the other hand, deamination of 5-HT in the rat heart and renal cortex revealed high K_m values, which were similar to those for ß-PEA in the heart (Table 1).

Inhibition studies (see Fig. 2) show that deamination of ß-PEA (1000 µM) in the rat heart was inhibited in a concentration-dependent manner by Ro 41-1049 (the selective MAO-A inhibitor) with a K_i value of 32 nM (22, 48; 95% confidence limits), but not by the selective MAO-B inhibitor lazabemide (up to 500 nM). Inhibition of 5-HT (1000 µM) deamination in rat heart homogenates by Ro 41-1049 was also concentration-dependent with a K_i value of 21 (16, 26) nM, while there was no effect for lazabemide.

As shown in Fig. 3, deamination of 5-HT (1000 μM) in the rat renal cortex was inhibited in a concentration-dependent manner by Ro 41-1049 with a K_i value of 12 (8, 17) nM. Deamination of ß-PEA in the renal cortex, in the presence of 250 nM Ro 41-1049, was inhibited by lazabemide with a K_i of 5 (3, 7) nM.

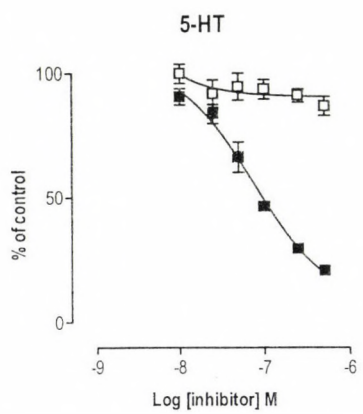
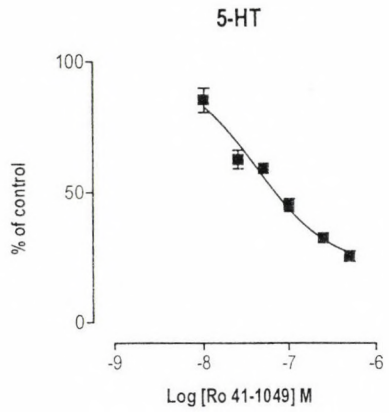
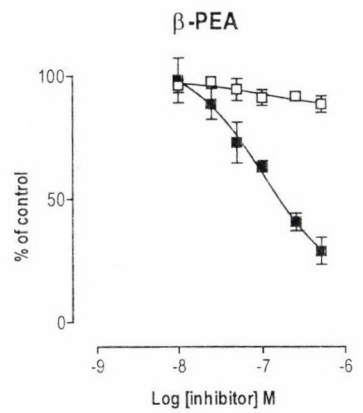


Fig. 2. Effect of increasing concentrations of Ro 41-1049 (■) and lazabemide (?) on rat heart deamination of 5-HT and ß-PEA. Symbols represent means of four experiments per group and vertical lines show SEM





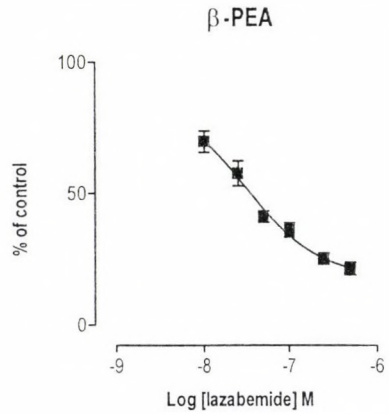


Fig. 3. Effect of increasing concentrations of Ro 41-1049 and lazabemide on the deamination of 5-HT and ß-PEA in rat renal cortex homogenates. Symbols represent means of four experiments per group and vertical lines show SEM

Table 1. V_{max} (in nmol mg protein^{-1} h^{-1}) and K_m (in µM) values for deamination of ß-PEA and 5-HT in homogenates of rat heart and renal cortex.

ß-PEA	V_{max}	K_m
	Heart	
Control	53±10	244±98
Lazabemide (250 nM)	42±6	191±59
Ro 41-1049 (250 nM)	10±1 *	229±68
	Renal cortex	
Control	9±1	19±6
5-HT		
	Heart	
Control	179±15	274±75
	Renal cortex	
Control	18±1	220±21

Values are means ± S.E.M. of four experiments per group.* significantly different from corresponding control values ($P<0.05$)

DISCUSSION

Classical classification of MAO forms has been made on the basis of substrate specificity and inhibitor sensitivity (6, 7). The suggestion that the two MAOs differ in their amino acid sequences came from peptide mapping analysis (25) and from immunoaffinity studies (26). The view that these two forms could arise from differences in post-translational modifications of a common enzyme precursor coded by a single structural gene locus (25), or even older assumptions that they resulted from a single enzyme existing in different membrane environments (27), were only abandoned after the isolation of MAO A and B cDNAs (3, 4). Although these isozymes have some different compositions they catalyze the same reaction, only preferring distinct amine substrates (19). However, it is also well known that substrate specificities vary with the species considered and with the tissue studied (2, 8). It has been previously shown (13) that the pattern of ß-PEA deamination in the rat heart is somewhat different from the usual one in that it is inhibited by the "A" inhibitor clorgyline. If tissue specificities simply reflect the ratio of MAO-A/MAO-B content, in the rat heart the specific binding of Ro 41-1049 and lazabemide in radioautography studies show A:B ratios ranging from 23 to 458 (9), or if they are the consequence of differences in the MAOs is not clear. In this study we tried to clarify some of these issues by using different substrates and inhibitors.

MAOs are flavoenzymes in that they require a flavin coenzyme to be active (1). For each MAO form there is a region containing a Cys residue (Cys406 and Cys397 in MAO-A and MAO-B, respectively) which covalently anchors flavin-adenine diphosphate (FAD) (28). In the rat heart, the investigation and characterisation of the active sites of both MAO forms using inhibitors has greatly relied on the use of the acetylenic inhibitors, clorgyline for MAO-A and selegiline for MAO-B (29), which form a 1:1 adduct by a covalent (irreversible) attachment to that same coenzyme (30). The use of another type of inhibitors, the N-(2-aminoethyl)arylcarboxamide derivatives lazabemide and Ro 41-1049, as probes for the biochemical characterisation of rat heart MAOs was, as far as we know, not done before. These compounds being converted into a product that is generally reactive and able to inactivate the enzyme, typically by covalent bond formation (19), turn to be very useful tools to study MAOs mechanisms of action.

As shown here, deamination of ß-PEA, classically considered a specific MAO-B substrate, in the rat heart was found to have an unusual pattern. This unusual pattern is mainly evidenced by high K_m values, approximately 10-fold that described for the same substrate in other tissues (2), and its insensitivity to lazabemide. Comparatively in the rat kidney and intestine, using this same substrate, we have recently shown that in both isolated renal epithelial cells and renal tubules and jejunal mucosa, deamination of ß-PEA proceeds through a high affinity mechanism (low K_m) and highly sensitive to lazabemide (20, 21). Another type of evidence suggesting that heart MAO deaminates ß-PEA by a process different from that observed in other tissues endowed with MAO-B activity is that K_i values for Ro 41-1049 using ß-PEA as a substrate were similar to those when 5-HT was used as substrate. This also indicates that Ro 41-1049 can no longer be regarded as a selective inhibitor of deamination of MAO-A substrates in the rat heart. However, if the A form in the rat heart has some affinity for the substrate ß-PEA, then we should have a similar sensitivity for the inhibitor lazabemide. Lazabemide has a very high degree of similarity with ß-PEA and shares the normal catalytic pathway, hence its presence should have some effect on ß-PEA deamination in this tissue. Because lazabemide lacked inhibitory effect on the deamination of ß-PEA, the real nature of the MAO form engaged in ß-PEA deamination in the rat heart remains questionable. However, K_m values for deamination of 5-HT, a specific MAO-A substrate, and its sensitivity to Ro 41-1094 in the heart were found not to differ from those in the kidney. This fits well the suggestion that MAO-A is the predominant MAO form in the rat heart (14, 29).

In conclusion, although these results suggest that in the rat heart the specific MAO-B substrate, ß-PEA, is deaminated by a form of MAO which most probably correspond to MAO-A, it may be worthwhile to further characterise the MAO isozymes in this tissue using a molecular approach.

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RESEARCH REPORT

STRUCTURAL APPROACH OF HUMAN MAO-A USING FOLD RECOGNITION (THREADING) TECHNIQUES

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The major goal of the present work is to further approach the structure of human monoamine oxidase A (MAO-A). A first partial three-dimensional model of human MAO-A has already been established using secondary structure predictions and fold recognition methods [Wouters and Baudoux, 1998]. In this modeled structure, a segment of the sequence (residues 369-393) located near the covalent linkage to the essential flavin cofactor, and potentially involved in the structure of the active site of the protein, could not be modeled. We here propose a possible fold for that segment, based on threading techniques. The identification of regions of the protein potentially involved in its dimerization was also undertaken by studying hydrophobic areas present at the surface of the structure.
Key words: type A monoamine oxidase (MAO-A), flavoproteins, membrane protein, fold prediction (threading), knowledge-based modeling

INTRODUCTION

The accurate prediction of the three-dimensional (3D) structure of a protein from its amino acid sequence is today one of the most ambitious goals in molecular modeling. Currently there are around 9000 different structures deposited at the Protein Data Bank (PDB, http://www.pdb.bnl.gov). On the other hand, we know hundreds of thousands of protein sequences. Although

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information in both databases in highly redundant, many sequences have still unknown structure and function. Aiming to reduce this gap, different methods have been developed to predict the 3D structure of proteins from their sequence among which, homology modeling (comparative modeling) and fold recognition (threading) methods.

Homology modeling [Browne et al., 1969; Greer, 1990] is based on the principle that proteins with homologous sequences have usually similar structures (and generally often related functions). In particular, an analysis of sequence alignments for proteins of known structure reveals that all proteins with more that 30% pairwise sequence identity [Eisenberg et al., 1984] have similar 3D structures (i.e. the essential fold of the two proteins is identical, details such as additional loop regions may vary). This is the basis of the comparative modeling technique. It consists basically in aligning the 'target' sequence (the sequence that one wants to model) against all the sequences of known protein structures. If some homologous proteins are found (sequence identity of the alignment over 30%), then those proteins are selected as 'template' structures for the modeling process. Based on the alignment of each pair of sequences (target and template), the target sequence is modeled on the template structure (Fig. 1). The accuracy of this technique depends directly on the degree of homology between the target and template sequences. In practice, in the cases where there is a very low or no significant similarity at the sequence level between a protein and all the proteins of known structure, it is not possible to use the technique of comparative modeling. In these cases, it is possible to use less accurate methods such as fold recognition or 'threading' to obtain a tentative model or general fold for these proteins.

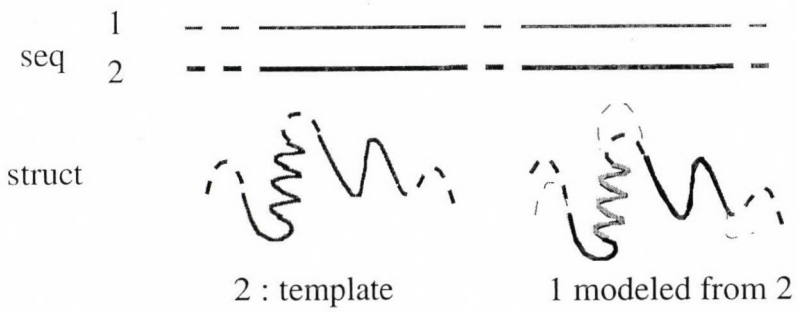

Fig. 1. General principle of homology (comparative) modeling. For details, see the text

Fold recognition techniques rest on the observation that two unrelated proteins at sequence, functional or evolutionary level can sometimes share a surprising similarity in some folding motifs or in their overall fold or architecture [Chothia and Lesk, 1986; Holm and Sander, 1993; Holm and Sander, 1995]. Actually, it seems that during evolution, only a limited number of building blocks of protein structures have been generated. Consistent with this is the fact that around 50% of the newly experimental structures appear to be related to already known folding motifs [Blundell and Doolittle, 1992]. Based on recent estimation, we currently know around 300 different (non-redundant) folds, for a total of about 500-600 possible folds [Blundell and Johnson, 1993; Chothia, 1992]. This knowledge gives the possibility to find known folds on which a particular protein sequence with unknown structure can fit in a good way. On this principle, threading or fold recognition techniques were developed. They consist in forcing the fold of a sequence on a library of different known folds. The sequence-structure alignments are evaluated on the basis of energies obtained using mean force potentials derived from databases of non-redundant folds [Melo, 1998; Bowie et al. 1991; Sippl and Weitckus, 1992; Jones et al. 1992; Wodak and Rooman, 1993] (Fig. 2).

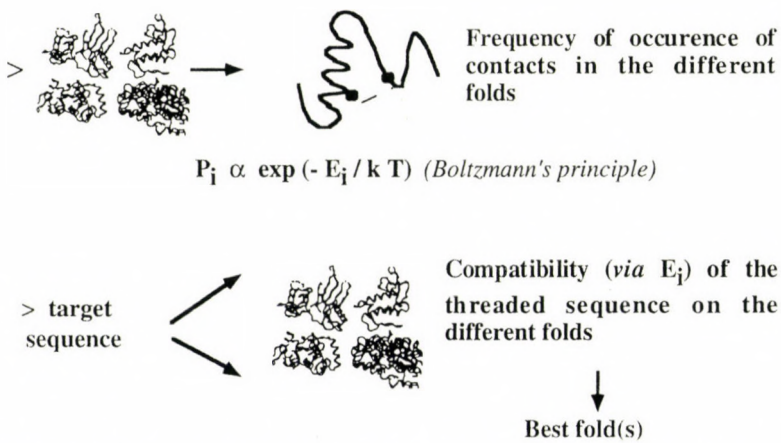

Fig. 2. General principle of fold recognition (threading) techniques. The frequency of occurrence of particular contacts between fragments in a given environment can be derived from a database of (non-redundant) folds. Those frequencies (probabilities) can be expressed in terms of 'energies' using Boltzmann's principle. Those 'energies' derived from the existing folds can be used to discriminate (rank, score) between folds generated by fitting (threading) a given target sequence on a set of existing folds. The best-threaded folds correspond to 'possible' structures for the target sequence

Although it has now been studied for a long time, there is still today a lack of information on the three-dimensional (3D) structure of monoamine oxidase (MAO flavin-containing, EC:1.4.3.4). MAO is a membranar flavoprotein that exists in two isoforms, MAO-A (527 residues) and MAO-B (520 residues) which share significant sequence homology and are therefore expected to display similar 3D structures. They present however differences in substrate preferences and inhibitory specificity, tissue and cell distributions [Berry et al., 1994; Kalgutkar and Castagnoli, 1995; Weyler et al., 1990; Wouters, 1998].

To our knowledge, no crystallographic nor NMR studies have elucidated the 3D structure of the enzyme. MAO-A and B do not display significant sequence similarity to other proteins of known 3D structure excluding homology modeling of the protein.

The implications of a successful structure determination of MAO are evident. Determination of the overall fold of the protein and of the architecture of the active site of MAO would facilitate the design of new potent and selective MAO inhibitors. In particular, because of the use of MAO-A inhibitors as antidepressants, knowledge of the tertiary structure of MAO-A could be of great value for the rational design of new effective drugs. Furthermore, there is a large body of data (biochemical and others) that awaits interpretation in terms of 3D structures of MAO-A and/or MAO-B.

The major goal of the present work is to study the three-dimensional structure of human monoamine oxidase A. A first partial three-dimensional model of human MAO-A had already been established by our group using secondary structure predictions and a combination of fold recognition methods [Wouters and Baudoux, 1998]. In this study, the C-terminal region of the protein was predicted to be responsible for anchoring the protein into the mitochondrial membrane and was not modeled. In the modeled structure, a segment of the sequence (residues 369-393) located near the covalent linkage to the essential flavin cofactor, and potentially involved in the structure of the active site of the protein, could also not be modeled. We here propose a possible fold for that segment, based on threading techniques. The identification of regions of the protein potentially involved in its dimerization was also undertaken by studying hydrophobic areas present at the surface of the structure.

MATERIAL

Sequence alignments [Depiereux et al., 1997], threading calculations [Sippl and Weitckus, 1992], model building and minimizations were executed on a Silicon Graphics Indigo^2 workstation running IRIX5.3. The three-dimensional coordinates of proteins were extracted from the Brookhaven Database (PDB; http://www.pdb.bnl.gov). Fold recognitions experiments were performed using PROFIT2.0 [Sippl and Weitckus, 1992]. The three-dimensional coordinates of template folds were assigned to the target sequences using the program MODELLER4 [Sali and Blundell, 1993]. Graphical displays and manipulations of the structures were performed with the *INSIGHTII* molecular modeling system of Biosym Technologies/MSI (San Diego, CA). Analysis of the hydrophobicity of the surface of the proteins was performed with the QUILT program [Lijnzaad et al., 1996].

The numbering retained in this work corresponds to the sequence of human MAO-A. Coordinates of the model of MAO-A can be obtained upon request to the authors (johan.wouters@fundp.ac.be).

METHODS

Because the target sequence of human monoamine oxidase (AOFA_HUMAN) is not significantly similar to any protein of known 3D structure, direct homology modeling must be excluded.

The starting point of this study was the preliminary model of human MAO-A that had been obtained previously [Wouters and Baudoux, 1998]. Fold recognition techniques were used to identify possible structures for the parts missing in the previous model. In particular, 1D/3D (1D : target sequence - 3D : sequence of the three-dimensional structure) alignments were produced for the segment corresponding to residues 369-393 and a model corresponding to this region was computed. A manual docking of this domain with the rest of the protein has also been performed.

In order to assess the quality of the overall structure and to help in the docking process, the hydrophobicity of the surface of the protein was calculated and hydrophobic patches were identified. Actually, when a correct fold is predicted, one should not find many hydrophobic (apolar) residues exposed to the solvent (at the surface of the protein). Hydrophobic patches thus represent either wrong folds or missing domains (interface domains) in the structure.

RESULTS AND DISCUSSION

A first partial three-dimensional model of human MAO-A has recently been established by our group using secondary structure predictions and a combination of fold recognition methods [Wouters and Baudoux, 1998].

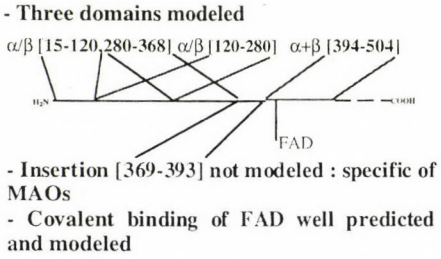

Fig. 3. Schematic representation of the first model of human monoamine oxidase A [Wouters and Baudoux, 1998]. The modeled structure presents three distinct domains that are shown along the primary sequence of the protein in this figure. The C-terminal region and a segment (residues 369-393) located near the flavin (FAD) cofactor were not modeled

The modeled structure (Fig. 3) shows three distinct domains: two α/β domains (the FAD-binding N-terminal and central domains) and an α+β domain (a rudimentary interface domain). The C-terminal region of the protein was predicted to be responsible for anchoring the protein into the mitochondrial membrane and was not modeled. In this modeled structure, a segment of the sequence (residues 369-393) located near the covalent linkage to the essential flavin cofactor, and potentially involved in the structure of the active site of the protein, could also not be modeled.

Interestingly, the missing segment (insertion) is located at the surface of the protein, in the 3D structure. This opens the possibility of adding a small domain compatible with the sequence to insert, without perturbing too much the overall fold of the existing model.

The hydrophobicity of the surface of the protein has also been calculated and three major hydrophobic patches were identified (Table 1, Fig. 4). Because hydrophobic residues are usually not exposed to the solvent, hydrophobic regions at the surface of the protein are often the indication of either a wrong, misfolded structure, or of incomplete domains. In the present study, all three hydrophobic regions where carefully analyzed.

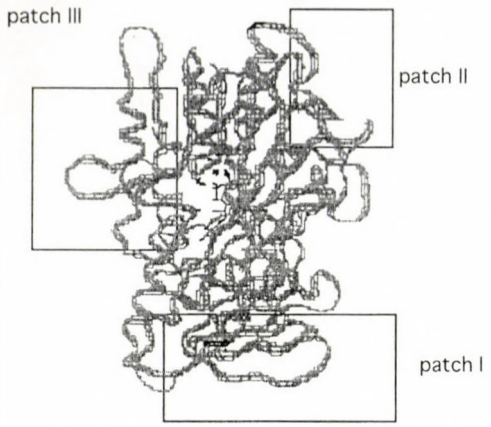

Fig. 4. Identification of hydrophobic regions (exposed hydrophobic amino acids) at the surface of the modeled structure of MAO-A.

One of those hydrophobic areas contains the loop where the residues 369-393 segment must be inserted. This is reasonable and suggests that when a suitable fold is found for this missing part, docking with the rest of the protein will mask this surface of the protein, burying the hydrophobic amino acids.

A second hydrophobic patch starts around the C-terminus of the model. This makes sense as we know that this part of the protein lacks a domain that was not previously modeled and that can be responsible for anchoring the protein to the mitochondrial membrane.

Table 1. List of residues forming hydrophobic regions at the surface of the modeled structure of MAO-A

PATCH I (area 1400.6)
D 15 - V 37 - S 38 - R 45 - G 49 - S 94 - E 95 - L 97 - V 98 - Q 99 - Y 100 - V 101 - K 102 - G 103 - T 105 - Y 106 - P 107 - F 108 - R 109 - A 111 - F 112 - P 114 - E 145 - Q 293 - I 295 - W 315 - K 316 - D 319 - Y 320 - C 321 - M 324 - H 365

PATCH II (area 1235.2)
V 115 - S 184 - Q 215 - L 277 - A 279 - I 281 - F 283 - R 284 - R 297 - L 298 - P 299 - M 300 - G 301 - A 302 - V 303 - I 304 - C 306 - M 307 - M 308 - Y 309 - Y 310 - E 312 - F 314 - W 315 - M 324 - I 335 - L 337 - K 341 - P 342 - R 360 - L 361 - L 364 - E 368 - K 395 - W 397 - P 426 - P 426 - V 427 - G 428 - R 429

PATCH III (area 1201.4)
V 198 - K 199 - C 201 - R 229 -I 230 - M 231 - L 233 - L 234 - G 235 - Y 410 - F 411 - P 412 - P 413 - G 443 - M 444 - E 445 - A 447 - V 448 - E 449 - E 452 - R 453

The third hydrophobic surface is located at the opposite side of the first. It covers a domain of the protein that corresponds to the 'interface domain' in other flavoproteins and that has been shown to participate in multimerization. We know that monoamine oxidase is not active as a monomer but acts as a dimer or oligomer [Kalgutkar and Castagnoli, 1995; Weyler et al., 1990; Wouters, 1998]. On the basis of the present analysis, we suggest that the third hydrophobic region is responsible for the dimerization of the protein, as is observed in other proteins belonging to the same glutathion-like flavin binding family of enzymes.

Table 2. List of folds corresponding to residues (369-393) and (369-403)

target 1 IRKKKICELYAKVLGSQEALHPVHYE
target 2 IRKKKICELYAKVLGSQEALHPVHYEEKNWCEEQYS

target 1										
PDB code	length	Sc 1	Sc 2	Sc 3	Sc 4	GS	GF	S%	F%	
1urn.A.-	1	96	-11	109	109	109	0	0	100	27
1chk.A.-	1	238	-46	92	92	92	0	0	100	10
1lin.-.-	1	146	62	67	60	60	0	1	92	16
1fua.-.-	1	206	46	55	55	55	0	0	100	12
2exo.-.-	1	312	7	48	48	48	0	0	100	8
target 2										
PDB code	length	Sc 1	Sc 2	Sc 3	Sc 4	GS	GF	S%	F%	
1urn.A.-	1	96	-30	217	217	217	0	0	100	37
3chy.-.-	1	128	-27	212	212	194	1	0	100	28
1pot.-.-	1	322	-13	191	191	191	0	0	100	11
1esl.-.-	1	157	38	181	181	165	1	0	100	22

A systematic search of folds compatible with the segment corresponding to the residues 369-393 was performed using threading programs. A series of fold emerged (Table 2.). They correspond to a general fold adopting an α helix followed by a turn and an extended strand. A similar search was performed on a longer segment containing the sequence missing in the first model (Table 2.). This strategy proved useful when trying to connect the different bits of the model together as both parts to be docked (the first model and the small missing segment) shared a few residues in common. From this study an interesting fold candidate emerged. It corresponds to part of the fold of a ribonucleoprotein (1urn). A manual docking of this domain with the rest of the protein is currently underway.

CONCLUSIONS AND PERSPECTIVES

The current modeled structure provides valuable information on the overall fold of MAO-A although its precision is not sufficient to study specific interactions of residues located in the active site (e.g. by docking with substrates and inhibitors). A similar modeling of MAO-B is currently under progress and will help in distinguishing both types of MAO activities. There is hope that efforts in this direction should allow, in the future, determination of the 3D structures of MAO isoforms and studies of the interaction between MAOs and their ligands.

Definite confirmation of the present partial model of MAO should await the crystal structure determination of the protein (or related soluble flavin-containing amine oxidases).

Further studies will also focus on the multimerization of the protein. In this perspective, close collaboration between experimentalists and theoreticians is appreciable

Acknowledgement. We thank the participants of the 8th Monoamine oxidase workshop for fruitful discussions and the organizer for their hospitality. Mr Guy Baudoux who participated in the modeling of the first structure is also very much acknowledged.

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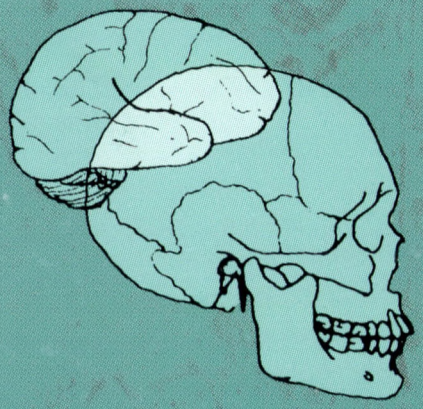

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RESEARCH REPORT

IS SEMICARBAZIDE-SENSITIVE AMINE OXIDASE IN BLOOD PLASMA PARTLY DERIVED FROM THE SKELETON?*

EKBLOM, J., GRÖNVALL, J.L.E., GARPENSTRAND, H., NILLSON, S. and ORELAND, L.

Department of Neuroscience (Pharmacology), Biomedical Center, Uppsala University, Uppsala, Sweden

Semicarbazide-sensitive amine oxidases (SSAOs) are widely expressed copper-containing enzymes. One enzyme of this family have high specific activity towards benzylamine and is present in human blood plasma. This enzyme is altered in several diseases, for instance in diabetes. Presently it is unclear where the plasma SSAO is synthesized. Previous autoradiographic studies have suggested that SSAO may be expressed in bone tissue. In the current study we have analyzed levels of SSAO in serum from cases with 'skeletal disease', i.e. patients with severe skeletal metastases of prostate cancer and subjects having recent fractures. Interestingly, subjects with metastases showed significantly elevated levels of SSAO in serum compared to individuals having prostate cancer without skeletal metastases. It is speculated that, at least in part, SSAO in the blood stream may be derived from bone tissue.

Keywords: benzylamine oxidase (BzAO), blood plasma, bone scintigraphy, cancer, copper-containing amine oxidase, prostatic adenocarcinoma

INTRODUCTION

Copper-dependent amine oxidases sensitive to semicarbazide (SSAO, E.C. 1.4.3.6) are expressed both by prokaryotes and eukaryotes (for a review, see Buffoni, 1993). The molecular biology of these enzymes is well characterized while their physiological functions in mammals are still unclear.

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It has been suggested that SSAOs may protect the organism from toxic exogeneous amines. Moreover, a variety of other physiological functions have been attributed to this enzyme family. For instace, in recent studies it has been demonstrated that SSAO may be involved in translocation of the glucose transporter Glu4 to the plasma membrane in adipocytes (Marti et al., 1998) and that the enzyme protein also might function as an adhesion molecule (Smith et al., 1998).

Most mammals, including humans, seem to have at least one SSAO activity in blood plasma that efficiently can oxidize benzylamine. This enzyme has been shown to be a marker for several diseases, e.g. diabetes mellitus, cardiac insufficiency and hernia diaphragmatica (Boomsma, 1995, 1997 and personal communication).

Today, the tissue origin of SSAO in blood plasma is unknown. It has been speculated that it may be synthesized in vascular smooth muscle and then released into the blood stream (Lyles and Singh, 1985). Nevertheless, it has been shown that one of the enzyme activities in bovine blood plasma is expressed in the liver (Mu et al., 1994).

In a recent study, we used an indirect autoradiographic method to map the distribution of SSAO in mice (Grönvall et al., 1998). Using this method we observed abundant radioactive accumulation in skeleton suggesting that SSAO activity may high in bone tissue (Ekblom et al., 1999). In the present study we have analyzed the activities of SSAO in cases with skeletal metastases of prostate cancer and in individuals with bone fractures. Such data may indirectly indicate whether or not SSAO is expressed in human bone tissue.

MATERIALS AND METHODS

Subjects

In the present study samples from twenty patients with prostate cancer and fifteen cases with femoral bone fractures were included. Blood samples were collected from the patients prostate cancer by venopuncture. The diagnoses of these subjects were determined by histology and metastases were verified with 99mTc-HDP bone scintigraphy. With regard to the patients having fractures, the samples were obtained 24–72 hours after the accident. The mean age in this group was 66.1 ± 21.7 years and 9 of these 15 patients were women. In five cases, the accidents were associated with additional fractures of hips and arms.

The control group consisted of 24 (6 men and 18 women) healthy volunteers, i.e. they had no known history of cardiovascular disease or any other condition

reported to influence SSAO activity. The mean ages of the two control groups were 67.4 ± 13.8 years and 71.7 ± 5.4 years, respectively. Patients who suffered from diabetes mellitus were from the beginning excluded from the study as were patients who were on medication with known SSAO-inhibitory substances.

SSAO activity estimations

Within 24 hours, the blood samples (5 ml) were centrifuged at 500 g for 10 minutes and 1 ml of the serum that was stored at -70°C for later use. SSAO activity estimations were then performed as previously described using ^{14}C-benzylamine as a substrate (Grönvall et al., 1998). Enzyme activities were expressed as nmol benzylamine oxidized/ml plasma/hour. All comparisons between groups were done by the use of both Student's t-test and factorial ANOVA.

RESULTS

Cases with skeletal metastases

SSAO activities were estimated in a group of patients having prostate cancer with severe skeletal metastases confirmed by bone scans. In order to exclude possible effects of pharmacotherapy, patients with prostate cancer without metastases were used as a control group. Moreover, SSAO-activities were also estimated in samples from a control group which consisted of samples from 24 healthy age-matched individuals. In the patient group having prostate cancer without skeletal metastases the mean specific activity of SSAO in serum was similar to that observed in samples of healthy controls (Fig. 1). In contrast, the mean specific activity of SSAO was significantly elevated in the group of patients with prostate cancer having skeletal metastases as compared to the other groups. The mean SSAO activity in this group was increased by 24–29 % when compared to healthy controls ($P < 0.007$) and patients with prostate cancer without metastases ($P < 0.013$), respectively.

Cases with bone fractures

SSAO activities were also estimated in samples collected from 15 individuals with femoral bone fractures. These samples were collected within three days after the accident. However, the mean SSAO activity of the patient group did not differ significantly from the activity levels observed in the group of healthy controls (Fig. 1).

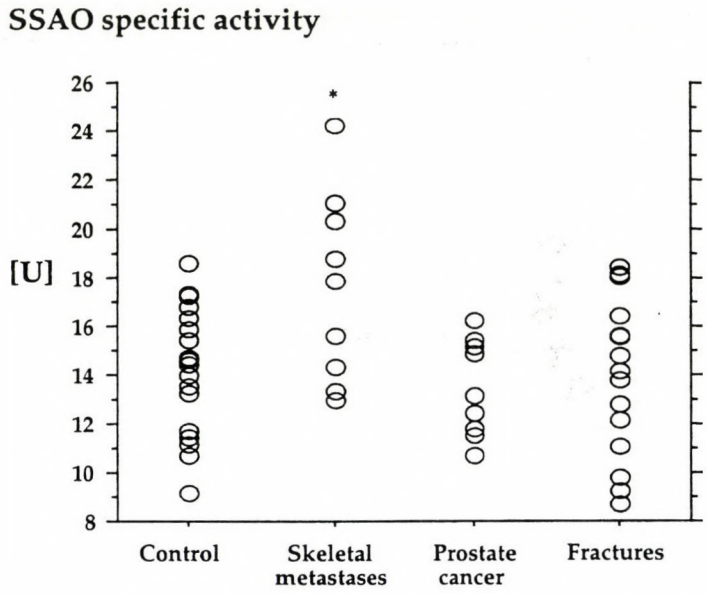

Fig. 1. Specific activities of SSAO in serum from i) healthy controls (n=24), ii) patients with prostate cancer associated with skeletal metastases (n=10), iii) patients with prostate cancer without any signs of skeletal (n=10) as well as iv) subjects with femoral bone fractures (n=15). The data represents mean + standard deviation. $P < 0.007$ Patients with skeletal metastases vs. healthy controls

DISCUSSION

The tissue distribution of SSAO activities has been mapped in several species by different approaches (for a review, see Buffoni, 1993). With regard to activities of benzylamine-metabolizing SSAO(s) in blood plasma there is a considerable variation between different mammalian species. For instance, high specific activities have been observed in blood plasma from goat and horse, while humans and other primates express relatively low levels (Boomsma et al., unpublished information). Moreover, in some species more than one SSAO activity is present in blood plasma and the origin of plasma SSAO enzyme(s) is presently unknown. One of the bovine plasma SSAOs (BSAO) has been cloned from a liver cDNA library (Mu et al., 1994) and Northern blotting analyses suggest that BSAO may be specifically expressed in the liver (Hogdall et al., 1998). The second bovine SSAO remains to be cloned and nothing is known

about its tissue of origin. In analogy, the source of human plasma SSAO is yet unknown.

Already in the 1960s it was shown by Tryding and coworkers (1969) that SSAO is elevated in diabetes mellitus. Recently, it has also been shown that the enzyme in blood plasma is altered in association with disorders such as congestive heart failure, pre-eclampsia and *hemia diaphragmatica* (Boomsma et al., 1995, 1997, unpublished results).

In a recent study, we provided evidence that SSAO activity may be present in bone by using an indirect 'metabolic' autoradiography in mice (Grönvall et al., 1998, Ekblom et al.). Methylamine is oxidized by SSAO to formaldehyde, which reacts very rapidly with the tissue where it is formed. Injection of ^{14}C-methylamine into mice resulted in an abundant accumulation of radioactivity in the skeleton. Moreover, this accumulation was inhibited in a dose-dependent fashion by hydralazine, an irreversible SSAO inactivator. These data may either suggest that there is abundant SSAO activity in bone or that bone tissue has an extraordinary capacity to react with formaldehyde produced in other tissues. The aim of the present study was to investigate if human 'bone disease' was associated with any changes in plasma SSAO activities. Significantly elevated levels were evident in cases having skeletal metastases of prostate cancer and these data may favour the hypothesis that SSAO is expressed in bone and that SSAO enzyme protein in the blood stream may be partly derived from this tissue. However, "the fracture" did not differ from healthy controls. Accordingly, if SSAO is expressed in the skeleton, it does not seem that the enzyme protein 'leaks out' into the blood stream after trauma. In the present study, samples were collected from the fracture cases within 72 h. It would be interesting to analyze activities of plasma SSAO at later time points after the fracture, i.e. at a stage coinciding with bone regeneration.

It has been proposed by many investigators that products arising from biological reactions catalyzed by amine oxidases may contribute to carcinogenesis, i.e. by production of reactive aldehydes and hydrogen peroxide. For instance, it has been shown that hydrogen peroxide produced by serum amine oxidases can cause *in vitro* spontaneous neoplastic transformation of cultivated murine cell (Parchment and Natarajan, 1992). In an investigation by Heston and collaborators (1981), it was shown that aminoguanidine, which is a potent inhibitor of copper-containing amine oxidases, affects the survival of prostate cancer cells in culture. In a previous study on human cancer, plasma SSAO levels were analyzed in a mixed group of patients being diagnozed with different malignancies, e.g., lung, breast and colon cancers (Lewinsohn et al., 1978). The mean specific activity of SSAO in blood plasma was considerably

lower in this heterogeneous group as compared with healthy age-matched controls. However, it was evident that some treatment regimes had effect on SSAO activities and no data was available on staging of the cancers.

With regard to the development of late diabetic vascular complications, it has been proposed that treatment with a selective SSAO inhibitor may have clinical value (for a review, see Ekblom, 1998). Clinical experience of long-term treatment with SSAO-inhibiting agents such as hydralazine, carbidopa and benzerazide suggests that future specific SSAO inhibitors should produce, if any, very limited side-effects.

In conclusion, the present study provides evidence for an involvement of SSAO in skeletal metastases of prostate cancer. The mechanism for increased specific SSAO activities in blood plasma from patients with metastases is unknown. Moreover, the results may suggest that a least a fraction of SSAO in the circulation may origin from the skeleton.

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RESEARCH REPORT

MODIFICATION OF DOPAMINE RELEASE BY SELECTIVE INHIBITORS OF MAO-B*

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Chronic low dose deprenyl treatment in rats causes an increase in striatal extracellular dopamine level, without significant reduction in deaminated metabolite formation. This effect could be the result of increased endogenous levels of the MAO-B substrate β-phenylethylamine, which is both a releaser of dopamine as well as an inhibitor of the neuronal membrane active dopamine uptake. In guinea pigs, however, striatal extracellular dopamine was not increased either by deprenyl or by clorgyline. Local infusion of the dopamine uptake inhibitor GBR-12909 caused a greater increase in striatal dopamine in microdialysate in rats than in guinea pigs. Intra-species differences in synaptic architecture or in density of dopamine transporter expression may account for these differences.
Keywords: deprenyl, clorgyline, dopamine, neuronal reuptake, β-phenylethylamine

INTRODUCTION

Administration of the selective MAO-B inhibitor eprenyl (selegiline) to Parkinsonian patients has a distinct if not large symptomatic effect (Olanow, 1996). Preliminary clinical data with a second selective MAO-B inhibitor, rasagiline (TVP-1012; N-propargyl-1(R)-aminoindan) indicates a similar phenomenon. Dopamine (DA) is an equivalent substrate for MAO types A and B (Fowler and Tipton, 1984). Since the subtype of MAO within the substantia nigral dopaminergic neuron in rodent and primate species is mainly

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or exclusively type A (Demarest et al., 1980; Westlund et al., 1988), the question remains how inhibition of MAO type B is able to exert a dopaminergic effect. One possibility which has been advanced by Paterson and coworkers (1991) is that the dopaminergic effect may be mediated by β-phenylethylamine (PEA), which accumulates following inhibition of MAO-B.

Phenylethylamine is produced from phenylalanine by decarboxylation by the enzyme aminoacid decarboxylase (AADC). There is normally a high production and an equally high rate of metabolism of PEA by MAO-B, resulting in a low normal tissue level of the amine (Paterson et al., 1990). Following inhibition of MAO-B, however, levels of phenylethylamine can increase considerably, by 330% in rat striatum (Paterson et al., 1991). Selective inhibition of MAO-B by deprenyl has been shown to cause a powerful potentiation of peripheral and CNS responses to exogenously administered PEA, as a result of decreased metabolism of the amine (Knoll, 1976), and the activity of endogenous PEA will be similarly potentiated.

The pharmacological actions of PEA are similar to those of amphetamine, including uptake into the dopaminergic neuron via the selective DA transporter molecule (DAT) and subsequent efflux of DA by non-exocytotic release, resulting from the reverse transport of DA out of the neuron by DAT (Parker and Cubeddu, 1988). However, PEA, by virtue of its affinity for the carrier sites on the DAT molecule, will also compete with DA for uptake. In vivo, therefore, PEA may be expected to show characteristics of uptake inhibitor and of release-inducer.

Since intraneuronal MAO is type A, inhibitors of MAO-A will potentiate the actions of PEA (Finberg and Youdim, 1985), and in the CNS dopaminergic neuron, this results from increase of DA in the cytoplasmic pool available for release by exchange transport. Inhibition of MAO-B will potentiate PEA effects mainly by inhibiting its metabolic breakdown.

In our previous study of the effect of chronic administration of deprenyl and rasagiline (which unlike deprenyl is devoid of intrinsic amphetamine-like action) on striatal extracellular DA levels in the rat, we observed that following chronic (21 days) but not single administration of both drugs, striatal extracellular DA level was increased (Lamensdorf et al., 1996). This increased DA level was not accompanied by a decrease in deaminated metabolites (DOPAC and HVA), by contrast to the effect of clorgyline, which increased levels of DA, but reduced levels of DOPAC and HVA. Steady-state tissue levels of DA are also increased by clorgyline but unaffected by deprenyl or rasagiline (in selective MAO-B-inhibitory doses). As a result, we felt that the

effect of MAO-B inhibition was possibly mediated by accumulation of endogenous PEA.

Since in the guineapig, as in primate species, brain MAO contains about 85% MAO-B (Azarro et al., 1985), and since PEA is a good substrate for MAO-B, we decided to look at the effect of chronic deprenyl administration on striatal extracellular DA in this species.

MATERIALS AND METHODS

Guineapigs (male, Hartley) were treated with deprenyl either once (2 mg/kg) or daily for 21 days (0.25 mg/kg sc daily). Other animals were similarly treated with clorgyline, once (4 mg/kg sc) or daily for 21 days (1 mg/kg sc). A microdialysis probe (cupraphane membrane, cutoff 17,000) was implanted in the left striatum under pentobarbitone/chloral hydrate anesthesia (12:60 mg/kg) on the last treatment day. Probes were perfused with artificial cerebrospinal fluid at a rate of 2 µl/min. Control microdialysate collections were commenced 24 h after the probe implantation, i.e. 24 h after the last drug dose. Microdialysate levels of DA and metabolites were determined by HPLC/EC.

RESULTS

Basal DA microdialysate level averaged 0.11±0.013 (n=14) pmol/10 min, and was not significantly increased by either MAO inhibitor, following either acute or chronic administration. DOPAC levels averaged 5.2±0.7 pmol/10 min, and were reduced 40% and 48% by clorgyline (acute and chronic treatments, $P<0.05$ and $P<0.01$, respectively), but not affected significantly by deprenyl. HVA levels averaged 12.3±1 pmol/10 min, and were reduced 54% and 65% by clorgyline treatments, but were increased 60% ($P<0.001$) by both acute and chronic deprenyl treatments. In order to examine whether the difference in behavior of rats and guineapigs could be releated to a difference in the influence of high affinity uptake on microdialysate levels, the powerful inhibitor of DA uptake, GBR-12909 (10 µM), was infused locally via the striatal microdialysis probe in rat and guineapig striatum. Dopamine microdialysate levels increased to 376% basal levels in rats, but only 171% in guineapigs.

DISCUSSION AND CONCLUSION

We have studied the effect of chronic deprenyl on extracellular DA in the guineapig, a species in which most CNS MAO is type B, as in the human. A previous study by Juorio et al., (1994) showed that doses of deprenyl which were selective for MAO-B increased tissue DA and PEA but had no effect on extracellular DA in an acute experiment. Non-selective inhibition of MAO-A and -B with pargyline, however, caused a large increase in extracellular DA. In view of our previous observation in the rat that chronic but not acute deprenyl treatment increased microdialysate DA, and because tissue PEA levels increase with time following MAO-B inhibition, we tested the effect of chronic and acute treatments in the guineapig. Surprisingly, chronic deprenyl treatment in the guineapig was not accompanied by an increase in striatal extracellular fluid DA level, although microdialysate HVA levels were increased, indicative of enhanced DA release.

The amount of DA detected in the extracellular fluid is dependent on the degree of spillover of the transmitter from the synaptic cleft. This is largely controlled by the activity of DAT in the perisynaptic structures, as well as by the synaptic architecture. The augmenting effect of local striatal perfusion with GBR-12909 on microdialysate DA was considerably less in the guinea pig than in the rat. A similar inter-species difference probably exists in the action of PEA. It may be that an altered synaptic architecture is responsible for this inter-species difference. Another possibility is that DAT is expressed to a lesser degree in perisynaptic areas in the guineapig striatum than in the rat. In this context, it is interesting that DAT was found to be distributed in intervaricose axonal segments in the prelimbic cortex, whereas in striatum, both varicose and intervaricose segments of dopaminergic neurons contained the transporter (Sesack et al., 1998). This could explain the finding of Timar et al., (1993) that deprenyl enhances release of DA from striatum but not from frontal cortex. Such a difference in distribution of DAT between rat and guineapig striatal neurons could also explain the observed species difference.

The current data indicate that MAO-B inhibition may increase DA release in both rat and guineapig, although the effect on extracellular DA concentration will be different.

In considering the effect of MAO-B inhibition on synaptic DA in the human, similar considerations of DAT distribution and synaptic architecture will apply. Paterson et al., (1995) found that deprenyl was effective in blockade of striatal DA metabolism in the macaque, and we have reported an increase in striatal

microdialysate in the rhesus monkey following MAO-B inhibition (Finberg et al., 1998).

Further studies are required to understand the intra-species differences in synaptic clearance of DA, and the involvement of endogenous PEA in the effect of MAO-B inhibitors, as well as the importance of chronic versus acute administration of the MAO-B inhibitors.

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RESEARCH REPORT

TRANSDERMAL FORMULATIONS OF DEPRENYL: GUINEA PIG AND PIG MODELS*

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The efficacy of many drugs relies on their presence at the site of action over a period of time. The retardation or programmed release capability of the conventional dosage forms like oral and parenteral are limited and toxic and undesired side-effects may occur after their applications. These problems may be solved using transdermal delivery systems. Transdermal systems are aimed for local, or systemic action. In the letter case controlling the rate of delivery or modulating the distribution in the organism. The selection of an adequate biological method of evaluating a new transdermal formulation is a critical point of the development. The *in vitro* methods can help in the characterization of the different formulas, but without an *in vivo* disposition study they cannot give relevant information about the expectable therapeutic behavior. We adapted and improved an *in vivo* test system for the evaluation of new transdermal particulate systems (patches) and liposomes containing deprenyl selegiline as active ingredient. The *in vivo* evaluation system consists of two steps: 1. Full biodisposition study on guinea pig, using isotope labeled selegiline. 2. Biodisposition studies on domestic pigs including dose, area, surface dependence and comparative bioavailability with traditional dosage forms and application moods. Specific examples of these studies and experimental technology are presented.
Keywords: MAO, deprenyl, transdermal application, pig

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For a memory to prof. Michael Mezei one of the father of liposome technology.

INTRODUCTION

In the past decade many of the major drug manufacturers have been working on the dermal route of administration of an old or new drug entities. There are specific problems that could be solve with these formulations, exclusively. Avoiding the gastrointestinal side-effects or the first pass metabolism, decreasing the central toxicity of a substance, producing an extended therapeutic potential by a program released skin-depot formulation, are typical examples of these problems. Transdermal systems are used for local, indeed superficial action or for systemic distribution. The development of available transdermal drug delivery system with a designed characteristic warrants a fundamental understanding of formulations, and requires an optimization of pharmacokinetic and pharmacodinamic parameters. For characterising preformulated products many of *in vitro* systems are available.

Miyajima, et al. have developed a model membrane system for estimating drug permeability of the skin by fixing liposomes composed of stratum corneum (SC) lipids (ceramides, palmitic acid, cholesterol, and cholesterol-3-sulfate) onto a supporting filter, Biodyne B. The model membrane and guinea pig skin behave similarly in terms of permeability. The influence of SC lipid composition on permeability was also investigated. A lipid composition similar to that of the skin SC showed the lowest permeability for the hydrophilic drug. This model membrane system was found to be widely applicable for drug permeation studies (Miyajima et al., 1994).

Measurement of the rate of delivery *in vitro* permits: (i) Control of the uniformity of industrial production, but not comparison of systems designed on the basis of different concepts. (ii) Study of the kinetics of delivery during the stage of pharmaceutical development.

It is a fact that the *in vitro* test does not permit a reliable conclusion regarding the performance of the system *in vivo*, in particular in term of rate and extent of transcutaneous absorption.

To overcome most of the disadvantages of current models to investigate percutaneous penetration of drugs or toxic substances, Lange and coworkers proposed a model, based on the isolated pig ear, which is obtained at the slaughterhouse, and perfused with oxygenated blood from the same pig. It is concluded that this technique offers an easy to handle, cost-efficient, and animal-saving model for skin penetration studies that lacks most of the disadvantages of existing models (De Lange et al., 1992).

This model was used by Chang et al. (1994) for xenobiotics. Srikrishna et al. (1992) compared the result of *in vitro* method with the *in vivo* absorption of paraquat.

In general the pig ear skin becomes a common model for studying the skin penetration (Delange et al., 1994; Van Rooij et al., 1995). In a series of other experiments, the *in vitro* permeability of pig ear skin was compared with human (abdominal) skin and rat (dorsal) skin using both hydrophilic (water, mannitol, paraquat) and lipophilic (aldrin, carbaryl, fluazifop-butyl) penetrants. Pig skin was found to have a closer permeability character to human skin than to rat skin, particularly for lipophilic penetrants. Electrical conductivity measurements across pig skin membranes showed that skin conductivity could be a useful method for assessing the integrity of membranes, particularly when used in conjunction with water permeability assessments (Srikrishna et al., 1992).

In vitro method can be used to measure changes in the barrier properties of skin caused by *in vivo* or *in vitro* topical exposure to different chemicals. The effect of a test chemical could be assessed *in vitro* by measuring the penetration of ^{14}C benzoic acid following the exposure, euthanizing the pigs and skin sections excised from the sites of application. Skin sections mounted in an *in vitro* penetration chamber the cumulative 24 h penetration of ^{14}C benzoic acid can be measured. This *in vitro* method, which quantify the transdermal transport of benzoic acid, can be used to assess the effect of chemical or physical agents on barrier function and time course of return to normal barrier function of skin (Kemppainen et al., 1993).

The combination of *in vitro* studies with animal skin and human skin and studies *in vivo* in animals permits an approximate prediction of the rate of delivery in man, a rate which will then have to be verified in the first clinical studies.

In a series of publication Moody and coworkers compared the transdermal parameters in six animal species. In summary, the *in vitro* data underestimated the dermal absorption observed *in vivo* and tentative explanations for this lack of agreement are discussed (Kemppainen et al., 1991; Moody and Nadeau, 1993, 1994; Moody et al., 1994). Various *in vivo* study methods have been considered, but no agreement could be reached with regard to possible prerequisites. On one hand the question arises about the skin permeability of different species and of the animal to be selected whose skin is similar to that of humans. On the other hand the metabolic potential of selected species can differ from human. Furthermore in the animal studies the

difficulty of active ingredient analysis came up: it is very often difficult to assay the absorbed drug and/or its metabolites at the very low concentration which result from percutaneous administration. Usually the distribution in the different organs can be measured only by labeled material (Foong and Green, 1988; Brennan et al., 1991; Monteiro Riviere et al., 1993).

In studies with tracer labeled materials it is better to use small rodent, reducing the necessity of labeled material, and simplify the measuring of radioactivity. In case of bigger animals the standard sampling of organs and the preparation of samples for counting are much more complicated, time and money consuming.

Surber published that the oral administration of retinoids provides a successful therapeutic approach in various cutaneous diseases characterized by disturbed keratinization, e.g. psoriasis. Nevertheless oral therapy is often associated with systemic adverse effects. This makes a topical form with desirably none or reduced systemic side-effects. Direct application of a topical acitretin formulation to the skin might result in therapeutic skin concentrations while minimizing systemic exposure. In the hairless guinea pig and in the rhesus monkey the percutaneous absorption of ^{14}C-acitretin from an isopropylmyristate formulation were investigated *in vivo*. After a 24 h exposure drug concentration in the skin was higher in the hairless guinea pig than in the rhesus monkey. A similar observation was made comparing the 24 h absorption data determined as amount of drug excreted (Surber et al., 1993).

Generally minipig (Brennan et al., 1991; Oberle et al., 1994; Jimbow et al., 1995) or pig (Han +39) exceptionally monkey (Surber et al., 1993) have been suggested rather than dog or other animals used in the daily practice in the pharmacokinetic studies. The pig is a good animal model in biomedical research because it has a physiological similarity, including the skin permeability, to the human. Morris reviewed the characteristic parameters of pig skin like structure of the epidermis, distribution of proliferative cells, mitotic and labeling indices, cell cycle phase duration, age related changes in cell kinetics, stem cells, and cell kinetic organization and compared briefly with the human skin (Morris and Hopewell, 1990). Dittmar and Jovic (1987) studied the behavior of excised human and pig skin in different test systems of mycosis. They compared the action of many commercial antimicotic preparations in the tissue activity test with direct inoculation, in the horny layer and the deeper skin parts. Based on the results of various comparative tests they declared that pig skin can be used as a substitute for human skin testing antifungal activity of transdermal products.

The pig is a good species for comparative pharmacokinetic studies, too, but the possible metabolic differences should be considered.

The pharmacokinetics and metabolism of diclofenac sodium (Voltaren) were studied in four Yucatan minipigs after intravenous and oral administration and the results compared to historical data in human. The absolute bioavailability after oral administration solutions were around 100%, compared to approximately 50% in human. The total plasma clearance in minipigs was five times slower than in humans. The plasma levels of the diclofenac metabolites were considerably lower in minipigs than in man after both i.v. and oral administrations. These results suggest slower metabolism and/or enterohepatic recirculation of the parent drug in minipigs. The volume of distribution of the central compartment was 40% less in humans than in pigs. The terminal half-lives of the parent drug were similar in pigs and humans (Oberle et al., 1994).

For our studies two animal species guinea pigs and pigs have been selected and combined the tracer radioactive labeling was combined with classic analytical determinations for evaluation of biodisposition and pharmacokinetic property of new transdermal deprenyl formulations. In these animal systems we measured the transdermal biodisposition of patches and liposome formulations.

MATERIALS AND METHODS

Materials

Standard materials

(-)-amphetamine hydrochloride (CH-191)
Batch No.: TZ-3137a Purity: 99.89% Certificate No: KKO-B-3/003
(-)-methamphetamine hydrochloride (CH-2285)
Batch No.: Sz-4648-53 Purity: 99.0% Certificate (date): 25.03.1991
(-)-demethylselegiline hydrochloride (CH-2589)
Batch No.: TZ-2932a Purity: 99.7% Certificate No: KKO-B-1/001
1-phenyl-2-penthylamine hydrochloride (CH-584)
Batch No.: TZ-2599a_3 Purity: 99.2% Certificate: KKO-AB-188/380
L(-)-N-(phenyl-isopropyl)-N-Methyl-N-propynyl ammonium chloride
Batch No.: Ch 900506 Purity: 99.4% Certificate: KKO-B-8/010/B
The above standard materials were prepared by the Synthetic Research Laboratory IV of Chinoin.

(±)-[$^2H_4$]Selegiline.HCl
Certificate (date): 604/94 (02.05.1994)
The above standard material was prepared by the Institute for Drug Research Ltd. Budapest.

Solvents and reagents
n-Hexane: Chemolab; HPLC grade, distilled
Toluene: Reanal; alt.
Dichloromethane: Merck; chromatographic purity
Water: bidistilled
NaOH: Reanal; alt.
NaCl: Reanal; alt.
2,3,4,5,6-pentafluoro-benzoyl-chloride: Fluka, purity >99%(GC)

Deprenyl tablet
Jumex
Produced by CHINOIN-Budapest
Batch number: 5220891
Active ingredient [L-(-)-N(phenyl-isopropyl)-N-Methyl-N-propynyl-ammonium-chloride] content: 5 mg/tablet

Transdermal formulation
Deprenyl in silicone resin
Produced by Technological University, Budapest
Batch number: S/27
Active ingredient (Selegiline) content: 7.557 mg/cm^2

Deprenyl in liposome
Produced by BioZone California
Batch number: Dlip/1,2,3
Active ingredient content 10.1%

Analytical determinations

A gas chromatographic (GC-NP) method for the determination of the N-dealkylated metabolites of Selegiline in plasma samples was elaborated. For the determination of unchanged compound we elaborated a GC-MSD method.

On the basis of Selegiline and its main metabolites plasma concentration - time data - where it was possible - we report the pharmacokinetic parameters were calculated by non-compartmental analysis of obtained concentration data using the TopFit software.

The radioctivity

Gas chromatographic methods

Determination of metabolites (GC-NP method)

Equipment: HP 5890 gas chromatograph-NP detector
Column: HP-2 Fused Silica 25 m × 0.2 mm ID
0.11 μm film of cross-linked
5% Ph-Me-silicone as stationary phase
Gases: Carrier gas: high purity Nitrogen (head pressure 15 psi)
Detector gases: Air: 100 $ml.min^{-1}$
Hydrogen: 3.8 $ml.min^{-1}$
Auxiliary gas: high purity Nitrogen, 20 $ml.min^{-1}$
Sample injection: splitless mode, delaying time 1 min.
Temperatures:
detector: 300 °C
injector: 250 °C
Under the above conditions the examined materials eluted with the following retention times:

amphetamine	20.7 min.
methamphetamine	20.9 min.
demethylselegiline	22.7 min.
internal standard	23.6 min.

Determination of Selegiline (GC-MSD method)

Equipment: HP 5890 SERIES II gas chromatograph
HP 5971A Mass Selective Detector
HP 7673 automatic injector and controller
HP ChemStation (Hewlett Packard)
Column: HP-2 Fused Silica, 25 m × 0.2 mm ID
0.11 μm film cross-linked
5% Ph-Me-silicone as stationary phase
Gases: Carrier gas: high purity Helium (Ultra plus 6.0)
(head pressure 15 psi)
Sample injection: splitless mode, delaying time 1 min.
Temperatures:
injector: 280 °C
detector(interface): 280 °C

Under the above conditions the examined material eluted with 3.4 min. retention time

Formulations

Due to the presence of ^{3}H isotope material in both of the patches and liposome formulas we could conduct complete biodisposition studies in the guinea pigs.

Producing small quantity of labeled formulations

There are specific problems in producing radiolabeled formulations for biodisposition studies. The limited amount of labeled compound demands micro technology but with identical product character to the manufactured product. In case of patches we used a microchamber for the polymerization of the active ingredient into a silicon polymer. This microchamber is adequate to produce 10-50 cm^2 patch surface. The liposome was produced in a small glass vial, under similar condition like those in manufacturing. A potential binding of the labeled material to the surface of the container always has to be checked. In case a valuable amount of radioactivity absorbed to the wall of the vial, a silicon coating of reaction chamber could be helpful.

Analytical characterizations

The dissolution parameters of patches can be measured in diffusion cell, based on radioactivity. In case of liposomes we cannot follow the original large scale-process, but we could control the main parameters of liposome (encapsulation rate, distribution of) produced.

Determination of specific activity in the formulas

For the calculation of the drug concentration in each organ tissue it is necessary to determine the specific radioactivity in the samples (patch or liposome). In case of silicon patches one cm^2 of polymerized material was weighted and after a chemical digestion the radioactivity was measured by liquid scintillation counter. In a control vial we measured a constant amount of active ingredient in the presence of the similarly treated but empty silicon polymer. This sample was used for the quench correction.

In case of liposomes the scintillation fluid destroys the structure of liposomes and the radioactivity could be measure directly. The same quench correction is also needed.

Biodisposition studies

Experiments with guinea pig

Animals

Albino guinea pigs (approx. 300-350 g in weight) from Charles-River line were housed in individual polyethylene boxes and were fed ad libidum with standard food and water. Certified bedding free from any chemical pollution has been used. The temperature of the animal rooms was regularly checked and varied between 20 and 26 °C with mean 23 °C.

Preparation of skin surface for the sample application

There are two different possibilities: shaving the skin surface or use chemical peeling.

The shaving can damage the surface of the skin causing an increased permeability of active ingredient. According to Moy et al. (1996) the phenol peel causes inflammation and nonspecific reactions, and in addition, a proportionate amount of new collagen deposition. Glycolic acid and pyruvic acid cause minimal nonspecific reaction. However, the collagen deposition caused by glycolic acid and pyruvic acid disproportionately increases suggesting a direct stimulatory effect of the two agents.

In our experiments before the application of transdermal products the hairs of guinea pigs were clipped off (but not shaved), and an area of 3.2 × 3.2 cm was marked on the back close to the head.

Doses

In the first series of experiments we applied 100 mg of multilamellar liposome to the back of animals on a piece of 2 cm^2 of Parafilm. This type of liposome was designed for a controled release of drug delivery over a longer period of time. In a second set of experiments the dose-activity relationship was studied using a much smaller dose range, i.e., 10-50 mg of the liposome product also on a piece of 2 cm^2 of Parafilm.

The Parafilm layer was covered by Tegaderm (produced by 3M) for fixing the samples. This covering was removed 2 hours after the beginning of treatment. The application surface was wiped out with ethanol moistened gauze. For determining the non-absorbed amount of liposome product we measured the radioactivity of the covering material (Parafilm and Tegaderm) and on the wiping gauze.

In a series of experiments we did not use any covering material, but one hour after the beginning of treatment we wiped out the remaining part of the liposome.

In case of silicon patches a piece of polymer containing 30 mg tracer labeled active ingredient was fixed to the back of animal using Tegaderm covering material. The covering material and the patch were removed 24 hours later at the end of the experiment. The non-absorbed amount of active ingredient was measured on the basis of remained radioactivity in the patch and on the covering material.

Sampling

The radioactivity content of blood, brain, lung, liver, spleen, heart, kidney, intestine, large intestine, stomach and the skin at the site of application was measured.

Chronic blood sampling

Blood samples were taken at various time intervals i.e. -0.5, 0, 6, 24, 48 and 144 hours from the corner of eye of animals and were collected into a tube containing 100 IU heparin. Three parallel aliquots of 50 µl were transferred into a cuvette containing 0.75 ml of Solune-350 and the same volume of isopropanol. After solubilization the samples were blenched by additing 0.5 ml of hydrogen peroxide and incubated at 60 °C for 1 hour. Twenty-four hours later 10 ml of Aquasol/0.5 M. HCl (9:1) liquid scintillation solution was added to the sample. The radioactivity was determined after the disappearance of chemiluminescence (on the following day). The remaining portion of plasma samples were transferred into ampoules, and were deep frozen (-20 °C) for later processing. At the end of the seventh day, 168 hours after the application, the animals were exterminated by deep ether narcosis. Blood samples were taken by direct punctation from the heart.

Preparation of organs

The removed organs and tissues were weighed and homogenized in an Ultra-Turax homogenizer with 4 volumes of physiological saline solution. There was 3 × 5 µl aliquots of homogenate pipetted into cuvettes containing 2 ml of Solune 350 and 0.5 ml of isopropanol. The following steps of sample processing were the same as those described for the blood previously. The radioactivity content of organs were determined by liquid scintillation counting, which represented the total amounts of parent compound and its

metabolites. The rest of the homogenates were frozen for further control analysis.

Preparation of skin
At the end of the experiment we dissected the skin at the surface of application. The tissue was weighed and homogenized as the organs and the sample was prepared the same way as in case of blood. Because the wiping process after removing the covering material, this sample represents the active ingredient deposited into the skin.

Experiments with pigs

These studies were performed in an FDA audited place, following the directions of GMP but not registered as GMP study.

Animals
Hungarian Big White pigs, bread on the animal farm of Csemô with a mean body weight between 20-25 kg were used for our initial experiments. The animals arrived this place one week before the experiment was started. The animals were healthy according to the veterinary observation, and according to the occasional laboratory investigation. They were housed in 1.8 m^2 individual polypropylene boxes with a stainless-steel, perforated floor. The special design of the floor protects against the coprofagia, which could disturb the result of experiments and permits the collecting of urine and faeces separately for further analysis if it is necessary. The animal boxes were cleaned daily, but at the beginning of the experiment were sterilized. The animals received constant pig food and tap water ad libidum.

Preparation of skin surface for the sample application
The samples were applied behind each ear on an area of approx. 5 × 4 cm and covered with Tegaderm. Before the application the hair was removed with a hair clipper from the area behind the ears. The surface of the skin was gently wiped out with an ethanol moistened gauze.

Doses
In the pig experiments we compared the bioavailability of 0.4 mg/kg i.v. dose and 2 mg/kg p.o. dose with 2.0 g transdermal dose of liposomal products.

I.v. treatment: The iv. dose was applied in two different ways. First by an injection directly to the vena cava cranialis in 1 ml solution. In a second series of experiment we used a chronically implanted heart catheter. The catheter was introduced in short-term (20 min.) anaesthezis one day before the experiment by a normal human subclavia set, through the dexter part of vena cava cranialis. This chronically implanted catheter permits administration of any volume and concentration of material in safely and under well-controlled condition directly into the heart.

P.o. treatment: The oral dose of material was applied in capsule or tablet form by an esophageal tube. The animals were fixed and through a mouth-dilator an esophagus tube was conducted directly into the verticulum. The capsules containing the test substances or the tablets were placed in the lumen of the tube and were transmitted into the gastrointestinal tract by flooding with 100 ml of tape water.

Transdermal treatment: The transdermal dose (2 × 1.0 g liposome) was applied behind each ears on an area of approx. 5 × 4 cm and covered with Tegaderm, containing 6 mg/kg active ingredient. The Tegaderm was fixed by a bandage around the neck. This bandage and the covering material Tegaderm was removed 2 hours after the beginning of treatment. That time the surface of treatment was wiped down once again by alcoholic gauze and this gauze and the covering Tegaderm were transmitted to the analytical laboratory for determination of the non-absorbed material.

Blood sampling

The blood was taken from the left or right side of vena cava cranialis by punction. For this process the animals do not need any adjuvant medication, namely they were held their back for 10-20 seconds. The stress caused, day by day decreased, and finally the animals tolerated easily the blood sampling procedure. In case of unwanted haemathomas another place the vena jugularis dex. or syn. is available. During our experiments we could collect easily 5-20 ml of blood samples 16 times without any side-effects or dangerous symptoms. The blood was collected into a tube containing 100 IU heparin. after the centrifugation the separeted plazma was deep-frozen for the HPLC determination of active ingredient and metabolit content.

RESULTS

Biodisposition study on guinea pigs

In a series of experiment we applied 100 mg of multilamellar liposome, formulated according to Mezei with tracer ^{3}H radioactivity label (containing 10% active ingredient) to the back of guinea pigs on a piece of 2 cm^2 of Parafilm covered by Tegaderm (produced by 3M). After the liposome encapsulation we determined the specific activity of the formula. Table 1 represents an example of these determinations. We measured the radioactivity of an aliquot of preformulated test material on two different radioactivity counter equipment. On bases of the average values of these measurements we calculate the specific radioactivity of formulas and the nominal applied dose on the animals.

The removed covering materials were extracted four times by alcoholic solvent and the radioactivity measured by liquid scintillation counting. On the basis of 27 sample the extraction efficacy was 71.23±11.4 after the first, 88.58±10.41% after the second, 97.82±12.7% after the third extraction. We can conclude that, after three consecutive extraction of covering material more than 97% of radioactive material content were extracted and the non-absorbed part of dose could be calculated. Relatively high amount (18.58±4.8%) of drug, expressed as percentage of dose, was remained on the Tegaderm (which was used to cover the site of application and was removed 2 h after the application). Other cases when we rubbed the liposome into the skin without the use of any covering material the liposomes penetrated the skin very fast and only 8.75±3% was the remaining extractable active substance on the wiping gauze. These results suggest, that in future experiments other films, or tapes should be tested, if any needed.

Comparison of different formulation

Due to modifying the constituent of liposome we can also influence the absorption and retardation of active substance. Fig 1 represents the result of a typical experiment for selection of liposome formulation on guinea pigs, measured at 168 h after a single application of a program released multilamellar liposomal formulations. The different formulations produced a different drug penetration profile into the skin and organs.

Table 1. Measurement of specific activity of different liposome deprenyl formulation and calculation of the applied nominal dose

Sample		mg/DPM	mg/DPM	Mean	Spec. act.	Animal	Dose	
	mg	Equip. 1.	Equip. 2.	(mg/DPM)	μCi/mg	No.	(mg)	μCi/animal
Formula I.	6.2	1349665	1486957			1	101.4	67.63
		1448136	1539014			2	103.5	69.03
		1451935	1582903			3	98.2	65.50
	12.8	1326028	1459883	1466280	0.667	4	100.5	67.03
		1437031	1538264			5	103.4	68.97
		1401797	1573750			6	99.4	66.30
Formula II.	3.9	1830795	1969689			7	103.3	88.11
		1895043	2040940			8	99.5	84.87
		1933333	2074359			9	100	85.30
	6.2	1656238	1815471	1877172	0.853	10	98.7	84.19
		1733925	1888369			11	102.7	87.60
		1762742	1925161			12	101.6	86.66
Formula III.	9.9	1512927	1668250			13	99.5	75.42
		1621953	1728552			14	98.9	74.97
		1683333	1738586			15	101.2	76.71
	7.2	1542752	1709841	1669608	0.758	16	102	77.32
		1628580	1752747			17	99.8	75.65
		1643611	1804167			18	102.1	77.39

The patches are designed for a shorter drug delivery because of their necessary presence on the skin surface during the treatment. To keep a patch on the body longer than 24 hours is inconvenient and because of the possible damages of the patch a change of constant rate of drug delivery can also occur.

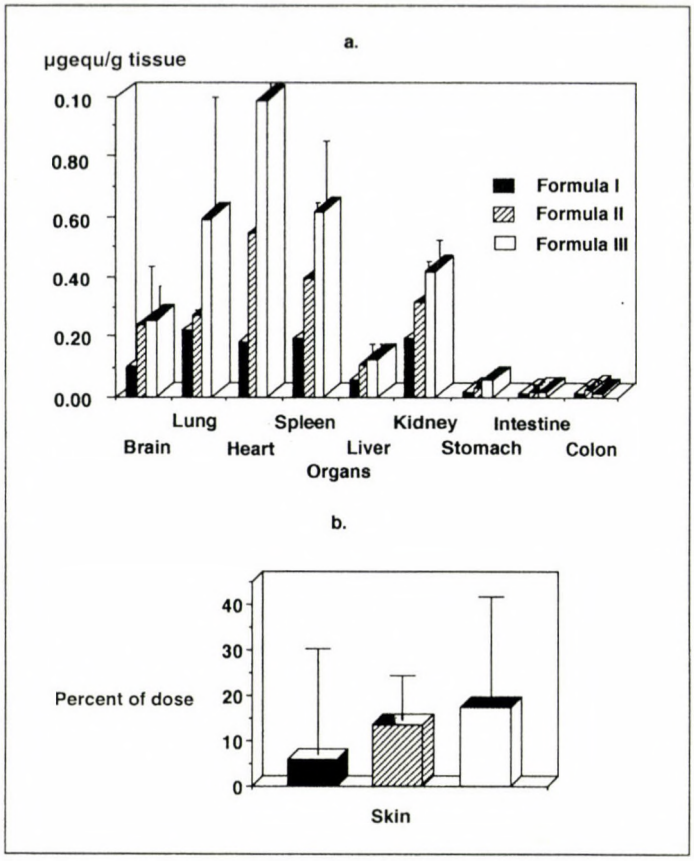

Fig. 1. Concentration of deprenyl in organs (a) and skin (b) of guinea pigs treated with 100 mg (10 mg active ingredient) dose of different multilammellar liposome formulations after 168 hours treatment

Figure 2 shows the result of a same experiment with a tracer labeled silicon patch formulation. This formulation ensures a similarly good drug targeting within 24 hours, but without any accumulation of active ingredient in the skin.

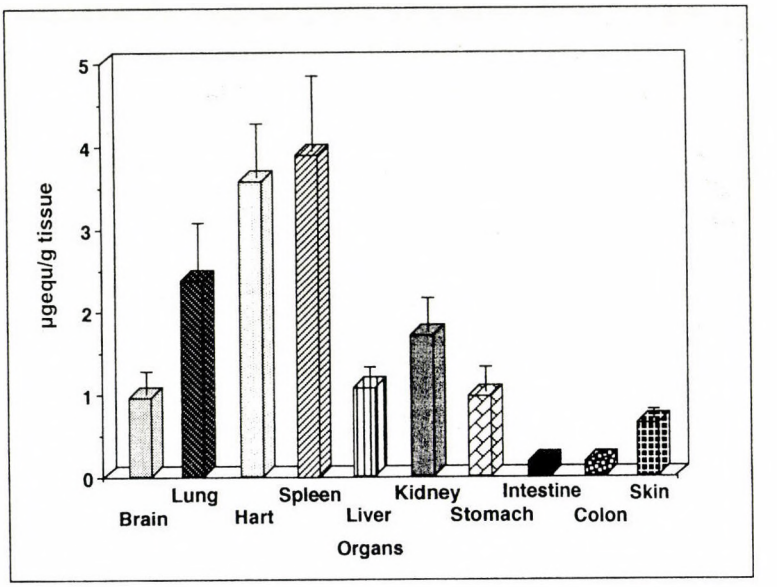

Fig. 2. Distribution of radioactive material (parent compound and metabolite) in the organs and skin of guinea pigs treated with a 2 cm^2 silicon patch (containing 30 mg deprenyl) 24 hours after the treatment

Table 2. Parent compound and metabolites content of pig plasma after a single dose (6 mg/animal) liposomal formulation of deprenyl

Hours	Deprenyl		Metamphetamine	Amphetamine	Desmethyl deprenyl
	Average	±SD	Average	Average	Average
0	0	0	N.D.	N.D.	N.D.
1	70.4	16	1	N.D.	N.D.
2	130.3	24	3	N.D.	0.9
4	156.5	18	4.8	N.D.	0.9
8	93.2	19	6.4	N.D.	N.D.
24	23.5	9.7	1.7	N.D.	N.D.
48	8.1	1	2.6	N.D.	N.D.
72	7.5	1.6	N.D.	N.D.	N.D.
96	17.3	4	2.9	N.D.	N.D.
120	9	6.9	0.8	N.D.	N.D.
144	26.9	6.2	1.1	N.D.	N.D.
168	2.92	5.2	1.6	N.D.	N.D.

(ND= Not detectable, below the detectation limit)

Dose dependence study on guinea pigs

This experiment was designed to test of dose dependence of liposomal transdermal products, in small doses like 10, 30, 50 mg (containing 10% active ingredient).

In Fig. 3 the active ingredient concentrations of blood are shown in the percentage of dose (a) and in μg equivalent (b). The equivalent means the sum of parent compound and possible metabolites content of the organs in absolute values. The same examination in case of brain, lung, spleen, liver and skin are shown in Fig. 4.

Comparing these two calculations it seems to be clear that the relative absorption is not proportional with the dose. The liposome formulation was absorbed better in a smaller dose than in a bigger one. It might be also influenced by the surface of application, because the larger dose needs a relatively large surface area and during our experiment this was constant, about 6-7 cm^2, limited by the covering material, Tegaderm.

Biodisposition studies on pigs

We compared the bioequivalece of the selected liposome formulation with the i.v. and oral dose of active ingredient. This study can give closer information about the expectable human profile of transdermal formulation. We treated six animals with 0.4 mg i.v. dose of active ingredient by chronically implanted catheter, another six animals with 2 mg/kg dose in capsule administered per os through esophageal tube.

The third group of six animals was treated with 2 g liposomal product containing 6 mg/kg active ingredient. The concentration of parent compound and metabolites after a single dose liposome formulation are summarized in Table 2. MAO-A and -B inhibition was measured in the blood platelet, in the brain and intestine (Table 3).

The pharmacokinetic parameters after different doses and surface area of application are collected in Table 4.

Figure 5 summarizes active ingredient contents of the blood in these comparative experiments. The plasma concentration clearly indicates the sustained release character of liposome product. The half-life ($t_{1/2}$) of the active ingredient was 0.085 hour by i.v. route, 2.4 hours at oral, and 26.7 hours by transdermal application.

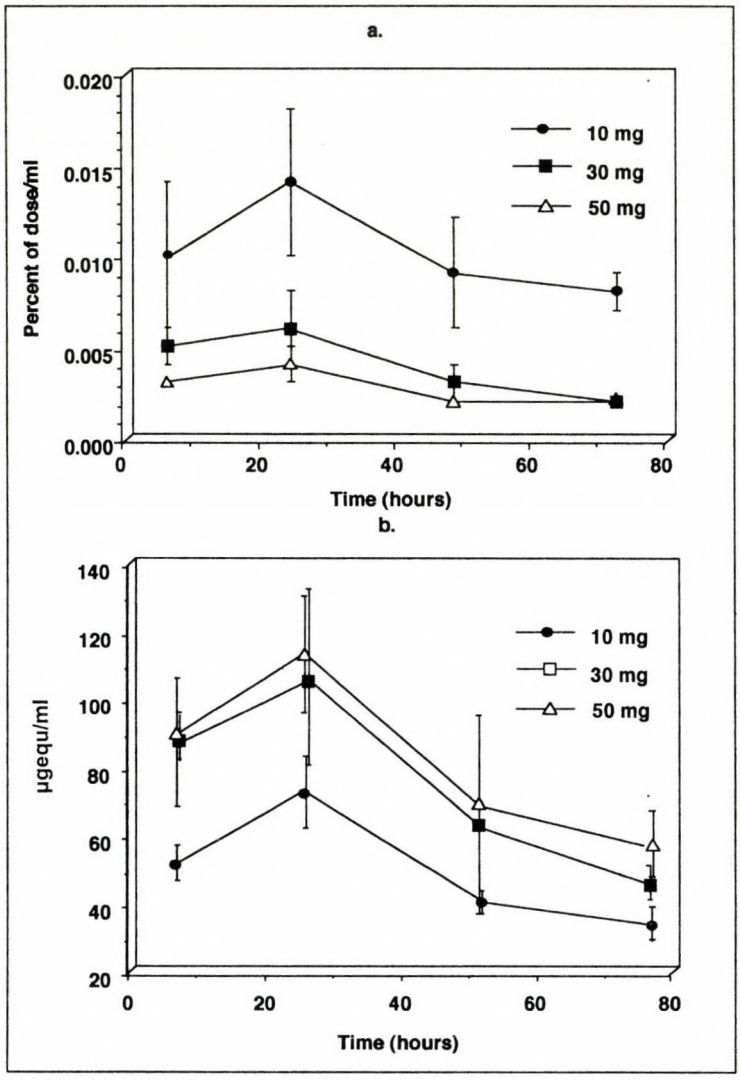

Fig. 3. The radioactivity content of guinea pig blood in percent of dose (a) and in µg(equ)/ml (b) after a treatment with different transdermal dose of liposomal preparation

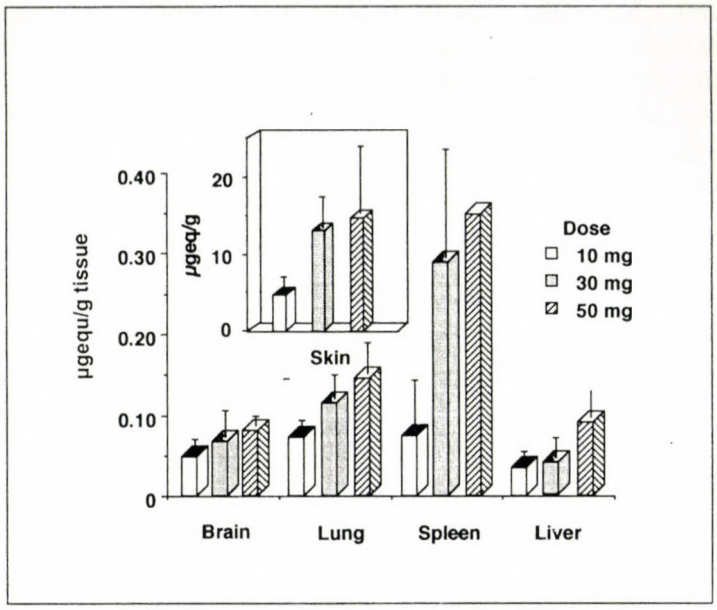

Fig. 4. The radioactivity content of guinea pig organs and skin in µg(equ)/g tissue after a treatment with different transdermal dose of liposomal preparation

Table 3. Inhibition of MAO-A and -B in pig organs after 168 hours of different dosage forms and doses of deprenyl

Dose	Brain		Liver		Small intestine	
Substrate	PEA	5-HT	PEA	5-HT	PEA	5-HT
50 mg/kg tablets p.o.	34.56±4.38	3.07±5.31	53.32 ±0.48	10.36 ±1.04	6.41±11.09	17.9±9.07
10 mg/ animal t.d.	51.71± 3.20	17.91± 3.98	46.07 ±7.41	11.81 ±5.99	1.27±2.19	7.25±9.05
20 mg/ animal t.d.	52.22±11.2	21.13±13.22	41.59 ±8.29	10.94 ±8.53	34.02 ±31.0	5.21±9.02
40 mg/ animal t.d.	53.80± 5.85	17.44±5.86	61.84 ±5.49	9.90±5.74	48.96±9.47	2.35±4.07
Control (mg /prot./h)	10.05± 0.39	4.78±0.29	122.3 ±4.56	12.68 ±0.55	19.93±8.87	22.86±4.62

(t.d.= transdermal)

Table 5 summarizes the relative blood concentrations 6 hours after the treatment (in percent of dose) in case of oral and transdermal dosage forms. Based on this comparison the absorption of liposomal product is not worse than the oral one, but the distribution is completely different. Considering these differences, in the level of organs the transdermal application is equivalent with the oral one.

CONCLUSION

We developed a system to analyze the biodisposition profile of different transdermal formulation. From the radio labeled studies on guinea pigs we can estimate the rate of absorption and the distribution in the body.

The topical liposomal form of active substances provided relatively high drug concentration in the brain, and in the different organs indicating that the liposome formulations are applicable not only for topical use. The liposomal formulas tested can provide sustained release property, consequently adequate drug concentration was maintained for a long period of time.

Table 4. The pharmacokinetic parameters after different doses and application surface of liposomal deprenyl

Dose Surface	AUC_{0-24}	AUC_{0-inf}	C_{max}	t_{max}	E rate	$t_{1/2}$
10 mg 32 cm^2	341.1 ± 82.5	453.4 ±50.7	34.4 ±13.9	1.0 ±0.0	0.059 ±0.016	12.2 ±2.9
10 mg 64 cm^2	359.0 ±191.3	453.1 ±257.4	28.3 ±9.2	4.9 ±3.1	0.072 ±0.009	9.7 ±1.1
20 mg 32 cm^2	535.6 ±119.3	701.2 ±176.3	42.6 ±10.2	5.7 ±4.0	0.063 ±0.005	10.9 ±0.8
20 mg 64 cm^2	481.6 ±60.8	612.8 ±90.1	37.1 ±6.5	4.7 ±3.1	0.066 ±0.003	10.2 ±0.4
40 mg 32 cm^2	685.7 ±137.6	851.4 ±234.4	53.1 ±5.7	4.0 ±0.0	0.068 ±0.007	10.3 ±1.1
40 mg 64 cm^2	752.3 ±302.5	967.6 ±417.4	60.1 ±24.0	4.7 ±3.1	0.067 ±0.009	10.4 ±1.5

(AUC= area under curve, C_{max}= maximal concentration, t_{max}= time of the maximal concentration, E rate = rate of elimination, $t_{1/2}$= half life)

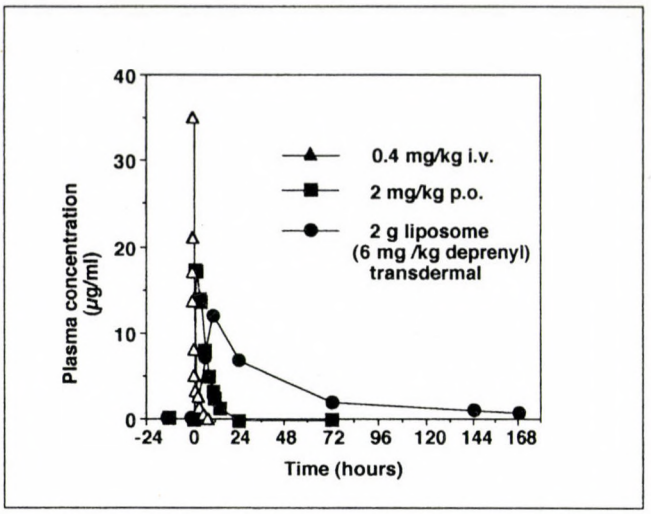

Fig. 5. The active ingredient content of the pig plasma after i.v., oral and transdermal route of administration

Table 5. Comparative pharmacokinetic parameters of different dosage forms of deprenyl applied on guineapigs, pigs and human orally and transdermally at 6 hours

Species	Mode of application	Dose mg	Weight	Dose mg/kg	Blood conc. (6 hours) ng/ml	D%/ml
Guinea pig	transderm.	5	300 g	16.6	89.32	1.78E-03
		3	300 g	10.0	86.41	2.88E-03
		1	300 g	3.3	53.01	5.30E-04
Pig	p.o.	5	25 kg	0.2	3.7	7.20E-05
Pig	p.o.	20	25 kg	0.8	11.0	5.50E-05
	p.o.	50	25 kg	2.0	26.8	5.36E-05
Pig	transderm.	200	22 kg	9.1	2.04	1.02E-05
		100	22 kg	4.6	10.4	1.04E-05
		50	22 kg	2.3	2.9	5.80E-06
Human*	p.o.	10	70 kg	0.14	12.0	1.20E-04

* Data from the literature.

One, who is knowledgeable in transdermal drug delivery, can expect a very low percentage of dose absorbed (e.g., the total drug absorption from Estraderm during one week of application is less that 15% of dose, and it is

still a very successful drug product!). As expected, low percentage of the transdermally applied doses appear in the blood. Due to their lipophylic property, they were quickly removed from the blood, to the more lipophylic tissues, since blood is an aqueous compartment. Obviously, the results, is that a relatively high drug level can be expected in the organs, even at 168 hours after a single application. The Fig. 1b and Fig. 4 insert illustrates that a valuable amount of the dose remained within the skin after 168 hours, producing a retard depot formation.

If this phenomena is considered, one should expect different relationship between drug concentration in the blood and other sites of the body, in liposomal form, compared to conventional dosage forms.

It is striking that a relatively low portion of dose appears in the gastrointestinal tract and in the liver after the transdermal application. This means, we can produce liposome formulation to replace oral dosage forms, thus avoiding gastrointestinal side-effects. The results also indicate advantages of transdermal route of administration avoiding the "first pass effect". The parent drug is not metabolized at the extent, as the orally administered one.

The second component of our evaluation system is the pig model. The 22-25 kg piglets were found adequate for a secondary screening of selected formulations. In accordance with the literature (Krishnan and Abraham, 1994) this medium sized animals are applicable for both comparative pharmacokinetic studies and also for measuring the efficacy of formulation by biochemical or pharmacological parameters.

Based on the blood curves the transdermal formulation satisfies the criteria of long-time retardation.

The present study clearly indicate the feasibility of developing a transdermal dosage form of deprenyl. The topical liposomal form of deprenyl provided relatively high drug concentration in the brain, which is the site of action.

All of the liposomal formulas tested provided sustained release, consequently adequate drug concentration was maintained for at least for 72 h in the blood and brain, while the oral tablet form may maintain concentration only for approximately 16 h.

The liposomal form avoid the “first pass effect" consequently the parent drug is not metabolized at than extent, than the orally administered one (rescue).

MAO-A and -B activities were favorably and selectively (more in the brain and less in the gastrointestinal tract) influenced by the liposomal transdermal form.

Compared to the oral tablet form, the liposome-encapsulated deprenyl has a greater affinity to the brain and other lipophilic tissues (because of its lipophilic property) the pharmacokinetic parameters, especially drug disposition favorably changed. Therefore we can expect a greater and wider action of the transdermally applied deprenyl that of the orally administered one.

According to the data of Table 5 the models are applicable for the preclinical evaluation of potential transdermal products.

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RESEARCH REPORT

AN AUTORADIOGRAPHIC METHOD OF VISUALISING SEMICARBAZIDE-SENSITIVE AMINE OXIDASE ACTIVITY IN MOUSE TISSUE SECTIONS

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Under the influence of semicarbazide-sensitive amine oxidase (SSAO), methylamine is deaminated to formaldehyde, which can react with various macromolecules and form irreversible adducts. We hereby present an autoradiographic method of visualising SSAO activity by measuring the *in vivo* formation of such adducts from ^{14}C-methylamine. Our results revealed high concentrations of radioactive deposits in the intestinal wall, brown adipose tissue, spleen and bone marrow. Hydralazine is a potent SSAO inhibitor and pretreatment with this irreversible inactivator resulted in a nearly complete loss of radioactive deposits in the tissues. By giving ^{14}C-methylamine at different time-points after irreversible inhibition of SSAO, it was also possible to determine the resynthesis rate of SSAO. Interestingly, the recovery rate of SSAO after such inactivation was tissue-specific. The possible therapeutic value of a specific SSAO inhibitory drug has been discussed.
Keywords: SSAO, BzAO, VAP-1, methylamine, formaldehyde, diabetes mellitus

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INTRODUCTION

The enzymes of the semicarbazide-sensitive amine oxidase (SSAO; EC. 1.4.3.6) family can be found in living organisms of any biological order, and in many species several different forms of SSAO have been reported to co-exist (for review, see Buffoni, 1993; Callingham et al., 1995). However, the kinetic properties and the substrate specificity of the enzymes differ markedly both between and within different species. The SSAO enzymes are characterised by being irreversibly inhibited by semicarbazide and other carbonyl agents and by requiring copper and TOPA-quinone as cofactors. Another characteristic is that they catalyse a double-displacement reaction to produce an aldehyde and hydrogen peroxide. This definition of SSAO defines a very heterogenous group of enzymes that includes not only the benzylamine oxidases (BzAO), but also the diamine oxidases (DAO). Commonly, the term SSAO is used synonymously with BzAO, i.e. DAO is not comprised in the term.

The wide distribution of SSAOs in nature suggests that these enzymes should have some important biological function. However, what this function or these functions are, is still largely unknown and we can only speculate in the matter. It has been observed that hydrogen peroxide produced in SSAO-catalysed reactions, is involved in the translocation of the glucose transporter, Glut4, to the plasma membrane in an insulin-mimicking way that may be of physiological importance (Enrique-Tarancon et al., 1998; Marti et al., 1998).

Presently, two forms of human SSAO - one placental (Zhang and McIntire, 1996) and one retinal form (Imamura et al., 1997) - are cloned. The placental form of SSAO has recently been shown to be identical to vascular adhesion protein 1 (VAP-1), cloned from smooth muscle of human ileum (Smith et al., 1998), and this has provided us with new aspects as to what the normal physiological function of SSAO might be. VAP-1 is a so-called homing protein and is expressed upon inflammation in order to attract lymphocytes to an inflamed area.

In certain pathological conditions, such as diabetes mellitus and uraemia, the activity of SSAO is significantly increased and it has been speculated that this might be deleterious for the patient (Ekblom, 1998). The speculations have arisen due to the fact that the end-products in SSAO-catalysed reactions often are more cytotoxic than the substrates. Both aromatic and aliphatic primary amines are substrates for human SSAO and among these are the endogenous substances methylamine and aminoacetone (for a review, see Callingham et al., 1995). In case of methylamine, the aldehyde produced is formaldehyde. *In vitro* studies have shown that methylamine in itself is relatively non-toxic towards

cultured endothelial cells, but that it becomes cytotoxic in the presence of SSAO (Yu and Zou, 1993). This is most likely the result of the formaldehyde-formation, since formaldehyde can bind covalently with macromolecules and cause crosslinking with proteins and DNA, forming adducts (Casanova-Schmitz et al., 1984). The levels of methylamine found in the circulation of normal healthy individuals are very close to the levels causing cytotoxicity *in vitro*. Methylamine levels have been shown to be increased in the blood of diabetic patients.

In the present work, we have used an autoradiographic method to study the metabolism of methylamine *in vivo*. To evaluate the autoradiographic method as an indirect method of visualising SSAO activity, we have compared the occurrence of radioactive deposits with the distribution of specific SSAO activity. Moreover, we have estimated the resynthesis time ($T_{1/2}$) for SSAO after irreversible inhibition of the enzyme by the antihypertensive drug, hydralazine.

MATERIALS AND METHODS

Animal experiments

All animal experiments were performed on male NMRI mice weighing 35-40 g (6-9 weeks old), purchased from B&K Universal AB (Sollentuna, Sweden). The animals were housed in wire cages with 3 mice in each cage, and they had free access to food and water. They were kept in a room with a window and were, therefore, exposed to normal day/night cycles. The experiments were carried out with the permission of the local animal ethical committee (No C56/97).

Determination of specific amine oxidase activities

To determine the specific activities of SSAO, monoamine oxidase A (MAO-A) and monoamine oxidase B (MAO-B), mice were treated with two i.p. doses of 2.0 mg hydralazine hydrochloride (Sigma Chemical Co., St. Louis, MO). Control animals were treated with saline in a similar way. The 2 doses were given 24 hours apart and the mice were killed 24 hours after the last injection of hydralazine hydrochloride. Different organs were dissected and immersed in cold, 50 mM Tris buffer, pH 8.0 (0.1 g tissue/ml buffer). The tissues were homogenised in this solution with an Ultra-Turrax homogeniser for 30 seconds and were thereafter kept at -70 °C until the time of analysis. The analyses were performed using a radiochemical assay as described previously (Tufvesson, 1969; Gronvall et al., 1998). Briefly, 50 µl tissue homogenate (10% w/v) was used in each reaction and 100 µM ^{14}C-benzylamine was used as a substrate. The

reaction was carried out in presence of clorgyline and deprenyl (10 μM each) for inhibition of MAO-A and MAO-B, respectively. Addition of the SSAO inhibitor, semicarbazide (1 mM), reduced the estimated activity with more than 90 %. Specific SSAO activities were calculated by subtracting the activity in the reaction with semicarbazide present, from the activity obtained in the reaction without this SSAO inhibitor.

Autoradiography

The mice used in the autoradiographic study were treated with hydralazine hydrochloride and saline as described above.

Twenty-four hours after the last injection of hydralazine hydrochloride, 3 of the hydralazine-treated mice were given an i.p. injection with 350 nmol, 20 μCi ^{14}C-methylamine (Amersham Life Sciences, Buckinghamshire, England). These mice are referred to as 'day 1'-mice. The following injections of ^{14}C-methylamine were done at day 2, 4, 7 and 14, with 3 mice each day. The controls that were not treated with hydralazine were injected with the ^{14}C-methylamine on the same day as the 'day 1' mice.

The mice were killed with CO_2 24 hours after the injection with ^{14}C-methylamine, and frozen at -20 °C. They were then frozen in cubes with carboxymethyl cellulose (CMC) and one day later, 20 μm thin sagittal tissue sections were cut in a cryomicrotome. The sections were collected on adhesive tape and dried at -20 °C for at least 24 hours before exposing them on X-ray film. The cryosectioning, autoradiography and image analysis was carried out essentially as described before (Ekblom et al., 1992; Gronvall et al., 1998).

Statistical analysis

The group data on the effect of hydralazine treatment on SSAO specific activity was statistically analysed by a two-tailed Student's t-test.

RESULTS

Specific amine oxidase activities in different tissues

Specific activities of SSAO were estimated in homogenates of tissues dissected from adult mice. We found that the activity was especially high in salivary glands and in the testicles and low in skeletal muscle, brain and liver. Intermediate activities were found in the heart, lungs, spleen, kidneys and pancreas (Fig. 1a). Treatment with hydralazine hydrochloride (2 × 2.0 mg/animal) resulted in a major reduction of SSAO activity ($P < 0.001$). The specific activities of MAO-A and MAO-B were not affected by hydralazine treatment (data not shown).

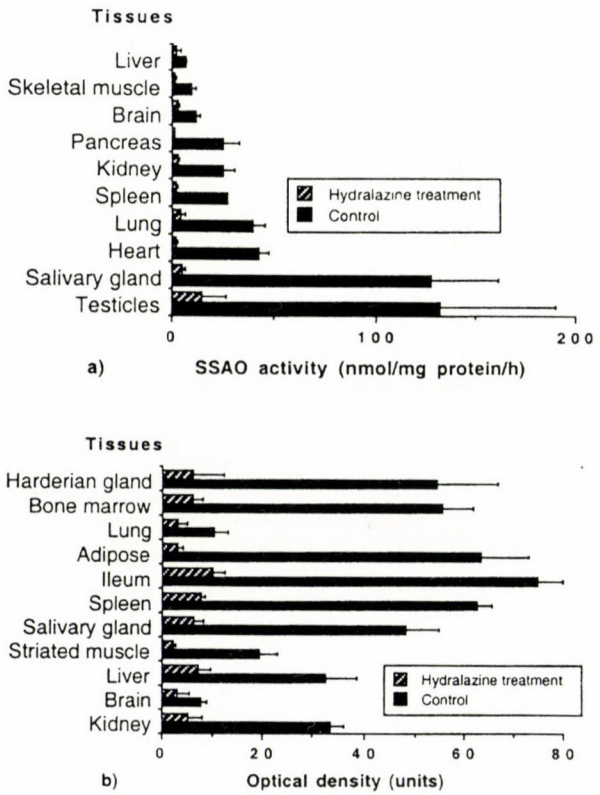

Fig. 1. Graphs showing the inhibition by hydralazine on a) SSAO activity and b) optical density

Tissue distribution of radioactive residues

The autoradiographic studies showed the presence of long-lasting residual radioactive deposits after administration of ^{14}C-methylamine (Fig. 2a). The amount of deposits varied strongly in the different mouse tissues. Densitometric analyses of the films showed high densities of radioactive deposits in ileum, brown adipose tissue, spleen, bone marrow, salivary gland and kidneys (Fig. 1b). Moreover, considerable amounts were also seen in the cortex of the adrenal gland and in the Harderian gland. The CNS was essentially devoid of deposits.

The effect of hydralazine treatment was highly evident at 'day 1' where the radioactive accumulation was close to the level of detection in most tissues (Fig. 1b).

Resynthesis of SSAO after irreversible inhibition

We were able to follow the recovery of SSAO activity after irreversible inhibition, by giving ^{14}C-methylamine at different time-points after the administration of hydralazine. The levels of radioactive residues were dependent on the time between inhibitor treatment and ^{14}C-methylamine injection as a reflection of enzyme activity normalisation. By analysing the optical density on the autoradiographic films, we found that the normalisation time for SSAO activity differs between different tissues. As a means of comparison, we estimated the time when half the original activity was restored ($T_{1/2}$). In ileum, $T_{1/2}$ was approximately 2.5 days, whereas in brown adipose tissue it was estimated to 9.2 days (Fig. 3).

The densitometric analyses also indicated that there might be a compensatory action of SSAO after inhibition. The optical density is generally higher in the 'day 14' mice than in the controls (Fig. 4). For some tissues, i.e. kidneys and ileum, these 'day 14' levels were statistically elevated as compared to controls ($P < 0.005$ and $P < 0.05$, respectively). However, since not all tissues have reached their control levels of optical density at 'day 14' (Fig. 3), and since the changes are relatively small, it is from our data impossible to statistically prove any compensation in most tissues investigated.

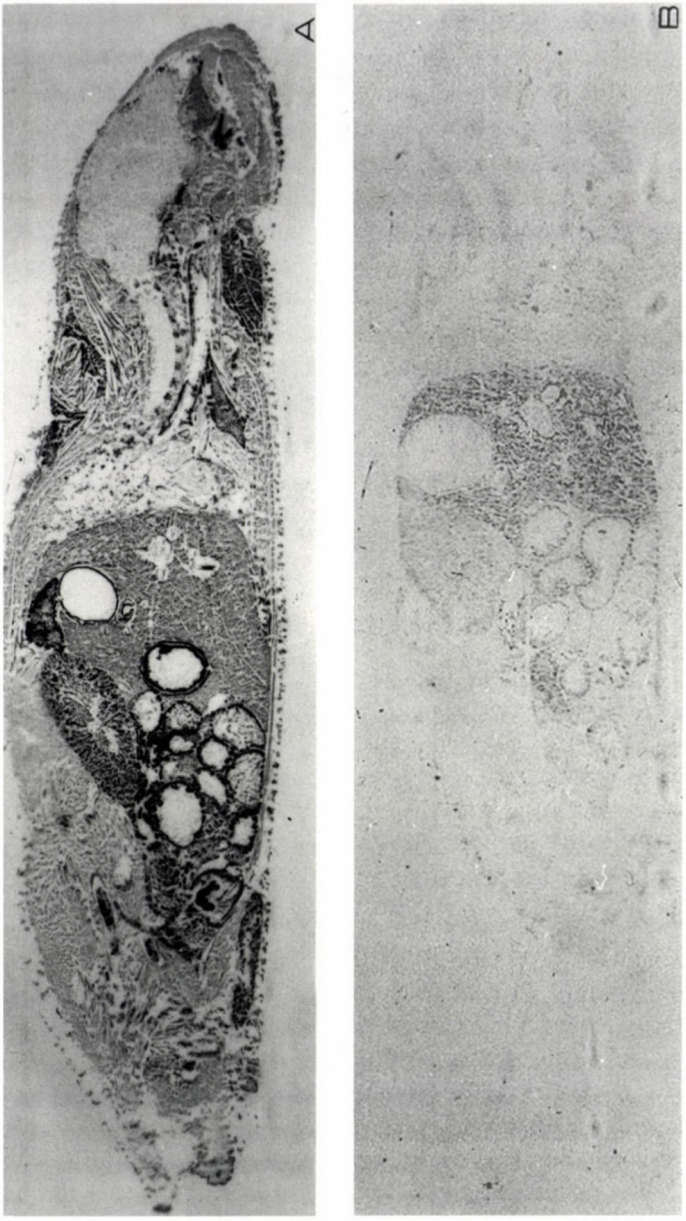

Fig. 2. Autoradiographic imaging of radioactive deposits in whole-body section of a) control and b) hydralazine-treated mouse

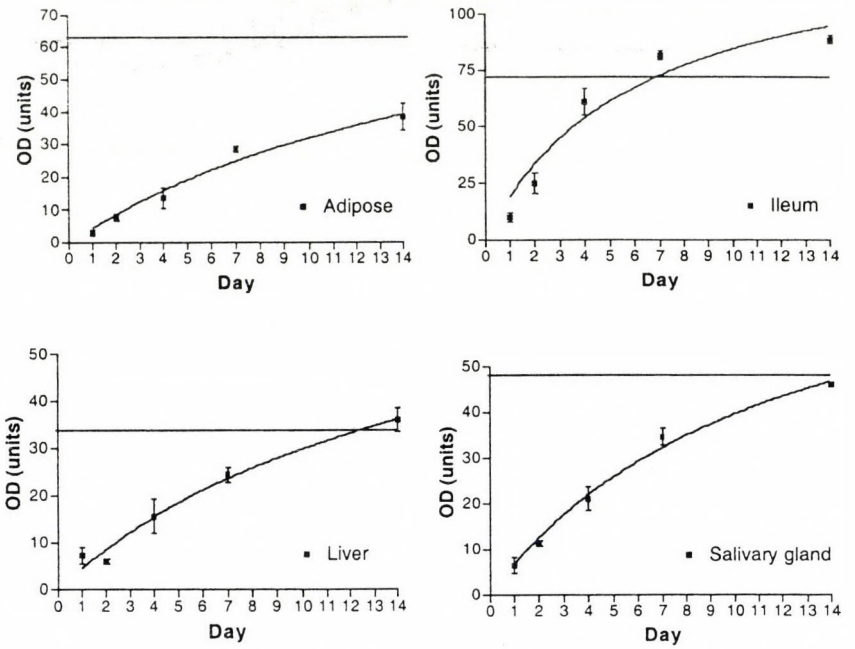

Fig. 3. Graphs showing the resynthesis of SSAO in different tissues after irreversible inhibition as measured by mean optical density ± standard deviation. The horizontal lines show the mean OD in the controls

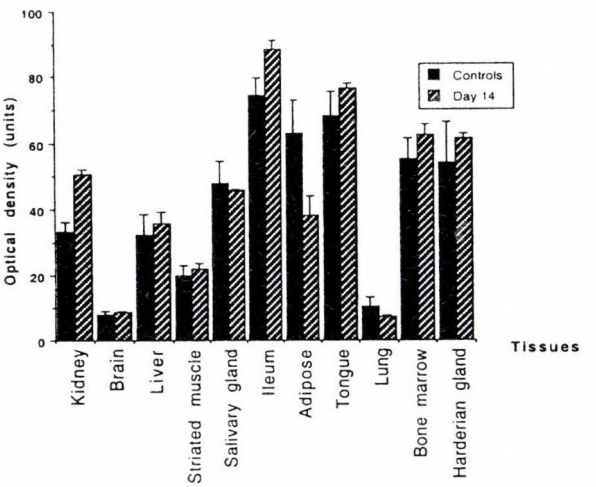

Fig. 4. Graph showing optical density in control animals and 'day 14' animals ± standard deviations

DISCUSSION

SSAO activities have in previous studies on rodents (mainly rat) been observed to be high in adipose tissue, eye, vascular smooth muscle and lung (Lewinsohn et al., 1978; Ryder et al., 1979; Cao Danh et al., 1985; Lyles and Singh, 1985). Data regarding activities in mouse tissues may not be directly compared with data of rat tissues since differences in the properties of SSAO between species have been reported to be considerable. Nevertheless, our findings are principally in accordance with findings reported by other investigators (for review, see Buffoni, 1993). We also estimated the activities in tissues not investigated before with regard to SSAO, such as the salivary gland, testicles and pancreas.

The autoradiographic method we present here showed a high degree of reproducibility and the sample-to-sample variability was below 10%. By using this whole-body autoradiography we obtained very detailed information regarding the distribution of formaldehyde deposits, since the spatial resolution was below 100 μm. However, a comparison with the SSAO activity measurements shows that high SSAO activities does not always coincide with high OD areas. Considering this, the autoradiographic method described here is not an appropriate method for determining absolute values for SSAO activity. Different organs may be exposed to varying amounts of substrate and/or product due to differences in blood perfusion, leading to differences in formaldehyde incorporation. Another possible explanation may be that the abundance of aldehyde dehydrogenases and glutathione (for glutathione-dependent aldehyde dehydrogenases) varies between different organs, and consequently the fraction of formaldehyde that is further metabolised is tissue-specific. It is also possible that the organs differ with regard to how efficiently formaldehyde forms adducts, i.e. differences in content of 'target molecules'. Nevertheless, studies to pinpoint the location of potentially harmful effects from SSAO may be monitored by this indirect autoradiographic method. It would be highly interesting to investigate if ^{14}C-methylamine autoradiography may be performed *in vitro*, since this would allow studies on postmortem human tissues.

By using autoradiography, we were able to follow the recovery-rate for SSAO in different tissues after an irreversible inhibition. This recovery of enzyme activity is presumably related to synthesis of new enzyme protein. The time for normalisation of SSAO activity was tissue-specific, e.g. it was restored rapidly in the smooth muscle of the ileum, whereas a much slower recovery was observed in brown adipose tissue. One may speculate that factors controlling the synthesis of SSAO differ in an organ-specific way. Furthermore,

considering that already two forms of human SSAO have been cloned, it cannot be excluded that the SSAO activities seen in these tissues represent different forms of enzymes, with different resynthesis rates.

Considering the apparent involvement of SSAO in diabetes, many investigators have proposed that treatment with a specific SSAO inhibitor may reduce or prevent development of late-diabetic complications such as retinopathies. More *in vivo* studies are needed to support such a hypothesis, but the circumstantial proof for the concept is growing stronger. The use of SSAO inhibiting drugs in other diseases suggests that inhibition of SSAO is not associated with serious side-effects. Carbidopa, benserazid and hydralazine are SSAO inhibiting drugs used clinically. However, these drugs lack specificity for SSAO and are therefore not suitable for treating diabetic patients.

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RESEARCH REPORT

(-)DEPRENYL (SELEGILINE): PAST, PRESENT AND FUTURE

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(-)Deprenyl (Selegiline), the N-propargyl analogue of (-)methamphetamine, is the only drug in clinical case which, by enhancing the impulse propagation mediated release of noradrenaline and dopamine in the brain (catecholaminergic activity enhancer, CAE, effect), keeps in small doses without side-effects the catecholaminergic brain system on a higher activity level. (-)Deprenyl stimulates the catecholaminergic neurons selectively in the brain because, in contrast to PEA and the amphetamines which induce the continuous release of noradrenaline and dopamine from their intraneuronal stores, (-)deprenyl is devoid of this property. It is due to the CAE effect that a) the maintenance of rats on (-)deprenyl during the postdevelopmental phase of their life slows the age-related decline of sexual and learning performances and prolongs life significantly; b) patients with early, untreated Parkinson's disease maintained on (-)deprenyl need levodopa significantly later than their placebo-treated peers, and when on levodopa plus (-)deprenyl, they live significantly longer than patients on levodopa alone; and c) in patients with moderately severe impairment from Alzheimer's disease, treatment with (-)deprenyl slows the progression of the disease. It is reasonable to expect that a prophylactic low dose administration of a safe catecholaminergic activity enhancer substance during the postdevelopmental phase of life will slow the age-related decline of behavioral performances, delay natural death and decrease susceptibility to Parkinson's disease and Alzheimer's disease.

Keywords: (-)deprenyl, selegiline, catecholaminergic activity enhancer (CAE) effect, catecholamine releasing effect, Parkinson's disease, Alzheimer's disease

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The pharmacological spectrum of (-)deprenyl

(-)Deprenyl was the first and is still the only CAE substance in clinical use free of the catecholamine releasing property. We may say in retrospect that the peculiar pharmacological spectrum of (-)deprenyl was clarified between 1965 and 1996 in three steps.

Step 1. It was proved that the compound, though a close structural relative of methamphetamine, is free of the catecholamine releasing property (Knoll et al., 1965,1968);

Step 2. It was proved that the compound is a highly potent and selective inhibitor of MAO-B (Knoll and Magyar, 1972);

Step 3. It was proved that the compound is a CAE substance, an enhancer of the impulse propagation mediated release of catecholamines in the brain (Knoll et al., 1996a).

The CAE mechanism and the role of (-)deprenyl in clarifying it

Food deprived rats in the late developmental phase of their life (2 months of age) are significantly more active than those in their early postdevelopmental phase (4 months of age) pointing to enhanced catecholaminergic activity during the developmental phase. In addition, both in male and female rats, the basic activity of the catecholaminergic and serotoninergic neurons in the brain was found to be significantly higher between weaning and the end of the 2nd month of age, i.e. during the crucially important developmental phase of their life, than either before of after that period (Knoll and Miklya, 1995). The finding that the brain constituents, â-phenylethylamine (PEA) and its indol-analogue, tryptamine, are both enhancers of the nerve stimulation induced release of ^{3}H-noradrenaline (Fig. 1), ^{3}H-dopamine (Fig. 2) and ^{3}H-serotonin (Fig. 3) from the isolated rat brain stem furnish ample evidence for the operation of a previously unrevealed 'catecholaminergic and serotoninergic activity enhancer (CAE/SAE)' mechanism in the central nervous system (cf. Knoll, 1994, 1997). Interestingly tryptamine is enhancing the release of ^{3}H-serotonin in a low, 0.25 µl/ml concentration and is ineffective in a tenfold higher concentration (see Fig. 3). CAE substances enhance the slow inward Ca^{2+} current in the sino-auricular fibers of the frog heart and regarding this effect we observed a similar concentration dependency. Further support for the hypothesis that a CAE mechanism works in the brain was the finding that high performing rats are more sensitive toward CAE compounds than their low performing peers (Knoll et al., 1996b).

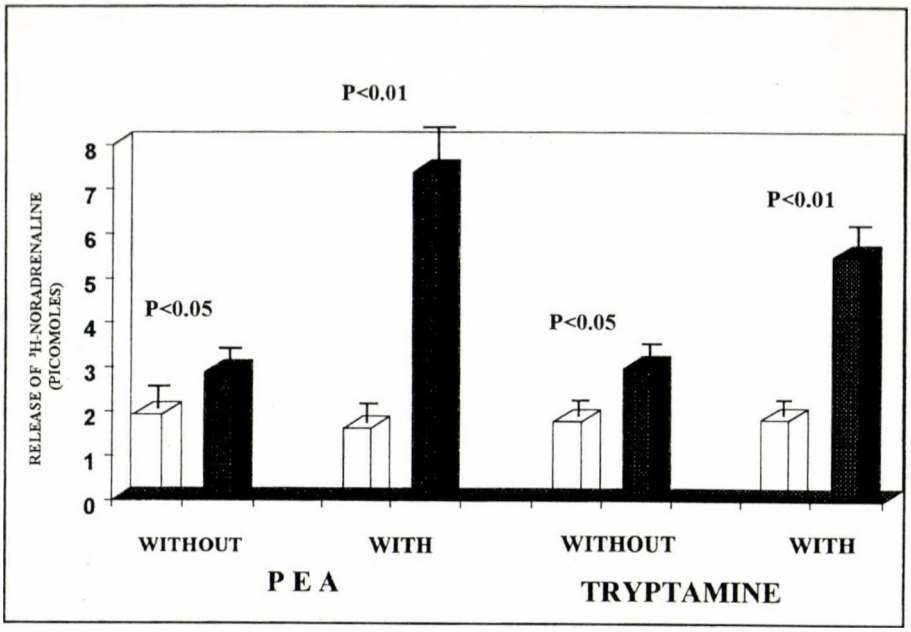


Fig. 1. Release of ^{3}H-noradrenaline from isolated rat brain stem before (open columns) and after (filled columns) electrical stimulation, in the absence and presence of 2.5 µg/ml PEA (N=8) and tryptamine (N=8), respectively. Each column represents the amount of ^{3}H-noradrenaline in picomoles released in a 3 min collection period (for methodological details see Knoll et al., 1996c). Vertical lines show S.E.M. Paired Students's t-test

We assume that a CAE/SAE mechanism plays a decisive role in brain activation, operates on a higher activity level from weaning to sexual maturity, drops back thereafter to the preweaning level and is subject during the postdevelopmental phase of life to a slow and continuous age-related decline until death (Knoll, 1994, 1997).

We demonstrated later that PEA and tyramine enhance in low concentration the impulse propagation mediated release of the catecholaminergic transmitters in the brain (CAE effect) and initiate the outflow of catecholamines from their neuronal stores (releasing effect) in higher concentration only (Knoll et al., 1996c). Amphetamine and methamphetamine, the PEA analogues with a long-lasting effect, act like their parent compound. It is characteristic to the amphetamines that the (-)enantiomers are more potent CAE substances than the (+)enantiomers, while, as well known, the contrary is true for the catecholamine releasing property of these drugs (Knoll et al., 1996c).

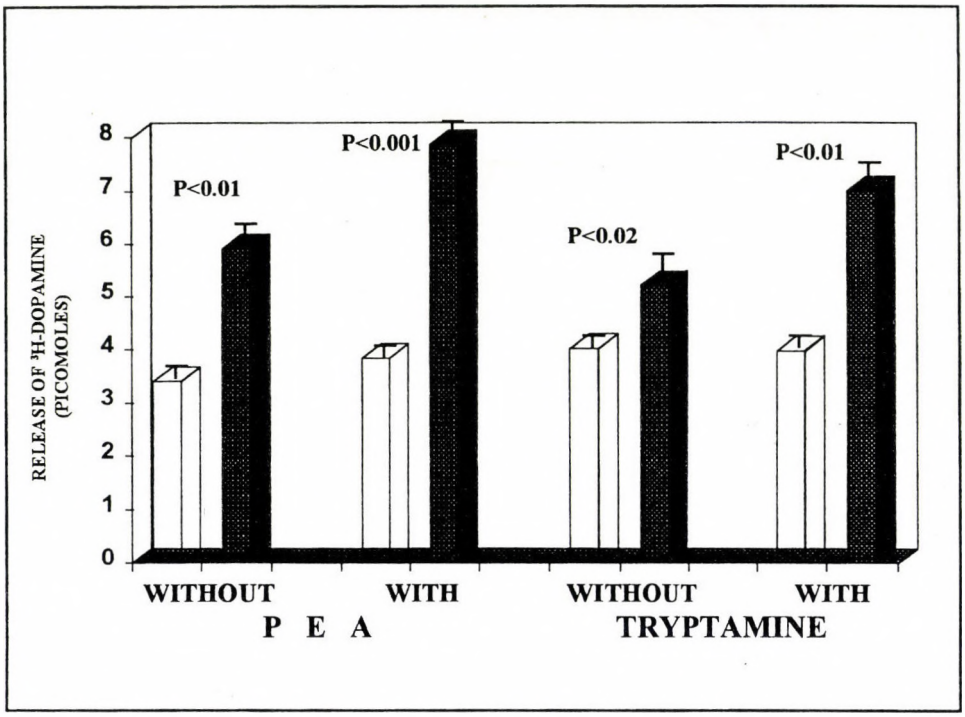


Fig. 2. Release of ^{3}H-dopamine from isolated rat brain stem before (open columns) and after (filled columns) electrical stimulation, in the absence and presence of 2.5 µg/ml PEA (N=8) and tryptamine (N=8), respectively. Each column represents the amount of ^{3}H-dopamine in picomoles released in a 3 min collection period (for methodological details see Knoll et al., 1996c). Vertical lines show S.E.M. Paired Students's t-test

The discovery of the CAE mechanism set our earlier experiences with (-)deprenyl in their true light. It is now obvious that when in the early 60s, aiming to develop a new MAO inhibitor (Knoll et al., 1965), we attached a propargyl group, a relatively bulky substituent, to the nitrogen in methamphetamine, we unintentionally eliminated the catecholamine releasing property of methamphetamine, by maintaining its CAE effect. At the same time, by adding a propargyl group to methamphetamine we also succeeded to produce a selective inhibitor of MAO-B, an experimental tool of crucial importance for research. (-)Deprenyl became world wide known as the first selective inhibitor of MAO-B and is still used as the reference substance for inhibiting this enzyme. Though thousands of papers were published on (-)deprenyl, the fact that this drug was the first and is still the only PEA/amphetamine derived CAE

substance in clinical use devoid of the catecholamine releasing property, remains to be generally realized and acknowledged. As an enhancer of catecholaminergic activity in the brain (-)deprenyl is more potent than (+)deprenyl (Knoll et al., 1996a).

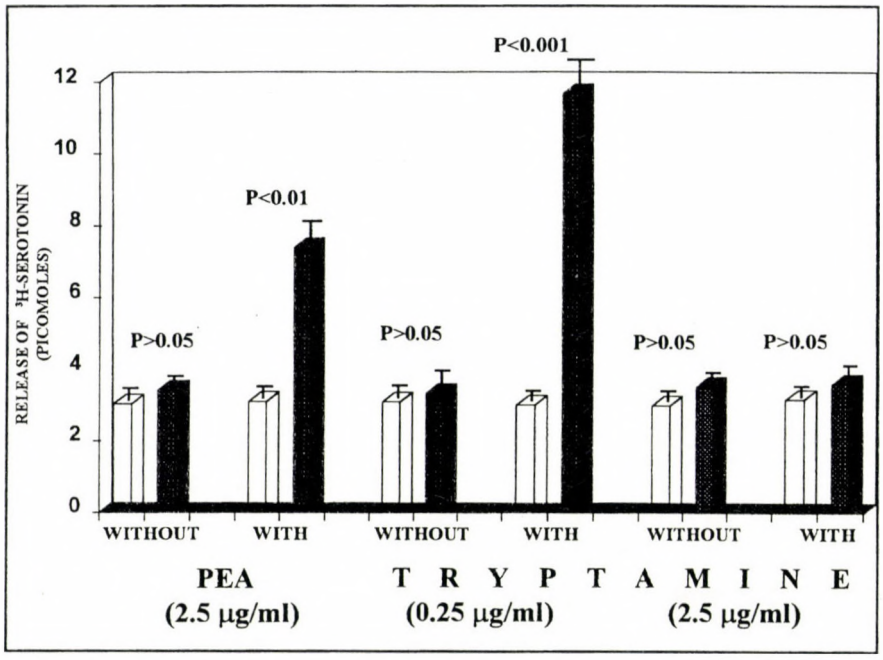
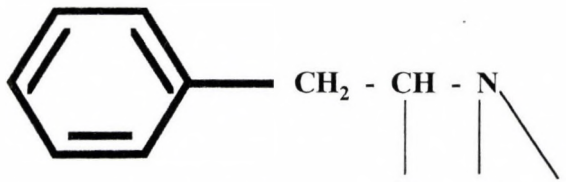


Fig. 3. Release of ^{3}H-serotonin from isolated rat brain stem before (open columns) and after (filled columns) electrical stimulation, in the absence and presence of 2.5 μg/ml PEA (N=8) and 0.25 and 2.5 μg/ml tryptamine (N=8 each), respectively. Each column represents the amount of ^{3}H-serotonin in picomoles released in a 3 min collection period (for methodological details see Knoll et al., 1996c). Vertical lines show S.E.M. Paired Students's t-test

Figure 4 shows the structure of PEA, amphetamine, methamphetamine, deprenyl and 1-phenyl-2-propylaminopentane (PPAP), the deprenyl analogue free of MAO inhibitory potency (Knoll et al., 1992a). PEA, a CAE substance and a releaser of catecholamines, is a short-acting substance because it is metabolized by MAO-B. Amphetamine and methamphetamine act like PEA, but because they are not metabolized by MAO, they are longer-acting CAE substances and releasers of catecholamines than PEA. (-)Deprenyl, the



				CAE effect	Catecholamine releasing effect	Relation to MAO
Phenylethylamine (PEA)	H	H	H	+	+	substrate to MAO-B
Amphetamine	CH_3	H	H	+	+	weak inhibitor
Methamphetamine	CH_3	CH_3	H	+	+	weak inhibitor
1-phenyl-2-methyl-N-methyl-propargylamine; (-)Deprenyl	CH_3	CH_3	C_3H_3	+	0	potent selective inhibitor of MAO-B
1-phenyl-2-propylamino-pentane; (-)PPAP	C_3H_7	H	C_3H_7	+	0	0

Fig. 4. Comparison of the chemical structure and pharmacological spectrum of PEA, amphetamine, methamphetamine, (-)deprenyl and (-)PPAP

methamphetamine variant containing a relatively bulky substituent, a propargyl-group, at the nitrogen, is a more potent CAE substance than methamphetamine but free of the catecholamine releasing property. As the propargyl-group in deprenyl makes a covalent binding with the flavin in MAO-B, the compound is a highly potent and selective inhibitor of this enzyme. It is clear by now that the attachment of a relatively bulky group to PEA at the α-carbon and/or nitrogen eliminates the catecholamine releasing property and the CAE effect survives. This conclusion is further supported by the finding that PPAP which differs from PEA by containing two propyl-groups, is a potent CAE substance. We developed PPAP in 1992 to demonstrate that the elimination of the MAO inhibitory capacity of deprenyl does not change the ability of the compound to keep the catecholaminergic neurons in the brain on a higher activity level.

(-)Deprenyl research enlightened the existence of the CAE/SAE mechanism, this previously unknown regulation of exocytosis in the catecholaminergic and serotoninergic neurons in the brain. PEA and tryptamine are examples of endogenous amines which enhance the impulse propagation mediated release of noradrenaline, dopamine and serotonin in the brain. Tryptamine represents an endogenous amine which is a CAE substance, but in contrast to PEA it is devoid of the catecholamine releasing property. (-)Deprenyl and (-)PPAP are the first examples of synthetic PEA derivatives which are free of the catecholamine releasing effect. Figuer 5 shows, as an example, the dopaminergic activity enhancer effect of (-)deprenyl in comparison to PEA. As a CAE substance (-)deprenyl is more potent its parent compound, methamphetamine.

An analysis of the pharmacological spectrum of (-)deprenyl clearly indicates that the remarkable benefits of the long-term, small dose administration of (-)deprenyl in animals (the slowing of the age-related decline of sexual and learning performances and the prolongation of life) and in patients with Parkinson's disease and Alzheimer's disease (the slowing of the progression of these illnesses) are due to (-)deprenyl induced enhanced catecholaminergic, mainly dopaminergic activity in the brain (for review see Knoll, 1998).

The finding that tryptamine is a potent CAE substance free of the catecholamine releasing property opens the way for the development of new CAE substances unrelated to the PEA/amphetamine structure.

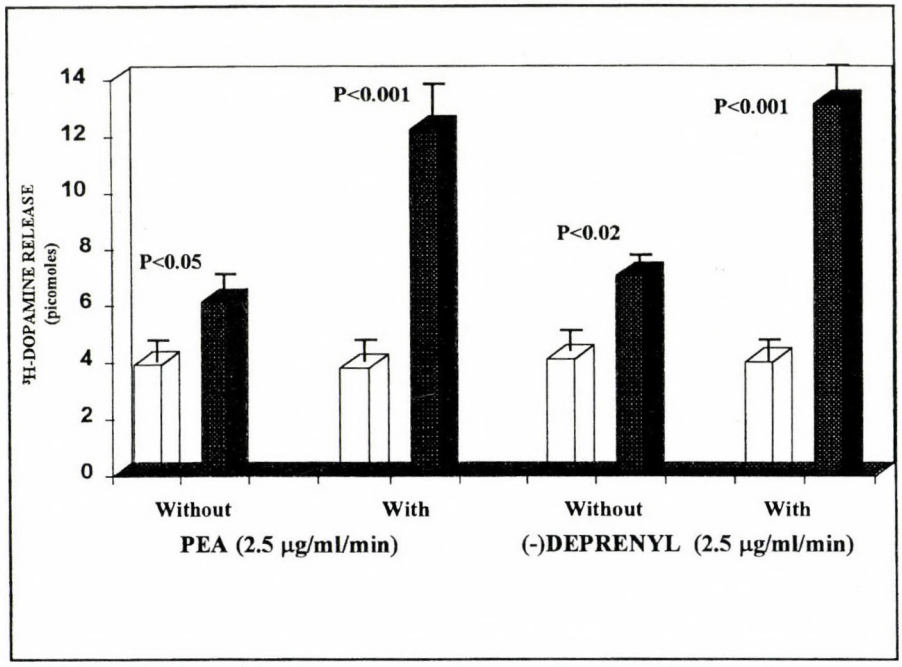


Fig. 5. Release of ^{3}H-dopamine from isolated rat brain stem before (open columns) and after (filled columns) electrical stimulation, in the absence and presence of 2.5 µg/ml PEA (N=8) and (-)deprenyl (N=8), respectively. Each column represents the amount of ^{3}H-dopamine in picomoles released in a 3 min collection period (for methodological details see Knoll et al., 1996c). Vertical lines show S.E.M. Paired Students's t-test

The age related decline of the nigrostriatal dopaminergic system and its functional consequences

In the human, striatal dopamine content and the number of nigrostriatal neurons are known to decline rapidly beyond the age of 45 years (cf. Birkmayer and Riederer, 1983). According to our present knownledge the nigrostriatal dopaminergic neurons are the most rapidly aging neurons in the human brain. The dopamine content of the human caudate nucleus decreases steeply, at a rate of about 13-15% per decade over age 45. We know that symptoms of Parkinson's disease appear if the dopamine content of the caudate sinks below 30% of the normal level.

About 0.1% of the population over 40 years of age develop Parkinson's disease, and prevalence increases sharply with age. Thus, normal aging of the system is slow enough to avoid the appearance of Parkinsonian symptoms within the average lifespan for 99.9% of the human population. In 0.1%,

however, the striatal dopaminergic system deteriorates at an enormously fast rate. This small part of the population crosses the critical threshold and manifests the symptoms classically described by James Parkinson in 1817. Parkinson's disease may be regarded as a premature rapid aging of the striatal dopaminergic machinery of unknown origin.

The onset of Parkinson's disease is extremely rare in the first decades of life. Only about 10% of patients begin having the disease before the age of 50 years. Parkinson's disease is the best example of an age-related neurological disease, and it is the only one for which the biochemical pathology, striatal dopamine deficiency, is firmly established. Viral infection, immunological abnormality, enzyme defects, and toxins have been proposed as the causes of Parkinson's disease, but the fundamental cause of the illness is still unknown. Is Parkinson's disease necessarily an ordinary disease caused by special pathogenic factor(s)? Based on the well-established nature of age-related deterioration of the striatal dopaminergic system, the answer to this question is not necessarily positive.

Because of the lack of constancy of physiological age, we know that the variance within a particular age group for any measurable parameter is large. In cross-sectional studies, no single age emerges as the point of sharp decline in function. Any individual may show different levels of performance, and the careful observer finds many dissociations between 'chronological' and 'physiological' age. A 70-year-old man, for example, may retain sexual performance equal to 40-year olds. Therefore, perhaps those who display the 'shaking palsy' represent that 0.1 % of the normal population who possess the most rapidly aging variants of nigrostriatal dopaminergic neurons and live long enough to pass the critical threshold for precipitating the symptoms we may search in vain for a specific cause of Parkinson's disease. Experiences with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are in complete harmony with this view. It was an accidental observation that the highly specific lesioning of the nigrostriatal dopaminergic neurons in young individuals by MPTP precipitated the full-scale symptoms of Parkinson's disease. The MPTP experience raised new hopes and initiated feverish activity to find the specific endogenous or exogenous 'MPTP-like' toxin which causes Parkinson's disease. The search was up-to-the-present unsuccessful.

With regard to the striatal system, dopamine ifself might play a main role in the unusually drastic age-related changes of the nigrostriatal dopaminergic neurons. Eighty percent of brain dopamine is localized in this system. The complex auto-oxidation of the large amounts of dopa and dopamine in the striatum, continuously generating substantial quantities of toxic free radicals and highly reactive quinones, create a permanent danger for the nigrostriatal

dopaminergic neuron, which has to mobilize its natural defensive measures against these toxic metabolites.

Neuromelanin, which is generated via the polymerization of oxidative products of dopamine with the evident aim of finally depositing waste products, is, in the substantia nigra, the visible sign of successful selfdefense of the neuron against the free radicals and quinones originating from dopamine metabolism. The sluggish depositing of neuromelanin in the human substantia nigra is in excellent agreement with this view.

Aging of the nigrostriatal dopaminergic neurons is essentially similar in rodents and in humans. Direct biochemical evidence for the age-related decline of striatal dopaminergic function in the rat is the loss of striatal D_2-receptors in the aging rat brain (cf. Knoll, 1998).

In looking for the functional consequences of the age-related decline of the nigrostriatal dopaminergic neurons, we have to consider the physiological role of these neurons in the continuous activation of the cerebral cortex (cf. Mink and Thach, 1993; Standaert and Young, 1996). This is realized via a highly complicated route of connections (cf. Albin et al., 1989). All in all, a more active nigrostriatal dopaminergic system means a more active cerebral cortex and vice versa, the physiological age-related decline of the nigrostriatal dopaminergic activity leads to an equivalent reduction in the activity of the cerebral cortex for review see Knoll, 1998). Under natural conditions proper functioning of the nigrostriatal dopaminergic system ensures that the animal will be ready to surmount every obstacle, even if life is in the balance, to seize its food or reach its sexual partner.

It is reasonable to conclude that the age-related decline of the nigrostriatal dopaminergic brain mechanism plays a significant role in the decline of performances with the passing of time. Aging of the dopaminergic system in the brain plays an undisputable leading role in the highly significant, substantial decline in male sexual activity and also in the more modest but still significant age-related decline in learning performance. In a human male study the median coital activity was the highest, 2.1/week, between ages 30-34 and decreased progressively with increasing age, sinking to 0.2/week ($P<0.001$) in the 65-69 year age group (Martin, 1977). We found essentially the same trend of changes in male rats in different series of experiments (Knoll 1988; Knoll et al., 1983, 1989).

We measured the age-related decay of sexual performance in male rats by selecting the best performing individuals from a huge population and copulatory activity was tested once a week during three consecutive 36-week periods. In a group of 49 rats an average of 14.04 ± 0.56 ejaculations were displayed during

the first 36-week period, dropping to 2.47±0.23 during the third 36-week period (Knoll et al., 1994). Also the learning performance of the same rats, tested in the shuttle box, declined significantly with the passing of time. The total number of conditioned avoidance responses displayed during the first 36-week period was 78.45±3.11 and decreased to 50.67±2.99 ($P<0.001$) during the third 36-week period.

Considering the functional consequences of age-related nigral changes it is reasonable to assume that safe and effective prophylactic medication is needed to slow these changes.

(-)Deprenyl protects the nigrostriatal dopaminergic neurons against selectively acting neurotoxins

A proof of the neurotoxic effect in rats treated with 6-hydroxy-dopamine is the enhanced release of acetylcholine from the striatum. We demonstrated that the administration of (-)deprenyl prevents the change in acetylcholine release in rats treated with 6-hydroxy-dopamine. This was the first experimental evidence that (-)deprenyl protects the nigrostriatal dopaminergic neurons against a selective catecholaminergic neurotoxin. (-)Deprenyl competes with 6-hydroxy-dopamine for the amine transporter and prevents the effect of this neurotoxin by inhibiting its uptake the sympathetic neuron (Knoll, 1978; Hársing et al., 1979). According to a recent study (-)deprenyl protects in young and aged rats also the peripheral sympathetic neurons from the toxic effect of 6-hydroxy-dopamine (Salonen et al., 1996). It was shown in 1984 that (-)deprenyl prevents, via inhibiting MAO-B, the formation of the toxic metabolite, MPP^+ protects thereby the striatum of monkeys from the effect of the highly selective striatal toxin, MPTP (Cohen et al., 1984), and a protection against MPP^+ was also described (Vizuete et al., 1993; Wu et al., 1993). (-)Deprenyl has a protective effect against the noradrenergic toxin DSP-4, too (Finnegan et al., 1990).

(-)Deprenyl facilitates scavenger function in the striatum and prevents age-related morphological changes in the neurocytes of the substantia nigra

One of the interesting and with all probability functionally highly important consequences of the (-)deprenyl-induced enhancement of dopaminergic activity is the finding that in (-)deprenyl treated rats the scavenger function of the nigrostriatal dopaminergic neurons is significantly enhanced (Knoll, 1988). We measured superoxide dismutase activity in the rat striatum. This enzyme is known to play a key role in the detoxification of free radicals resulting from auto-oxidation of the endogenous metabolites of dopamine. We found that daily administration of (-)deprenyl for 3 weeks significantly enhanced the activity of

superoxide dismutase in the striatum of both male and female rats in proportion to the dose given. This effect of (-)deprenyl is unrelated to MAO inhibition as clorgyline inhibits striatal superoxide dismutase activity (Knoll, 1988, 1990).

(-)Deprenyl has no direct effect on brain superoxide dismutase activity in general. Using the cerebellum as a reference tissue, we showed that the superoxide disutase activity in this area did not change in a statistically significant manner in (-)deprenyl-treated male and female rats (Knoll, 1989). Thus, it is reasonable to assume that the enhanced scavenger function in the striatum is just an expression, a corollary of the (-)deprenyl-induced enhanced activity of the nigrostriatal dopaminergic neurons.

Our finding that (-)deprenyl treatment increases scavenger function in the striatum of rats was first confirmed by Carrillo et al. (1991) who also corroborated that this effect of the drug is selective to the striatum; (-)deprenyl did not change the superoxide dismutase and catalase activity in the hippocampus (Carrillo et al., 1992).

Morphological evidence in rats for the long-term consequences of (-)deprenyl induced enhanced scavenger function in the nigrostriatal dopaminergic neurons is the complete prevention of characteristic age-related changes in neuromelanin, a biological marker of aging, in the neurocytes of the substantia nigra (Knoll et al., 1992b). We developed a method, using a TV-image analyzer, for measuring the number, area, and density features of melanin granules in the substantia nigra of young and old male rats. We found that the majority of the neurocytes in 3-month-old rats contained numerous, small-sized neuromelanin granules, whereas in the majority of the neurocytes of 3-year-old rats, smaller numbers of large-sized neuromelanin granules were detected (Tóth et al., 1992). We treated 3-month-old rats with saline and 0.25 mg/kg (-)deprenyl, respectively, three times a week for 18 months. In the neurocytes of the substantia nigra of 21-month-old saline-treated rats the number of melanin granules decreased significantly and the area of the melanin granules increased in comparison to the neurocytes of 3-month-old untreated young rats. There was, however, no significant difference in the number, total area and area of one pigment granule between the neurocytes of the substantia nigra of 3-month old young rats and of 21-month old (-)deprenyl-treated rats (Knoll et al., 1992b). These data give convincing morphological evidence that long-term (-)deprenyl medication protects the nigrostriatal dopaminergic neurons against age-related changes. Our results are in harmony with the finding that the number of medial nigral neurons was greater and the number of Lewy bodies fewer in those Parkinsonian patients who had been treated with (-)deprenyl in combination

with levodopa when compared with patients who had received levodopa alone (Rinne et al., 1991).

(-)Deprenyl treatment slows aging of the catecholaminergic system in the rat brain

The continuous, slow age-related decrease in the catecholaminergic, mainly in the nigrostriatal dopaminergic function, resulting in a decay of general activity, a substantial decline in sexual potency and to a lesser extent in learning ability, deteriorate the quality of life in the aged. The administration of (-)deprenyl during the postdevelopmental phase of life works against these changes. It was shown by us that the maintenance of rats on 0.25 mg/kg (-)deprenyl, three times a week, keeps the rats on a higher activity level and prolongs their life significantly (Knoll, 1988; Knoll et al., 1989). This unique effect of (-)deprenyl in rats was confirmed by others (Milgram et al., 1990; Kitani et al., 1992). In a second longitudinal study we selected 94 sexually 'low performing' and 99 'high performing' rats from a population of 1600 males. Half of the rats were treated with saline and half with (-)deprenyl. The 'high performing' rats lived significantly longer than their 'low performing' peers. The (-)deprenyl treated rats lived in both groups significantly longer than the saline treated ones. Furthermore, the (-)deprenyl treated males maintained in both groups their ability to ejaculate for a significantly longer period and remained better performers in the shuttle box than their saline treated peers (Knoll et al., 1994). Treatment with (-)deprenyl prolongs the life in elderly dogs too (Ruehl et al., 1997). The shift of the time of natural death in (-)deprenyl treated animals is obviously a consequence of the peculiar CAE effect of this drug. (-)Deprenyl keeps the catecholaminergic, mainly the dopaminergic neurons in the brain on a higher activity level (Knoll et al., 1996a), enhances scavenger function in these neurons (Knoll, 1988), protects the neurocytes in the substantia nigra from age-related morphological changes (Knoll et al., 1992b), thus, counters the age-related deterioration of the dopaminergic system in the brain. It slows, accordingly, the aging induced decline in general activity and the decay in sexual and learning performances. As a final result of all these effects the animals live longer (cf. Knoll, 1988; Knoll et al., 1989, 1994).

(-)Deprenyl and Parkinson's disease

There is a quantitative difference only between the physiological age-related decline of the dopaminergic input and that observed in Parkinson's disease. In the healthy population the calculated loss of striatal dopamine is about 40% at the age of 75, which is the average life time; the loss of dopamine in Parkinson's

disease is 70% or thereabout at diagnosis and over 90% at death. The drastic reduction of the dopaminergic output in Parkinson's disease leads evidently to an accordingly drastic reduction of cortical activity.

Diagnosing Parkinson's disease the neurologist selects subjects with the most rapidly aging striatal dopaminergic system (about 0.1% of the population). As symptoms of Parkinson's disease become visible only after the unnoticed loss of a major part (about 70%) of the striatal dopamine and further deterioration is irresistible, Parkinson's disease is, according to the sense, incurable. Prevention is the only chance to fight off Parkinson's disease. We need to slow the age-related functional decline of the striatal dopaminergic neurons in due time. According to physiological and pharmacological considerations (cf. Knoll, 1994; Knoll and Miklya, 1995) catecholaminergic activity enhancer substances which enhance the activity of the nigrostriatal dopaminergic neurons by facilitating the impulse propagation mediated release of dopamine could serve this aim. They increase dopaminergic activity without stimulating D_1 or D_2 receptors, without mobilizing dopamine from the stores or changing the function of the amine transporter, i.e. without changing substantially the physiological activity of the neuron and its surrounding (Knoll et al., 1996a).

That (-)deprenyl is capable to slow the rate of the functional deterioration of the nigrostriatal dopaminergic neurons was proved not only in the rat (for review see Knoll, 1998), but also in patients with early, untreated Parkinson's disease. Age-related deterioration of the striatal machinery is a continuum and any precisely determined short segment of it is sufficient to measure the rate of decline in the presence or absence of (-)deprenyl. As a matter of fact a segment of this continuum, the time elapsing from diagnosis of Parkinson's disease until levodopa was needed, was properly measured in the DATATOP study of the Parkinson Study Group (1989) in untreated Parkinsonian patients and the effect of (-)deprenyl versus placebo was compared on this segment. It was first shown by Tetrud and Langston (1989) that (-)deprenyl delayed the need for levodopa therapy. The average time until levodopa was needed was 312.1 days for patients in the placebo group and 548.9 days for patients in the (-)deprenyl group, clearly indicating that (-)deprenyl, which enhanced the activity of the surviving dopaminergic neurons, kept these neurons on a higher activity level for a longer duration of time.

The design of the DATATOP study was unintentionally the same we used in rat experiments with (-)deprenyl since 1980. We selected male sexual activity as a quantitatively measurable rapidly aging dopaminergic function, compared the effect of (-)deprenyl versus saline treatment on the age-related decline of this function, and demonstrated that (-)deprenyl treatment slows significantly

the age-related decay of sexual performance (Knoll, 1982, Knoll et al., 1983). This effect of (-)deprenyl is unrelated to the inhibition of MAO-B, as (-)PPAP, a derivative of (-)deprenyl free of MAO-B inhibitory property (Knoll et al., 1992a), is enhancing dopaminergic activity in the brain like (-)deprenyl (for review see Knoll, 1998). By now it is clear that if we select a quantitatively measurable dopaminergic function and measure its age-related decline by fixing an exact end point, a shift of this end point in time in (-)deprenyl treated rats shows the dopaminergic activity enhancer effect of the drug. For example male rats, due to the physiological aging of the striatal dopaminergic system, lose finally their ability to ejaculate. We found that saline treated rats reached this end point at the age of 112±9 weeks, whereas their (-)deprenyl treated peers lost the ability to ejaculate only at the age of 150±12 weeks (Knoll, 1992). The design of the DATATOP study was essentially the same. The authors knew that after diagnosis of Parkinson's disease the next stage downward is levodopa need, and they measured the (-)deprenyl induced delay in reaching this end point.

Although Tetrud and Langston and other authors of the DATATOP study were not aware of the CAE effect of (-)deprenyl, their study was the first convincing evidence that (-)deprenyl keeps the nigrostriatal dopaminergic neurons on a higher activity level also in human. In addition, this effect of (-)deprenyl was detected in a selected human population with the lowest striatal dopaminergic activity. The highly significant effect of (-)deprenyl and the ineffectiveness of tocopherol during the first years of the DATATOP study was clear proof that the drug acted via the enhancement of the activity of the nigrostriatal dopaminergic neurons. The patients selected for the study with early, untreated Parkinson's disease were ideal for demonstrating this effect. The subjects still had a sufficient number of dopaminergic neurons the activity of which could be enhanced by (-)deprenyl, thus, the need for levodopa therapy was delayed. Tocopherol, devoid of a dopaminergic activity enhancer effect, remained ineffective. As drug effects in Parkinson's disease are necessarily transient in nature, it is obvious that parallel with further decay of the striatal dopaminergic system, the responsiveness of the patients toward (-)deprenyl decreased with the passing of time (cf. Parkinson Study Group 1996). (-)Deprenyl, though it is still not used for this purpose, is primarily destined to be used as a prophylactic agent in the healthy population for slowing the physiological age-related decline of the striatal dopaminergic neurons. It is reasonable to assume that if patients who developed Parkinson's disease had taken, preceding the precipitation of the symptoms of the disease, just 1 mg of (-)deprenyl per day during the whole postdevelopmental period of their life,

they would have either avoid reaching within their life time the low level of striatal dopamine needed for the manifestation of the disease (as 99.9% of the human population avoid it) or at least would have crossed the critical threshold substantially later.

In patients with Parkinson's disease tre~ levodopa (-)deprenyl has evidently two effects. It is a dopaminergic activity enhancer substance and, as an MAO-B inhibitor, it has also a levodopa sparing effect. (-)Deprenyl was introduced by Birkmayer in 1975 for treating patients with levodopa need in the hope that the compound, an MAO inhibitor devoid of the 'cheese effect', will exert a levodopa sparing effect without dangerous side-effects (cf. Birkmayer and Riederer, 1983). Birkmayer used the 10 mg daily oral dose of (-)deprenyl which was calculated by us, on the basis of the MAO-B inhibitory potency of the compound, in the mid-1960s for the first clinical trial carried out by Varga in 1965 to check the antidepressant effect of (-)deprenyl (for review see Knoll, 1998). This amount is still the worldwide used daily dose of (-)deprenyl in therapy, which inhibits completely MAO-B in human platelets and brain and allows to decrease in Parkinson's disease the dose of levodopa by 20-50%.

Multicenter clinical trials (Parkinson Study Group, 1989, 1993, 1996; Allain et al., 1991; Myttyla et al., 1992) with (-)deprenyl as initial treatment in *de novo* patients with Parkinson's disease revealed the safeness of the long-term administration of 10 mg (-)deprenyl daily. It is commonly assumed that (-)deprenyl by itself is an exceptionally safe compound. In patients with levodopa need, however, there is always a risk that the administration of (-)deprenyl will enhance the side-effects of levodopa, which can only be avoided by decreasing the levodopa dose properly according to the individual sensitivity of the patient. An example of a multicenter clinical trial in which the improper combination of levodopa with (-)deprenyl led to confusion and misinterpretation is the one performed by the Parkinson's Disease Research Group of the United Kingdom (PDRG-UK). Quite unexpectedly this group published an alarming paper claiming that Parkinsonian patients treated with levodopa combined with (-)deprenyl show an increased mortality in comparison with the patients treated with levodopa alone (Lees, 1995). This finding was in striking contradistinction with all other experiences published in different countries. Birkmayer et al. (1985) found even an increased life expectancy resulting from addition of (-)deprenyl to levodopa treatment in Parkinson's disease. The 'idiosyncratic prescribing' (Dobbs et al., 1996) of (-)deprenyl in combination with levodopa in the PDRG-UK study led to the misconclusion of the authors. Comments (Dobbs et al., 1996; Knoll, 1996; Olanow et al., 1996) pointed uniformly to the substantial overdosing of levodopa as the cause of the

observed deaths with (-)deprenyl as an adjuvant in this trial. Anyway, in the light of recent experimental studies the optimum (-)deprenyl for exerting the dopaminergic activity enhancer effect in Parkinson's disease needs to be reconsidered. It might be substantially lower than 10 mg per day. In the rat the catecholaminergic activity enhancer effect of (-)deprenyl is exerted in one tenth of the dose needed for the inhibition of MAO-B in the brain (Knoll and Miklya, 1994; Knoll et al., 1996a).

(-)Deprenyl and Alzheimer's disease

Beneficial effects of (-)deprenyl treatment in Alzheimer's disease, the other serious age-related neurological illness, was demonstrated from 1987 in a number of studies with small sample size (for review see Knoll, 1998). The first double-blind, placebo-controlled, randomized, multicenter clinical trial conducted with (-)deprenyl in a total of 341 patients with Alzheimer's disease of moderate severity was published in 1997. The authors concluded that in patients with moderately severe impairment from Alzheimer's disease, treatment with (-)deprenyl slows the progression of the illness (Sano et al., 1997).

The future

It still has to be generally realized that (-)deprenyl is not only the first selective inhibitor of MAO-B described in literature and the historical experimental tool used to identify and analyze MAO-B, but also the first CAE substance in clinical use devoid of the catecholamine releasing property, which can be safely used in humans to slow the age-related decline of the nigrostriatal dopaminergic neurons.

(-)Deprenyl, the PEA/methamphetamine derived selective inhibitor of MAO-B, is a CAE substance metabolized to methamphetamine and amphetamine. The development of (-)PPAP, the first amphetamine derived CAE substance which is not metabolized to amphetamine and is devoid of the MAO inhibitory property, was the first step forward to surpass (-)deprenyl. The recent discovery that tryptamine is possessing the CAE property, opens now the way for the development of highly potent and selective CAE substances, structurally unrelated to PEA and the amphetamines.

The real challenge for future pharmacological and clinical research is to take up the fight against the physiological age-related activity decline of the catecholaminergic system in the healthy brain, via long-term, small-dose administration of a proper CAE substance, like (-)deprenyl, during the postdevelopmental phase of life. It is reasonable to expect that preventive medication with a safe, small dose of a CAE substance will slow the age-related

decline of behavioral performances, delay natural death and decrease susceptibility to Parkinson's disease and Alzheimer's disease.

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RESEARCH REPORT

SELECTIVE INHIBITORS AND COMPUTER MODELLING OF THE ACTIVE SITE OF MONOAMINE OXIDASE*

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MAO inhibitors can be employed for computer modelling of the active site of MAO A and B. Competitive fully reversible MAO inhibitors with rigid structure and limited number of conformers are preferential compounds for these studies. Among various isatin analogues with nearllanar structure selective MAO B inhibitors fit to 3D box of 8.5 ×5.1×1.8 Å, whereas 3D box of 14.2×5.6×1.8 Å accommodates selective MAO A inhibitors. Validity of these data was tested using a series of pyrazinocarbazoles, analogues of short-acting antidepressant pirlindole. Rigid analogues exhibiting potent and selective inhibition of MAO A have 3D size limits of 13×7×4.4 Å. Flexible analogues also demonstrated potent inhibition of MAO B and in contrast to rigid analogues their inhibitory activity did not show any dependence on 3D sizes. 3D-QSAR with CoMFA of isatin and pirlindole analogues of MAO A and B revealed differences in the models of MAO A and B.

Keywords: monoamine oxidase, inhibitors, computer modelling, active site, CoMFA, 3D-QSAR

INTRODUCTION

A decade passed after the estimation of deduced amino acid sequences of monoamine oxidases (MAO) A and B (Bach et al., 1988). Subsequent molecular biology manipulations constructing various chimeric proteins revealed important regions of the polypeptide chains of MAO A and MAO B essential for manifestation of catalytic activity and preferential substrate

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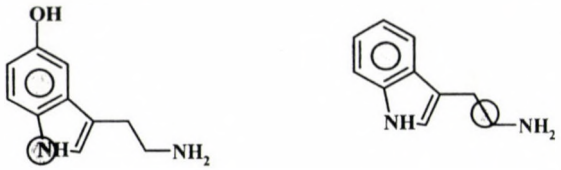
selectivity of these enzymes (Shih et al., 1998; Cesura et al., 1998). However, three-dimensional structures of MAO A and MAO B remains unknown. Even impressive results by Tsugeno and Ito (1997) demonstrating «a key amino acid responsible for substrate selectivity of monoamine oxidase A and B» can account for only MAO A vs MAO B differences with respect to some substrates (serotonin and tyramine) and inhibitors clorgyline and deprenyl. A single mutation of Phe-208 in MAO A for the corresponding amino acid in MAO B, Ile, and the mutation of Ile-199 in MAO B for the corresponding amino acid in MAO A, Phe, did not influence catalytic properties of the mutant enzymes assayed with other substrates (2-phenylethylamine and tryptamine) (Tsugeno and Ito, 1997). This suggests that more than one amino acid is responsible for specific substrate and inhibitor binding (Veselovsky et al., 1998). In fact, our recent pharmacophore analysis of classic substrates and inhibitors suggests the involvement of more than one amino acid into the ligands binding (Fig. 1). Computer-aided prediction of the secondary structure (Veselovsky et al., 1998) did not reveal any possible influence of the «key amino acid» mutation on the secondary structure in the region (~100 amino acids) important for catalytic properties of MAO A and MAO B (Tsugeno and Ito, 1997; Shih et al., 1998) (see Fig. 2). Thus, the development of the models of MAO A and MAO B, which can provide satisfactory explanation of known differences between these enzymes, still requires crystal structures of both enzymes. However, high aggregability of MAO A and MAO B, the integral proteins of the outer mitochondrial membrane seriously complicates this problem. So besides chimeric molecules some other «bypasses» have been designed: (a) employment of soluble amine deaminating enzyme of microbial or fungal origin as evolutionary precursors of animal MAOs (Singer et al., 1997); (b) computer-aided studies of MAO interaction with substrates and inhibitors (Altomare et al., 1992; Efange and Boudrean, 1991; Kneubuhler et al., 1995; Thull et al., 1995). The latter approach is also very useful for the development of new selective MAO inhibitors.

MATERIALS AND METHODS

Determination of MAO activity and its inhibition. Rat liver mitochondria were used as a source of MAO A and MAO B. The activity of MAO was assayed radiometrically as described previously (Medvedev et al., 1995, 1998).



MAO A **MAO B**

Fig. 1. Proposed pharmacophore models for MAO A and MAO B substrates. Circles show the parts of molecules important for binding substrates in the active sites MAO A and MAO B

MAO A (rat)	120	**AYLDYNNLWRTMDEMGKEIPVDAPWQARHAQEWDK**	154
MAO $A_{Phe208Ile}$	120	**AYLDYNNLWRTMDEMGKEIPVDAPWQARHAQEWDK**	154
MAO A (rat)	155	**MTMKDLIDKICWTKTAREFAYL***FVN***INVTSEPHEV**	189
MAO $A_{Phe208Ile}$	155	**MTMKDLIDKICWTKTAREFAYL***FVN***INVTSEPHEV**	189
MAO A (rat)	190	**SALW***FLWYV***RQCGGT***ARIFSVT***NGGQ***ERKFV***	220
MAO $A_{Phe208Ile}$	190	**SALW***FLWYV***RQCGGT***ARIISVT***NGGQ***ERKFV***	220
MAO B (rat)	120	***R*TMDEMGQEIPSDAPWKAPLAEEWDYMTMKELLDK**	154
MAO $B_{Ile208Phe}$	120	***R*TMDEMGQEIPSDAPWKAPLAEEWDYMTMKELLDK**	154
MAO B (rat)	155	**ICWTNSTKQI***ATLFV***NLCVTAETHEVSALWFL***WYV***	189
MAO $B_{Ile208Phe}$	155	**ICWTNSTKQI***ATLFV***NLCVTAETHEVSALWFL***WYV***	189
MAO B (rat)	190	***K*QCGG***TTRII*****S***TTNGGQERKFIGGSGQV***SER***	220
MAO $B_{Ile208Phe}$	190	***K*QCGG***TTRII*****S***TTNGGQERKFIGGSGQV***SER***	220

Fig. 2. Secondary structure prediction for MAO A and B (wild and mutant strains) in the substrate binding region. Amino acids predicted in alpha-helix marked by bold font, in beta-sheets – italic font. Mutated amino acids are underlined. Amino acid sequence was taken from Tsugeno and Ito (1998)

Computer modelling. Most of calculations were carried out on Silicon Graphics Workstation Indigo-2 (R4400, XZ) using Sybyl 6.3 software (Tripos Inc., USA). The molecular models were constructed and their geometries were optimised using the standard Tripos force field. The atomic charges calculation and geometry optimisation of molecules were made by semiempirical AM1 method. They were used in the subsequent analysis. Linear sizes of pirlindole

and its derivatives were calculated using HyperChem 4.5 software (Hypercube Inc., Canada) by constructing boxes around molecules. Sizes of minimal box in which given compound fits were considered as sizes of the molecule. Conformational analysis was done using Random search program of Sybyl. For 3D-QSAR with CoMFA analysis the inhibitors were aligned by fitting indole part of molecules structure atom by atom. The region was generated automatically by the program. The grid size had a resolution of 2 Å. Both steric and electrostatic fields were taken into consideration. The steric and electrostatic potentials were generated using a sp^3 carbon probe and a + 1 charge. QSAR analysis was carried out in two steps using PLS technique. In the first analysis, using 5 components and a number of cross-validation groups equal to a number of compounds, the optimal number of components was determined. The optimal number of components for the final 3D-QSAR model was chosen as the number of components that corresponds to the minimum cross-validated standard error of estimate (s_{cv}) and $R^2_{cv} > 0.4$. The second run was performed without cross-validation, using the optimal number of components previously determined. The results of the second analysis were used for drawing the coefficients' contour maps.

Criteria for the use of MAO inhibitors for computer modelling studies

The development of modification of QSAR method using three-dimensional structure of ligand molecules (3D-QSAR) provided perspective tool for modelling of the enzyme-inhibitor interactions. Distribution of 3D-fields around molecules can reveal regions essential for non-covalent binding of the inhibitor with the enzyme. This approach was employed for the development of 3D-QSAR models describing three-dimensional distribution of steric, electrostatic, and hydrophobic regions in the active site of MAO A and MAO B determining interaction with substrates - MPTP analogues (Altomare et al., 1992) and inhibitors –isoquinoline derivatives (Thull et al., 1995) and 5H-indeno[1, 2c]piridazines (Kneubuhler et al., 1995).

However, in our viewpoint, the employment of this method meets some difficulties:

1.) Lack of information on ligand location in the active sites of enzymes complicates universal analysis of MAO interaction with inhibitors from various chemical groups (principles of alignment are unknown);

2.) Mutual influence of enzyme and inhibitor (especially in case of mechanism-based inhibitors) can alter spatial organisation of the protein and this question correctness of the resultant field distribution;

3.) Lack of precise information on the preferential inhibitor conformation during its binding at the active site of the enzyme can influence 3D-models of field distribution. Using flexible molecules with number of rotation points of substituents we may obtain various models of the active site, however, it is nearly impossible to determine which model reflects reality (Veselovsky et al., 1997).

Taking into consideration these limitations of 3D-QSAR method we are using the following criteria for selection of compounds for computer-modelling studies:

1.) Reversible competitive inhibitors, which do not undergo MAO-dependent metabolism, are preferential compounds for 3D-QSAR;

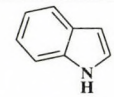
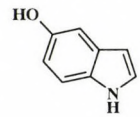
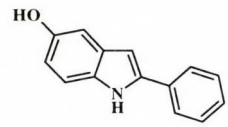
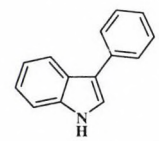
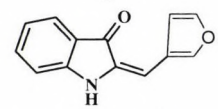
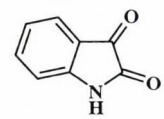
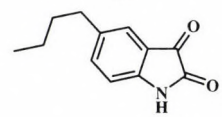
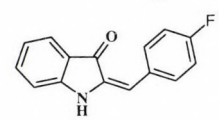
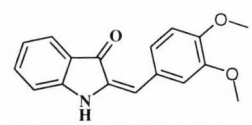
2.) These molecules must be relatively «rigid» with limited number of conformers;

3.) In case of rigid molecules steric factors are crucial for enzyme-inhibitor interaction because they determine a possibility of active site occupation.

3D-QSAR with CoMFA analysis of MAO inhibition by isatin and pirlindole analogues

Isatin (indoledione-2, -3) and its derivatives are competitive and readily reversible MAO inhibitors possessing relatively rigid structures (Table 1) (Medvedev et al., 1995; 1996a). Most of isatin analogues have planar structures and the «length» and «width» of most potent MAO A inhibitors did not exceed 14 and 6 Å, whereas selective MAO B inhibitors have smaller sizes (8.5 and 5.0 Å, respectively) (Fig. 3). Some isatin analogues possessing 3D structure with the «thickness» of molecules not more than 4.5 Å demonstrated more potent inhibition of MAO A than isatin. These data suggest that a size of molecules competitively interacting with either type of MAO can be used for evaluation of active site volume of these enzymes. A model based on 3D-QSAR with comparative molecular field analysis (CoMFA) (Fig. 4) exhibited good prediction not only for MAO inhibitory activities of the compounds tested in our laboratories (Table 1) (Medvedev et al., 1992, 1995) but also for isatin analogues tested elsewhere (Rajesh et al., 1994).

Table 1. Inhibitory activity (IC_{50}, μM) and size (Å) of isatin analogues (adapted from Medvedev et. al., 1996b)

Structure	MAO A		MAO B		Size
	exper.	pred.	exper.	pred.	(X×Y×Z)
	200	245	100		6.7×5.0×0
	600	650	600	600	6.8×5.0×0
	71	20	>1000	708	11.4×5.0×0
	4	5	20	21	11.0×5.0×0
	4	4	447	372	11.3×5.1×0
	56	19	8	5	6.7×5.0×0
	6	4	54	59	10.5×5.1×1.8
	4	4	>1000	1148	12.1×5.0×0
	45	45	20	23	13.5×5.5×4.5

Applicability of the constructed «image» of MAO inhibitors, isatin analogues has been investigated using analogues of pirlindole and tetrindole, the effective short-acting antidepressants (Mashkovsky, 1993; Bruhwyler et al., 1997). Pirlindole and tetrindole are selective inhibitors of MAO A exhibiting properties of tight-bound inhibitors and used in clinic (Medvedev et al., 1992a, 1994, 1998). Inhibition of MAO after administration of these compounds to rats or incubation with isolated mitochondria persisted through mitochondria isolation and wash.

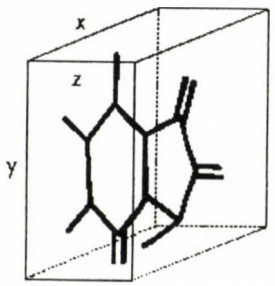
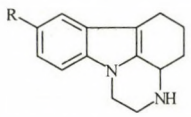


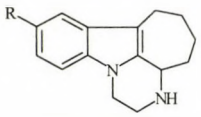
Fig. 3. Three-dimensional box for isatin analogues sizes determinations

Evaluation of 3D sizes of pirlindole analogues revealed that the length of rigid compounds exhibiting potent and selective inhibition of MAO A are within limits previously determined for isatin analogues, selective inhibitors of MAO A (Table 2). However, we should emphasise that all isatin analogues tested have sizes along Y-axis 5.0-6.0 Å, whereas these sizes in most pirlindole analogues were a bit longer (6.9-7.2 Å). In contrast to rigid compounds flexible analogues possessing substituents at C-8 with rotation points (Table 2, molecules 2, 3, 15) did not demonstrate such dependence between sizes of molecules and the inhibitory activity. The most probable explanation of these discrepancies consists in differences of binding sites for pirlindole analogues in MAO A and MAO B molecules. The active site of MAO A has enough space to accommodate rather «long» rigid inhibitor molecules (Fig. 5) whereas geometry of MAO B active site does not allow to accommodate them. If this hypothesis is correct, an increase in the MAO B inhibitory activity of flexible analogues can be explained by the possibility of such conformation that corresponds to the geometry of the active site of MAO B (Fig. 5). Many MAO B inhibitors are flexible compounds and limitation of flexibility often

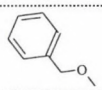
Table 2. Inhibitory activity (IC_{50}, μM) and size (Å) of pirlindole analogues (adapted from Medvedev et al., 1998a)



P1-P11, P13-P16



P12

		MAO A		MAO B		Size		
Name	R	exp.	pred.	exp.	pred.	X	Y	Z
P1		0.11	0.11	>>100	97.7	10.894	6.959	4.355
P2		0.04	0.04	20	19.1	13.462	7.031	3.498
P3		12.6	13.0	>178	182	11.433	7.121	2.854
P4		3.98	3.80	>178	182	8.791	6.912	2.829
P5		0.071	0.08	158	182	9.746	6.909	2.828
P6		7.9	8.1	250	245	9.871	7.027	2.849
P7		0.79	0.83	180	174	12.991	7.198	4.448
P8		4.0	3.8	>1000	912	9.532	6.894	2.827
P9		13.0	13.2	16.0	15.5	12.704	7.089	3.521
P10		0.26	0.24	12.5	11.8	13.286	6.829	4.151
P11		0.5	0.5	>500	525	11.784	6.969	4.006
P12		0.53	0.5	29.0	30.2	13.051	7.019	4.393
P13		0.08	0.07	160	160	12.340	7.043	4.360
P14		0.13	0.14	7.5	7.5	13.002	7.064	2.869
P15		0.14	0.13	0.14	0.14	14.944	7.093	2.869
P16		0.03	0.03	79.4	79.4	12.070	7.096	3.031

decreases their MAO B inhibitory activity. For example, long and flexible oxodiazolones are very potent inhibitors of MAO B whereas shorter but rigid analogues were less potent (Krueger et al., 1995).

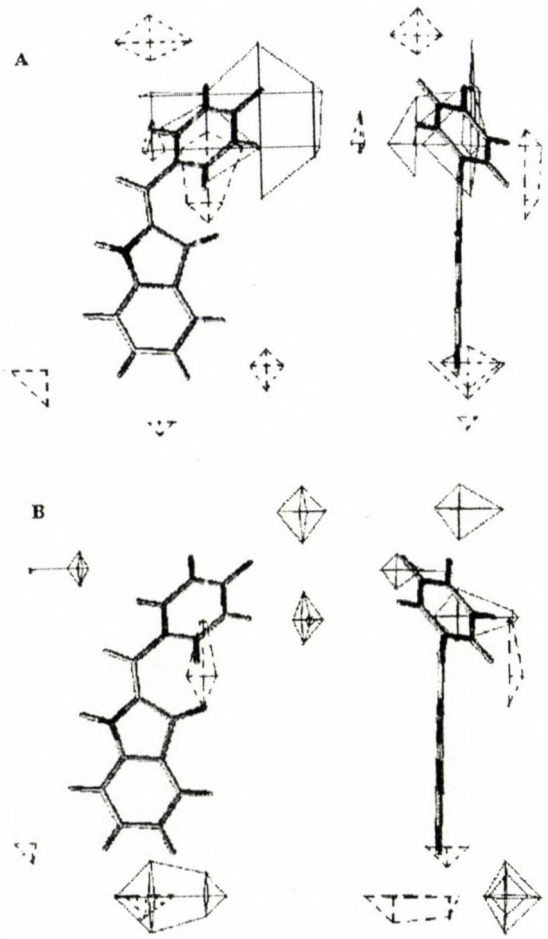


Fig. 4. Standard dev*coefficients contour plot of CoMFA steric fields of MAO A (A) and MAO B (B) for isatin analogues. Sterically unfavourable areas (contribution level of 20%) are represented by solid lines; sterically favourable areas (contribution level of 80%) – by dashed lines

3D-QSAR + CoMFA models for MAO A and MAO B based on pirlindole analogues provide further evidence for steric differences of the active sites of these enzymes (Fig. 6). Comparison of steric fields of pirlindole analogues as

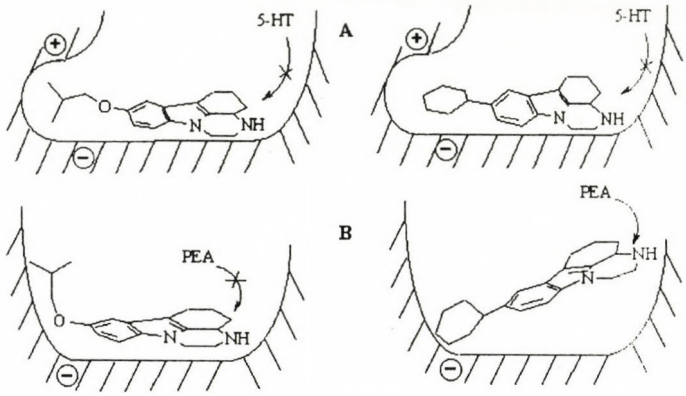


Fig. 5. Schemes of MAO A (A) and MAO B (B) active site structures and putative positions of P2 (left) and tetrindole (right) molecules. Arrows show that phenylethylamine (PEA) can displace tetrindole, but not P2. Serotonin (5-HT) cannot displace both molecules

MAO A and MAO B inhibitors suggests the existence of steric obstacle at C-8 (Fig. 5) in MAO B molecule which might explain inability of long rigid pirlindole analogues to potent inhibitors of MAO B. Also 3D-QSAR + CoMFA models suggest that the efficacy of MAO A inhibitors is determined by certain limits in size of molecules and the «thickness» of (rigid) molecules must be less than 4.5 Å (Table 2). The distribution of steric fields suggests that the active site of MAO A could represent relatively narrow slot whereas the active site of MAO B is shorter but wider. Steric and electrostatic fields of these models suggest that different amino acids of the active site region of MAO A and B are involved into the interaction with C-8 substitutents. So negatively charged amino acid residues in the active site of MAO B probably determine limitation of the length of substituents at C-8. In MAO A the cavity accommodating substituents of pirlindole analogues is formed by negative and positive charges amino acids.

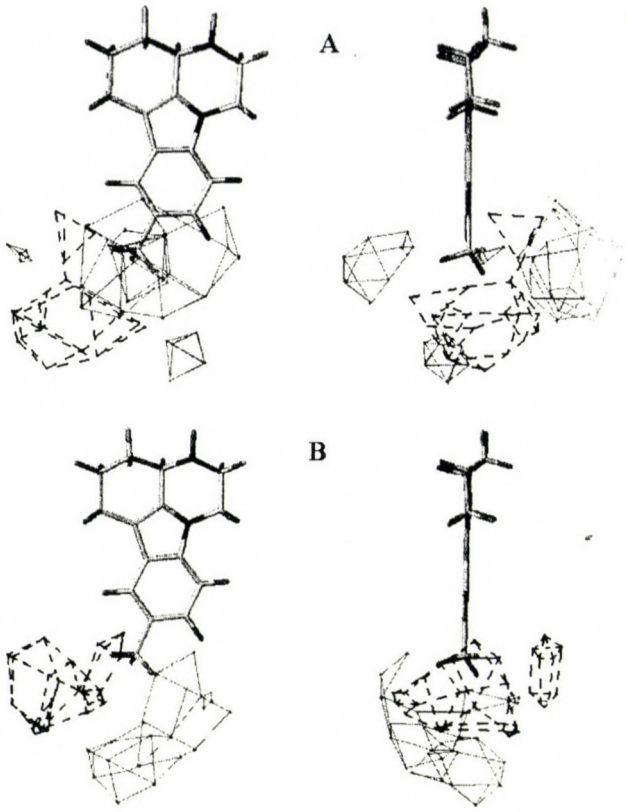


Fig. 6. Standard dev*coefficients contour plot of CoMFA steric fields of MAO A (A) and MAO B (B) for pirlindile analogues. Sterically unfavourable areas (contribution level of 30%) are represented by solid lines; sterically favourable areas (contribution level of 70%) – by dashed lines

CONCLUSIONS

The employment of relatively rigid inhibitor molecules for computer modelling of the active site of MAO revealed good predictability of the resultant models. Modelling of the action of reversible inhibitors allows to consider only factors involved into the enzyme-inhibitor interaction (steric, electrostatic, lipophilic, etc.) leaving out of consideration such events typical for mechanism-based inhibitors as possible bio-activation of the inhibitor, formation of reactive products and mode of their interaction with the active site. Use of molecules with limited conformation flexibility also overcomes numerous problems of

determination of the actual conformer involved into the interaction with enzyme. However, the rigidity of molecules limits sizes of molecules, which can be used in such analysis, and this is obvious limitation of such approach. At the same time rigid molecules, exhibiting properties of reversible and selective inhibitors of MAO could serve as a skeleton for subsequent superimposition of flexible inhibitors for preparation of the «mould» of the active site of MAO (Fig. 7).

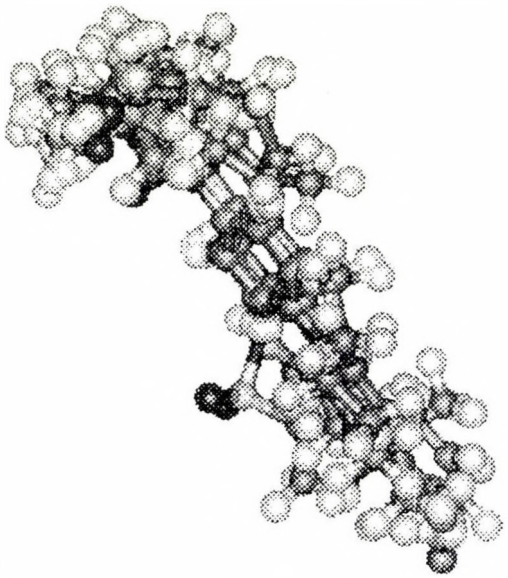


Fig. 7. Drawing a mask of the active shape by superimposing parts of various MAO-interacting substrates essential for MAO inhibition; potential problems of alignment of the inhibitors

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RESEARCH REPORT

INHIBITOR SENSITIVITY OF HUMAN SERUM AND VASCULAR SEMICARBAZIDE-SENSITIVE AMINE OXIDASES*

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Recent data suggest that elevated serum semicarbazide-sensitive amine oxidase activity (SSAO) may cause endothelial injury. Formation of cytotoxic metabolites (especially formaldehyde) and increased oxidative stress might lead to initiation or progression of atherosclerosis. Effective and selective inhibitors of human SSAO might exert cytoprotective effect on endothelial cells. To compare the inhibitor sensitivity of human serum and vascular tissue SSAO enzyme, the inhibitory effect of semicarbazide and MDL 72974A was investigated. Serum and vascular SSAO activity has been determined using ^{14}C-benzylamine as a substrate. The IC_{50} values of semicarbazide were estimated to be 5×10^{-3}M and 5×10^{-4}M for SSAO from human serum and saphenous vein, respectively. MDL 72974A amine oxidase inhibitor was more than thousand times more effective than semicarbazide. The IC_{50} values were 10^{-7}M and 10^{-8}M for SSAO from human serum and saphenous vein, respectively. This finding supports the hypothesis that soluble and membrane-bound vascular SSAO enzymes might have similar structure.

Keywords: semicarbazide, amine oxidase, serum, smooth muscle

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INTRODUCTION

Human plasma and tissues contain various monoamine oxidases that are inhibited by semicarbazide. Recent findings suggest that elevated activity of these semicarbazide-sensitive amine oxidases (amine:oxygen oxidoreductase EC 1.4.3.6, SSAO) may be involved in the pathogenesis of atherosclerosis and diabetic vascular complications by increasing oxidative stress and formation of cytotoxic metabolites [Yu and Zuo, 1993; Boomsma et al., 1995; Yu and Zuo, 1996; Yu et al. 1997].

SSAO enzymes deaminate primary amines producing aldehyde, ammonia and hydrogen peroxide. They metabolize the synthetic amine benzylamine particularly well [Buffoni and Blaschko, 1964]. The conversion of the xenobiotic aliphatic amine, allylamine to the cardiovascular toxin, acrolein, and the production of formaldehyde and methylglyoxal from endogenous aliphatic amines, methylamine and aminoacetone, respectively, suggest that high SSAO activity may cause endothelial injury [Hysmith and Boor, 1988; Lyles et al. 1990; Lyles and Chalmers, 1992]. Aminoacetone, an amine derived from glycine and threonine metabolism, is one of the best substrates of SSAO in men [Lyles, 1996]. In diabetes mellitus, serum concentrations of methylamine and aminoacetone are elevated, which might lead to the excessive production of cytotoxic aldehydes by SSAO [Yu and Zuo, 1993].

SSAO is found in the plasma membrane of various cells (e.g. vascular smooth muscle cells, endothelial cells, adipocytes, chondrocytes, fibroblasts, etc.), and is also present in soluble isoform in the human serum. The membrane bound endothelial isoform of SSAO is identical to vascular adhesion protein 1 (VAP-1), a lymphocyte adhesion receptor. Its expression on the cell surface is increased under inflammatory conditions [Smith et al., 1998]. Elevated plasma SSAO activity has been reported in insulin dependent diabetes mellitus, chronic heart failure, and uraemia [Boomsma et al., 1995; Boomsma et al. 1997; Levinsohn, 1988]. These conditions are often associated with accelerated atherosclerosis. On cell cultures, SSAO inhibitors can prevent endothelial injury caused by human serum and methylamine [Yu and Zuo, 1993].

In our previous study of patients with non-insulin dependent diabetes mellitus, positive correlation was found between human serum SSAO activity and several risk factors of atherosclerosis (body weight, body mass index, fasting plasma glucose, hemoglobin A_{1c}, serum triglyceride, total cholesterol level) [Mészáros et al., 1999].

This correlation suggests, that potent and selective inhibitors of human SSAO enzymes might exert cytoprotective effect on endothelium, and delay the progression of atherosclerosis and late diabetic vascular complications.

Hydrazine compounds, such as semicarbazide, are relatively weak inhibitors of SSAO. Recently, a new, highly potent, MAO-B inhibitor (E)-2-(4-fluorophenethyl)-3-fluoroallylamine hydrochloride, (MDL 72974A) was found to be a potent SSAO inhibitor [Zreika et al., 1989; Yu and Zuo, 1992]. This compound is the most effective known inhibitor of SSAO in human serum and human umbilical artery.

The aim of the present study was to compare the activity and inhibitor sensitivity of SSAO from human serum and human saphenous vein. The total amine oxidase activity, and monoamine oxidase (MAO) activity were also determined.

MATERIALS AND METHODS

Twenty-four patients with varicosity, nursed at the Department of Vascular Surgery, Central Military Hospital, were included in the study. The patients underwent detailed medical examination. Informed consent was obtained before enrollment in the study. Subjects with a history of malignancy, recent infection, diabetes mellitus, renal failure, hepatic cirrhosis, chronic heart failure or alcoholism (more than three drinks daily) were excluded from the trial. The study was approved by the Ethical Committee of the Central Military Hospital.

Ten milliliters of venous blood was drawn from each subject. The samples were collected in Vacutainer tubes without anticoagulant, stored at +4 °C during delivery, and centrifuged at 2500 g for 10 min. The sera were mixed and stored at –80 °C until SSAO assay.

After varicotomy, saphenous vein pieces (0.1-0.5 g) were rinsed thoroughly with saline, sliced into small pieces, and homogenized (Ultra Turrax, IKAWERK, Staufen, Germany) in chilled 0.01 M phosphate buffer (pH=6.8). The crude homogenates were centrifuged at 800 g for 10 min and the supernatants were further centrifuged (20,500 g, 40 min). These final supernatant enzyme preparations were stored at –80 °C.

A radiometric procedure was adapted to determine human serum and saphenous vein SSAO activity [Yu and Zuo, 1992]. The radioenzymatic assay is based on the extraction of ^{14}C-benzaldehyde formed by the enzyme from ^{14}C-benzylamine.

Benzylamine, tyramine, chlorgyline, Coomassie Brillant Blue, phosphoric acid and semicarbazide were purchased from Sigma-Aldrich Ltd. (Germany). ^{14}C-benzylamine was obtained from Amersham International plc (United Kingdom) (Specific activity: 2.18 GBq/mmol). ^{14}C-tyramine was obtained from DuPont Co. (USA) (Specific activity: 2.2 GBq/mmol). MDL 72974A was provided by Marion Merrel Dow Inc. (USA). ULTIMAGOLD liquid scintillation cocktail was obtained from Packard Co. (The Netherlands). All other chemicals were of analytical grade.

The SSAO enzyme preparations were preincubated with chlorgyline (10^{-4}M) at room temperature for 20 min to ensure that any MAO activity, if present, was completely inactivated. The enzyme was then incubated in the presence of ^{14}C-benzylamine (5×10^{-5}M, 0.1 μCi) in a final volume of 200 μl at 37 °C for 40 min. Adding of 200 μl 2M citric acid stopped the enzyme reaction. The oxidized products were extracted into 1 ml toluene:ethylacetate 1:1 v/v mixture, of which 600 μl was then transferred to a counting vial containing 5 ml ULTIMAGOLD fluid. Radioactivity was assessed using a liquid scintillation counter (Beckman LS5000 TA, USA). Protein content of the samples was determined according to Bradford [Bradford, 1976]. Serum SSAO activity was expressed in the following unit: pmol oxidized substrate/hour/mg protein.

Platelet rich plasma was prepared as follows:

Two milliliters of venous blood were withdrawn from the patients, into Vacutainer tubes containing 0.17 M EDTA-K_3 anticoagulant. Following a two-hour sedimentation at +4 °C, the supernatant was used for the MAO assay.

Platelet MAO-B activity was determined using 80 μM ^{14}C-tyramine as substrate in the presence of 1 mM semicarbazide [Watanabe et al., 1990]. After a twenty-minute preincubation period at room temperature, the reaction was started by ^{14}C-tyramine substrate added to the incubation mixture containing 20 μl platelet rich plasma, 0.05 M phosphate buffer (pH=7.4), 1 mM semicarbazide in 100 μl final volume. After a 20 minute incubation period at 37 °C the reaction was stopped by 40 μl 2 M citric acid. The formed product was extracted using 1 ml of toluene:ethylacetate 1:1 mixture, and the samples were centrifuged (3000 g, 1 min). An aliquot of 0.5 ml supernatant was added to 5 ml liquid scintillation cocktail and radioactivity was measured using a liquid scintillation counter (Beckman LS5000 TA, USA). Platelet count was determined immediately after sedimentation using CELL-DYN® 900 Hematology Analyzer (Sequoia-Turner Co., USA). Platelet rich plasma MAO-B activity was defined as: nmol oxidized substrate/hour/10^9 platelets.

Plasma amine oxidase activity (total activity and MAO-B) was determined using 80 μM ^{14}C-tyramine as substrate, vascular SSAO activity (total activity and SSAO) was determined using 5×10^{-5}M ^{14}C-benzylamine. One mM semicarbazide was applied to inhibit SSAO in human plasma, and 0.1 mM chlorgyline inhibited MAO nonselectively in vascular tissue.

The enzyme preparations were preincubated for 20 min at room temperature (+20 °C) in the presence of various concentrations of the inhibitors, before addition of substrates.

RESULTS

In vitro inhibitory effect of MDL 72974A and semicarbazide on human serum and vascular SSAO is summarized in Table 1. The enzyme activity is expressed as percent of control SSAO activity. (Control tubes contained 0.1 mM chlorgyline for MAO inhibition, but not other inhibitors.) The relative enzyme activitites (%) were estimated from three experiments.

Table 1. SSAO activity (percent of control) in the presence of SSAO inhibitors (values are expressed as mean±S.D.)

		Inhibitor concentration						
Inhibitor	Source	10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
MDL 72974A	serum	NA	0	0	5.7±2.6	67.1±11.6	82.3±11.5	NA
MDL 72974A	vein	NA	0	0	0	4.4±0.9	64.0±6.0	98.9±4.7
Semicarbazide	serum	18.0±5.8	100.8±12.3	106.9±4.0	98.2±19.6	111.5±15.1	99.9±9.4	NA
Semicarbazide	vein	0	31.3±0.6	83.1±13.6	94.5±6.1	96.6±6.5	NA	NA

0: no detectable enzyme activity, NA: determination was not performed

The IC_{50} values of semicarbazide were estimated to be 5×10^{-3} M and 5×10^{-4} M for SSAO from human serum and vein, respectively. MDL 72974A was more than thousand times more effective inhibitor than semicarbazide. The IC_{50} values were about 10^{-7} M and 10^{-8} M for SSAO from human serum and saphenous vein, respectively.

SSAO and MAO activities of pooled control enzyme preparations were also determined. The mean amine oxidase activity of three experiments is indicated

in Figure 1. (Standard deviation was less than 10% of the mean, in each case.) Mean serum SSAO activity was 91.46 pmol/hour/mg protein. Saphenous vein SSAO activity of the same patients was more than 15 times higher; 1393.95 pmol/hour/mg protein.

Vascular MAO activity (MAO-A and -B together) was 316.50 pmol/hour/mg protein. Platelet rich plasma MAO-B activity was 39.58 nmol/hour/10^9 platelets corresponding to 275.72 pmol/hour/mg protein.

In vascular tissue preparations SSAO activity predominated; it reached 81.50% of total amine oxidase activity (Fig. 1.). However in platelet rich plasma MAO-B activity was considerably higher than that of SSAO; it was up to 75.09% of total plasma amine oxidase activity.

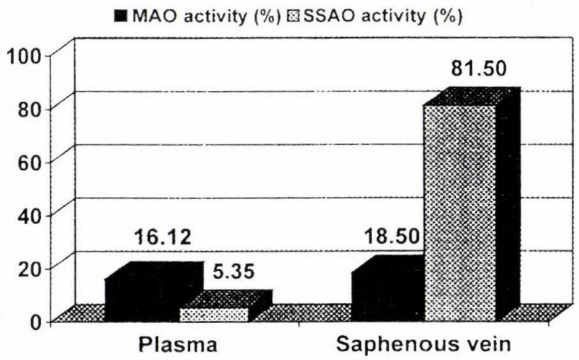

Fig. 1. SSAO and MAO activity in human platelet rich plasma and saphenous vein (percent of total vascular amine oxidase activity)

DISCUSSION AND CONCLUSIONS

Our results support the findings of Yu et al. [1992] that MDL 72974A is capable of inhibiting human semicarbazide-sensitive amine oxidases. The inhibition by MDL 72974A was irreversible and time dependent in that study. The IC_{50} values of MDL 72974A were similar in human umbilical artery and human saphenous vein. Bovine and human serum were relatively insensitive to MDL 72974A and semicarbazide compared to vascular tissues. However, human serum SSAO activity was considerably lower than that of the vascular tissues. This finding supports the hypothesis, that an endogenous SSAO inhibitor may be present in human serum [Banchelli et al., 1988].

Remarkably, in human saphenous vein SSAO exhibited higher activity compared to MAO type A and B using ^{14}C-benzylamine as a substrate. These vascular amine oxidases (especially MAO-A) are responsible for extraneuronal deamination of noradrenaline [Osswald, 1990], and 5-hydroxytryptamine [Vanhoutte, 1983]. Monoamine oxidase activity is not uniform throughout the cardiovascular system [Cohen et al., 1979]. In cystic artery the deamination of biogenic amines is mainly carried out by SSAO with a lower participation of MAO-B. In colonic arteries a low MAO-A activity was also detected [Figueiredo et al., 1998]. The exact physiological role and main substrate of vascular SSAO have not been elucidated so far.

Platelet rich plasma MAO-B exhibited considerably higher activity than that of SSAO. However, increased activity of SSAO in human serum might be more dangerous than increased platelet MAO-B activity. Catalase, peroxidase and aldehyde dehidrogenase enzymes, which protect against hydrogen-peroxide and aldehyde toxicity, are confined to the intracellular compartment, and cannot protect endothelial cells from products of serum SSAO enzyme. It is possible, that effective and selective inhibitors of human SSAO enzymes might exert cytoprotective effect on endothelial cells.

The potency of the known SSAO inhibitors (hydrazine derivatives, 2-phenyl-3-haloallylamines, 4-picolylamine analogues, aromatic α-methyl-monoamines, etc.) is determined by the species and tissue source of the enzyme [Lyles, 1996]. Thus, in vitro measurements on human tissues are necessary during development of selective SSAO inhibitors.

The similar SSAO inhibitory effect of MDL 72974A and semicarbazide on SSAO enzymes from human vein and serum support the hypothesis that soluble and membrane-bound vascular human SSAOs might have similar structure and inhibitor sensitivity. This characteristic of human SSAO enzymes might be beneficial in the development of cytoprotective SSAO ihibitor drug candidates.

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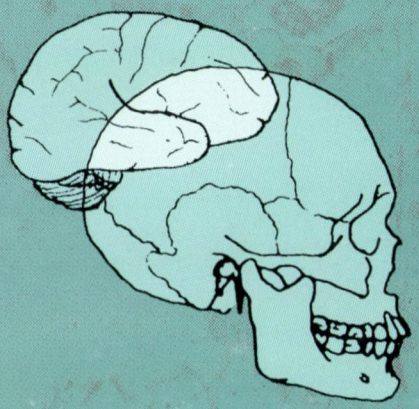

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RESEARCH REPORT

THE EFFECT OF ETHANOL CONSUMPTION ON THE SENSITIVITY OF RAT BRAIN MONOAMINE OXIDASES TO THE INHIBITION BY PARGYLINE *IN VIVO* AND *IN VITRO**

PANOVA, N.G., AXENOVA, L.N. and MEDVEDEV, A.E.

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The effect of ethanol consumption on the sensitivity of rat brain mitochondrial monoamine oxidases to the inhibition by pargyline *in vivo* and *in vitro* was investigated. Administration of pargyline (10 mg/kg, s.c.) produced significantly higher inhibition of MAO-A in alcoholised rats, whereas MAO-B inhibition did not differ from that observed in control animals. The concentration-response curve for the inhibition of brain mitochondrial MAO-A and MAO-B by pargyline *in vitro* did not reveal higher sensitivity of MAO from alcoholised rats to pargyline. This probably means that more pronounced inhibition by pargyline of brain MAO-A in alcoholised rats *in vivo* can be attributed to decreased content of compounds reversibly interacting with the its catalytic site. Taking into consideration some ethanol-induced decrease of brain tribulin content we suggest that the reduced level of endogenous inhibitors (tribulin components?) may have some importance in the development of alcoholism.

Keywords: ethanol, brain monoamine oxidase, tribulin, sensitivity to endogenous inhibitors, pargyline

INTRODUCTION

The development of alcoholism is accompanied by alterations in metabolism of monoamine neurotransmitters [Anokhina et al., 1987]. Changes of monoamine oxidase (MAO) activity in alcoholism are still a matter of controversy. Previously we had found that the most pronounced inhibition of rat liver mitochondrial MAO was detected during acute administration of a large dose of ethanol whereas chronic ethanol feeding caused less pronounced effect

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[Medvedev et al., 1995]. Rats characterised by alcohol preference had higher MAO activity and [^{3}H]pargyline binding than water preferring rats, whereas urinary output of monoamine oxidase inhibitory activity (tribulin) was higher in water preferring rats [Medvedev et al., 1996].

Tribulin, endogenous monoamine oxidase inhibitory activity extractable into ethyl acetate has been found in many mammalian tissues and body fluids [Glover and Sandler, 1993; Glover, 1998]. It contains several components selectively inhibiting MAO-A and MAO-B, respectively [Medvedev et al., 1992; Glover 1998]. Augmentation of tribulin content was observed in a variety of conditions associated with stress and anxiety [Glover and Sandler, 1993, Glover 1998]. Some evidence exists that endogenous regulation of MAO by tribulin (components) may have biological importance. For example, in conditions characterised by increased tribulin output the administration of antidepressant phenelzine to rats produced smaller MAO inhibition compared to the control [Clow et al., 1989]. These data suggest endogenous MAO inhibition by tribulin *in vivo* which decreases the effect of exogenously administered inhibitors [Glover and Sandler, 1993]. In fact, the administration of isatin, the major MAO-B-inhibitory component of tribulin, attenuated irreversible inhibition of MAO-B *in vivo* caused by phenelzine administration [Panova et al., 1990].

However, in our viewpoint much evidence for the involvement of endogenous factors reversibly interacting with MAO *in vivo* could be obtained during the comparison of MAO inhibition *in vivo* and *in vitro*. If certain (stress) influences affect only the content of endogenous inhibitors (tribulin components, etc.) we may expect altered sensitivity of MAO to exogenous inhibitors only *in vivo* but not *in vitro* (especially after mitochondria wash).

Inhibition of brain MAO by tribulin during alcohol intoxication was not investigated yet and so we have investigated the effect of ethanol consumption on the sensitivity of rat brain monoamine oxidases to the inhibition by pargyline *in vivo* and *in vitro*.

MATERIALS AND METHODS

Protocol of chronic ethanol feeding of rats was described previously [Medvedev et al., 1995]. One day before experiment (pargyline administration) ethanol solution was substituted for water. Pargyline (10 mg/kg, s.c.) was injected 1.5 hours before decapitation.

Isolation of rat brain mitochondria and assay of MAO activity were described previously [Medvedev et al., 1994].

For the isolation of tribulin fraction rat brains were homogenised in 1 N HCl using glass homogeniser and a teflon pestle and centrifuged at 3,000 g during 15 min. Clear supernatant was extracted with 2 volumes of ethyl acetate. The organic phase was evaporated to dryness under nitrogen and the final residue was reconstituted in phosphate buffer, pH 7.4 and aliquots were used for determination of inhibition of rat liver mitochondrial MAO-A and MAO-B, using 0.1 mM [14-C]5-hydroxytryptamine or 0.005 mM [14-C]phenyl-ethylamine, respectively [Medvedev et al., 1996].

Data were statistically treated by Student's t-test.

RESULTS

Chronic ethanol feeding of rats (during 3 months) had minor influence on the activity of brain mitochondrial MAO-A (+17%) and MAO-B (-3.5%) ($p > 0.2$-0.5). This is consistent with the recent observation from the other laboratory [Della Corte et al. 1994]. There was a small but statistically significant reduction in the content of MAO-A and MAO-B inhibitory components of tribulin in the brain of alcoholised rats (Table 1). Administration of pargyline (10 mg/kg, s.c.) to control rats caused the inhibition of brain mitochondrial MAO-A and MAO-B by 33 and 85%, respectively (Table 2). The same treatment of alcoholised rats produced significantly higher inhibition of MAO-A (54%, $p<0.01$) whereas MAO-B inhibition did not differ from that observed in control animals (90%).

There were no significant differences in the sensitivity of brain mitochondrial MAO from control and alcoholised animals to inhibition by pargyline *in vitro* (Fig. 1).

Table 1. The influence of chronic alcoholisation of rats on the content of MAO-A -and MAO-B-inhibitory components of brain tribulin (MAO-AI and MAO-BI, respectively)

Group of animals	MAO-AI	MAO-BI
Control	81.0 + 1.1	88.3 + 0.1
Alcoholisation	68.2 + 2.4	78.3 + 0.4
p	<0.02	<0.02

Tribulin activity was expressed as per cent of MAO inhibition in the test system.

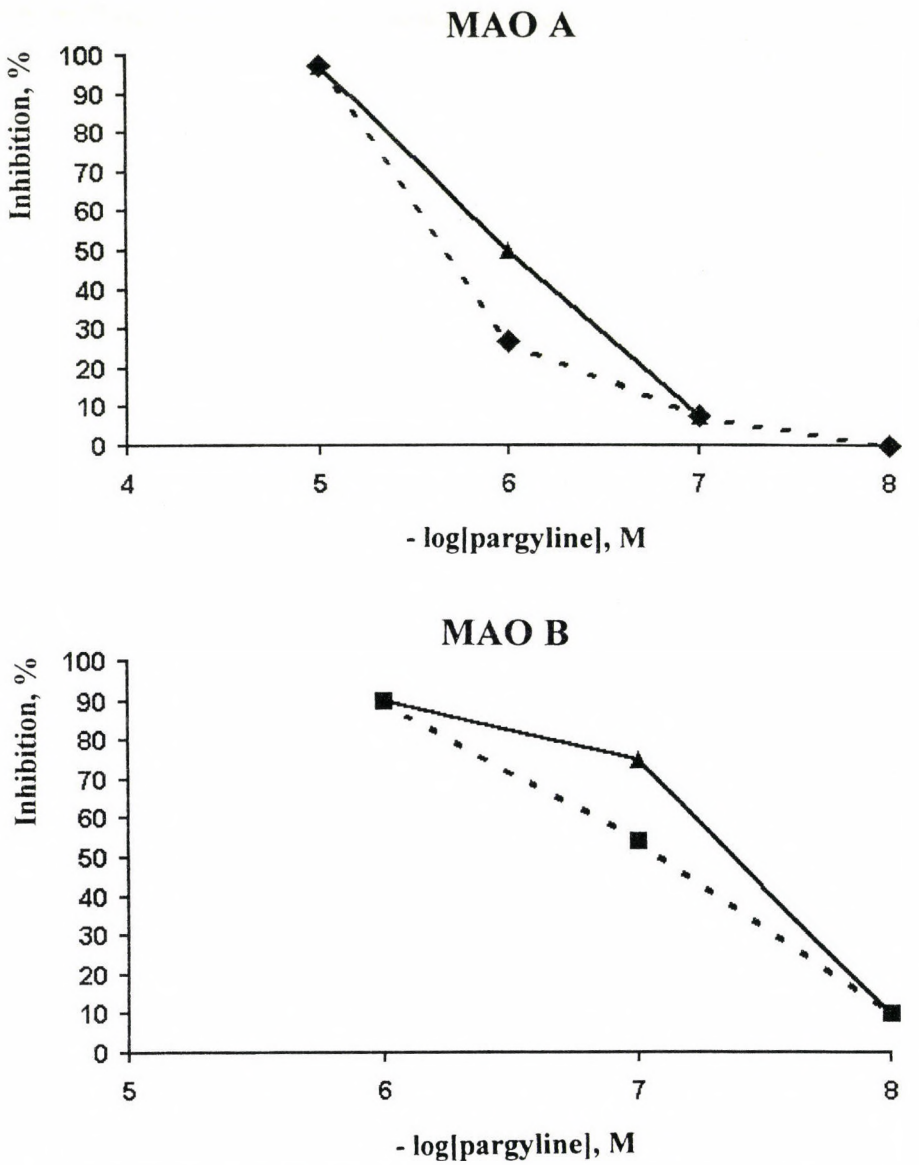

Fig. 1. The influence of pargyline on the activity of MAO-A and MAO-B *in vitro*. Mitochondria were preincubated with pargyline for 30 min at 37 °C. Solid line - control, punctured line - alcoholised rats

Table 2. The influence of pargyline administration on the inhibition of brain MAO-A and MAO-B

Group of animals	MAO-A	MAO-B
Control	33.4 + 3.9	85.2 + 3.1
Alcoholisation	53.6 + 3.2	89.7 + 0.6
p	<0.01	n. s.

Data expressed as percent of MAO inhibition. MAO activity in the groups of intact animals (both control and alcoholised) was considered as 100%. In each group n=5–7

DISCUSSION

There are at least three types of changes in MAO activity induced by alcohol consumption: 1. Oxidation of MAO molecules induced by LPO-dependent changes in membranes during acute alcohol intoxication; 2. Alteration of compartmentalisation of MAO during chronic alcohol consumption; 3. Alteration in the content of endogenous regulators (tribulin, carbolines, etc.) [Medvedev, 1996].

Various endogenous factors can influence MAO activity *in vivo* and isolation of mitochondria for determination of the enzyme activity *in vitro* usually removes most of them. So the comparison of sensitivity of MAO to exogenous inhibitors *in vivo* and *in vitro* is crucial for the evaluation of a possible role of endogenous regulation of MAO under some (patho)physiological conditions.

MAO in brain mitochondria of control and alcoholised rats exhibited the same *in vitro* sensitivity to pargyline. Consequently, more pronounced inhibition of brain MAO-A by pargyline in alcoholised rats *in vivo* can be attributed to decreased content of compounds reversibly interacting with the its catalytic site. This is consistent with some reduction of MAO-A-inhibitory component of tribulin induced by chronic ethanol feeding. It is possible that potent inhibition of MAO-B by pargyline *in vivo* did not allow us do detect the influence of chronic ethanol feeding (which caused some reduction of MAO-B-inhibitory component) on pargyline effect.

Since some tribulin components may attenuate effects of mechanism-based inhibitor(s) on MAO *in vivo* [Clow et al., 1989, Panova et al., 1997] we suggest that the increased sensitivity of brain MAO-A to pargyline *in vivo* but not *in vitro* may reflects ethanol-induced deficit of tribulin components and other endogenous MAO inhibitors. Such reduction of some endogenous MAO inhibitors can be crucial for the development of alcoholism.

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RESEARCH REPORT

NEUROPROTECTIVE ASPECTS OF A NOVEL MAO-B INHIBITOR PF9601N*

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PF9601N is an acetylenic tryptamine derivative devoid of amphetamine-like properties, that behaves as suicide MAO-B inhibitor more potent than l-deprenyl. It is highly selective towards MAO-B and it neuroprotects from the neurotoxicity induced in C57Bl/6 adult mice by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). PF9601N also shows *in vitro* antioxidant properties by inhibiting the dopamine autoxidation. A potential therapeutic use in Parkinson's disease treatment is proposed for this compound.
Keywords: MAO-B inhibitor, antioxidative, neuroprotection

INTRODUCTION

Monoamine oxidase (MAO) is a FAD-dependent enzyme located on the mitochondrial outer membrane which deaminate endogenous amines and some xenobiotics. Two MAO isoforms has been reported according to pharmacological criteria. MAO-A selectively metabolizes serotonin and is sensitive to specific inhibition by acetylenic inhibitor clorgiline, whereas MAO-B has greater affinity towards 2-phenylethylamine and is selectively inhibited by l-deprenil (Johnston, 1968).

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MAO-B inhibitors, including l-deprenyl, have showed to prevent MPTP-induced Parkinson-like neurotoxicity in animals, because the pro-toxin is not metabolized by MAO-B to MPP^+ (Heikkila et al., 1984). Furthermore, l-deprenyl has been shown to prevent dopamine autoxidation process (Chiueh et al., 1994), which was suggested to be important in dopaminergic neuron degeneration (Fornstedt, 1990).

Recently, it has been shown that the acetylenic tryptamine derivative N-(2-propinyl)-2-(5-benzyloxyindol)methylamine, the PF9601N compound, is a potent selective and irreversible MAO-B inhibitor (Pérez et al., 1998). The present work was undertaken to study the PF9601N neuroprotective role in MPTP-lesioned mice, and its antioxidant effect on dopamine autoxidation.

Our results show that this IMAO-B is able to decrease the nonenzymatic dopamine autoxidation process and is also able to prevent the MPTP-toxicity in C57Bl/6 mice.

MATERIALS AND METHODS

To determine the neuroprotective effect of PF9601N, C57Bl/6 male mice (8 week) were injected intraperitoneally with MPTP-HCl (30 mg/kg) either alone or in combination with PF9601N (29.4 μmol/kg), or with the specific IMAO-B l-deprenyl (29.4 μmol/kg) for comparative proposes. Twenty-four hours later, the mice were injected with a second MPTP dose and were sacrified by decapitation 48 hours after the first injection. The brains were removed and the striata was dissected. The tissue was weighed and then sonicated in a medium containing 250 μM EDTA, 100 μM $Na_2S_2O_5$ and 250 μM $HClO_4$ and were centrifuged for 10 minutes. The supernatants were used for measurement of dopamine and metabolites, performed by HPLC using an ESA Coulochem II electrochemical detector.

The dopamine antioxidant effect of PF9601N was assayed by the Chiueh method (1994). Briefly, the rate of dopamine autoxidation in pH 7.4 Earle's balanced salt solution (EBSS) without glucose, was measured spectrometrically (450 nm) by assaying dopamine melanin formation. Thus, 5 mM dopamine either alone or with 2.5 mM PF9601N or 2.5 mM l-deprenyl, in presence of 5 mM MPP^+ were added in borosilicate glass vials contained 3 mL EBSS (volume final). At different times, the nonenzymatic dopamine autoxidation by melanin formation was measured in these samples.

RESULTS

The administration of MPTP in C57Bl/6 male mice showed a decrease in striatal dopamine (DA) and their metabolites DOPAC and HVA. Furthermore, as shown in Table 1, the DA decrease by MPTP toxicity was 60%, approximately, when compared with the striatal dopamine present in the control mice.

Table 1. Dopamine and metabolite contents in striatal mice tissue

Group	n	Dopamine	DOPAC	HVA
(1) Controls (*)	11	9441 ± 1645	1159 ± 311	1578 ± 489
(2) MPTP (30 mg/kg) x 2	10	2849 ± 686	661 ± 151	1171 ± 214
(3) FA-73 (29.5 mmols/kg) + MPTP (*)	7	8719±2170	1370±253	2045±589
(4) FA-73 (19.4 mmols/kg) + MPTP (*)	3	9570±357	624±97	1072±62
(5) FA-73 (12.8 mmols/kg) + MPTP (*)	3	9858±398	673±32	1025±97
(6) FA-73 (8.47 mmols/kg) + MPTP (*)	8	9377±1526	713±153	1103±162
(7) FA-73 (5.59 mmols/kg) + MPTP (**)	7	6971±413	1020±91	1288±446
(8) FA-73 (3.68 mmols/kg) + MPTP (**)	5	5651±1107	601±163	1238±278
(9) FA-73 (2.42 mmols/kg) + MPTP	6	4140±455	464±153	999±220
(10) FA-73 (1.5 mmols/kg) + MPTP	3	2141±171	516±44	1011±72
(11) FA-73 (0.354 mmols/kg) + MPTP	3	1875±483	646±3	1109±177

Catecholamines were determined in striatal mice tissue after i.p. treatment of MPTP (30 mg/kg) alone or combined with PF9601N or with l-deprenyl. Data are expressed in pmols catecholamine/mg striatal tissue ± SD. (*) Significantly different from MPTP-group as analyzed using Scheffé test after ANOVA. $p<0.05$

When the IMAOs B PF9601N or l-deprenyl were administered with the MPTP-treatment, they prevented completely the depletion of striatal DA induced by repeated i.p. administration of MPTP (30 mg/kg) in C57Bl/6 male mice. Thus, the striatal dopamine content in treated mice with thous IMAOs B (29.5 μmol/kg) plus MPTP (30 mg/kg) showed similar values related to control (see Table 1). This data indicate that these compounds are able to prevent the mice MPTP-toxicity, showing a neuroprotector effect on this dopaminergic neurotoxin (Fig. 1).

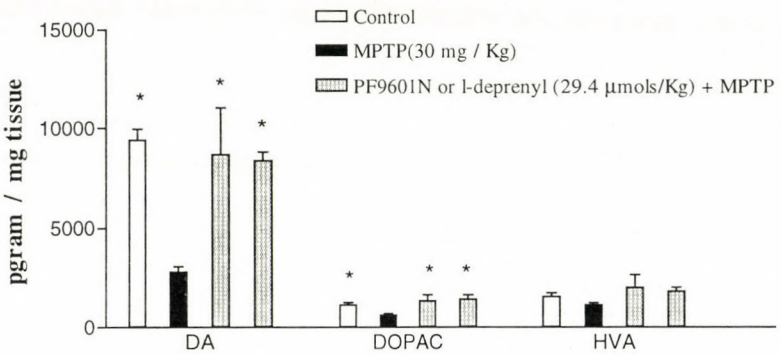

Fig. 1. Neuroprotection by PF9601N and l-deprenyl from MPTP-toxicity. Dopamine and metabolites were determined in striatal tissue after 24 hours from the last MPTP-mice injection alone or combined with both IMAOs B PF9601N, or l-deprenyl. (*) Significantly different from MPTP-group as analyzed using Scheffé test after ANOVA. $p<0.05$

Dopamine melanin formation was evaluated by the PF9601N or l-deprenyl effect. When the MPP^+ was added to dopamine, the rate of oxidation of this neurotransmitter was increased 15%, approximately, whereas using the same assay conditions, the IMAOs B PF9601N or l-deprenyl were able to disminish dopamine autoxidation in a time-dependent fashion (Fig. 2).

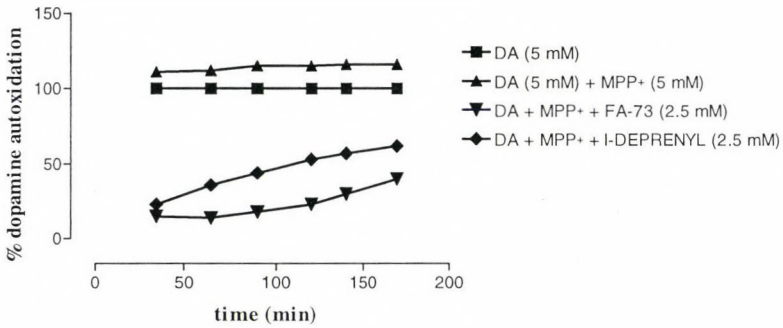

Fig. 2. Time-dependent antioxidant properties of the PF9601N. Dopamine melanine formation after incubation at different periods of time in presence of 2.5 mM PF9601N or l-deprenyl

Thus, these compounds at 2.5 mM concentration showed a protection of dopamine autoxidation after 35 minutes of reaction (15% and the 23% for PF9601N and l-deprenyl, respectively). When the dopamine melanin formation was measured at 170 minutes of the reaction start, these values increased to 40% of controls for PF9601N compound and 62% for l-deprenyl (Table 2).

Table 2. Time-dependent dopamine antioxidative effect by PF9601N or l-deprenyl

	% Autoxidation of dopamine					
	35 min	65 min	90 min	120 min	140 min	170 min
DA	100	100	100	100	100	100
([a]) DA + MPP^+	111 ± 2	112 ± 2	115 ± 1	115 ± 2	116 ± 1	116 ± 1
([b]) DA + MPP^+ + FA-73 (2.5 mM)	15 ± 1	14 ± 1	18 ± 2	23 ± 1	30 ± 1	40 ± 1
([b]) DA + MPP^+ + deprenyl (2.5 mM)	23 ± 1	36 ± 1	44 ± 1	53 ± 2	57 ± 2	62 ± 2

Dopamine melanine formation was measured at different periods of time in presence of 2.5 mM concentration of PF9601N or l-deprenyl.

DISCUSSION

MPTP has been described as Parkinson-like toxin (Langston, 1984). In the present study we demonstrated that the acetylenic tryptamine derivative PF9601N compound, a novel IMAO-B (submitted), showed neuroprotective properties, preventing MPTP-toxicity in C57Bl/6 male mice. Such neuroprotective effects have been described for other IMAOs B (Heikkila et al., 1984). The neuroprotective role of PF9601N was similar to that showed by l-deprenyl in this parkinsonism animal model. Furthermore, the effect elicited by l-deprenyl is in agreement with that reported by other authors (Fredriksson et al., 1997).

The present *in vitro* study, demonstrates that dopamine melanin formation is also partially supressed by acetylenic MAO-B inhibitors PF9601N and l-deprenyl. These data are in agreement with that published by others authors about propargyl MAO inhibitors (Yu et al., 1994). It has also been described the antioxidant effects of l-deprenyl in the nonenzymatic dopamine autoxidation (Chiueh et al., 1994).

In addition the antioxidant effects observed are comparable between both compounds at 35 minutes of the reaction start time, whereas, after 170 minutes,

PF9601N showed a decreasing of 20% of dopamine autoxidation when compared with l-deprenyl.

In summary, the protective properties showed by the new MAO-B inhibitor PF9601N in the Parkinson-like MPTP-toxicity model, and their antioxidant properties in the dopamine autoxidation process, suggest that this compound, devoid of amphetamine-like properties, would be interesting in the study of dopaminergic transmission and might be proved useful as potential therapeutic agent in Parkinson's disease.

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RESEARCH REPORT

INVOLVEMENT OF CA^{2+} IN DOPAMINE RELEASE IN STRIATAL RAT SLICES BY PF9601N AND L-DEPRENYL*

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Rat striatal slices were incubated 1 hour in Krebs buffer with carbogen by continuos perfusion. When both MAO-B inhibitors PF9601N and l-deprenyl were added in the incubation medium, at 200 μM concentration, a diminution in the dopamine content was observed. The decrease in dopamine content was partially calcium dependent and showed a different mechanism between both compounds. When 1 mM concentration EGTA was added at the Krebs incubation medium without calcium, dopamine content was partially recovered, being not affected in case of l-deprenyl. When 50 μM dantrolene (a calcium vesicular release inhibitor) was added in the Krebs incubation medium without calcium, dopamine was partially recovered for l-deprenyl, being not affected in case of PF9601N. These data indicate that dopamine release is extracellular calcium dependent in case of PF9601N, whereas l-deprenyl depends on calcium intracellular origin.
Keywords: MAO-B inhibitor, antioxidative, neuroprotection.

INTRODUCTION

The acetylenic tryptamine derivative N-(2-propinyl)-2-(5-benzyloxy-indol)methylamine, the PF9601N compound, is a selective and irreversible MAO-B inhibitor which shows a K_i value for this enzyme of 0.75 nM, a 22.4 times lower value than that obtained for l-deprenyl (Pérez et al., 1998). Both compounds showed neuroprotective effects on dopaminergic toxicity in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) lesioned mice.

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Furthermore, both PF9601N and l-deprenyl are able to stimulate contralateral rotation in the turning behaviour animal model (Fuxe and Ungerstedt, 1974) showed by L-DOPA administration (data not shown). This behaviour could be due to a release of striatal dopamine produced by these MAO-B inhibitors. In this context, we have used rat striatal slices to investigate dopamine release by PF9601N and l-deprenyl, and the involvement of the Ca^{2+} ion in this process. Our studies indicate that although both derivatives are able to produce dopamine release, this is partially-dependent on external calcium in case of PF9601N, whereas the dopamine release by l-deprenyl is partially-dependent on internal calcium.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (200 to 250 g) were used for all experiments. The care and use of these animals were in accordance with the policy on the use of animals in neuroscience research published by the Society for Neuroscience.

Striatal slices preparation

Rats were killed by decapitation. The right and left striata were quickly chopped into 0.3 mm sections and incubated at 37 °C in 1 mL Krebs-Ringer bicarbonate buffer, pH 7.2, in a shaking water bath under an atmosphere of 95% O_2 / 5% CO_2.

The medium consisted of 119 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 23.8 mM $NaHCO_3$, 5.5 mM glucose and 25 mM MOPS (Vyas et al., 1986).

In the extracellular calcium-dependence experiments, calcium was omitted in the Krebs incubation medium and 1 mM EGTA was added. In case of the studies on the dependence of intracellular calcium, 50 μM dantrolene a calcium vesicular release inhibitor, was added to the medium and the slices were pre-incubated 10 minutes before PF9601N or l-deprenyl were added.

Incubations were carried out in all cases for 1 hour. The slice protein content was measured by the Bradford method (Bradford, 1976).

Dopamine measurement

Slices were homogenized by sonication in 200 µL of medium containing 250 µM EDTA, 100 µM $Na_2S_2O_5$ and 250 µM $HClO_4$ and were centrifuged at 12,000 g for 10 minutes. The supernatants were used for dopamine measurement, performed by HPLC using a ESA Coulochem II electrochemical detector.

RESULTS

When the rat striatal slices were incubated in the Krebs medium 1 hour (basal and no depolarizing conditions), dopamine content in this tissue was of 2.17 ± 0.18 nmols/mg of prot., and this was reduced approximately, to 70%, approximately, after incubation in presence of 200 µM PF9601N. This effect in dopamine content was similar to that observed when the slices were incubated with 200 µM l-deprenyl. In the later case, the dopamine content was reduced to 35%, approximately (Table 1).

Table 1. Dopamine content in rat striatal tissue

Group	n	Slice dopamine content (nmols / mg prot)	%
Control	3	2.17 ± 0.18	100
PF9601N (1 µM)	3	1.69 ± 0.07	78
(*) PF9601N (200 µM)	3	0.63 ± 0.12	29
(*) l-deprenyl (200 µM)	3	0.74 ± 0.04	34

Striatal slice dopamine content after 1 hour of incubation with PF9601N or l-deprenyl. Values are the mean ± SD (n=3) and 12 slices for experiment. (*) Significantly different from control, as analyzed using Scheffé test after ANOVA, $p< 0.05$

The dependence on external calcium of the dopamine release was assayed, omitting the Ca^{2+} ion in the Krebs incubation medium containing 1 mM EGTA as chelator. In these conditions, when this tissue was incubated with PF9601N or l-deprenyl, striatal slice dopamine content was affected in a different manner. Indeed, the dopamine release due to 200 µM PF9601N incubation, was only reduced to 40%, approximately, recovering a 100%, when comparing with the values observed in presence of calcium ion (Table 2).

Table 2. Effect of extracellular calcium in dopamine release

Group	n	Slice dopamine content (nmols / mg prot)	%
Control	3	2.17 ± 0.17	100 ± 7.8
(*) PF9601N (200 μM) (+) Ca^{2+}	3	0.63 ± 0.12	29.03 ± 5.5
(*) l-deprenyl (200 μM) (+) Ca^{2+}	3	0.74 ± 0.04	34.1 ± 1.8
(*)(**)PF9601N (200 μM) (-) Ca^{2+}	3	1.32 ± 0.08	60.8 ± 3.6
(*) l-deprenyl (200 μM) (-) Ca^{2+}	3	0.89 ± 0.03	41.0 ± 1.4

Striatal slice dopamine content after 1 hour of incubation in a Krebs medium, which contained or not calcium, with PF9601N or l-deprenyl. Values are the mean ± SD (n=3) and 12 slices for experiment (*) Significantly different from control, (**) Significantly different from values obtained by the same compound in a medium with calcium, as analyzed using Scheffé test after ANOVA, $p< 0.05$

Furthermore, in case of l-deprenyl no significant differences were observed when the striatal slices were incubated in presence or absence of extracellular calcium (Table 2).

The incubation of striatal slices with 50 μM dantrolene affected dopamine release by l-deprenyl, whereas dopamine depletion observed by the PF9601N was not altered. Thus, slice dopamine content after 1 hour incubation in presence or absence of dantrolene was 60%, approximately, compared to control slices (Table 3).

Table 3. Effect of intracellular calcium in dopamine release

Group	n	Slice dopamine content (nmols / mg prot)	%
Control	3	2.17 ± 0.17	100 ± 7.8
(*) FA-73 (200 μM) - dantrolene	3	1.32 ± 0.08	60.8 ± 3.6
(*) l-deprenil (200 μM) - dantrolene	3	0.89 ± 0.03	41.0 ± 1.4
(*) FA-73 (200 μM) + dantrolene	3	1.49 ± 0.24	68.6 ± 11
(*)(**) l-deprenyl (200 μM) + dantrolene	3	1.31 ± 0.14	60.3 ± 6.4

Striatal slice dopamine content after 1 hour of incubation in a Krebs medium, which contained or not the vesicular calcium release inhibitor 50 μM dantrolene, PF9601N or l-deprenyl. Values are the mean ± SD (n=3) and 12 slices for experiment. (*) Significantly different from control. (**) Significantly different from the values obtained by the same compound in a medium with calcium, as analyzed using Scheffé test after ANOVA, $p< 0.05$

Furthermore, in the presence of 50 μM dantrolene in the incubation medium without calcium, the striatal dopamine content showed by l-deprenyl increased a 50%, approximately, when compared with the values obtained in absence of dantrolene (Table 3).

DISCUSSION

Striatal dopamine depletion showed by l-deprenyl is in agreement with the data previously reported (Kalasz et al., 1990, Neusch et al., 1997). This effect was similar to that elicited by the PF9601N at same concentration, despite that not all IMAOs B are dopamine releasers (Lupp et al., 1993).

The recovery of the striatal slice dopamine content showed by PF9601N, in absence of extracellular calcium, was not 100%, indicating that this effect is partially-dependent on extracellular calcium, as it has been described before (Levent, 1997, Elverfors et al., 1997, Nankai et al., 1998). Furthermore, dopamine depletion in nondepolarizing conditions due to l-deprenyl, showed to have an extracellular calcium independent mechanism. These results are different to those described by Neusch et al. (1997) who related a dopamine release effect by l-deprenyl with the inhibition of ATP-dependent potassium channels. This difference could be explained by different experimental conditions in the evoked-dopamine release used by these authors, since their slices had been depolarized.

The inhibition of vesicular calcium release affected only the dopamine depletion observed by l-deprenyl, while it was not observed in case of PF9601N. In the first case the striatal slice dopamine recovery was not 100%, indicating that the dopamine release could be due to a calcium independent process in which the dopamine transporter could be involved (Kalisch et al., 1996; Elvefors et al., 1997; Kantor et al., 1998).

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RESEARCH REPORT

MAO-A AND MAO-B LOCALISATION IN HUMAN LUNG AND SPLEEN*

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Localisation of MAO-A and -B in human lung and spleen was studied by immunohistochemistry. The primary antibodies used were mouse monoclonal anti-human MAO-A (6G11/E1) and anti-human MAO-B (3F12/G10/2E3). Samples of lung and spleen were obtained from 6 routine autopsy cases. Both immunoreactivities showed a homogeneous distribution in lung, where all cell types had both MAO-A and -B staining. In spleen MAO-A and -B showed a very weak immunoreactivity, which was restricted to smooth muscle cells and reticular cells of the white pulp. These data represent the most comprehensive study of MAO-A and -B localisation in the two tissues.
Keywords: monoamine oxidase (MAO), human, lung, spleen, immunohistochemistry

INTRODUCTION

Monoamine oxidases (MAO; EC 1.4.3.4) are the main degradative enzymes of monoamine hormones, neurotransmitters and trace amines. Two MAO isoenzymes named MAO-A and MAO-B, have been identified (Johnston, 1968) which differ in inhibitor sensitivity and substrate selectivity (Waldmeier, 1987; Cesura and Pletscher, 1992). They also present different cellular localisation in the human CNS (Saura et al., 1996). Thus, MAO-A is found

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in the brain in adrenergic and noradrenergic neurones (Major et al., 1979; O'Carroll et al., 1986; Strolin Benedetti and Dostert, 1992); whereas MAO-B is present in astrocytes and both serotoninergic and histaminergic neurones (Levitt et al., 1982; Waldmeier et al., 1977; Westlund et al., 1988).

In contrast to the human brain, little is known about the localisation of MAO-A and MAO-B in human peripheral tissues. In a recent paper we described the distribution and localisation of MAO-A and MAO-B in six human peripheral tissues by means of quantitative autoradiography (Saura et al., 1996). The absence of cellular resolution of the technique did not allow us to identify the cell types expressing MAO-A and MAO-B. In the present study, immunohistochemistry with specific anti-human-MAO-A and MAO-B antibodies was conducted to answer this question in lung and spleen.

MATERIALS AND METHODS

Human lung and spleen samples, in accordance with local institutional guidelines, were taken from 6 routine autopsy cases (3 males and 3 females). The age was 73.6 ± 4.9 years (mean ± SEM,) and the *post mortem* delay 12.3 ± 2.5 hours (mean ± SEM). Immediately after autopsy, samples were fixed by immersion in 2% paraformaldehyde in 0.1 M phosphate buffer at 4 °C for 4 hours. After fixation, tissues were immersed for 72 hours in 15% sucrose in 0.1 M phosphate buffer for cryoprotection, frozen in dry ice and stored at -30 °C. Twelve µm cryostat sections were obtained and mounted on gelatinized slides.

The peroxidase-antiperoxidase (PAP) technique was used for immunohistochemistry. As primary antibodies for the detection of MAO-A and MAO-B the mouse monoclonal anti-human MAO-A (6G11/E1) (Yeomanson, 1990; Church et al., 1994) and anti-human MAO-B (3F12/G1012E3) (Yeomanson and Billett, 1992) were used diluted 1:50 and 1:75, respectively, in normal swine serum. Incubation was performed at 4 °C for 24 hours. After washing, sections were incubated for 30 min in rabbit anti-mouse IgG (1:100), rinsed and washed in PBS, and incubated for 30 min in mouse/PAP (1:100). Sections were developed for 15 min in a 0.05 M Tris solution containing 0.03% DAB and 0.006% H_2O_2.

RESULTS

Lung

The same localisation of MAO-A and MAO-B immunoreactivity (ir) was found in lung. Ciliated and Clara cells, both from blonchiole, showed a strong staining for MAO-A and MAO-B antibodies. A strong signal was also observed in alveoli, where both irs were found in all pneumocytes.

Spleen

MAO-A and MAO-B staining also had the same pattern of localisation. The immunoreactivity observed was weak but present in the cells of the capsule and trabeculae and in some cells in the white pulp. In contrast, no signal was detected in the red pulp. It is interesting to note that spleen lymphocytes were negative for both immunoreactivities.

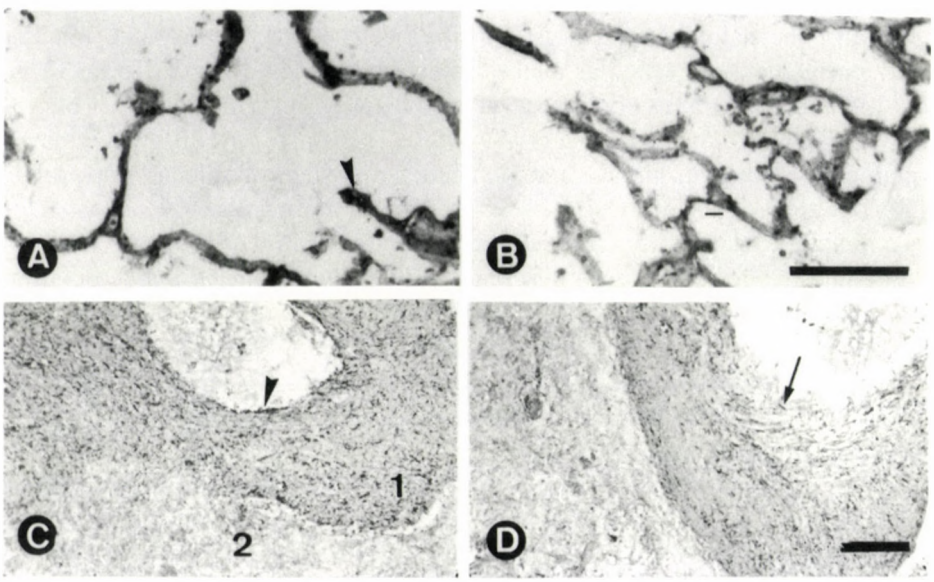

Fig. 1. MAO-A and -B immunostaining in adjacent sections of lung and spleen. (A) Uniform MAO-A ir in epithelial cells of the alveoli (arrow head). (B) MAO-B ir in an adjacent section of lung. (C) MAO-A ir in the spleen, staining was present in white pulp (1) and veins (arrow head) but not in the red pulp (2). (D) MAO-B ir presented the same pattern of localization; fibroblasts of arteries also had MAO ir (arrow) Calibration: 50 μm

DISCUSSION

The homogeneous localisation of the two enzymes in lung contrasts with human brain where both isoenzymes differ in cellular localisation and are resticted to some cellular types (Levitt et al., 1982; Strolin Benedetti and Dostert, 1992; Saura et al., 1997; Waldmeier et al., 1997). However, this widespread distribution and the co-localisation of MAO-A and MAO-B observed in lung is a commun feature in peripheral tissues since it is also described in liver, duodenum and kidney (Saura et al., 1996). The physiological role of pulmonary MAO, i.e. deamination of locally-released noradrenaline and of circulating dopamine, noradrenaline and adrenaline (Bryan-Lluka and O'Donnell, 1992), seems consistent with their homogeneous localisation.

In spleen, MAO-A and MAO-B also had an identical cellular distribution, which is consistent with our previous report (Saura et al., 1996). MAO of the capsula and trabeculae are found in the smooth muscular elements, and their role is probably the deamination of sympathetic noradrenaline. We were unable to identify the nature of the MAO positive cells in the white pulp, but they could correspond to reticular cells. Spleen lymphocytes were negative for MAO-A and MAO-B in contrast to lymphocytes in human blood that express MAO-B but not MAO-A (Balsa et al., 1989). This important difference may be explained by either an inmature or non-functional state of spleen lymphocytes, which could block the MAO-B gene expression in these cells.

To conclude, the widespread cellular distribution shown by MAO-A and -B in lung and the co-localization described in both tissues are in marked contrast with human brain tissue, where most cells are devoid of MAO and co-localisation of both isoenzymes is extremely rare (Westlund et al., 1985). However, the physiological substrates of MAO-A and -B in these tissues are unclear and further studies are required to ascertain the physiological role of MAO in both tissues.

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RESEARCH REPORT

APOPTOTIC AND ANTIAPOPTOTIC EFFECT OF (-)DEPRENYL AND (-)-DESMETHYL-DEPRENYL ON HUMAN CELL LINES*

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SUMMARY

The antiapoptotic effect of (-) deprenyl on human phaeochromocytoma cells after serum deprivation has been reported by earlier. Two melanoma (M-1 and HT-2058) cell lines were used in our experiments. Serum deprivation for five days resulted in excessive number of apoptosis in the cell cultures. Very low doses of (-)-deprenyl (10^{-7} - 10^{-13} mol) caused an approximately 2 days delay in the onset of apoptosis. At the same time, +deprenyl was ineffective. In further experiments (-)-deprenyl and (-)desmethyl-deprenyl was administered in higher doses (10^{-2}, 10^{-3} and 10^{-4} mol) to A-2058 melanoma and HT-1080 fibrosarcoma cells in culture. In these experiments no serum deprivation was applied and the treatment was started 24 hours after plating. Total eradication of the A-2058 cells was caused by 10^{-2} mol (-)-deprenyl and (-)-desmethyl-deprenyl. The type of cell death appeared to be apoptosis. Sixty percent apoptotic ratio was seen 24 hours and 72 hours after 10^{-3} mol (-)-desmethyl-deprenyl treteatment. The same dose of (-)-deprenyl caused 50% apoptosis an 72h. Only (-)-desmethyl-deprenyl induced apoptosis (20%) at 24 hours, in the dose of 10^{-4} mol. Interestingly (-)-deprenyl treatment resulted in 60% apoptosis

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48 hours and in 20% apoptosis. Seventy-two hours after the administration of this compound, in case of HT-1080 fibrosarcoma cells. Sixty per cent apoptosis was found in the culture of the same cell, tpye 72 hours after treatment with (-)-desmethyl-deprenyl. (-)-desmethyl-deprenyl, in the dose of 10^{-2}, eradicated this cell culture by apoptosis. On the other hand, 10^{-2} mol (-)-deprenyl caused so-called cytoplasmic, vacuolar, non-lysosomal active cell death (Clarke type III) 24, 48 hours after (-)-deprenyl administration. *In vivo* treatment of A-2058 melanoma xenografts (growing in immunodeprived mice) with 0.2-2-20 mg/kg daily subcutaneous dose of (-)-desmethyl-deprenyl resulted in dose-dependent growth inhibition of this tumor. Our results show that - deprenyl and its metabolite may influence apoptosis and other type of active cell death dose dependently. The role of caspases in this phenomenon should be investigated.

(-)-deprenyl (Selegilin) a known MAO-B inhibitor is used in the therapy of Parkinsonism, Alzheimer's disease and other neurodegenerative dysorders (1). The neuroprotective and neuronal rescue effect of this compound cannot be explained by the MAO-B inhibitory action of this substance. *In vitro* studies on cultured cells of neuronal origin pointed to the fact, that (-)-deprenyl prevents the apoptosis-inducing effect of serum deprivation and other damaging agents in such a low dose which does not exhibit MAO-B inhibitory effect (2). The antiapoptotic action of (-)-deprenyl has been first described on human phaeochromocytoma cells (3) and confirmed among others on human melanoma cells by our group (4). At the same time (+)deprenyl and a metabolite of (-)deprenyl, (-)-desmethyl-deprenyl proved to be ineffective in our experiments. As the antiapoptotic effect of (-)-deprenyl is dose-dependent in a paradox way - i.e. very low doses (10^{-7} - 10^{-9} - 10^{-12} mol) are more effective than higher doses (10^{-5} - 10^{-3} mol), the question emerged whether higher doses of (-)-deprenyl as well as (-)-desmethyl-deprenyl inhibit or enhance apoptosis in cell cultures.

MATERIALS AND METHODS

Cell culture

M-1 human melanoma (5), A-2058 human melanoma (ECACC 91100402) and HT-1080 human fibrosarcoma (ATCC CL 121) cells were cultured in G-well Grainer (Kremsmünster, Austria) plates containing glass cover slips. The culture medium was RPMI (GIBCO), supplemented with 10% calf serum (Bioproduct, Gödöllő, Hungary). Cell number at plating was 1.5×10^5/well. The plates were placed into a humidified 5% CO_2 incubator at 37 °C.

Treatment schedule No1
The medium was changed 48 hours after plating and no more serum supplementation was given since that time. Five days after changing the medium. (-)-deprenyl was given to the cell cultures to reach the final concentrations of 10^{-3}, 10^{-7} and 10^{-13} mol. M-1 and A-2058 cell cultures were also treated with the same doses of (+)-deprenyl. Samples were taken 24, 48 and 72 hours after treatment. Duplicate cover slip cultures per dose and per day, including 2-2 untreated controls were stained with Hematoxylin and Eosin. The percentage of apoptosis was determined taking into consideration the morphological signs of apoptosis (6).

Treatment schedule No2
A-2058 and HT-1080 cell cultures were treated 24 hours after plating with 10^{-4}, 10^{-3} and 10^{-2} mol (-)-deprenyl and (-)-desmethyl-deprenyl, respectively. Medium was changed at treatment, but no medium change was carried out following treatment. Triplicate samples were taken 24, 48 and 72 hours after treatment. Apoptotic and mitotic ratio was determined as described above.

Human tumor xenograft
A-2058 human melanoma, maintained in our Institute in immunodeprived 8 weeks old CBA/CA male mice was used. Treatment of tumor-bearing mice (10/group) was started 7 days after tumor inoculation when the diameter of the subcutaneously growing tumors reached 2-3 mm. The animals were treated daily, subcutaneously, with 0.2 - 2.0 - 20.0 mg/kg (-)-desmethyl-deprenyl, respectively, for 20 days. On day 21 the animals were exsanguinated under anesthesia, and the weight of tumors was measured. Body weight and two diameters were determined continuously in course of treatment.

RESULTS

Serum deprivation caused increasing number of apoptotic cell death in both M-1 and A-2058 melanoma cell cultures, which reached its peak 72 hours after treatment (8 days after serum deprivation). Low doses of (-)-deprenyl caused significant decrease in apoptosis, compared to the control, 10^{-13} and 10^{-9} mol being more effective than higher concentrations. This effect was observed in both melanoma cell cultures. Figure 1 shows the response of M-1 cells to serum

deprivation and (-)-deprenyl treatment. On the other hand, (+)-deprenyl was totally ineffective in prevention of apoptosis caused by starvation of the cells.

When A-2058 and HT-1080 cells were treated with (-)-deprenyl and (-)-desmethyl-deprenyl and serum deprivation was not performed, the dose of 10^{-2} mol resulted in total cell loss in case of A-2058 melanoma. 10^{-2} mol (-)-deprenyl treatment caused apoptosis in one-tenth of HT-1080 fibrosarcoma cells but the signs of cytoplasmic vacuolar active cell death was observed in 90% of the cells 24, 48 and 72 hours after treatment. (-)-deprenyl caused apoptosis in high per cent of the cells in 10^{-3} mol concentration, in case of A-2058 melanoma 72 hours after treatment and in the case of HT-1080 fibrosarcoma 48 and 72 hours after treatment. (-)-desmethyl-deprenyl showed the same effect in 10^{-3} and 10^{-4} mol concentration at hour 24 and in 10^{-3} mol concentration at hour 72 in case of A-2058 melanoma. High ratio of apoptosis of HT-1080 cells after 10^{-3} mol concentration of (-)-desmethyl-deprenyl was found 72 hours after treatment (Table 1). The mitotic index of the treated cell cultures decreased in parallel with the increase of apoptosis. The results of the *in vivo* studies are shown in Tables 2 and 3.

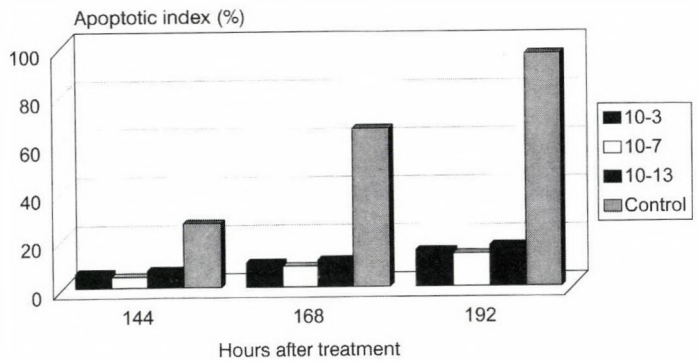

Fig. 1. The effect of (-)-deprenyl on apoptosis of M-cell cultures

(-)-desmethyl-deprenyl treatment of the tumor-bearing mice resulted in dose-dependent retardation of tumor growth. This effect proved to be significant when the changes in tumor volume were examined.

Table 1. Effect of (-)-deprenyl and (-)-desmethyl-deprenyl on A-2058 melanoma and HT-1080 fibrosarcoma cells in culture

Time table	A-2058		HT-1080	
	(-)-deprenyl	(-)-desmethyl-deprenyl	(-)-deprenyl	(-)-desmethyl-deprenyl
24 h				
10^{-2}	total cell loss	total cell loss	90%C-3, 10/ apoptosis	total cell loss
10^{-3}	4-5% apoptosis	60 % apoptosis	2% apoptosis	3% apoptosis
10^{-4}	1-2% apoptosis	20 % apoptosis	2% apoptosis	3% apoptosis
48 h				
10^{-2}	total cell loss	total cell loss	90%C-3, 10/ apoptosis	total cell loss
10^{-3}	5% apoptosis	5% apoptosis	60% apoptosis	3% apoptosis
10^{-4}	2% apoptosis	3% apoptosis	3% apoptosis	2% apoptosis
72 h				
10^{-2}	total cell loss	total cell loss	90%C-3, 10/ apoptosis	total cell loss
10^{-3}	50% apoptosis	60% apoptosis	20% apoptosis	60% apoptosis
10^{-4}	3% apoptosis	3% apoptosis	3% apoptosis	4% apoptosis

C-3 = Clarke type 3 (cytoplasmic vacuolar) active cell death

Table 2. Changes in tumor volume in course of L-desmethyl-deprenyl treatment of HT-18 xenografts

	04.20.	04.24.	04.27.	04.28.	04.29.	04.30.	05.04.
Control	43.6 ± 27.5	78.8 ± 40.6	233.9 ± 106.4	294.4 ± 133.9	355.6 ± 125.0	391.8 ± 140.9	511.5 ± 169.7
0.2 mg/kg	41.9 ± 23.3	105.6 ± 83.0	193.6 ±113.6	222.8 ± 127.9	284.4 ± 150.4	348.9 ± 175.2	377.2 ± 181.6
2.0 mg/kg	31.9 ± 14.6	92.7 ± 36.1	222.0 ± 104.9	235.4 ± 113.8	278.1 ± 118.2	303.8 ± 127.1	331.3 ± 125.5 $p < 0.05$
20.0 mg/kg	38.4 ± 19.3	99.0 ± 46.1	202.7 ± 122.4	209.0 ± 116.6	210.2 ± 122.2 $p < 0.05$	250.8 ± 139.0 $p < 0.05$	282.9 ± 139.9 $p < 0.01$

Table 3. Changes in tumour weight after L-desmethyl-deprenyl treatmnet of HT-18 xenografts (g)

Control	2.33 ± 1.05
0.2 mg/kg	2.20 ± 1.02
2.0 mg/kg	1.93 ± 0.92
20.0 mg/kg	1.78 ± 0.52

DISCUSSION

The apoptosis-preventing effect of (-)-deprenyl may explain the neuroprotection and neural rescue observed in clinical and experimental studies. Our experimental investigation showed that melanoma cells, which are of neuroectodermal origin, can be also rescued by (-)-deprenyl, from the apoptosis-inducing effect of serum deprivation. The optical configuration of deprenyl is important, because the (+) isomer is in effective. Our previous studies showed that (-)-desmethyl-deprenyl was also ineffective in the serum-deprivation model, suing melanoma cells.

On the other hand, doses of (-)-deprenyl and (-)-desmethyl-deprenyl, higher than 10^{-4} mol and without serum deprivation, caused cell death *in vitro* and (-)-desmethyl-deprenyl treatment resulted in tumor growth retardation *in vivo*. This finding is in accordance with the observation of Tatton et al. (2) who reported enhanced apoptosis of PC-12 cells after 10^{-3} mol (-)-deprenyl. The cell death induced by (-)-deprenyl and (-)-desmethyl deprenyl showed the morphological signs of apoptosis, but cell death, characterised by morphological features of Clarke type 3 vacuolar cytoplasmic active cell death (7) occurred in the majority of HT-1080 fibrosarcoma cells after high dose of (-)-deprenyl treatment. At the same time, lower dose caused apoptosis of the cells of the same cell line.

Little is known about the mechanism of antiapoptotic action of (-)-deprenyl. It may be presumed that caspase 3 activity, which is needed for certain forms of apoptosis, is influenced by this compound. According to Hickman (8), if caspase 3 is paralysed during the process of apoptosis, cytoplasmic vacuolar cell death occurs. This may be also the case of high dose (-)-deprenyl treatment. How (-)-deprenyl induces and inhibits caspases in lower doses is the task of further studies.

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RESEARCH REPORT

EFFECT OF MAO INHIBITORS ON THE HIGH-AFFINITY REUPTAKE OF BIOGENIC AMINES IN RAT SUBCORTICAL REGIONS*

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The noradrenaline (NA), dopamine (DA) and serotonin (5HT) reuptake inhibitory potency of deprenyl, the highly selective and irreversible inhibitor of MAO-B, methamphetamine enantiomers, and some other MAO inhibitors (clorgyline, J-508, J-511, J-512, J-516, LK-63, U-1424, 2-HxMP) was compared.

In vitro hypothalamic NA reuptake was inhibited by (+)-, and (-)-methamphetamine, (+)-J-508 and (+)-deprenyl (IC50: 0.35, 3.5, 17.0 and 17.8 μmol/l, respectively), and U-1424, J-512, J-516, LK-63 and 2-Hx-MP showed IC50 >1000 μmol/l. Striatal DA reuptake was inhibited by (+)-, and (-)-methamphetamine, (+)-, and (-)-deprenyl and clorgyline with IC50 of 0.6, 42.0, 24.0, 98.6 and 27.0 μmol/l, respectively, however the other compounds were ineffective. Hippocampal 5HT reuptake was slightly inhibited by clorgyline (IC50 205.0 μmol/l), while the other MAO-inhibitors were devoid of potency.

Data suggest that potency and selectivity of MAO inhibition and reuptake inhibition are independent features of the compounds, and metabolism of deprenyl results in increased noradrenaline and dopamine reuptake inhibition.

Keywords: MAO inhibitors, reuptake, biogenic amines, rats

INTRODUCTION

R(-)-deprenyl, the irreversible and selective inhibitor of the B-form of the monoamino oxidase (MAO, EC 1.4.3.4) has complex pharmacological profile that can only be partly related to the MAO-B inhibition. It was widely

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demonstrated that (-)-deprenyl facilitates nigro-striatal dopaminergic system (Knoll, 1987) without influencing mesolimbic dopaminergic activity (Tímár et al., 1993) and having dopamine sparing effect is used in the treatment of Parkinson's disease (Birkmayer et al., 1985; Tetrud and Langston, 1989). R(-)-deprenyl strongly antagonises the effects of tyramine (Knoll and Magyar 1972), enhances the noradrenaline content of the rat heart (Magyar, 1970) and has catecholamine reuptake inhibitory effect (Tekes et al., 1988; Fang and Yu, 1994).

Pre-treatment with (-)-deprenyl can protect dopaminergic neurones from 6-hydroxy-dopamine (Knoll 1987) or MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine), noradrenergic neurones from DSP-4 [(N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine] (Magyar, 1991, 1994) as well as cholinergic neurones against AF64A (methyl-β-acetoxyl-2-chloroethylamine) induced neurodegeneration (Ricci et al., 1992). It was recently demonstrated that in as low as 10^{-13} mol/l concentration it can effectively protect apoptosis in M-1 human melanoma cells (Magyar et al., 1996).

Metabolism of R(-)-deprenyl results in (-)-methamphetamine and amphetamine known as compounds having psychostimulant activity (Szökő and Magyar, 1996).

In the last years several deprenyl analogues were synthesised and structure-activity relationship determined (Magyar, 1994) for MAO inhibitory potency and selectivity.

In our present study the noradrenaline, dopamine and serotonin reuptake inhibitory potency of these MAO inhibitors and metabolites of deprenyl were measured and correlation with MAO blocking activity and/or selectivity was examined.

MATERIALS AND METHODS

Materials

L-(7,8-^{3}H)-noradrenaline (Amersham, spec. act. 1.63 TBq/mmol), (7,8-^{3}H)-dopamine (Amersham, spec. act. 1.27 TBq/mmol), 5-Hydroxy-(G-^{3}H)-tryptamine creatinine sulphate (Amersham, spec. act. 666 GBq/mmol), R(-)- and S(+)-deprenyl, R(-)- and S(+)-methamphetamine, U-1424 (N-methyl-N-propargyl-(2-furyl-1-methyl)-ethyl ammonium), J-508 (N-methyl-N-propargyl-(1-indanyl)-ammonium), J-511 (1-cyclopenthyl-isopropyl-methyl-propargyl-amine, J-512 (1,2,3,4-tetrahydro-α-naphtyl)-methyl-propargylamine, J-516 (4-

hydroxy-5-(N-methyl-N-(3-propargyl))-amino-4,5,6,7-tetrahydro-benzo-(b)-tiophen, LK-63 (N-methyl-N-propargyl-2-methyl-4,5,6,7-tetrahydro-4-benzo-(b)-furyl-ammonium chloride, 2-HxMP (N-(2-hexyl)-N-methylpropargyl-amine), imipramine (RBI), nomifensine (RBI) and clorgyline. The MAO inhibitors were sythesised and provided by CHINOIN Pharmaceutical Works as the hydrochloride salts, clorgyline was kindly donated by May and Baker Ltd., 2-HxMP was a present from Prof. P.H. Yu.

Compounds were tested in 10^{-8} - 10^{-3} mol/l concentration range and IC_{50} values calculated from data of three independent experiment run in duplicate by linear regression.

Male Wistar rats weighing 150-180 g were kept under 12:12 light/dark cycle at 22 ^{0}C, sacrificed by decapitation, brain removed immediately and hypothalamus, striatum and hippocampus dissected over an ice-cold aluminium surface. Tissue was homogenised in 10 volume of 0.32 mol/l saccharose, spun down at 1000 g for 10 min at 0 ^{0}C. Uptake measurements were carried on according to Snyder and Coyle (1969) with minor modifications at 0.1 μmol/l substrate concentration. Protein determinations were made as described by Peterson (1977).

RESULTS

In the hypothalamic noradrenaline (NA) reuptake studies imipramine was used as reference compound. As summarised in Table 1 deprenyl enantiomers and S(+)-J-508 showed slight inhibitory potency. Clorgyline and R(-)-J-508, even in 10^{-3} mol/l concentration, could not result in 50% inhibition, so their relative potency was compared to IC_{25} value of imipramine. Table 1 also shows that U-1424, J-512, J-516, LK-63 and 2HxMP were inactive in inhibiting NA reuptake having IC_{50} value >1000 μmol/l. The most potent inhibitory potency was shown by S(+)-methamphetamine and R(-)-methamphetamine being one order of magnitude higher than that of R(-)-deprenyl.

In respect of striatal dopamine (DA) inhibition (Table 2) S(+)-methamphetamine was the most potent followed by (-)-J-508, (+)-deprenyl, clorgyline and (-)-methamphetamine, respectively, however (-)-deprenyl and (+)-J-508 showed very slight potency. J-511, J-512 and U-1424 were still less potent as even in the highest concentration studied 50% inhibition could not be achieved. As shown in Table 2 J-516 as well as LK-63 were free of DA reuptake inhibitory effect.

Table 1. ^{3}H-Noradrenaline reuptake inhibition by MAO inhibitors (rat hypothalamus)

	IC_{50} (μmol/l ± SEM)	Relative potency
Imipramine	0.081±0.004	1000
R(-)-deprenyl	36.1 ± 2.9	2.2
S(+)-deprenyl	17.0 ± 0.5	4.8
R(-)-methamphetamine	3.5 ± 0.4	23.1
S(+)-methamphetamine	0.36 ± 0.07	225.0
Clorgyline	13.2 ± 0.9*	0.97**
S(+)-J-508	17.8 ± 1.3	4.8
R(-)-J-508	35.1 ± 2.4*	0.37**
2Hx-MP	>1000	-
U-1424	>1000	-
J-512	>1000	-
J-516	>1000	-
LK-63	>1000	-

Control value: 0.325±0.04 pmol × mg $prot^{-1}$ × min^{-1}
* IC_{25} value, ** relative potency compared to IC_{25} value of imipramine (12.9 nmol/l)

When hippocampal serotonin (5HT) reuptake inhibitory potency of the MAO inhibitors and methamphetamine enantiomers was compared to that of imipramine (Table 3), it could be seen, that only clorgyline had some very slight potency. In case of the other compounds practically no 5HT inhibitory activity was observed.

Table 2. ^{3}H-Dopamine reuptake inhibition by MAO inhibitors (rat striatum)

	IC_{50} (μmol/l ± SEM)	Relative potency
Nomifensine	0.77 ± 0.04	1000
R(-)-deprenyl	98.6 ± 4.1	7.8
S(+)-deprenyl	23.9 ± 11.0	32.2
R(-)-methamphetamine	42.0 ± 3.5	18.3
S(+)-methamphetamine	6.0 ± 0.1	128.3
Clorgyline	27.1 ± 0.9	28.4
S(+)-J-508	118.3 ± 12.1	6.5
R(-)-J-508	12.4 ± 1.3	62.0
J-511	54.7 ± 2.7*	14.0**
J-512	30.3 ± 2.1*	25.4**
U-1424	18.8 ± 1.4*	40.9**
J-516	>1000	-
LK-63	>1000	-

Control value: 1.168±0.14 pmol × mg $prot^{-1}$ × min-1
* IC_{25} value, ** relative potency compared to IC_{25} value of nomifensine (100.4 nmol/l)

Table 3. ^{3}H-Serotonin reuptake inhibition by MAO inhibitors (rat hippocampus)

	IC_{50} (μmol/l ± SEM)	Relative potency
Imipramine	0.42 ± 0.02	1000
R(-)-deprenyl	109 ± 6.4*	0.6**
S(+)-deprenyl	64.4 ± 4.4	1.1
R(-)-methamphetamine	>1000	-
S(+)-methamphetamine	>1000	-
Clorgyline	205.0 ± 13.5	2.0
S(+)-J-508	44.2 ± 2.6*	1.6**
R(-)-J-508	908.5 ± 62.7*	0.1**
J-512	30.3 ± 2.1*	25.4**

Control value: 0.623±0.05 pmol × mg prot^{-1} × min^{-1}
* IC_{25} value, ** relative potency compared to IC_{25} value of imipramine (0.07 μmol/l)

DISCUSSION

As shown in Table 1 the hypothalamic NA reuptake inhibitory potency of compounds studied is very slight compared to imipramine, as reference substance. However, stereoselectivity could be observed, S(+)-isomers being more potent, also shown by Iversen (1975) and Knoll and Magyar (1972). Data presented do not confirm Hendley and Snyder (1968) hypothesising that difference in antidepressive potency of MAO inhibitors is related to their catecholamine reuptake inhibitory activity as no correlation between selectivity of MAO inhibitory effect and NA reuptake inhibitory effect was observed. The IC_{50} values for NA reuptake inhibition of deprenyl and J-508 enantiomers is two to three order of magnitude higher than for MAO inhibition and U-1424, J-512, J-516 and LK-63 having IC_{50} for MAO inhibition in the range of 10^{-6} - 10^{-8} mol/l (Magyar et al., 1980) are free of NA reuptake inhibitory effect. In respect of 2HxMP we could confirm Yu et al., (1994) demonstrating that this very potent MAO inhibitor is devoid of NA reuptake blocking activity. In spite of the methodological differences NA reuptake inhibitory potency of deprenyl enantiomers was found very similar (IC_{50} 10 - 50 μmol/l) by most of the authors (Braestrup et al., 1975; Lai et al., 1980; Tekes et al., 1988; Bondiolotti et al., 1995), however, in case of clorgyline we have observed much lower hypothalamic NA reuptake inhibitory potency than Lai et al (1980) on rat frontal cortex synaptosomes. Differences may be due to dissimilarities in brain regions studied and subcellular preparations as well as different methods used.

As shown in Table 2 MAO inhibitors studied possess very weak inhibitory potency on striatal DA reuptake compared to nomifensine as reference compound. Remarkable stereoselectivity was observed in case of J-508 and methamphetamine stereoisomers R(-)-J-508 and S(+)- methamphetamine being ten times more potent than the corresponding isomers, however, in case of deprenyl stereoselectivity was less pronounced. It is also noteworthy that S(+)-methamphetamine, metabolite of (+)-deprenyl (Schachter et al., 1980; Szökő and Magyar, 1996) exhibited the highest DA reuptake inhibitory effect. In respect of R(-)-deprenyl and clorgyline our data are in good correlation with Azzaro and Demarest (1982). Considering structure-activity relationship it can be seen, that changing phenyl ring of deprenyl to furyl one (U-1424) both potency and MAO-B blocking selectivity of the molecule remains similar to deprenyl (Magyar, 1980), however, DA reuptake inhibitory potency is lost. The structure of J-511, J-516 and LK-63 are common in lacking aromatic ring and are devoid of DA reuptake inhibitory activity giving further evidence to Koe (1976) demonstrating that aromatic ring is required for binding of the molecule to the uptake site.

As Table 3 shows neither deprenyl and derivatives having strong MAO inhibitory effect nor methamphetamine enantiomers possess inhibitory potency on hippocampal 5HT reuptake in reasonable concentrations. Data are in line with that of Bondiolotti et al. (1995).

CONCLUSIONS

R(-)-deprenyl, the highly selective and irreversible inhibitor of MAO-B has complex pharmacological effect that only be partly related to enzyme inhibition. From the several analogues synthesised in the last years we have chosen those ones that are very potent and selective MAO inhibitors and measured their noradrenaline, dopamine and serotonin reuptake potency. Methamphetamine enantiomers were also tested, known as metabolites of deprenyl.

Data presented show that potency and selectivity of MAO inhibition and reuptake inhibition are independent properties of the molecules and some minor modification in the structure of deprenyl results in complete loss of reuptake inhibitory effect.

It can also be concluded that metabolism of deprenyl enantiomers results in increased noradrenaline and dopamine reuptake inhibition.

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PRELIMINARY NOTE

THE LACK OF NURR1 DOES NOT EFFECT CHOLECYSTOKININ mRNA EXPRESSION IN THE VENTRAL MIDBRAIN IN NEWBORN MOUSE

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Nurr1 is a transcription factor which belongs to the nuclear hormone receptor superfamily (5). These receptors comprise a group of structurally related transcription factors that coordinate physiological and developmental responses to a variety of chemical signals (3). Family members include receptors for steroids, retinoids, vitamin D, thyroid hormone, and a growing number of orphan receptors (4). Although the physiological function of some orphan receptors has been uncovered, their putative ligands still remain unknown. Recently, disruption of the mouse Nurr1 locus by homologous recombination revealed that Nurr1-null mice developed normally in utero, but were unable to feed, and died within 24 hours after birth (2, 6, 9). Immunohistochemical and *in situ* hybridization analysis of their brain revealed that in the absence of Nurr1 dopamine biosynthesis was abolished in the substantia nigra and ventral tegmental area of the midbrain (2), but not in other dopaminergic neurons in the central nervous system (1).

Cholecystokinin (CCK), one of the most common brain peptides, has facilitatory modulating effects on the inhibitory actions of dopamine in the mesolimbic pathway (8). It is known from the literature that CCK coexists with dopamine in a large proportion of the ventral tegmental and substantia nigra neurons in rodents (7). Since the lack of Nurr1 (Figures 1A and B) affects the dopamine biosynthesis, we investigated the expression of CCK mRNA in the ventral tegmental area and the substantia nigra of the Nurr1 knockout mice, and compared them to the same areas of their wild-type littermates. We performed *in situ* hybridization on frontal sections at the level of the midbrain using ^{35}S-labeled riboprobes to detect Nurr1, tyrosine hydroxylase (TH) and CCK mRNA in the above mentioned areas.

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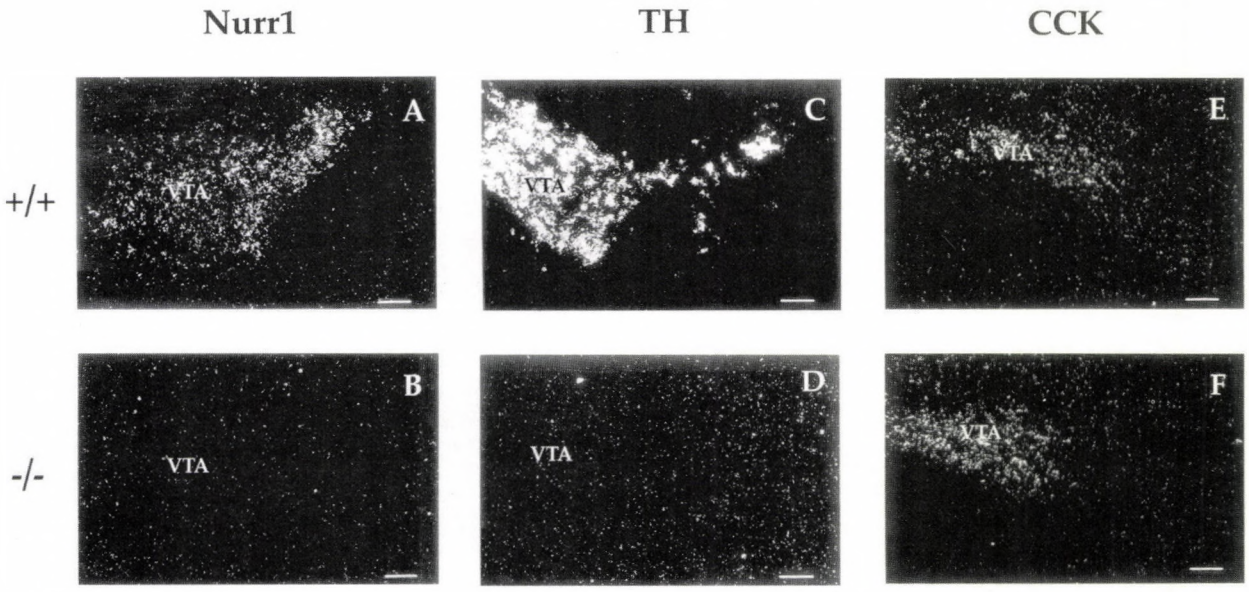

Fig. 1. *In situ* hybridization signals in the ventral tegmental area and the substantia nigra of the newborn wild type (+/+) and Nurr1 knockout (-/-) mice. Dark-field pictures: (A and B) antisense Nurr1 mRNA, (C and D) antisense TH mRNA and (E and F) antisense CCK mRNA. Abbreviation: VTA - ventral tegmental area. Scale bars = 100 μm

Our results show that while the TH mRNA expression was completely disrupted both in the ventral tegmental area and the substantia nigra of the Nurr1 knockout mice (Figures 1C and D), the expression of CCK mRNA was maintained in both areas at the same distributional pattern as it is in the wild type (Figures 1E and F). These results suggest that Nurr1 does not influence the CCK expression in the ventral tegmental area and substantia nigra of the midbrain in the newborn mice.

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PRELIMINARY NOTE

SEROTONIN TRANSPORTER, SEROTONIN-2A RECEPTOR AND TRYPTOPHAN HYDROXILASE GENE POLYMORPHISMS IN DEPRESSED SUICIDE VICTIMS

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Family, twin and adoption studies suggest that genetic factors are involved in the etiology of major depressive illness and suicidal behavior. Genes encoding proteins involved in regulating serotonin (5-HT) synthesis, release and uptake, metabolism or receptor activation are major candidates in association studies of depression and suicidal behavior. Several polymorphisms in the serotonin transporter (5-HTT), serotonin-2A (5-HT_{2A}) and tryptophan hydroxilase (TPH) gene or polymorphisms involved in its expression have been reported. Among these polymorphisms, of particular interest relation to the vulnerability to affective disorders, is a 44 bp insertion/deletion functional polymorphism in the promoter region of the gene which regulates expression of 5-HT transporter.

The present investigation tested the hypothesis that the observed changes in serotonin transporter, and 5-HT_{2A} receptor density in depressed suicide victims are the trait rather than state markers and are associated with a polymorphism in respective candidate genes. Another aim of this study was to determine whether there are differences in genotype and allele distribution of the 5-HTT, 5-HT_{2A} and tryptophan hydroxylase (TPH) gene polymorphism in *post mortem* brain samples from suicide and control subjects of the same ethnic background.

Brain from 32 depressed suicide victims and from 57 subjects who died suddenly from causes not directly involving the central nervous system and without documented evidence of mental illness were obtained at the autopsy in the Department of Forensic Medicine of Semmelweis University of Budapest. Brains were obtained at 1-48 hours after death. The genotyping was carried out in all suicide victims and control subjects. The densities of brain 5-HTT and 5-

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HT_{2A} receptors were determined by measuring ^{3}H-paroxetine and ^{3}H-ketanserin binding in prefrontal cortex samples (Brodmann area 9). DNA was extracted from *post mortem* brain tissue by using the method describe by Gemmell and Akiyama. Four polymorphic gene loci were examined: two polymorphic variants of the 5-HT_{2A} receptor gene (102T/C and His452Tyr), a functional polymorphism in the 5' regulatory region of the 5HTT gene (5-HTTLPR) and the 218A/C polymorphism in the 7 intron of TPH gene. The functional polymorphisms were genotyped by the polymerase chain reaction (PCR).

Allele and genotype frequencies as well as homo-heterozygotes distribution of gene polymorphism between depressed suicide victims and controls were compared by a chi-square test (two-tailed).

There were significant differences between frequencies and genotype distribution of long allele in 5-HTT gene promoter region. The frequency of the long allele was increased in suicides group in comparison to the controls (67.7 % vs 47.4 %, χ^2=6.7, p=0.009). The frequency of subject with an L/L genotype in depressed suicide victims was double of that found in the control group (48.4 % vs 24.6 χ^2=6.1, p=0.048) The odds ratio (OR) for the L allele associated with depressed suicide was 2.3 (95 % CI 1.2-4.5). No significant difference between controls and depressed suicide victims in 5-HT2A and TPH polymorphisms were detected (Table 1).

The major finding of this study was a significant difference in the frequency of the long allele in 5-HT transporter gene between suicide victims and control subjects. This is to our best knowledge, the first evidence suggesting that a functional polymorphism in the promoter region of serotonin transporter gene may be associated with suicide in depressed subjects. This finding suggest that serotonin transporter may be a susceptibility gene in depressed suicides. The association between 5-HTT genotype and suicide support the hypothesis that genetic factors can modulate suicide risk by influencing serotonergic activity.

Table 1. Genotypes and allele distributions of the 5-HTT, 5-HT_{2A} receptor and tryptophan hydroxylase gene polymorphism in suicide victims and control subjects

5-HT transporter	Depressed suicides (n=31)	Controls (n=57)	$\chi 2$	p
Genotype				
L/L	15 (48.4%)	14 (24.6%)	6.1	0.048
L/S	12 (38.7%)	26 (45.6%)		
S/S	4 (12.9%)	17 (29.8%)		
Allele				
L	42 (67.7%)	54 (47.4%)	6.7	0.009
S	20 (32.3%)	60 (52.6%)		
Tryptophan hydroxylase				
Genotype				
A/A	4 (12.5%)	3 (5.3%)	1.6	0.46
A/C	23 (71.9%)	43 (75.4%)		
C/C	5 (15.6%)	11 (19.3%)		
Allele				
A	31 (48.4%)	49 (43.0%)	0.49	0.48
C	33 (51.6%)	65 (57.0%)		
	n= 24	n=31		
5-HT_{2A} receptor [a]				
Genotype				
T/T	3 (12.5%)	3 (9.7%)	0.12	0.94
C/T	10 (41.7%)	13 (41.9%)		
C/C	11 (45.8%)	15 (48.4%)		
Allele				
T	16(33.3%)	19 (30.6%)		
C	32 (66.7%)	43 (69.4%)	0.09	0.76
5-HT_{2A} receptor [b]				
Genotype				
His/His	20 (83.3%)	20 (64.5%)	2.42	0.12
His/Tyr	4 (16.7%)	11 (35.5%)		
Tyr/Tyr	0	0		
Allele				
His	44 (91.7%)	51 (82.3%)	2.03	0.15
Tyr	4 (8.3%)	11 (17.7%)		

[a]102T/C polymorphism, [b]452His/Tyr polymorphism

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PRELIMINARY NOTE

A MOLECULAR ELEMENT OF NEURONAL RESPONSE TO LYSOLECITHIN-INDUCED FOCAL, EXPERIMENTAL DEMYELINATION IN RAT

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Multiple sclerosis (MS), is the prototype of primary demyelinating diseases. Recent progress in imaging [De Stefano et al., 1998] and pathologic [Trapp et al., 1998; Lovas et al., 2000] studies revealed that besides failure in remyelination, decreased axonal density is also a basic feature in MS [Lovas et al., 2000]. The latter is believed to cause the development of irreversible neurological deficit signs [De Stefano et al., 1998] which are characteristic for the later stages of the disease. Although the axonal injury in MS is basically related to the activity of the ongoing inflammation [Trapp et al., 1998], the neuronal involvement in MS draws attention on our limited knowledge about the effects of demyelination on neuronal integrity, and about molecular mechanisms that might be involved in neuronal response to demyelination process.

Certain inducible transcription factors are known to be expressed in various central nervous system axotomy/injury models [Herdegen et al., 1997]. Inducible transcriptions factors are immediate early gene products that are key regulators of different intrinsic cellular programs [Herdegen et al., 1993]. In particular, the Jun and Fos families have been shown to play crucial role in various types of neuronal damage models. Protooncogene c-Jun is documented to be expressed long-term following neuronal injury, like axotomy in the septohippocampal pathway [Herdegen et al., 1993]. Other members of the Fos or Jun transcription factor family as c-fos, junB or fos-related antigens were

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neither expressed nor up-regulated in such models [Butterworth, Dragunow, 1996].

The primary objective of this study was to investigate whether the expression of protooncogene c-Jun is involved in neuronal response to experimental demyelination. The well-characterised lysolecthin-induced focal demyelination model [Woodruff, Franklin, 1999] was used in the study. The model was chosen because: i) there is no primary inflammation before demyelination, ii) it has well-characterised time course [Woodruff, Franklin, 1999], iii) besides demyelination it hardly damages the axons [Allt et al., 1988] iv) the demyelinated target area within the CNS can be precisely selected.

Lysolecithin (1%) was injected into the pontocerebellar and the septohippocampal pathways in Sprague-Dawley rats. The survival was determined in 6 days because i) in the time course of lysolecithin-induced demyelination model, this period is in the end of demyelination (remyelination starts just 2-3 days later) [Woodruff, Franklin, 1999], ii) high c-Jun expression is documented at this time-point in most of CNS injury models [Herdegen et al., 1993].

The animals were transcardiacly perfused, the brains were removed and further processed according to immunohistochemistry protocols using c-Jun polyclonal primary antibody. Following the pontocerebellar injection, pronounced c-Jun positivity was detected in neurons of the contralateral pontine nuclei and only a slight reaction was detected on the ipsilateral side. In the septal region, following septohippocampal lesion, specific nuclear c-Jun expression was found in the ipsilateral nucleus of the diagonal tract (both in the vertical and in the horizontal limbs). Non-specific, bilateral c-Jun positivity was observed over some cortical areas, especially in the piriform cortex. Rarely c-Jun immunopositive nuclei were detected in control animals, either in the pontine nuclei, or in the mediobasal forebrain.

In our experiments, lesion specific expression of the c-Jun protein was observed in neurons whose axons transverse lysolecithin-induced demyelinated lesion. The expression of inducible transcription factor c-Jun in the neuronal nuclei might represent one step in the complex sequence of neural response to demyelination (Fig. 1).

Although lysolecithin-induced demyelination primarely destroys the myelin sheath and preserves the axons [Allt et al., 1988], other demyelination models (e.g. anti-myelin protein antibody injection) should also be investigated in the future.

Further understanding of the neuronal response is required to clarify what molecular and functional sequence follows demyelination, and how does it affect oligodendroglial (remyelination?) or neuronal (degeneration?) functions.

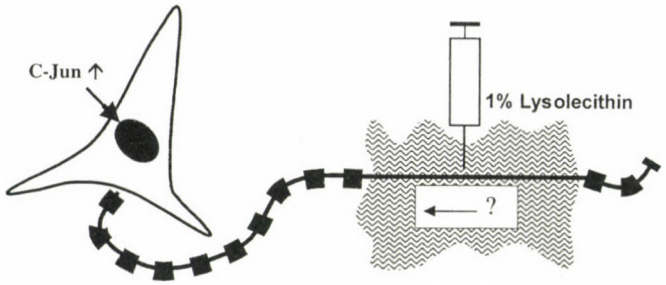

Fig. 1. Lysolecithin injection into long neuronal pathways (the olivocerebellar tract and septohippocampal pathway, respectively) has resulted pronounced nuclear c-Jun protein expression (detected by immunohistochemistry) in the corresponding neuronal nuclei. The signal cascade, resulting c-Jun expression is unknown

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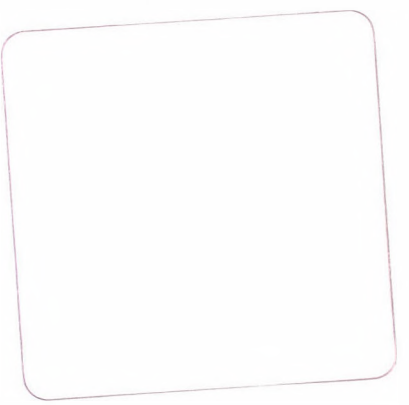

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PRELIMINARY NOTE

NEUROCHEMICAL CHARACTERIZATION OF KIDNEY REGULATING BRAINSTEM NEURONS IDENTIFIED BY PSEUDORABIES TRANSNEURONAL LABELING

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Neurotropic herpes viruses are useful tools for tract-tracing experiments, since they amplify themselves in every infected neuron, and cross the synaptic-cleft without signal reduction, thus they can label long neuronal-chains. These viruses code a virion host shut-off protein (which is an RNase) and other cytotoxic components, causing rapid degradation of the cellular macromolecules when they enter the cell. Therefore, cellular mRNAs have been disrupted at the time when viral antigens appear in the neuron, leading to difficulties in the characterization of the infected cell (2).

The participation of brainstem biogen amine-regulating neurons in the innervation of the kidney was examined by double-labeled immunohistochemistry using genetically engineered pseudorabies virus (Prv), Ba-DupLac, combined with the detection of catecholamine or serotonin markers (4). Ba-DupLac was designed to express a high amount of β-galactosidase at a very early period after penetration of the virus to the cell. This feature of the virus makes possible the detection of cellular mRNAs.

10-10 µl of virus solution (titer: 10^9 PFU/ml) was injected 2 mm under the surface of the upper and lower pole of the kidney of male Sprague-Dawley rats (body weight 250-300 g). After 4 days the animals were sacrificed by transcardiac perfusion of 4% paraformaldehyde solution. After removal, selected regions of the brain were paraffin-embedded, 5 µm paraffin sections were cut on a microtome and mounted on silane-treated slides.

Infected cells in the A1/C1 cell groups of the ventrolateral medulla were identified using double-label fluorescence immunohistochemistry. In these

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experiments TH were detectable either in Prv or β-gal containing cells, and there was no significant reduction in the signal.

Using radioactive *in situ* hybridization histochemistry (5), TH-containing virus-infected cells were identified in the A1, A5, A6 catecholaminergic cell groups (1). Since A5 neurons directly (monosynaptically) innervate preganglionic neurons in the intermediolateral cell column of the thoracic spinal cord, the virus reached the A5 cell group first. This region was heavily infected 4 days after inoculation, and the neurons here were degraded to a great extent. At that stage of the infection, the cells expressed only viral proteins, not β-galactosidase (Fig. 1C). Compared it to the control sections (Fig. 1A), we could not detect TH signals in this region (Fig. 1B).

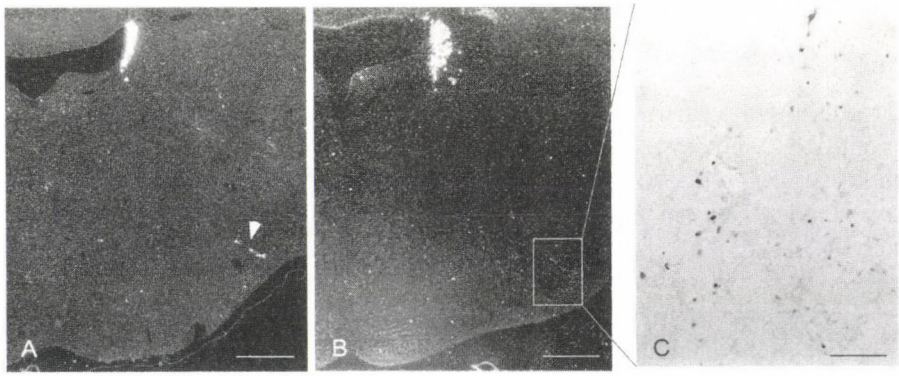

Fig. 1. A. TH *in situ* hybridization signal in the A5 region (white arrowhead) of a control section.
B. The TH signal was not detectable in the A5 region of virus-infected animals, after 4 days. Bar scales (A, B): 1 mm.
C. This cell group is heavily infected 4 days after inoculation, the neurons are degraded at a great extent, and express only viral proteins. Bar scale: 300 μm

The virus may reach the A1 and A6 (locus coeruleus) cell groups later, presumably trans-synaptically through the A5 region. Therefore, neurons in these cell groups were less infected. Many β-galactosidase-positive cells containing TH mRNA were observed in these regions, while Pry antigen positivity was hardly seen (if any) in cells, co-localized with *in situ* hybridization signals (Fig. 2).

The serotonin transporter (5HTT) was detected in the infected cells of the raphe nuclei (nucleus raphe magnus and pallidus) also by performing *in situ* hybridization histochemistry (3). Like TH mRNA, the 5HTT mRNA was only detectable in β-galactosidase positive cells (Fig. 3).

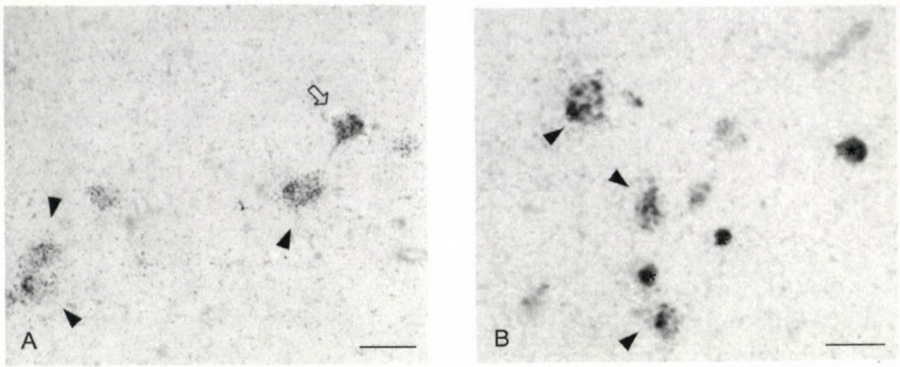

Fig. 2. A. Colocalization of the TH mRNA and β-galactosidase immunoreactivity in A1 catecholaminergic cell group. Beside non-infected catecholaminergic cells (arrowhead) double-labeled cells (open arrow) were observed here. B. In the same region, the TH signal (arrowheads) did not appear in the viral protein containing perikarya (stars). Bar scales: 80 μm

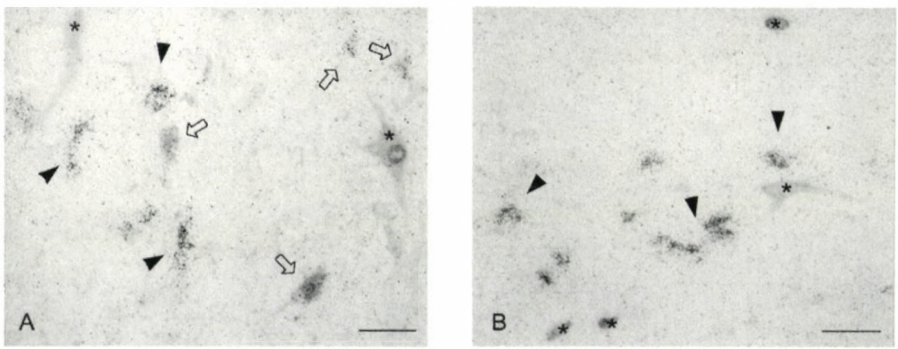

Fig. 3. A. 5HTT mRNAs are still present in β-galactosidase positive cells in the nucleus raphe magnus. (5HTT mRNA – arrowheads; β-galactosidase positivity – stars; double labeled cells – open arrows). B. Alike TH mRNA, the 5HTT mRNA was also not detectable in virus-positive cells (stars). Bar scales: 85 μm

In summary, using genetically engineered pseudorabies virus, due to the strong and very early β-galactosidase expression, the neuronal infection is detectable immediately after the virus enter the perikaryon. At this stage the cell has intact mRNAs, thus, it can be characterized neurochemically by *in situ* hybridization. Later, it means after few hours, the cytotoxic effect of the virus

results in a rapid degradation of mRNAs. Consequently, we can only get information about the character of the cell by using antibodies against such proteins (e.g. tyrosin-hydroxilase) which are abundantly present in the perikaryon.

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PRELIMINARY NOTE

NEURONAL PROJECTIONS FROM THE LIMBIC CORTEX TO THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS: TRANSNEURONAL RETROGRADE LABELING WITH PSEUDORABIES VIRUS

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The hippocampus is strongly involved in the inhibitory control of the hypothalamo-pituarity-adrenal (HPA) axis. Hippocampal afferents to the hypothalamus arise mainly from the ventral subiculum (VSub) (1, 4, 6). The subiculum receives a strong projection from the entorhinal cortex which represents the primary source of extrinsic inputs to the hippocampus (7).

Anterograde tracing studies failed to demonstrate significant direct neuronal input from the VSub to the paraventricular nucleus (PVN) which occupies a central position in the HPA-axis (1). taken the advantage of the transneuronal tracing technique, the exact location of the PVN-projecting hippocampal (HI) (ventral subicular) neurons has been investigated in the present study. This technique allows us to label neurons through the entire neuronal chain retrogradely. By this way, entorhinal cells that innervate PVN-projecting subicular neurons can be also visualized and localized in the limbic cortex.

A recombinant derivative of Bartha strain of Pseudorabies virus (Prv-Ba), termed Ba-DupLac (titer: 10^9 PFU/ml; volume: 50 nl) was injected into the PVN (Fig. 1a and b) of adult female Sprague-Dawley rats (body weight: 250-280 g). After three days of inoculation, rats were sacrificed by transcardiac perfusion of a Zamboni fixative solution. After removal, the forebrains were cut in 50 μm thick coronal serial sections.

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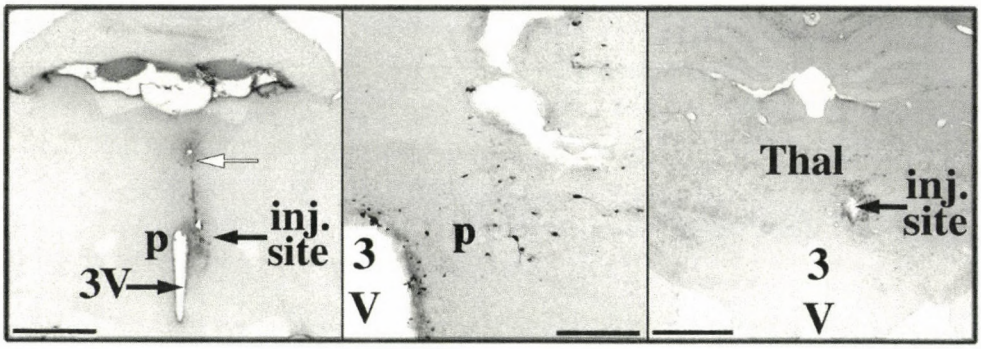

Fig. 1. (a) Virus-injection into the paraventricular nucleus (injection site: 1.7 mm caudal to the bregma). Some infected neurons (white arrow) along the needle channel. (b) High power magnification of Fig. a. infected cells in the PVN. (c) Virus-injection into the thalamus (injection site: 2.4 mm caudal to the bregma). In these animals, no labelled cells were seen in the PVN. Abbreviations: p: PVN, 3V: third ventricle, Thal: thalamus, inj. site: injection site. Bar scales: 2000 pm (1a, 1c), 500 μm (1b)

Anterograde and retrograde neuronal tract-tracing studies have demonstrated that axons originating in the VSub may reach the hypothalamus through the medial corticohypothalamic tract directly (1,4), or they relayed in the bed nucleus of the stria terminalis (NIST) (2). We found dense neuronal labelling in the medial (anterior, ventral, posterior) and in the posterior lateral subdivisions of the NIST (not shown).

Strongly labeled cells were found in a well-outlined portion of the VSub at rostro-caudal levels between 5.4 and 6.4 mm caudal to the level of the bregma, with an ipsilateral dominance (Fig. 2a and b). In the close dorsal vicinity of the VSub, some CA1 neurons were also labelled. In these levels, a group of cells were densely labelled in the lateral entorhinal (LEnt) cortex (Fig. 2a and b). Although the virus was unilaterally injected into the PVN, neurons in the LEnt cortex appeared on both sides with an ipsilateral dominance (Fig. 2a and b). This observation correlates well with previous anterograde tracing studies which show that the entorhinal cortex projects to the contralateral hippocampus (7).

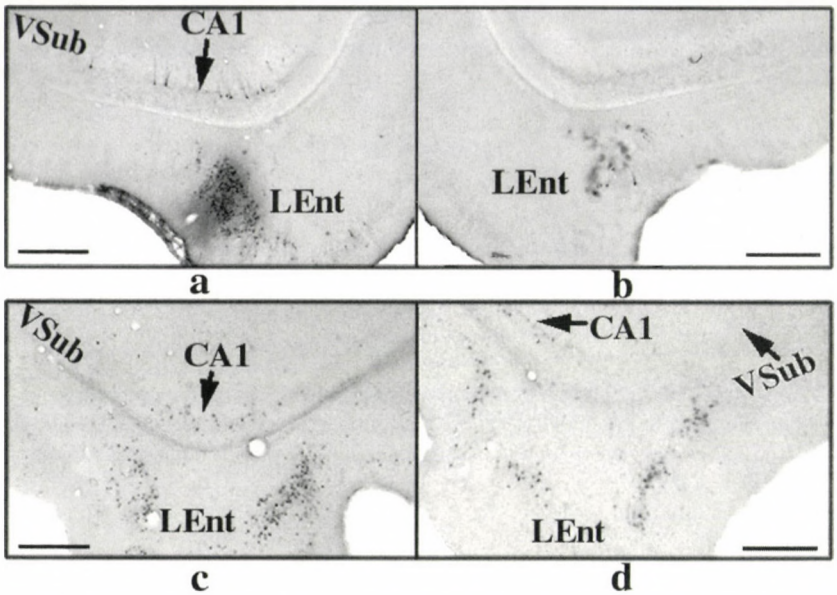

Fig. 2. Virus-infected cells in the VSub, hippocampus CA1, and the entorhinal cortex. Labelled cells (a) ipsilateral, and (b) contralateral to the site of the PVN-injeclions. Labelled cells (c) ipsilateral, and (d) contralateral to the site of the Thal-injections. Abbreviations: VSub: ventral subiculum, CA1: CA1 region, LEnt: lateral entorhinal cortex. Bar scales: 800 μm (a-d)

After the PVN-injection of the virus, some infected neurons were also seen in the thalamus (Thal), presumably due to the leakage of the virus along the needle track. It is known that thalamic neurons, especially some intralaminar and midline thalamic nuclei, receive subicular projections (3, 6). Therefore, in a control experiment, Ba-DupLac was injected into the Thal (Fig. 1c). Infected cells appeared in same brain regions as after PVN-injections but in different topographical patterns (Fig. 2c and d). The majority of Thai-projecting neurons in the VSub were detected more caudally than PVN-projecting neurons (6.0-6.8 mm caudal to the level of bregma). The virus injected into the Thal infected some neurons not only in the ventral (like after PVN-injections) but the dorsal CA1 region (not shown). In addition, labelled cells were seen in the retrohippocampal, perirhinal, occipital and temporal cortical areas, in a good correlation with previous observations obtained from anterograde tract-tracing (5).

In summary by using a retrograde transneuronal labelling technique, neurons with PVN projections were localized in the VSub. Virus-labelled cells in the

LEnt cortex may represent second ordered neurons which may participate, through the hippocampus, in the limbic control on the activity of the HPA-axis.

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ABSTRACTS

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CELL FORMATION IN THE HUMAN CEREBELLUM AND HIPPOCAMPUS BETWEEN THE 24TH GESTATIONAL WEEK AND THE FIRST POSTNATAL YEAR

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Cell formation has been examined using the cell proliferation marker, Ki-67, in brains of children whose death was not related to genetic diseases, head injury or neurological disorders. The external granular layer (EGL) of the cerebellum displayed the highest number of labelled cells (43-45%) between the 24th week and term, when labelling started to decrease. However, the EGL contained at least 30% labelled cells until its disappearance between the 5th and 8.5th postnatal months. The internal granular layer (IGL) contained fewer labelled cells. The ratio was highest from the 24th to the 28th weeks (6-7%), but rapidly decreased to 1%, or less, after the 32nd gestational week. Labelled cells mainly occurred among or below the Purkinje cells in all age groups, including the 5 and 8.5 months old children.

In the hippocampus, Ammon's horn did not display labelled neurons after the 24th gestational week, since all labelled cells could be identified as glial or endothelial cells. The hilus contained the highest number of labelled cells, but their percentage was less than 1% in all age groups. The labelled cells were granule cell, glial precursors or endothelial cells. Most migrating cells lacked immunolabelling. In conclusion: large number of neurons is formed in the human cerebellum postnatally, whereas neuron formation in Ammon's horn is an early prenatal event. Similarly, the overwhelming majority of granule cells of the dentate gyrus is formed before the 36th gestational week.

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ULTRASTRUCTURE AND CALCIUM DISTRIBUTION OF SPINAL MOTONEURONS OF RATS EXPOSED TO EXCITOTOXINS *IN VIVO*

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Excitotoxicity has been postulated to play a role in the pathomechanism of ALS, the most frequent motoneuron disease. In acute excitotoxicity there is a destabilization of intracellular Ca^{2+} homeostasis which activates a cascade of harmful metabolic events. To investigate the

ultrastructural changes and distribution of calcium under such conditions, we subdurally injected DL homocysteic acid, an excitotoxic analogue of glutamate that is particularly effective in triggering motoneuron degeneration. At various survival intervals we analyzed the evoked pathological changes by electron microscopy. *In situ* calcium distribution was preserved with oxalate-pyroantimonate fixation. The main cytopathological features were: *(i)* motoneuronal perikarya with dark cytoplasm and numerous vacuoles containing calcium precipitates. *(ii)* swollen dendritic and axonal processes with amorph electrondense material and increased calcium in the cytoplasm and mitochondria. The morphology of motoneuronal injury appeared to show both apoptotic and necrotic features. These findings demonstrate that increased intracellular calcium plays a key role in excitotoxic motoneuron injury.

EFFECTS OF PERINATAL LESIONS ON BRAIN DEVELOPMENT-ROLE OF MIGRATION DISTURBANCES?

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Consequences of brain injuries are usually tought to be caused by destruction of pathways and death of the affected neuronal groups, while failure of regeneration is attributed to the reactive gliosis. Present study demonstrates the outcome of such pre- and neonatal injuries which are not followed by reactive gliosis. The effect of these lesions may be attributed to disturbances of cell migration at least in part. The injuries were paramedian incisions penetrating the developing cortex, corpus callosum and hippocampus. The lesioned area was analysed using immunohistochemical stainings against neurofilament, GFAP, extracellular matrix proteins, NCAM and nestin on parallel sections, as well as by staining according to Nissl. The main morphological alterations were as follows: i, when the lesion formed a new surface, the arrangement of the cortical and hippocampal layers was adapted to it; ii, when no new surface appeared, in the hippocampus the layer formation was oriented by the lesion track; iii, cell groups could get trapped in the subventricular or intermediate zone of the developing cortex, these cell groups seemed to be responsible for the growth failure of callosal fibers; iv, masses of cortical neurons could migrate up to the meningeal surface, this phenomenon may be attributed to the destruction of the cell population (Cajal-Retzius), which is supposed to stop the migration.

Further investigations are needed, however, to prove the existence and mechanism of an actual migratory disorder. Until now, investigations concerning the composition of the extracellular matrix (laminin, fibronectin, chondroitin sulfate) or those with NCAM and nestin, the marker of immature neuroepithelial cells, have failed to provide satisfying information.

DESCENDING CONTROL OF SENSORY INFORMATION PROCESSING IN THE SPINAL CORD

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In certain life-threatening situations, inhibition of nociceptive information is of fundamental importance in animal survival strategies. A growing body of experimental evidence indicates that this internal antinociceptive mechanism of the brain functions as a complex highly interacting apparatus the activities of which converge on the spinal dorsal horn where it exerts a powerful inhibition on nociceptive neurons. It appears that in addition to the spinal dorsal horn, the most important constituents of this internal pain attenuation system are the periaqueductal gray matter (PAG), rostral ventromedial medulla (RVM), pontine noradrenergig cell groups (A6-7) and the ponto-bulbar reticular formation. It is highly probable that this complex antinociceptive neural apparatus is activated by descending projections of various areas of the limbic system to the ventrolateral aspect of the PAG. Evoked neural activities of the PAG then may influence the nociceptive information processing mechanisms of the spinal cord through an intricate interneuronal circuit. Most probably signals from the PAG first and foremost influence the excitation level of the medial subdivision of the ponto-medullary reticular formation. Here they may activate neural circuits that forward the signals towards the spinal cord, or more likely towards the rostral ventromedial medulla and pontine noradrenergic cell groups. A second, much smaller group of PAG efferents terminate within the rostral ventromedial medulla, locus subcoeruleus and A5 cell group on non-serotonergic and non-noradrenergic neurons. These incoming volleys are presumably further processed by interneuronal circuits, and after further processing the signals may be transmitted to spinally projecting neurons. A third group of efferent fibers, the smallest in number, may establish monosynaptic contacts with serotonergic and noradrenergic neurons among which there might be a few spinally projecting cells. The spinally projecting neurons then presumably integrate the monosynaptic inputs and signals from the activated intra- and extranuclear interneuronal circuits, and in case of suprathreshold activation they may conduct volleys to the spinal dorsal horn. In the superficial dorsal horn, the terminals of descending fibers may release serotonin, noradrenalin, GABA and glycine, and the released neurotransmitters may evoke inhibition in spinal neural circuits underlying nociceptive information processing, that results in analgesia and attenuation of pain behavior.

DISTRIBUTION OF NK1R, GLUR1 AND GLUR2/3 IMMUNOREACTIVITY IN THE LUMBAR SPINAL DORSAL HORN OF RATS

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It is well established that substance-P and glutamate are released by C and Aδ primary afferents in the superficial spinal dorsal horn in response to painful peripheral stimuli. The released substance-P acts on neurokinin-1 receptors (NK1R), whereas the effect of glutamate is mediated by various receptors including GluR1 and GluR2/3 receptors. Thus, spinal neurons that express NK1R, GluR1 and GluR2/3 are supposed to play crucial roles in the spinal transmission of nerve signals evoked by various noxious stimuli. Therefore, we studied the distribution of NK1R, GluR1 and GluR2/3 immunoreactivities in the lumbar spinal dorsal horn of rats using diaminobensidine- and nanogold-based preembedding immunocytochemical techniques at the light and electron microscopic levels. We demonstrated that the segmental, laminar, intracellular and synaptic-perisynaptic distribution of NK1R, GluR1 and GluR2/3 immunoreactivities show a number of distinct features in the superficial spinal dorsal horn. NK1R immunoreactivity was confined to lamina I, whereas, with the exception of a few large presumably supraspinally-projecting neurons, laminae II-III were free of labelling. In contrast to this, GluR1 and GluR2/3 immunoreactivities were distributed throughout the entire dorso-ventral extent of laminae I-II and laminae I-III, respectively. At the level of L3, L4 and L5 spinal segments in the medial aspect of the superficial dorsal horn, however, only a faint immunostaining was observed for GluR1 and GluR2/3 in laminae I-II. At these areas, immunoreactivity for both GluR1 and GluR2/3 were mostly confined to lamina III. At the cellular level, immunostaining for NK1R was associated with the outer plasma membrane of perikarya and dendrites, whereas immunoreactivity for GluR2/3 was mostly confined to the cytoplasm of perikarya. The immunostaining of the outer plasma membrane and the cytoplasm of perikarya were equally strong for GluR1. Investigating the synaptic and perisynaptic distribution of the receptors, immunogold particles marking receptor molecules were encountered in the most peripheral 20-40 nm wide outer rim of the postsynaptic membrane thickening of asymmetric synaptic contacts and at perisynaptic location in a 20-200 nm distance from the edge of the synaptic apposition. Within this territory, GluR1s were somewhat closer to the synaptic apposition than NK1Rs. Immunolabelling for GluR2/3 was only occasionally found at synaptic-perisynaptic location.

FIRING PROPERTIES RECORDED *IN VIVO* ARE ALTERED IN HIPPOCAMPAL CA1 PYRAMIDAL CELLS 10-12 MONTHS AFTER GLOBAL FOREBRAIN ISCHEMIA

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Ca^{2+} buffering in ischemia is of interest, because calbindin decrease or loss in surviving cells has been reported in several studies. An increase in adaptation of action potential firing and post-spike after-hyperpolarization (AHP) was found in calbindin knock out mice when the relation between Ca^{2+} buffering and recovery from ischemia was studied.

We also found an increased AHP and altered action potential (AP) shape in 8 putative pyramidal cells from the hippocampal CA1 area. The resting membrane potential and the somatic input resistance, recorded *in vivo*, did not differ in these cells compared to control measurements, and were 62±4 mV and 57±36 MΩ, respectively. The AHPs of the APs increased and their halfwidth decreased both for APs that occurred spontaneously as well as for evoked ones.

Several studies support the observation that calbindin content fails to protect neurons against ischemic insult. On the contrary, surviving neurons lose, or at least decrease, their calbindin content. The enhanced AHP and altered AP shape in the pyramidal cells surviving ischemia can be the result of the absence or decrease of the fast Ca^{2+} buffering system. Alternative explanation may be the altered cable properties of the surviving neurons, since they are swollen in most of the cases. Anatomical investigations of the recordeed (and intracellularly filled) neurons is in progress.

THE ROLE OF NURR1 IN THE DEVELOPMENT OF DOPAMINERG CELLS IN THE VENTRAL MIDBRAIN

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The orphan nuclear receptor Nurr1 is a transcription factor that belongs to the steroid/thyroid hormone receptor superfamily and it is being expressed in many regions of the brain. To investigate the physiological role of Nurr1, mice were generated with null mutation in the Nurr1 gene. Nurr1-null mice seem to develop normally but die within 12 hours after birth. Immunohistochemical and *in situ* hybridization analysis revealed the absence of tyrosine hydroxylase the key enzyme in the dopamine biosynthesis in the substantia nigra/ventral tegmental area of the Nurr1 knockout mice. However, the normal ventralization of neuroepithelial cells to the ventral midbrain, their differentiation into neurons (confirmed by the neuronal nuclear specific marker NeuN) and the establishment of their projections to the striatum, (shown by nigrostriatal transport of a fluorescent tracer, DiI) are not affected by the

loss of Nurr1. Furthermore, Ptx3, a transcription factor, specific for the mesencephalic dopaminergic neurons and cholecystokinin, a co-transmitter of ventral tegmental dopaminergic neurons are being expressed by these neurons. The untranslated exon 1 and 2 of the Nurr1 gene remains intact after homologous recombination, which proves the presence of dopaminergic precursors in the ventral midbrain of the Nurr1-null mice. These results provide evidence that Nurr1 is essential for terminal differentiation of the dopaminergic neurons in the ventral midbrain, but the early steps of their neurogenesis, migration, and striatal projection are not effected by the lack of Nurr1.

THE EFFECTS OF ANGIOTENSIN II AND LOSARTAN MICROINJECTIONS INTO THE ZONA INCERTA AND THE AMYGDALA ON FLUID BALANCE

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Thirst and salt appetite are satisfied by ingesting water and salty substances. Behavioral, endocrine and neural responses to losses of body fluids are critical for reestablishing homeostasis. The brain renin-angiotensin-system has a pivotal role in the regulation and modulation of this action. The influences reaching the brain require integrative neural network including the structures of the lamina terminalis, the amygdala and the zona incerta (ZI). These regions receive afferent sensory input and process information related to hydromineral balance. It is supposed that angiotensin II (AII) acts on through these structures via its receptors (AT1 or AT2) to stimulate thirst and sodium appetite under conditions of hypovolemia. These receptors have a prominent heterogenity in distribution, but both can be involved in dipsogenesis. Binding studies showed in the ZI mainly of the AT1, but in the central amygdaloid nucleus (ACE) both, but the AT2 subtype in a higher percentage. Other reports showed high concentration of AII immunoreactive terminals in these regions. In our experiments the effects of AII and its AT1 receptor antagonist losartan injected into the ZI and the ACE have been studied on thirst. One hundred ng/0.5 µl doses of AII and 90 ng/0.5 µl AT1 antagonist losartan or vehicle microinjections were applied in male CFY rats after 23 h water deprivation. Liquid intake was registered in every 5 min for 30 min and in the 60th min as well when AII was injected. AII was effective in increasing water intake in both structures. Furthermore, losartan eliminated the AII induced drinking response in the ZI, while it was ineffective in the ACE. As the effects of AII - in this paradigm - have not been tested in the ZI, the finding that water intake increased in water-deprived animals suggests that AT1 receptors in the ZI play an important role in the regulation of water intake. Considering our results in the ACE, AII has a significant role in reestablishing fluid balance after deprivation in rats, but its influence can be effective through the AT2 receptor. Further experiments with specific AT2 antagonist are required to prove this hypothesis, however.

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LIQUID CHROMATOGRAPHIC DETERMINATION OF DIADENOSINE-5',5'''-P_1,P_4-TETRAPHOSPHATE AND ITS APPLICATION TO ISCHEMIC NEUROTRANSMITTER STUDY IN TISSUE OVER FLOW FLUID

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An on-line automatic sample enrichment process of diadenosine-5',5'''-P_1,P_4-tetraphosphate [Ap_4A], which are formed on backreaction of the amino acid activation step, has been developed in various tissue overflow fluid. It was separated by reversed-phase HPLC and detected at specific absorption wavelength of adenine nucleotides (254 nm). This potassium phosphate buffer without ionparing reagent with electrochemical detection (EC) was useful in endogenous noradrenaline [NA] analysis, too. The optimum of one portion of stripping volume was 247.8±19.1 µl. The detection limit of [Ap_4A] was ca. 5 pmol/ml in tissue superfusate when 1000 µl of the sample was applied in four portions to the column. Using the aqueous buffer in a higher ion concentration decreased the absorbance of residual of matrix (Krebs-bicarbonat buffer). The highest recovery 91.6 ± 2.8% was obtained by using one washing step. The relative standard deviation of intra-assay reproducibility was 8.3%. The release of [Ap_4A] and [NA] from adrenal medulla induced by ischemia or carbachol increased from 93.8±17.3 pmol/ml; 56.2±7.9 pmol/ml to 130.8±9.9 pmol/ml; 94.8±19.7 pmol/ml, respectively. The present paper shows that HPLC-UV, HPLC-EC on-line sample enrichment allows a sensitive and specific quantification of endogenous nucleotides and biogenic amines same tissue overflow sample.

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CHLORIDE DISTRIBUTION IN THE CA1 REGION OF NEWBORN AND ADULT HIPPOCAMPUS BY LIGHT MICROSCOPIC HISTOCHEMISTRY

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The fast inhibitory effect of GABA is exerted by binding to the postsynaptic $GABA_A$ receptors by making them permeable to chloride ions. Thus, depolarizing, or excitatory effects of GABA, experienced in early postnatal life or in certain regions and/or conditions of the adult brain, is thought to depend on a reversed transmembrane chloride gradient. However, there is only limited direct information about the correlation of the actual excitatory versus inhibitory effects of GABA and the local chloride distribution. We adapted and characterized methods, based on trapping tissue chloride with silver ions during freeze-

substitution or aldehyde fixation, to visualize the chloride distribution in hippocampal slices. The freeze-substitution protocol was superior in chloride retention while with aldehyde fixation tissue preservation was better. Both methods were qualitative in nature, had limited applicability to the superficial 20-30 μm of slices, but were able to demonstrate a reduced extracellular-to-intracellular chloride gradient in the CA1 pyramidal neurons of the newborn hippocampus as compared to adult animals. In the 4-aminopyridine model of epilepsy the redistribution of chloride from extracellular to intracellular space could also be demonstrated.

IS AMINOXYACETIC ACID PROCONVULSIVE OR ANTICONVULSIVE?

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In some epilepsy models aminoxyacetic acid (AOAA) was found to be anticonvulsive, however AOAA was also reported to induce seizures. AOAA is a non-specific aminotransferase-inhibitor, blocking the GABA-catabolizing enzyme GABA-transaminase (GABA-T) as well as the kynurenic aminotransferase enzyme, which syntheses the endogenous excitatory aminoacid antagonist, kynurenic acid. AOAA may exerts anticonvulsive effects through action on GABA metabolism and reuptake that may potentiate synaptic inhibition, while proconvulsive effects and insufficient neuroprotection may be explained by reduced formation of kynurenic acid. Since the convulsive or anticonvulsive action of AOAA is unclear, in this study the effect of AOAA was investigated on the 4-aminopyridine (4-Ap)-induced cortical seizure activity in anaesthetised adult rats.

Local pretreatment of the cortex with AOAA-crystal for 3 hours alone, induced neither characteristic epileptiform discharges, nor obvious changes in the ECoG pattern. However, it completely blocked the induction of epileptiform activity by 4-Ap. On the other hand, pre-treatment with 100 μM AOAA for 1 h, did not preventing but modified the pattern of epileptiform activity induced by 4-Ap. Generalised ipsilateral paroxysmal discharges appeared after a very short latency, characterised mostly by facilitated occurrence of slow, interictal-like (0.8-1.2 Hz) discharges. Later, progressively, periodic ipsilateral activity developed, with addition of higher frequency discharges (10-15 Hz), the propagation of which was almost completely blocked through the corpus callosum. Only fewer epileptiform discharges occurred in the contralateral hemisphere with longer latency and smaller amplitudes.

The effect of AOAA seems to be time- and concentration-dependent. Treatment with lower concentrations and shorter duration may result in an elevated brain excitability ipsilaterally, reflected in a prolonged interictal-like pattern progressing into an enhanced ictal activity. This proconvulsive effect may be explained by the rather prompt reduction in the level of kynurenic acid resulting in hyperactivation of NMDA-receptors or by impariring intracellular energy metabolism. The anticonvulsive effect of AOAA indicated by the

suppressed generation of high frequency discharges and their restricted interhemispheral propagation, might require a threshold level of GABA at the presynaptic terminals, which develops gradually.

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EVIDENCE FOR THE INVOLVEMENT OF ATP, BUT NOT OF VIP/PACAP OR NITRIC OXIDE, IN THE EXCITATORY EFFECT OF CAPSAICIN IN THE SMALL INTESTINE

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Capsaicin-sensitive neurons release neurotransmitters not only in the spinal cord but also at their peripheral endings. In the guinea-pig small intestine, these neurotransmitters activate myenteric plexus neurons that then contract the smooth muscle. In the present experiments the possible involvement of ATP (acting at P2 purinoceptors), as well as its relation to tachykinins (TK; such as substance P) in the motor effect of capsaicin was assessed.

Capsaicin (2 μM) caused half-maximal longitudinal contraction. This was reduced by a combination of TK receptor antagonists (GR 82334 for TK NK1 and SR 142801 for TK NK3 receptors). The P2 purinoceptor antagonist PPADS, but not the PACAP/VIP receptor antagonist PACAP(6-38) or an inhibitor of NO synthase, in combination with the TK receptor antagonists further inhibited the motor response with capsaicin. Given alone, PPADS (and also the PACAP/VIP antagonist or the NO synthase blocker) were ineffective. PPADS blocked the myenteric neuronal stimulation caused by ATP and alpha-beta-methylene ATP and PACAP(6-38) inhibited that evoked by VIP or PACAP.

It is concluded that an endogenous P2 purinoceptor stimulant (ATP?), but not PACAP/VIP or NO, is included in the activation of myenteric neurons in the course of capsaicin's effect, possibly as a sensory neurotransmitter.

AXON ORIGIN OF THE NEURONES IN THE SPINAL CORD

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The site of origin and the course of the initial, unmyelinated portion of the Golgi-impregnated spinal cord neurones were studied in adult rats. The spinal cord was impregnated according to the Golgi-Kopsch procedure and sectioned in the transverse plane.

Impregnated neurones were sampled mostly in the intermediate zone and in the ventral horn of lumbar segments. Selected neurones were either drawn with camera lucida or traced with the aid of NEUROLUCIDA system. Numerical data were collected either by direct measurement or captured automatically by NEUROLUCIDA.

The initial portion of the axon could be unequivocally detected at about 30% of the impregnated neurones. The length of the initial portion measured 15 to 40 μm in length. A bayonett-like inflection was often seen on the initial portion.

The axon origin was found either on the perikaryon, or in the immediate vicinity of one of the main dendritic trunks, or far away from the soma, along a dendrite. The axon of the large size motoneurones in the ventral horn originated from the perikaryon. Among the medium and small size neurones all three forms of axon origin could be seen in identical proportions. The initial portion of the axon of the dorsal horn neurones was directed ventrally. That of the neurones in the intermediate zone showed a random orientation.

It is suggested that in more than half of the spinal cord neurones the axon originates from one of the dendrites or dendritic trunks instead of the perikaryon. It is probable that in these neurones the initiation of the action potential will be determined more by the synaptic input impinging on the dendrite in question than by that of the other dendrites that are separated from the axon origin by the perikaryon.

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ORGANIZATION OF EFFERENT VESTIBULAR NEURONS IN THE FROG

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The localization and morphology of the vestibular efferent neurons of the frog (*Rana temporaria*) was studied by retrograde labelling of vestibular nerve branches innervating individual semicircular canal and otolith organs with biocyitin and fluorescent dyes. The soma and the dendrites of efferent neurons were intensely labelled with biocytin and in most cases the axons were also detected. The cell bodies were located in the reticular formation of the rhombencephalon between the motor nuclei of the VIIth and IXth nerves. Efferent neurons labelled from the semicircular canal nerves were located more caudal and ventral than efferent neurons labelled from otolith organs. Based on the serial reconstruction of the dendritic trees and the shape of their soma the efferent neurons could be divided into two groups. In the first group the soma was fusiform with two or three dendritic trees with few long branches running into medial and lateral direction. In the second group the cells presented large polygonal soma with 3-6 stem dendrites and the dendritic trees formed a dense meshwork close to the soma.

Labelling of the individual branches of the vestibular nerve with biocytin resulted in a few labelled axons and axon terminals in the untreated nerve branches and vestibular endorgans. In order to test the possibility that individual efferent neurons may supply different labyrinthine endorgans we applied two different fluorescencent dextran amines to the anterior and the posterior rami of the vestibular nerves. The experiments revealed that about 50% of the vestibular efferent neurons gave off collaterals within the vestibular nerve and innervated more then one vestibular endorgans.

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THYROID HORMONE LEVEL AFFECTS NITRIC OXIDE SYNTHASE CONTENT IN THE DEVELOPING POSTNATAL RAT BRAIN

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Thyroid hormone (TH) level is important to keep normal metabolic function of the brain during the lifetime, and essential for the right construction of developing neuronal connections. THs have significant impact on migration, differentiation of neurones and synaptogenesis by the regulation of genes encoding growth factors and by the modulation of structural proteins. In spite of the expanded investigation of this field, up to date, there is only few data available whether any functional relation exists between THs and nitric oxide synthase (NOS). Recently it was established that NOS is highly expressed in certain maturating neurones, however, the exact role of NOS in neural development is controversial. Therefore, in this study, the thyroid status of rat pups were chronically changed by the daily application of thyroxin (T_4) and the hypothyroidism resulting chemical, propylthiouracil (PTU) for two weeks from the 7th postnatal day. The NOS level was followed up in the cerebellum, cerebral cortex, hippocampus and hypothalamic nuclei by the localisation of NADPH-diaphorase (NADPH-d) and neuronal NOS (nNOS), respectively. In the cerebellum the axon growth cones of granular cells were found at the upper third of the molecular layer and expressed NOS extensively at the bottom in nontreated animals. The fibres of NOS containing granular cells reached the top of the molecular layer and gave moderate, homogenous staining for NOS in case of T_4 treatment, whereas only short and sparse axon processes within weak NOS content could be detected on the tissue slices of animals exposed to PTU. Hypothyroidism significantly attenuated, whereas hyperthyroidism upregulated the staining of several immunopositive neurones in the cerebral cortex, compared to control. Number of pyramidal cells and granular cells of the dentate gyrus stained slightly in the hippocampus of control animals, discernible increase of the nNOS content in PTU treated, and enhanced staining intensity in T_4 treated rats were notable. Increased NOS activity was observed in the neuroendocrine cells of the paraventricular and supraoptic nucleus in the presence of high T_4 level, whereas pale reactivity occurred in the

case of PTU treatment, compared to control. NADPH-d was also demonstrated in blood vessels in the whole examined brain area. PTU provoked marked, whereas T_4 resulted weak NADPH-d activity, and moderate staining was found in blood vessels of control. These results suggest, that NO level possibly changes during chronic up-, or downregulation of thyroid hormones in the developing central nervous system. NO may have some role in the formation of cerebellar synaptic contacts under thyroid hormone control. Moreover, metabolic alterations evoked by high or low level of plasma T_4 may differently regulate NOS isoforms responsible for brain blood supply.

LOCALIZATION OF GLUTAMATE/ASPARTATERGIC PROJECTION NEURONS IN THE NEURAL STUCTURE OF THE SEPTUM: COMBINATION OF [^{3}H]D-ASPARTATE AUTORADIOGRAPHY AND IMMUNOCYTOCHEMISTRY

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The septal formation plays an important role in interconnecting the limbic system with the hypothalamus and brainstem. It establishes several reciprocal connections with the hippocampal formation, diencephalic and brainstem structures. The lateral septum participates in a number of functions involving the hypothalamus. A variety of septal projection neurons characterised by their calcium-binding protein and/or neurotransmitter content participates in these connections.

In the present study our aim was to identify glutamate/aspartatergic projection neurons in the area of the septum. The investigations were based on the high-affinity selective uptake of [^{3}H]D-aspartate by glutamatergic terminals and the retrograde transport of the tritiated amino acid to parent cells. [^{3}H]D-aspartate was injected into different hypothalamic areas; the paraventricular nucleus, the anterior hypothalamic area and the ventromedial nucleus. These experiments were carried out in combination with counterstaining for toluidine blue and/or with immunostaining for calretinin (CR) and calbindin (CB).

Our investigations demonstrate that following the [^{3}H]D-aspartate administration in different parts of the hypothalamus a large number of autoradiographically labelled neurons is present in the intermediolateral, laterodorsal and lateroventral part of the lateral septum.

These autoradiographically labelled cells never colocalised with CR immunocytochemistry but a well detectable part of them were positive for CB.

These results indicate that a considerable glutamate/aspartatergic cell population exists in the septum. Our data suggest also that the calbindin positive cell population in the septum considered to be mainly GABAergic is heterogenous as a part of these cells are glutamate/aspartatergic.

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LOCAL INJECTION OF SUBSTANCE P CONJUGATED SAPORIN SELECTIVELY ELIMINATES SUBSTANCE P RECEPTOR EXPRESSING NEURONS

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Selective damage of subpopulation of neurons is an effective tool for revealing their function. Here a ribosome inactivating protein, saporin, conjugated to substance P (SP-SAP) was used to selectively damage substance P receptor-expressing interneurons in the dentate gyrus of the rat and mouse hippocampus. Three different doses (50 ng, 25 ng and 5 ng) were tested in animals surviving 1, 2, 3, 5 and 10 weeks. Immunohistochemistry (parvalbumin, calbindin, calretinin, SPR, GluR2 and mGluR1α) was carried out to examine specific or nonspecific damage.

High (25 ng and 50 ng) dose eliminated SPR elements in an approximately 2×3 mm area, but it also caused nonspecific damage around the center. In rats injected with 5 ng nonspecific damage to granule cells and mossy cells was not observed whereas SPR-immunoreactive interneurons were selectively eliminated a far as 0.7 mm from the center of the injection. The loss of SPR immunoreactive cells was clearly visible already after two weeks.

In the mouse, the SPR antibody stained a subpopulation of interneurons different from that of the rat. Injections of SP-SAP selectively eliminated these large interneurons. Damage to SPR-neurons caused abnormal electrical patterns within 3 days. Between days 5 and 10 postinjection 40% of the mice had behavioral and/or electroencephalographic seizures.

The selective lesion of a well-defined subgroup of hippocampal interneurons can reveal their physiological role in normal function. Furthermore, the permanent and selective absence of interneurons may be an effective tool for creating focal epilepsy. Supported by NIH, NIMH and Eötvös fellowship.

THE EFFECT OF ENDOMORPHIN-1 ON BEHAVIOR AND PITUITARY-ADRENAL ACTIVATION

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The effects of the recently identified opioid peptide, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2), on the behavioral response and on the hypothalamo-pituitary-adrenal system (HPA) were investigated in mice. The locomotor activity was measured in an "open-field" apparatus and characterized by the total number of the squares explored by the mice. The number of rearings, groomings and defecation boluses were also measured. Different doses of the endomorphin-1 (250 ng-5 μg) were administered intracerebroventricularly (icv.) to the mice. Thirty min after the treatment the peptide caused a significant change in the locomotor activity. The dose-response curve showed an bell-shape response having a maximum at 1 μg and a decline with the larger dose of 2 and 5 μg. The most effective dose of the tetrapeptide (1 μg) elevated the number of the rearings as well. In case of grooming no difference was observed between the control and the peptide-treated groups. Furthermore, the effect of the opioid peptide on the basal corticosterone secretion was investigated. The plasma corticosterone level was assayed by fluorescence method. The administration of the peptide produced a significant increase in the corticosterone level at the dose of 5 ?g. These results suggest that endomorphin-1 might play a role in the regulation of both locomotion and stress response.

LOCALIZATION OF GLUTAMATE/ASPARTATERGIC NEURONS PROJECTING TO THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS: COMBINATION OF [^{3}H]D-ASPARTATE AUTORADIOGRAPHY AND IMMUNOCYTOCHEMISTRY

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Several data indicate that the excitatory amino acid glutamate is a major regulator of synaptic activation in the hypothalamic paraventricular nucleus (PVN). The purpose of the present study was to investigate the localization of glutamate/aspartatergic (Glu/Aspergic) nerve cells terminating on PVN neurons. The investigations were based on the high-affinity

selective uptake of [^{3}H]D-aspartate by glutamatergic terminals and the retrograde transport of the tritiated amino acid to parent cells. [^{3}H]D-aspartate was injected into the PVN and the different brain areas were examined for labelled neurons detected autoradiographically.

On the basis of our results it appears that almost all hypothalamic nuclei and areas send Glu/Aspergic afferents to the PVN except for the arcuate nucleus. Telencephalic regions known to project to this hypothalamic cell group are the amygdala, bed nucleus of the stria terminalis, lateral septum, medial and lateral preoptic area and hippocampus. We detected retrogradely radiolabelled neurons in all these structures except the hippocampus. We did not find any retrogradely labelled Glu/Aspergic neurons in the brain stem. This suggests that the brain stem presumably does not contribute to the glutamatergic innervation of the PVN.

Our observations provide direct morphological evidence for the hypothesis that glutamatergic fibers arising from various brain structures represent a significant part of the neural input to the PVN.

The studies were supported by the Hungarian Academy of Sciences (AKP 98-12/3,2/42 to J.K.), the National Research Foundation (OTKA T-029004 to B.H. and OTKA T-023989 to J.K.) and the Ministry of Health (ETT 478/96 to B.H.).

FINE STRUCTURE AND POSTEMBRYONIC DEVELOPMENT OF THE PINEAL ORGAN OF DJUNGARIAN DWARF HAMSTER (*PHODOPUS SUNGORUS*)

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The pineal organ plays a role in the seasonal timing of several functions like gonadal activity, moulting periods, pelage composition, etc. The pelage of the Djungarian dwarf hamster is grey on the backside and white on the other ventral side during summer and it changes to white on the whole body in winter. This is presumably controlled by the pineal organ according to the environmental light circumstances because this change of colour may be reproduced by artificial illumination characteristic for wintertime. It is generally accepted that light information that reaches the pineal organ comes from the retina via the peripheral adrenergic innervation of the organ. In contrast, submammalian pinealocytes are photoreceptors being directly sensitive to light. Since in some mammals, like the gerbil and ferret, immunoreactive rhodopsin was demonstrated in pinealocytes some authors suppose direct light sensitivity of mammal pinealocytes.

Due to the pelage colour changes, the Djungarian dwarf hamster seems to be a good object for studying this problem. The structure of the pineal organ of this species is not completely known. Therefore, in the first step of our study we wanted to clarify the finestructural basis of a supposed neural and hormonal efferentation as well as the possible way of the light information to the pineal organ.

First we examined the light microscopic structure of the pineal organ. The hamsters studied were in different ages as one day, three days, one week and five weeks old. We have found that the pineal organ of this species is attached to the inner surface of the skull through

the confluence of sinuses. Presumably, this connection has some importance in the hormonal efferentation of the organ.

Fine structurally pinealocytes, glial cells and neurons compose it. Pinealocytes may have light or dark cytoplasm containing some granular vesicles. They bear 9x2+0-type cilia. The glial cells contain microfilaments and multiform dark nuclei. Intrapineal nerve cells have light cytoplasm, rough-surfaced endoplasmic reticulum and receive axosomatic and axodenritic synapses. Axon-like profiles may contain synaptic ribbons. In the vascular spaces of the organ myelinated and unmyelinated fibers were found. Similar nerves are present in the nervous tissue of the proximal part of the organ. These elements are present in the first postnatal period of this species.

The electron microscopic organization shows that the structural basis of hormonal as well as neural efferentations is present, furthermore, there is a peripheral and central nerve connection of the organ. In further investigations we want to study whether the central or peripheral nerve connections are involved in the afferentation of light information needed for the function of the organ with special attention to the circannual colour changes of the pelage. We also want to examine the possibility of direct light sensitivity of the pinealocytes.

ROLE OF THE NGF-C-jun SYSTEM IN THE REGULATION OF NEUROPEPTIDE EXPRESSION OF PRIMARY NOCICEPTIVE NEURONS

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Transection of a peripheral sensory nerve as well as crush or blockade of the retrograde axoplasmic transport are known to induce depletion of the neuropeptides substance P and CGRP from lamina I and IIo, and of the enzymes FRAP and TMP from lamina IIi of the ipsilateral, segmentally related portion of the superficial spinal dorsal horn [1]. Depletion is preceded by the expression of the immediate early gene c-jun in nuclei of the related dorsal root ganglion cells [2] and followed by appearance of VIP [3], galanin and NPY [4]. The role of the nerve growth factor (NGF) in the plasticity was studied with immunohistochemical methods.

Application of a perineural anti-NGF cuff resulted in expression of c-jun in dorsal root ganglion cells and in plastic alterations of neuropeptides and enzymes, in a manner similar to axotomy. On the other hand, the appearance of noradrenergic axons around nerve cells of the dorsal root ganglion, a novel aspect of nociceptive neuroplasticity: [5], which is regarded as a major cause of chronic pain [6] could be elicited only by axotomy and not by blockade of the retrograde axoplasmic transport. It is assumed that pericellular sprouting of noradrenergic axons is due to the axotomy-induced revival of paracrine NGF production characterizing embryonic satellite cells [7].

It is concluded that the NGF-c-jun system plays important role in the pathomechanism of chronic, intractable pain. If so, our studies may open up new possibilities in the gene therapy of chronic pain.

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ROLE OF CERTAIN SIGNAL MOLECULES IN THE REGENERATION OF THE NERVOUS SYSTEM IN OLIGOCHAETES

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The central nervous system of Oligochaetes posseses a good regenerative capacity. The regeneration is controlled by external and internal factors. The reorganization of neuronal systems containing certain signal molecules was studied in the newly forming brain of *Eisenia fetida*. The new brain is formed by the 80th day and this period can be divided into six stages. Stage 1: The body wall is reorganized. Stage 2: A loose wound tissue is formed in place of the removed brain. Stages 3-4: Fibres and cells containing neurotransmitters/modulators appear in the wound tissue. Stage 5: A capsule develops around the preganglion. Stage 6: The neurons of the preganglion migrate to their final positions. From the 56^{th} postoperative day the formation of different cell groups can be observed and by the 80^{th} postoperative day the total cell number of the brain can be estimated.

The neurons of the newly formed brain originate from the neuroblasts situated on the inner and outer surface of the intact ganglia and from the mitotic proliferation of the pharyngeal neurons.

During regeneration the monoaminergic elements appear earlier than the peptidergic ones. It is supposed that serotonin and dopamine play a role in the proliferation of the neuroblasts and, indirectly, the migration of the neurons. GABA and FMRFamide accelerate the differentiation and proliferation as well as the migration of the neurons. At the beginning of the regeneration the exogenously applied GABA has an inhibitory effect on the non-neural elements, therefore the wound tissue develops slower than normally, but GABA does not prolongate effect the differentiation and proliferation of the nervous elements.

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PH-DEPENDENT ACTIONS OF ALUMINATES ON PASSIVE MEMBRANE PROPERTIES AND ACTION POTENTIALS OF SNAIL NEURONES

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Aluminium is one of the elements, which most frequently subjected to experiments, however, the experimental results are rather conflicting. The cause of this controversality is recently unknown thus, the exact relation of the aluminium with some disorders such as Alzheimer's disease, dialysis encephalophathy, Parkinsonism-dementia of Guam is unclear.

We studied the influence of the pH-dependent actions of $AlCl_3$ solutions (Al) on the membrane potential, input resistance and action potential of *Helix pomatia L.* neurones at pH 7.7, 7.2, and 6.8 by use of one-electrode current clamp technique.

We reported significant differences in the actions of equimolar Al suspensions, which were applied at pH 7.7, 7.2, or 6.8 at room temperature. 0.3 mM, freshly prepared and applied Al-suspension caused a significant decrease of the interspike interval at pH 7.7 and 6.8 but not at pH 7.2. Also the Al suspension caused a significant and dose-dependent depolarisation, and a concomitant decrease of the input resistance and time constant of the membrane at pH 7.7. Similar, but significantly decreased depolarisation, input resistance and time constants were found at pH 7.2. Changes of the measured parameters of the neuronal membrane at pH 6.8 were the same as at pH 7.7 when equimolar Al-suspensions were applied. A significant increase of the action potential duration at 50% amplitude (APD_{50}) and attenuation of the overshoot (OS) and peak potentials (PP) were recorded after administration of 0.3 mM Al solution at pH 7.7. However, these parameters did not alter in equimolar Al suspension at pH 7.3. Significant decreases of the threshold potential, OS and PP were recorded in 0.3 mM Al containing media at pH 6.8.

We suppose that changes of the aluminum speciation spectrum by the pH could modify the concentration and composition of the biologically active aluminum compounds (aluminates) which might play a role in the controversality of the results published during electrophysiological observations.

ELECTROPHYSIOLOGICAL AND PHARMACOLOGICAL CHARACTERIZATION OF SOME IDENTIFIED NEURONS AND THEIR SYNAPSES IN THE TERRESTRIAL SNAIL, *HELIX POMATIA L.*

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Neurons, such as left (L) and right (R) Parietal 1, 2, 3; L and R Pleural 1; Visceral 1, LPa4 and Br have been identified in *Helix pomatia* L. preparations on the bases of membrane potential (E_m), input resistance (R_{in}), time constant of the membrane (τ) action potential pattern. Electrophysiological and pharmacological properties of synaptic transmission were also studied by extracellular stimulation of the intestinal, anal, L and R pallial, and cerebro pleural connectives.

We found significant differences between the E_m of the basal LPa4 and Br neurons (46.8±5. mV and 43.5±3 mV) and the E_m of investigated apical neurons (49-51±4 mV). Similar differences were found between τ of Br (82.9±3 ms) and other apical cells (39-56±4 ms). Topological differences were not found in the R_{in} of these cells.

We recorded potential-dependent and compound EPSPs in the investigated neurons by stimulation (6V, 1ms) of the above-mentioned nerves. IPSPs or ILD were recorded in the RPl1 neuron by stimulation of LPl nerve. Other identified neurons responded with slow compound EPSPs after stimulation of the main nerves. Reversal potentials of EPSPs were between –40.1 and –18.7 mV in all cases, which shifted to left in decreased K^+ containing extracellular medium. Decreased extracellular concentration of Na^+ and Ca^{++} markedly attenuated the amplitude of the EPSPs.

Furthermore, we studied the ACh, GABA and glutamate responses of these neurons. We classified the cells according to the desensitization of their responses. We found two types of glutamate responses: one desensitizing and one non-desensitizing, and three types of ACh responses: one non-desensitizing and two desensitizing (fast and slow desensitizing responses). It seems that the GABA responses of the V1 neuron seasonally changing. At spring it has an inhibitory and at autumn it has an excitatory GABA responses.

SEX DIFFERENCES IN THE AGING PROCESS OF RATS WITH A SPECIAL VIEW TO THE COPULATORY BEHAVIOR, WITH AND WITHOUT (-) DEPRENYL TREATMENT

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Previously we carried out a lot of experiments with (-)deprenyl in correlationship between sexual activity and life span. In this presentation we compare the male and female rats' aging process.

1. The life span of saline treated sexually inactive females is significantly longer than that of sexually inactive males.

2. (-)Deprenyl treatment (0.25 mg/kg s.c. three times a week till decay) does not change the life span of sexually inactive females, while (-)deprenyl administration enhances the sexually inactive male's life span.

3. With saline treatment sexually active females have a shorter life span than sexually active males.

4. (-)Deprenyl treated sexually active females live just as long as the saline-treated group but (-)deprenyl treatment does not influence the life span of males.

5. In sexually active males there is an age-dependent decline of copulatory activity which can be reversed with continuous (-)deprenyl treatment. Age-dependent decline of copulation could not be observed in female rats.

The above data suggest that there are differences between the two sexes in their aging process in relation to sexual behavior.

PLAY BEHAVIOR REDUCES ANXIETY LEVEL IN ADULT AND AGING RATS

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Male Wistar rats aged 3, 15, 24 months were housed 4 per cage and exposed to inanimate objects. The objects induced social play in all age-groups. Play behavior consisted of chewing and transportation of the objects around the home cage. The rats were housed individually for 72 h prior to subjection to the objects and the plus-maze test. The object-induced increased, exaggerated locomotor activity lasted for 20 to 30 minutes and showed a decrease both in intensity and duration by age. One hour after the start of exposure, the animals were tested in elevated plus-maze to measure anxiety level. It was found that the activity elicited by the exposure to the inanimate objects (two objects for each rat) resulted in a significant reduction of anxiety level both in adult and in aging rats. Rats in all age groups spent significantly more time in the open arm of the elevated plus-maze than the corresponding controls. The number of entries to the open or to the dark arm, however, did not show statistical differences indicating that the novel object-induced elevated locomotor activity did influence the activity in the plus-maze.

FATE OF EMBIYONIC NEURAL PROGENITOR CELLS IN THE FOREBRAIN OF ADULT MICE

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Despite of the permanent neuron-production (1) by the subventricular neural stem cells, the majority of decaying neurons cannot be replaced in the adult mammalian brain. Progenies of the subventricular stem cells migrate along the outer wall of the ventricles, in the rostral migration stream or along large fiber tracts, The precursors develop into small intenieurons *of* the olfactory bulb, dentate gyrus (2) or the ftonto-parietal cortex (3). The restricted migration and fate of "adult" precursors might be due to the narrowed potential of stem cells, or might be governed by environmental factors provided by the mature brain. The role of the adult brain milieu was stadied by implanting neural progenitor cells of embryonic origin into the adult forebrain. NE-4C cells, cloned derivatives of forebrain stern cells of 9-day-old mouse embryos (4), had been transfected with the genes coding green fluorescent protein (GFP). The GFP-4C subclone were implanted into the lateral ventricles, and the striata of adult mice. Small aggregates of grafted cells attached to the inner wall of ventricles, choroid plexus and striatal tissue. The number of embryonic progenitors increased during the first three weeks of implantation, indicating that the milieu did not prevent their proliferation. After 4-6 weeks, however, only a few surviving cells were found, mainly in the wall of ventricles, in large fibre tracts and in the fibrous parenchyme of the lateral septum.

Embryonic progenitors behaved similarly to "adult" precursors indicating that the restricted neuron-production in the adult forebrain might be a result of the non-permissive conditions provided by the mature neural tissue.

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EEG-RELATED ACTIVITY OF IMMUNOHISTOCHEMICALLY IDENTIFIED BASAL FOREBRAIN NEURONS

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Monosynaptic innervation originating from cholinergic and GABAergic neurons in the basal forebrain reaches cerebral cortex and most other parts of the telencephalon. This corticopetal projection has been claimed to participate in different behavioral processes ranging from sleep induction to arousal, memory, and attention. However, anatomical complexity of this area renders direct functional analysis of basal forebrain neurons very difficult.

The aim of the present experiments in urethane-anesthetized rats was to record EEG-related neurons in the basal forebrain with extracellular micropipettes and to label them with juxtacellularly ejected biocytin. Immunohistochemical detection of biocytin enabled the localization of the recorded cells, which were then tested successively for the presence of choline-acetyltransferase, parvalbumin and NPY. Neuronal activity was analyzed during spontaneous EEG changes and also following noxious stimulation (tail pinch). Two of the recorded fast-wave-active (F-cell) neurons were proved to be cholinergic while one of them contained parvalbumin, thus was GABAergic. Two cells displayed opposite relationship with the EEG and were silenced by tail pinch (slow-wave-active or S-cell). They were also GABAergic neurons, but were positive for NPY as well. We are convinced that electrophysiological recording combined with immunohistochemical identification will enable the reconstruction of the basal forebrain circuitry and will lead to the understanding of the functional significance of this system.

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EFFECTS OF URIDINE AND ADENOSINE ON NEURONAL ACTIVITY PATTERNS AND EXTRACELLULAR METABOLITE LEVELS

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Both adenine nucleotides and their nucleoside analogue adenosine are neuroactive compounds. Since receptors of uridine nucleotides have recently also been found, we investigated the role of uridine in the brain function. Previously, we demonstrated the release of uridine in response to depolarisation. Here we present data concerning the electrophysiological and biochemical effects of uridine in the hippocampus and thalamus of anaesthetised rats. Uridine administered via microdialysis probes attached to microelectrodes

was demonstrated to decrease multiple unit activity in the rat hippocampus but not in the thalamus. When we were able to separate activity of single hippocampal pyramidal cells, the inhibitory effect of uridine was almost complete. The inhibitory effect of uridine was not accompanied with changes of extracellular concentrations of purine nucleosides and amino acids. Adenosine was also tested in the same experimental conditions. Adenosine completely blocked neuronal activity in both the hippocampus and the thalamus, increased the extracellular concentrations of purine nucleosides and some amino acids including GABA. The effect of uridine on neuronal activity and its release by depolarisation support the role of uridine in brain function. The selective cytoprotective role or more general neuromodulatory actions of uridine are also discussed.

STREPTOZOTOCIN MICROINJECTION INTO THE VENTROMEDIAL HYPOTHALAMUS EVOKES DIABETES-LIKE METABOLIC CHANGES

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Streptozotocin (STZ) is known to specifically destroy insulin-producing β-cells of the pancreatic Langerhans-islets, and thus, to cause diabetes-like symptoms. The ventromedial hypothalamic nucleus (VMH) accommodates specific, so-called, glucose-monitoring (GM) neurons whose activity is facilitated by microelectrophoretically applied glucose. On the basis of electrophysiological similarities of pancreatic β-cells and these GM neurons, 1) acute and chronic homeostatic-metabolic consequences of STZ administration in the rat VMH, and 2) direct effect of STZ on VMH neurons were studied in complex, behavioral-biochemical experiments, and by means of the single neuron multibarreled microiontophoretic technique. Bilateral STZ microinjection elicited diabetes-like elevation of plasma glucose levels both in acute or chronic (10 min or 4 wk after STZ microinjection, respectively) glucose tolerance tests. The peak of the blood glucose curve in the STZ administered animals was found approx. at the 60^{th} min after the start of glucose load, 39-42 min later than in controls, with pathologically high value even at the 120^{th} min. Microiontophoretic application of STZ in the electrophysiological study not only exclusively facilitated but also proved to be toxic for the GM neurons whose firing irreversibly stopped after repeated administration of STZ. Histology of the pancreas showed normal islet structure in all animals. Our findings provide evidence for that STZ disintegrated the ventromedial hypothalamic GM neural network and thus, caused diabetes-like metabolic-homeostatic changes. The results, along with our previous data, substantiate the validity of a new central model of metabolic diseases, with particular emphasis on diabetes mellitus.

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ULTRUSTRUCTURAL ASPECTS OF PEPTIDERGIC MODULATION IN THE PERIPHERAL NERVOUS SYSTEM OF GASTROPODS

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The ultrastructure of peptidergic peripheral contacts in the snail, *Helix pomatia*, was investigated, with special attention to the innervation of the heart, buccal mass and salivary gland by *Mytilus* inhibitory peptide (MIP)-immunoreactive (IR) neurons. Following the application of correlative light- and electron microscopic pre-embedding immunocytochemistry, the peripheral tissues revealed a rich innervation by MIP-IR elements, based on a similar pattern of distribution of labeled profiles in them. MIP-IR neurons established three types of neuromuscular contacts in the heart and buccal mass: (i) close (16-20 nm) unspecialized membrane contacts; (ii) contacts with a relative wide (40-100 nm) intersynaptic cleft; and (iii) labeled varicosties located freely in the extracellular space, far (0.5-several μm) from the muscle cells. In the salivary gland, the immunoractive profiles contacted both the muscular and glandular elements with close (type i) and wider (type ii) membrane attachments. The great majority of MIP-IR profiles contained an ultrastructurally uniform population of large (120-150 nm) electron dense granules. The ultrastructural features of the innervation by MIP-IR elements were compared with those established by immunogold labelled FMRFamide-containing profiles in the heart and salivary gland. These latter displayed similarities in forming the different kinds of intercellular contacts, and differencies in the morphological variability of the content of granules in the immunolabeled profiles. The results suggest diverse, non-synaptic, modulatory roles of neuropeptides in the peripheral nervous system of *Helix pomatia*, including both localized membrane and neurohormonal-like remote control effects.

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INVESTIGATION OF TEMPERATURE EFFECTS OF DOPAMINERGIC AGENTS BY TELEMETRIC SYSTEM

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The dopaminergic system plays an important role in the central regulation of body temperature, but the nature of the involved receptors is not clear at present. The major role of dopamine D_2 receptors in the mediation of hypothermia has long been assumed (Salmi et al., Eur. J. Pharmacol. 253:67, 1994), but recently it was suggested that activation of the closely related D_3 receptors also can induce hypothermia. In these and other studies (Millan et al.,

Eur. J. Pharmacol. 260:3, 1994, Baric et al., Pharmacol. Biochem. Behav. 60:313, 1998) antagonists at the D_3 receptor inhibited the action of agonists but did not modify core temperature when given alone.

In the present study we examined the effects of two D_2/D_3 agonists, 7-OHDPAT (0.3 mg/kg sc.) and PD-128907 (0.5 mg/kg sc.) as well as that of the partial D_3 agonist BP-897 (1 mg/kg ip.) on body temperature of rats. U-99194A (12 mg/kg sc.), a selective D_3 antagonist was tested against the hypothermia induced by 0.5 mg/kg PD-128907. Finally, we investigated the effect of RGH-1756 (20 mg/kg ip.), a potent D_3 partial agonist *in vitro*, on core temperature of rats after acute as well as during subchronic (5 days) administration (3 mg/kg p.o. daily).

Changes in body temperature were monitored by a radiotelemetric system. For comparison, we measured the effect of 24 mg/kg sc. RGH-1756 by the traditional method (rectal probe), too.

The D_2/D_3 agonists, 7-OHDPAT showed rapid onset significant hypothermia. The maximal effect of -1.5 °C was measured at 1 hour after injection and hypothermia was maintained up to 2 hours. PD-128907 elicited 0.5 °C hypothermia, which appeared within 10 min after drug injection and lasted for cca. 1 hour. This short, but significant hypothermia was antagonised by concomitant administration of U-99194A.

BP-897, the partial D_3 agonist induced slight (0.3 °C) hypothermia.

At a single dose of 20 mg/kg ip., RGH-1756 induced 0.7 °C hypothermia, which appeared 30 min after drug injection and lasted for cca. 2 hours. However, after injection of 24 mg/kg sc. no change in body temperature could be measured by the traditional rectal method.

During repeated administration at daily doses of 3 mg/kg p.o. of RGH-1756 a slight but significant hypothermia developed following the second administration, and this effect was sustained on days 3 and 4, too. The maximal decrease in temperature was 0.4 °C measured about 60 min after drug treatment. The total duration of hypothermia was cca. 40 min. This effect disappeared after the fifth administration, suggesting the development of tolerance.

These results show that the radiotelemetric measurement of core temperature in animals (in combination with other physiological parameters like locomotor activity) can be used as a sensitive tool for detecting slight and transient temperature changes such as those caused by the D_3 (partial) agonists in this study.

This noninvasive technique is also very suitable for monitoring physiological functions and pharmacological effects in long-term studies.

POSTALIMENTARY HYPERTHERMIA IN ARTIFICIALLY FED RATS

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Fasting causes falls in metabolic rate (MR) and core temperature (T_c). Upon re-feeding, these falls can be quickly normalized or converted to postalimentary elevations. Abdominal chemo- and/or mechano-sensory afferent nerves are presumed to carry signals to elicit this conversion. Other state of these signals may be responsible for the falls in fasting.

We measured MR and T_c of cold-adapted rats, at the thermoneutral 25 °C, following 48-h fasting, and after 3-h oral re-feeding by either the usual chow, or saccharine-sweetened $CaCO_3$ tablets. In order to avoid oro-facial afferents, in other cases either a calorie-rich substance (generally used by body-builders), or a calorie-free one (X-ray contrast material) was given to fasting animals (while measuring MR and T_c), through a pre-implanted gastric cannula, in injections of 30 ml/kg thick fluid. In still other cases, 40% glucose was infused (4 ml within 2-h), through a pre-implanted jugular cannula, for calorie replacement without interfering with abdominal afferents.

Fasting caused a decrease in resting MR and T_c. Both parameters increased to near-normal levels following 3-h re-feeding with chow, and the increase was still significant when the animals ate calorie-free tablets. Re-feeding through the gastric cannula elicited an immediate increase in MR and T_c in cases when the calorie-rich formula was used, and similar increase with about 1-h delay when the calorie-free substance was used. No changes, or a fall, in RMR and Tc accompanied glucose infusions.

In the fasting-induced falls in RMR and T_c not caloric deficit, rather some regulatory alteration is decisive, due to various neural signals of abdominal origin. Altered signals may contribute to the development of postalimentary hypermetabolism and hyperthermia.

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THE ROLE OF THE K^+/Cl^- COTRANSPORTER KCC2 IN THE MAINTANCE OF LOW Mg^{2+} INDUCED EPILEPTIC ACTIVITY

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In a number of *in vitro* models of epilepsy a deterioration of the inhibitory mechanisms has been suggested to contribute to the progression of bursts in the rat hippocampal slice (Whittington 1995, et al. J. Physiol. 486:723). We have recently shown that the K^+/Cl^- cotransporter KCC2 is essential for the fast hyperpolarizing effect of $GABA_A$ receptors (Rivera et al. 1999, Nature 397:251). In this study we have examined the changes in the reversal potential of evoked $GABA_A$ IPSPs recorded from CA1 hippocampal pyramidal cells and the expression level of KCC2 at different time points after the onset of spontaneous activity in the low magnesium model of epilepsy. One hour after the application of 0 Mg^{2+} solution a positive shift in the reversal of $GABA_A$ IPSPs was found, and it was accompanied with a down-regulation in KCC2 expression. Thus epileptogenesis occurs in 0 Mg^{2+} solution resulting from an alteration of inhibitory mechanisms caused by a reduction in the transmembrane Cl^- gradient together with enhanced NMDA receptor-mediated synaptic conductances.

DISTRIBUTION OF NK1R, GLUR1 AND GLUR2/3 IMMUNOREACTIVITY IN THE LUMBAR SPINAL DORSAL HORN OF RATS

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It is well established that substance-P and glutamate are released by C and Aδ primary afferents in the superficial spinal dorsal horn in response to painful peripheral stimuli. The released substance-P acts on neurokinin-1 receptors (NK1R), whereas the effect of glutamate is mediated by various receptors including GluR1 and GluR2/3 receptors. Thus, spinal neurons that express NK1R, GluR1 and GluR2/3 are supposed to play crucial roles in the spinal transmission of nerve signals evoked by various noxious stimuli. Therefore, we studied the distribution of NK1R, GluR1 and GluR2/3 immunoreactivities in the lumbar spinal dorsal horn of rats using diaminobensidine- and nanogold-based preembedding immunocytochemical techniques at the light and electron microscopic levels. We demonstrated that the segmental, laminar, intracellular and synaptic-perisynaptic distribution of NK1R, GluR1 and GluR2/3 immunoreactivities show a number of distinct features in the superficial spinal dorsal horn. NK1R immunoreactivity was confined to lamina I, whereas, with the exception of a few large presumably supraspinally-projecting neurons, laminae II-III were free of labelling. In contrast to this, GluR1 and GluR2/3 immunoreactivities were distributed throughout the entire dorso-ventral extent of laminae I-II and laminae I-III, respectively. At the level of L3, L4 and L5 spinal segments in the medial aspect of the superficial dorsal horn, however, only a faint immunostaining was observed for GluR1 and GluR2/3 in laminae I-II. At these areas, immunoreactivity for both GluR1 and GluR2/3 were mostly confined to lamina III. At the cellular level, immunostaining for NK1R was associated with the outer plasma membrane of perikarya and dendrites, whereas immunoreactivity for GluR2/3 was mostly confined to the cytoplasm of perikarya. The immunostaining of the outer plasma membrane and the cytoplasm of perikarya were equally strong for GluR1. Investigating the synaptic and perisynaptic distribution of the receptors, immunogold particles marking receptor molecules were encountered in the most peripheral 20-40 nm wide outer rim of the postsynaptic membrane thickening of asymmetric synaptic contacts and at perisynaptic location in a 20-200 nm distance from the edge of the synaptic apposition. Within this territory, GluR1s were somewhat closer to the synaptic apposition than NK1Rs. Immunolabelling for GluR2/3 was only occasionally found at synaptic-perisynaptic location.

ELECTROPHYSIOLOGICAL EXAMINATION OF THE COMPLEMENT REGULATORY MOLECULE CD59 (HRF-20)

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In order to prevent self-cells from the complement attack, the host cells including neurons express membrane-bound regulatory molecules, for example CD59. One function of the CD59 is to block the membrane attack complex (MAC, C5b-9) to become larger by inhibiting insertion of extra C9 molecules into the C5b-9 complex. Using two cell lines either expressing or being deficient of CD59, we studied another function of CD59. Whole cell clamp electrophysiology revealed, that if the cell does not express CD59 on its surface, the MAC forms a non-specific ion channel in the membrane. Flow cytometry showed that in the presence of CD59, the MAC can also insert into the membrane forming a channel. However, electrophysiology demonstrated that the channel does not function, since no ions could pass through the formed MAC channel. From these results we deduce that in addition to inhibiting insertion of extra C9 molecules into the C5b-9 complex, CD59 can block the C5b-9 channel either by plugging the pore or by causing conformation change in the three-dimensional structure of the complex making the ions impossible to pass through.

EFFECTS OF TGF-? AND BMP-4 ON EARLY DIFFERENTIATION OF TYROSINE-HYDROXYLASE POSITIVE NEURONS IN CULTURES OF RAT VENTRAL MESENCEPHALON

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The early specification of dopaminergic (DA) neurons during vertebrate CNS development relies on signals produced by organizing centers. Later on, growth factors, such as members of the TGF-ß superfamily, are necessary for their maintenance and survival. TGF-βs are expressed at early stages of neural differentiation in close proximity to developing DA neurons in the ventral midbrain. This raises the question whether these factors can also influence the specification of DA phenotype. As a model we used a very low density culture system. Low density cultures from the ventral mesencephalon were treated with various members of TGF-ß family for 24 h and thereafter double-stained for tyrosine-hydroxylase (TH) and different transcription factors. We show that TGF-β2 and 3 increase numbers of

TH-positive neurons, whereas BMP-4 completely abolishes TH-immunoreactivity. Antibodies that block TGF-β signaling were used to show that TGF-β activity is required for the induction of TH. The alterations in the number of TH-positive neurons can not be explained by changes in cell proliferation rate since we did not find any significant differences in BrdU labeling following growth factor treatments. On the other hand, both TGF-β and BMP-4 enhanced the number of nuclei positive for En-1, a transcription factor regulating differentiation of specific neuronal phenotypes both in the ventral and dorsal neuroepithelium. However, only TGF-β was able to induce ventral markers such as FP3 and HNF3β. Furthermore, TGF-β diminished the appearance of MSX1/2, a dorsally expressed transcription factor and important downstream mediator of BMP-4 signaling. In good correlation, BMP-4 induced a robust (5 fold) increase in the number of MSX1/2-positive nuclei.

Our results provide strong evidence that TGF-βs have an important impact as morphogen factors determining the TH-positive neuronal fate in the developing midbrain.

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TOPOGRAPHIC RELATION OF THE DEVELOPING HUMAN PINEAL ORGAN AND THE BRAIN SINUSES

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The pineal organ releases - among other hormone-like compounds - melatonin and serotonin. In some mammals the pineal is attached to the confluence of sinuses, while in others the hormonal release structures of the organ face the internal cerebral veins and the vein of Galen. As the blood of these veins is collected by the sinus rectus, the meningeal sinuses seem to have a general role in mediating the hormonal efferentation of the organ. In addition, the wall of the vein of Galen was found to be the site where the transitory physiological invasion of lymphocytes reaches the pineal tissue in young animals.

The production of melatonin in the pineal organ is inhibited by environmental light. The daily fluctuation of melatonin is supposed to entrain genetically fixed circadian rhythmicity of the organism. It is generally accepted that light information reaches the mammalian and human pineal from the retina via its peripheral adrenergic innervation by a hypothetic system of pathways. Some authors have found a central fiber connection of the organ from the retina-related brainstem nuclei via the pineal stalk.

In contrast, submammalian pinealocytes are photoreceptors being directly sensitive to light of various wavelengths. Mammalian pinealocytes bear photoreceptor outer segment-like cilia in some species only e.g. in the bat or in the ferret. Immunoreactive visual pigments were demonstrated in some pinealocytes of the gerbil and ferret. On the basis of morphological, biochemical and electrophysiological data some authors do not deny the direct light sensitivity of mammalian pineal.

Concerning a supposed direct light sensitivity of the human pinealocytes, the main problem is how the light may reach the pineal organ through the skull. In several vertebrates examined, the skull is transparent for short wavelengths of light. In our earlier studies, the human skull was found to be transparent at the sulci of the sinuses, at the holes of granulations of Paccioni and at lateral quamal parts of bones. Therefore, the venous sinuses may have a role not only in the hormonal release and lymphocytic invasion but also in the supposed direct light sensitivity of the organ.

Studying the relation of the human pineal organ to the cerebral veins and meningeal sinuses in the present work, we investigated human skulls of different developmental stages. We have found that from the early fetal life on there is a close relation between the pineal organ, internal cerebral veins, vein of Galen and sinus rectus. Examining the system of sinuses by artificial illumination of the skull, we cannot exclude the possibility that sinuses – like microilluminators – convey some light to the human pineal organ hidden below the caudal part of the corpus callosum in the adult brain.

NPY AND ITS ANTAGONISTS IN REGULATION OF FOOD INTAKE IN RATS OF DIFFERENT THERMAL ADAPTATION

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Neuropeptide Y (NPY) elicits food intake (FI) and suppresses metabolic rate (MR) and body temperature (T_c). Fasting-induced low MR and T_c may indicate high endogenous NPY activity. Cold-adapted (CA) rats, as compared with non-adapted (NA) ones, are hyperphagic, and their FI response to NPY or fasting is enhanced. In this hyperphagia, a role for NPY has been assumed. However, neither MR nor T_c is low during stay in the cold without fasting.

In the present studies CA and NA rats were either given NPY into their lateral cerebral ventricle (ICV) through a pre-implanted cannula, or they had a period of fasting. To modify the FI response to these manipulations, NPY antagonists were applied: D-Tyr^{27,36},D-Thr^{32}-NPY(27-36) (peptide-antagonist, H-3328), or α-1,2,6-trinositol (functional antagonist, α-T) was given ICV. The aim was to clarify whether endogenous NPY contributes to the hyperphagia of CA animals, or to the regulatory differences of CA vs. NA rats.

H-3328 effectively antagonized FI to injections of NPY. In presence of H-3328 the FI response to short-term fasting also decreased. In NA rats this was significant; with more severe fasting, and in CA rats, the H-3328-antagonism on FI was moderate. Basically similar, but more pronounced effects were seen with the application of α-T.

It is concluded that the effects of exogenous/endogenous NPY are stronger and less easily antagonized in CA than in NA rats. Although, on this basis, enhanced responsiveness to endogenous NPY may contribute to the enhancement of FI in CA rats, it is likely that other factors also participate in the development of hyperphagia.

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NEUROMEDIN C MICROINJECTION INTO THE AMYGDALA INHIBITS FEEDING

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Experimental data show that endogenous bombesin (BN) like peptides play physiological role in the control of food intake. Twenty-seven amino acid long-gastrin releasing peptide [GRP1-27, (GRP)], the GRP18-27 [neuromedin C (NMC)], and neuromedin B (NMB) belong to this peptide family. Accordingly, there are two distinct receptors for GRP/NMC family and for the NMB family. After i.c.v. or intracerebral hypothalamic microinjection, and in our recent experiments after intraamygdalar application of GRP caused significant decrease in food intake. Nevertheless, the possible effects of NMC in the amygdala were not studied yet. It is known, however, that high dose i.c.v. NMC injection caused alteration in behavioral activity. According to immunohistochemical and autoradiographic data the amygdala contains GRP/NMC receptors in high density. In the amygdala HPLC studies showed that interstitial levels of NMC/GRP markedly increased during food ingestion and during the postprandial period. In the present experiments NMC (N-6388, Sigma) was bilaterally microinjected into the central part of the amygdala (ACE) in male CFY rats. Liquid food (milk, 136.45 kj/100 ml) intake was measured every five min for 30 min, and at the 40th and 60th min following either NMC, or vehicle micronjection. NMC was injected bilaterally in three different doses (120, 60, 30 ng dissolved in 0.5 µl 0.15 M sterile NaCl, respectively). In the ACE direct application of NMC resulted in transient inhibition of food intake. When administered in low dose (30 ng) it significantly suppressed food consumption for 5 min, however, food intake transiently increased in the fifth 5 min observation period. High doses (120 or 60 ng) were not effective on consumption. The effects on food intake were prevented by prior application of a BN antagonist (B-1025, Sigma). After NMC microinjecions animals were videomonitored in their homecage and the time spent with feeding, resting, exploration, grooming, and scratching has been analised in 5 min intervals for 30 min. Data showed that the inhibitory effect of NMC on feeding could not be explained by changes in stereotype activity or by alteration of general behavior. The results of the present experiments indicate that NMC may act as a specific satiety signal in the ACE.

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EFFECT OF NEONATAL DEXAMETHASONE TREATMENT ON HIGH AFFINITY NEUROTENSIN RECEPTOR BINDING AND MRNA EXPRESSION IN THE RAT BRAIN DURING DEVELOPMENT AND AGING

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Neurotensin (NT) is a neurotransmitter in the brain which plays important role in dopamine-related behaviors. Evidence supports that glucocorticoids can influence the central actions of NT. At a critical period of brain development, on postnatal days 1, 3 and 5 rats were treated with a synthetic glucocorticoid, dexamethasone (DEX). NT receptor density and NT receptor mRNA expression were visualized at postnatal day 10 and 21, at adult age (4 months) and at old age (25 months) using *in vitro* autoradiography and *in situ* hybridization techniques. DEX induced transient increase of both receptor density and mRNA expression in several brain areas at young ages, while attenuated the physiological decline of NT receptors in the limbic cortex and amygdala of aged animals. In the nigrostriatal pathway of adult rats DEX reduced the NT receptor expression and density. The facilitated development of NT receptors in young animals may be related to the increased neurogenesis by DEX. The region-specific decrement in NT receptor content of adults as well as the specific changes of NT receptor content in aged rats are likely a result of a long-term neuroendocrine effect of DEX via hypothalamo-pituitary-adrenal axis. The permanent changes in NT receptor density following neonatal DEX treatment can elicit functional changes in dopamine-related behaviors of adult and aged animals.

PATCH CLAMP ANALYSIS OF THE EFFECT OF SUBUNIT SELECTIVE NMDA ANTAGONISTS ON RECOMBINANT RECEPTORS

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NMDA receptors are glutamate activated ion channels, widespread in the central nervous system. The endogenous glutamate or the specific agonist N-methyl-D-aspartate evokes an influx of calcium and sodium ions into neurons. The channel is composed of four subunits. There are two types (NR1 and NR2) of subunits and the latter has four subtypes (2A, 2B, 2C, 2D). Those hetero-oligomers that contain the NR2B are involved in pain transmission. The inhibition of these receptors is thought to be suitable for the treatment of neurogenic pain, while inhibition of other receptor subtypes may cause various side effects. Our aim is to develop subtype-specific inhibitors. One of the methods we applied for studying selectivity is whole cell patch clamping. HEK293 cells were transiently transfected with rat NMDA

subunit genes, the NR1 gene, one of the NR2 genes and the gene of the Green Fluorescent Protein (GFP). GFP was used for visualizing the successfully transfected cells. We measured the peak amplitude of 100 μM NMDA (EC_{80}) evoked inward current. The non-selective competitive NMDA antagonist APV reduced the peak current in all receptor subtypes studied. Two subtype selective compounds, erythro-ifenprodil and CP-101,606 were investigated. They blocked the current at low micromolar concentrations in NR1/2B transfected cells, while there was no significant blocking effect in the other subtypes (NR1/2A, NR1/2C, NR1/2D) up to 30 μM. In conclusion, application of patch clamping on recombinant receptors proved to be a suitable experimental system for studying NMDA receptor subtype selectivity.

MARKERS OF CHRONIC ACTIVATION IN THE STRESS-RELATED NEURONAL CIRCUITRY

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c-fos is a widely used functional anatomical marker to identify neurons acutely activated by various extracellular stimuli. The major disadvantage of c-fos based activity mapping is the transient induction of this immediate early gene (IEG), that do not label neurons activated by chronic challenges. In the stress-related circuitry c-fos is significantly induced by different acute stressors, but neither c-fos mRNA or c-Fos protein could be detected after adrenalectomy (ADX), which induces synthetic and secretory activity of the hypothalamo-pituitary-adrenocortical (HPA) axis. Fos-related antigens (FRAs) are a stable splice variants of FosB that accumulate in brain after chronic treatment because of their stability and long half-life. To test the hypothesis if FRAs are induced in response to ADX, we followed the time course of Fra-2 immunoreactivity (-ir) in the hypothalamus. Rats were perfused at intervals ranging from 12, 24, 48, 72 h to 7 days following ADX and brain sections were processed for Fra-2-ir using an antibody that does not cross-react with cFos, Fra-1, and FosB. Sham-operated animals did not display any Fra-2 in the paraventricular nucleus (PVN) of the hypothalamus. Numerous Fra-2 positive cell nuclei were found in the parvocellular PVN of ADX rats all times after surgery. To characterize interaction of FRAs with consensus DNA sequences that confer AP-1 responsiveness, gel mobility shift assays were performed using hypothalamic extracts of rats that were intact, ADX or exposed to stress. Nuclear extracts displayed significant binding affinity to AP-1 consensus sequences, which was further increased following ADX. Supershift analysis using anti-Fra, anti-Fos antibodies revealed that AP-1 complex contained FRA and cFos proteins under basal, acute and chronic conditions. These data provide evidences that Fra-2 is induced by adrenalectomy in the stress-related parvocellular neurosecretory neurons and might be used as a cellular marker of chronic cellular activation in the stress-related circuitry.

AB1-42 PEPTIDE INHIBITS POTASSIUM-EVOKED GABA RELEASE FROM RAT BASAL FOREBRAIN SLICES

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Some structural and biochemical observations suggest that GABAergic neurons, which play important roles in inhibitory circuits in the brain, are affected in Alzheimer's disease (AD). The basal forebrain (BF) is one of the functionally most important ascending subcortical projections and has also been critically implicated in AD. The BF comprises ACh-containing neurons which are the primary sources of eholinergic innervation to the limbic system and neocortex. Anatomical and physiological evidence supports that these cholinergic neurons in the BE are transynaptically regulated by GABAergic inputs. The sources of this input include local interneurons, axon collaterals of GABAergic projection neurons and neurons from the n. accumbens. Other lines of evidence indicate that amyloid-β peptide (Aβ) deposits, which are invariant features associated with AD, are initiating and/or contributory factors in the pathogenesis of the disease. The present study revealed that Aβ peptide can potently inhibit GABA release, probably by acting on the GABAergic terminals in the rat BF. BF slices were superfused in 50 mM potassium-containing Krebs-Ringer buffer in either the presence or the absence of 10 μM Aβ1-42. Al] alone did not evoke direct GABA release. The peptide was freshly dissolved or aggregated for 1, 4 or 7 days. The degree of inhibition was increased by aging the peptide. Similar results were found by others in connection with ACh release from cholinergic terminals in rat brain slices. Our observations demonstrate that Aβ peptide can modulate the transmitter release from both GABAergic neurons and cholinergic neurons. This effect is therefore not specific for cholinergic neurons (whose preferential sensitivity in AD is well established), as was presumed previously. Supported by OTKA (T022683) and ETT (584/1996).

INVOLVEMENT OF $GABA_A$ RECEPTOR-MEDIATED NEUROTRANSMISSION IN THE AMINOPYRIDINE-INDUCED ICTAL EPILEPTIFORM ACTIVITY

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The aim of this study was to investigate whether disinhibition plays a causative role in the generation of 4-Ap-induced ictal activity. To examine the involvement of $GABA_A$ receptor-mediated neurotransmission we applied $GABA_A$ receptor antagonist, bicuculline to the surface of the somatomotor cortex at the site of the primary focus (Pf) of adult anaesthetised rats in *in vivo* experiments. In one group of animals, bicuculline was applicated 30 minutes prior-, in other group, bicuculline treatment started 60 min. after induction of periodic,

rhythmic ictal activity. The surface electrocorticogramm (ECoG) was recorded at the site of 4-Ap application and of the mirror focus as well as at two distant cortical points.

Pretreatment of the cortex by bicuculline before local application of 4-Ap by itself did not induce ictal seizure activity, although, in a few cases, synchronous interictal-like, sharp spikes were observed in the ECoG. On the other hand, the induction of epileptiform discharges seemed to be facilitated, although a specific type of seizures were induced by 4-Ap, when the endogenous GABA transmission in this region was previously compromised by focal application of bicuculline. Paroxysmal discharges of lower frequencies (4-9 Hz) appeared to be dominant, while those of higher frequency (10-15 Hz), seemed to be strongly suppressed in their spatio-temporal expression. In most cases they failed to develop into full blown ictal periods, or to propagate to the contralateral hemisphere. Application of bicuculline to the already active Pf seemed to facilitate synchronisation of neuronal activity, resulting in significant increases in the amplitudes of the ictal discharges. During observation for one hour, there was a shift in the ratio of discharges patterns in favour of 4-9 Hz, as well as a remarkable increase in the duration of the individual ictal periods. In the present of bicuculline the propagation of epileptiform discharges to the overall cortical surface was highly facilitated.

In conclusion, our data suggest that, $GABA_A$ receptor-mediated function is differentially involved in the induction, maintenance and propagation of 4-Ap-induced ictal discharges of different frequencies; while it seems to be necessary to the generation and spread of rhythmic discharges of high frequency, its luck of activity may facilitate the expression of discharges of lower frequencies. In the presence of bicuculline, the frequency of synchronised epileptiform discharges in the contralateral hemisphere, is depends not only on the primary epileptiform events, but also on the successful propagation through the neural circuit.

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EFFECTS OF D3-LIGANDS IN THE PLACE CONDITIONING MODEL

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Recent theories of addiction emphasize the relation between the positive reinforcing properties of drugs and sensitization of brain dopamine systems. Pharmacological, human postmortem and genetic studies point out the important role of D_3 receptors in drug addiction. Caine and collegues (NeuroReport, 8, 2373-2377 (1997)) observed that the relative potencies of dopamine agonist to decrease cocaine self-administration were highly correlated with their functional potencies to stimulate mitogenesis in cells expressing D_3 but not D_2 receptors. Until recently, however, in consequence of lack of selective and high-affinity ligands there has been difficult to see clearly the role of the D_3 receptors in drug abuse.

Our aim was to study the effect of D_3-ligands in a widely used animal model of drug-dependence, the place conditioning paradigm and their ability to inhibit cocaine place preference.

Conditioned place preference/aversion (CPP/CPA) testing is a behavioural method considered to be capable of measuring the affective (positive, neutral or negative) properties of psychoactive drugs. Cocaine injection in rats reliably produces a positive place preference. Drugs that attenuate or block this effect of cocaine have obvious potential for developing treatments to address cocaine addiction.

In our experiments we used a rectangular plexiglas chamber that had three compartments separated by removable partitions as a testing apparatus (place conditioning box, PCB)

The procedure required 4 phases: *handling* (two days), *preconditioning* (15 min free moving in the three compartments of the PCB for three days), *conditioning* (animals received drug injection than they were placed in the drug-paired, white compartments for 30 min; four hours later they got saline injection and were placed in the saline-paired, black compartment for 30 min. This pairing one compartment with drug and the other with saline was repeated for four days.), and the *postconditioning test* (15 min free access in the three compartments of the PCB without any injection on the last day). The time spent by rats on the saline-conditioned and the drug-conditioned side was measured on the three days of preconditioning phase and in the postconditioning test.

The D_3 agonist 7-OHDPAT produced either conditioned place aversion (1 mg/kg sc.) or had no effect (0.5 mg/kg sc.). The more selective agonist PD-128907 (1 mg/kg sc.) induced conditioned place aversion. The D_3 partial agonist BP-897 (1 mg/kg ip.) was not effective. Ten mg/kg ip. cocaine produced conditioned place preference. 7-OHDPAT (0.5 mg/kg sc.) administered 30 min prior to the daily cocaine injection was not able to inhibit cocaine induced place preference. Pretreatment with BP-897 (1 mg/kg ip.) prevented place preference, moreover the animals displayed a mild, but nonsignificant place aversion.

Our results suggest that partial agonist but not full-agonist may attenuate the addictive potential of cocaine, therefore they would be a hopeful starting-point in the pharmacological development to cure cocaine-addiction.

EFFECTS OF HISTAMINE AND H1 ANTAGONIST PYRILAMINE ON THE DOPAMINE TRANSMISSION IN THE NUCLEUS ACCUMBENS: *IN VIVO* STUDY IN ANAESTHETISED RATS

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Several data shows the existence of the close relationship between the activity of the mesolimbic dopaminergic system (MLDS) in the nucleus accumbens (NAC) and reward related processes. Data of neuropharmacological and behavioral studies suggests the

inhibitory role of neuronal histamine (HA) originating from the tuberomammillary nucleus in the control of reinforcement. Futhermore, inhibition of H1 receptors produced potentiated effects on drug induced reward (e.g. psychostimulants and opioids) and on self-stimulation. Modulation of dopamine transmission in the NAC is a crucial event in reward modulating effects of H1 antagonist, however, the underlying mechanisms on dopaminergic activity in the NAC are not understood. Therefore, in this study dopamine (DA), DOPAC and HVA levels of NAC were detected by ED-HPLC methods. We examined the effect of HA (in doses of 0.1 pmol/µl, 1.0 pmol/µl, 10.0 pmol/µl and 1 nmol/µl) used unilaterally by reverse dialysis. In a second experiment, H1 antagonist pyrilamine (PYR 10 mg/kg, 20 mg/kg i.p.) was injected before the HA (1 nmol/µl) administration. To check the influence on the level of DA and its metabolits, PYR (0.1 pmol/µl, 1.0 pmol/µl, 10.0 pmol/µl) was dialysed unilateral into the NAC. Present data showed a definite increase in the extracellular DA level in every dose. The peak of elevated DA levels was found to be delayed after the HA administration. Biphasic changes (decrease and increase) were observed in DOPAC and HVA levels. The HA induced DA release was fully antagonised by higher PYR doses. In addition application of PYR led to mild enhance in DOPAC and HVA levels. Intra-accumbens administration PYR resulted in a decrease in DA, and increase in DOPAC and HVA levels. The present data are the first to show in *in vivo* experiments, that HA enhances the DA transmission in the NAC. Furthermore, the reward potentiated effect of H1 antagonist does not depend on the local effect on DA transmission in the NAC, which was hypothetised in early studies.

ANALYSIS OF N SEIZURE ACTIVITY EVOKED BY BICUCULLINE OR 4-AMINOPYRIDINE IN BRAIN SLICES PREPARED FROM YOUNG AND ADULT RATS

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In the present study the seizures activity developed after bicuculline (BIC) or 4-aminopyridine (4-AP) application was investigated in slices prepared from two-week-old and adult rats. The extracellular recording electrodes were positioned systematically from the layer II to the layer VI in four different places in each slices and the characteristics of the discharges were compared.

Bath application of the two convulsants evoked spontaneous seizure activity which were different in their mean frequency, duration and amplitude. Usually two-phase discharges were detected, a quick short component was followed by a long-lasting negative wave. The direction of the waves changed usually in the layer II in the case of BIC and deeper in 4-AP. However, the seizure were less complex in BIC as in 4-AP. The inhibitory effect of AMPA antagonist GYKI 52466 was stronger on the BIC activated seizures, while the NMDA antagonist APV was more effective on the 4-AP evoked seizures. In young animals compared to the adults seizure discharges were more frequent but similiar in shape in the case of BIC in contrast of 4-AP, where more complex waves were observed with a lower

frequency. The effect of the glutamate antagonists were slightly stronger in BIC but controversially much weaker (NMDA-type) in 4-AP treated young rats.

ALTERED RESPONSE TO PRESYNAPTICALLY ACTING STIMULATORS IN MDMA INDUCED NEUROTOXICITY IN RATS

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MDMA (methylendioxymethamphetamine) or "Ecstasy" is a mood-altering drug used illicitly for recreational purposes. Repeated administration of MDMA causes neurotoxicity in serotonergic (5-HT) system of the brain resulting in reduced concentration of 5-HT and 5-HIIA and activity of the synthetising enzyme tryptophan-hydroxylase.

Previously we proved that presumably in consequence of above-mentioned biochemical changes the spontaneous motility was significantly lower in MDMA pretreated animals measured on the 3rd postinjection day, however, there was no difference on the 13th day. In the present experiments we examined the effect of parachloroamphetamine (PCA) and the MDMA challenge as presynaptically acting drugs in the animals pretreated with neurotoxic dose of MDMA (4 × 10 mg/kg sc. in every 2nd hours). The intensity of 5-HT syndrome induced by PCA (2 mg/kg ip.) was significantly decreased in MDMA pretreated animals on the 3rd , 14th and 30th postinjection days, while PCA-induced hypermotility was significantly lower only and on the 3rd postinjection day. 5-HT syndrome induced by MDMA challenge (10 mg/kg ip.) proved to be significantly lower on the 3rd and 14th postinjection days, too, however, there was no difference on the 30th day.

On the basis of above data we may conclude that 5-HT syndrome, which is evoked by 5-HT release seems to be suitable for detecting the impairment of presynaptic function following neurotoxic MDMA treatment.

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BEHAVIOURAL EFFECTS OF RGH-1756, A D_3 DOPAMINE RECEPTOR LIGAND

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The D_3 dopamine receptor shows a peculiar distribution in the mammalian brain. The regions with the highest density of the receptor are the major island of Calleja, a small nucleus which supposedly takes part in the regulation of body temperature, and the nucleus accumbens, which has long been known as the centre of the "reward system" of the brain.

Opposite to the function of the D_2 receptors, the accumbal D_3 sites mediate hypolocomotion and induction of climbing. Compounds with D_3 agonist activity, like 7-OHDPAT or PD-128907 also induce hypothermia and yawning at doses at which they do not occupy the D_2 receptors.

RGH-1756 (1-(2-methoxy-phenyl)-4-(4-[4-(6-imidazo [2.1-b]thiazolyl)-phenoxy]-butyl)-piperazine) showed high affinity to human cloned D_3 receptors (K_i=0.12 nmol/l) exhibiting 100-fold selectivity over the D_2 subtype. The aim of our study was to characterize the *in vivo* D_3 activity of RGH-1756.

In contrast to the D_3 agonist 7-OH-DPAT, which caused dose-dependent hypomotility (ED_{50}=0.055 mg/kg sc.) in mice, RGH-1756 showed only slight effect on motor activity administered either orally or subcutaneously. In a study addressing the possible *in vivo* D_3 antagonist action of RGH-1756 the compound (0.1-3 mg/kg sc.) did not prevented 7-OH-DPAT (0.1 mg/kg sc.) induced hypolocomotion, while the D_3 antagonist U-99194A, used as a reference drug, dose-dependently antagonized it.

RGH-1756 (1, 3, 6 mg/kg sc.) induced significant, dose-dependent decrease in the rectal temperature of mice, and at the dose of 3 mg/kg sc. it prolonged the hypothermia induced by 7-OH-DPAT (0.2 mg/kg sc.). U-99194A significantly and dose-dependently inhibited the hypothermia induced by either RGH-1756 (3 mg/kg sc.) or by 7-OHDPAT.

However, RGH-1756 showed no influence on normal body temperature of rats in a broad dose range (0.3-24 mg/kg sc.). The compound at sc. doses of 0.3, 1, and 3 mg/kg significantly but only partially (cca. 45 % at all doses tested) inhibited the hypothermia induced by 1 mg/kg sc. PD-128907 in rats, while U-99194A showed significant and dose-dependent protective effect against the PD substance.

RGH-1756 only partially (cca. 50 %) and nondose-dependently inhibited the 0.05 mg/kg sc. 7-OH-DPAT-induced yawning behaviour either after oral or subcutaneous administration (0.03-3.0 mg/kg) in rats. U-99194A dose-dependently inhibited 7-OH-DPAT-induced yawning (ED_{50}=0.66 mg/kg sc.). RGH-1756 itself caused a weak increase in yawning responses at 1 mg/kg sc. dose which could be inhibited - although nonsignificantly - by the D_3 antagonist U-99194A (3 mg/kg sc.).

Provided that the effects 7-OH-DPAT and U-99194A on motility and temprature are mediated via D_3 receptors we can conclude that RGH-1756 did not show D_3 antagonist like effect in any of the models applied. On the basis of the results from the temperature but not from the motility experiments carried out in mice the action of the compound can be characterized as agonist like. The partial and nondose-dependent inhibitory effect of RGH-1756 against D_3 agonist-induced hypothermia and yawning in rats suggests that it may act as a partial D_3 agonist. Obviously, further studies are needed in order to precisely clarify the *in vivo* nature of the compound.

EFFECT OF PCB 153 IN RAT HIPPOCAMPUS

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We have examined the effects of gestational and lactational exposure to 2,4,5,2',4',5'-hexachlorobiphenyl (PCB 153) on the magnitude of long-term potentiation (LTP) observed in the CA1 region of hippocampal brain slices prepared from rats at 30 days of age, and have compared these actions to those observed when PCB 153 is dissolved in normal Krebs-Ringer solution and perfused on slices from control rats of the same age. *In vivo* exposure was at three dose levels from gestational day 3 through weaning at postnatal day 21. While responses to low-frequency stimulation of the Schaffer collateral pathway in exposed animals were not different from controls, there was a significantly reduced LTP induced after tetanic stimulation, even at the lowest dose studied. A comparable depression of LTP was observed when control slices were perfused with Krebs-Ringer that had been equilibrated with PCB 153 in a generator column. Neither *in vivo* nor *in vitro* exposure significantly altered the input-output curves obtained prior to tetanic stimulation, but both suppressed the increase in response observed in controls following tetanic stimulation.

Our observations indicate that low concentration of PCB 153 results in a reduction of LTP without major alteration of general synaptic transmission. This may be at least a part of the basis of the effects of PCBs on IQ.

PRESYNAPTIC CANNABINOID CB1 RECEPTORS MODULATE HIPPOCAMPAL GABAergic TRANSMISSION

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Immunostaining with an antibody developed against the C-terminal portion of cannabinoid CB1 receptor gave a similar expression pattern in the hippocampus as previously reported (Katona et al., 1999) but with the greater sensitivity. Besides the somatic staining of cholecystokinin-containing interneurons, electron microscopy confirmed that approximately half of the boutons giving symmetrical (GABAergic) synapses were strongly immunoreactive for CB1 receptor, but those establishing asymmetrical (glutamatergic) synapses were always negative.

Whole-cell patch-clamp technique has been used to investigate the effect of presynaptic CB1 receptor activation on hippocampal inhibition. Inhibitory postsynaptic currents (IPSCs)

were recorded from hippocampal principal cells in the presence of 3 mM kynurenic acid, an ionotropic glutamate receptor blocker. Bath application of selective CB1 receptor agonists (WIN 55,212-2 or CP 55,940) suppressed IPSCs evoked by electrical stimulation in the perisomatic region of principal cells. This effect could be prevented or reversed by the CB1 receptor antagonist, SR 141716A. We also examined the effect of CB1 receptor agonists on the action potential independent IPSCs (mIPSCs). Both agonists increased the frequency, but not the amplitude of mIPSCs. This increment was absent when Cd^{2+} was co-applied with CP 55,940 or when the recordings were done in the Ca^{2+} free extracellular buffer with 1 mM EGTA. In addition, we mimicked the firing of basket cells during the theta rhythm of hippocampal EEG when these neurons rhythmically fire a burst of, on average, 4 action potentials at ~ 20 Hz (intraburst frequency). In the control period, the first evoked IPSC was small followed by 2 or 3 times larger ones. Application of CP 55,940 increased the amplitude of the first IPSC but strongly reduced the others. Similar changes were also observed by applying of Thapsigargin, which increases the Ca^{2+} level in the boutons. Thus, activation of presynaptic CB1 receptor can modulate the action potential driven IPSCs most likely by increasing the Ca^{2+} concentration in the terminals, which can alter the IPSC amplitude and pattern during theta rhythm.

SEROTONERGIC INNERVATION OF CHEMICALLY CHARACTERIZED NEURONS IN THE BASAL FOREBRAIN

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The basal forebrain (BF) territories are populated by cholinergic, GABAergic and peptidergic neurons. The BF cholinergic system is implicated in cortical arousal, attention, sensory processing, motivation, learning and memory as well as in several neuropsychiatric disorders. Several lines of evidence indicate that serotonergic raphe neurons of the brainstem modulate cortical activity both through their axon terminals in the cerebral cortex as well as via the BF (e.g. Záborszky and Luine, 1987) where serotonin may act directly on cholinergic cells or indirectly via GABAergic or peptidergic interneurons. Recently, ultrastructural evidence has been found that median raphe serotonergic neurons innervate parvalbumin-containing cells of the septal complex (Léránth and Vertes, 1999).

In the present study, immunocytochemical double labeling was conducted to find out whether serotonergic axons may also innervate cholin-acetyltransferase- (ChAT), calbindin- (CB), calretinin- (CR) or somatostatin- (SOM) containing neurons in BF areas. Our results demonstrate that scattered serotonergic fibers are distributed throughout the BF. Serotonin-immunoreactive axons form a dense network in the substantia innominata (SI) and these fibers establish symmetric synaptic contacts. Axo-somatic and axo-dendritic close contacts suggestive of synaptic input have been revealed between serotonergic fibers and ChAT-,

CB-, CR- and SOM-immunoreactive structures of the SI and ventral pallidum. These results suggest that serotonin may regulate cortical activity by controlling cholinergic functions at the level of basal forebrain.

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DETERMINATION OF THE FUNCTION OF DIFFERENT CA^{2+} REMOVAL MECHANISMS ON FRESHLY ISOLATED DCN PYRAMIDAL NEURONES

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There are numerous bodies of evidence that in neurones the Ca^{2+} pump located on both the surface membrane and on the endoplasmic reticulum, as well as the Na^+/Ca^{2+} exchange of the surface membrane play significant roles in the elimination of the free cytoplasmic Ca^{2+}. The aim of the present study was to assess the significance of these Ca^{2+} removal mechanisms in the Ca^{2+} handling of freshly isolated pyramidal cells of the dorsal cochlear nucleus of rats following depolarizations evoked by the elevations of extracellular $[K^+]$. The pyramidal cells were isolated by a combined collagenase/pronase treatment and a subsequent mechanical trituration. The cells were loaded with the esther form of the fluorescent Ca^{2+} indicator dye fura-2 (3 μM).

The decay of the Ca^{2+} transients was fitted to the sum of two exponential functions, yielding a faster (3.1±0.2 s; n=81) and a slower (21.3±1.2 s; n=71) time constant. When trying to evaluate the relationship of these rate constants to the intracellular Ca^{2+}, only the faster component showed moderate Ca^{2+}-dependence. To demonstrate the significance of the Ca^{2+} pump in the surface membrane, $LaCl_3$ (3 mM) was applied following the application of K^+. In the presence of La^{3+} the rate of the Ca^{2+} elimination was significantly decreased. However, the removal of the extracellular Na^+ had little effect on the decay of the Ca^{2+} transients, indicating a somewhat less significant role of the Na^+/Ca^{2+} exchange in the Ca^{2+} extrusion. To prevent the Ca^{2+} uptake of the intracellular Ca^{2+} stores thapsigargin (50 nM), the selective blocker of the Ca^{2+} pump in the endoplasmic reticulum was applied. In these experiments not only the rate of the decay but the amplitude of the Ca^{2+} transients was also decreased.

We conclude, therefore, that all principal Ca^{2+} extrusion mechanisms are involved in the removal of the depolarization-activated Ca^{2+} transients in isolated pyramidal neurones of the dorsal cochlear nucleus.

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MECHANISM OF THE INHIBITORY EFFECT INDUCED BY TT-232 AND ANANDAMIDE ON SENSORY NEUROPEPTIDE RELEASE

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Chemical or electrical stimulation of the capsaicin-sensitive afferent nerve terminals results not only in pain sensation but a release of sensory neuropeptides. Calcitonin gene-related peptide (CGRP) is responsible for vasodilatation and substance P (SP) for plasma protein extravasation in the innervated areas, furthermore somatostatin (SOM) exerts systemic anti-inflammatory effect. TT-232 is a stable and selective heptapeptide SOM analog with strong tyrosine kinase inhibitory action, which has been proved to have anti-inflammatory and anti-nociceptive activity in rats *in vivo*. Anandamide is an analgetic CB_1 cannabinoid receptor agonist and since it is structurally related to capsaicin, it may have interaction with vanilloid receptors, too. The aim of the present study was to investigate the effect of TT-232 and anandamide on SP, CGRP and SOM release measured with RIA from isolated rat tracheae and to examine their mechanism of action using the G-protein blocker pertussis toxin (PTX) and the tyrosine kinase inhibitor genistein. TT-232 (200 and 500 nM) dose-dependently inhibited the release of the peptides induced both by capsaicin (10^{-7} M) and electrical stimulation (10 Hz, 120 s) and this inhibition was prevented by 100 ng/ml PTX. Genistein (50 μM) and PTX by itself did not influence electrically-induced peptide release. Anandamide (10^{-5} M) decreased the basal release and this action was also abolished by PTX. Therefore, the effect of both agents is mediated via G-protein coupled receptors. Since TT-232 is free of endocrine actions, participation of SOM 1 and/or 4 receptor subtype in peptide release inhibition is suggested. As genistein was ineffective, the tyrosine kinase inhibitory mechanism is unlikely in these studies. Anandamide in the present case is supposed to act on CB1 receptors and not directly on ion channels.

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CHANGES OF BIOELECTRIC PROPERTIES DURING *IN VITRO* NEUROGENESIS

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Neuroectodermal stem cells in the primary ventricular zone (VZ) give rise to neurons and macroglial cells of the developing CNS. The regulation of the cell production by primary progenitor cells is far from clear. Recent studies (1) indicated that, depolarising agents (KCl, GABA, glutamate) can decrease the proliferation in the VZ: the cell-production might be controlled by depolarising compounds released by neurones already functioning close to the VZ.

The effects of depolarisation on the neurogenesis have been investigated *in vitro*, using a neuroectodermal progenitor cell line, NE-4C (2) derived from the VZ of embryonic (9t}l day) mouse forebrain. Non-induced, epithel-like NE-4C cells divide continuously, but acquire neuronal features within 7 days, if induced by retinoic acid (RA) (3). During the first 2 days of induction, the cells sustain their proliferation-activity. The majority of the cells exit the cell cycle by the 5th day of induction.

The membrane potential of non-induced cells are between –20 and –40 mV. In the first day of induction, continuously proliferating cells adopt more negative (–60 and –100 my) resting potential. The intracellular Ca-level cannot be increased by 30 mM KCl. At this time, the majority of the cells are coupled by gap junctions. From the 4th day of induction, primitive form of voltage-dependent Na^+-currents and KCl-induced Ca-responses can be elicited in a few cells. Neuron-specific spike potentials and intracellular Ca-responses are regularly evoked by depolarisation from the 7th day on.

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INVERTEBRATE NEUROPEPTIDES IN THE CENTRAL NERVOUS SYSTEM OF *PORCELLIO SCABER*: CRUSTACEAN CARDIOACTIVE PEPTIDE

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Several (112) crustacean cardioactive peptide immunoreactive CCAP-IR somata, characterised by various soma size and shape, were found in the central nervous system (CNS) of *Porcellio scaber* (Crustacea, Isopoda). A few small cells occurred in the proto- and tritocerebrum, and in the subesophageal ganglion while the deuterocerebrum did not contain any IR structures. In the thoracic ganglia and the terminal ganglion an unequal distribution of labelled cells and fibres was observed. Thoracic ganglia from the first to the fifth one have only four or six stained perikarya, respectively. Except of the first thoracic ganglion no fibre-like structures could be followed neither in these ganglia nor in their segmental nerves, however, each of their connectives contained a CCAP-IR fibre. In the sixth and seventh thoracic ganglia strongly stained neurons with contralateral projection occurred. The largest proportion of immunoreactive structures, both perikarya and nerve fibres was observed in the fused mass of the 8th thoracic and terminal ganglion, further stained axons were also seen in the 3rd-6th nerves of the terminal ganglion. These findings suggest that most of the labelled cells could be interneurons and only a small part of them has peripheral connections supposedly regulating the contractions of visceral muscles.

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INVERTEBRATE NEUROPEPTIDES IN THE CENTRAL NERVOUS SYSTEM OF *PORCELLIO SCABER*: STRUCTURES WITH ALLATOSTATIN-LIKE IMMUNOREACTIVITY

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The distribution pattern of Dip-Allatostatin-like immunreactive structures was investigated in the central nervous system (CNS) of the *Porcellio scaber* (Crustacea, Isopoda). Immunoreactive structures, both perikarya and nerve fibres, occurred in all ganglia. Most of the labelled neurons were situated in the protocerebrum. In the 3rd, 4th, 5th and terminal ganglion cells of considerably larger diameter than in other ganglia were also identified. Longitudinal AST-like immunoreactive pathways from the cerebral to the terminal ganglion

and fibres connecting the neighbouring ganglia were observed both on the dorsal and ventral surfaces of the CNS as well. Only a low number of labelled fibres were found in segmental nerves, however, fine meshworks of thin fibre-branches were seen in large areas of the ganglia. This arrangement could be explained by the parasynaptic liberation of this neuropeptide.

The large number of AST-like immunoreactive cells in the terminal ganglion further the presence of labelled fibres in its 3rd-6th segmental nerves may refer to the role of AST in the regulation of the muscle contraction in the abdominal segments. Comparing the AST-IR pattern in the CNS of *P. scaber* to those observed in other crustacean species we could establish a considerable homology which may be a mark of an anatomically conserved arrangement.

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TOPOGRAPHIC PATTERNS IN THE GASTROPOD BRAIN

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This study describes the topographic patterns of cerebral efferent neurons with different neurochemical characters as well as the termination of afferent pathways from different head areas in the cerebral ganglia of *Helix pomatia*. The simultaneous Co and Ni-lysine backfills through cerebral nerves show that efferent neurons are labeled and concentrated in seven representation foci and nearly each head area are represented in each focus. At a gross level, the representation of the different head areas in the representation foci shows a topographic arrangement. Each focus is constituted by neurochemically different groups of neurons. The combination of backfills through cerebral nerves with the immuno-labeling of serotonin, dopamine, FMRFamide and CARP-containing neurons revealed that the different head areas are topographically represented on both serotonergic and dopaminergic groups of neurons, and they are represented also on CARP and FMRFamide immunoreactive groups of neurons in all of the foci.

Combination of degeneration of afferent pathways and anterograde labeling through cerebral nerves shows that afferent pathways terminate in a mild topographic pattern along the procerebrum. Pathways from the anterior and posterior tentacles terminate rostrally and medially whereas caudally from the mouth and lip indicating the important role of procerebrum in the sensory information processing. These observations suggest that the patterned organization of cerebral efferent neurons can be considered as efferent subsets of different central pattern generator networks which generate patterned outputs to different sets of efferent neurons during an ongoing behavior

CHANGES OF NEURAL ACTIVITY IN THE PREFRONTAL CORTEX DURING INCENTIVE LEARNING IN THE RAT

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The prefrontal cortex (PFC) is widely reported to contain a population of neurons exhibiting gustatory responsiveness. Also, in other studies, the prelimbic subregion (PrL) of the PFC is claimed to participate in visceral adjustment functions, such as learned autonomic responses. The presence of the above two functions in the same neural population in the PrL, however, has not been tested before. Therefore, in our present experiments, extracellular single unit recordings from PrL neurons, together with monitoring heart rate and breathing activity, was recorded in anaesthetized CFY rats during (a one-trial) learning of taste aversion. Glass capillary microelectrodes (filled with neurobiotin) were lowered into the PrL. Data collected continuously during the whole session was digitized and analyzed off line. At the end of behavioral testing, neurons were juxtacellularly labeled with neurobiotin by means of injecting current pulses through the electrode tip. Activity of the neurons was continuously monitored during tracer injection, as well. After histological procedures, location and projections of the labeled neurons were revealed. According to our results, bimodal taste/visceral response type PrL neurons (5 of 54, 9%) were detected and their activity changes during learning were subsequently analyzed. The present findings indicate the involvement of bimodal prelimbic neurons in the formation of single association memories between external (taste) and internal (gastrointestinal discomfort) environmental cues. Our results also demonstrate methodological aspects of juxtacellular labeling of cortical neurons *in vivo*.

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OCTOPAMINE RECEPTORS IN INSECTS

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Octopamine (OA) receptors are widely distributed in insect tissues and were classified in OA_1 and OA_2 receptor subtypes. To identify and characterize the OA receptors in the brain and in the optic lobes of locust, *Locusta migratoria migratorioides,* we investigated the presence of OA stimulated adenylate cyclase and the binding properties of ^{3}H-OA and ^{3}H-yohimbine. A concentration dependent stimulation of the adenylate cyclase by OA were found in both brain and optic lobes, suggesting that the cAMP system is involved in the

effect of OA. Synephrine and demethylchlordimeform (DCDM) stimulated also the cyclase activity, whereas mianserin and phentolamine inhibited the OA simulated adenylate cyclase activity. ^{3}H-OA binds specifically and reversibly to the membrane fragments of the brain and optic lobes. However, the bound ^{3}H-yohimbine was not dissociable, and therefore it cannot be used as a ligand to the characterization of OA receptors. The Scatchard analysis of the saturation curve revealed a single high affinity binding site in both the brain and optic lobes. The receptor is stereospecific. The affinity of synephrine, DCDM and naphazoline to the receptor is the same as that of OA, whereas dopamine, adrenaline and noradrenaline have low affinity. Mianserin and phentolamine have high a affinity, gramine, cyproheptadine and metoclopramid have a moderate affinity, and yohimbine has a low affinity to the receptor. This pharmacological profile suggests the presence an OA_2 type receptor in both the brain and optic lobes.

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BINDING SITES OF AN ENDOGENOUS TETRAHYDROISOQUINOLINE, SALSOLINOL, IN RAT BRAIN REGIONS AND IN THE ANTERIOR LOBE OF THE PITUITARY GLAND

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Tetrahydroisoquinolines have been reported as naturally occurring substances in the human, cat, mouse and rat brain. Among these products, (R)-salsolinol (1(R)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) has been detected in high concentration in the striatum, hypothalamus and in the neuro-intermediate lobe of the pituitary gland. Furthermore, it has also been shown that salosolinol is enzymatically synthesized in the brain. Our laboratory has recently discovered that salsolinol can profoundly elevate the level of plasma prolactin. This effect is very rapid and is supposedly receptor mediated. The existence of salsolinol in the striatum has suggested that this area of the brain may also have binding sites. Therefore, receptor-binding assays with ^{3}H-salsolinol have been performed on homogenates prepared from the striatum, cortex, hypothalamus and the anterior lobe of the pituitary gland. Binding of salsolinol could be detected in all these regions. Besides salsolinol, dopamine, L-DOPA and methyl-L-DOPA can also displace the tritiated compound. In contrast, specific agonists or antagonists of D_1, D_2, D_3, D_4 or D_5 receptors have no effect except apomorphine. Moreover, displacement of ^{3}H-salsolinol by L-DOPA has been largely affected by lactation. Our results suggest that *(1)* specific binding sites for salsolinol exist in the CNS, *(2)* it differs from the known dopamine receptors, *(3)* it can be influenced by the physiological stage of the animal. In summary, these results further support that salsolinol is a putative hypophysiotropic prolactin-releasing factor.

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SUBTYPE-SELECTIVE NMDA ANTAGONISTS AS POSSIBLE ANALGESIC AGENTS

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There is a great unmet medical need for pain relief especially in some chronic pain states such as neuropathic pain (NP). One of the key elements in pathomechanism of NP, i.e. central sensitisation of spinal cord pathways, is an NMDA receptor-dependent process. However, clinical use of currently available NMDA antagonists has been hindered by undesirable side-effects.

There is a novel group of subtype selective NMDA antagonists (SSNA) that block only receptor subtype containing the NR2B subunit. The prototype of these is the β_1 antagonist ifenprodil (IF). Followers include Pfizer's CP101606 (CP), which is under clinical trial for stroke. It is free of motor side effects or memory impairment. This feature of SSNAs have been attributed to the restricted distribution of NR2B subunit containing NMDA receptors in CNS.

Two recent papers reported that SSNAs are promising analgesic compounds with excellent side-effect profile. To further examine the usefulness of SSNAs as analgesics we used the following rat tests: mechanical hyperalgesia in partial nerve ligation (PNL) model of neuropathic pain; carrageenan test (inflammatory hyperalgesia); formalin test (phase 2). As an *in vitro* efficacy index, IC_{50} for NMDA-induced rise in intracellular calcium concentration in cultured cortical neurons was determined. Possible side-effects were characterised by β_1 binding *in vitro*, and by rat rotarod test *in vivo*.

Compounds examined include IF, CP, three novel SSNAs of CoCensys without published analgesic effect, and memantine (ME), a non-selective channel blocker NMDA antagonist. All SSNAs had anti-hyperalgesic effect in PNL model with ID_{80} values of 6-8 mg/kg ip., except a CoCensys compound with $ID_{80}<2.5$ mg/kg. In carrageenan and formalin tests only IF was potent ($ID_{80}\approx10$ mg/kg, ip.), SSNAs were less effective. ME had only weak, if any, effect in all tests. In rotarod test, ID_{50} values were ≥100, 17 and 9 mg/kg for SSNAs, IF and ME, respectively.

These results further support that SSNAs have a potential therapeutic value in neuropathic pain without side-effects of classical NMDA antagonists.

ANTINOCICEPTIVE EFFECTS OF CONTINUOUS INTRATHECAL ENDOMORPHIN-1 IN RATS

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Endomorphin-1 and endomorphin-2 were recently postulated to be endogenous μ-opioid receptor agonists. Our earlier results have shown that they have a very short-lasting antinociceptive effects in acute and inflammatory pain tests. The purpose of the present study was to investigate the antihyperalgesic effects of endomorphin-1 on inflammatory pain sensations after continuous intrathecal administration in awake rats.

Methods: After institutional ethical approval had been obtained from our animal care committee intrathecal (i.t.) catheters were implanted into male Wistar rats and they were allowed to recover for at least 4 days before use. For the inflammatory pain test (paw withdrawal test) the rats were placed on a glass surface in a plastic chamber and allowed to acclimatize to their environment for 15-30 min before testing, and baseline hindpaw withdrawal latencies (pre-carrageenan values at -180 min) were obtained. The heat stimulus was directed onto the plantar surface of each hindpaw and on the tail. Unilateral inflammation was induced by intraplantar injection of 2 mg carrageenan in 0.1 ml physiological saline into the right hindpaw. The paw withdrawal and tail-withdrawal latencies were obtained again 3 h after carrageenan (post-carrageenan baseline values at 0 min), and then every 10 min for 130 min. The drug administration begun with a 10 μg bolus dose (in 5 μl volume) and continued with different doses (0.3, 1 or 2 μg/min) for 70 min. Control experiments were carried out with the vehicle delivered intrathecally. Drugs were injected at a rate of 1 μl/min. Groups were compared by ANOVA with $P<0.05$ considered significant.

Results and discussion: In the control group a significant decrease in the paw withdrawal latency was observed at the injured side during the whole period. Continuous administration of endomorphin-1 dose-dependently decreased the thermal hyperalgesia. 2 μg/min endomorphin-1 totally relieved the hyperalgesia. After the cessation of the infusion the hyperalgesia reappeared. None of the doses of endomorphin-1 influenced the paw withdrawal latencies of the non-injected paws, although dose-dependently increased the tail-withdrawal latencies. No side-effects could be observed. Our results demonstrate that continuous intrathecal administration of the short-lasting endomorphin-1 is an effective method for relieving the thermal hyperalgesia in awake rats.

INNERVATION OF THE OLIVOCOCHLEAR CELLS STUDIED BY TRANSSYNAPTIC TRACING IN THE GUINEA PIG AND RAT

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The olivocochlear projection constitutes the last stage of the descending auditory system in the mammalian brain. The medial olivocochlear cells reside in the ventral nucleus of the trapezoid body of both sides of the brain and innervate outer hair cells in the cochlea. Lateral olivocochlear cells are associated to the lateral superior olive, innervate predominantly the ipsilateral cochlea and terminate beneath the inner hair cells.

We injected the Bartha's strain of Aujeszky's disease virus (pseudorabies) into the cochlea. Following different survival times the animals were sacrificed and labeled neurons in the brain were identified by routine immunoperoxidase staining. Specific polyclonal antiviral antibody was used in the immunohistochemical procedure.

The first labeled neurons were observed in the superior olivary complex (primarily in the periolivary region and in the lateral superior olive) at 25-35 h after the infection. The number of the labeled neurons in these nuclei further increased by the 72-96 h survival times. Moreover, neurons of other auditory-related nuclei (ventral cochlear nucleus, inferior colliculus) and of the brainstem autonomic system (nucleus raphe magnus and dorsalis, the ventral and dorsal parabrachial nuclei, as well as cells in the periaqueductal central gray area) were abundantly labeled.

These data provide evidence for the existence of direct neuronal inputs from brainstem autonomic nuclei to the descending auditory system.

HORMONAL REGULATION OF GAP-43 EXPRESSION IN THE OLFACTORY BULB

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Growth-associated protein GAP-43 is a membrane-bound phosphoprotein which has been implicated in axonal elongation and synaptogenesis. In the perinatal period GAP-43 is expressed in all neurons, by maturity most neurons no longer express detectable levels, although GAP-43 level is still moderately high in the adult hippocampus and olfactory bulb. Since estrogen is thought to influence neuronal connectivity and synaptic remodeling, the present study investigated estrogen modulation of GAP-43 expression in the olfactory bulb of the rat. This area is a very good experimental model for such studies because a permanent remodelling of the synaptic connections takes place in adult animals.

By Western blotting technique we could demonstrate that the GAP-43 expression is sexually dimorphic; in the first two months we measured higher amounts in males which was reversed after sexual maturity.

In intact females we found cyclic changes: the GAP-43 was elevated during proestrus afternoon and returned to the normal level on the following days. In ovariectomized animals sc. injection of 17ß-estradiol (100 µg/100 g body wt. dissolved in sesame oil) resulted in an increase of GAP-43 expression reaching a maximum 9 h after the treatment.

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DETECTION OF THE "β" BUT NOT THE "α" ISOTYPE OF ESTROGEN RECEPTOR IN LUTEINIZING HORMONE-RELEASING HORMONE NEURONS OF THE RAT BRAIN

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Dual-label *in situ* hybridization (ISH) was used to determine whether the messenger ribonucleic acids (mRNAs) encoding the classical estrogen receptor (ER-α) as well as the recently-cloned β isoform of estrogen receptor (ER-β) are expressed in luteinizing hormone-releasing hormone (LHRH) neurons of the rat forebrain. Sections containing the organum vasculosum of the lamina terminalis (OVLT) and the medial preoptic area (MPOA) of female rats were chosen for these studies based on previous data that ovarian steroids regulate the expression of LHRH mRNA, galanin and c-Fos by LHRH neurons located in these areas. While we found no evidence for the expression of ER-α mRNA in LHRH neurons, an observation that corroborates previous immunocytochemical data, the dual-label ISH approach revealed the presence of ER-β mRNA within a large population of LHRH neurons in both ovariectomized and estradiol-primed female rats.

The detection of ER-β mRNA in LHRH neurons of the rat re-addresses the direct estrogen-responsiveness of the LHRH neuronal system which appears to be mediated, at least in part, by the recently-discovered β type of ER. Future studies will need to determine the target genes that are regulated by ER-β within LHRH neurons.

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IN SITU HYBRIDIZATION DETECTION OF ESTROGEN RECEPTOR α (ER-α) AND β (ER-β) MESSENGER RIBONUCLEIC ACIDS (MRNAS) IN NEURONS OF THE HYPOTHALAMIC SUPRAOPTIC (SON) AND PARAVENTRICULAR NUCLEI (PVN)

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The regulation of magnocellular neuronal functions by estradiol is well documented, however, the receptorial mechanisms are controversial. The present studies used highly sensitive *in situ* hybridization (ISH) approaches to readdress the expression of both known estrogen receptors (ER-α; ER-β) in the paraventricular and supraoptic nuclei (PVN; SON) of the rat hypothalamus. Although, previous reports agreed in that the classical ER-α is absent from both regions of the rat hypothalamus, our present ISH data indicate that low cellular levels of ER-α mRNA can be revealed within the majority of neurons in the PVN and the SON. In addition, the wide distribution of the recently discovered ER-β mRNA was established in the SON as well as both the parvi- and magnocellular subdivisions of the PVN. Triple-label ISH studies demonstrated that ER-β mRNA is expressed in both oxytocin and vasopressin neurons of the SON, PVN and accessory magnocellular cell groups of the hypothalamus. Although, distinct neuronal subsets of the PVN contained highly variable amounts of ER-β mRNA, the hybridization signal was not expressed preferentially in either oxytocin or vasopressin neurons, which finding contrasts previous observations from other laboratories.

Our data suggest that large populations of paraventricular and supraoptic neurons can be regulated directly by estradiol. Because merely all subdivisions of the PVN express both ER-α and ER-β, the contribution of the ER isotypes to the molecular regulation of parvicellular and magnocellular neuronal functions needs further clarification.

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EFFECTS OF COLD AND/OR FASTING ON CIRCADIAN BODY TEMPERATURE RHYTHM IN RATS

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Circadian body temperature rhythm and general activity have been studied in unrestrained female Wistar rats held either under thermoneutral conditions (22-25 °C), or in cold (4 °C). To influence energy state food was either freely available or the rats were exposed to

complete fasting for 5 or 2 days at thermoneutrality or in cold environment, respectively. The animals were implanted under general anaesthesia with intra-abdominal telemetric transmitters (MINIMITTER, Sunriver, OR) allowing continuous monitoring of core temperature and general activity.

Circadian core temperature rhythm developed some days after transmitter implantation. When applied at thermoneutrality, 5 days fasting resulted in an augmentation of the amplitude of circadian body temperature oscillations. Transfer of the rats to cold resulted in the appearance of an infradian oscillation of body temperature (with a cycle-length of 3-4 hours and an amplitude of about 1 ^{o}C) superimposed on the circadian rhythm. Two days lack of food in the cold led to a significant and reproducible increase of amplitude of core temperature cycles with mesor (mean value) either remaining unchanged or decreasing slightly.

It is concluded that during cold exposure rats can maintain or even increase the rhythmicity of their core temperature, the latter result being interpreted as a sign of a modified body temperature regulation resembling the phenomenon of broad band control, but the mesor or the night maxima of core temperature could be maintained even when food was not available for two days during cold exposure. The mechanism of cold-induced, high frequency oscillation of body temperature (and of general activity observed simultaneously) cannot be explained so far.

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NEUTRALIZATION OF TGF-βS *IN OVO* IMPAIRS THE EARLY DEVELOPMENT OF SEROTONERGIC NEURONS IN THE CHICK EMBRYO

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Despite the general importance of the brainstem serotonergic system in a number of psychiatric and physiological functions, little is known about the molecular (intrinsic and epigenetic) mechanisms that control the generation and early phase of the serotonergic neuron development. During development, serotonergic neurons are first generated in two major groups (rostral and caudal) in the ventral part of the developing CNS, in the immediate vicinity of the CNS midline floor. A network of transcription factors and secreted signaling molecules (e.g. sonic hedgehog /Shh/), emanating from the midline structures are thought to be involved in different steps of precursor cell induction, fate decision and differentiation of serotonergic neurons. We have tested whether TGF-βs (secreted, pleiotropic cytokines) play a role in the early serotonergic neuron development. In the developing chick CNS TGF-β3 is expressed in the ventral neural tube and TβRII (a key receptor component of the TGF-β signal transduction) is strongly expressed in the ventral midline of the developing CNS. To test the function of TGF-βs in serotonergic neuron development, we have interrupted TGF- β signaling by treating early chick embryos with pan–anti TGF-β neutralizing antibody (10 μg/embryo for 3days) *in ovo*. Neutralization of all three TGF-β isoforms resulted in a nearly

complete loss of the caudal serotonergic neuron population and a significant reduction in the rostral serotonergic cell population as demonstrated by tryptophan hydroxylase immunoreactivity. Since neutralization of TGF-βs did not affect HNF3β expression in the floor plate, we hypothesize that TGF-βs can represent a parallel or downstream mechanism to the sonic hedgehog signaling network in elaborating serotonergic development. Here, we have demonstrated that we can effectively interfere with serotonergic neuron development other than the direct manipulation of the well described Shh signaling pathway by interrupting TGF-β triggered mechanisms.

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EXPRESSION OF $VPAC_2$ RECEPTOR MRNA IN THE SUPRACHIASMATIC NUCLEUS OF THE AGEING FEMALE RAT

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Aged rats often display abnormal circadian activity rhythms; this seems to be a consequence of the deterioration of a central circadian light-entrainable pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Receptors showing equal affinity for VIP and PACAP ($VPAC_2$) have been demonstrated in many SCN cells, including VP and VIP neurons. We hypothesised that age-related changes in rhythmicity are associated with alterations in the patterns of expression of $VPAC_2$ receptor (R).

The 24-h profile of $VPAC_2R$ mRNA expression in the SCN was analysed in young (4-6 months) and old (20-24 months) ovariectomized female rats. Five groups of animals were maintained on a 12:12 LD cycle and three groups on a reversed 12:12 DL cycle (lights on, Zeitgeber time [ZT] 0, at 07.00 h or at 19.00 h, respectively). Animals (n=5) were killed at each of 8 time points over the 24 h cycle and processed for *in situ* hybridization using a 481 bp cRNA probe complementary to a portion of the $VPAC_2$ receptor mRNA.

Quantitative analysis of the autoradiographs indicates that there is a significant variation within the SCN in the expression of the mRNA for the $VPAC_2R$ across the light/dark cycle in the young rats. There is a biphasic variation with significant peak levels at the beginning of the day (ZT 0) and night (ZT 12), respectively. In aged rats there is also a significant variation within the SCN. The biphasic pattern of mRNA levels is still present, but in contrast to the younger rats the peak levels appear at "mid"-day (ZT 6) and "mid"-night (ZT 18). The old animals have a significantly lower signal for $VPAC_2R$ mRNA compared to the younger animals. The disturbed function of the SCN with ageing may be associated with the altered pattern of $VPAC_2R$ mRNA expression reported here. These changes in transcription

may contribute to the alterations observed in the production and/or entrainment of circadian rhythms in aged animals.

COMPARATIVE STUDY OF GFAP-IMMUNOPOSITIVE STRUCTURES IN VERTEBRATES

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The astroglia have similar characteristics in the most specialized amniote brains: i) the predominant elements are astrocytes; ii) the distribution of the GFAP-positive structures is uneven, iii), the vimentin disappears almost completely from the adult glia. These features appear both in mammals and in birds, while they are missing from reptiles, although crocodiles exhibit such local glial specializations, which resemble avian brain. Considering that mammals and birds develop from different branches (synapsid and diapsid) of the amniotes, the aforementioned features are supposed to be results of parallel evolutions. The different structure of the perivascular and surface-limiting glia of mammals and birds supports this opinion. Teleost fishes have also mainly ependymoglia, in quite even distribution (except the brain stem) and they lack GFAP positive astrocytes. Among Chondrichthyes, in the skate brain astrocytes occur widely, and large areas are free of GFAP immunopositivity, while in the squalomorph sharks radial glia are the ubiquiter, predominant structure, and the GFAP-immunopositivity is distributed evenly throughout the brain. These data suggest that the thickening of the brain wall and the growing complexity are concommittant with the predominance of astrocytes and the regression of the GFAP-immunopositivity. Up on request, however, GFAP can be expressed, at least in mammals and birds. It can be supposed that the appearance of astrocytes promotes the repression of the unnecessary GFAP-production in most areas of the brain. A strict correlation, however, cannot be found between the characteristics of the astroglia and either the actual thickness of the brain wall, or the "laminar" or "elaborate" type of the brain.

INVESTIGATION OF BRAIN VASCULARISATION BY IMMUNOHISTOCHEMICAL STAINING AGAINST LAMININ

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The present results suggests that the newly formed brain vessels can be distinguished by the proper immunohistochemical staining of laminin. Two models were studied: i) the vessels of the intact brain, in adult rats and in the first two postnatal weeks; ii) the vessels after brain lesion, in the same ages. The lesions were cortical stab wounds, performed in adults after

drilling the skull, but percutaneously in the pups, in deep ketamine-xylazine or ether anaesthesia, respectively. After one-week survival period, the animals were overdosed with ether and transcardially perfused with 4% phosphate-buffered paraformaldehyde solution. Parallel floating coronal sections were processed for immunochemical staining against laminin or GFAP, antibodies were obtained from Sigma and Boehringer, respectively. In intact newborns the vessels were intensely immunopositive to laminin in the whole sections. This immunopositivity disappeared almost completely during development. In operated adults, the vessels were intensely laminin immunopositive around the lesion, in the territory of the glial reaction, as it was detected by immunochemical staining against GFAP. In newborns, vessels could penetrate the cortex deeply from the lesions, independently from the presence or absence of glial reaction. During development, the vascular reaction became similar to that seen in adults. This latter results suggests that pial vessels starting from a wound can vascularize the cortical tissue only until a stage of cortical maturation. The reason of that the laminin antibodies prefer newly formed vessels to older ones, both in cortical maturation and wound healing, is not clear, but the phenomenon did not depend from the working dilution between 1:100 and 1:1000.

EXPRESSION OF PRESENILIN-1, PRESENILIN-2 AND AMYLOID PRECURSOR PROTEIN IN DIFFERENT NEURONS AND THEIR PROCESSES IN THE CENTRAL- AND PERIPHERAL NERVOUS SYSTEM. EFFECTS OF MECHANICAL LESION ON THE AXONAL TRANSPORT OF THESE PROTEINS

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Alzheimer's disease (AD) is an age-related disorder. The neuropathologieal changes involve the degeneration of different neuronal cell bodies and their processes. Some cases of AD are caused by the mutations in presenilin-1 (PS-1) and amyloid precursor protein (APP), which are integral membrane proteins. It has previously been demonstrated that PS-1 and PS-2 are localized in the neuronal perikarya and at the synaptic sites. APP has been detected in the neuronal cell bodies, the axon terminals and the astrocytes. APP reaches the synaptic sites by fast axonal transport. There are no data, however, as to how the PSs reach the synaptic region.

Our aim, therefore, was to localize PS-1, PS-2 and APP in the different neurons and axonal processes by immunohistochemical means and to clarify their transport from the cell body to the axon terminal. This investigation was concentrated mainly on the sciatic nerve of adult rats. To study the axonal transport of the different membrane proteins, the sciatic nerve of rats was ligated for 24 h. The immunohistochemical data were supported by the Western blot technique. The results revealed that the proteins that play a significant role in AD can be found not only in the perikarya of the neurons, but also in their axons. In the sciatic nerve of rat, APP is transported mainly. In the anterograde direction, whereas PS moves in both anterograde and retrograde directions. This is the first study to demonstrate that PS can be

found in the axon and the APP and PS are not strictly related to each other. During axonal transport, these proteins may be separated and their transport may be differently regulated.

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ANXIOLYTIC EFFECT OF GALANIN IN THE ELEVATED PLUS-MAZE ON MICE

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The neuropeptide galanin can be found in the central nervous system, and it can play a role in the mechanism in the regulation of animal behavior. The purpose of this study was to investigate the effect of galanin on anxiety.

An elevated plus-maze model was used to assess the anxiety of CFLP mice. Different doses of galanin were administered intracerebroventricularly - the most efficient dose was 800 ng. Galanin significantly increased the ratio of entries into open arms over the total number of entries and prolonged the time spent in the open arms. To determine if the anxiolytic effect of galanin is a receptor mediated event, the effects of picrotoxin (0.1 mg/kg, ip.), pentylenetetrazole (20 mg/kg, ip.), atropine (0.5 mg/kg, sc.), propanolol (5 mg/kg, ip.), phenoxybenzamine (1 mg/kg) and haloperidol (1 mg/kg, ip.) were examined. Receptor blockers had no effect upon entries compared to the vehicle treated control animals. Picrotoxin, atropine, phenoxybenzamine significantly reduced the galanin-induced anxiolytic effect. Haloperidol and pentylenetetrazole had no influnce on the effect of galanin.

These results suggest that galanin may play a role in the reduction of anxiety and this effect can be mediated by multiple neurotransmitter system.

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LOCALIZATION OF CB1 CANNABINOID RECEPTOR AND MODULATION OF PRESYNAPTIC GABA RELEASE BY CANNABINOIDS IN THE HUMAN HIPPOCAMPUS

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Activation of CB1 cannabinoid receptors has been shown to underlie most, if not all, behavioural effects of exo- and endocannabioids. Since cannabinoids have a well-known disrupting effect on memory consolidation, localization of CB1 receptors was investigated in

control and epileptic human hippocampus by immunocytochemistry. Expression of CB1 was confined to interneurons of the hippocampus. Most of these cells contained the neuropeptide cholecystokinin as well, but they never expressed the calcium-binding protein parvalbumin, another characteristic marker of hippocampal interneurons. CB1-positive cell bodies were distributed in all layers, whereas immunostaining of dendrites was not observed. CB1-immunoreactive axons covered the entire hippocampus and corresponded to GABAergic axon terminals verified by electron microscopic investigations. These CB1-positive boutons contained cholecystokinin as well. This unique pattern of expression and putative functional localization sites correspond to our previous results in the rat, which suggests a conserved role for CB1 in controlling presynaptic GABA and/or cholecystokinin release. Since epileptic human hippocampal tissue deriving from human temporal lobectomy samples showed no major differences in these patterns compared to postmortem human hippocampus, tissues from the same patients were used to demonstrate the characteristic reduction of ^{3}HGABA release by the CB1 receptor agonist WIN 55212-3 (1 μm). These data suggest that cannabinoids may have a role in the presynaptic modulation of GABA release. As a consequence, the disruption of synchronized oscillatory patterns of the hippocampus may underlie the confusion in memory acquisition observed during marijuana smoking.

THE EFFECT OF CELLULAR PHONE IRRADIATION ON THE LATENCY OF SHORT-TERM MEMORY AND MOTOR REACTION TIME

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From the total output power of commercial (GSM) cellular phones, 40-60 % is immediately absorbed in the head of the user. Mostly the auditory organ and the adjacent cortical zones, involved in hearing, are affected by the irradiation. Our previous results, based on audiometry and analyses of evoked responses from the cochlear system and the brainstem, indicate that the auditory threshold values together with latencies and amplitudes of cochlear and brainstem evoked responses are negatively affected by radio frequency (RF) irradiation. Since RF irradiation affects not only the auditory system, but presumably the whole hemisphere, our examinations have been expanded to investigate the P300 component of the cognitive auditory evoked potential, which is accepted to be an indicator of short-term/working memory functions. The amplitude and latency values of P300 potentials, elicited by discriminative auditory cues, were recorded on a 16 channel-EEG device in an auditory odd-ball paradigm. Motor reaction times (RTs) of the same subjects were also recorded and analysed. To create a simplified animal model for the effects of RF waves on brain function, extracellular single/multiple unit activity was recorded in 5 anaesthetised male CFY rats before, during, and after RF irradiation. According to the results obtained

from 30 volunteered medical students, the auditory P300 was altered after 20 min GSM RF irradiation. The amplitude of the P300 decreased significantly, and its shape changed, too. Furthermore, onset latency of P300 was found to increase by 50-100 ms. The most profound effects were detected in the P4 recording position. However, the same effects could be detected, with varying extent, in the whole ipsilateral hemisphere and also in contralateral areas. In RT studies, increases of response latencies were observed as consequences of delays in cognitive information processing, depending on the age of the subject. In our animal model experiments, long-lasting (up to 2 h), reversible increase of spontaneous firing rates of single/multiple units was observed in the TAud area of the rat. Our present results indicate that RF irradiation caused by GSM cellular phones elicits dramatic changes in the auditory cognitive P300 potential indicating impaired cognitive and/or memory functions, which is accompanied by delayed latencies of RTs. Results of the animal model experiments indicate that changes of the P300 or RT values may be caused by the increased excitability of cortical neurons. Due to the RF irradiation, these neurons become unable to attend to their previous functions. In addition, the dynamics of recovery of basal neural firing rates may reflect on the time course of the underlying intracellular biochemical mechanisms.

THE NEURONAL NETWORK UNDERLYING THE GENERATION OF RHYTHMIC FEEDING BEHAVIOUR IN *LYMNAEA*

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In the pond snail *Lymnaea stagnalis*, the neuronal network generating feeding, a three-phase rhythmic behaviour, consists of central pattern generating (CPG) interneurones, motoneurones and modulatory interneurones. The three phases of feeding (protraction, rasp and swallow) are generated in the buccal ganglia, by three main types of CPG interneurones known as N1, N2 and N3. The feeding motoneurones (B1 - B10) of the buccal ganglia receive chemical synaptic inputs from the CPG interneurones and are classified as either protraction, rasp or swallow phase neurones, defined by the phase in which they become active. Some of the motoneurones form electrotonic synapses with CPG neurones active in the same phase and therefore are able to modulate these. The three main modulatory neurones of the feeding network are the slow oscillator (SO), the cerebral ventral 1 (CV1) and the cerebral giant cell (CGC) interneurones. Both the SO and the CV1 cells can drive fictive feeding activity in the CPG when they are depolarized and this is similar to fictive feeding triggered by the appropriate chemosensory inputs. The CGCs have an important gating/frequency modulating role in the generation of rhythmic feeding.

More recently, hybrid CPG/modulatory (N1L) and motor/modulatory neurones (OC) as well as new subtypes of CPG interneurones also have been described. A variety of classical (5-HT, Ach, DA, OA) and peptide transmitters (MyoM, SCP) and the gaseous transmitter NO have been found in neurones of the feeding network, which was also found to undergo plastic changes as a result of classical conditioning. The feeding network of *Lymnaea*

therefore offers an excellent model system in which to study mechanisms of pattern generation and transmitter action as well as cellular and molecular mechanisms of sensory information processing and learning.

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THE EFFECT OF NGF, BDNF, AFGF, BFGF AS WELL AS CELL DENSITY ON THE EXPRESSION OF NPY IN RAT DORSAL ROOT GANGLION CULTURES

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Adult rat dorsal root ganglion (DRG) cultures were used as a model to study the effect of neurotrophic factors on neuropeptide-Y (NPY) expression after peripheral nerve injury.

Immunohistochemical studies showed no effect of NGF, BDNF or bFGF on NPY expression during 72 h incubation of the dispersed DRG cultures while aFGF increased the number of NPY - immunoreactive (-IR) neurons by 1.6-2.0-fold. Using the more sensitive nonradioactive *in situ* hybridization method no significant effect of NGF or BDNF treatments was observed. However, bFGF induced a 1.8-fold (significant) and aFGF a 2.25-fold (highly significant) increase in NPY mRNA positive neurons.

In high-density cultures an inhibition of NPY expression could be observed both under control condition and with treatment of any of the four trophic factors.

The fact that NPY IR could be detected in 16%-20% and NPY mRNA in 27% of DRG neurons in culture supports the hypothesis that DRG neurons in culture are in an axotomized state, providing a model system for *in vitro* studies of nerve injury.

TOPOGRAPHICAL ORGANIZATION OF PROJECTIONS IN BRAINSTEM AUDITORY NUCLEI

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The cochlear nucleus (CN) sends fibers directly or via the nuclei of the medial (MSO), lateral (LSO) superior olive and of the lateral lemniscus (NLL) indirectly to the central nucleus of the inferior colliculus (CNIC). The aim of the present study was to determine the distribution of the afferent terminals on postsynaptic sites in the auditory nuclei. In our experiments microinjections of BDA and HRP were administered with iontophoresis into the auditory nuclei. After adequate survival time the sections were processed according to immunocytochemical protocols and examined in light and electronmicroscope. Following

anterograde tracer injections into the CN labelled terminals with round vesicles, making multiple asymmetrical synapses on the soma and principal dendrites oriented towards the side of injection were observed in the ipsilateral LSO and in the MSO of both sides. In the NLL the labelled terminals were in contact with dendrites without any preference to their direction. In the CNIC the labelled fiber bundles were making synaptic contact with both soma and proximal dendrites. Injections into the MSO and LSO revealed fine labelled fibers establishing asymmetrical synaptic contact with the peripheral dendrites in the NLL and CNIC. In combined experiments the labelled ascending terminals were in synaptic engagement with both the labelled relay and unlabelled interneurons with similar distribution. The descending fibers from the CNIC had preference for the soma and proximal dendrites of neurons in the lower auditory relay nuclei.

MODULATION OF ION CHANNELS BY MOLLUSCAN NEUROPEPTIDES

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Cell membrane ion channels are integral parts of signaling pathways in almost all cells. A number of extracellular ligands including neuropeptides, membrane potential, phosphorylation, ions themselves and second messengers are well established regulators of the channel activity. A wide variety of kinetically and pharmacologically distinct K channels, including several voltage-dependent and -independent ion channels can be modulated by neuropeptides. Here, we summarize some of the experimental data, with emphasis on the MIP (*Mytilus* inhibitory peptide), concerning the types of K currents, the role of G proteins involved in coupling the neuropeptide receptors to the ion channels and the mechanism whereby the G-proteins exert their control. K channel types controlled by neuropeptides carry inwardly rectifying ($I_{K(IR)}$), Ca-dependent ($I_{K(Ca)}$) and serotonin sensitive ($I_{K(S)}$) K-currents. Recently, two additional K-conductances were described following application of the neuropeptides. One is similar to $I_{K(S)}$ but voltage dependent (Kits et al. 1997), and the second is a Na-activated K-current (Kiss et al. 2000).

Activation of K channels by neuropeptides involves either the direct interaction of G proteins with the channels, or the intervention of enzyme systems and second messengers.

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ECTO-ATPDASES IN CAVEOLAE OF MULTIDRUG RESISTANT CANCER CELLS

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Ecto-ATPDase is an integral membrane protein, hydrolyzing extracellular nucleotide-tri and diphosphates, one of the most intensively studied members of the ecto-NTPDases enzyme family. Caveolae are specialized, detergent-insoluble membrane invaginations, enriched in cholesterol and sphingolipids. Caveolae serve as docking places in the plasmalemma for numerous proteins involved in signal transduction (e.g. G-proteins, eNOS, phospholipase D2) and mediate clathrin-independent endocytosis and cholesterol efflux. In several cell types which possess caveolae, ecto-ATPDase is preferentially targeted to these membrane microdomains. In the present study we focused on MCF-7 human breast adenocarcinoma cells and their derivative, adriamycin-resistant MCF-7-AdR cells. MCF-7 cells do not express caveolins (main structural proteins of caveolae) and are devoid of caveolae, whereas MCF-7-AdR cells express caveolin-1 and caveolin-2 and possess caveolae. Enzyme histochemical determination demonstrated that although both MCF-7 and MCF-7-AdR cells show some ecto-ATPDase activity in the plasmamembrane, outstandingly high enzyme activity is present in the caveolae of MCF-7-AdR cells. While the function of ecto-ATPDase in caveolar function and/or dynamics remains unknown, the presence of these "message centers" in the multidrug-resistant cells only, supports the hypothesis that caveolae contribute to the multidrug resistance phenotype.-

GLUTAMATERGIC INTERNEURONS IN THE PARAVENTRICULAR NUCLEUS OF RAT HYPOTHALAMUS: COMBINATION OF [^{3}H]D-ASPARTATE AUTORADIOGRAPHY AND IMMUNOCYTOCHEMISTRY

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Several data indicate that the excitatory amino acid glutamate is a major regulator of synaptic activation in the hypothalamus. The localization of glutamatergic nerve cells terminating on hypothalamic neurons is not known. The purpose of the present study was to investigate the paraventricular nucleus (PVN) for glutamatergic neurons. The investigations were based on the high-affinity selective uptake of [^{3}H]D-aspartate by glutamatergic terminals and the

retrograde transport of the tritiated amino acid to parent cells. [^{3}H]D-aspartate was injected into the PVN and the cell group examined for labelled neurons detected autoradiographically.

A considerable number of autoradiographically labelled neurons were observed in the PVN, mainly in the parvocellular but also in the magnocellular subdivisions. Radiolabelled cells of different shape could be distinguished in these areas: some were rounded or multipolar, others elongated fusiform, occasionally a short proximal portion of a dendrite could be recognized. The majority of the radiolabelled cells were accumulated mainly in the medial parvocellular and periventricular subdivisions. A large number of labelled cells were also seen in the lateral part of the posterior magnocellular division.

The findings provide the first morphological evidence for the presence of glutamatergic neurons (interneurons) in the PVN. There are numerous glutamate/aspartatergic neurons in the PVN, both in the parvocellular and magnocellular part which are interneurons or at least have axon collaterals terminating on other neurons of the same cell group.

The studies were supported by the Hungarian Academy of Sciences (AKP 98-12/3,2/42 to J.K.), the National Research Foundation (OTKA T-029004 to B.H.) and the Ministry of Health (ETT 478/96 to B.H.).

THE EFFECT OF SELECTIVE NR2B ANTAGONISTS ON SPINAL REFLEXES *IN VITRO*

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The dorsal root evoked ventral root potential (DR-VRP) consists of an initial sharp positive peak, which corresponds to a monosynaptic population action potential (MSR), followed by a motoneuronal EPSP-related long-lasting tonic potential. It is mediated primarily by AMPA and NMDA type glutamate receptors: AMPA receptors have a greater role in the early part of the potential, while NMDA receptors mediate mainly its late components. Formerly we found that NMDA receptor antagonists (APV, MK-801, CPP) did not take part in the mediation of the monosynaptic reflex potential *in vitro*, but have a significant role in the mediation of late part of EPSP. The aim of this study was to investigate the role of the NR2B subunit containing NMDA receptors in the mediation of early and medium-late parts of the reflex potential (up to 180 ms), using specific antagonists. *In vitro* experiments were performed on hemisected spinal cords isolated from 6-day-old rat pups. DR-VRP from the L_5 ventral root was recorded following stimulation of the corresponding dorsal root.

DR-VRP Ifenprodil inhibited MSR, and exerted a weaker inhibitory effect on EPSP. The novel NR2B specific drug, CP 101,606 did not influence DR-VRP. Results with CP 101,606 show that NR2B subunit do not play a role in the mediation of the first 180 ms of the reflex. The reflex inhibitory action of ifenprodil is attributable to its voltage gated calcium channel blocking action. Other cation channel antagonists such as tolperisone, silperisone and lidocaine have a similar pattern of effect in this model.

Further experiments are needed to investigate the possible role of NR2B receptors in mediation of the later reflex potential components (180 ms-5 sec) of DR-VRP.

CHARACTERISATION OF SUBUNIT SELECTIVE NMDA ANTAGONISTS: INHIBITION OF THE NMDA-EVOKED ELEVATION OF INTRACELLULAR CALCIUM IN PRIMARY CORTICAL CELL CULTURE

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The NMDA receptor consists of different subunits named NR1, NR2A-D. The NR2B-subunit containing NMDA receptors are considered to be involved in the transmission of specific types of pain (e.g. neuropathic pain). Antagonists selective for this subunit lack the typical side-effects of nonselective NMDA antagonists so these compounds could be useful drugs to treat pain. Thus our aim is to find new NR2B selective compounds. We established a medium throughput method based on the fluorometric measurement of intracellular calcium ion concentration ($[Ca^{2+}]_i$) for screening of compounds with NMDA antagonist activity. With this method we characterised a number of NR2B specific compounds and compared our results to those in the literature. We used a fluorescent plate reader to monitor changes of $[Ca^{2+}]_i$ upon NMDA receptor activation in rat cortical cell cultures in 96-well plates. NMDA caused a dose dependent elevation of $[Ca^{2+}]_i$ with an EC_{50} of 12.1 μM. The nonselective NMDA antagonists MK-801 and APV almost completely inhibited the $[Ca^{2+}]_i$-rise evoked by 40 μM NMDA, with an IC_{50} of 130 nM and 30.8 μM, respectively. In contrast, all the NR2B-subunit selective antagonists tested (threo- and erythro-ifenprodil; CP-101,606; CP-101,581 and some other new compounds) showed incomplete inhibition: maximal inhibition was in the range of 55-75%. This is mostly due to the fact that the cortical cells in primary culture contain multiple types of NMDA receptors with different NR2 subunits. The IC_{50} values we determined on cortical cells for these compounds are comparable to those obtained with different methods (including tests on recombinant receptors) and published in the literature.

THE ROLE OF PRELIMBIC CORTEX IN GOAL DIRECTED LEARNING BEHAVIOR IN THE RAT

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In our present experiments, prefrontal cortical (PFC) mechanisms concerning the regulation of goal directed, (instrumental) behavioral actions for altered reward values were examined. To investigate functioning of the rat PFC, excitotoxic microlesions were bilaterally performed into the prelimbic (PrL) cortical area by means of kainic acid iontophoresis. Microlesions were done after animals were successfully conditioned to a single pedal press/water association. After recovery, animals were exposed to a novel saccharin (*Experiment 1*) or citric acid (*Experiment 2*) reward as a replacement of water in the schedule. This was followed by a single injection of sickness inducing LiCl. After recovery, instrumental behavior of animals was tested in the same environment. Lesioned animals in *Experiment 1* showed enhanced neophobia and reduced instrumental behavior for saccharin when it was newly introduced. In the devaluation test, however, there was no statistically significant difference in pedal presses between novel and altered rewarding situations in lesioned animals but not in their controls, where reduction of pedal presses was observed. In *Experiment 2*, animals behaved similarly to those of *Experiment 1*, except to that they did not exhibit reduced pedal press behavior to the novel reward. Our results indicate that the PrL is involved in monitoring both inherently positive and negative preference values and adjust them to altered circumstances. Furthermore, our results suggest the importance of the PrL in directing the execution of instrumental actions guided by the altered preference values of different rewarding substances.

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REGIONAL DISTRIBUTION OF THE NUCLEOSIDES IN THE WHITE AND GREY MATTER OF THE HUMAN BRAIN

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Nucleotides (e.g. UTP and ATP) are extracellular signaling substances and nucleosides (e.g. uridine, adenosine) could be neuroactive in the human brain. Although several pyrimidine receptors have been cloned recently we do not know the concentration distributions of

nucleotides and nucleosides in the brain. Present study reports measurement of the nucleoside concentrations of the 13 areas in the human brain. Nucleosides concentrations of the microdissected samples were analysed by a chromatography-based assay for nucleosides from tissue homogenates. Human brains were removed from sudden death and traffic accident victims' skull at 2 hours after death. 251 samples were taken out from the 13 areas of 39 brains. Five areas were selected from cerebellum and eight areas from the cerebrum. The measured concentrations of the nucleosides demonstrate differences in regional distribution of nucleoside metabolism.

The most important result is that nucleosides have uneven distribution in white and grey matter of human brain. In addition to nucleosides concentrations of the grey matter of the human brain structures (cerebrum, cerebellum) were very different but the samples of white matters were very similar. Thus our results suggest that uneven distribution of nucleosides can be correlated with anatomically different construction of white matter and cortical areas in the human brain.

SYNAPTIC INTERACTIONS BETWEEN GALANIN IMMUNOREACTIVE NEURONS AND AXON TERMINALS IMMUNOREACTIVE FOR TYROSINE HYDROXILASE AND DOPAMINE B HYDROXILASE IN THE BED NUCLEUS OF THE STRIA TERMINALIS IN THE RAT

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The limbic-hypothalamo-pitiutary-adrenal (LPHA) system plays an important role in the regulation of stress responses. The bed nucleus of the stria terminalis (BST) was suggested as a relay nucleus between hypothalamic, limbic and brainstem centers. The BST exhibits numerous galanin-ir nerve cells, as well as a dense network of TH-ir and DBH-ir fiber terminals. The distribution of galanin-ir neurons and that of the catecholaminergic fibers overlaps suggesting the existence of synaptic connections between them.

Studying light microscopic sections several TH- and DBH-ir axon terminals were observed adjacent to galanin-ir cell bodies. Examining semithin sections, TH- and DBH-ir axon terminals were juxtaposed to galanin-ir neurons suggesting synaptic contact sites between catecholaminergic fibers and galaninerg perikarya. At the electronmicroscopic level we found axosomatic as well as axodendritic synaptic contacts between TH- and DBH-ir fibers and galanin-ir nervous structures.

The coexpressin of vasopressin and galanin in the BST was described. The galaninerg cells exhibit several androgen receptors. The androgens are known to modify stress responses, but neither the vasopressin- nor the corticotrop releasing factor (CRF)-ir cells in the BST posess adrogen receptors. The catecholamins are known to play an important role in the stress regulation. The galanin-ir cells, influenced by catecholaminergic inputs can convey androgen inputs onto CRF and vasopressin-ir neurons, thus modifying endocrine, behavioral and physiological responses during stress. These neuropeptide interactions can be central in

the fine tuning of endocrine, behavioral and autonomic changes during stress thereby facilitating a better adaptation to stress.

DYNAMIC MEMBRANE PORES ON ASTROCYTES AND OSMOREGULATION IN THE BRAIN

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Due to the very narrow intercellular clefts in the brain, neuronal activity results in highly relevant osmotic changes in the neuronal-glial cellular microenvironment. In addition, neurotransmitters leaking out from the synaptic cleft contribute to the perturbation of local cell volume regulation which may affect capillary blood flow. Therefore, the prompt rectification of the neuronal microenvironment, performed by the transport machinery of astroglia concomitant with neuronal firing, is essential for functioning of the central nervous system (CNS). A large amount of data has been collected to uncover the mechanisms behind this astroglial function, however, no sufficient explanation has been propounded yet. Particularly, the very rapid changes of astroglial volume (in the range of sec), e.g., at the very beginning of the onset of ischemia, seem to be rather enigmatic.

Recently, the possibility has been revealed that non-lethal membrane attack complexes (MAC) provided by the astroglial complement system may lead to dynamic membrane pore formation and to subsequent prompt volume changes in the CNS under either physiological or pathological conditions.

The objective of our present work has been to test this hypothesis by studying the Na^+ transport and Ca^{++} signalisation in cultured rat Type-I astrocytes exposed to MAC. As source of terminal complement (C) complexes, fresh rat sera were applied. As C-fixing antibody (Ab) sheep anti-rat 5'-nucleotidase was used kindly provided by Prof. B.P. Morgan (Univ. of Wales, Cardiff). The C-fixing Ab together with either homologous or heterologous serum significantly increases $22Na^+$ uptake by cultured astroglia. The start of increased $22Na^+$ uptake is preceded by the appearance of prompt cytoplasmic Ca^{++} signals in astroglia as revealed by laser scanning confocal microscopy. Fluo-3 fluorescent dye was used to load the astrocytes for monitoring the Ca^{++} signals.

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THE ROLE OF ASTROGLIAL COMPLEMENT SYSTEM IN THE REGULATION OF ION AND WATER HOMEOSTASIS IN THE BRAIN UNDER PHYSIOLOGICAL OR PATHOLOGICAL CONDITIONS

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There is at present no sufficient explanation available for the extremely rapid and well orchestrated osmotic regulatory activities of astroglia, particularly, for their early swelling in brain ischemia (1). Specific signals triggering the astroglial membrane to initiate the transport machinery should be considered. It has been proved by the work of B.P. Morgan's group and others that both classical and alternative pathways of the complement (C) system are expressed by astroglia and microglia in the mammalian brain (2). Preceding these studies, it has been demonstrated first in B.P. Morgan's laboratory and then in other laboratories that in eucaryotic cells of non-CNS origin, non-lethal membrane attack complex (MAC) causes the appearance of specific Ca^{++} signals, transient cell swelling and changes of Na^{+} and K^{+} transport.

Investigations have been reported to test the possible effects of non- lethal MAC or T cell perforin on ion transport and volume regulation of brain cells. Analysis of Ca^{++} signals elicited in oligodendrocytes by non-lethal MAC or perforin has been published. The role of Ca^{++} signal generation by non-lethal MAC or T cell perforin has not been studied in astrocytes, although it may be of particular significance because cytoplasmic Ca^{++} plays a central role in the ion transport and volume regulation of astroglia. Ion transport and cell volume changes of brain cells caused by non-lethal MAC or T cell perforin may be especially significant under ischemic conditions. Both complement and perforin expression are upregulated in the brain in diverse pathologies, including ischemia.

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ACTIVATION OF MICROGLIA IN VARIOUS EXPERIMENTAL SITUATIONS

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Activation of microglia is a sign of injuries in the central nervous system. A stereotype response is upregulation of CR3 complement receptor specific for microglia. We used a monoclonal antibody (OX-42) to visualize the CR3 receptor with an immunohistochemical technique, and studied early microglia reaction *in vivo* as an effect of mild forebrain ischemia, and *in vitro* in hippocampal slices.

After bilateral occlusion of the common carotid arteries in rats for 10 min activation of microglia could be detected. Twenty min ligation without reperfusion caused strong microglial reaction. The effect was similar in hippocampal slices. Replacing oxygen with nitrogen in the gas phase of the recording chamber caused depression of neuronal function and the activation of microglia. Neuronal function recovered during reoxygenation, but microglial reaction could further be detected. Hyperactivity of neurons evoked by the application of kainic acid, activated microglia in a dose dependent manner. One μM kainate applied for 5 min caused mild, while 20 μM of the drug brought about very strong glial reaction. Since both hypoxia and neuronal hyperactivity is accompanied by the elevation of extracellular K^+ level, we replaced Na with K partially or completely in the bathing solution to study the effect of potassium in normoxyc condition. The result was again a time and dose dependent activation of microglia.

We suppose that the primary factor in microglial activation is the elevated extracellular potassium level.

PACAP-LIKE IMMUNOREACTIVITY IN THE NERVOUS SYSTEM OF OLIGOCHAETA

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Pituitary adenylate cyclase activating polypeptide (PACAP) was first isolated from ovine hypothalami, and is a member of the secretin/glucagon/VIP family. The primary structure of PACAP is identical among all mammalian species examined, and its amino acid sequence in lower vertebrates (frog, chicken, fish) differs from human PACAP only in 1-4 amino acids. This remarkable similarity suggests that PACAP is a highly conserved peptide during phylogenesis. No studies have yet demonstrated its presence in invertebrate species.

In the present study, we investigated the distribution of PACAP38-like immunoreactivity in the nervous system of 3 oligochaete species (*Lumbricus terrestris, Lumbricus polyphemus, Eisenia fetida*), by means of immunohistochemistry. The distribution pattern of immunoreactivity was similar in all three species. The cerebral ganglion contains numerous immunoreactive cells in the dorsal cell mantle, and fibers are intensively stained in the central neuropil. The subesophageal and ventral cord ganglia also displayed immunoreactivity, although the number of cells was much less. In the peripheral nervous system, immunoreactivity was found in the enteric nervous system, in epidermal sensory cells and in the clitellum.

LIGHT AND ELECTRON MICROSCOPIC ANALYSIS OF ESTROGEN RECEPTOR ß IMMUNOREACTIVE STRUCTURES IN THE RAT FOREBRAIN

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The recent discovery (Kuiper et al., Proc. Natl. Acad. Sci. USA, 1996; 93: 5925-5930) of the novel, ß-subtype of estrogen receptor (ER- ß opened a new field in studying the signalling mechanisms of the ovarian steroids, estrogens. In comparison with estrogen receptor α, ER-ß shows a wider distribution in the rodent brain (Shughrue et al., J. Comp. Neurol, 1997; 388: 507-525) and regulates neuroendocrine events directly at the hypothalamic level (Hrabovszky et al., Endocrinology, 1998; 139; 2600-2604).

In order to reveal the neural sites of ER-ß synthesis and the subcellular localization of the receptor, an antibody was generated against the last 19 amino acids at the carboxy terminus of the rat ER-ß sequence and used for the immunocytochemical mapping of the receptor. Intact, adult female rats were perfused with a 1% paraformaldehyde and 1% glutaraldehyde solution. Vibratome sections prepared from the forebrain were immunostained for ER-? using silver-intensified colloidal gold and diaminobenzidine chromogens.

The distribution of ER-ß immunoreactivity in most forebrain regions was similar to the expression of the ER-ß mRNA (Shughrue et al., J.Comp. Neurol, 1997;388:507-525), although, a stronger signal was detected in the cerebral cortex, the hippocampal formation and the striatum by means of immunocytochemistry. At the light microscopic level, the majority of the receptor was found in association with the cell nuclei. A weak cytoplasmic labelling was also detected in the primary dendrites of pyramidal neurons of the neocortex and hippocampal formation and in some glial cells. Ultrastructurally, the receptor was predominantly confined to the chromatin of the cell nuclei. Ribosomes and vesicles of the Golgi-complex were labelled to a lesser extent. In pyramidal neurons, the dendritic-tree – including the dendritic spines - possessed the receptor. The immunolabel was also detected in association with the cell membrane and post-synaptic specializations. Oligodendroglia and

astrocytes were immunopositive, too. The foot-processes of astrocytes located around blood vessels also exhibited ER- ß immunoreactivity.

The findings indicate that ER-ß may transmit the humoral signal of estrogen via both the traditional genomic mechanisms and membrane-coupled regulatory channels. The ultrastructural results support the view that the receptor may be involved in the mechanisms of synaptic plasticity, learning and memory.

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DEVELOPMENTAL CHANGES IN MEMBRANE PROPERTIES AND EXCITATORY POSTSYNAPTIC CURRENTS OF MORPHOLOGICALLY IDENTIFIED MOSSY CELLS OF THE RAT DENTATE HILUS

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Hilar mossy cells are known to be one of the most vulnerable cell type in the hippocampus. Brief periods of ischaemia or kainic acid (KA) treatment cause hilar cell loss, including mossy cells, in the adult rats. In contrast, hilar neurons of the immature hippocampus, before the 15th postnatal day are resistant to kainate-induced cell death *in vivo*. In recent experiments we tested whether this age-dependent difference of mossy cell death could be found *in vitro*, and whether presumed changes of the intrinsic membrane properties or the increase of the excitatory synaptic input, associated with the development, could account for this vulnerability. Intracellular and whole cell patch-clamp recordings were made in three age groups (PD7-8, PD 12-14 and PD 22-24) of rat hippocampal slices. Mossy cells (n=29) were identified by morphological confirmation of biocytin labeling and distinct electrophysiological characteristics different from other cell types of the hilus and CA3 pyramidal region. Exposure to mossy cells of age PD12-14 (n=5) for kainic acid (500 nM) promoted spontaneous firing first with low rate and followed by a frequency increase, and these cells were considerably stable during KA application. In contrast, progress of kainate-induced excitotoxicity was rapid in mossy cells of age PD22-24 (n=5) as indicated by high frequency bursting, baseline depolarization and more often complete block of firing and evoked responses. Input resistance of mossy cells decreased markedly from PD12-14 to PD22-25. Mossy cells at all examined postnatal ages were capable of generating (TTX-sensitive) action potentials with similar amplitudes and gradual decrease of duration with the age. The mean frequency of the CNQX-sensitive spontaneous excitatory postsynaptic potentials (sEPSPs) increased more than eightfold during development associated with a significant increase of mean amplitude of sEPSPs, indicating a developmental increase of synaptic strength at mossy cell-MF synapse. Interestingly, transient potentiation of sEPSPs elicited by short trains of depolarizing current pulses could be evoked at all age groups. Taken together, our findings provide direct evidence of age related difference of mossy cell vulnerability for KA-treatment. We propose that the maturation of intrinsic properties of the

mossy cells contribute to the age-dependent increase of their vulnerability after the 15th postnatal day.

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INCREASED C-JUN EXPRESSION IN NEURONS AFFECTED BY EXPERIMENTAL DEMYELINATION IN RATS

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The aim of the study was to assess the expression of protooncogen c-jun in neurons after focal demyelination in the distal region of their axonal projection. Demyelination was induced by local injection of lysolecithin (*i*) into the pontocerebellar and (*ii*) into the septohippocampal pathways. Six days following injections the expression of c-jun has been visualised by immunohistochemistry. Unilateral, lesion specific protooncogene expression was observed in neuronal cell nuclei of the pontin nucleus and the vertical and horizontal limbs of the nucleus of the diagonal band. Lysolecithin injection into the inferior cerebellar peduncle elicited moderate c-jun expression in the contralateral inferior olive. Lysolecithin evoked demyelination induces selective of c-jun expression in the perikarya of the affected neurons. The activation of c-jun gene caused by demyelination could represent one step in a complex process of neuronal response which may lead to either apoptosis or to the expression of factors involved in induction of remyelination.

ASTROCYTES AND NEURAL PROGENITORS: CELLULAR INTERACTIONS *IN VITRO* AND *IN VIVO*

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Evidences of the last decade indicate that neuron-production in the CNS is sustained for the whole life of mammals. In the forebrain, the subventricular zone (SVZ) contains proliferating progenitors which can produce both neurons and astrocytes and provides neuronal precursors mainly for the olfactory bulb, dentate gyrus (1) and the associative cortex (2). The phenotype of such cells, however, seem to be restricted to the type of small, local interneurons. The question was raised whether the phenotypic restriction was due to the

narrowed potential of "adult" progenitors, or it is determined by the milieu of the mature brain.

As an approach, embryonic neuroectodermal (NE-4C) progenitor cells with nonrestricted developmental potential were implanted into the forebrain of adult mice and were co-cultured with astrocytes. NE-4C neuroectodermal progenitor cells (3) had been cloned from the forebrain vesicles of 9-day old mouse embryos. The continuously proliferating cells develop into Golgi I or Golgi II-type neurons, and later on, to astrocytes, if induced by retinoic acid. Green fluorescent protein or galactosidase enzyme-expressing subclones of NE-4C were established and used to study the interactions with primary brain cells. In co-cultures with fetal or perinatal astrocytes, NE-4C cells produced neurons without any exogenous inducer, The labeled progenitors survived also in cultures of differentiated astrocytes, but neuron-formation was not detected. Implanted GFP-labeled NE-4C progenitors survived and migrated in the forebrain, similarly to the progenies of the intrinsic "adult" progenitors. Principal-like neurons developed only inside of self-aggregates. According to the data, the fate of neural progenitors are governed mainly by the milieu and the cellular interactions provided by the host tissue.

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FUNCTIONAL REORGANISATION OF THE EPILEPTIC HUMAN DENTATE GYRUS

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In order to shed light on alterations in inhibitory processes, the distribution, size, dendritic morphology, and synaptic connections of calbindin-, calretinin-, parvalhumin- and substance P receptor-immunostained interneurons and fibres have been examined in control and epileptic human dentate gyrus. In the epileptic dentate gyrus, calbindin-containing interneurons are preserved, but their dendrites become elongated and spiny, and several cell bodies appear hypertrophic. The relative laminar distribution of calretinin-containing cells did not change, but their number is considerably reduced. The calretinin-positive axonal bundle at the top of the granule cell layer originating from the supramammillary nucleus expanded, forming a dense network in the entire width of stratum moleculare. The number of parvalhuminimmunostained cells is reduced, their termination pattern is changed, their boutons more frequently synapse on axon initial segments of granule cells than in control.

Substance P receptor-immunopositive cells are partially lost in epileptic samples, and in addition, the laminar distribution and dendritic morphology of the surviving cells differ considerably from the controls. Their synaptic input is also changed. The extent of individual pathological abnormalities correlates with each other in most cases. Our data suggest that, although a large proportion of inhibitory intemeurons is preserved in the epileptic human dentate gyrus, their distribution, morphology and synaptic connections differ from controls considerably. These functional alterations of inhibitory circuits in the dentate gyrus are likely to be compensatory changes with a role to balance the enhanced excitatory input in the region.

DISTRIBUTION OF VERTEBRATE-TYPE Ca^{2+}-BUFFER PROTEINS IN THE NERVOUS SYSTEM OF *EISENIA FETIDA*

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In this study we investigated the localization of four Ca^{2+}-binding proteins (or their homologs) in the nervous system of earthworm *Eisenia fetida*. In vertebrates three of them, calbindin, calretinin and parvalbumin, are found to be typical neuronal markers whereas S-100 protein is known as a glial constituent. Only two of the four proteins (calretinin and S-100) can be found in the cerebral ganglion of the earthworm, while parvalbumin was present in the ventral cord ganglia. Previous and the present experiments for detecting S-100 protein (or its homologs) in the nervous system of invertebrates utilized polyclonal antibodies produced against S-100 protein of vertebrates. It is now obvious that an S-100-like protein exists also in invertebrates, however, not in glial but in neuronal elements of the nervous system. This complementary location of S-100 proteins in case of vertebrate and invertebrate nervous system raised new questions. As for the distribution we found that the S-100 immunopositive cells are located mostly in the superficial-dorsal layer of the cerebral ganglion previously described as "small cell layer". Parvalbumin and calretinin immunopositive cells were found in small number in the ventral cord ganglia and calretinin immunopositive cells were found in the "small cell layer" of cerebral ganglion. There are only few clues regarding intracellular functions of calretinin, parvalbumin and S-100 protein (or their homologs) but it is certain that S-100 is able to bind the largest amount of Ca^{2+}. Therefore now we can include S-100 among the neuronal Ca^{2+}-buffer proteins in invertebrates.

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CENTRAL CONNECTIONS OF THE LATERAL VESTIBULAR NUCLEUS IN THE RAT

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The dorsolateral part of the brainstem contains the four vestibular nuclei receiving fibers from the labyrinthine organs. Although numerous studies were performed to elucidate the different functions of individual vestibular nuclei, our knowledge is limited in many aspects about their role played in the maintenance of equilibrium. The functional differences of these nuclei can be related to their different connections with the brainstem and spinal cord structures.

The neurobiotin was injected into the lateral vestibular nucleus (LVN) in order to study its ascending and descending projections. The following areas of termination could be discerned: (1) In the diencephalon the labeled fibers were detected bilaterally mainly in the ventral posteromedial nucleus of the thalamus. (2) At the level of the mesencephalon the terminals were found bilaterally in those areas which are involved in the eye movements. Labeled terminals were also detected in the red nucleus. (3) At the level of the rhombencephalon the other three vestibular nuclei received fibers bilaterally. A large number of fibers terminated in the nucleus of prepositus hypoglossi, nucleus of solitary tract, inferior olive and the dorsal part of the reticular formation. (4) The descending fibers could be followed mainly in the ipsilateral anterior funiculus of the spinal cord as far as the sacral levels. The terminals were distributed in the Rexed laminae V, VI, VII, VIII, and IX. Some of the descending fibers were found in the lateral and posterior funiculi. The retrogradely labeled cells were found in the following areas: vestibular nuclei, reticular formation, contralateral inferior olive, central cervical nucleus and nucleus intermediomedialis of spinal cord.

Our findings are in good agreement with the results of the physiological experiments.

This work was supported by FKFP 025/1999 and OTKA T 020257 grants.

MIGRATION OF MULLER GLIAL CELLS ON EXTRACELLULAR MATRIX: DYSTROPHIN IMMUNOREACTIVITY AND MOTION ANALYSIS BY VIDEOMICROSCOPY

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Absence or faulty expression of dystrophin protein leads to the syndrome known as Duchenne muscular dystrophy (DMD). DMD is mainly characterized by abnormal structure of neuromuscular junctions, however it might also be coupled with cognitive impairment and abnormal retinal functioning.

The dystrophin gene (DMD gene), located on chromosome X, gives rise to a number of dystrophin proteins (Dps) with different molecular masses, synthesized in various cell types. Little is known about the physiological function of these isoforms.

In the retina, Muller glial cells play an important role in metabolic and ion homeostatic processes associated to neuronal processing of light stimuli mainly due to their cell-cell interactions.

In this study, we used retinal primary Muller glial cultures as a model system. We examined cells' interaction with extracellular matrix molecules via videomicroscopic motion analysis and subsequent immunocytochemical staining. We used monoclonal antibody to localize one of the isoforms of the 71 kDa dystrophin proteins (Dp71f).

We observed that laminin-covered surface promotes adhesion and subsequent motility of cells. In cells identified by videomicroscopic motion analysis, the localization of Dp71f has distinct patterns. In resting cells, immunoreaction has symmetric distribution. In rapidly migrating cells however, no Dp71f staining is observed at the leading edge, whereas the strongest staining is seen in the perinuclear area and the uropodium. In all cases, Dp71f immunoreaction has a dot-like pattern. In double-labeling experiments, we observed Dp71f molecules associated to actin stress filaments, whereas others remained non-associated.

Findings published here as well as our future studies on other cell surface receptors (such as integrins) will contribute to a molecular level understanding of adhesion and migration of cells.

This work has been supported by the grants FKFP 0672/1997 and OTKA T030330.

HYPOPHYSIOTROPIC THYROTROPIN-RELEASING HORMONE-SYNTHESIZING NEURONS OF THE HUMAN HYPOTHALAMUS ARE INNERVATED BY AXONS CONTAINING NEUROPEPTIDE-Y, α MELANOCYTE-STIMULATING HORMONE AND AGOUTI-RELATED PROTEIN

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We have recently demonstrated that three arcuate nucleus derived peptides, neuropeptide Y (NPY), agouti-related protein (AGRP) and alpha melanocyte-stimulating hormone (α-MSH) are contained in axon terminals that heavily innervate hypophysiotropic TRH neurons in the rat brain and may contribute to the altered setpoint of the hypothalamic-pituitary-thyroid axis (HPT) axis during fasting. To determine whether a similar regulatory system exists in human brain, we performed a series of immunohistochemical studies using antiserum against NPY, AGRP, α-MSH and TRH in adult hypothalami obtained within 15 h of death. A similar distribution pattern was found for NPY- and AGRP-IR neurons in the arcuate nucleus, whereas α-MSH-IR cells appeared to form a separate cell population. By double-labeling fluorescent immunohistochemistry, 82% of NPY neurons co-contained AGRP, and 87% of AGRP neurons coexpressed NPY. No colocalisation was found between α-MSH- and AGRP-IR neurons. Both NPY- and AGRP-containing axons densely innervated the hypothalamic paraventricular nucleus, embedding TRH-synthesizing neurons in a rich fiber network. α-MSH-IR beaded fibers were also present in the paraventricular nucleus, but mainly in the lateral portions. NPY axon varicosities were found in close juxtaposition to approximately 80% of TRH-synthesizing cell bodies and dendrites and particularly prominent at the anterior and mid-level of the PVN. AGRP-IR varicosities were juxtaposed to approximately 64% of TRH, and in some instances, enveloped these neurons in a fiber basket outlining the cell surface. α-MSH-IR fibers were closely apposed to 75% of TRH-positive cells and dendrites, primarily at the mid-level of the PVN.

These studies demonstrate that the NPY-, AGRP- and α-MSH-IR neuronal systems may be involved in regulating the HPT axis in the human brain.

A KINETIC ANALYSIS OF ACETYLCHOLINE- AND CHOLINE-ACTIVATED α7-CONTAINING NICOTINIC RECEPTOR CHANNELS IN HIPPOCAMPAL NEURONS

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At the neuromuscular junction, nicotinic receptor (nAChR) activity is terminated primarily by hydrolysis of acetylcholine (ACh) into choline and acetate. The recent finding that choline acts as a full agonist of α7-like nAChRs, however, suggests that the kinetics of activation and inactivation of α7-like nAChRs exposed to ACh and choline may play a major role in controlling synaptic functions mediated by these nAChRs. In the present study, the interactions of choline and ACh with α7-like nAChRs were analyzed. To this end, the whole-cell and the outside-out modes of the patch-clamp technique were applied to rat hippocampal neurons in culture. The time-to-peak and the decay phase of whole-cell currents evoked by ACh were similar to those of currents evoked by equieffective concentrations of choline; the currents only differed in their decay to the baseline after removal of the agonist. The conductance and mean open time (τ_{open}) values of α7-like nAChR channels activated by choline and ACh were similar. However, the recovery of α7-like nAChRs from desensitization was faster when choline was the agonist both in whole-cell and outside-out patches. Thus, the predominant difference between the two agonists is in their affinity for α7-like nAChRs, not in the gating process. The process of desensitization was further analyzed in neurons under whole-cell configuration using two U tubes. At 10–40 μM ACh and 100–300 μM choline, the desensitization vs. cumulative charge flow relationship was close to linear. At high agonist concentrations, however, it deviated from linearity, and the deviation was not due to agonist-induced block of nAChR channels. We evaluated these results in terms of kinetic models for α7-like nAChRs. This suggests the existence of a fast alternative pathway towards desensitized state, which is only activated at high concentrations of agonists. According to our findings, the total charge flowing through α7-like nAChRs is higher with moderate agonist concentrations than with high agonist concentrations. Our results support the concept that perisynaptic α7-like nAChRs can respond well to agonists diffusing from the synaptic cleft, and that synaptic and/or perisynaptic levels of choline are likely to control the activity of native α7-like nAChRs.

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FUNCTIONAL SYNAPTIC PLASTICITY OF THE GABAERGIC INNERVATION TO THE PVN

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Stress cascade is initiated by a discrete group of neurons localized in the hypothalamic paraventricular nucleus (PVN). Inhibition of the hypothalamo-pituitary-adrenal (HPA) axis to limit the magnitude and duration of stress responses is accomplished by the glucocorticoid negative feedback and/or by inhibitory neuronal inputs originating from extrahypothalamic glucocorticoid-receptive sites. Hippocampus, which contains the highest level of type I corticosteroid receptors mediates tonic inhibition to the HPA axis. Limbic inputs are relayed to GABAergic interneurons in the bed nucleus of stria terminalis, preoptic area and local hypothalamic circuitries that provide inhibitory signals to the PVN. Pharmacological and electrophysiological evidences have been accumulated favouring the role of local GABAergic inputs in regulation of stress-related neurons in the PVN, the morphological basis however not fully characterized at ultrastructural level.

Our aim was to identify GABAergic synapses on CRH secreting neurons and to characterize the functional impact of this innervation. Serial ultrathin sections from the dorso-medial parvocellular subdivision of the PVN were examined using combination of pre-embedding immunocytochemistry for CRH and postembedding immunogold reaction for GABA. In intact animals for the medial parvocellular subdivision of the PVN 9730 GABAergic synapses per mm^3 of tissue were calculated using the dissector method. 3/4 of these contacts were axo-dendritic, 1/4 were axo-somatic type. 79% of the all GABAergic boutons in the medial parvocellular subdivision were terminated on CRH neurons. The number of GABAergic synapses, as well as the size of the boutons significantly increased seven days after adrenalectomy although the ratio of GABAergic inputs to the CRH-positive profiles was not changed. Dexamethasone treatment resulted in 38% increase of the number of GABAergic synapses in the medial parvocellular subdivision.

These ultrastructural data provide the morphological basis for GABAergic control of the HPA function.

TIN AND CADMIUM EFFECTS ON GABA AND GLUTAMATE ACTIVATED ION CURRENTS OF *LYMNAEA STAGNALIS* NEURONS

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The goal of this investigation was to determine whether toxic heavy metals alter the GABA and glutamate activated membrane processes.

The acute effect of tin and cadmium have been studied on GABA and glutamate activated responses and ion-channels on identified neurons of *Lymnaea stagnalis* L. Current clamp and two microelectrode voltage clamp techniques were applied. Heavy metals (trimethyltin chloride - $(CH_3)_3SnCl$, tin chloride - $SnCl_2$, and cadmium chloride - $CdCl_2$) were added directly into the external solution.

In case of semi-intact preparation application of inorganic tin modified the biphasic response of GABA, and elimination of GABA effect was observed after $CdCl_2$ application.

In voltage clamp experiments tin was less effective even in higher concentration then cadmium. Cadmium proved to be an effective chloride channel blocker in higher concentration. Biphasic effect was detected by low Cd^{2+} concentrations. Adding of Cd^{2+} in lower concentration resulted proportionally lower inhibition after a slight potentiation of the ligand activated ion current.

The results suggest that toxic metals modify the effect of transmitter by specific inhibition or potentiation of ion permeability of the neuron membrane. In the mechanism of the effect both the binding of transmitter molecule, and the channel gating (activation and inactivation processes) can play a role. These alterations have an effect in the activity of the neuron, in the relations between neurons and in the control function of the central nerve system.

FINE-FIBRED POLYSEGMENTAL GABA-IMMUNOREACTIVE INTERNEURONS IN THE CENTRAL NERVOUS SYSTEM OF *LUMBRICUS TERRESTRIS*

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Putative inhibitory interneurons, both perikarya and nerve fibres, were identified by means of GABA immunocytochemistry in the ventral nerve cord ganglia of the adult specimens of *Lumbricus terrestris* (Annelida, Oligochaeta). Small groups of strongly-stained neurons are situated at the dorsolateral parts of all ganglia behind the first segmental nerves. Each group consists of 3 neurons that send their axon ramifications, in both the ipsi- and contralateral direction, to the dorsomedial- (DM) and dorsolateral- (DL) interneuron tracts (ITs) and to a distinct part of the medial fibre bundle lying between the large interneurones and medial dorsal giant axons. The longitudinal fine-fibred GABA-IR interneuron tracts run without interruption from the terminal to the cerebral ganglion and their fibre number remains the same in all ganglia, suggesting that their organisation is syncytial. Some GABA-IR axons come into close proximity to the axons of the large segmental interneurones moreover axons and collaterals of the giant motoneurons which cross the neuropile on their way to the contralateral segmental nerves. Further close connections between GABA-IR nerve fibres and nerve processes of medial and lateral dorsal giant axons were also occurred. These findings strongly suggest that the above described fine-fibred polysegmental interneuron tracts have an inhibitory effect on giant axon systems, large interneurons and giant

motoneurons thus they can act as specific interneuronal inhibitory pathways controlling both locomotion and escape reflexes of the earthworms.

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PERIPHERAL PATHFINDING OF A VENTRAL UNPAIRED MEDIAN NEURON BY EXTRACELLULAR LUCIFER YELLOW FILLING

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In this study an extracellular iontophoresis of Lucifer Yellow CH (LY) has been applied to the cut end of the 3rd-6th segmental nerve of the terminal ganglion of *Porcellio scaber* (Crustacea, Isopoda) to trace possible targets of a ventral unpaired median (VUM) neuron. The exact anatomy of the filled neuron and its terminals in the peripheral targets were compiled from the results of *in situ* observation and investigation of the serial sections of tracer-containing samples. A VUM-neuron, located at the border of the eighth thoracic ganglion and the first abdominal neuromere, was constantly traced via one of the finest medial rami of the 3rd-6th segmental nerve of the terminal ganglion. This neuron was always brightly labelled with LY and the side branches of its intact axon was also traceable not only in the terminal ganglion and its contralateral segmental nerve but also in peripheral organs. Two collateral processes of the intact axon were identified: one of them entered the hindgut and sent several transversal processes to its muscular layer, while the second one innervated the musculature of the uropod exopodit. Our results show that extracellular LY backfilling could also be a suitable method to identify peripheral targets of a neuron that has symmetric projections to the periphery.

This study was supported by the Hungarian National Research Fund (OTKA No. T 026652) and the Adaptation Biology Research Group of Hungarian Academy of Sciences and University of Pécs.

GLUTARALDEHYDE-RESISTANT NADH-DIAPHORASE CONTAINING NEURONAL STRUCTURES IN THE CENTRAL NERVOUS SYSTEM OF *PORCELLIO SCABER*

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A special form of the NADH-diaphorase was found in the CNS of the wood louse *Porcellio scaber* (Crustacea, Isopoda) which proved to be insensitive against strong, long-term glutaraldehyde fixation (2.5% glutaraldehyde in 0.1 M phosphate buffer, for 24 hours at 24 °C). The activity of the enzyme was demonstrated by a standard incubating solution (freshly

prepared mixture of 2.5 mg Nitro Blue Tetrazolium, 10 mg β-NADH and 10 ml 0.1 M phosphate buffer). In control experiments the substrate was omitted from the incubating solution or replaced by α-NADH. No staining of samples occurred in controls.

Staining with β-NADH gives Golgi-like staining in neurons thus even the finest fibres of stained cells can be followed not only in sections but also in whole mounts. Though some unexplained inconsistencies were revealed in this technique e.g. intensity of staining was heterogeneous and variable signal-to-noise ratio occurred in samples even if they derived from the same incubation, a consistent, distinct neuron group could always be identified with this method. The distribution pattern of labeled neurons did not correspond to the occurrence of any single neurotransmitter or neuroactive molecule system thus the function and physiological significance of this glutaraldehyde resistant NADH-diaphorase still remained unknown.

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DIMENSIONAL REDUCTION ASSOCIATED WITH THE OCCURRENCE OF THE P3 EVENT-RELATED POTENTIAL COMPONENT IN HUMANS AND IN CATS

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The analytic algorithms derived from non-linear deterministic models may be more sensitive to differences in physiological data than those based on linear stochastic models. Among the non-linear algorithms the time-dependent dimensional ones appear to be the most sensitive discriminators. In the present study event-related auditory potentials (ERPs) were analyzed with the point correlation dimension (PD2) method in humans and in cats using the classical oddball-paradigm. Subjects had to press a button upon the occurrence of a rare stimulus which in the animal experiments was reinforced with an unavoidable shock.

Significant PD2 decrease was found to accompany the occurrence of the P3 wave the pattern of which in the human studies showed characteristic regional and task-dependent differences. In the animal studies the PD2 decrease was most prominent in the auditory cortex while in the dorsal hippocampus the pattern of PD2 change was clearly different.

The PD2 decrease – corresponding to a decrease in the dimensional complexity of the analyzed signal – is interpreted as a sign of increased cooperativity among the areas involved in the processing of the meaningful stimulus.

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EFFECT OF ETHANOL-ADAPTATION AND WITHDRAWAL ON CALCIUM-HOMEOSTASIS OF NEURONS

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Cellular mechanisms underlying neuronal adaptation to ethanol are only partially understood. To answer the question whether physical dependence can develop in cultures of neurones and for investigation of the neuronal processes involved in development of alcohol-dependence and withdrawal symptoms, cultures of cortical neurones were adapted to alcohol. Morphological characteristics of neurones were not altered during the chronic (3-day) repeated (once per day) ethanol (50-100 mM) treatment, mM treatment, whereas obvious signs of neuronal damages such as granulation, shrinkage as well as disintegration of neuritic processes and cell bodies were seen after the following 24 hours of alcohol-withdrawal. The extent of the damage, quantitated by measuring the release of lactate dehydrogenase (LDH), was dependent on the concentration of ethanol during the adaptation period. LDH-release induced by alcohol-withdrawal was significantly reduced by re-addition of ethanol, as well as by administration of the L-type voltage sensitive calcium-channel blocker nimodipine (10 μM) or NMDA receptor antagonists (MK-801, 1 μM or threo-ifenprodil, CP-101,606, 10 μM), whereas the effect of the $GABA_A$ receptor agonist muscimol (10 μM) was not significant. Withdrawal of the chronic ethanol treatment potentiated the NMDA induced neurotoxicity, and the inhibitory effect of acute alcohol on NMDA induced LDH-release in alcohol-treated cultures was stronger then in control cultures. In relation with these observations, continuous, but marginal increase in basal intracellular Ca^{2+}-level or in NMDA-induced Ca^{2+}-mobilisation was detected during alcohol-adaptation, whereas it was considerable (222% and 164% of initial value respectively, $p<0.005$) after withdrawal.

According to these results, i) the phenomenon of alcohol-dependence could be observed at the level of neurones; and ii) changes in calcium-homeostasis seem to play central role in development of ethanol dependence and in neurotoxicity induced by alcohol-withdrawal.

AMINERGIC REGULATION OF THE FEEDING BEHAVIOUR OF LOCUST

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The locust salivary gland (SG) contains dopamine (DA) and serotonin (5-HT) and the gland is directly innervated from the suboesophageal ganglion (SOG) by DA (SN1) and 5-HT

(SN2) containing neurons. These monoamines are supposed to be involved in the stimulation of excretion mechanisms in the SG. Based on the effect of DA and 5-HT on the saliva secretion from the isolated gland, and the stimulation of adenylate cyclase by DA and 5-HT, the presence of receptors of these transmitters is supposed.

The aim of our present work was to investigate the concentration of monoamines and their metabolites in the SOG and to monitor their changes in the course of the feeding behaviour. The presence and pharmacological properties of the D_1 and D_2 receptors in the SOG and in the SG were also studied.

According to our findings, the SG contains a considerable amount of DA (1.8-2.2 pmol/mg), N-acethyl-DA (20.4-26 pmol/mg) and 5-HT (0.5-1.2 pmol/mg), whereas trace amounts of N-acethyl-5-HT can be detected. From the start of feeding, the 5-HT content increases in both SOG (2.8-3.9 pmol/ganglion) and SG (0.5-1.2 pmol/mg). After 20 minutes of feeding, the DA and N-acetyl-DA contents are markedly enhanced in both the SOG (3.3-3.9 pmol DA/ganglion and 1.7-2.5 pmol N-acetyl-DA/ganglion) and SG (1.8-2.2 pmol DA/mg and 20.4-26 pmol N-acetyl-DA/mg). Investigating the binding properties of DA specific radioligands (^{3}H-SCH23390 and ^{3}H-spiperon), both D_1 and D_2 receptors of vertebrate type can be identified in the SOG, whereas D_1 receptor type occurred in the SG.

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IMPAIRMENT OF NEUROGENIC INFLAMMATION ELICITED SYSTEMIC ANTI-INFLAMMATORY RESPONSE IN EXPERIMENTAL DIABETES

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Neurogenic inflammation has been implicated in various pathological processes. More recently, it has been demonstrated that a primary neurogenic response triggers a systemic anti-inflammatory effect on a subsequent inflammatory challenge mediated by somatostatin of neural origin. The present work was undertaken to study the influence of 4- or 8-week streptozotocin-induced diabetes on the systemic anti-inflammatory action in rats.

The primary and the secondary neurogenic plasma extravasation were elicited by electrical stimulation (20 V, 0.5 ms, 5 Hz, for 5 min) of the left and right peripheral stumps of the cut sciatic nerves respectively, with a time difference of 5 min both in control and diabetic animals. The resultant plasma extravasation was quantified by using Evans blue dye accumulation method. In addition, somatostatin plasma levels at baseline and after bilateral sciatic nerve stimulation were measured by means of radioimmunoassay.

Our results show that (i) plasma extravasation due to the second stimulation was decreased by 52.7±3.1 % ($p<0.01$) in control animals, and by 29.7±2.2 and 18.1±1.5 % in 4- or 8-week diabetic rats, respectively, compared to values of the first excitation; (ii) bilateral sciatic nerve stimulation increased plasma somatostatin levels from 6.4±0.3, 11.7±1.4 and

16.8±3.8 to 28.3±2.9 ($p<0.01$), 17.9±3.7 and 25.1±1.7 pmol/l in normal and in 4- or 8-week diabetic rats, respectively.

The systemic anti-inflammatory response elicited by a primary neurogenic inflammation is impaired under diabetic condition. We conclude that the reduced extent of somatostatin release of neural origin may be responsible for the effect observed. The relevance of the increasing baseline somatostatin plasma level accompanying the development of the diabetic state is not yet clearly understood.

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EFFECT OF GABA ON THE CONCENTRATION OF INTRACELLULAR CA^{2+} AND SECRETION OF β-ENDORPHIN OF THE MELANOTROPE CELLS

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Freshly isolated melanotrope cells from both adult and 2-day-old pituitaries and whole intermediate lobes from adult rat pituitaries were investigated. Previous studies described two distinct types of GABAergic receptors on the intermediate lobe melanotropes: the $GABA_A$, and the $GABA_B$ receptors. In this study we tested the relative importance of both $GABA_A$ and $GABA_B$ receptors in the regulation of intracellular free calcium ($[Ca^{2+}]_i$) and β-endorphin secretion of the melanotropes. $[Ca^{2+}]_i$ was microspectrofluorimetrically measured by the Fura-2 method, secretion of β-endophin was measured by a superfusion system.

In the adult melanotropes, GABA resulted in a dose dependent (10^{-6}-10^{-4} M) and complex effect on $[Ca^{2+}]_i$: firstly a rapid increase then a long-lasting decrease that was often followed by a rebound effect. The long-lasting decrease of the hormone secretion was observed by superfusion, too. The selective $GABA_A$ agonist muscimol (10^{-6}-10^{-4} M) caused a robust increase and after it a smaller decrease, while the the selective $GABA_B$ receptor agonist baclofen (10^{-4}-10^{-6} M) markedly decreased $[Ca^{2+}]_i$. In the superfusion system both drugs decreased the secretion of β-endorphin. In contrast to this, GABA and muscimol in the young melanotropes elevated $[Ca^{2+}]_i$, while baclofen had a more complex effect.

We conclude that GABA plays a significant role in the regulation of the signal transduction and secretory processes of the melanotropes, but the mechanism of the regulation differs between the youg and the adult pituitaries.

EXPRESSION OF NEUROGENETIC AND REGION-SPECIFIC GENES DURING IN VITRO INDUCED NEUROGENESLS

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During neurohistogenesis, various atonal- and Ashaete-homologue neurogenetic genes are switched on and off, while region-specific positional genes are continuously expressed. The expression of positional and neurogenetic genes was investigated in the course of the *in vitro* neurogenesis by NE-4C embryonic neuroectodermal progenitor cells (1). Retinoic acid (RA)-induced neuron-production was followed by immunocytochemical methods (2), and the mRNAs of several neurogenetic genes (MASH-1, neurogenin 1, NeuroD, MATH-2) was checked in defined stages of neurogenesis by RT-PCR.

Non-induced NE-4C cells expressed *En* and *Otx* positional genes, indicating the anterior origin of the cell line. If induced by RA, the expression-pattern of positional genes changed: the amount of *Otx*-mRNAs decreased, *En*-mRNAs increased. The observation indicated that RA could exert porteriorising effects on isolated cells. Non-induced N-E-4C cells expressed several atonal-homologue genes: NeuroD, Math2 and neurogenin. The amount of the transcripts increased with the advancement of neuronal differentiation, and decreased if the proportion of the non-committed progenitors enhanced. Mash I-mRNAs were not detected in non-induced cells, but appeared on the second day of induction and increased up to the 4th day, when the first cells with neuronal phenotype appeared.

RA caused opposite changes in the expression of the forebrain-specific Otx and the neurogenetic gene, MASH1. The observation might indicate some causal relations between the positional and neuronal determination.

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ULTRASTRUCTURAL ALTERATIONS AND CHANGES IN CALCIUM HOMEOSTASIS IN IMMUNE-MEDIATED MODELS OF MOTOR NEURON DISEASES

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Sporadic amyotrophic lateral sclerosis (SALS) is the commonest motor neuron disease with degeneration of upper (cortical) and lower (ponto-bulbo-spinal) motoneurons. IgG from the sera of patients directing to Ca-channels injected intraperitoneally (ip) in mice caused increased vesicle number in motor axon terminals and in boutons synapsing on spinal motoneurons. Using the oxalate-pyroantimonate technique for calcium precipitation, these antibodies produced dose dependent calcium increases either in axon terminal synaptic vesicles and mitochondria, or in rough endoplasmic reticulum, mitochondria and Golgi complex of spinal motoneurons.

Multifocal motor neuropathy (MMN) is a motor syndrome that can closely mimic SALS. However, in this disease anti-GM1 ganglioside antibodies cause conduction block influencing sodium channels at the node of Ranvier. Serum samples from these patients injected ip. in mice did not increase the intraterminal vesicular volume, decreased the vesicular calcium content and had no influence on the content and distribution of calcium in the perikarya of spinal motoneurons.

The different effect of antibodies on the calcium homeostasis of motoneurons may explain the relative sparing of motoneurons in MMN.

EFFECT OF OPIOID PEPTIDES ON THE AP-1 DNA BINDING DURING SEXUAL DEVELOPMENT IN RAT HYPOTHALAMUS

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Several data indicate the role of opioid peptides in the regulation of GnRH secretion in the hypothalamus. Recent study using GTI-1 cells, an immortalized LHRH cell line, demonstrated that these cells express oestrogen receptor alpha (ERβ) as well. These observations, combined with our previous results, indicating that the oestrogen induced changes in the DNA binding of AP-1 proteins in the oestrogen sensitive areas of the hypothalamus can be affected by opioid peptides, provide the possibility of intracellular

cross talk between the opioid peptide and oestradiol receptor system at the transcriptional level in the GnRH secreting cells.

The aim of the present experiments was to investigate the effect of opioid peptide on the oestrogen induced changes in the binding of AP-1 proteins to DNA in oestrogen sensitive areas of developing rat hypothalamus.

Five-, 14- and 21-day-old female male and two-month-old ovariectomized rats were used. The animals were treated with D-met^2-pro^5-enkephalinamide (ENK) at a dose of 100 µg/100 g b.w. and 15 min later by oestradiol (OE; 10 µg/100 g b.w.) Binding of AP-1 proteins to DNA was determined by electrophoretic mobility shift assay (EMSA) after two hours of OE injection.

The changes in DNA binding of AP-1 proteins were age and sex dependent. In adult rats, on the effect of OE, in agreement with the previous data, the OE decreased the AP-1 DNA binding, which was augmented on the effect of ENK. In immature age groups of females an increased AP-1 binding was detected in both OE and OE + ENK treated groups.

In male hypothalamus, significant changes in the AP-1 DNA binding were found in the youngest, 5-day-old groups. OE administration resulted in a decrease, which was more pronounced by the concomitant administration of ENK.

As the results show, the sensitivity of AP-1 binding to DNA in the hypothalamus to both oestradiol and opioid peptides significantly differs during sexual maturation and the effects are sex dependent. We suppose that the observed changes might play role in the age and sex specific function of the hypothalamus.

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ENDOTHELIAL PROTEIN KINASE C ALTERATIONS INDUCED BY HUMAN AMYLOID ß PEPTIDE

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Vascular amyloid deposition resulting in cerebral amyloid angiopathy is widely considered to be a common feature of Alzheimer's disease (AD). Defects in postreceptor signalling pathways, including protein kinase C (PKC), have also been observed in AD. The first goal of this study was to examine whether the reduced PKC activity in the cerebral microvessels in AD is related to amyloid deposition. A further question that arises concerns which PKC isoform is reduced occurs by the action of amyloid ß peptide (Aß). Since the 40-amino-acid form of Aß (Aß1-40) is the predominant form of cerebrovascular amyloid, rat cortical endothelial cultures were treated with 20 µM human Aß1-40 for 2, 24 or 48 h. The PKC and PKCα levels in the control and Aß1-40-treated endothelial cells were analysed by Western blotting. Treatment with 20 µM Aß1-40 increased the cytosolic PKC and PKCα levels and reduced the membrane-bound PKC and PKCα immunoreactivities. The alterations involving

PKC and PKCα could be observed at 48 h after treatment. The reduction in the PKCα level in the membrane-bound fraction of the endothelial cells was much higher (21.1% of the control value) than the decrease in the membrane-bound PKC level (57% of the control value). The translocation of PKC and PKCα from the membrane to the cytosol suggests the down-regulation of these enzymes by Aß1-40. These findings indicate that vascular amyloid deposition may play a role in the decreases in the PKC and especially the PKCα levels and activities in AD. The similar changes in PKC and PKCα in cortical endothelial cultures and in the microvessels of AD patients underscore the possibility that study of this family of enzymes may be a useful means of investigating the cellular pathophysiology of AD.

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PARALLEL INVESTIGATION OF LIGHT INDUCED EXTRACELLULAR AMINOACID LEVEL CHANGES AND ERG IN THE RAT EYE USING *IN VIVO* EYECUP PREPARATION

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The retina is an excellent model for the investigation of neurochemical background of physiological and pathophysiological processes. Only a small number of citations can be found in the literature, where *in vitro* even the *in vivo* preparations were applied for neurochemical analysis using microdialysis or push-pull perfusion technique. All the interpretations of these data have been difficult since the vascularization is removed and the composition of the extracellular space, as well as the environment of the retina is dramatically changed and extended.

Here we describe a technique, the *in vivo* eyecup preparation, where the eye muscles are cut, the cornea, the iris, the lens and the vitreus are removed, but the major blood vessels of the eye remain untouched. The retinal cell's condition largely effects the extracellular space composition, so this preparation offers us a better and more suitable way to do neurochemical investigations. The main goal of this preparation is that it provides the possibility of parallel recording of ERG and neurochemical analysis using microdialysis probes. The looped shaped microdialysis probe is placed on the surface of the retina and superfused with Ringer's solution. The eyecup is fixed to a Teflon ring with six stitches or with surgical acril glue. The light stimuli come from a 3 mm diameter LED, which fit and fulfils the space of the eyeball. This technique also prevents the retinal environment from the extension. The recording electrode of the ERG is stick to the front of the LED, while the reference electrode can be either a plate electrode placed under the skin, or a simple Teflon coated stainless steel electrode touched to the outer wall of the sclera. The ground electrode is placed in the scull over the cerebellum.

We applied our technique to investigate the effect of light adaptation processes as the most physiological processes in the retina. Our results support the data known from the literature that the retinal taurin level is high in the microdialysis control (dark) samples

compared with other brain regions. The taurin, the glutamine and the alanin levels selectively decreased during light period. The ERG also showed the normal dark and light adapted waveforms. We can conclude that the *in vivo* eyecup preparation is a suitable technique to investigate neurochemical and electrophysiological processes in the retina.

SUPRAORBITAL VIBRISSAL TRANSECTION REVEALS SUBPOPULATIONS OF VIP-, AND NPY-IMMUNOREACTIVE PRIMARY AFFERENTS IN THE TRIGEMINAL BRAINSTEM NUCLEAR COMPLEX OF RATS

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Dissecting both supraorbital (SO) vibrissae in rats, the spatial appearance and temporal distribution of VIP-, and NPY-ir nerve fibers were investigated in the trigeminal brainstem nuclear complex (TBNC) postoperatively. VIP-immunoreactive SO vibrissal primary afferents appeared first on postoperative day 6 and terminated exclusively in the parvocellular layer of the caudal subnucleus of the spinal trigeminal nucleus (spVc). On postoperative day 10, two further VIP-ir SO vibrissal primary afferent fiber populations became appearent: One was found in the magnocellular layer, while the other was present around the border of lamina II and III of the spVc. One or both of these VIP-ir fiber populations gave rise to few collaterals to the interpolar subnucleus of spinal trigeminal nucleus (spVi) and to the trigeminal principal sensory nucleus (TPSN). On postoperative day 10, NPY-ir SO vibrissal primary afferents were present around the border of lamina II and III of the spVc, as well as in the spVi, and TPSN. Peripheral nerve transection results in an altered pattern of temporal distribution and spatial appearance of VIP and NPY expression in SO primary afferents of the TBNC. Based upon these results, the existence of at least four different subpopulations of SO vibrissal sensory neurons is suggested.

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NEURONAL PATHWAYS OF PAIN-EVOKED STRESSFUL STIMULI

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Noxious stimuli elicit pain sensation and also act as stressors. They activate spinal reflexes, supraspinal sensory-discriminative and motivational-behavior mechanisms, and evoke stress responses. Signals to these regulatory circuits are carried by different neuronal pathways. To localize them, two techniques have been applied: 1) Fos immunohistochemistry in combination with immunostaining for various types of neurotransmitters, and 2) anterograde (*Phaseolus vulgaris* leucoagglutinin) and transneuronal (viral) tract-tracing methods. Rats were subjected to various stressors like formalin-induced pain (4% formalin, s.c.), restraint, cold exposure, audiogenic stress, hemorrhage, insulin hypoglycemia. Stressors were applied both acute and chronic (repeated stress) conditions. In addition to pain-related spinal reflex loops, six ascending pathways can be localized (four of them are pain-related) which transfer neuronal inputs to the brainstem, hypothalamic, thalamic, limbic and neocortical areas. In contrast to other stressors, all of the pain-related stressful stimuli activate the central catecholaminergic system. Depending on the nature, the intensity and the duration of stressful stimuli, stress responses show certain specificity (inconsistent with Selye's "non-specific stress response" theory): different stressors use different output pathways and activate different humoral (hypothalamo-pituitary-adrenal axis) or neuronal mechanisms (hypothalamic and limbic projections to autonomic preganglionic neurons). In addition to specific stress responses, painful stressors activate brainstem neurons to inhibit pain at the spinal cord level.

CHANGES OF CORTICAL SENSORY POTENTIALS EVOKED BY A STIMULUS TRAIN IN RATS WITH SUBCHRONIC NEUROTOXIC EXPOSURE

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Human populations can be exposed to a variety of neurotoxic substances which points to the need of search for means of early detection of harmful effects. Sensory evoked potentials are readily recorded in experimental animals and in humans and have been shown to be sensitive of toxic effects. Our aim was to see if the fatigue seen during a series of cortical somatosensory evoked potentials is reproducible and sensitive to the substances used. Evoked potentials recorded earlier from rats, treated for 12 weeks with 5% ethanol in the drinking water, with low dose organophosphate (dimethoate) or heavy metal (lead or mercury) daily by gavage, or with both, were re-evaluated. The evoked potentials were recorded in urethane anesthesia with electric shocks (ca. 4 V, 0.1 ms, 1 Hz) delivered to the whiskery skin area as stimulation and potentials recorded from the exposed cortical surface

in the barrel field. The first and last five potentials from a series of 50 were averaged and latency and amplitude of the main wave components was measured. Of the parameters investigated, the latency of the wave components of the evoked potential showed negligible changes during a stimulus series and this was not altered by administration of the substances mentioned. There was, however, a measurable decrease in the amplitude between the first and the last evoked potentials - considered as fatigue - in each animal. In the control rats, the decrease was moderate and was also not much altered by alcohol treatment. In the rats given dimethoate, lead or mercury, however, the decrease was significantly stronger. Combination with alcohol increased the fatigue caused by lead but not that caused by dimethoate or mercury. Four weeks without dimethoate administration ("wash out") after the 12 weeks of treatment resulted only in a partial recovery.

This kind of evaluation showed that the fatigue of the sensory evoked potential, as defined here, can be calculated from simple measurements and seems to be sensitive to certain toxic influences. As sensory evoked potentials are also easily recordable in humans, measurement and calculation similar to that above can be a promising candidate for a functional biomarker of neurotoxic effect.

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IN VITRO EFFECTS OF HUMAN AMYLOID-BETA PEPTIDE (Aβ1-42) AND ITS FRAGMENTS ON THE VESICULAR ACETYLCHOLINE TRANSPORTER AND SYNAPTOPHYSIN IMMUNOREACTIVE AXON TERMINALS

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Acetylcholine (ACh) is synthesized by choline acetyltransferase and transported into the synaptophysin (SYN) containing synaptic vesicles by the vesicular ACh transporter (VAChT) in the cholinergic cell bodies and axon terminals. Both proteins are located in the synaptic vesicles, but only VAChT is characteristic for the cholinergic neurons. It is not known whether in neuronal tissue cultures the two synaptic proteins are expressed in the same developmental stage or not. In the etiology of Alzheimer's disease (AD), a neuropathological role has been attributed both to the Aß peptide and to the cholinergic system. The aim in the investigation was to demonstrate the *in vitro* effects of human Aß1-42 and its fragments on basal forebrain neuronal tissue cultures. After different periods of Aß treatment, the presence or absence of AChVT and SYN in the cholinergic cell bodies and their axon terminals were studied immunohistochemically. The results revealed that the SYN immunoreactivity appeared first in a large number of axon terminals. The VAChT immunoreactivity appeared later, in far fewer cells and far fewer nerve fiber terminals. The neurotoxic effect of Aß (20 μM) is very rapid and can be detected within the cholinergic

cells and their terminals. The disappearance of the VAChT immunohistochemical reaction preceded that of the SYN immunoreactivity.

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PLASTIC CHANGES IN THE ARCUATE NUCLEUS: HORMONAL EFFECTS

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Earlier studies have shown that sex steroids play a decisive role in neuronal development and plasticity by modulating the size, morphology and synaptic density of gonadal steroid-responsive structures in the central nervous system. In the arcuate nucleus of adult females a continuous cyclic synaptic remodelling takes place and more recently we have demonstrated that in ovariectomized animals estradiol treatment results in a decrease of GABAergic axo-somatic synapses.

In the present work we studied the changes in the numerical density of axo-dendritic synapses in the arcuate nucleus of ovariectomized adult female rats under different experimental conditions. Following estradiol injection we found a significant increase in non-GABAergic spine synapses, while the number of both GABA+ and GABA-synapses on dendritic shafts increased only moderately. In agreement with earlier electrophysiological data, the morphological observations indicate a shift in the balance of excitation and inhibition of arcuate neurons.

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A VIP AXONAL TERMINATION FIELD IN THE RAT MESENCEPHALON: INTERPEDUNCULAR NUCLEUS REVISITED

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Correlated light and electron microscopic immunocytochemical experiments were carried out for the localization of the neuropeptide vasoactive intestinal polypeptide (VIP) in the rat midbrain. In addition to areas described earlier such as the periaqueductal gray and median

raphe nuclei, a distinct VIP-immunopositive area was revealed in the dorsal part of the interpeduncular nucleus (IPN) at the mid-caudal level of the IPN, with a staining pattern completely different from that of the above-mentioned areas: this paired "cap-like" area had a very dense, diffuse VIP staining with no light-microscopically identifiable structures. The ultrastructural analysis revealed that the immunolabelled structures were mainly axons, however their arborization pattern showed a unique and intricate complexity: longitudinally sectioned axons run in the closely packed neuropil in a helicoid manner embracing dendrites, or other, immunonegative axon profiles. Their contours were irregular resembling rather astrocytic processes than bouton-laden axons. They often established very intimate glomerule-like connections with their postsynaptic targets, especially with small-calibre dendrites and vesicle-filled axon profiles: the multiple synaptic contacts were mainly symmetrical, however occasionally asymmetrical synapses also occurred. Synapses between VIP-ergic elements were not encountered. Since the studied area contained only a few VIP-immunoreactive perikarya, the elucidation of the origin and functional role of the dense axonal meshwork needs further studies.

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE-INDUCED HYPERTHERMIA. ROLE OF NEUROTRANSMITTERS

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Our laboratory recently reported hyperthermic effect of centrally administered pituitary adenylate cyclase-activating polypeptide (PACAP-38) in rats. The ability of the pyrazolone derivative, noraminophenazone to abolish the PACAP-induced hyperthermia suggested a possible role of cyclooxygenase products to play in this effect, however the mediation of the hyperthermia induced by PACAP remains unclear. The present experiments were undertaken to investigate the possible involvements of several neurotransmitters in the mediation of the PACAP-38-induced hyperthermia.

Intracerebroventricular (ICV) administration of PACAP-38 in a dose of 1 μg elevated the body temperature of male Wistar rats 2, 3, 4, 5 and 6 h after injection. Haloperidol-pretreatment (20-40 "4g/kg, IP) 30 min before peptide administration had a significant and dose-related diminishing effect on PACAP-38-induced hyperthermia, whereas IP pretreatment with a number of other inhibitors (bicucullin, 1 mg/kg; propranolol, 10 mg/kg; phenoxybenzamine, 2 mg/kg; atropine, 2 mg/kg) of neurotransmission did not modify the hyperthermic effect of PACAP-38.

These data suggest that dopaminergic transmission might participate in the mediation of the PACAP-38-induced hyperthermia, while the GABA-ergic, ?-and ?-adrenergic or cholinergic transmission is not involved in the hyperthermic action of PACAP-38.

EFFECT OF NEONATAL MATERNAL DEPRIVATION ON THE BEHAVIOUR OF ADULT RATS

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The hypothalamo-pituitary-adrenal axis (HPA axis) plays a central role in the stress response, and also participates in the control of the feeding behaviour. A number of central and peripheral components of the HPA axis regulates the energy consumption, as well as the quantity and quality of food intake. Our aim was to investigate how the alteration of HPA axis reactivity influences the feeding behavior.

For the alteration of the HPA axis reactivity we used a strong neonatal physiological and psychological stress, the maternal deprivation. We have already shown that this neonatal stress causes a long-term increase in the ACTH response to restraint, parallel to a long-term decrease in the food intake. The present study aimed to assess the influence of maternal deprivation on food choice. Maternal deprivation (MDEP) was performed by separating experimental litters from their mothers for 24 h on the postnatal day 5 or 14, at 35 °C and 40% humidity, while control litters were not separated. From the postnatal day 75, two experiments were parallelly performed. The nutrient choice was firstly analysed in a three-nutrient paradigm: the consumption of *ad libitum* carbohydrate, protein and lipid (with vitamins and minerals added) was measured. In a second set of experiments, the effect of a highly palatable, highly caloric "cafeteria" diet on body weight gain was studied in rats that were provided with *ad libitum* banana, liver pate, bacon, sweet biscuits, and three nutrients. At 125 days of age, animals were sacrificed, and weights of ependymal, perirenal and subcutaneous white adipose tissue measured. Body weights were measured twice or three times a week in both experiments.

The results showed that the nutrient choice was altered by maternal deprivation depending on the day of the separation. MDEP day 5 rats ate significantly less carbohydrate than controls (-21%), their lipid intake increased (+59%), and this was significant during the light period. Total energy intake was significantly decreased. MDEP day 14 rats did not differ from controls in carbohydrate (-3%), lipid (-1%) or total energy intake. Protein intake of the three treatments was similar. "Cafeteria" diet significantly increased the body weight gain of MDEP day 5 rats during the first 18 days of the experiment compared to controls, but remained equal to controls later. For the MDEP day 14 rat group, weight gains did not differ from controls. At the end of the experiment, subcutaneous fat tissue of MDEP day 5 rats weighed significantly more than that of control rats; no such difference was found for MDEP day 14 rats. Furthermore, the stress differently affected the HPA reactivity when it was performed oth the 5th or 14th day of the life. It restrictively prolonged the induced-release of ACTH in adult when it was performed on the 5th day.

In conclusion, as expected, the early stress modified both food intake and nutrient preference of the adult rats, but the effects depended on the time of the stress application.

When applied during the "hyporesponsive period" of the life (5th day after birth), the stress decreased CHO consumption and increased the lipid diet intake of the adults. It also enhanced the response to the highly palatable diet (cafeteria) by increasing the body weight gain. On the contrary, the stress became inefficient when it was applied later (14th day of life). We hypothetized that the decrease of the feed-back effect of glucocorticoids can be involved in the modifications of food preference as it was likely involved in the prolongation of the ACTH response.

NERVE INJURY-INDUCED CHANGES IN THE SOMATOSENSORY AND MOTOR SYSTEMS OF THE RAT

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It is well known that representations in the primary somatosensory (SI) and motor cortices (MI) can be altered by peripheral manipulations. In adults, for example, partial deafferentation of the face (infraorbital nerve transection) is followed by changes in the receptive fields of the neurons within the deprived portion of the VPM and SI. Similarly, partial deefferentation of the MI (unilateral transection of the facial nerve: n7X) results in ipsilateral vibrissal movements in response to intracortical microstimulation. The denervation-induced changes in both SI and MI may be considered to occur in at least two phases. The initial phase is probably due to reductions in cortical inhibition, and is generally referred to as the disinhibition or, „unmasking" of latent inputs. Our recent results suggest that n7X induces disinhibition in the MI in both hemispheres, a procedure mediated by the somatosensory system. All these data lead us to hypothetize a loop functioning between the somatosensory and motor systems (as parts of the CNS) and the muscles of the vibrissae (as effectors). Data observed recently suggest that injury of this loop induces immediate cortical disinhibition (tested by a double pulse stimulation protocol). During this early period of changes, subpopulations of neurons in related cortex areas displayed a reverse effect to GABA and muscimol (facilitation instead of inhibition) applied juxtacellularly, suggesting that denervation may cause GABA-A receptor-dependent disinhibition in related cortex areas.

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COLD-ADAPTATION VS. HYPERTHYROIDISM: METABOLIC AND THERMOREGULATORY RESPONSIVENESS

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Cold-adapted (CA) rats, as compared with non-adapted (NA) ones, give exaggerated metabolic responses both to acute cold exposure (the metabolic overshoot resulting in "paradoxical" rise of core temperature /T_c/ in the cold), and to central injection of prostaglandin E (PGE) (resulting in enhanced PGE-hyperthermia). These differences between CA and NA rats might possibly be explained by the high thermogenic capacity of peripheral tissues of CA animals. Alternatively, the central regulation may differ in CA and NA rats.

If enhanced tissue thermogenesis is indeed a crucial factor in inducing the characteristic metabolic responses of CA rats, similar reactions might be expected to occur in other hypermetabolic states, e.g., in hyperthyroidism.

NA rats were injected daily with 100 µg thyroxine s.c., for a period of 2 weeks (T_4 group). A metal guide cannula was implanted into the lateral cerebral ventricle (ICV) of each T_4 rat, as well as of non-treated NA and CA rats. Metabolic rate (MR) and T_c were measured at thermoneutrality (25, 30, and 20-25 °C, for CA, NA, and T_4 groups, respectively), during cold exposure (5 °C for CA and T_4, 15 °C for NA rats), or following ICV injections of 100 ng PGE_1.

The order for resting MR was: T_4>CA>NA, for resting T_c it was: T_4>NA>CA. Upon cold exposure, MR rose comparably in all rats, T_c rose ("paradoxically") in CA, but fell in NA and T_4 rats. PGE_1 induced a rise in both MR and T_c, in the order of CA>NA>T_4.

In conclusion, the large thermogenic capacity of CA rats, in itself, cannot explain the enhanced responsiveness to cold or PGE, adaptation-induced regulatory changes must be assumed.

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CYCLOOXYGENASE PRODUCTS ARE INVOLVED IN THE BRADYKININ-INDUCED SENSITIZATION TO HEAT OF RAT CUTANEOUS NOCICEPTORS, *IN VITRO*

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Bradykinin (BK) is known to sensitize nociceptors to noxious heat and to stimulate phospholipase A_2, but it is unclear whether the BK-induced sensitization involves *de nova* eicosanoid synthesis. This question was addressed by investigating the effect of two

enantiomers of the cyclooxygenase inhibitor flurbiprofen (flu): S-flu has a much higher affinity for cyclooxygenase than R-flu. Racemic flu (10^{-6} M) has been shown to abolish PGE_2 synthesis in the rat skin. Single unit activity was recorded from C-mechano-heat-sensitive fibers in the isolated rat skin-saphenous nerve preparation. BK (10^{-5} M for 5 min) induced an 1.95±0.37-fold increase in the number of spikes evoked by the noxious heat stimulus (a rise of temperature from 32 to 46 °C during 20 s) in a reproducible manner. S-flu (10^{-6} M) abolished the BK-induced noxious heat sensitization but did not alter the unconditioned heat response itself. Under R-flu (10 M) BK still induced heat sensitization which was non-significantly reduced (by 25±6%) while the unconditioned heat response was unaffected. The inhibition of the BK-induced heat sensitization by S-flu could partially (by 73±13%) be overcome by exogenously applied PGE_2 (10^{-5} M). It is concluded that i) the BK-induced nociceptor sensitization to noxious heat depends on cyclooxygenase activity in the rat skin, *in vitro;* ii) PGE_2 seems to be a likely but perhaps not the only important cyclooxygenase product involved; iii) R-flu may have a mild inhibitory effect on the sensitization independent of cyclooxygenase blockade and a similar additional action for S-flu cainot be excluded; iv) neither S- nor R-flu inhibit the unconditioned noxious heat response of the C-mechano-heat-serisitive nociceptors.

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PROPRIOSPINAL CONNECTIONS IN THE SUPERFICIAL SPINAL DORSAL HORN OF RATS

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The propriospinal connections of the medial and lateral subdivisions of the superficial dorsal horn differ from each other in many respect: (a) A 300-400 μm long section of the medial aspect of laminae I-IV projects to and receives afferent fibres from the gray matter of the adjacent spinal segments exclusively, whereas the same length of the lateral aspect of laminae I-IV projects to and receives afferent fibres from the entire rostrocaudal extent of the lumbar spinal gray matter. (b) The medial aspect of laminae I-IV projects extensively to the lateral aspect of the superficial dorsal horn, but, with the exception of a few fibres at the segmental level, does not receive afferents from this area. (c) There is a substantial direct commissural connection between the lateral aspects of laminae I-IV on the two sides of the lumbar spinal cord. The medial part of laminae I-IV does not establish any direct connection with the gray matter on the opposite side of the spinal cord. (d) The lateral aspect of laminae I-IV appears to be the primary source of fibres projecting to the ipsi- and contralateral ventral horns and to supraspinal brain centers. Terminals of the medio-medial, medio-lateral, and latero-lateral short projections form mostly asymmetric synaptic contacts, whereas the terminals of commissural fibres establish mostly symmetric synaptic appositions with the postsynaptic targets. Most of the terminals and their postsynaptic structures of all four

projection systems appear to be negative for both GABA and glycine, suggesting that volleys in the medio-medial, medio-lateral, and latero-lateral short projection systems may increase the level of excitation of spinal cord neurons. In contrast to this, the commissural fibres interconnecting the lateral subdivisions of the superficial dorsal horn may exert inhibition on neural circuits underlying sensory information processing in the spinal dorsal horn of rats.

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CARRAGEENIN, ZYMOSAN AND INTERLEUKIN-1β-INDUCED NEUTROPHIL ACCUMULATION IN NK_1 RECEPTOR KNOCK OUT MICE

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In this study we have used neurokinin 1 (NK_1) receptor knock out mice (-/-) and their wild-type controls (+/+) developed to learn more about the link between NK_1 receptors and neutrophil accumulation in normal naive skin, as compared to inflamed skin. We have determined a lack of role of the NK_1 receptor in interleukin-1β-induced neutrophil accumulation in normal mouse skin; but a contributory effect of added NK_1 agonists, which by themselves have no effect on neutrophil accumulation in normal skin. The results support the concept that NK_1 agonists such as substance P cannot act on their own to mediate neutrophil accumulation in naive skin. By comparison, the results provide direct evidence that in inflamed skin, under certain circumstances, the NK_1 receptor can play a pivotal role in modulating neutrophil accumulation during the ongoing inflammatory process. We have investigated the responses to two standard inflammatory stimuli (carrageenin and zymosan). Neutrophil accumulation was significantly attenuated in carrageenin-induced (but not zymosan-induced) inflammation in NK_1 knockout mice. The carrageenin-induced cellular response was inhibited by a NK_1 receptor antagonist, SR140333 in the wild-type group. Bradykinin receptor antagonists desArg^9[Leu^8]-bradykinin and Hoe 140 also attenuated the leukocyte accumulation to carrageenin in wild-type animals, but did not cause further reduction in the suppressed response of knock out mice. The results provide evidence that B_1 and B_2 kinin receptors participate in NK_1 receptor dependent neutrophil accumulation in inflamed mouse skin.

THE DISTRIBUTION OF GABAERGIC NEURONS AND ITS ALTERATION IN THE CENTRAL NERVOUS SYSTEM OF *PORCELLIO SCABER*

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The overview of the GABA system in the CNS of the woodlouse, performed by the aid of immunocytochemistry, demonstrated several distinct clusters in each ganglion. This cluster organisation was already obvious in the investigated juvenile age-groups, however, the position and size of the single clusters was modified by the post-hatching developmental alterations, up to the 5 months old stage, when the animals became mature and produced first the adult pattern. The majority of the strongly labelled large neurons had already been present in the early stages, while the number of the moderately labelled cells together with the area of the stained neuropile was gradually increased up to the maturation. As an exception, the number of the stained cells in the terminal ganglion scarcely changed. The consequent differences in the immunostaining show the presence of putative GABA-synthesising and GABA-sequestering cells. It may refer to an accurate regulation of GABA level, significance of which may be explained by the role of this substance in morphogenetic actions and synapse formation both in the developing and mature CNS. Compared to the investigated insect species, where almost all the GABA-immunoreactive neurons were present by hatching, in the woodlouse a large number of cells were seen to appear during the post-hatching development of juveniles. To reveal the background of this neurotransmitter expression pattern needs further studies.

This study was supported by the Hungarian National Research Fund (OTKA No. T 026652) and the Adaptation Biology Research Group of Hungarian Academy of Sciences and University of Pécs.

KAPPA-OPIOID RECEPTORS ON SOMATOSTATIN-CONTAINING INTERNEURONS IN THE RAT HIPPOCAMPUS

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Previous immunocytochemical studies revealed similarities between the distribution of somatostatin- and kappa opioid receptor-immunoreactive nerve elements of the rat hippocampus: both cell populations are the most numerous in the hilus of the dentate gyrus and in the stratum oriens of the CA1 area. The aim of this study was to reveal, whether the two immunoreactive cell groups represent distinct, fully or partially overlapping cell populations.

Pre-embedding immunocytochemistry was used on vibratome section pairs, alternately processed either with somatostatin antiserum (DiaSorin Inc.) or mab KA8 antibody

(Maderspach et al., 1991). On the surface of matching section pairs, the two halves of the cut cells were scrutinized for double labelling. The somatostatin immunoreactivity was restricted mainly to the soma of the neurons, whereas the mab KA8 stained the proximal dendrites as well. Double-labeled immunoreactive elements were found in the hilus, strata oriens and pyramidale of the CA1 area only in a low amount. In other layers no double-labeled structures were detected.

These results indicate, that there is only a restricted overlap between the somatostatinergic and kappa opioid receptor-immunopositive interneuron populations. Thus, further studies are needed to decide about the neuropeptide content and possible role of the rest of interneurons decorated with kappa-receptors in the hippocampal neuronal circuitry.

Reference

Maderspach et al. (1991) J. Neurochem. 56, 1897-1904.

KAPPA-OPIOID RECEPTORS IN THE RODENT HIPPOCAMPUS: A COMPARATIVE IMMUNOCYTOCHEMICAL STUDY IN THE RAT, MOUSE, HAMSTER AND GERBIL

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Pre-embedding light microscopic immunocytochemistry using a monoclonal antibody (mab-KA8) raised against a frog brain kappa receptor preparate recognising selectively the kappa opioid receptor (Maderspach et al., 1991) was used for studying the occurrence, distribution and species-specificity of the kappa opioid receptor in the hippocampal formation of four rodent species (rat, mouse, hamster and gerbil).

Mab-KA8 immunoreactivity was detectable in each studied animal, however the distribution of the labeled structures was found to be heterogeneous. In the rat and hamster the hilus of dentate gyrus and the stratum oriens of the CA1 area contained immunoreactive cell bodies and proximal dendrites. In the mouse the occurrence of the immunoreactive cells was restricted to the stratum oriens of CA1 area and all layers contained immunopositive elements resembling to microglial or oligodendroglial processes. In the gerbil mab-KA8 immunopositive cell bodies were recognisable in the stratum radiatum of the CA1 and CA3 areas. In the hamster varicose axon-like elements were also detected in the CA3 pyramidal layer. In each studied species the subiculum contained a remarkable amount of immunoreactive multipolar perikarya.

The results confirm the high degree of species-specific heterogeneity characterising the distribution of opioid peptides and their receptors in the hippocampal formation. The receptor was found in most cases postsynaptically, however the immunopositive varicose axons may refer to presynaptic localisation in the hamster.

Reference
Maderspach et al. (1991) J. Neurochem. 56, 1897-1904.

INHIBITION OF CHOLINESTERASES BY METRIFONATE DOES NOT CHANGE THE BLOOD-BRAIN BARRIER

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One of the theories concerning the etiology and pathogenesis of Alzheimer's disease (AD) is that a blood-brain barrier (BBB) dysfunction is the primary event of the disease.

Acetylcholinesterase (AChE, EC 3.1.1.7) is the enzyme responsible for terminating the action of acetylcholine (ACh) at the cholinergic synapses. The physiological function of the structurally related enzyme butyrylcholinesterase (BuChE, EC 3.1.1.8) is not known, but it may serve as a scavenger in the detoxification of certain chemicals. BuChE is present in high concentrations in the wall of all those microvessels equipped with a morphological BBB, and the involvement of BuChE in the BBB function has been suggested (Joó and Csillik, 1966, Joó et al., 1967). Therefore, the aims of the present study were: i) to determine whether chronic cholinesterase inhibition by MTF interferes with the synthesis of ACh, ii) to establish whether MTF exhibits any preference for the inhibition of AChE or BuChE, and iii) to investigate the effects of chronic cholinesterase inhibition on the BBB.

After treatment with the irreversible organophosphate cholinesterase inhibitor metrifonate (MTF) for 2 or 5 weeks (100 mg/kg daily i.p.), both the AChE and BuChE activities were substantially inhibited in the rat brain (by 60-80%). *In vivo* MTF treatment did not interfere with the synthesis of ACh, because it did not have a significant effect on the choline acetyltransferase activity. Damage to the BBB was assessed via the extravasation of Evan's blue dye (EB). EB was injected into the femoral vein of control and of 5-week chronic MTF-treated rats. Fluorometric quantitation of EB was performed 1 or 2 h after EB injection in 3 brain regions: the cerebral cortex, the hippocampus and the striatum. No extravasation of EB was found in the brain. Our data indicate that chronic MTF treatment has no effect on the BBB. Therefore, it is reasonable to hypothesize that the cholinesterase activity plays no role in the BBB permeability.

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CELL TYPE- AND SYNAPSE-SPECIFIC VARIABILITY IN SYNAPTIC $GABA_A$ RECEPTOR OCCUPANCY

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The benzodiazepine agonist, zolpidem, was used to investigate the degree of postsynaptic $GABA_A$ receptor occupancy. Zolidem is known to increase the affinity of receptors for GABA at room temperature (Perrais and Ropert, 1999, J. Neurosci., 19:578-588) leading to an enhancement of synaptic current amplitudes, if the postsynaptic receptors are not fully occupied by the released transmitter. We recorded mIPSCs from eight different cell types in three brain regions of rats and mice. Zolpidem (10-20 μM) prolonged the decay of all mIPSCs (151-184% of control), confirming that receptors in every cell type were benzodiazepine-sensitive. The amplitude of the $GABA_A$ receptor-mediated events was significantly enhanced in dentate granule cells, in CA1 pyramidal cells, in hippocampal GABAergic interneurons, in cortical layer V pyramidal cells, in cortical layer V interneurons, and in cortical layer II/III interneurons. An incomplete postsynaptic $GABA_A$ receptor occupancy is thus predicted in these cells. However, zolpidem had no significant effect on mIPSC amplitudes recorded from layer II/III pyramidal cells, suggesting full $GABA_A$ receptor occupancy. Moreover, we were able to find different degrees of receptor occupancy at distinct GABAergic synapses on a given cell. For example, the amplitude of only one type of the two distinct populations of zolpidem-sensitive mIPSCs recorded in olfactory bulb granule cells was significantly enhanced by the drug. Thus, at synapses that generate mIPSCs, postsynaptic receptor occupancy is cell type- and synapse-specific, reflecting local differences in the number of receptors or in the transmitter concentration in the synaptic cleft.

DELAYED SYSTEMIC ADMINISTRATION OF PACAP38 IS NEUROPROTECTIVE IN MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT

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Many substances have been shown to reduce brain damage in models of stroke, but mainly when given either before or shortly after the onset of ischemia. Delayed systemic administration of pituitary adenylate cyclase-activating poplypeptide (PACAP) has been shown to attenuate the neuronal damage in the hippocampus in a model of global ischemia in rats. The present study examined the neuroprotective action of delayed systemic

administration of PACAP38 in a model of transient focal ischemia produced by middle cerebral artery occlusion (MCAO) in rats.

PACAP38 was given as an intravenous bolus (20 nmol/kg body weight) followed by an intravenous infusion for 48 hours using a micro-osmotic pump at a rate of 160 pmol/μl/hour, beginning 4, 8 or 12 hours after a 2-hour transient MCAO using a filament model. The size of the infarct was determined by examining 2-mm-thick brain sections stained with triphenyltetrazolium chloride, followed by image analysis. Control animals received intravenously 0.1% bovine serum albumin in 0.9% saline as a bolus and infusion at the same time intervals.

The administration of PACAP38 beginning 4 hours after MCAO significantly reduced the infarct size by 50.88%. Treatment with PACAP38 starting 8 or 12 hours after the onset of ischemia did not result in a significant reduction of the infarct size, although infarct volumes tended to be smaller than in the control groups.

Systemic administration of PACAP38 should be clinically useful for reducing brain damage resulting from stroke even when administration is delayed for several hours.

NEUROCHEMICAL CHARACTERIZATION OF KIDNEY-REGULATING BRAINSTEM NEURONS IDENTIFIED BY PSEUDORABIES TRANSNEURONAL LABELING

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The participation of brainstem biogen amine-regulating neurons in the innervation of the kidney was examined by double-labeled immunohistochemistry using genetically engineered pseudorabies virus, Ba-DupLac, combined with the detection of cathecholamine or serotonin markers. Neurotropic viruses have a special virion host shut-off protein (which is an RNase) and other cytotoxic components, causing rapid degradation of the cellular components when they enter the cell. Therefore cellular mRNAs have been disrupted at the time when viral antigens appear in the neuron. Ba-DupLac was designed to express β-galactosidase at a very early period after penetration of the virus to the cell. This feature of the virus makes possible the detection of cellular mRNAs.

Infected cells in the ventrolateral medulla were identified using anti-viral, anti-β-galactosidase, and anti-tyrosine-hydroxylase (TH) antibodies. In these experiments TH colocalized with either PrV or β-gal antigens, and there was no significant reduction in the signal. Using *in situ* hybridization histochemistry, TH and serotonin-transporter (5-HTT)-containing virus-infected cells were identified in the A1, A5, A6 catecholaminergic cell groups and the nucleus raphe magnus and pallidus, respectively. With this technique - compared to the immunohistochemical detection - mRNAs of either TH and 5-HTT were

detectable only in β-gal positive infected cells, while PrV antigen positivity was not coupled with *in situ* hybridization signals of both mRNAs. This result confirms, that virally-infected neuronal cells can only be characterized by *in situ* hybridization at an early stage of infection.

DISTRIBUTION OF NO-ERGIC NEURONES AND POSSIBLE ROLES OF NO IN THE GASTROINTESTINAL TRACT OF PULMONATE GASTROPODS

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Presence of neuronal nitric oxide synthase (nNOS) has been well described in the myenteric plexus of several vertebrate species. Nitric oxide (NO) is known as a relaxation provoking agent at the smooth musculature of different segments in the vertebrate alimentary tract. Up to date, however, there is no data about a similar regulatory role of NO in the gut motility of lower phyla, such as Molluscs.

In our study, first, the distribution of nNOS was investigated in the gastrointestinal tract of Basommatophoran (*Lymnaea stagnalis, Planorbarius corneus*), and Stylommatophoran (*Helix pomatia, Cepaea nemoralis, Limax maximus, Arion subfuscus*) Pulmonate snails by enzyme histochemical localisation of NADPH-diaphorase (NADPH-d), a marker for nNOS. Secondly, the *ex vivo* effects of NO liberating chemicals [sodium nitroprusside (SNP), glycerine trinitrate (GT)] were tested on the isometric tension of different gut segments of *Helix*.

The NADPH-d reactivity appeared in the buccal and pharyngeal areas of all investigated species. Particularly, NADPH-d positive primary sensory neurones in the lips, nerve terminals on the radular epithelium and on the circular musculature of the prooesophagus were notable. Staining could be detected nowhere else in the rest of the alimentary tract of Basommatophoran snails. In *Helix*-like Stylommatophorans (*Helix* and *Cepaea*) a NADPH-d reactive plexus was found in the wall of caecum, numerous unipolar neurones and a rich neural network within. Circular musculature of the caecum was found to be innervated by NADPH-d reactive terminals. In *Limax*-like Stylommatophorans (snails with rudimentary domus) the demonstration this caecal plexus was failed by NADPH-d. SNP and GT in micromolar concentration range provoked dose dependent relaxation on K^+ elicited contracted circular musculature of the prooesophagus and caecum, and increased the tonus of the caecal longitudinal muscles during peristalsis, whereas had no effect on gaster.

Based on other studies and our observations it can be concluded, that NO feasible takes part in some molecular events of chemoreception, regeneration of the radular teeth, and the relaxation of the prooesophageal sphincter in all investigated Pulmonate snails. Moreover, in *Helix*-like Stylommatophorans NO was proved to be a relaxing agent in the caecum, which

motility has special importance in the transport of digestive enzymes and nutrition between the alimentary tract and the midintestinal gland. The lack of this kind of regulation of NO in other snails may be explained by some anatomical or functional differences between groups of Pulmonate snails.

MULTIPLE CELLULAR MECHANISMS MEDIATES THE EFFECT OF LOBELINE ON THE RELEASE OF NOREPINEPHRINE

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The complex effect of lobeline on [^{3}H]norepinephrine ([^{3}H]NE) release was investigated in this study. Lobeline-induced release of [^{3}H]NE from the vas deferens was strictly concentration-dependent. In contrast, electrical stimulation-evoked release was characterized by diverse effects of lobeline depending on the concentration used: while at lower concentration (10 μM) it increased the release, using high concentration (100 and 300 μM) the evoked-release of [^{3}H]NE was abolished. The effect of lobeline on the basal release was $[Ca^{2+}]_o$-independent, insensitive to mecamylamine, a nicotinic acetylcholine receptor antagonist, and to desipramine, a noradrenaline uptake inhibitor. However, lobeline-induced release was temperature-dependent: at low temperature (12 °C), when the membrane carrier proteins are inhibited, lobeline failed to increase the basal release. Lobeline dose-dependently inhibited the uptake of [^{3}H]NE into rat hippocampal synaptic vesicles and purified synaptosomes with an IC_{50} values of 1.19 ± 0.11 μM and 6.53 ± 1.37 μM, respectively. Lobeline also inhibited Ca^{2+}-influx induced by KCl depolarization in sympathetic neurons measured with the fura-2 technique. In addition, phenylephrine, an a_1-adrenoceptor agonist, contracted the smooth muscle of vas deferens and enhanced stimulation-evoked contraction. Both effects were inhibited by lobeline. Our data can be best explained as a reversal of the monoamine uptake by lobeline that gets support by the increased intracellular NE level after lobeline blocks vesicular uptake. At high concentration lobeline acts as a non-selective Ca^{2+}-channel antagonist blocking pre- and postjunctional Ca^{2+}-channels serving as a counterbalance for the multiple transmitter releasing actions.

ULTRASTRUCTURAL STUDY OF THE ROLE OF FCγRI RECEPTORS IN IMMUN-MEDIATED MOTONEURON INJURY

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Immunoglobulins from amyotrophic lateral slerosis patients passively transferred to mice have been shown to increase the number of synaptic vesicles and to raise intracellular calcium level in motor axon terminals. Similar changes could also be induced with injection of a number of antibodies against different motoneuronal compartments. These findings lead to the assumption that Fc-receptor mediated mechanisms might play a role in the above-mentioned IgG effect. To test this hypothesis, attempts were made to demonstrate the presence of high-affinity Fc receptor (FcγRI) in the spinal cord of wild type mice, furthermore, passive transfer experiments were performed with genetically modified mice lacking the γ subunit of the Fc receptor. In these FcR –/– mice the uptake of ALS IgG, as well as its ability to enhance intracellular calcium and acetylcholine release, were markedly attenuated. Ultrastructural analysis of the neuromuscular junctions of FcR –/– mice revealed an increased Schwann cell envelopment of the motor nerve terminals compared to the wild type controls. This modified conformation of the neuromuscular junctions, regardless of the presence of the FcγRI receptors on the motoneurons, might contribute to the observed reduction of the effect of ALS IgG in the FcR –/– mice.

NOISE REDUCING PROCEDURES IN DIGITAL MICROPHOTOGRAPHY

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Antecedents and object of the study: Structural analysis on the dendritic tree arborization of HRP labeled Purkinje cells of the cat, subserving functional modeling, was carried out with the aim of an automatic computer software package of our own. (Histological samples were kindly provided by G. Bishop, J. King and also by J. Takács.)

Source image files for the forthcoming computer analysis were grabbed by two systems of different technical capability:

a, Zeiss Axioskop with achroplan objectives - Mintron OS65D CCD camera - Adobe Photoshp 3.0,

b, Zeiss Axiophot with plan-neofluar objectives - KODAK DCS 400 C camera - Adobe Photoshop 4.0.

Grabbing procedures: Aiming the best possible image file, first the optimal grabbing procedure has been elaborated:
a, a Köhler-type illumination adjustment was performed before each grabbing session,
b, software package has been developed in order to
i, access the optimal setting parameters of camera (gamma, shutter, thermal stability),
ii, select the relevant menu-offers of grabbing software (i.e. for color correction, sampling saturation etc.).

Noise reduction, software package : As an offline facility to refine the grabbed pictures, the following procedure was followed up by the relevant software:
a, instead of taking one picture, a series of ten (in some cases up to sixty) RGB files were grabbed and transformed into 24-Byte .BMP files.
b, all frames from a series were averaged pixel-by-pixel to get a mean picture.
c, the difference between the sample items and their average were then computed to show up the two kinds of noises, which had been eliminated by the averaging procedure:
i, Global thermal noise resulted by uncertain setting of pixel bit values,
ii, structured noise elicited by diffraction at edges of neural processes.
d, statistical variance and standard deviation of average picture has been computed concerning saturation of the individual frames.
e, the distribution of saturation indicates a homogenous group of files, which are to be processed to get a clearer mean output file.

Conclusions, suggestions:
a, at optimal illumination, the shorter the shutter time is (1/10 000 Sec for the Mintron camera e.g.) the less noise will be generated by the CCD elements.
b, in density-based studies a gamma=1 camera adjustment, otherwise $\gamma <> 1$ is proposed,
c, The most powerful tool of noise reduction is the online averaging of homogenous group of original frames, indicated by density distribution.
d, Linear anisotropic distortion of the pixel array could be measured by double analysis with 90 degree rotation of a test (e.g. electronmicroscopic) grid.

COMPARATIVE STUDY ON RETINOGRAMS OF TYROSINE HYDROXYLASE MUTANT MOUSE STRAINS

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Thyrosine hydroxylase is the rate limiting enzyme of dopamine synthesis and controlled by Quantitative Trait Loci which can be transferred from one mouse strain to the other. This model allows investigating the role of dopamine levels in behaviour and diseases. Retinogram is generated by ion currents of retinal cells including Muller glia cells, which express large number of dopamine receptors. Consequently we assume that retinogram reflecting dopaminergic influences, can be successfully applied for testing the dopamine mutant mouse strains. Retinal dopamine level can influence the stimulus duration

dependence and adaptation properties of retinograms. For combined behavioural and electrophysiological studies we developed a mouse implantation model for simultaneous recording of retinogram and cortical event related potentials from freely moving mouse. Investigation of BALB/cJ and CXBI mouse strains, which have high and low tyrosine hydroxylase activity respectively, we observed strain dependent changes in the wave shapes of retinograms in stimulus duration series and light adaptation series. The light adaptation significantly changed in the CXBI strain, which showed no adaptation in B-wave amplitude while BALB/cJ strain had normal adaptation process. In conclusion we developed an electrophysiological method using retinogram and cortical evoked potentials on mouse for investigation dopamine mutant strains.

GANGLION DEVELOPMENT IN *EISENIA FETIDA*: A NEUROHISTOCHEMICAL STUDY

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Total cell number and the distribution pattern of some transmitter specific neurons were investigated in the ventral nerve cord ganglia, situated in the same body segment, of the juvenile and mature specimens of *Eisenia fetida* (Annelida, Oligochaeta). Applying proliferating cell nuclear antigen antibodies it has been proved that not only glial cells but also neurons have mitotic activity during posthatching development of the earthworm. The number of cells showing NADPH-diaphorase activity, serotonin- and GABA-like immunoreactivity, respectively, increased till the maturation of the animals. However, a marked decrease in the number of those GABA-IR neurons which were positioned in distinct neuron sets at the dorsal and dorsolateral parts of all ganglia behind the arising of the first segmental nerves, had been established. In juvenile worms 7-9 while in adults usually 3 neurons occurred in these neuron sets and their processes form the same longitudinal fine-fibred polisegmental interneuronal tracts of the ventral nerve cord in both investigated age groups. Parallel with the decrease of the number of GABA-IR interneurons formation of several new processes of remaining cells was observed, to reach their axon ramifications characteristic for them in adult worms. These results provide evidence at the *in situ* single-cell level for the ways in which the cell phenotype (morphology and maybe transmitter expression) could be modified. This finding is consistent with the pattern seen in many vertebrates, that interneurons differentiate last, and are thus must susceptible to the influence of environmental and other factors.

This study was supported by the Hungarian National Research Fund (OTKA No. T 026652) and the Adaptational Biology Research Group of Hungarian Academy of Sciences and University of Pécs.

THE EMBRYONIC FORMS OF GLUTAMIC ACID DECARBOXYLASE ARE EXPRESSED IN THE REACTIVE ASTROCYTES OF THE TRAUMATIZED ADULT SPINAL CORD

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Spinal cord injury initiates a cascade of events that leads to cell death, wound repair and to some extend functional recovery. Identification the factors that are auto-destructive or neuroprotective will help to clarify the mechanisms of secondary tissue damage and may led to novel therapeutic approaches.

γ-aminobutiric acid (GABA), the principal inhibitory neurotransmitter of the CNS also acts as a trophic/morphogenic factor during early CNS development, and as a neuroprotective agent after brain trauma. GABA is synthesized by the enzyme glutamic acid decarboxylase (GAD). The two forms of GAD (GAD65 and GAD67) are encoded by distinct genes and are co-localized in almost all GABAergic neurons at different levels. In embryo two additional truncated forms of GAD67 are also present. GAD25 that corresponds to the N-terminal segment of GAD67 probably exerts unknown regulatory functions, whereas GAD44 that is identical with the C-terminal catalytic region of the enzyme may synthesize the non-synaptic GABA with developmental function.

In this study the expression of different adult and embryonic GAD forms was followed in a spinal cord injury model during a 5-week self-recovery period. We found that both truncated embryonic GADs, GAD25 and GAD44, are expressed in the traumatized spinal cord close to the crush site. GABA was also detected, which is co-localized both with the embryonic GAD25 and the astrocytic marker GFAP.

Our results suggest the GABA in the reactive astrocytes that is synthesized by the embryonic GAD44 may be involved in the partial functional recovery of the spinal cord after injury.

C-FOS PROTEIN EXPRESSION IN THE RAT HIPPOCAMPUS FOLLOWING 4-AMINOPYRIDINE SEIZURES

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4-aminopyridine (4-AP), as specific blocker of the voltage dependent K^+ channels, is commonly used convulsive substance. Due to the mechanism of action and fast serum clearance (Lemeignan et al., 1984), 4-AP proved to be applicable for the investigation of epileptogenesis.

We determined the distribution of c-fos protein expression in the rat hippocampus, following generalized, acute seizure. The experiments were carried out on 180-200 g Wistar rats. Seizures were induced by intraperitoneal administration of 5 mg/kg 4-AP. Within a few minutes, 4-AP caused generalized tonic-clonic convulsion. The animals were anaesthetised with ether and perfused with 4% phosphate-buffered formaldehyde 1, 3 and 5 hours after the injection of 4-AP. Series of coronal plane frozen sections were processed for c-fos immunohistochemistry. C-fos immunoreactivity was observed in the hippocampus, dentate gyrus, thalamus and other subcortical structures and in the cerebral cortex. The immunoreactive nuclei in the hippocampus (CA1, CA2, CA3) were counted at 400X magnification using a digital microscope camera and the Image Pro Plus 4 morphometry program. C-fos induction in CA1, CA2, CA3 sectors of the hippocampus was present at 1, 3 and 5 hours after 4-AP injection. Maximal c-fos immunoreactivity has been detected in cornu ammonis (CA1 and CA3) at 3 hours. A significant decrease was observed at 5 hours in these areas.

The distribution of stained nuclei and the time course of their number suggest a long-lasting neuronal activation.

CHANGES OF THE OPTIC CHIASM EVOKED POTENTIALS DURING THE SLEEP WAKING CYCLE IN FREELY MOVING RATS

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We have been studying the visual system of rats with implanted electrodes on the cornea, in the optic chiasm, and on the visual cortex. Stimuli are delivered through a small light emitting diode glued to the skull. The rat is free to move inside a small box tethered only by the stimulus and recording wires attached to a plug on its head.

Recordings obtained from rats awake (W), in slow wave sleep (SWS), and in REM sleep will be displayed and compared. Flash-evoked optic nerve activity recorded at the chiasm level-the retinal ganglion cell response-has the form of a triphasic wave lasting about 400 ms. Potentials recorded during SWS differ significantly from those recorded during W and REM. SWS amplitudes are larger and internal details of the waveshape differ. W and REM histograms resemble each other. The cortical responses covary in details with the histograms, but all electroretinograms (the corneal traces) look alike.

We interpret these findings as showing efferent fibers reach the rat retina from the brain and modulate its output during sleep.

FINE STRUCTURE OF THE RETINA IN BATS

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The retina of bats has a special role due to the nocturnal life of these animals. In several species the photolocator function of the eye is restricted by the presence of additional echolocating orientation. Comparing the retina of several bat species, in the present work we investigated the organization, fine structure and cytochemistry.

The microchiroptean species investigated by us have small sized eyes and reduced retinae. The outer segments appear rod-shaped; the inner segments contain several mitochondria. The outer nuclear layer is well developed large, while there are only scattered cells in the ganglionic cell layer. Some of the large ganglionic cells are situated among bipolar cells.

The photoreceptors and neuronal cells exhibited weak-to-medium immunoreaction for the excitatory amino acids glutamate and aspartate. The synaptic zones contained ribbon-type synapses. Presynaptically, immunoreactive glutamate was accumulated in these synapses. There was no essential difference between the studied bat species in the pattern of immunoreaction of excitatory amino acids.

In the fruit eating macrochiropteran *Cynopterus sphinx* and *Rousettus niloticus*, the photoreceptor layer of the retina is well developed, and contains rods with long outer segments. The photoreceptor layer, inner and outer segments develop folds and crypts. The row of the bipolar cells follow this folding of photoreceptors, but other layers of the retina e.g. inner synaptic zone, ganglionic cells, zone of optic fibers are not folded.

There are no pigment granules in the pigment epithelium of these animals; instead, the cytoplasm of the epithelial cells is filled with lipid droplets of various sizes. Several rows of melanophores are attached to the pigment epithelium on the choroidal side and are also introduced into crypts of the photoreceptor layer.

The follicular organization of the retina is similar to that of the submammalian pineal organ serving as a "photodensitometer" of the environmental light periodicity. Since a folded retina is not viable for a screen in decoding two-dimensional images, we suppose that this peculiar organization of the retina – similarly to the pineal organ is connected to the significant photometer task of the eye of these species that are active at night.

EFFECTS OF GLUTAMATE-RELATED LIGANDS ON THE ELECTRORETINOGRAM (ERG) OF THE XENOPUS RETINA

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Vertebrate photoreceptors utilise glutamate as a neurotransmitter, releasing more glutamate in the darkness than during the light. The second order retinal neurons, bipolar and horizontal cells respond to glutamate and glutamate agonists and antagonists. There are two subtypes of bipolar cells: the ON depolarizing and the OFF hyperpolarizing bipolar cells. The postsynaptic receptors for glutamate are different in the two bipolar cells. In OFF bipolar cells glutamate binds to an ionotropic glutamate (AMPA) receptor that directly opens a non-specific cation channel. In this case the reduction in glutamate release by light hyperpolarizes the OFF-bipolar cells. In ON bipolar cells glutamate work through metabotropic (G-protein coupled) receptors that are linked to a cGMP second messenger system. In darkness the high glutamate level results in a low cGMP level. In light the diminished glutamate release allows the cGMP to rise and the cGMP gated channels open. This results a depolarization of the ON-bipolar cells in the light.

New electrophysiological and immunocitochemical evidence, however, suggests the existence of other glutamate receptors on the bipolar cells besides AMPA or metabotropic receptors. Considering these works we investigated the effect of selective kainate and AMPA receptor ligands on the Xenopus laevis electroretinogram (ERG).

The components of the ERG are believed to result from different subtype cells of the retina. The a-wave is generated largely by the photoreceptors. The b-wave reflects directly the activity of the ON bipolars or through the regulation of the extracellular K^+ by Muller glia cells.

We recorded ERG responses to bright red flashes superimposed on a steady-state green background light to suppress rod mediated responses. Our results suggest that the d-wave and partly the a-wave can be blocked by the selective AMPA receptor antagonist, whereas the b-wave can be influenced by both AMPA and kainate receptor specific ligands. Our data support the idea that; 1, OFF bipolar cells contribute to the ERG a-wave 2, kainate could not increase the b-wave through activating any channel on the ON bipolar cells, since the effect would be to oppose the light-evoked decrease in glutamate release. Because the kainate opens the ion channels of the AMPA receptors in a continuous way, the kainate evoked increase of the b-wave is rather due to a restriction of extracellular space. But when we put on L-AP-4 and then see a small and slower b-wave after adding kainate, we think this is the Muller cell contribution.

STOCHASTIC ASSOCIATIONS MEASURED BY INFORMATION-THEORETIC METHODS IN LOW-[MG^{2+}]-INDUCED CA^{2+} BURSTS IN ORGANOTYPIC HIPPOCAMPAL SLICE CULTURES

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We show here that information-theoretic, entropy-based analyses revealed differences in associations of free intracellular Ca^{2+} ($[Ca^{2+}]_{f,i}$), external Ca^{2+} ($[Ca^{2+}]_e$) and field potential (fp) bursts induced by low external [Mg^{2+}] in cultured rat hippocampal slices. Since the traditional measures of association (like cross-correlation function) do not take into account nonlinear interdependencies of signals we quantified the stochastic dependence between them by their mutual entropy and uncertainty coefficients. The progress of spontaneous bursting activity can be resolved into sequential *pre*burst, burst-efflux, burst-influx and *post*burst periods which can be characterized by distinguishable levels of stochastic associations between $[Ca^{2+}]_{f,i}$, $[Ca^{2+}]_e$ and fp signals, displaying maximum and minimum in the burst-influx and *post*burst periods, respectively. The physiologically most significant result of our observations is that the dynamics of external and internal [Ca^{2+}] changes come coupled under epileptic conditions.

REALISTIC COMPUTER MODELING OF IPSCs ELICITED BY DIFFERENT TYPES OF HIPPOCAMPAL INHIBITORY INTERNEURONS

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Distinct classes of inhibitory interneurons in the hippocampus target different regions on the surface of principal cells. This may probably play a role in the functional differentiation of these interneurons. It is difficult to estimate the physiological properties of the specific synaptic connections, due to the voltage and space-clamp errors associated with the use of a somatic electrode to measure current from dendritic synapses.

We used experimentally measured inhibitory postsynaptic currents (IPSCs) evoked by identified GABAergic interneurons, located in str. oriens and innervating distinct domains of pyramidal cells (Maccaferri et. al.). We tried to reproduce this data and quantify the synaptic

current distortion under voltage-clamp conditions using compartmental models (in GENESIS) of a range of morphologically reconstructed hippocampal CA1 pyramidal cells (n=7), originating from two sources (Mainen et al., 1996; Pyapali et al., 1998). We found that the dendritic diameters were significantly different between the two data set.

Generally the model could fit the experimental data only if the membrane resistivity was high and the cytoplasmic resistivity was low. Even in this case the simulated IPSC rise-time for synapses located in str. lacunosum-moleculare (str.lm.) was slower than the measured values. The apparent IPSC kinetics were too slow for all synaptic positions in cells with small dendritic diameters. Simultaneous activation of many synapses, distributed on different branches but at same electrotonic distance from the soma, did not accelerate substantially the IPSP rise-time. Our passive model predicts for distal synapses (e.g. str.lm.) an IPSC amplitude attenuation of approx. 4-fold in cells with thicker dendrites and 10-fold in cells with thinner dendrites. Adequate space clamp is not possible for most of the synapses. In the subsynaptic membrane of the distal branches the voltage can deviate up to 25 mV from the holding potential, if the dendritic diameters are small.

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EGIS-9933 A NEW ANXIOLYTIC DRUG WITH SEROTONERGIC ACTIVITY

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In the last couple of years more and more attention has been focused on the 5-HT_2-receptor family, as these receptors play an important role in physiological and pathophysiological processes of the CNS. There is a substantial body of evidence showing that 5-HT_{2C}-receptor-subtype mediated effects are involved in the pathomechanism of various CNS disorders. The potential therapeutic targets of 5-HT_{2C}-selective compounds have been identified as anxiety, obsessive-compulsive disorder, depression, migraine and anorexia. To elucidate the potential advantages of selective 5-HT_{2C} ligands in CNS disorders, a series of new pyrimidine-coupled piperazines were synthesized, and the most selective compounds were tested in *in vivo* animal models of anxiety.

Here we summarize the results obtained with a recently developed selective compound EGIS-9933 (a HCl-salt of EGIS-8465) in receptor-binding assays, and a wide range of anxiolytic models.

EGIS-9933 showed strong selectivity to rat 5-HT_{2C} receptors in binding studies. It profoundly inhibited mCPP-induced hyperthermia and hypomotility in rats. The compound exhibited significant anxiolytic activity in three rodent tests of anxiety with a minimum effective dose of 1, 20 and 10 mg/kg, respectively: in elevated plus maze (increase in time spent in the open arms and number of entries into the open arms), Vogel punished drinking (increase in number of punished licks) and social interaction (time spent with social investigation). Unlike the reference compound diazepam, EGIS-9933 did not inhibit the spontaneous motor activity in rats and mice, up to 100 mg/kg dose.

The high effectiveness in anxiolytic tests and the low psychosedative potential of this compound suggest good therapeutical perspectives in man.

INTERACTING EFFECTS OF CAPSAICIN AND ANANDAMIDE ON INTRACELLULAR CALCIUM OF THE RAT'S ISOLATED TRIGEMINAL NEURONES

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The cloned-capsaicin (CAPS) receptor, the VR-1 is a nonselective cation channel which can be activated by CAPS-type pungent agents and noxious heat stimuli, and is expressed selectively in noxious heat sensitive nociceptive primary afferent neurons. Anandamide (ANA), a natural endocannabinoid is a potent antinociceptive agent and acts on CB1 receptors. The aim of the present study is to reveal the interactions of CAPS and ANA on cultured trigeminal neurons of the rat using the intracellular free Ca^{2+} measurement with a fluorescence technique.

Primary cultures of TRG neurones with nerve growth factor were made from Wistar rat pups of 1-7 days old. Technique of $[Ca^{2+}]i$ measurement with the fluorescent indicator fura-2 AM was used. Dye loaded (1 μM fura-2 AM, 15 minutes) cells were illuminated alternately at 340 and 380 nm light and the 340/380 fluorescence ratio was measured in 3-12 cells simultaneously. The percent of CAPS-responsive cells to 100 nM, 330 nM and 1 μM CAPS were 47.4%, 45.3%, and 70.4%, respectively. There was no significant difference in the peak R values obtained with 100 nM, 330 nM and 1 μM CAPS, being the respective mean and SD values 0.254 ± 0.23 (n-93), 0.242±0.20 (n-16) 0.291±0.19 (n=13). The effect of 200 nM ANA was tested on the CAPS induced responses. In the CAPS-sensitive cells, CAPS exposure (330 nM for 3 sec) produced R = 0.242±0.20, n=16 while in the presence of ANA the same CAPS exposure induced only R= 0.04±0.07 n-14. The effect of ANA on the intracellular calcium concentration was measured, too. Seventeen % of the tested cells responded with R increase to 200 nM ANA, and including these cells, 27% of the cells responded to the 10 μM concentration. The effect of ANA on verified CAPS-responsive cells were also examined. The fluorescence responses evoked by the two agonists did not change in parallel by the preceeding exposures.

The results suggest that activation of CB1 receptors induce calcium influx and that 200 nM ANA inhibits the calcium influx produced by activation of the CAPS receptor ion channel complex.

DEVELOPMENT OF THE CEREBELLAR CORTEX IN CATS

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We have studied the postnatal development of the cerebellum from birth to young adulthood in the cat by the help of metabotropic glutamate receptor mGluR1a immunoreactivity. This receptor is present at high density in cerebellum, especially in Purkinje cell (PC) dendritic spines, but is also present in the interneurons both of the molecular and of the granular layer. mGluR1a appears early during development and its expression and cellular distribution is an inherent (genetically determined) property, independent of epigenetic signals of the Purkinje cells (PCs). Sagittal and horizontal serial sections were immunostained by monoclonal mGluR1a antibody of the cerebella and the brainstem of kittens at different postnatal ages: P0 (the day of birth), P1, P8, P15, P22, P42, P62, P72, P90, P135 and also in young adult (1-2 years old) cats. The differentiation, migration and integration of cerebellar cortical neurons were investigated on the basis of morphological characteristics by the help of their Golgi impregnation-like immunostaining both in the brainstem and also in the white matter of the cerebellar cortex. In contrary to the view that PCs do generate predominantly - if not exclusively - during the prenatal embryonic period in the cat, we have observed considerable neurogenesis of these cells within and beneath the ependymal layer of the IVth ventricle and also on the lateral surface of the brainstem corresponding to the embryonic corpus pontobulbare mostly during the perinatal period. The genesis and migration of PCs was also considerable and characteristic during the first postnatal week, especially in the cerebellar hemispheres and also in the late developing vermal lobules (V-VI), targeting the borderline between the molecular and internal granular layers (territories from which the PCs were still missing). Migrating basket neurons - alone or together with PCs - were also frequently seen during this developmental period, occupying their characteristic place in the cerebellar cortical cytoarchitectonics, preceding often their synaptic partner in the integration. However, the number of migrating and integrating PCs was surprisingly high during the first and second postnatal months too, and the level of morphological differentiation of generating and migrating Pcs corresponded to those being integrated to their lamina. The number of migrating forms was always higher in the white matter at deeper cerebellar regions (near and/or around the cerebellar nuclei, in the cerebellar peduncles and also in the brainstem) and less numerous at the deep cerebellar lobular invaginations and also at the more distal parts of the cerebellar lobules and sublobules. This spatio-temporal distribution pattern could be the consequence that rate of the migration could probably speeding somewhat up during the migratory course of PCs, terminating with a relatively shorter neuronal integration period

throughout the (internal) granule cell layer into the PC lamina. Beside PCs, a considerable number of all migrating cerebellar cortical interneurons - basket cells, small and large Golgi cells, Lugaro cells, unipolar brush cells - were also frequently seen in the white matter not only during the period considered as the cytoarchitectonic built up of the cerebellar cortex of the cat (at about two months) but also - although in gradually decreasing number - during the whole time scale of the present study, and most surprisingly in young adult animals, too. We suggest that the neurogenesis, migration and integration of the cerebellar cortical neurons is not restricted to the embryonic, perinatal and relatively short postnatal developmental periods, but - although slowing down considerably - persist continuously in the cerebellum of young adult cats.

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CO-TARGETED GAP JUNCTIONS AND GABAERGIC SYNAPSES SYNCHRONISE CORTICAL INTERNEURONS

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Neural networks in the cortex can engage in synchronous oscillations in the gamma frequency range, a coherent activity likely to subserve a code in neural information processing. Such cortical synchronization can result from internal coordination of spike timing which is not simply caused by stimulus driven discharge. We performed simultaneous whole-cell recordings from pairs of interneurons in rat somatosensory cortical slices and found a mechanism intrinsic to interneuronal networks capable of synchronizing the firing at gamma frequency. Unitary connections mediated by combined gap junctional and GABAergic synaptic coupling instantly phased postsynaptic firing with a precision of less than 3 milliseconds. Temporal accuracy is due to the curtailment of gap junctional potentials and minimization of spike latency jitter by consecutive action of GABAA receptors. Connections mediated solely by gap junctions phased postsynaptic firing with a substantial (~10 ms) phase lag and interactions via GABAAergic synapses only result in theta frequency (~7 Hz) postsynaptic discharge. Precision of timing in dual connections is further emphasized by spatial specificity as unitary gap junctions and chemical synapses target neighbouring sites on the postsynaptic cells. Such oscillating interneuronal networks of spatiotemporal accuracy might serve as a reference for cortical information coding.

EFFECTS OF AMPA ANTAGONISTS ON SPINAL MOTOR FUNCTIONS

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Glutamate plays a crucial role in the mediation of both spinal segmental reflexes and higher motor functions. GYKI 52466, a non-competitive antagonist of AMPA-type glutamate receptors with 2,3-benzodiazepine structure has a central muscle relaxant effect and depresses both monosynaptic and polysynaptic spinal reflex potentials at low doses (Tarnawa et al., 1989, Eur. J. Parmacol. 167: 193; Farkas and Ono, 1995, Br. J. Pharmacol. 114: 1193). In the present experiments we investigated the effects of some novel, more potent 2,3-benzodiazepine derivatives on spinal functions, both *in vivo* and *in vitro*.

2,3-Benzodiazepines inhibited dorsal root stimulation-evoked ventral root reflex potentials in hemisected spinal cords isolated from young rats. The monosynaptic action potential-related early peak component was completely blocked, while the motoneuronal EPSP-related tonic component was inhibited only partially. When the compounds were tested for depression of spinal reflex potentials *in vivo*, in spinalized adult rats, all reflex components were blocked by low intravenous doses of these compounds, but the polysynaptic reflex potentials were slightly less affected than the earlier reflex components. The 3-N-methylcarbamoyl analog of GYKI 52466 was 9-fold, while the acetyl derivative, was about 2.5-fold more potent than the parent compound for inhibition of flexor reflex in anesthetized cats. Both of these derivatives are racemic compounds. The active isomers were about twice as potent as the corresponding racemates. The differences between the compounds were smaller, and their intravenous ED_{50} values were higher in the rat flexor reflex test, which is probably due to an extremely rapid metbolism of the compounds in rodents. When applied to conscious mice, 2,3-benzodiazepine AMPA antagonists deteriorated the performance of mice in the inclined screen and rotarod tests, inhibited the effect of the tremorigen compound GYKI 20039, and prevented the development of Straub tail following morphine application.

The rank orders of potencies in the different assays are in agreement with the AMPA antagonist potencies in whole-cell voltage clamp experiments, supporting the view that the blockade of AMPA receptor-mediated functions is responsible for the muscle relaxant effect of these compounds.

RELEASE OF SOMATOSTATIN AND CGRP FROM CAPSAICIN-SENSITIVE SENSORY NERVE TERMINALS *IN VITRO* AND *IN VIVO*

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Activation of the capsaicin-sensitive primary afferents elicits a Ca^{++} dependent release of neuropeptides [calcitonin-gene related peptide (CGRP), somatostatin, substance P] stored in the nerve terminals. The aim of the present study was to analyse the release of CGRP and somatostatin in rats in response to chemical stimulation (capsaicin, resiniferatoxin (RTX)) *in vitro* and *in vivo*, combined with pharmacological modulation by systemic nociceptin and anandamide pretreatment. *In vitro release study*: Dissected tracheae of two adult Wistar rats were perfused in an organ bath (1.8 ml) with oxygenated Krebs solution at 37 °C for 60 min. After equilibration the solution was changed three times for 8 min (prestimulated-stimulated-poststimulated fractions) and the CGRP and somatostatin content of each fractions was measured with RIA. Chemical stimulation (capsaicin 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} mol/l, and RTX 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} mol/l) induced concentration dependent peptide release, in which RTX was 100 times more potent. Six fractions were collected after RTX 10^{-8} mol/l to demonstrate the slower kinetic and longer duration of its action compared to capsaicin. For *in vivo experiments* Wistar rats were used, anaesthetised with sodium thiopentone 100 mg/kg i.p. RTX (0.1, 0.3, 0.6, 1, 3 µg/kg, i.v.) was injected and arterial blood samples (5 ml/animal) were collected 5 min and 60 min after the RTX administration. RTX provoked a dose-dependent elevation in plasma level of CGRP and somatostatin at 5 min, and lasted for 60 min. Pretreatment with the endogenous CB1 ligand anandamide (100 µg/kg i.v.) significantly inhibited the release of both neuropeptides induced by RTX (0.6 µg/kg, i.v.), while the inhibitory effect of the ORL-1 ligand nociceptin (20 µg/kg, i.p.) reached significant level only on somatostatin release. These data support the acut excitatory effect of RTX on capsaicin-sensitive nerve terminals resulting a dose-dependent and long-lasting release of CGRP and somatostatin, which action can be modified by systemic anandamide and nociceptin administration.

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BEHAVIOURAL AND BIOCHEMICAL CONSEQUENCES OF MDMA-INDUCED NEUROTOXICITY IN RATS

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Repeated administration of high doses of amphetamine derivatives is neurotoxic in rat brain both to the dopaminergic (DA) and serotonergic (5-HT) systems. According to our previous results, methylendioxymethamphetamine (MDMA, ecstasy), a popular illicit drug of abuse, administered in a high dose (15 mg/kg four times in every two hours) significantly reduced spontaneous motility of the animals on the 3rd but not on the 13th postinjection day. Aim of the present series of experiments was to check whether a lower dose of MDMA (10 mg/kg) results in similar behavioural changes, and if it does, how it correlates with the biochemical consequences. The results were as follows.

1. 10 mg/kg MDMA given four times in every two hours significantly decreased the spontaneous motility of the animals on the 3rd postinjection day, however, no difference was observed in the later testing time.

2. Significantly lower DA and 5-HT contents were measured in the hippocampus on the 3rd and 7th postinjection days but the values returned to the control levels 2 months later.

3. In the striatum, however, DA and 5-HT contents were significantly reduced not only on the 3rd and 7th postinjection days but they remained decreased for 2 months.

Correlation of the behavioural and biochemical results indicates that the biochemical changes induced by the neurotoxic dose of MDMA result in a decreased spontaneous activity at the beginning, however, later the adaptive responses may correct the behavioural deficit.

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DENERVATION-INDUCED PLASTIC CHANGES IN THE ADULT MAMMAL CORTEX

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Plastic changes in the primary somatosensory cortex (SI) of adult mammals were first detected in nerve-injury studies where remapping of the remaining active inputs was observed. It soon turned out that the somatotopic representation map of the muscle system in the primary motor cortex (MI) on both sides may also be modified, e.g. by unilateral transection of the facial nerve (n7X). These changes take hours to days to achieve completion and persist as long as muscle reinnervation is prevented. However, cortical reorganization begins with a much shorter latency (3-5 min) after n7X. Little is known about

the underlying mechanisms. Data observed recently suggest that a sensory and facial nerve injury (and even a sham operation, but for a much shorter period) transiently decreases the normal inhibition level of the cortex and hence unmasks cortico-cortical inputs to the MI of both hemispheres. Electrophysiological and histochemical methods were recently combined in order to follow changes in the cerebral cortex between 5 min and 2 weeks after n7X. During the transient disinhibition, a new histochemical technique visualized a transient chloride accumulation in subpopulations of pyramidal cells. In parallel with this phenomenon, a small percentage of the pyramids exhibited a reversal effect (facilitation vs. inhibition) following GABA or muscimol application juxtacellularly. These observations suggest that denervation may cause GABA-A receptor-dependent disinhibition in the related cortices during the primary reaction.

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IN SITU MONITORING OF THE EFFECT OF FREE RADICAL SCAVENGERS ON THE PMA-GENERATED SUPEROXIDE-DEPENDENT CHEMILUMINESCENCE (CL) IN CULTURED RAT ASTROCYTES

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Receptor-mediated phosphoinositide hydrolysis leading to PKC activation is a fundamental signal transducing mechanism in the brain. Several of the stimuli inducing glial activation use signal transduction pathways involving PKC activity. GFAP and Vimentin the two main glial cytoskeletal proteins are both substrates for PKC and their phosphorylation is stimulated by the synthetic diacylglycerol analog phorbol ester PMA. In cerebral ischemia, early astroglia activation may contribute to neuronal damage by producing reactive oxygen species.

In our study free radical production of the protein kinase C activator PMA was examined in primary cultures of type-1 rat astrocytes. Astrocytes exhibited a significant superoxide-dependent CL response to PMA which was almost completely abolished by SOD and AETU. DMSO was partially effective in blocking PMA-evoked free radical generation. Catalase, Pyruvate and Mannitol was ineffective in scavanging PMA-induced free radical generation. It can be supposed that in response to PMA beside the primarily produced dominant free radical superoxide, a mixture of related free radicals could be generated. Our data demonstrate a neurotoxic role for astroglia in CNS injury.

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CONNECTIONS BETWEEN THE DIENCEPHALON AND THE HIPPOCAMPUS IN THE RAT: A RETROGRADE TRACT-TRACING STUDY USING PSEUDORABIES VIRUS

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Hippocampal afferents may reach the hypothalamic paraventricular nucleus (PVN) through direct (medial corticohypothalamic tract) or indirect projections – relayed by neurons in the bed nucleus of stria terminalis (BST) – arising mainly from the ventral subiculum (VSub). The exact locations of PVN-projecting hippocampal (HI) neurons and the fine topography of their path have been investigated in the present study.

We used a recombinant derivative of Bartha strain of Pseudorabies virus (PrV-Ba), termed Ba-DupLac, as a retrograde transsynaptic tract-tracer to reveal the neural connections between the hippocampus and the PVN. PrV was injected to the PVN (PVN-inj) of female Sprague-Dawley rats. After three days of post-inoculation rats were killed by transcardiac perfusion. Dense labelling was observed in the BST, in the lateral entorhinal area (LEnt), in CA1 regions, and in the VSub, with an ipsilateral dominance. Some infected neurons were also seen in the thalamus (Thal) – mainly in the anteromedial (AM) and interanteromedial (IAM) nucleus. This labelling was presumably due to the leakage of the virus along the needle track, since injecting Ba-DupLac into the thalamus (Thal-inj), infected cells were seen mainly ipsilaterally in the BST, in retrohippocampal and neocortical regions, such as LEnt, perirhinal, occipital, and temporal areas, and also in CA1 and in subicular regions.

Labelled cells in the VSub were located at rostrocaudal levels between 5.4 and 6.8 mm caudal to bregma. The majority of Thal-projecting subicular neurons were detected more caudal (6.0-6.8 mm) than PVN-projecting cells (5.4-6.4 mm). CA1-neurons projecting to the PVN were observed only in the ventral part of this region, while Thal-inj resulted in infected dorsal and ventral CA1 cells. The relay neurons in the BST occupied several divisions, such as the anterior medial, ventral medial, posterior medial, and posterior lateral divisions. Virus-labelled cells in the LEnt may represent second ordered neurons infected by HI cells transsynaptically (PVN-inj), and direct AM/IAM-projecting neurons (Thal-inj).

EXPRESSION OF SUBSTANCE P RECEPTOR IN HUMAN CALCIUM BINDING PROTEIN-CONTAINING INTERNEURONS

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GABAergic inhibitory cells in the hippocampus may contain neuropeptides or calcium binding proteins. These neurochemical markers were shown to label functionally different

subsets of interneurons with distinct termination pattern. A new subclass of inhibitory cells expressing substance P receptor (SPR) was described recently in the rat. These cells were proved to be morphologically heterogeneous. They formed partially overlapping populations with calcium binding protein- and neuropeptide-containing cells. Morpho-functional identification largely relies on efferent connectivity of interneurons, but in this case it is difficult since substance P receptor is not present in axons. Therefore, colocalization with neuropeptides and calcium binding proteins may help the identification of their function in the hippocampal network. In a recent human study SPR-immunostained cells were shown to be sensitive to epileptic injury, thus a need for their functional classification has emerged. In the present study fluorescent double immunostaining was carried out against SPR and three calcium-binding proteins (calbindin, parvalbumin, calretinin), and the results were compared to rats. We found that most of the parvalbumin- and calretinin-immunopositive cells do not express SPR, whereas large number of the calbindin-stained interneurons showed SPR-immunopositivity in the CA1-CA3 region. In contrast, a large proportion of the parvalbumin- and calretinin-containing cells showed substance P receptor-immunopositivity in the rat. Distribution of the calbindin-SPR double-stained cells was similar both in rats and humans. We conclude that parvalbumin-containing perisomatic inhibitory cells (basket and axo-axonic cells) and calretinin-containing inhibitory interneurons (responsible for the dendritic inhibition and the inhibition of interneurons) are not among the substance P receptor expressing cells in human hippocampus.

EXPRESSION OF GAD-ISOFORMS DURING *IN VITRO* INDUCED NEUROGENESIS

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In the adult nervous system, two glutamic acid decarboxylase enzymes (GAD65 and GAD67 proteins) are responsible for the GABA-production. From the GAD67 gene, proteins with molecular weight of 25 kDa and 44 kDa are translated only in the embryonic CNS (1, 2).

The expression of the adult- and embryo-specific GAD-isoforms and the appearance of the GABAergic phenotype have been analyzed during the *in vitro* induced neurogenesis of a neuroectodermal clone, NE-7C2. In the cultures of NE-7C2 cells, neurons and astrocytes developed if induced by alltrans retinoic acid (ATRA).

Our data demonstrate that GAD65 and the embryonic forms of GAD67 genes are expressed at hardly detectable levels by non-committed NE-7C2 cells. Treatment with ATRA resulted in an elevated level of embryonic GAD67 nIRNAs together with the appearance of N-tubulin immunreactive cells. On the 4th day of induction, the level of embryonic GAD67 mRNAs reached a plateau. The expression of embryonic GAD67 forms decreased with the progression of neurogenesis, while the expression of the GAD65 form raised with the increasing number of neurons in cultures. Cells with high GABA-content appeared at a time when embryonic and adult forms of GAD were expressed. The early and

transient presence of the embryonic GAD67 isoforms indicates that the proteins play role in neuron formation - for instance - by synthesizing morphogenetic GABA.

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IN SITU HYBRIDIZATION ANALYSIS OF ADRENOCEPTOR SUBTYPES WITHIN HYPOPHYSIOTROPIC THYROTROPIN-RELEASING HORMONE NEURONS OF THE RAT HYPOTHALAMUS

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The adrenergic effects upon plasma thyrotropin (TSH) levels are well characterized and appear to be mediated by the hypophysiotropic thyrotropin-releasing hormone (TRH) neuronal system. Nevertheless, little is known about the receptorial mechanisms whereby adrenergic afferents regulate the secretion of TRH into the hypophysial portal blood. To determine whether TRH neurons located in the hypothalamic paraventricular nucleus (PVN) express adrenoceptors (AdrR), furthermore, to potentially identify the receptor subtypes expressed, we used a dual-label *in situ* hybridization (ISH) approach in which ^{33}P-labeled cRNA probes complementary to specific AdrR (α-1B, α-1D, α-2A and β-1) mRNAs were hybridized to tissues simultaneously with a digoxigenin-labeled cRNA probe complementary to proTRH mRNA. All four AdrR transcripts exhibited relatively high levels of expression in the cerebral cortex and hippocampus, but occurred at low-to-moderate levels in hypothalamic areas, including the PVN. The highest degrees of co-localization with proTRH mRNA in the PVN as well as in the reticular nucleus of the thalamus were revealed following the application of the α-1B and the α-1D AdrR-specific hybridization probes. Using either one of the four AdrR probes, subsets of proTRH mRNA-expressing neurons remained unlabeled. The identification of distinct AdrR subtypes in TRH neurons of the PVN might reveal the receptorial mechanisms whereby cold exposure activates the hypophysiotropic TRH neuronal system.

DEVELOPMENT OF UNIPOLAR BRUSH CELLS IN THE CEREBELLUM OF CAT

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UBCs, the recently characterized glutamatergic interneurons in the cerebellar cortex of mammals (Harris et al. 1993, Mugnaini and Floris, 1994) and birds (Takács et al. 1999) express metabotropic glutamate receptor 1a (mGluR1a) abundantly (Jaarsma et al. 1995, Mugnaini et al. 1997, Takács et al. 1999). The postnatal developmental distribution pattern of mGluR1a immunoreactive unipolar brush cells (UBCs) was studied in the cerebellar cortex of kittens at postnatal days P0 (on the day of birth), P1, P8, P15, P22, P42, P62, P72, P92, P132. NeuroLucida drawings were made from the immunoreacted sections at about the midsagittal vermal plane, at the transitory area between the vermis and the neocerebellum and at the level of cochlear nucleus and the distribution of immunoreactive UBCs were marked. In order to reveal the medio-lateral migration of UBCs, some horizontal sections were also analysed. The relative number of mGluR1a ir UBCs was compared in the three preferential planes of various age groups.

On the day of birth (P0) UBCs were already present in the white matter in lobule X of the vermis, and started their migration to the deeper region of the internal granular layer. By the end of the first week (P8) UBCs already have invaded lobules IX, VIII, I, and II of the vermis, and they spreaded in the transitory area medio-laterally from the vermis towards the cerebellar hemispheres. By P15, UBCs appeared in lobules III and VII of the vermis, as well as in corresponding lobules of the neocerebellum, with especially high number in lobule VII. By P22, UBCs migrated further following their medio-lateral course in the neocerebellum, and began to invade lobules V and VI. At P62 the amount of UBCs in midsagittal planes of early developing lobules (I, II, VII-X) resembled to the P132 or adult pattern. The medio-lateral migration of UBCs slowed down in the late-developing cerebellar lobules V and VI, in which it was completed only by P132. At this time, the spatial distribution of UBCs was comparable to that of seen in the young adult cat. Although by P70 the postnatal migration of the majority of UBCs was completed, a few migrating UBCs could still be observed in the white matter of the cerebellar lobules, and beneath the ependyma of the fourth ventricle in the cerebellum of adult cats. It is suggested that during ontogenesis the migration course of UBCs follows the developmental sequence of cerebellar lobules.

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EFFECTS OF MODULATORY COMPOUNDS ON AMPA/KAINATE RECEPTORS TESTED ON HIPPOCAMPAL SLICES, PRIMARY TELENCEPHALON CULTURES AND A CHO CELL LINE EXPRESSING GLUR1 (FLOP) RECEPTOR SUBTYPE

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AMPA/kainate receptors have a major role in learning, memory and also in neuronal cell loss which may follow brain damage. Establishing a model from a set of *in vitro* tests which is suitable to identify the mechanism of action of drugs on these receptors and also to reflect on the results of the specific features of the tests can be a useful strategy in drug research. Our model is composed of *i*) whole-cell patch clamp studies of AMPA (10 μM) and kainate (250 μM) evoked currents in neurones from rat telencephalon cell cultures and in *ii)* CHO cells expressing the flop splicing variant of AMPA/kainate receptor GluR1 subtype, as well as *iii)* evoked field potential recordings from the CA1 region of rat hippocampal slices.

Both in cultured neurones and in CHO cells GYKI 52466 and EGIS 8332 inhibited kainate-evoked currents and also blocked the amplitude of population spikes in the slice preparation. The flip-variant selective cyclothiazide (10 μM) produced tenfold elevation in AMPA evoked currents in neurones, but had only a negligeable effect on currents in CHO cells and on the population spike amplitude (at concentrations as high as 100 μM). Aniracetam (2 mM) an agent on the flop variant, only slightly enhanced AMPA evoked current in neurones, but displayed remarkable effect on CHO cells, and also increased the amplitude of population spike.

The effects of these modulatory compounds are in accordance with the fact, that even in individual neurons a population of different heteromeric and homomeric AMPA/kainate receptors may be expressed and, on binding of the agonist, an average of currents is obtained. Therefore, beside tests carrying out on traditional preparations the expression of and studies on specific receptor subunit gene products are probably essential to the development in new and more specific drugs.

RETINAL DISTRIBUTION OF CALCIUM BINDING PROTEINS IN A GAD67 TRANSGENIC STRAIN OF MICE

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The gene of the 67 kDa isoform of glutamic acid decarboxylase (GAD67) has been inserted into mice. Retinal tissues from control and transgenic animals were collected at E14, P60, P300 and P600, fixed in 4% paraformaldehyde. Sections were cut in cryostat and processed for parvalbumin, calbindin and calretinin immunocytochemistry. Control and heterozygous animals had well-developed eyes and showed identical labeling patterns for calcium-binding proteins. Parvalbumin could not be detected, while calbindin was found in horizontal, bipolar, amacrine and ganglion cells. Calretinin was restricted to the inner retina (amacrine and ganglion cells). The eyes of the homozygous animals were already appreceably smaller at P60 than those of controls. By P300 the eyes of the homozygous animals were microphthalmic and by P600 the retina could not be found in the eye, only the stump of the optic nerve could be dissected. At the microscopic level, the retina of the E14 animals seemed to be identical to the controls. By P60, however, enormous changes occurred. Though the calcium-binding proteins were still present as described for the controls, the photoreceptor layer mostly disappeared. Other retinal layers were also thinner, due to secondary degeneration after photoreceptor loss. By P300, this process continued, and the retina was very thin, but still contained some neurons positive for calretinin and calbindin. It seems that retinal cells that contain calcium-binding proteins are more resistent to degeneration than those without.

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DIFFERENTIATION OF LUGARO CELLS IN THE CEREBELLUM OF DEVELOPING CAT

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The Lugaro cell (LC) is a relatively rare and negligated cell type in the cerebellar cortex, possibly a feedback interneuron on the corticocerebellar output (Laine and Axelrad, 1996). Lugaro cells (LCs) are present throughout the cerebellum, characterised by a fusiform cell body located slightly below the Purkinje cell layer with thick, horizontally oriented dendrites extending in the parasagittal plane. LCs express metabotorpic glutamate receptors mGluR1a and mGluR5 in mouse and rat (Négyessy et al. 1997). We have studied the appearance and developmental dynamics of LCs by using mGluR1a immunocytochemistry in the cerebellar

cortex of kittens from birth to postnatal P135 days. LCs express mGluR1a receptor as early as the day of birth, and could be identified with the aid of an enhanced penetration of immunoreagent. This results in a Golgi-like immunostaining of the large fusiform cell body and the bipolar dendrites of LCs migrating in the white matter of the cerebellum including the vermis, the transitory zone and the neocerebellar territories. Migrating LCs can be identified and followed up to the border of white matter and internal granule cell layer in all cerebellar lobules and sub-lobules. By counting LCs in series of vibratome sections we made and approximation of the total number of migrating Lugaro neurons in cerebella of kittens at postnatal days P0, P1, P8, P15, P22, P42, P62, P72 and P135, respectively. We have found only a few of these cells on the day of birth, but the differentiation and migration of LCs increased rapidly (at about two thousand and five thousand migrating forms at P1 and at P8, respectively) which showed a peak at about two weeks after birth (nearly fifteen thousand migrating LCs in the whole kitten's cerebella at P15). The production and migration of Lugaro neurons continued but decreased continuously (at about seven and half thousand, three thousand and two thousand migrating cells at P22, P42 and P62/72, respectively). The production of this cell type is not concluded by the time of the cytoarchitectonic built up of the kitten's cerebella (between 60-70 postnatal days): at about one thousand migrating cells of this neuronal type were present in the cerebellum of kitten at P135, too. In conclusion: the birth of Lugaro cells can be followed during the whole postnatal developmental period in the kittens. The highest rate of production and migration was found at about two weeks postnatally, which slowed down considerably by P62/72, but did not finish even at P135, providing the first experimental evidences that the birth, differentiation and migration of Lugaro cells appears much longer than suspected earlier.

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ANALYSIS OF SPREADING DEPRESSION MEASURING INTRINSIC OPTICAL SIGNALS

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Recent studies indicate that spreading depression (SD) represents the pathophysiological signal contributing to the enlargement of the infarct area following focal cerebral ischemia. We used *in vitro* optical recording techniques to analyse the generation, propagation and pharmacology of KCl-induced SD in somatosensory cortical slices from adult rats. Slices were illuminated in a 45° angle from above and the reflected light was measured with a CCD camera in an *in vitro* interface-type slice chamber. Following drop application of 3 M KCl, intrinsic optical signals clearly revealed a SD, which coincided with an extracellular DC deflection by 10-20 mV and which propagated with 3 mm/min across the whole slice. The SD could be elicited most easily in layers II/III and either invaded lower layers or was restricted to supragranular layers during horizontal propagation. Application of the

unspecific gutamate antagonist kynurenic acid (KA, 500 μM) blocked the propagation, but not the initiation of the SD. Drop application of KA (1 mM) caused a local blockade of the SD propagation. Our preliminary results using carbenoxolone (20 μM) indicate that gap junctions play a minor role in the propogation of the SD.

A NOVEL METHOD IN QUANTITATIVE *IN SITU* HYBRIDIZATION: CALCULATION OF THE MAXIMAL HYBRIDIZATION CAPACITY FOR MULTIPLE CALMODULIN MRNAS

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In estimations of mRNA copy numbers per volume unit, quantitative *in situ* hybridization (ISH) is expected to be performed at saturating probe concentration. In practice, however, this condition can scarcely be fulfilled when medium-to-high amounts of transcripts exist and/or in large-scale experiments. To resolve this problem, a double-step procedure has been developed and tested involving the use of calmodulin (CaM) I, II and III [^{35}S]-cRNA probes on adult rat brain sections. (1) By means of hybridization at increasing probe concentrations for a time sufficient for saturation, saturation curves were created for, and a maximal hybridization capacity (Hmax) value was assigned to, selected brain areas. The Kd values of different brain areas did not show significant differences, allowing the creation and use of one calibration graph showing Hmax values vs hybridized [^{35}S]-cRNA values for all brain areas for a given probe concentration. (2) Large-scale ISH experiments were carried out for the quantitation of multiple CaM mRNAs by utilizing a medium probe concentration. A calibration graph corresponding to the employed probe concentration was created and Hmax values (expressed in copy no./mm^3 units) for several brain regions were calculated via the calibration. The usefulness of the method described here was demonstrated by simultaneous quantification of the total accessible multiple CaM mRNA contents of several brain areas in a precise and economic way.

BUILDING BLOCKS OF A POPULATION MODEL FOR SWIMMING IN THE *XENOPUS* TADPOLE

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The basic behaviour of the *Xenopus* tadpole at stage 37/38 is that it can swim. This locomotion requires a neural network (Central Pattern Generator, CPG) that produces a co-ordinated alternating output to the muscles on the two sides of the body. The aim of this work was to create the building blocks of a computer model for the tadpole's CPG that will allow us to increase the number of neurones to get a population model for swimming in the tadpole. We modelled the neurones as single compartments since we found that the length of dendrites should exceed

100 μm to get significant differences between the one- and multi-compartment models and the size of tadpole dendrites is less than this limit. GENESIS software was used and run on a Pentium III PC to build neurones and a basic network of interneurones which produces sustained alternating activity closely resembling that found in experiments during swimming. The details of the construction of model neurones, synaptic connections and the operation of the network will be presented. We conclude that our model can serve as a basis for a population model of the tadpole's CPG, in which the number of neurones would approach that in the real animal. Such a model may increase our general understanding of CPGs.

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ULTRASTRUCTURAL STUDIES OF THE PINEAL COMPLEX OF THE GUINEA FOWL (*NUMIDIA MELEAGRIS*)

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Numida meleagris is a comparatively ancient species in the avian evolutionally rank. Compared with birds which are evulotionary younger its pineal complex is presumably more ancient. The guinea fowl has constant sexual photoperiodicity and besides producing melatonin its pineal organ may have direct light sensitivity as well.

The avian pineal originating from the diencephalic roof is located just under the skull and the meninges. It has a connection with the brain by a long pineal stalk. The structure of the pineal of *Numida meleagris* consists of tiny lobules with labyrinthic lumina that are

communicating with the third ventricle. The wall of the organ contains pinealocytes, glial cells and neurons.

Pinealocytes have inner and outer segments protruding deeply in to the lumina and contacting the pineal liquor. The outer segments are well developed by the end of embryonal age and in young birds. Later, due to fixation difficulties (caused by the large size of the organ) and degeneration their structure becomes hardly examinable. The dendritic process of pinealocytes abounds in microtubules. Our microscopic studies revealed ependymal cilia of the 9x2+2 pattern in the lumina of the pineal organ. Pinealocytes have relatively large dark nuclei. They are arranged in groups separated by septa of connective tissue, which are continuous with the meninges.

The septa contain many blood vessels (arterioles and venules), further myelinated and naked nerve fibers surrounded by collagen bundles. Some of these nerve fibers seem to innervate arterioles. The perikarya of pinealocytes contain granular vesicles also present in the basal process extending to the vascular surface. Among pinealocytes small ependymal and glial cells can be seen with dark multiform nuclei. Beside of kinocilia already mentioned, the ependymal cells bear microvilly on their luminal surface. The glial cells are sitting among the perikarya of pinealocytes. By their endfeet the peripheral processes of ependymal and glial cells form the glial limiting membrane of the vascular surface of the pineal tissue. Near the vascular surface there are large neuronal perikarya with light nuclei and rough surfaced endoplasmic reticulum. Axons running around the perikarya form axosomatic and axodendritic synapses. Further investigations are needed to clarify whether the hormonal (neurohormonal terminals of pinealocytic processes) or neural efferentation (synaptic contacts with pineal neurons) is the dominating output of the organ.

THE ROLE OF GLUTAMATE IN THE RELEASE OF ADRENOCORTICOTROP HORMONE

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The hypothalamo-pituitary-adrenal axis (HAA) is regulated by neuropeptides (corticotrop releasing hormone (CRH), arginin vasopressin (AVP)) which originate in the nucleus paraventricularis hypothalami (PVN) and play a prominent role in the stress response. We examined the role of glutamate in the release of adrenocorticotrop hormone (ACTH), the hypothalamic component of the HPA. Glutamate can act through distinct receptor types from which we studied the ionotropic N-methyl-D-aspartate (NMDA) and kainate receptors.

We destroyed the CRH and AVP containing PVN of male Wistar rats with a special knife; and used homozygous Brattleboro rats as a model of isolated AVP deficiency (this strain has a mutation). Under ketamin-xylazin anaesthesia we implanted a cannula into the right jugular vein for drug administration and blood sampling.

In control rats (sham lesion or heterozygous Brattleboro) both intravenous NMDA (5 mg/kg) and kainate (2.5 mg/kg) administration caused significant ACTH-elevation in 5

minutes. The PVN lesion alone did not influence the basal ACTH secretion but abolished the ACTH-elevation caused by the glutamate agonists. The lack of AVP in homozygous Brattleboro rats did not influence either the basal or the NMDA/kainate-induced ACTH-release.

Our results suggest that glutamate, acting through NMDA and kainate receptors can activate the CRH-containing neurons in PVN which - in turn - lead to ACTH-elevation in plasma. The two receptor-types can have different functional role as the time-course of ACTH-elevation was different. NMDA may have a role in short-term changes, while kainate might play a role in long-term changes.

HISTOCHEMICAL CHARACTERISATION OF SENSORY LONGITUDINAL AXON BUNDLES IN THE CENTRAL NERVOUS SYSTEM OF *LUMBRICUS TERRESTRIS*

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Earlier we demonstrated that NADPH-diaphorase (NADPH-d) activity and GABA-like immunoreactivity were found in primary sensory cells that have central afferent to the ventral nerve cord ganglia but its anatomical position in the central nervous system has not been identified yet. Therefore, in this study the pattern of the above-mentioned neuroactive substances was determined in the sensory pathways of the ventral nerve cord ganglia of *Lumbricus terrestris* (Annelida, Oligochaeta).

All sensory longitudinal axon bundles (SLBs) contained NADPH-d positive fibres. The strongest staining was found in the ventrolateral (VL), ventromedial (VM) and intermediomedial (IMM) SLBs while the intermediolateral (IML) and dorsolateral (DL) SLBs were moderately labeled. In the VM- and VL-SLBs strong GABA-like immunoreactivity, while in the other ones (IMM-, IML- and DL-SLBs) no immunostaining was occurred.

Our results show the histochemical heterogeneity of SLBs in the earthworm CNS suggesting the existence of a multitransmitter system in the perception of the environmental stimuli. In this study we report first on the occurrence of GABA in the central sensory pathways of earthworms, however, its role in sensation has not been clear yet.

This study was supported by the Hungarian National Research Fund (OTKA No. T 026652) and the Adaptation Biology Research Group of Hungarian Academy of Sciences and University of Pécs.

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BOOK REVIEWS

DNA TOPOISOMERASE PROTOCOLS. VOLUME I: DNA TOPOLOGY AND ENZYMES

in "Methods in Molecular Biology"series, Vol. 94
ED. BY BJORNSTI, M-A. AND OSHEROFF, N., Humana Press 1999

Among the numerous enzymes that act on DNA, DNA topoisomerases comprise a family of increasing importance. They intervene in those processes where the highly organized structure of the duplex DNA pose topological constraints, DNA replication, transcription and nucleosome assembly being the most important ones. Several key features of these processes has been understood on the basis of recent studies on DNA topoisomerases.

The first volume of "DNA topoisomerase protocols" provides background information and experimental protocols for researchers willing to study the topology of DNA as well as for those interested in DNA topoisomerases.

Special attention is paid to the different aspects of the higher-order structures of the double-stranded DNA; supercoiling, knotting and catenation. Techniques are given for the purification and analysis of DNAs of particular topological form as well as for their use in *in vitro* topoisomerase assays. The description of the classical way of there solution of DNA molecules by one- and two-dimensional agarose gel electrophoresis is followed by more specific techniques treating e.g. the analysis of cruciform DNA, the purification of supercoiled plasmid DNA or the isolation of the kinetoplast DNA network of the protozoan parasite *Crithidia fasciculata*. Camilloni et al. (Chapter 6) provides a clear compilation of the methods for the generation and purification of DNA minicircles of different linking numbers, together with their possible applications. Four ways of analyzing DNA bending (ligation ladder, circular permutation, phasing and ring-closure experiments) are described and critically evaluated in Chapter 12 by J.D. Kahn. The detailed protocol given for coating DNA with RecA protein for visualization of DNA structure in the electron microscope is worth of mentioning (Zechiedrich and Crisona, Chapter 11).

The next ten chapters consider the production, purification and properties of several different DNA topoisomerases. Powerful techniques are described for the overexpression of these enzymes in a multitude of the expression systems (baculovirus, bacterial, yeast and mammalian). A method is given for the isolation of topoisomerases from native sources as well (*Drosophila melanogaster*, Chapter 21), in order to check if the enzymes expressed in non-native systems are identical with those in the native species.

Chapters 24-28 describe techniques and data of general interest, ranging from affinity chromatography techniques applicable for the purification of DNA topoisomerases to the compendium of the amino acid sequences known. In conclusion, this book is a useful compilation of reliable techniques described in a detailed, reproducible way by well-known experts of the field for the purification and analysis of DNA and DNA topoisomerases.

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Akadémiai Kiadó, Budapest

A NEW REGULATORY FUNCTION IN THE NERVOUS SYSTEM

ED. BY BAULIEU, E-E., ROBEL, P. AND SCHUMACHER, M.
in Neurosteroids, Humana Press 1999

The nervous system is a target of steroid hormones. Several of these hormones - termed neurosteroids – are synthesized de novo within the neuronal and glial components of the central and peripheral nervous system (CNS and PNS) from their precursors. Since the discovery of this fact in 1981, numerous aspects of their physiological importance and pathological effects have been clarified. The first book devoted entirely to neurosteroids ("Neurosteroids. A new regulatory function in the nervous system" (ed. by Baulieu, E-E., Robel, P. and Schumacher, M. Humana Press 1999) provides an interesting, detailed and up-to-date comprehensive review of these achievements.

The first chapters provide basic information on the subject, including e.g. the biochemistry and metabolism of the neurosteroids, as well as the developmental, regional, and tissue-specific regulation of the expression of the steroidogenic enzymes within the CNS and PNS. Subsequent discussions concentrate on the physiological functions of neurosteroids. It is now well established that these hormones affect brain function by acting on neurons and on glial cells as well. They exert their effects both via classical intracellular receptors and by interacting with cell-surface neurotransmitter receptors, the first mechanism providing a possibility for regulation of gene expression, while the second results in the modulation of neuronal excitability. This latter seems to be achieved primarily by the modulation ofGABAergic neurotransmission, but regulation of other ligand-gated channels (ionotropic glutamate receptors, nicotinic acetycholine receptor) or the voltage-dependent and -independent calcium channels are also of great importance. Excellent reviews describe the molecular, physiological and behavioral correlates of these phenomena. The reader is guided through the role of neurosteroids in CNS development, sex differentiation of the brain, stress, learning, memory and brain aging, just to mention some of the most important aspects. Information on the regulation of synaptic and glial plasticity by gonadal hormones and neurosteroids are also extensively and critically evaluated. Description of behavioral studies and evidences pointing to the psychopharmacological potential of neurosteroids complete the scope of the subjects covered.

The amount of references cited is remarkable facilitating further orientation for the interested reader. The referee is also impressed by the fact, that descriptions are frequently concluded by new questions emerging on the basis of the data mentioned, indicating the dynamism of this nascent scientific field.

This monograph is a fundamental reading for neurobiologists, endocrinologists, pharmacologists - or anybody else who wishes to gain deeper insight into the multiple facets of the physiological and pathological roles of steroid hormones in the nervous system.

V. Jancsik

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