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J. Ben-Gurion

An international symposium on NEURONS AND NEURON ASSEMBLIES sponsored by the Hungarian Academy of Sciences and by the Hungarian Association of Anatomists was held at Pécs, Hungary, August 30 - September 1, 1982. The invited speakers paid tribute to Professor János Szentágothai whose 70th birthday was celebrated on that occasion. By publishing a considerable portion of the papers delivered at the above symposium the Editorial Committee of the present journal wants to congratulate Professor Szentágothai and wishes many productive years for his scientific activity.

CONTENTS

THE BANQUET

This is a great occasion to honour János Szentágothai, *whom we are so happy to recognize as our friend and as our inspiration. He is a true scientist in an age where the customary performance is to publish data - measurements and observations with a minimum of interpretation because ideas and interpretations may be disproved and one does not dare to be wrong! This can be seen in so many neuroanatomical publications where great details of neuronal structure and connections are depicted, but no attempt is made to give a comprehensive diagram, say of the basic neuronal circuitry of the cerebral cortex, in which the discovered details achieve a wider significance. By contrast look at one of John's papers - on the spinal cord - on the cerebellum - on the cerebral cortex - and you will find in addition to the experimental findings a superb diagram drawn by John, who is a skilled artist. You will see his creative imagination in action. He dares to draw a detailed structure of neurones and their connectivities as he envisages them in the light of his knowledge, knowing full well that the maze of cells and connectivities is but a provisional picture and will in due course be corrected in the light of the new knowledge that is provided by experiments testing his hypothesis; and correct them he does in the light of new discoveries. But that is the essence of the scientific enterprise - to put up creative ideas or hypotheses that challenge experiment and falsification. And so we progress in our scientific undertaking in the manner so effectively described by Karl Popper. John's beautiful diagrams have spoken to me and given the guidance in my years of studying the cerebellum and then the cerebrum, and I am sure that I can speak for you all. Of course there have been criticisms by the "hard science" school who believe that one should do no more than report what we see. So John has been described by a distinguished neuroanatomist:*

"The last of the romantic anatomists".

On the contrary I believe that John has made neuroanatomy an exciting adventure. The study of the brain is the greatest scientific challenge confronting mankind. By contrast the great successes of space travel, for example, are trivial. We have to be romantic in this tremendous adventure. I hope for a succession of romantic anatomists. Ramon y Cajal was a great pioneer, and John Szentágothai is a worthy successor of Cajal.

Sir John C. ECCLES

Aug. 31, 1982

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THE FOCAL TONIC CORTICAL CONTROL OF INTRALAMINAR THALAMIC NEURONS MAY INVOLVE A CORTICO-THALAMIC LOOP

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The effects exerted by the cortex on thalamic neuronal activity were studied using the technique of cortical spreading depression. Glass micropipette recordings were made simultaneously in the thalamus and cortex and we found that the activity of a portion of the thalamic neurons was suppressed when the cortical spreading depression arrived at a particular and localised cortical area, which was different for different thalamic nuclei. The suppression of spontaneous activity was longer and more frequently observed for cells of the intralaminar thalamic nuclei.
To determine if the action of the cortex on the intralaminar thalamic nuclei, demonstrated by these experiments, involved a monosynaptic pathway we used both electrophysiological and anatomical methods. When recording in the cortical area which we had found to control intralaminar thalamic activity we observed that, antidromic activation and also a pause of cortical cells was produced by intralaminar stimulation. When HRP was injected into the intralaminar nuclei we found retrogradely labelled cells in the same cortical area.
To determine if a reciprocal connection exists we injected HRP into the same cortical area; retrogradely labelled cells were subsequently found in the intralaminar nuclei.
A reciprocal cortico-thalamic connection thus appears to exist between the controlling cortical area and the intralaminar thalamic region under this control. The possibility that this loop is involved in the facilitatory descending influence exerted on intralaminar nuclei is discussed.

In order to examine the role played by the cortex in the activity of thalamic structures we have transitorily removed the cortical influence acting at this level. Different tech-

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niques can be used to produce such a reversible block. In cats and monkeys, cooling techniques or cortical blockade through local application of KCl were used to study similar problems [6, 15]. As we were working in rats we were able to use the technique of spreading depression, first described by Leão [10] and subsequently by Bures et al. [5], to investigate cortical subcortical relationship. This technique provokes a transient and reversible block which is propagated over the entire cortical surface.

We have recently reported [1] using this technique, the existence of a tonic cortical facilitatory control over thalamic nuclei and we have shown that intralaminar nuclei were the most significantly affected. We also reported evidence for the existence of a topographic organisation of this cortico thalamic control. In this paper we will give more evidence for this topical organisation and through electrophysiological and anatomical techniques describe our search for a reciprocal cortico-thalamic loop that may be responsible for some of these focal effects.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 280 gr were used in these experiments, 71 for the electrophysiological section and 5 for the HRP studies.

For the electrophysiological experiments the rats were anaesthetised intramuscularly with Ketamine (80 mg/kg), tracheotomised and placed in a conventional stereotaxic apparatus. The skull

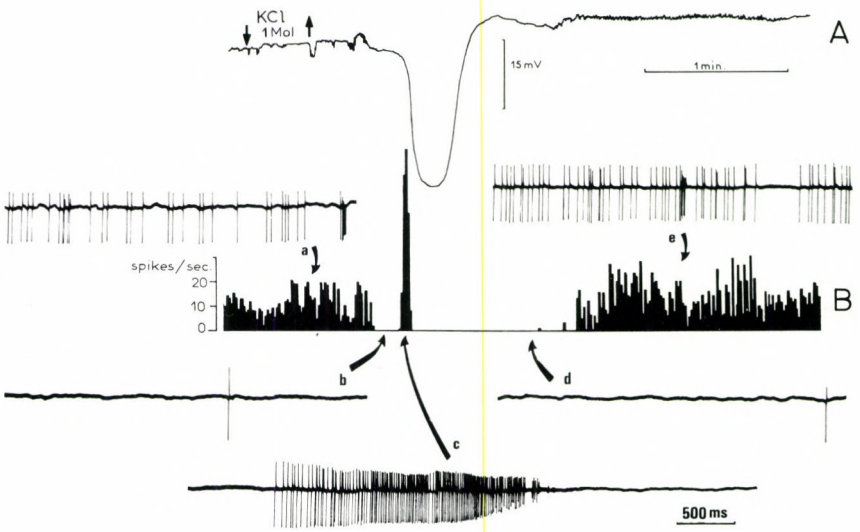
was exposed and trephine openings were made in order to introduce the stimulating and recording electrodes and also to produce the cortical spreading depression. The animals were then paralysed with Gallamine triodoethylate (Flaxedil) (15 mg/kg) and artificially ventilated. Heart rate was monitored continuously and body temperature maintained at about 37 ^{o}C by a hot water circulating pad.

The stereotaxic coordinates (thalamic and cortical) were taken from the atlas of D. Albe-Fessard et al. [3] and Sapienza et al. [14].

Extracellular recordings were made using one, two or four microelectrodes (micropipettes filled with KCl (0,6 M) and pontamine blue (4 %). The electrode impedance was between 8 to 10 MΩ. For each microelectrode, recordings were obtained from a DC amplifier with a large frequency band. The recorded activity was filtered in order to separately derive DC phenomenon and spikes. The spikes were recorded on tape or film, or digitalized and automatically counted to construct time frequency histograms or raster dot displays.

To trace pathways we have used antidromic activation techniques and HRP retrograde labelling of cells. To recognize antidromic activation of cells we employed the three classical tests described by Paintal [12] , Darian-Smith et al. [8] , Albe-Fessard et al. [2] and many others: fixed latency, ability to follow a high frequency stimulation and collision with spontaneous spikes. For central stimulation, bipolar concentric stainless steel electrodes (50 to 100 KW) were implanted in thalamic nuclei using stereotaxic coordinates and electrophysiological

the posterior region (Ant. 1.5). The application of KCl lasted approximately 1 minute after which the dural surface was carefully washed with physiological saline solution. Recording with a single microelectrode, connected to a DC amplifier, it was observed that the cortex anterior to the site of KCl application was invaded by a DC wave which was accompanied by the silence of the given cell recorded. This wave of blockade of the cellular activity travelled at a speed of 4.8 mm/min. Figure 1 shows that the DC shift is preceeded by a pause (b), then a burst of spike activity (c) which accompanied the onset of the DC shift and a long pause in spike activity commenced after the onset of the prolonged negative DC shift. In a single electrode tract we observed similar phenomena for all the cortical cells encountered (Fig. 2). In brief the activity of a column of cells was blocked when the GSD arrived at its level. During this period the responsiveness of cortical cells to afferent impulses as well as to antidromic activation disappeared. We have verified this fact by activating cells through pyramidal stimulation.



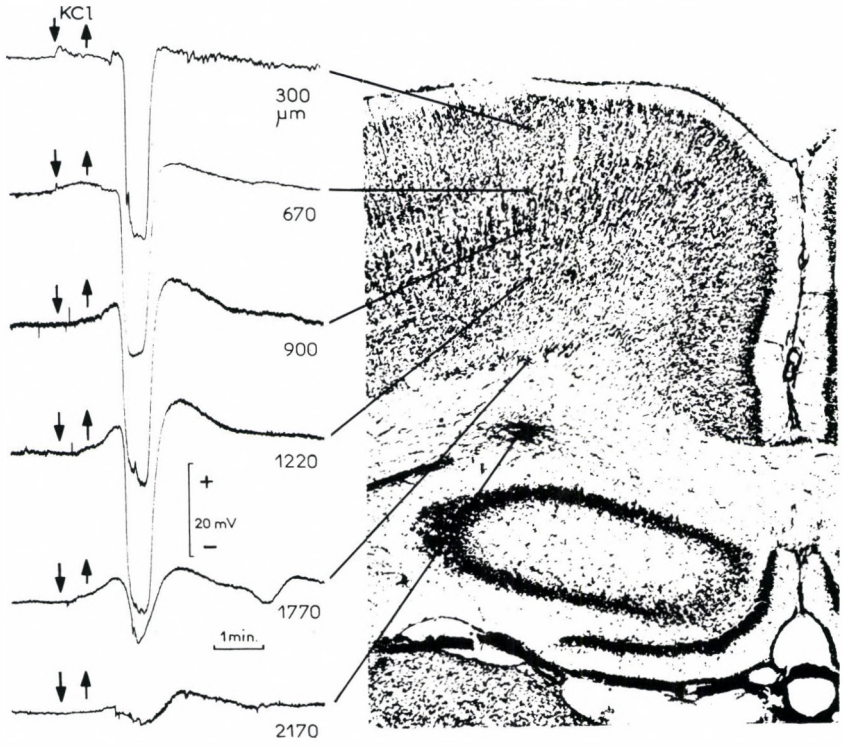


Fig. 2. Recordings taken from a cortical microelectrode trajectory (depth in µm) in wich the activity of 5 cells (5 superior traces) was recorded during the arrival of successive spreading depressions provoked by the application of KCl at exactly the same posterior cortical area (arrows). Only DC changes are presented but for all 5 cells the spikes had the behavior presented in Fig 1. The lower record was performed at the level where the DC shift disappeared

Fig. 1. Events appearing at cortical cell level on arrival of a spreading depression produced by the application of KC on posterior cortical zone (arrows). Recordings were performed with a glass micropipette, they are treated in three different ways. Upper trace : DC changes are recorded on an ink-writer, the spike activity being filtered. A downward deflection corresponds to a negativity of the recording electrode. Middle trace: The spike activities deprived of their DC component are automatically counted and a frequency histogram is presented at the same speed as the DC changes (1 min). Spike tracings: A few examples of the spike activity are presented at a more rapid speed (500 msec). They correspond to the different phases (a, b, c, d, e) of the frequency histogram, and the correspondences are underlined by arrows. For the spikes a downward deflection corresponds to a positivity of the microelectrode

RESULTS

Behavior of cells in different thalamic areas during cortical spreading depression

In normal conditions, cortical spreading depression is not transmitted to thalamic areas. This point is well demonstrated in Figs 3 and 4 where no DC shift was observed in the thalamic

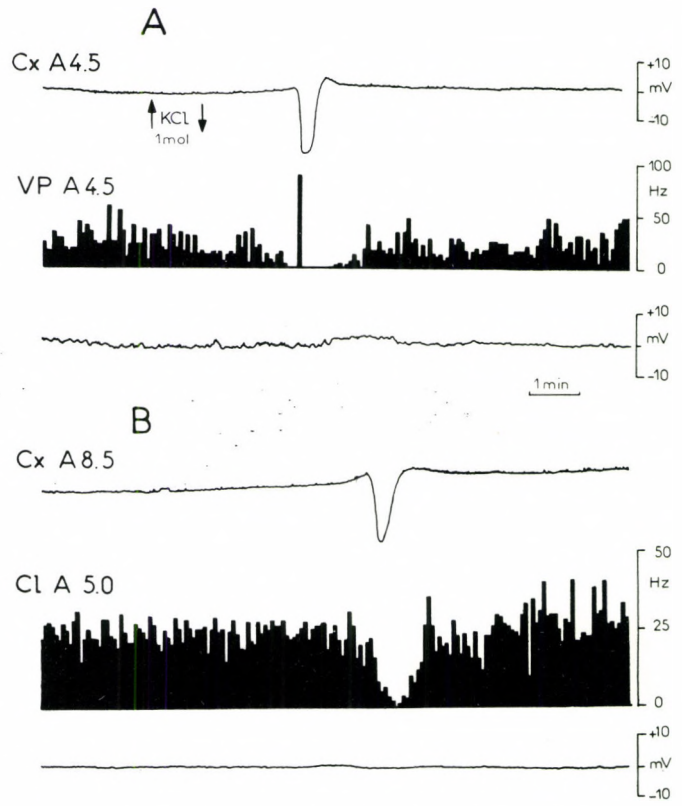


Fig. 3. Events appearing in two different thalamic nuclei when a spreading depression propagates in the cortex. Four simultaneous microelectrodes recordings were used : two in cortical regions (1st and 4th line), two in thalamus, one in ventralis posterior (VP, 2nd and 3rd line), one in centralis lateralis (Cl, 5th and 6th line). For the cortical recordings only DC shifts are presented, for thalamus spike frequency histograms and DC recordings are given. Note that spike activity is modified at thalamic level when spreading depression arrives at a given cortical focus and that no negative DC shift appears in the thalamus

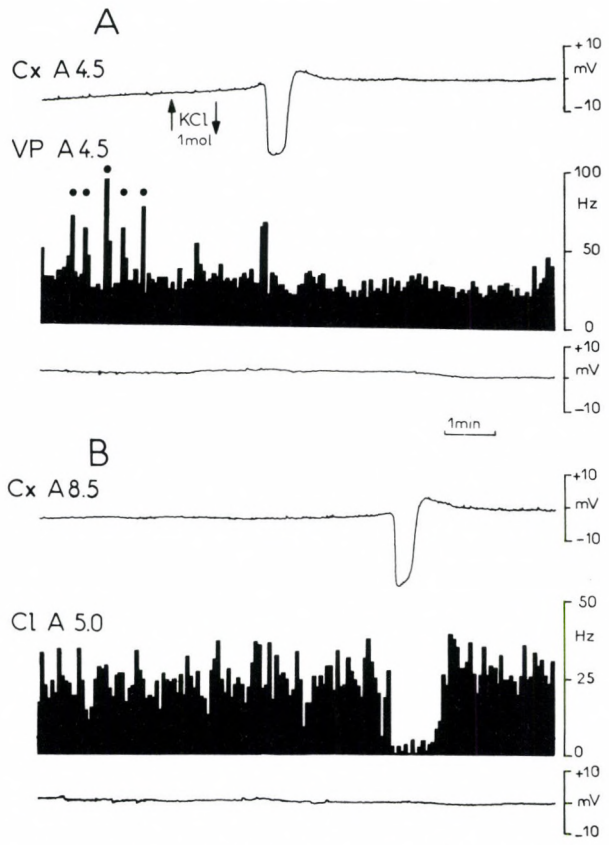


Fig. 4. Same type of experiment as for Fig. 3. Note that in this case the VP cell activity is not depressed during the cortical spreading depression and only a short burst of activity appears. This VP cell was driven by tactile natural stimulation applied to the forelimb (black dots)

controls. To mark the stimulating electrode at the end of the experiment an electrolytic deposit of iron was made by passing a current of 100 μA anodal for 10 sec and stained using a ferric-ferrocyanide reaction. To verify the position of the microelectrode recordings, an ejection of pontamine blue was performed at the terminal point of each trajectory using 15 μamp cathodal for 15 minutes. The brain was perfused with formalin 10 % and frozen sections were stained with the Nissl technique. HRP injections were performed in the cortex and in intralaminar

nuclei with a syringe connected to a micropipette filled with a solution of 40 % HRP in distilled water. A volume of 0.03 µℓ was injected in the cortex and in the thalamus 0.01 to 0.0ℓ µℓ were injected. After a survival time of 48 hours the animals were anaesthetized and perfused intracardially with a solution of 2 % paraformaldehyde, 2 % glutaraldehyde in 0.1 M phosphate buffer. The brain was removed immediately and left in the perfusion solution overnight then transfered to a phosphate buffer with a 5 % glucose solution for a further 48 hours. The brains were then sectioned on a freezing microtome at 40 µm and treated with DAB reaction.

The sections were mounted and counter-stained with Nissl stain and examined with a light microscope.

Cortical spreading depression (CSD)

The CSD was provoked by placing a small piece of filter paper (4 mm^2) soaked in 1 molar KCl onto the dural surface in recordings when spreading depression appeared at different cortical levels. However the same figures demonstrate that cells in different thalamic nuclei undergo changes when spreading depression invades the cortex. These changes are presented in Fig. 3 for a ventralis posterior (VP) and a centralis lateralis (CL) cell. Four main points from this expreiment can be deduced:

- In both cases a reduction of spike activity was observed in both cells and it was of about the same duration.

- Only in the VP cell did a burst of discharge appear during the first phase of cortical spreading depression.

- The reduction of spiking was similar in duration to that produced by CSD in a cortical cell.

- The reduction of spiking did not appear at the same time in different nuclei.

In Fig. 3, we have presented the CSD's which corresponded to VP cell change (Ant. 4.5) and to CL cell change (Ant. 8.5).

A similar experiment is presented in Fig. 4, but here the VP cell did not undergo as clear a reduction in spiking as the CL did and only a short burst of spikes appeared during CSD. In this case the VP cell could be driven by tactile stimuli. We have found that VP cells not controlled by the cortex often had this property.

Figure 7B presents a map of the position of the cells studied in the thalamic areas which presented the three different types of behavior described above. The only long lasting effects observed were a reduction of spiking. The other clear phenomenon are a short excitatory burst of spikes of about the same duration as that accompanying the onset of cortical spreading depression. The reduction of spiking can be easily explained if we accept that a descending facilitatory effect coming from cortical cells is acting permanently on certain thalamic cells and that these facilitatory pathways are topically organised.

The burst of discharge can be explained in two ways, either it is the result of the excitation of the facilitatory pathways by the phasic discharge which just preceeds the onset of CSD or, it is the sign of a phasic suppression of an inhibitory mechanism.

The technique of cortical blockade which we have employed does not allow us to determine if the facilitation or inhibition just proposed are mediated through a direct or a relayed pathway, we will deal with this problem in the following section.

Direct descending connections between cortex and intralaminar nuclei

We have searched for these connections using both electrophysiological and anatomical techniques, both results are presented in Fig. 5.

(a) Recording in the cortex at the level from which the cortical control exerted on CL cells was found (Ant. 8), we studied the responses to bipolar stimulation of Cℓ. Seventy-two cells were studied, among them 9 presented a short latency excitatory response. Four of them were confirmed by the collision test to be antidromic responses (Fig. 6A). The latencies of responses corresponded to axon conduction velocities of between

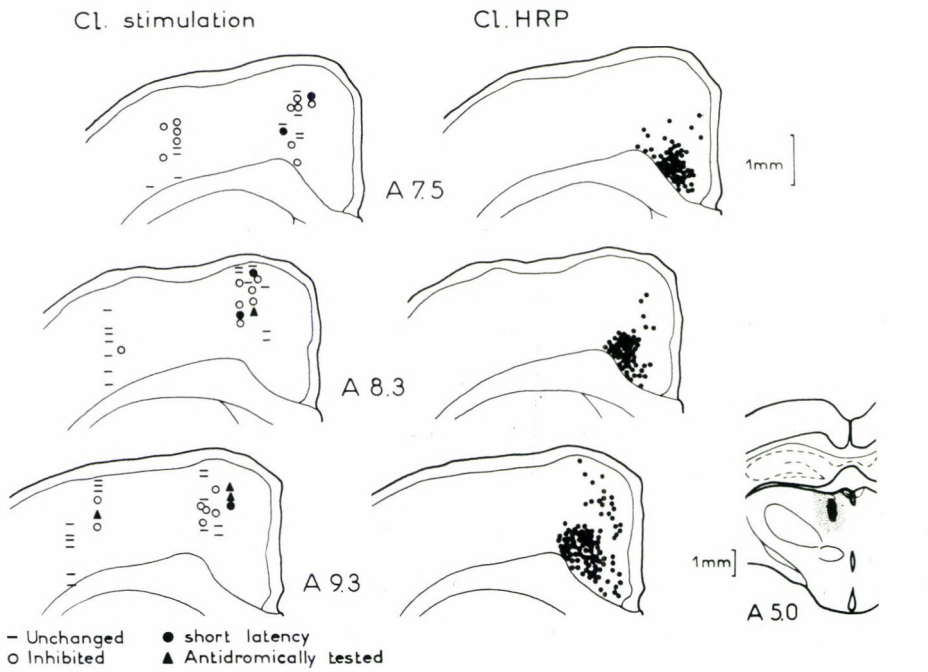


Fig. 5. Electrophysiological and anatomical results obtained at cortical level when stimulation (at left) or injection of HRP (at right) were applied at Cℓ thalamic level in the area in which the majority of cells presenting a pause during cortical spreading depression were found (diagram lower right corner). Note that cells antidromically activated by Cℓ stimulation are in the same area as cells at the origin of a cortico-Cℓ pathway. At this same level a portion of the cells are inhibited or not affected

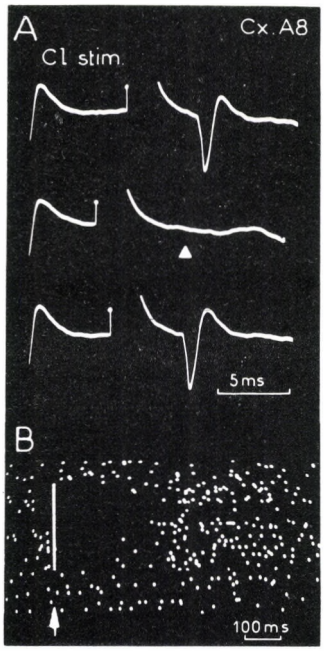


Fig. 6. Examples of responses of cortical cells produced by Cℓ stimulation. A : cell antidromically activated. Only the collision test with a spontaneous spike (which trigger the sweep) is presented here (triangle designates the place of the collide antidromic response). B : another cell presenting a pause after Cℓ stimulation (arrow). Successive pauses are presented on a raster display

2 and 5 m/sec. The other 5 cells responded to Cℓ stimulation with too short a latency for the collision test to be conclusive. The collision time may have been the refractory period. The 9 cells excited at short latency also showed a subsequent decrease in their spontaneous activity. A further 25 cells presented only a decrease in their spontaneous activity. The duration of the pause had a mean value of 144 msec ± 42, a typical pause response is presented in Fig. 6B. The 38 remaining cells studied had their activity unchanged by CL stimulation. As can be seen in Fig. 5 (at left) the majority of the unresponsive cells were found in the more lateral cortical explorations.

(b) Injections of HRP into the Cℓ were performed in 2 rats. These injections were placed at the same level as where the Cℓ was excited in the electrophysiological experiments. The positions of the cells retrogradely labelled are presented in Fig. 5 (at right). This region corresponds to the area where short latency responses were found with electrophysiological technique.

These experiments have demonstrated that a dire t pathway exists between cortex and Cℓ which may be the support of the facilitatory process which is suppressed by the CSD wave.

The pause which follows the excitation produced by CL stimulation can be explained in two ways. It is due to the activation of an inhibitory intra-cortical neuron, either by a collateral of the descending axon or, by an ascending pathway coming from CL. To make a choice between this two hypotheses we had to determine if a CL-cortical pathway exists.

An ascending pathway between CL and cortex

Injections of HRP were performed in the cortex at the anterio-medial level where antidromically activated cells were found after CL stimulation (Ant. 8, Lat. 1). The position of the thalamic cells labelled in 3 such experiments are presented in Fig. 7A. They were found in majority in CL, in the lateral nucleus and a small part of ventralis lateralis (VL). It is interesting to note that in the same nuclei cells generally present a pause in their activity during the passage of CSD in the same cortical area as the injection. Figure 7B shows for comparison, the place where cells presenting only a decrease of activity during CSD (black dots) were found. In Fig. 8 two examples taken from two

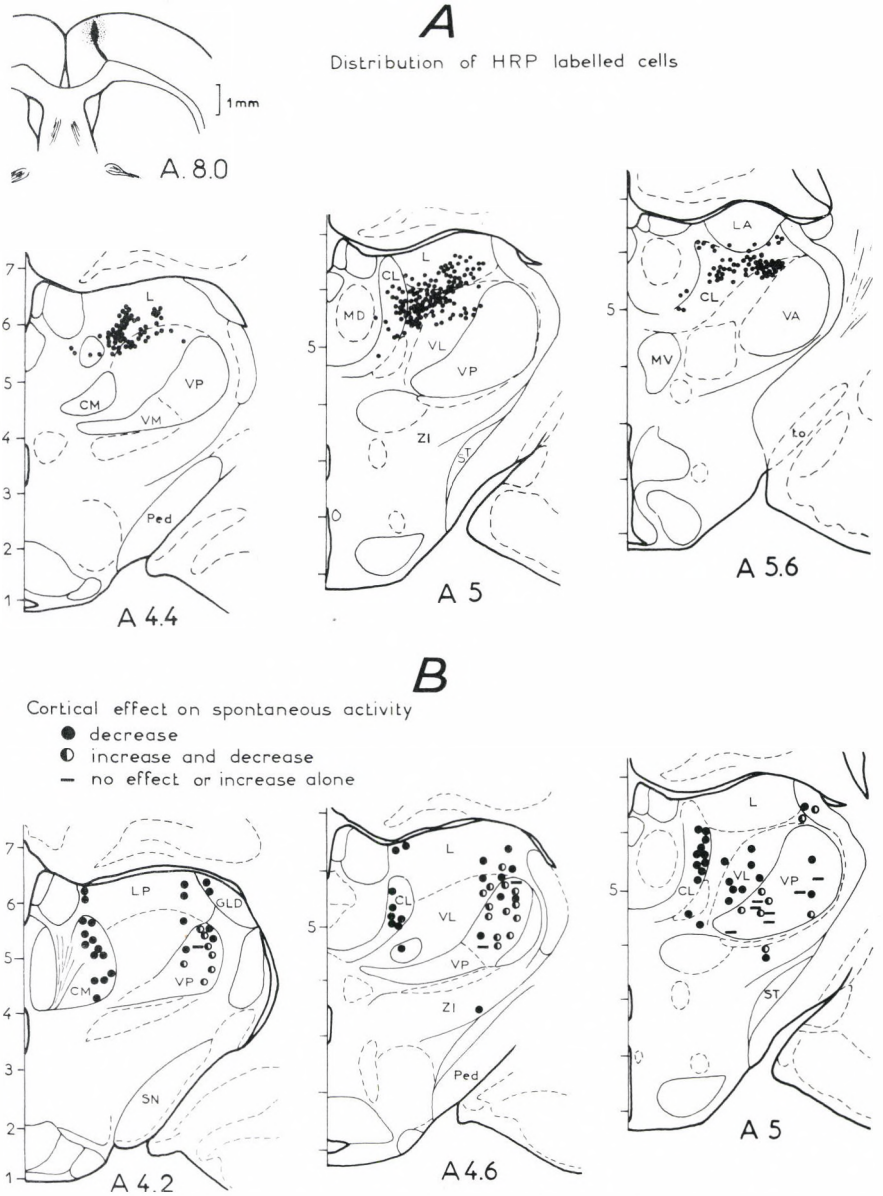


Fig. 7. A : distribution of the cells of origin of a thalamo-cortical pathway determined at 3 anterior levels. An injection of HRP was performed at Ant. 8 (diagram upper left hand corner) in the zone where a cortical spreading depression was shown to be simultaneously accompained by a pause in CL cell activities. The majority of the cells which where labelled are in centralis lateralis (CL), nucleus lateralis (L) and a part of ventralis lateralis (VL). B : for comparison the distribution of cells whose reaction during cortical spreading depression were studied is shown (1)

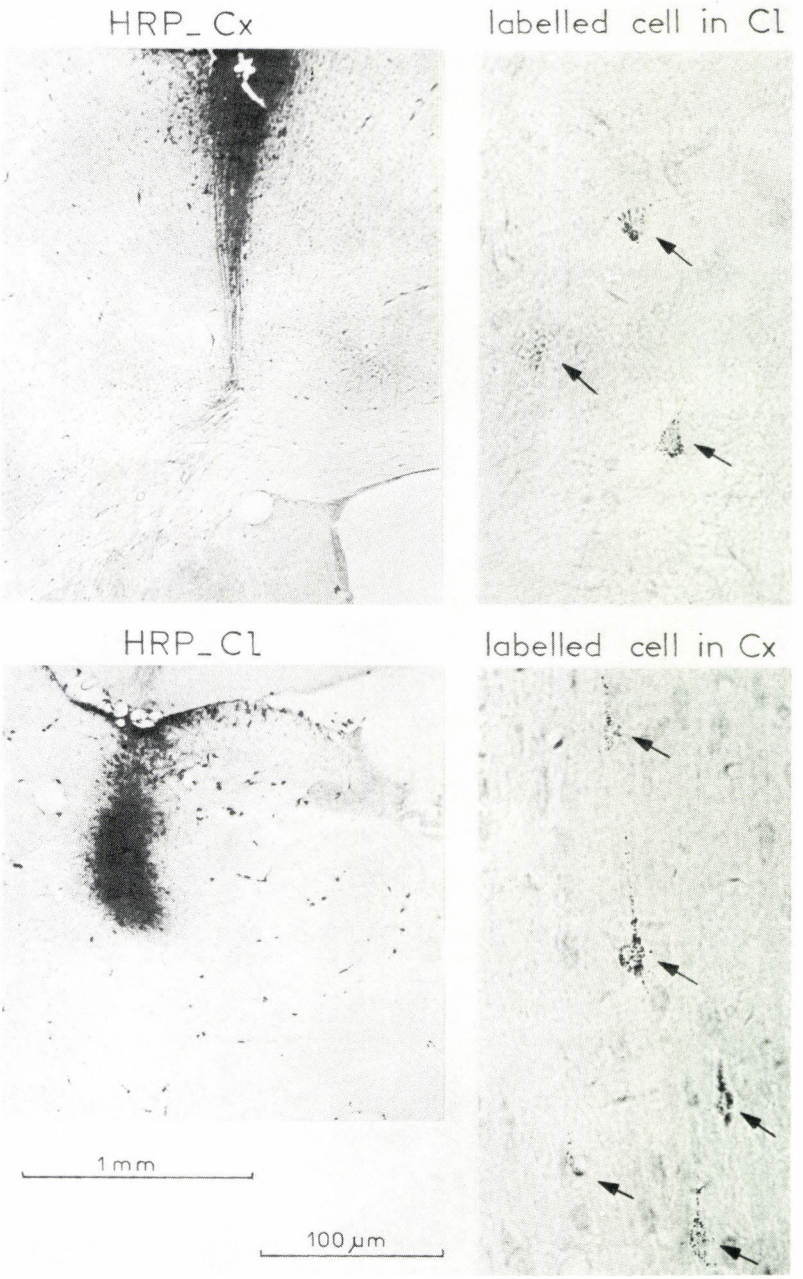


Fig. 8. In two different animals the HRP injection sites are presented at left and examples of the corresponding labelled neurons at right (arrows). First row the injection was in cortex the labelled cell in Cl. Second row, the injection was in Cl, labelled cells in cortex

different animals are given, they show the reciprocal connections between CL and the cortex at anterior 8, medial level.

DISCUSSION

We have confirmed in this paper, using the CSD technique, that cortical areas have a permanent tonic facilitatory influence on certain thalamic cells. This tonic control is clearer in the intralaminar region and the cortex involved is in the medial area of plane Ant. 8. From the maps drawn by Sapienza et al. [14], this region corresponds to the limit of motor and premotor cortex.

1. This facilitatory effect seems to involve a direct descending pathway, the existence of which is demonstrated in our work. However the definitive proof of the involvement of the direct descending pathway in this facilitation would be the demonstration that the descending cortico-thalamic pathway is excitatory. This point has not yet been demonstrated in the rat. The existence of a descending pathway from cortex to medial thalamus has been previously reported by Rinvik [13] in cats with the use of silver impregnation. He showed that the caudal portion of the cingulate gyrus projects to CM-CL and that a topographical organisation exists.

2. A reciprocal connection exists between the cortically controlled intralaminar nuclei and the cortex of origin of this control. The ascending pathway from CL to cortex was found in the cat by electrophysiological [2] and anatomical techniques [9,4,11]. In the rat, Cesaro et al. [7] have also confirmed its existence.

Our results are in close agreement with previous reports which show the existence of a cortico-thalamo-cortical loop for intralaminar nuclei. They give moreover, a functional role to this loop which seems to play an important role in spontaneous activity at thalamic and probably also at cortical level.

3. It is difficult to decide with the experiments we have performed if the short burst of activity which appears in certain thalamic cells (in particular in VP cells) is due to an activation of the facilitatory pathway by the burst of activity which signals the onset of CSD or, if it represents the suppression of a descending phasic inhibitory control. We are in favor of the first explanation. This opinion is based on the experiment in cats of Waller and Feldman [15] which compared the effects of KC1 blockade and of cooling, and reported that it is only with spreading depression, where the cortical blockade is preceeded by an excitation, that the thalamic excitatory burst appeared in VP.

4. If we accept that the origin of the first burst is an excitation of the facilitatory descending pathway, it is difficult at first sight to explain why this first burst is not present in intralaminar cells, the place where the facilitatory tonic cortical pathway seems to have a primordial role. This fact can be explained however, if we consider that all descending facilitatory impulses may activate an ascending inhibitory circuit acting on the cells in which the descending facilitatory action originates. This inhibitory retroactive process would then limit the frequency at which a CL cell can follow a descending facilitatory message. The cortico-thalamo-cortical loop would thus exert a sort of filtering on the

descending facilitatory circuit. This phenomenon must be absent in the case of VP cells where the first burst appears but is not followed by a pause in activity. Experiments are now being performed to examine if this is tenable.

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DEVELOPMENT OF VISUAL SYSTEM - COMPARISON OF MONKEY AND MAN

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In Golgi preparations of the lateral geniculate nucleus of adult humans and monkeys several types of neuron are described. Multipolar neurons have a "radiate" or "tufted" dendritic arbor. The next commonest class is the bipolar neuron with two or three thick dendrites arising from opposite poles of the soma. A few examples of rare medium-sized neurons with beaded dendrites are found. There are also small neurons with fine "axon-like" dendritic processes. Some have long, untapered dendrites and others shorter dendritic arbors. A class of large, capsular neurons is found in the circumgeniculate capsule.

Maturation of lateral geniculate nucleus neurons in baby humans and monkeys was studied in Golgi preparations. They pass through post-natal stages characterised by dendritic growth cones and a profusion of spine-like protuberances on dendrites and somata. The mature form is found by the second month in monkey and about one year in man.

The morphological changes in the thalamus are paralleled by synaptogenesis in the visual cortex. In man synaptic density reaches a maximum at about one year of age, declines later in childhood and stabilises at adult levels by about 11 years of age.

In both monkey and man the period of morphological maturation in the visual pathways corresponds to a time of increasing visual acuity when visual deprivation is most likely to have permanent harmful effects.

Study of the development of the visual pathways of normal and visually deprived animals has helped us understand some of the patho-physiological processes in human visual defects, especially amblyopia. Of the animals used the one most likely to allow extrapolation to man is the monkey. We here show that there is a close relationship between monkey and man in some

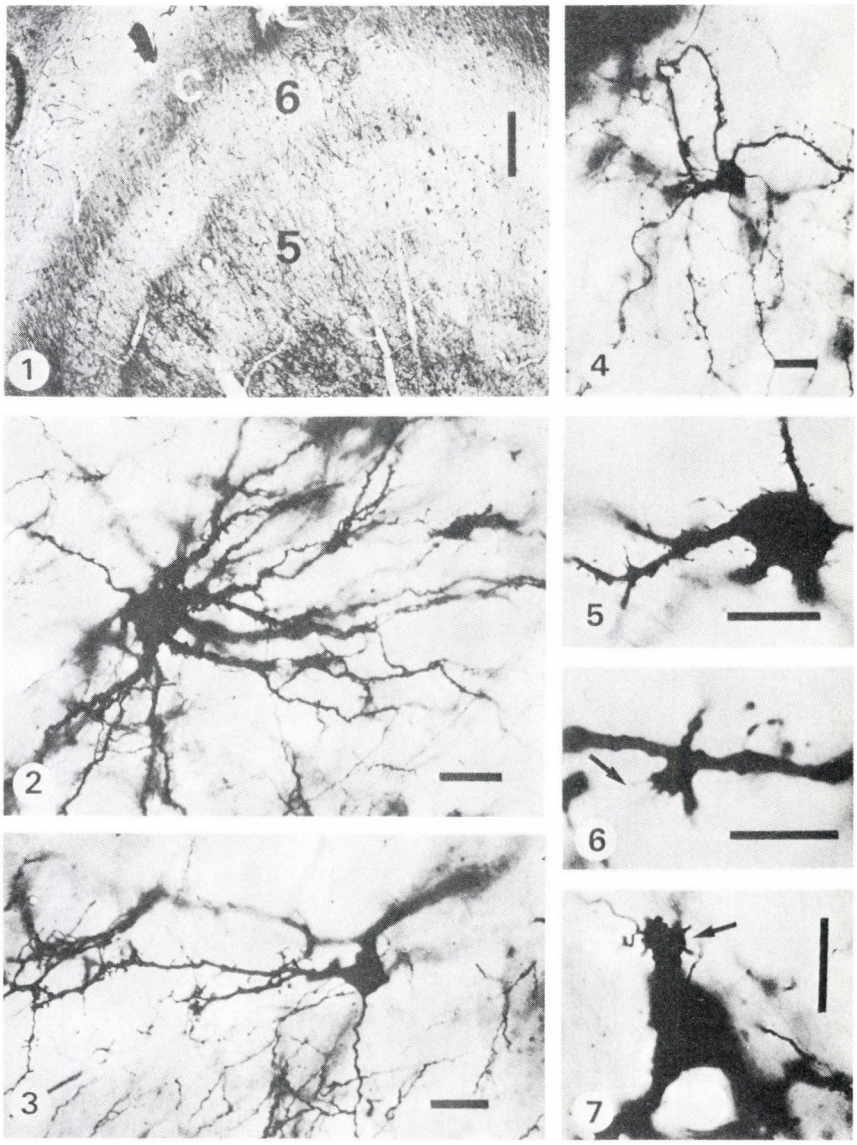
aspects of the morphological maturation of the visual pathways.

A rapid increase in the size of neuronal somata and the number of synapses in the lateral geniculate nucleus (LGN) has been shown in animals, including the monkey, in the first weeks of life [10, 14, 18] when visual acuity is increasing and during which visual deprivation can cause morphological and functional defects [3, 4, 17, 21, 22, 26, 28, 29]. A postnatal increase in LGN soma size also occurs in man during the first two years of life [17], when he is also highly sensitive to the effects of visual deprivation [2, 27]. The visual system remains sufficiently "plastic" to recover if corrective action is taken during this time [4, 5, 11, 13, 24, 26].

Maturational changes also affect the dendrites and axons of LGN cells in the monkey. In material impregnated by the method of Golgi the LGN laminae are easily visible (Fig. 1) and various cell types can be identified [25]. They have similar features in all monkeys examined by us including several old-world, and one new-world, species. The commonest type of neuron in the monkey LGN is the multipolar, with several dendrites originating from the soma either radially, or in an asymmetrical "tuft". Next

Fig. 1. Part of LGN of a late monkey foetus. Laminae (5 and 6 are labelled), interlaminar zones and circumgeniculate capsule (C) are clearly visible. Scale: 0,5 mm
Fig. 2. Multipolar neuron in a one-day-old monkey. The dendrites are well developed, but bear many hair-like and spiny processes. Scale (Figs 2-7 : 25 μm)
Fig. 3. Neuron with long, cylindrical dendrites in a one-day-old monkey. Hairs and spines are rare on such neurons.
Fig. 4. Another form of neuron with cylindrical dendrites in a 14 day monkey
Fig. 5. Hairs and spines on dendrites and soma of multipolar neuron in 14 day monkey
Fig. 6. Growth bud with filopodia (arrow) on a dendrite of a foetal monkey
Fig. 7. Growth cone with filopodia on a dendrite of a 5 day monkey

commonest is the bipolar neuron, with two diametrically opposed stem dendrites. The "triangular" neuron is a variant, with one dendrite at one pole and two at the other. Much rarer are neurons with very long, untapered ("cylindrical") dendrites and "axon-like" dendritic processes, perhaps interneurons. Two other,



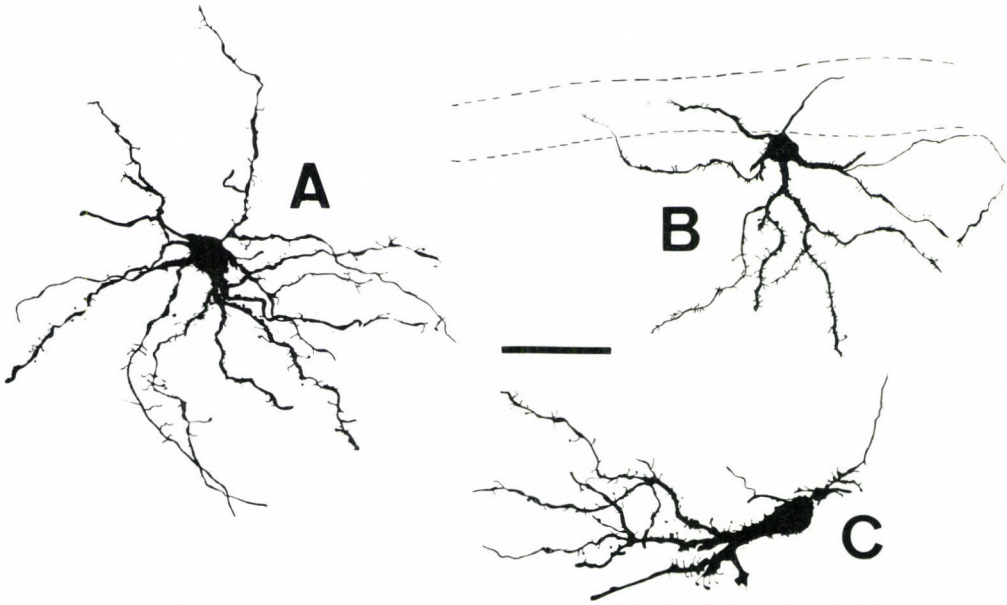


Fig. 8. Scale: 50 μm A. Typical multipolar neuron with numerous hairs and spines in a foetal monkey B. Tufted multipolar neuron in a 14 day monkey, with most dendrites in lamina 6, but a few entering the circumgeniculate capsule (dotted lines) C. Spines, hairs, growth buds and cones, and filopodia on a neuron of a 14 day monkey

possibly interneuronal, classes are small multipolar cells with axon-like dendrites and neurons with beaded dendrites. Finally, there are "capsular" neurons with their soma in the circumgeniculate capsule and their dendrites in lamina 6.

In the late foetus dendrites and axons bear growth cones and filopodia. A few days postnatally growth cones disappear and neurons develop a multitude of spiny and hair-like processes on their soma and dendrites (Figs 2 to 8). By the end of the first month after birth these excess spines and hairs have regressed from most neurons, which take on their adult morphology [15].

Using autopsy material from humans of both sexes aged from 35 weeks gestation to 82 years we have examined LGN sections impregnated by the rapid Golgi or the Golgi Cox method [7, 8]. LGN lamination is easily visible in young humans and most neuronal

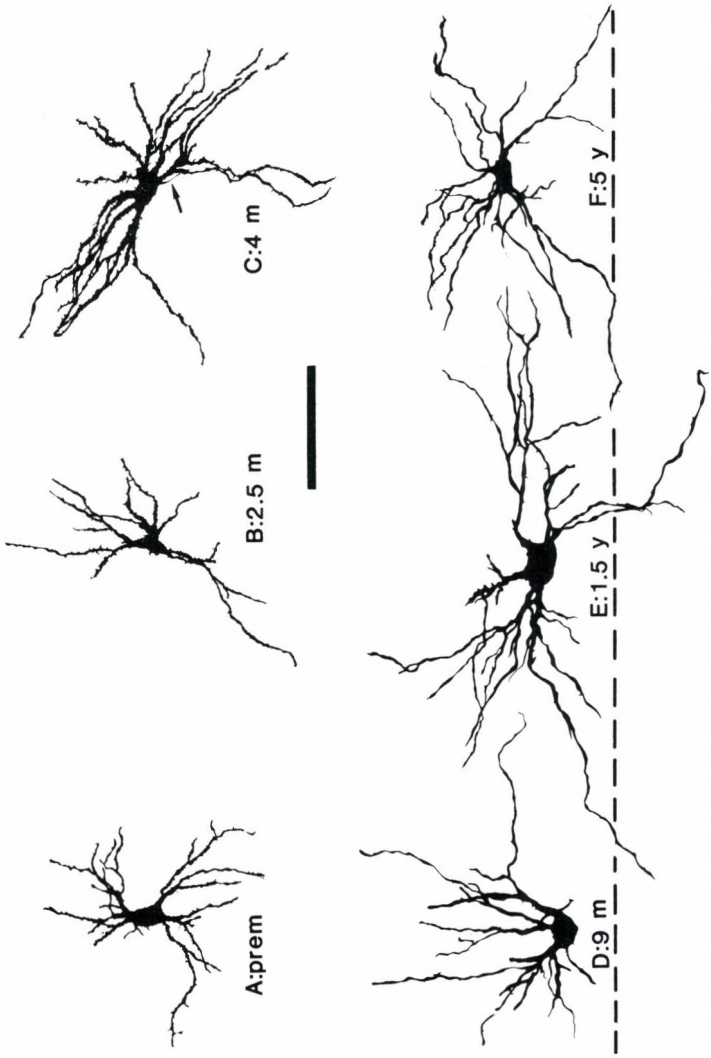


Fig. 9. Scale; 50 μm. Montage of multipolar magnocellular neurons of human infants at different ages. A. 35 week gestation permature, with numerous filopodia arising from growth cones and buds. B. 2.5 month infant: note the greater number of spines and hair-like processes. C. 4 month child, with even more numerous dendritic and somatic processes (arrow indicates axon). D. 9 month child: note the abrupt loss of immature features compared to the neuron in C. E, F, more mature magnocellular neurons at 1.5 and 5 years

types found in the adult LGN are distinguishable even before birth. The same types of neuron are found in the LGN of man as described above for monkey. During the first months of life human LGN cells bear a multitude of spiny and hair-like processes on their dendrites and soma (Fig. 9), just as in monkeys [7, 8, 15]. This contrasts with adult human LGN neurons which, as in monkey, have few dendritic or somatic spines [25]. Hairs and spines are abundant at birth, but by the age of 4 months in man they are even commoner, although they have regressed in the monkey. By 7 months in man spine density has decreased and hair-like processes are rare. At the end of the first year even fewer spines are visible on dendrites and somata and at this stage the LGN looks like that of an adult. Not all neurons show such exuberant hairs and spines. The cells with long, cylindrical dendrites (the supposed interneurons) are less affected than multipolar and bipolar cells. Until the age of 5 months neurons in the human LGN have swellings along their dendrites, like the growth buds and growth cones found in the monkey in the first days of life [15].

So, most neurons in the monkey LGN look mature by the second postnatal month. Visual acuity increases rapidly during the early months of the monkey's life, susceptibility to deprivation is high and recovery from deprivation is difficult if allowed to persist later [4, 11, 24, 26]. Man's LGN undergoes similar maturational changes during the first year or so of life, which is also a period of rapid increase in visual acuity when visual deprivation may lead to amblyopia [7, 8, 12].

In view of these age-related changes in the visual thalamus, we have recently examined the human visual cortex for signs of

structural changes over similar per_ods [9, 23]. We studied area 17 in human brains from 28 weeks gestation to 71 years old. Measurements of the total volume of area 17 were made from serial celloidin sections stained with methylene blue. The extent of area 17 is clearly defined by the stria of Gennari even in the foetus. Tissue was also prepared for electron microscopy by the ethanolic phosphotungstic acid (EPTA) method [1] which stains synaptic profiles selectively. The distribution of synapses was recorded in strips of cortex extending from pia to white matter and mean synaptic density calculated for the whole cortical depth and for individual layers.

The total volume of area 17 was found to increase rapidly after birth reaching its adult volume at about 4 months. Synaptic density increases rapidly between 2 and 8 months postnatally decreasing thereafter to an adult value of about 60 % of the maximum, reached by about 11 years of age (Fig. 10).

If one compares the data for synaptic density and volume, it would appear that the total number of synapses in area 17 decreases sometime after the first year of life, reaching its adult value by the age of about 11 years. Thus there is a rapid production of synapses in the human visual cortex followed by a decline during the first months of life, corresponding well with the period of maturation of LGN neurons described above . The Golgi study of human LGN has shown that loss of dendritic spines occurs there with the appearance of adult morphology by about 9 months [7, 8]. It may, therefore, be supposed that the number of synapses in the LGN could be decreasing during the same first few months of life.

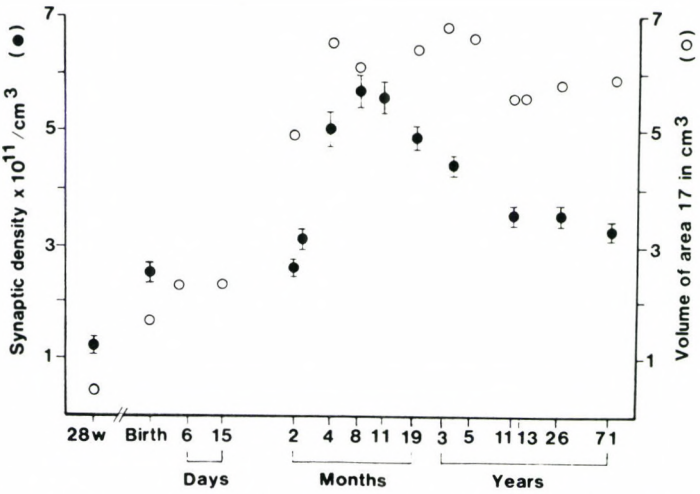


Fig. 10. Graph of changes in synaptic density (filled circles) and volume of area 17 (open circles) with age in human visual cortex. Age is plotted logarithmically. Bars on plots of synaptic density represent standard errors of means of 6 separate synapse counts in each brain

Visual alertness, acuity and stereopsis increase rapidly in these first few months [11], and the visual system is in a period of high vulnerability to abnormal conditions. Visual deprivation [3, 4, 17, 21, 22, 26, 28, 29] or experimental strabismus [20] lead to extensive changes in the functional organization of the visual cortex in animals. In man, childhood strabismus or early visual deprivation as in congenital cataract lead to permanent visual impairment unless corrected quickly [2, 27]. Cataract removal tends to result in near normal visual acuity only if performed before the age of 2 months [16], that is just prior to the period of the most rapid synaptogenesis in the cortex. Thus, normal visual input seems to be important during this period of synapse formation.

ACKNOWLEDGEMENTS

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Several of the illustrations are taken from references 8 and 15, with the permission of Springer Verlag.

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ELECTRICAL SIGNS OF ACTIVITY IN ASSEMBLIES OF NEURONS: COMPOUND FIELD POTENTIALS AS OBJECTS OF STUDY IN THEIR OWN RIGHT

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A case is made for the study of evoked and ongoing brain potentials as signs of activity in organized assemblies of neurons. Whereas the study of single unit activity, usually spike activity only, will continue to be a major window onto brain function, it cannot explain or predict much of the compound field potential recorded from assemblies even those with small populations via semimicroelectrodes. We need as many different windows as we can look through; each gives insight on a different set of integrative mechanisms. Evoked and ongoing potentials reveal a number of features and have advantages for a variety of questions not otherwise as readily addressed.

I. INTRODUCTION

If you want to understand how Budapest or New York work, it helps to study single individuals but it cannot suffice. You have to use some techniques that smear individual differences and lump categories. The same is true if we want to understand how the human body works; single cell studies are important but cannot suffice.

The gap in mutual appreciation is regrettably wide between workers dealing with single neuronal units and those who study compound field potentials of assemblies of neurons. This gap is unnecessary and counter-productive; neither approach can hope to tell the whole story. Even with both approaches working together

and each extended in various ways, especially in multichannel recording, we will be hard pressed for a long time to understand what is really going on in the brain. The present occasion seems an auspicious one and the aim of this essay is to improve the understanding of the assembly approach. Since the relations are not symmetrical and the space of this essay is insufficient to discuss both approaches, I will concentrate here on some reasons for reaching out beyond the recordings of single unit activity, even many simultaneous channels of such recording to include the compound field potentials, especially from extracellular semimicroelectrodes. I will consider both the activity evoked by controlled stimuli and the background activity which is ongoing without intentional stimulation. I have in mind throughout the three prime questions of biology: what is there (the natural history and morphology)?; how does it work (the reductionistic physiology)?; and how did it get that way (the development and evolution)? Woven through the polemics, are suggestions for new research.

II. REASONS A PRIORI FOR STUDYING ASSEMBLY FIELDS

The sheer existence of a form of activity should attract scrutiny until we have a good picture of the natural history. After all, it is only an hypothesis that this form of activity is attributable to the sum of independent activity of neuronal units. Current concepts of membranes, of intercellular compartments and of glia encourage speculation on sources of local currents other than conventional transmembrane e.m.fs., at least for slower events. Even for the presumably major contribution of

neuronal activity to the vector sum, the summation of unit activity is so complex that the resultant is not at all trivial. Each neuron is an extended three dimensional array of smaller .nd larger generators. The volume conductor for the resulting fields of current is far from isotropic, the intercellular space is often small and labyrinthine. Even if intracellular recording were much easier than it is and if we could get into the fine processes as well as into somata and large processes, we could not expect to predict or to explain the composite field potential of the assemblage of local units and parts of distant units.

The composite field is a window onto the neural activity; it only partly overlaps with the intracellular unit window. Most unit recording however is extracellular and confined to the spike form of activity. This is especially true of recording with many microelectrodes simultaneously - which is one of the most hopeful frontiers for major technical developments in the future. This restriction to the spike form of activity is a severe one, since so much of the interesting integrative activity of cells, dendrites and axonal terminals is graded, slow and generally small. In many places units with good spikes are difficult to find, are clearly not representative, or are even absent, as in much of the retina, many invertebrate ganglia and I suspect rather widely in the brains of vertebrates.

I would certainly not argue - as some people do - that single unit recording is irrelevant to understanding the higher levels of brain function. Even when it is confined to extracellular spike activity, it will continue to be a major window, not only into lower but also into higher levels of neural physiology. With the advent of new methods of microelectrode fabrication,

placement, and data processing, new horizons that we cannot yet delimit are opening up for further insights. My argument is simply that, for all its obvious attractions, the approach of looking at one or a few units as samples is by itself an inadequate window; we cannot see enough of what is happening. The field potential of the assemblage is also a severely limited window because it sums the activity of distinctly different kinds of neurons, but it permits seeing things we cannot see otherwise and it should not be shunned just because the questions it addresses are different and the findings are difficult to interpret in terms of units. We need both of these and all the windows we can put our eyes to.

III. IMPORTANT FEATURES OF ASSEMBLY POTENTIALS

I propose now to document the point that field potentials of assemblies of neuronal units have emergent properties, by listing some of the notable and unpredictable features that have impressed me in my own experience with a variety of species, including higher and lower vertebrates and invertebrates.

A. Evolution: paucity of correlates with phylogeny, brain size, or lamination

It would be a reasonable presumption that the EEG we are familiar with from the human subject must be different in some ways from the ongoing activity in other primates or other mammalian orders, and especially from that in nonmammalian vertebrates, with much smaller brains, no neocortex and much less lamination.

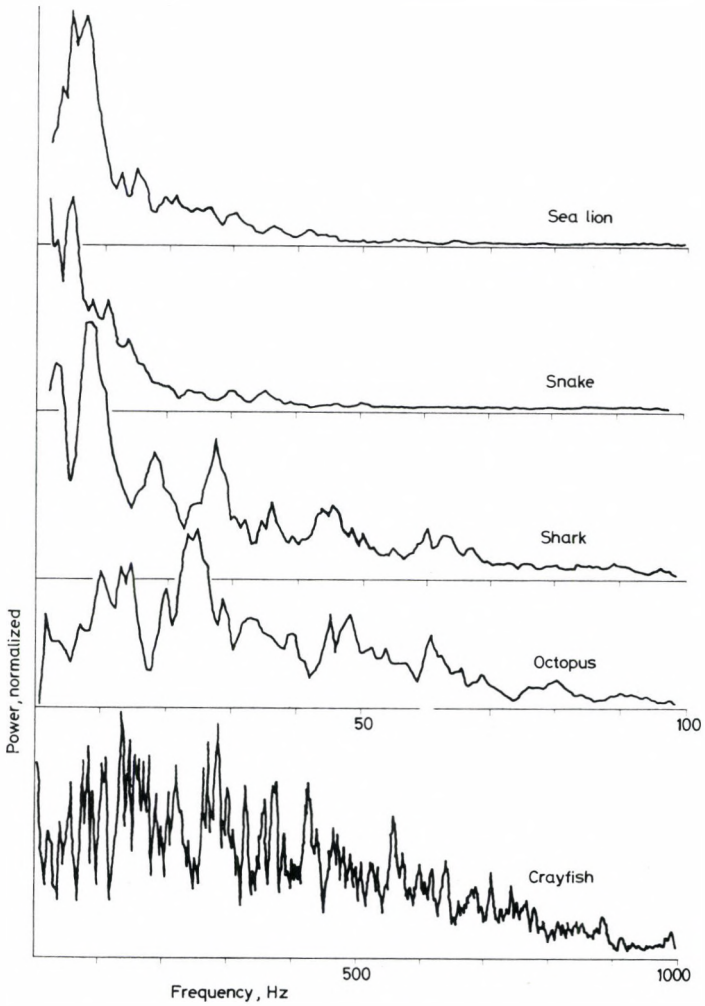

Fig. 1. Power spectra of the electroencephalogram of several species. The vertebrate ongoing activity was recorded from the cerebrum (sea lion, Zalophus and rattlesnake, Crotalus) or tectum (shark, Carcharhinus) in the quiet, unanesthetized, awake state with bipolar electrodes. The Octopus activity was from the vertical lobe in a similar state. Crayfish (Cambarus) activity was from the circumesophageal connectives near the brain. Note the difference in scale on the abscissa

The surprising finding, and one that I think must be telling us something significant, is that the EEG shows no systematic differences in a series of vertebrate species ranging from large to small mammals, to frogs, fishes and sharks - when

comparisons are made as nearly as possible in the same state and from comparable parts of the brain (Fig. 1). At least there are no differences at the crude level of gross wave form. Differences within the individual between states or places are large, but as between species of vertebrates the size of the brain or brain region and even its degree of differentiation and lamination are of minor importance to the overall form and spectrum of the ongoing activity. It remains a major challenge to find the correlates of the enormous advances in evolution of the nervous system and its functional capacity.

Invertebrates, however, generally exhibit a very different kind of ongoing activity. Instead of the smooth, slow waves mostly below 30 Hz that we are used to with the vertebrate EEG, gastropods, insects, crustaceans and worms show a predominantly much faster record with many large spikes, even with gross

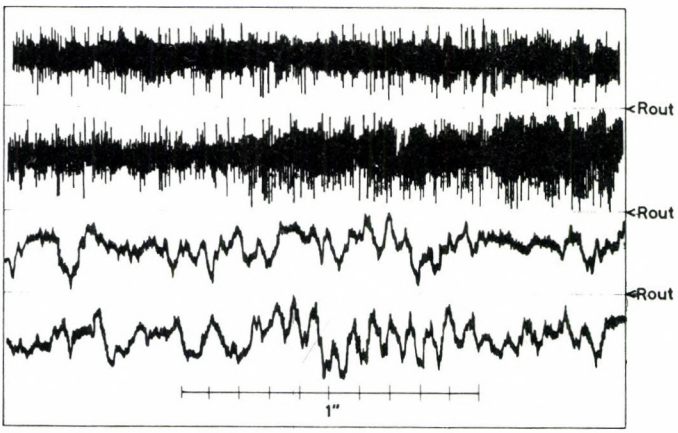

Fig. 2. Upper two traces are crayfish ongoing activity recorded as in the lowest record of Fig. 1; lower two traces are from a cat cortex, with the same time scale. The spikiness of the crayfish and its small slow waves are typical of records from insects, worms and snails and do not depend on the type of electrode or size of the brain

electrodes (Fig. 2). This is not a question of size or numbers of cells, but something intrinsic to the way the neuropile functions. The numbers of cells in a large lobster or *Limulus* or *Aplysia* brain are far higher than those in small fish or in the isolated one tenth milligram piece of the olfactory lobe that Gerard [5] showed many years ago can still exhibit brain waves, about 4 to 6 Hz, much like those in the intact animal.

The situation is not so simple as a dichotomy, with the vertebrates having ongoing activity whose power spectrum peaks below 20 Hz and falls to virtual noise level by 50 Hz, and the invertebrates a power spectrum that extends into the hundreds of Hz, most energy being above 50 Hz. The latter, or else intermediate spectra can be found in the vertebrate spinal cord or medulla with some kinds of electrodes. Short epochs with large slow waves of 15 to 30 Hz can be found in insect optic ganglia after flash stimuli, as also in the vertebrate retina. Octopus, at least in its higher centers, acts in an intermediate fashion, but much more like a vertebrate (Fig. 1). All these assertions are still very tentative and a broad ranging comparison of different phylogenetic levels is still much needed.

Evoked potentials likewise show no simple correlations: they can be brief or long, early or late, simple or complex in invertebrate brains large or small and in structures with or without lamination. Auditory brainstem responses can be similar among taxa from fish to primates (Fig. 3). Evoked potentials can be virtually absent even when units are quite active. This is not only true quite generally for invertebrates - with some exceptions, but also for vertebrates. Evoked waves are a facultative form of summation that presumably requires certain geometric

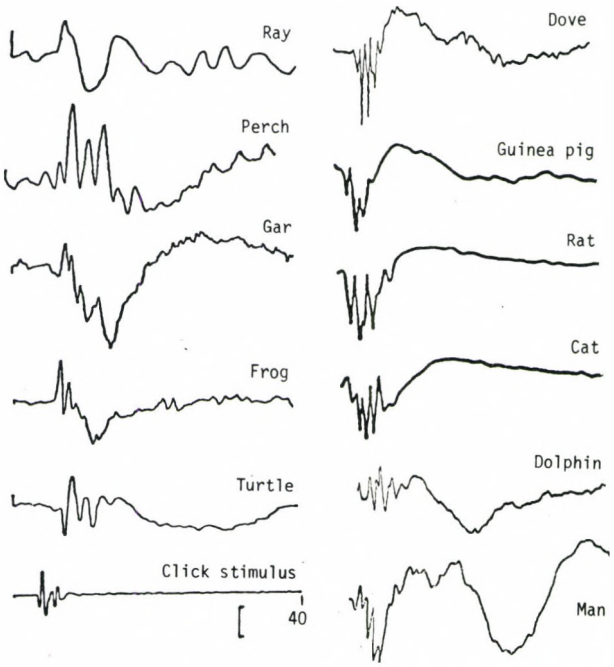

Fig. 3. The ABR (auditory brainstem response) in a series of vertebrates, recorded outside the brain by averaging from 64 to 2000 responses to airborne clicks at low repetition rate (<10/s). This is one of the few references in this paper to far-field recording from large volumes of tissue. A microphone record of the click used for several of the species is shown at lower left but the arrival time is not precisely the same for all species. As long as the main initial wave is above 1 kHz the exact composition of the click is unimportant in determining the ABR form; for the ray the click has to be <400 Hz and the response wave form is sensitive to its composition. Recording electrodes in the left column and in the bird were just intracranial via fine midline holes through the cranium above the posterior cerebellum and above the rostral end of the cerebrum; rostral electrode negative = upwards deflection. In the mammals electrodes were near the vertex and the mastoid extracranially; vertex negative = upwards deflection. All records 40 ms long. Voltage scale mark = ca. 2 μV for ray, perch, rat, guinea pig, cat and dolphin, 5 μV for dove; ca. 0.5 μV for man. Amplifier filters: 10-3000 Hz except dolphin, 1-5000 Hz. Modified from Bullock [2]; Corwin et al. [4]; Merzenich et al. [6]

and temporal conditions, still poorly known. For example, the inferior colliculus and nucleus of the lateral lemniscus of the dolphin show no evoked potentials to sounds below 5 kHz, whereas units must surely be active since the cerebral cortex

shows evoked potentials to such sounds. The same is true of the auditory thalamus of the dove - it shows no evoked waves to sounds that evoke good waves in the cortex. Though we don't understand these facts in terms of volume conductor theory, it is clear that evoked potentials are, so to speak, an option used by some neural centers. Some neurons may be active but invisible to the field electrodes. By extension, it is likely that different neuronal components of the assembly contribute unequally to the compound field seen by a certain configuration of electrodes.

B. Temporal organization: complexity of wave forms and power spectra

The time course or morphology of the succession of waves, although now and then correlated with the activity of certain units, is in general an unpredictable, emergent property. This is true for both ongoing and for evoked wave forms. I will speak mainly about the latter and mainly about recording with semimicroelectrodes. Evoked potentials can be a rather complex series of larger and smaller waves even when we are recording the response to a single brief event in a primary sensory nucleus in the medulla (Fig. 4). For example a brief, physiological stimulus to electroreceptors in electric fish causes a sequence of four or five peaks and valleys in its cranial nerve nucleus, extending out to at least 130 ms. A single light flash causes a sequence of waves in the optic tectum which, in an elasmobranch, can extend out to more than one second (Fig. 4B); this is due to a sequence of discrete volleys from the retina, the last of which peaks at 850 ms.

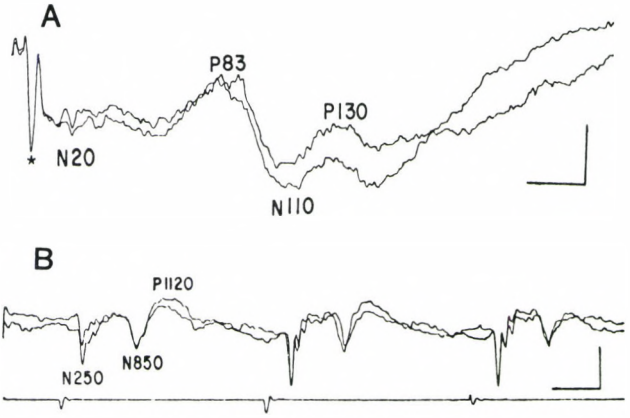

Fig. 4. A. Example of complex sequence of evoked waves in a brainstem nucleus. Record from the contralateral anterior medial tectal surface of the ray, Torpedo following stimulation by a 5 ms, 100 μV/cm electric pulse in the bath, exciting some and inhibiting other electroreceptors in the skin. Average of 4 responses. Asterisk: stimulus artifact. Calibrations: 25 ms, 50 μV. Note late, slow waves, because of which the early negative peak at 20 ms is amplified very little.
B. Example of late, slow waves in response to 3 flashes of submaximal diffuse light in the contralateral eye, at 0.5/s, recording from the superficial tectum. Average of 2. Calibrations: 500 ms, 100 μV. Note the change in small deflections after N250. Modified from Platt et al. [10]

If, instead of the raw wave form, we compare power spectra - a particularly suitable form of first order analysis for the ongoing EEG, we find that typically the power spectrum of field potentials seen by small electrodes on or in the brain is not narrow but at least several octaves broad, with a more or less distinct maximum. For the general case it is a mistake to think of brain waves as having a characteristic or dominant frequency; only in the human subjects with a strong alpha wave - which is not usual among mammals, is the power spectrum normally a sharp peak. I find it interesting that there is such a wide divergence among authors: some regard the alpha wave as the principal component of the EEG, some emphasize the origin of the EEG from a myriad

of generators of different frequencies. It seems important from the comparative viewpoint that we visualize the ongoing activity in the forebrain of mammals as being typically a mixture of many oscillations including energy at all frequencies in a band from about 50 Hz down to some very low frequency, usually limited arbitrarily by our amplifiers. Although the power spectrum is broad, it is quite different as between certain states such as sleep and arousal and as between certain regions of the brain. Comparing, for example cerebellum, spinal cord, different laminae of the optic tectum and of the cerebrum, the frequency of maximum power can be strikingly different - from <8 to >150 Hz. It looks as though we are not dealing with a single phenomenon in respect to sources, underlying events, synchrony or other mechanistic variables.

C. Spatial organization: diversity in geometry and synchrony of fields

When the neural substratum is clearly laminated histologically, the field naturally reflects this. Nevertheless, it is not always explicable why some evoked waves have reversal depths and others do not, or why some spread so far as to be detectable for centimeters whereas others are only detectable within a millimeter. Some waves can be represented as equivalent dipoles with a certain orientation and polarity and these can be significantly different for the several components of a response. For example, the successive waves of the well known auditory brainstem response have quite different axes of orientation as well as loci of the equivalent dipole [12]. These and other differences in geometry are usually empirical and not adequately accounted for by known anatomy and

unit physiology. Still less understood are the ongoing waves from various parts of the brain, apart from a few of the larger waves in the mammalian cerebral cortex. Volume conductor theory is well developed but has not helped much to predict or to explain observed field potentials.

In view of the enormous literature one would suppose that we have a good picture of the spatial organization of the EEG, at least the cortical activity in higher mammals. This is far from the case except in terms of very gross structure - on a scale of centimeters. A conspicuous need is for descriptive study of the three dimensional structure of the EEG on the millimeter scale; this means multichannel recording with semimicroelectrodes in known positions, as well as efficient means of data reduction. I illustrate with some preliminary results using a measure of microstructure designed to quantify the degree of synchrony between the assembly field potentials of small volumes of neural tissue. This is the plot of coherence as a function of distance (Fig. 5). We calculate coherence at each frequency over a band from 2 to 40 Hz, for each of several distances between electrodes. We find, as one might have intuitively expected, that coherence tends to fall as frequency rises - but by no means always and not steadily. Coherence also falls with distance between unipolar recording electrodes, referred to a common, distant, inactive reference electrode. The steepness of this fall varies but commonly coherence is appreciably below 1.0 already at a separation of 0.5 mm and is down to about 0.5 at only 3 to 5 mm. Such numbers should be measured for a wide sample of states, places, depths and species and with many more channels to permit revealing the structure in three planes and over greater distances. Other sorts of analysis

should also be added such as reexamining the findings of the early toposcopic display techniques that surface waves often travel over the cortex for some distance, die out or clash with others, arise from loci and radiate or exhibit other spatial patterns that might have meaning.

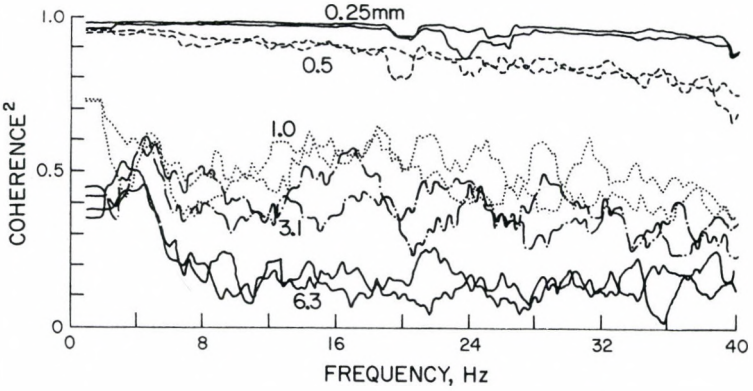

Fig. 5. Coherence between recording loci at different separations; ongoing EEG from cortical surface of the rat under light barbiturate, plotted for each frequency from 1 to 40 Hz. Each trace is the average of 15 epochs of 2 secs each. Separation of the active electrodes, all in a line, shown in millimeters; common reference in nasal sinus. The power spectra for the several channels (not shown) are similar to that in Fig. 1 (top)

D. Coupling functions: variety in input-output relations

Another domain in which empirical findings of composite assembly potentials could hardly be predicted from a limited knowledge of single units is that of how output changes with input changes, in each of the significant parameters such as intensity, duration, frequency, part of the receptive field, and rate of movement. These functions are widely variable with loci of the brain, state variables, concomittant stimuli and temporal factors. They constitute a major part of the characterization of the various parts of the brain. That is to say, in addition

to the characterization by lists of afferent and efferent projections and by intrinsic connections, histological and neurochemical differentiation, cell types and arrangement, significant descriptors include the physiological properties of the assemblage as an excitable system, even though these overall response properties are the composites of diverse unit responses.

So much for my list of special features of assembly potentials considered as excitable systems to be examined phenomenologically in their own right. The message of this list of variables which cannot be predicted from even an extensive knowledge of unit behavior, or even explained after the fact, is not that assembly potentials are a hopeless quagmire - which is one common reaction - but instead, that they are an empirical domain, rich in information, deserving to be studied in their own right, as signs of the underlying activity in the assembly - just as we study another set of signs that we call behavior.

IV. QUESTIONS FOR WHICH EVOKED POTENTIALS ARE ADVANTAGEOUS

I turn now to a group of questions for which evoked potentials in particular are advantageous end points of measurement. These are a kind of basic science equivalent of the clinical use of evoked potentials in diagnosis.

A. Distinguishing central afferent systems and subsystems

The functional counterpart of the anatomist's brain nucleus should be a collection of neurons defined by some kind of commonality of function or property - without any requirement that all the

neurons are alike in all respects. Diversity of cell types in such an assembly is usual, perhaps even universal. Unit by unit study can bring out this diversity and sometimes, as in the case of columns or slabs, can define a functional assembly - where all the units share some feature. Certainly the effort is worthwhile to test, unit by unit, whether the reality of columns or small groups of similar (not identical) cells, as functional units, extends widely to lower levels of the nervous system and to lower vertebrates. But however that turns out, the somewhat larger entity corresponding to a cortical area or to a subcortical nucleus embraces a considerable diversity of cells within a defineable commonality. Evoked potentials are not the ultimate basis for this definition but they can often be the first and most obvious sign that a center or focus of such and such function exists and that it is distinct from a neighboring area because of consistent differences in wave form or dynamic properties. This kind of question and use of compound evoked potentials is most readily exploited in sensory systems. A conspicuous case is the demonstration that electroreception as a central modality occurs in xenomystine fish and in lampreys, chimaeras, lungfish, sturgeons and others (Fig. 6), much more easily by recording evoked potentials in the torus semicircularis of the midbrain than by single unit searching, centrally or peripherally, or by behavioral tests.

A related situation is the demonstration that the center for evoked activity in response to electroreception is adjacent to but distinct from the center for response to another lateral line, hair cell submodality - for mechanoreception, and that in turn is distinct from the center for acoustic reception. In

some of these situations it is so far very difficult to record from single units, yet the evoked potential is quite reliable and simple. With a little trouble it can be accurately localizing, without questions of current spread from somewhere else, with reproducibility, ample time of maintained response - at least hours - and ready quantification of each of several parameters - as I will now spell out.

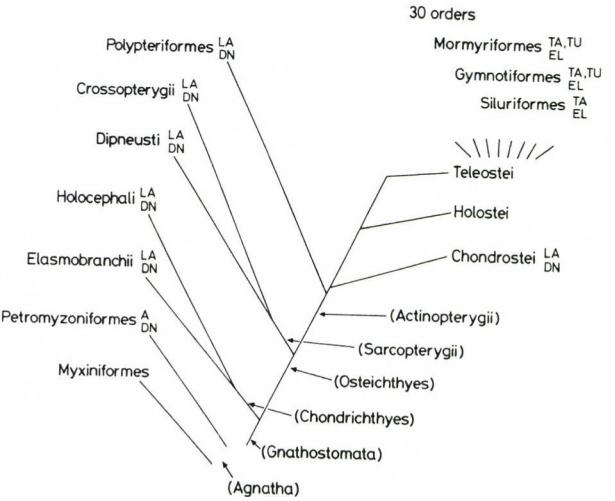

Fig. 6. Phylogenetic dendrogram of major taxa of fishes showing the distribution of electroreception as revealed by evoked potentials from specific brainstem centers. Small letters indicate the taxa that possess electroreception, the major types of receptors and the type of first order medullary nucleus for this afferent system. A, ampullae of undetermined type are the electroreceptors; DN, dorsal octavolateral nucleus; EL, electroreceptive lateral line lobe (an alternative nucleus to DN); LA, Lorenzinian ampullae are the receptors; TA, teleost-type of ampullae; TU, tuberous electroreceptors. By these criteria electroreception is absent in Mysinformes (hagfish), Holostei (gars, bowfin) and presumably in the majority (30 orders, not listed) of Teleostei. Bullock et al. [3]

B. Characterizing compound responses physiologically

I have already referred to the possibilities of using physiological properties of evoked potentials for distinguishing

between modalities and submodalities, but this was in the context of validating an anatomical separation or a sensory subsystem. For these purposes it may not require more than the first or second test of properties to establish the difference. Now I want to call attention to the difficulty of being the "compleat" physiologist, that is of asking whether we really have a full knowledge of the physiological characteristics, for their own sake, with respect to each distinguishable response type or subsystem. Very rarely has there been an effort to be as thorough as we take for granted in an anatomical study! To make the point I will give a partial list of the candidate properties that might belong in an adequate physiological characterization.

The wave form and time course of the successive phases of the response are of course a first order series of descriptors,

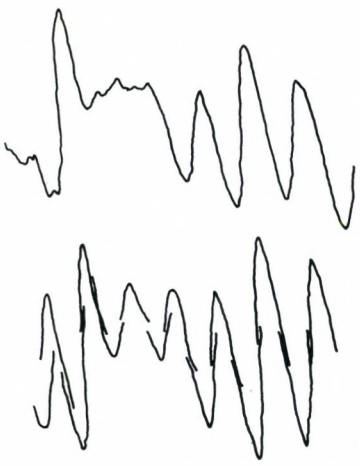

Fig. 7. Upper trace: average visual evoked potential, human; 1 flash/3 sec; whole sweep = 500 ms. Lower trace: LCA = latency corrected average. Note the time base is interrupted between the computer-identified peaks each of which is sought with a template filter on a second pass through the raw data and latency shifted to a mean on the hypothesis that there is independent jitter of latencies. Note several peaks emerge which were not present in the average evoked potential. Controls show that these do not come from unevoked EEG. Modified from Aunon [1]

including not only the latencies of peaks and valleys but their amplitudes and the steepness of slopes. In some cases it aids the eye to record, besides the raw wave form, a first derivative with respect to time - thus bringing out steepness. Another measure that can be useful is a continuous plot of variance of amplitude at each moment of time, since it is found that this can be different for the successive landmarks of the evoked potential. Especially when there are several peaks and subpeaks it may be useful to assume that the variance of latency is different among them; this can be exploited by computer programs such as Aunon (1) has given us, that identity and group peaks, using temporary templates, then go back through the raw data again, look for peaks in each trial at the expected places, and superimpose them by distorting the time axis between these landmarks. This can bring to light consistent details of the response hidden in ordinary averaging by the smear of latency play (Fig. 7).

As I already mentioned, the different components of the response can have different sources and sinks and orientations of the equivalent dipoles. These can move over time as part of the characteristics of the response. To uncover these important aspects of the signs of activity requires high resolution recording methods, such as are not often used. I am thinking of two in particular. One approach is exemplified by the micromapping studies of the cerebellar granule cell layer of Welker and his associates [11], with scores of electrode tracks per square millimeter, as close as 50 μm apart, each track permitting stops at several depths. These authors concentrated on fast, spikey multinuit activity. The other approach is exemplified by the current source density recording technique for which Nicholson

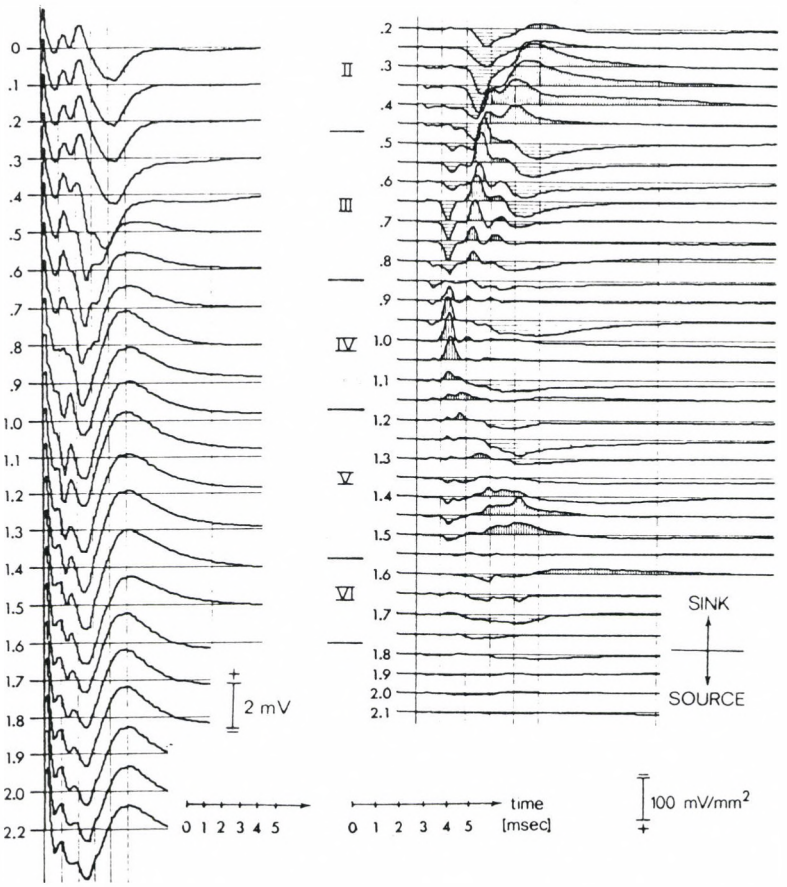

Fig. 8. The current source density (CSD) display (right column) compared to the average evoked potential (AEP) (left column). Recording at the depths shown in succession, with a single micropipette, from area 18 of the cat. Stimulating by a 50 μs electric shock to the optic radiation at the moment of the first vertical line. Each AEP trace is the average of 20. CSD traces computed offline from the accumulated AEPs, as the second spatial derivative, with 50 μm recorded steps, assuming the tissue conductivity does not change with depth, the cortex is symmetrical horizontally, and the brain is in a stationary state over the duration of the recording. Mitzdorf and Singer [7]

and Freeman [8] have given us a practical, 7-electrode on-line device and Mitzdorf and Singer [7] have used a simplification for laminar structures such as the cortex (Fig. 8). The exploitation of these methods has an urgency for future work, since no other

methods - with single units or microanatomy - can reveal the actual organization, in time as well as in space, of the integrative subthreshold activity of neural assemblies.

I turn now from the microstructure of assembly response to another major domain of characterization, the influence upon response functions when stimulation is repetitive at short intervals. Usually components of the complex wave form change with successive stimuli independently or at least differentially. For maximizing the information learned about the system, it is not the best strategy to use continuous repetition of stimuli at various rates, giving a steady state, average response. Much better is the use of short trains of about 5 or 10 stimuli, with rest periods between trains, since this reveals the succession from first response through facilitated or/and depressed second, third and later responses toward the steady state (Fig. 9). The

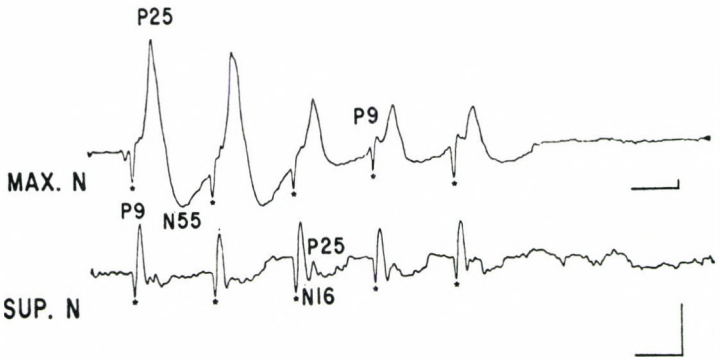

Fig. 9. Example of the use of a short train of stimuli to show some of the dynamics of responses in two different modalities. Recording from the contralateral, anterior tectal surface in Torpedo following five direct shocks to nerves. Max. n = maxillary nerve, from electroreceptors in ampullae of Lorenzini; sup. n = supraoptic nerve from cutaneous receptors other than electric or lateral line. Shock trains separated by relatively long rest periods. Averages of 16 responses. Calibrations: 50 ms, 25 μV. Note differences between the two modalities and between earlier and later waves, as seen in the same tectal locus, in respect to latency, facilitation and antifacilitation. Modofied from Platt et al. [10]

short train, studied as a function of frequency of stimuli within the train, is more insightful than the method of paired stimuli (conditioning and test stimuli at different intervals) which treats the dynamics as little more than a recovery cycle. The short train is one way to reveal the combination of earlier and later phases of facilitation and depression, the multiple, interacting components of the total response. These may represent important integrative processes and they may require certain temporal patterns of input.

Interactions with preceding, simultaneous, or even shortly following input in other pathways form another important category, seldom examined with any completeness. This includes masking and priming, multimodal convergence, heterofacilitation and occlusion. It should lead into consideration of the effects of time of day, circadian rhythms, seasonal changes, behavioral readiness and other forms of state dependence.

C. Revealing anatomical organization

A realm of questions deals with the anatomical segregation of stimulus defined or response defined functions. Of course, evoked potentials are far from the last word or the universally best method - but no method is very good for this and we need to use all those available. In some situations evoked potentials are probably the best if not the only means available for the first, crude, topographic segregation.

When we begin to investigate the large and seemingly little differentiated telencephalon of relatively primitive vertebrates, such as elasmobranches, units are rare and usually uninterested in anything we do. Lesions or electrical stimulation of the brain

can be insightful if we have the normal ethogram of the species, quasi-natural aquarium conditions, plenty of patience and fortunate choices of loci. HRP and the axon-transport techniques are of limited help until we know the modality specificity or the connections of the sources of input or the targets of output of each forebrain area. Evoked potentials can sometimes give reliable and discriminating answers, even though crude ones in terms of eventual understanding of the transactions going on. For example, we can get our first clue that there are two, or more, distinct auditory areas in the primitive telencephalon, that they are probably organized sequentially, and that they are not simply points in a physiologically undifferentiated diffuse nucleus.

Even in a lower brainstem system evoked potentials can reveal, more readily than single unit recording or experimental anatomy, whether the separate octaval nuclei or the cerebellar auricle or corpus or parts of the torus semicircularis receive input from particular modalities of stimulation such as acoustic, vibrational, or pitch or yaw stimuli [9].

D. Correlating with endogeneous events

We cannot omit from this short list the class of questions concerned with physiological signs of the state or the activity of the brain during or preceding significant events arising from within. Examples are cognitive processes associated with recognition of the expected but rare stimulus, that give rise to the so-called P300 wave in humans, and the processes that lead to voluntary movements, the contingent negative variation and others. I want only to make one point about this well established and active field of exploitation of evoked potentials: the method has been

used very little on nonhuman subjects, especially on nonprimates and perhaps not at all on nonmammalian species. It may not be easy and the results may be hard to compare with those from human subjects, but I am guessing that there is a rich frontier of application, given ethological guidance, to animals as diverse and far from mammals as teleosts, elasmobranches and cephalopods and probably also to insects, crustaceans and gastropods.

The technical and conceptual challenges mentioned herein, or others not listed, will surely yield to new developments and concerted effort, thus opening up still further challenges. But even before new break-throughs there is much we can learn from available methods by studying the compound potentials of assemblies of neurons as signs of integrative neural activity.

ACKNOWLEDGEMENT

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THE MAIN PRINCIPLES OF THE ORGANIZATION OF IONIC CHANNELS WHICH DETERMINE THE EXCITABILITY OF THE NEURONAL MEMBRANE

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The results obtained during last decades in the investigations of the excitability of neuronal membranes have definitely shown that the latter is based on the presence of discrete ion-conducting structures - ionic channels - which can change their permeability under the action of the transmembrane electric field. Of special importance are the ionic channels which produce an inward flux of cations; the positive charges carried by them across the membrane decrease the existing transmembrane potential difference (the so-called resting potential) and thus create conditions for the activation of new ionic channels and the generation of a regenerative (self-supporting) cellular response.

A detailed description of the function of channels which pass selectively sodium ions has been obtained from the studies on axonal membranes (first of squid giant axons, later on of the Ranvier nodes of myelinated fiber of the frog). At the same time it became obvious that the sodium channels are not a sole mechanism for the depolarization of electrically excitable membranes. In several cases the membranes also contain ionic channels which pass selectively calcium but not sodium ions. The comparison of the organization of different types of ionic channels responsible

for the depolarization of the neuronal membrane may be of great importance and may help to understand the general principles of the synthesis and assembly of such channels in the nerve cell.

However, until recently an extensive study of the ionic conductance of the nerve cell membrane was difficult because of the lack of adequate technical approaches. The intracellular perfusion technique developed in our laboratory opened the way for complete separation and exact measurement of individual ionic currents in the somatic membrane of isolated nerve cell necessary for the description of the corresponding ion-conducting molecular structures.

Table I summarizes the data about relative permeabilities of sodium channels in different excitable membranes towards monovalent cations obtained from the measurements of the reversal potential shifts for the corresponding currents produced by the relevant changes in the ionic composition of the extracellular medium. Permeability for Na^+ is taken as 1.

Table I

Relative permeabilities of sodium channels to different monovalent cations

Object	Na^+	Li^+	$N_2H_5^+$	NH_4^+	K^+	Reference No
Squid axon	1.0	1.10	-	-	0.08	[2]
Frog axon	1.0	0.93	0.59	0.16	0.09	[4,5]
Frog muscle fiber	1.0	0.96	0.31	0.11	0.05	[1]
Rat nerve cell soma	1.0	0.79	0.43	0.33	0.18	[10]
Snail nerve cell soma	1.0	1.04	0.44	-	0.10	[9]

One can see that the obtained permeability series are similar and show only some quantitative differences. These series can be adequately explained by the assumption that all sodium channels have an "ion-selecting filter" of a definite size (3.1x5.1Å) which pass a definite group of monovalent cations together with some hydrating water molecules due to steric separation [4]. The formation of hydrogen bonds between the penetrating ions and oxygen atoms in the channel wall can be an additional factor which facilitates their passage through the channel.

Table II summarizes the data about the relative permeabilities of calcium channels. As these channels pass only inward-going ions, the presented values were obtained from the comparison of the maximal current values at equimolar substitutions of the corresponding ions in the extracellular solution. Permeability to Ca^{2+} is taken as 1.

Table II

Relative permeability of calcium channels to different divalent cations

Object	Ba^{2+}	Sr^{2+}	Ca^{2+}	Mg^{2+}	References
Snail nerve cell soma	2.8	2.6	1.0	0.2	[3]
Rat nerve cell soma	1.8	1.3	1.0	-	[10]

In both cases no detectable penetration of monovalent cations through the calcium channels has been found.

The sequences of relative permeabilities for divalent cations in both cases are qualitatively similar, although they

differ quantitatively. This difference may be due to the fact that maximal current values are influenced not only by differences in channel permeability, but also by other factors like changes in the near-membrane ionic concentration created by the membrane surface potential. For a more detailed evaluation of the properties of the corresponding channels we performed measurements of the concentration dependence of the transmembrane currents induced by different carrier ions. This dependence showed a definite saturation and could be described by Langmuir's isotherm, from which the dissociation constants K_d and the $pK = (-\lg K_d)$ for the complex of penetrating ion with the binding group in the channel could be calculated. Table III presents the obtained values corrected for the possible changes of the near-membrane ionic concentration. This table also presents the pK for the effects exerted on the calcium channels by other divalent cations which contrary to Ba^{2+}, Sr^{2+} and Ca^{2+} do not pass through but block the channels. This blocking effect can be described by Langmuir's isotherm and therefore can be considered as a result of competitive binding of these ions to the same binding group. The table also gives for comparison the data about binding of divalent cations to a carboxylic group (glycin) in aqueous solution.

It follows from the data presented that divalent cations can be arranged in a continuous series according to the capability to bind themselves to the calcium channel; this series corresponds to that for ion binding to a carboxylic group. All divalent cations which bind to the carboxylic group weaker than Ca^{2+} pass through the channel the better, the weaker is their binding.

Table III

pK of divalent ion complexes with calcium channels

Object	Ba^{2+}	Ca^{2+}	Co^{2+}	Ni^{2+}	Cd^{2+}	References No
Snail neuron somatic membrane	1.0	2.0	3.2	3.5	4.3	[6]
Rat neuron somatic membrane	0.6	1.3	2.5	2.6	4.3	[8]
Carboxylic group	0.8	1.4	3.2	3.2	4.8	[12]

All divalent (and as well polyvalent) cations which bind to the carboxylic group stronger than Ca^{2+} block the channel, the effectiveness of blocking being the higher, the stronger is their binding.

The results obtained bring us to the conclusion that the organization of the "ion-selecting filter" in the calcium channels is, in principle, different from that in the sodium channels. The capability of the former to select certain divalent cations depends on the binding characteristics of the corresponding binding group in the channel; binding is a necessary step in the passage of the ion through the channel.

A question arises why the calcium channels do not pass monovalent cations, for instance Na^{+}, which weakly binds to the carboxylic group but resembles Ca^{2+} in size. A clue for the understanding of this important question has been given by detection of reversible transformation of "calcium" channels into sodium ones after complete removal of divalent cations from the extracellular solution (by addition of calcium-chelating substances to the latter, cf. [7]). The selectivity of the transformed channels for monovalent cations has the sequence

$Na^+ : Li^+ : N_2H_5^+ : NH_4^+ = 1.0 : 0.8 : 0.5 : 0.1$ which is slightly different from that of typical sodium channels [9]. At the same time the transformed channels are not sensitive to tetrodotoxin (a specific blocker of sodium channels) but can be blocked by verapamil and niphedipine like original calcium channels. This finding indicates that divalent cations in the extracellular solution by themselves are the reason why calcium channel excludes monovalent cations. One may suggest that the high probability of the presence of a bound divalent cation within the channel prevents monovalent cation from passing through. However, a special study of the dependence of the transformation of calcium channels upon the concentration of divalent cations in the extracellular medium has shown that the pK for the transition is about 4 orders higher than the pK for the above described divalent cation binding within the channel. Obviously, the calcium channels have one more external binding group which binds divalent cations in a highly effective way; this group, being in a complex state, somehow prevents monovalent cations from entering the channel.

The hypothesis about the presence of two different binding sites in the calcium channel is supported by the finding of a special type of sodium channels in the somatic membrane of some dorsal root ganglion neurons of rat [10, 11]. The permeability of these channels towards monovalent cations can be described by the series $Na^+ : Li^+ : N_2H_5^+ : NH_4^+ : K^+ = 1.0 : 0.98 : 0.47 : 0.42 : 0.26$, what is quite close to the permeability series for typical sodium channels (cf. Table I). These channels are not affected by tetrodotoxin but can be blocked by verapamil and divalent cations in the same way as calcium channels. In other

words, they have an inner "ion-selecting filter" which is similar to that of calcium channels, but they lack the outside structure which hinders monovalent cations to enter the channel.

In summary, we may conclude that the quite complicated problem of separation of mono- and divalent cations and generation of corresponding specific ionic currents is solved in the excitable neuronal membrane by using at least three different physico-chemical mechanisms based on different structural components of the respective ionic channel. These structural components can operate in various combinations in different types of channels and are probably synthetized by the cell in the form of separate subunits which further combine during reconstruction in the membrane in a new molecular entity - the ionic channel.

Considering the functional meaning of specialized calcium ionic channels characteristic for the nerve cell somatic membrane, one should discuss their possible participation in the generation of an active membrane response and in the coupling of membrane and cytoplasmic processes. If present in sufficient density, the calcium channels can produce an inward transmembrane current which will effectively recharge the membrane capacity and generate a regenerative response in the form of a propagating impulse. Moreover, the calcium component of the inward current can exceed the sodium one, and the action potential may be of calcium nature at normal composition of electrolytes in the extracellular medium. Usually this is the case for invertebrate neurons related to some ecological conditions for the corresponding organisms.

However, it should be emphasized that even if the generation of a propagating impulse in the nerve cell soma is based

mainly on the functioning of calcium ionic channels, in the axon of the same cell it is changed completely to the sodium ones. The reason why calcium ionic channels are used for the same purpose only in the somatic (and, probably, dendritic) membrane but not in the axonal membrane is not yet clear; probably, this difference is related to some peculiarities of the intracellular synthesis of the corresponding channel-forming proteins and their transport to the sites of reconstruction in the surface membrane. It should be noted that in some cases the possibility of the generation of propagating "calcium" spikes in the axon has been observed.

Nevertheless, in most excitable membranes the inward currents produced by the system of sodium channels are more than sufficient for the generation of an active membrane response, and the participation of calcium channels is manifested only in some modifications of the time-course of the spike. This finding definitely indicates the predominant role of membrane calcium conductance in other cellular functions, which may be connected to such special physico-chemical features of Ca^{2+} as its high coordination number and irregular coordination geometry essential for the effective binding to biological molecules irregular in structure.

The intracellular transport of substances from the cell soma into dendrites and the axon is one of well defined calcium-regulated process. It is known that the volume of transported substances largely changes during cellular activity. The transport of labelled amino acids from the soma of dorsal root ganglion neurons along their axons in peripheral nerves decreases by 60 % in cases when the ganglion is placed in calcium-free

solution; a similar procedure applied to the axons has no effect on the transport. In parallel with the change in the amount of transported amino acids a similar change also occurs in the transport of labelled Ca^{2+} along the axons. It is quite possible that the described changes reflect the role of inward calcium fluxes during cellular activity in the regulation of cytoplasmic transport processes, especially in the maintenance of certain correspondence between the level of cellular activity and the amount of transported substances.

The liberation of neurotransmitters, neuromodulators and neurohormones accumulated within the cells as a result of intracellular transport is another well known calcium-dependent process. Quantitative studies of the role of inward calcium fluxes in liberation of transmitter from the nerve terminals proved to be the most effective on giant synapses of the stellate ganglion of squid because of very large size of both pre- and postsynaptic elements allowing the introduction in each of them of 2 microelectrodes. Similar studies of other forms of calcium-regulated nerve cell secretion are still lacking.

Finally, a specific physiological function of the inward flux of Ca^{2+} during cellular activity is its recurrent effect on different types of ionic conductances of the membrane. It may affect certain types of potassium channels rendering them into an activated state, as well as the calcium channels switching them out of action. The intracellular feed-back links created by these actions of Ca^{2+} ions obviously are of great importance in the functioning of the nerve cell as an integrated system.

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ANATOMICAL ORGANIZATION OF THE SPINOCEREBELLAR SYSTEM, AS STUDIED BY THE HRP METHOD

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Using the HRP method we have identified eleven groups of spinocerebellar tract (SCT) neurons in the cat, and determined their projection areas in the anterior and the posterior lobes.

Neuronal groups projecting to lobule VIII are located in the central cervical nucleus (CCN), Clarke's column and the medial part of lamina VII of L6 to the caudal segments while those projecting to the paramedian lobule are lamina V neurons of C8 to L4, marginal neurons of Clarke's column and spinal border cells. In the anterior lobe the CCN projects to lobules I to V, most abundantly to lobules I and II. The medial lamina VII group of L6 to the caudal segments projects mainly to lobules I and II. Spinal border cells project to lobules II to V, but predominantly to lobules III and IV. Clarke column neurons project to lobules I to V. The medial lamina VI group and the central lamina VII group of the cervical segments project to lobules III and IV. Marginal neurons of Clarke's column and lamina V neurons of the lower cervical to the lumbar segments project to lobules III to V.

The present study suggests that the SCT neurons project to different sagittal zones of the cerebellar cortex, with various quantities in the rostrocaudal direction.

The spinocerebellar system has been established to consist of four major tracts; the dorsal, ventral and rostral spinocerebellar tracts and the cuneocerebellar tract [3, 11]. The dorsal and the ventral spinocerebellar tracts convey input from the hindlimb regions whereas the cuneocerebellar and the rostral spinocerebellar tracts convey input from the forelimb regions as functional equivalents of the dorsal and the ventral spinocerebellar

tracts [11]. Recent anatomical studies using the horseradish peroxidase (HRP) method identified various kinds of spinocerebellar tract (SCT) neurons in the cat, rat, dog and monkey [see refs. 7,8 for review]. Electrophysiological studies also identified SCT neurons in the cervical [5] and lumbar segments [1, 2, 13]. The present communication deals with an anatomical aspect of the spinocerebellar system revealed by the HRP method in the cat; classification of SCT neurons [8] and their topographic projections to the cerebellar cortex [7, 9, 10].

CELLS OF ORIGIN OF THE SCTs AND THEIR CLASSIFICATION

SCT neurons were identified in the whole length of the spinal cord following extensive injections of HRP into the cerebellum. In another experimental group the spinal cord was hemisected at cervical levels (between C1 and C2 or between C5 and C6) prior to HRP injections, in order to determine whether the axons of the identified SCT neurons cross within the spinal cord [8].

Six groups of labeled neurons were found on the side ipsilateral to a hemisection; they give rise to crossed ascending axons (Fig. 1, right side). In C2 to C4 labeled neurons were concentrated in the central cervical nucleus (CCN). Rostral to the hemisection the CCN neurons were labeled bilaterally. A few labeled neurons were seen in lamina VIII of the cervical segments. Similar labeled neurons in lamina VIII of the thoracic and lumbar segments were tentatively regarded as belonging to the same group. In the lumbar segments many labeled neurons

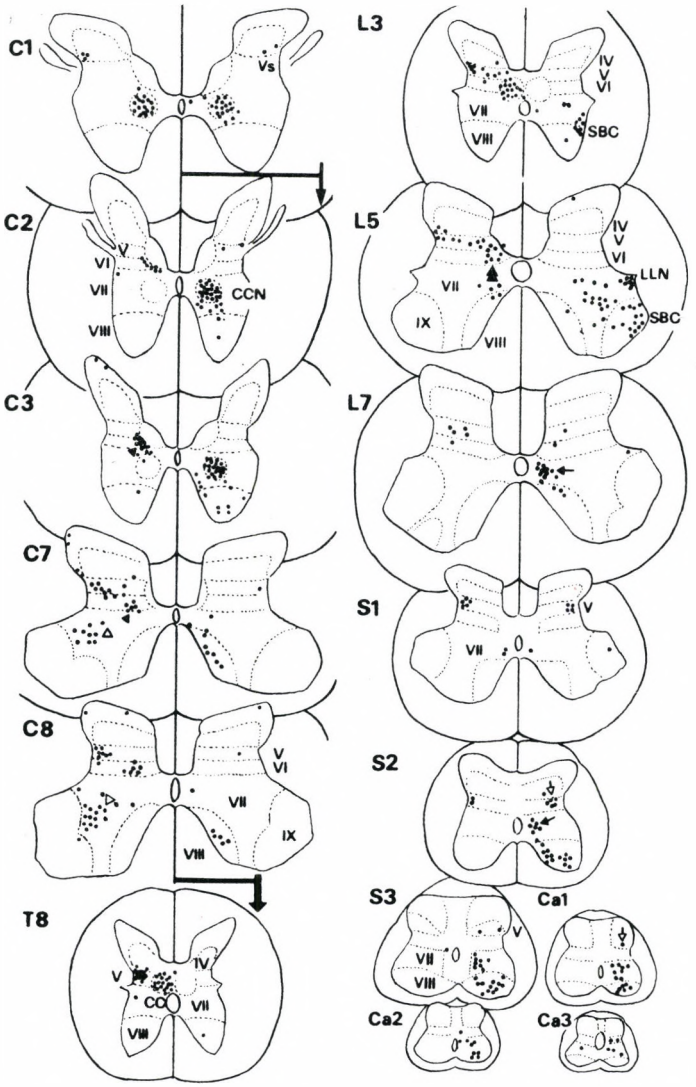


Fig. 1. Diagram showing the location of HRP-labeled spinocerebellar tract neurons in C1 to C8 in a case with a hemisection between C1 and C2 and in T8 to Ca3 in a case with a hemisection between C5 and C6. The hemisections are made on the right side (large arrows). Solid arrows, neurons in the medial part of lamina VII of L6 to Ca3; open arrows, neurons in the dorsal horn of the sacral-caudal segments; solid arrowheads, neurons in the medial part of lamina VI of C2 to T1; open arrowheads, neurons in the central part of lamina VII of C6 to T1; double arrowheads, neurons in the medial part of lamina VI of L5 and L6. (Modified from ref. 8)

were seen in the lateral border of the ventral horn (Fig. 1, SBC). They were most numerous at L4 to L6, and were identified as spinal border cells [2]. Caudal to L6 a distinct group of labeled neurons was found in the medial part of lamina VII. It consisted of medium-sized and large multipolar neurons, and persisted to the caudal segments in the corresponding location (Fig. 1, L7-Ca3: arrows). In addition, two groups of labeled neurons were found in the sacral-caudal segments: one in the dorsal horn and the other in the ventral horn (Fig. 1, S1-Ca3). The neurons of the former group were very large and medium-sized, and appeared in the border region between laminae IV and V (Fig. 1, open arrows). In the ventral horn (laminae VII and VIII) many labeled multipolar neurons were found; they were often intermingled with the neurons in the medial part of lamina VII.

Five groups of labeled neurons were found on the side contralateral to a hemisection; they give rise to uncrossed ascending axons (Fig. 1, left side). Of these, two were present in the cervical cord; one in the medial part of lamina VI of C2 to T1, corresponding to the nucleus centrobasalis [12] and the other in the middle part of lamina VII of C6 to T1 [5]. The neurons of the former group were mainly medium-sized spherical or fusiform neurons, and were distributed dorsal to the CCN in C2 to C4 or lateral to the internal basal nucleus (Fig. 1, arrowheads). The neurons of the latter group were large to medium-sized, and were distributed mainly dorsomedial to lamina IX (Fig. 1, open arrowheads). In the thoracic and the upper lumbar segments many labeled neurons were found along the border of Clarke's column, corresponding to marginal neurons of Lenhossék and Cajal. Furthermore, many labeled fusiform, oval or multipolar

neurons were found in lamina V and the adjacent layers. They were distributed mainly laterally in the thoracic segments but in the lumbar segments they were distributed in the entire mediolateral extent of lamina V. Neurons belonging to this group were found in the border region between laminae V and VI of C6 to T1. In L5 and L6 another group of labeled neurons was seen in the medial part of lamina VI or along the lateral border of the dorsal cord (Fig. 1, double arrowheads). The labeled neurons were large, multipolar or medium-sized, and different from Clarke column neurons in their morphology [1]. Recently, a few neurons with uncrossed ascending axons were found in the ventral horn of the lumbar to the caudal segments and the dorsal horn of the sacral-caudal segments [4].

On the basis of these findings SCT neurons were classified into eleven groups, which give rise to six crossed and five uncrossed ascending SCTs (Fig. 2). The crossed SCTs are (1) the CCN-SCT arising from the central cervical nucleus (CCN), (2) the medial lamina VII-SCT arising from neurons in the medial part of lamina VII of L6 to the caudal segments, (3) the lamina VIII-SCT arising from neurons scattered in lamina VIII of the cervical to the lumbar segments, (4) the border cell-SCT arising from spinal border cells, (5) the dorsal horn-SCT arising from neurons in the base of the dorsal horn of the sacral-caudal segments, and (6) the ventral horn-SCT arising from neurons in laminae VII and VIII of the sacral-caudal segments. The uncrossed SCTs are (7) the cervical medial lamina VI-SCT arising from neurons in the medial part of lamina VI of C2 to T1, (8) the cervival central lamina VII-SCT arising from neurons in the central part of lamina VII of C6 to T1, (9) the lamina V-SCT

arising from neurons in lamina V of the lower cervival to the lumbar segments, (10) the Clarke's column-SCT, and (11) the lumbar medial lamina VI-SCT arising from neurons in the medial part of lamina VI of L5 and L6.

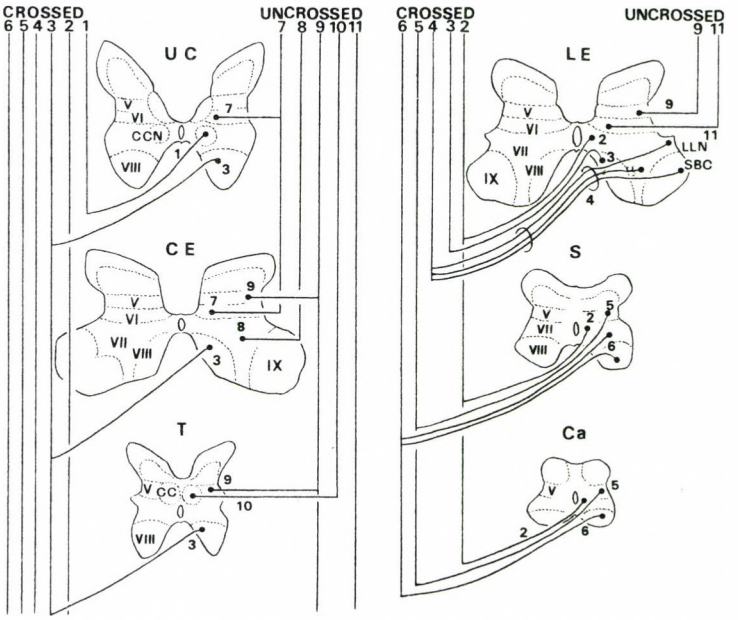


Fig. 2. Diagram showing the location and fiber course of cells of origin of the spinocerebellar tracts (SCTs). 1-6 are crossed SCTs; 7-11 are uncrossed SCTs. (1) the CCN-SCT arising from the central cervical nucleus, (2) the medial lamina VII-SCT arising from neurons in the medial part of lamina VII of L6 to the caudal segments, (3) the lamina VIII-SCT arising from neurons in lamina VIII of the cervical to the lumbar segments, (4) the border cell-SCT arising from spinal border cells, (5) the dorsal horn-SCT arising from neurons in the dorsal horn of the sacral-caudal segments, (6) the ventral horn-SCT arising from neurons in the ventral horn of the sacral-caudal segments, (7) the cervical medial lamina VI-SCT arising from neurons in the medial part of lamina VI of C2 to T1, (8) the cervical central lamina VII-SCT arising from neurons in the central part of lamina VII of C6 to T1, (9) the lamina V-SCT arising from neurons in the lower cervical to the lumbar segments, (10) the Clarke's column-SCT, and (11) the lumbar medial lamina VI-SCT arising from neurons in the medial part of lamina VI of L5 and L6

SCT NEURONS PROJECTING TO THE ANTERIOR LOBE

SCT neurons projecting to lobules I to V were identified following restricted injections into each lobule (Fig. 3) [7,10].

Injections into lobules I and II labeled many CCN neurons whereas injections into lobules III to V labeled a smaller number of CCN neurons. Many neurons were labeled in the medial part of lamina VII of L6 to the caudal segments following injections into lobules I and II. A few such neurons were labeled following injections into lobule III but only occasionally were they labeled following injections into lobule IV. Spinal border cells were labeled following injections into lobules II to V, but most abundantly following injections into lobules III and IV. In the sacral-caudal segments labeled neurons were seen in the dorsal (lamina V) and the ventral horns (lamina VIII) following injections into lobules I and II. A few neurons of these groups were labeled following injections into lobules III and IV.

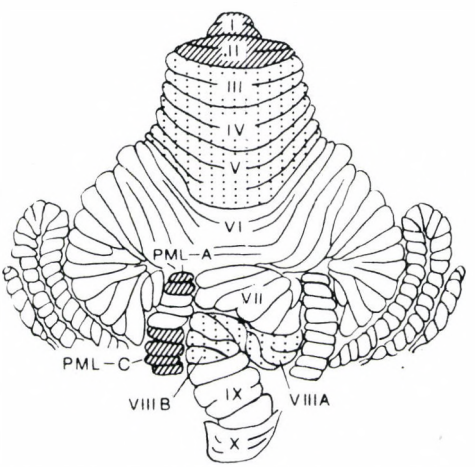


Fig. 3. The sites of HRP injections in the anterior and the posterior lobes (hatched and stippled)

SCT neurons in the medial part of lamina VI of C2 to T1 and in the middle part of lamina VII of C6 to T1 were labeled following injections into lobules III to V. However, no significant number of these neurons was labeled following injections into lobules I and II. Lamina V neurons of the lower cervical to the lumbar segments were labeled following injections into lobules III to V. They were also labeled at L3 to L5 following injections into sublobule IIB, but only occasionally were they labeled in the thoracic and lumbar segments following injections into sublobule IIA. Many Clarke column neurons and marginal neurons of Clarke's column were labeled following injections into lobules III to V, but only a small number was labeled following injections into lobules I and II.

SCT NEURONS PROJECTING TO THE POSTERIOR LOBE

SCT neurons projecting to lobule VIII (the posterior vermis) and the paramedian lobule were identified following injections into each folium of these lobules [9]. Injections into sublobule VIIIB (Fig. 3) labeled many neurons in the CCN and the medial part of lamina VII of L6 to the caudal segments. A few neurons were labeled in the medial part of lamina VI of C2 to T1. Clarke column neurons were also labeled; they were more numerous in the lumbar segments than in the thoracic segments.

Injections into the paramedian lobule (Fig. 3, PML-C) labeled neurons in the border region between laminae V and VI of the cervical segments. Similar labeled neurons were present in lamina V of the thoracic segments. Furthermore, labeled neurons were

seen along the lateral border of Clarke's column without labeling the neurons within the column. On the other hand, many labeled neurons were found in lamina V, and along the border of or within Clarke's column. Labeled spinal border cells were found from T10 to L5.

PROJECTIONS TO THE VERMIS OF THE ANTERIOR LOBE

Restricted injections into the midline region of lobules III and/or IV labeled many CCN neurons, and Clarke column neurons in the thoracic and lumbar segments [7]. A few labeled neurons were found in the medial part of lamina VI of the cervical cord, the medial part of lamina VII of L6 to the caudal segments, and the dorsal and the ventral horn of the sacral-caudal segments. An injection into the middle region of lobules I and II labeled many CCN neurons, but a few Clarke column neurons and neurons in the medial part of lamina VII of L6 [10].

PROJECTIONS TO THE INTERMEDIATE-LATERAL REGIONS

Following injections into the intermediate-lateral regions (regions more than 2.0 mm lateral to the midline) of lobules III and/or IV labeled neurons appeared mainly ipsilaterally to the injections [7]. In the thoracic segments a few lamina V and Clarke column neurons were labeled while in the lumbar segments many Clarke column neurons including marginal neurons, lamina V neurons and spinal border cells were labeled.

Similarly, spinal border cells and Clarke column neurons, mainly of the lumbar segments were labeled following an injection into the intermediate region of sublobule IIB and lobule III.

CONCLUDING REMARKS

Using the HRP method we have identified various kinds of SCT neurons, which include classically known Clarke column neurons and spinal border cells. Restricted injections into the medial or lateral regions of the lobules in the anterior lobe revealed some aspects of the mediolateral projection of SCT neurons (Fig. 4). The CCN neurons appeared to project to zone A [14] or the first compartment [15], since many CCN neurons, but few or no Clarke column neurons were labeled in cases with a limited injection into the midline region of lobules I and II. The results are in agreement with those of the autoradiographic [16] and physiologic experiments [6].

It has been shown that the dorsal spinocerebellar tract axons project to the intermediate region and the adjacent strip of the vermal region of the anterior lobe [3, 11]. In cases with injections into the midline region of lobules III and/or IV labeled neurons were seen in the CCN and Clarke's column of the thoracic and lumbar segments, while in cases with injections into the intermediate-lateral regions labeled Clarke column neurons were many in the lumbar segments, but only a few in the thoracic segments [7]. Similar findings were made of the projections to the posterior lobe; Clarke column neurons of the thoracic segments project to the vermis but not to the

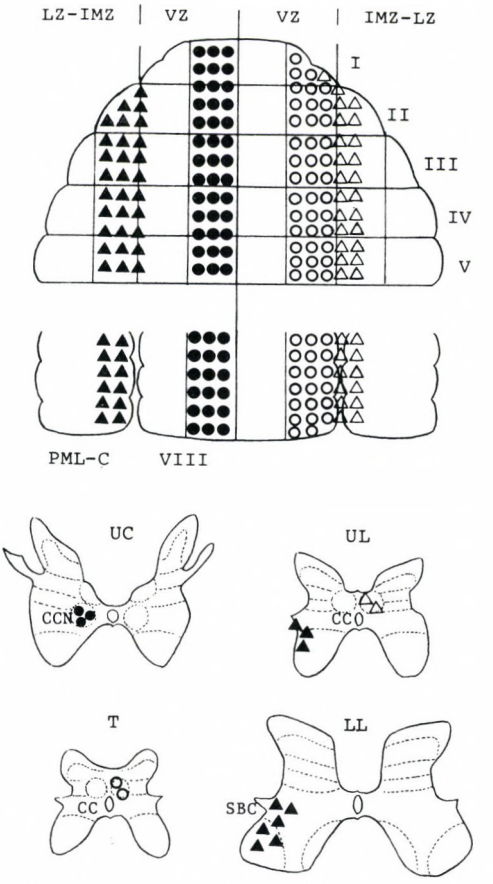


Fig. 4. Zonal projections of the major spinocerebellar tract neurons in the anterior and the posterior lobes. The central cervical nucleus neurons project to the medial part of the vermal zone. Spinal border cells project to the most lateral part of the vermal zone and the intermediate-lateral zones of the anterior lobe, and the paramedian lobule. Clarke column neurons of the thoracic segments project to the lateral part of the vermal zone while those of the upper lumbar segments project to the lateral part of the vermal zone and the intermediate-lateral zone of the anterior lobe, and the paramedian lobule

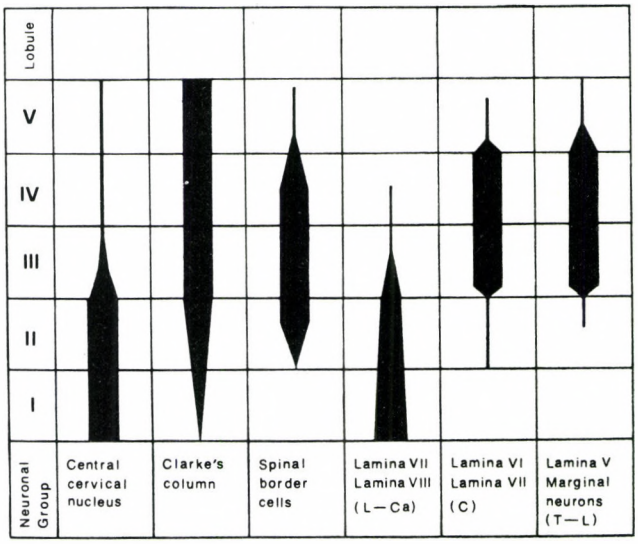
paramedian lobule whereas those of the lumbar segments and marginal neurons of Clarke's column project to both the vermis and the paramedian lobule [9]. From these findings it seems that in the anterior lobe Clarke column neurons of the thoracic segments project to the lateral part of the vermis (zone B or

the second compartment), and those of the lumbar segments as well as marginal neurons project to zones B and C (the intermediate region or the third compartment). In addition, it has been suggested that the medial lamina VII group of L6 to the caudal segments, and the dorsal and the ventral horn neurons of the sacral-caudal segments project to the medial part of the vermis [7, 10].

As suggested previously [2,3], spinal border cells also project to the lateral part of the vermis and the intermediate regions since many spinal border cells were labeled after injections into these regions, without labeling CCN neurons [7, 10].

The present study reveals another feature of the spinocerebellar projections. The main sites and quantity of projections are different according to the groups of SCT neurons (Table I). Lobules I and II are the main projection sites for CCN neurons, the medial lamina VII groups of L6 to the caudal

Table I. Extent and quantity of the major spinocerebellar projections in the anterior lobe. Note that the difference in the quantity among the neuronal groups is not considered



segments, the medial lamina V-and lamina VIII-SCT neurons of the sacral-caudal segments. Lobules III and IV are the main projection sites for the cervical SCT neurons (the medial lamina VI group and the middle lamina VII group), spinal border cells, Clarke column neurons, marginal neurons of Clarke's column and lamina V neurons of the thoracic and lumbar segments.

As these findings show, the SCT neurons are considered to project to different longitudinal (sagittal) zones of the cerebellar cortex, with various quantities in the rostrocaudal direction.

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NEURONAL ORGANIZATION IN THE DORSAL HORN OF THE SPINAL CORD

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Two major output systems from the dorsal horn, the spinocervical tract (SCT) and the post-synaptic dorsal column (PSDC) pathway, have been characterized by electrophysiological methods and the injection of horseradish peroxidase into single neurones. Neurones of both systems have somata in laminae III, IV and V but they differ in their physiology and anatomy. SCT neurones have few inputs and their receptive fields are simple. PSDC neurones show several patterns of excitatory convergent input and have more complex receptive fields. Structurally, SCT cells are fairly homogeneous with dendrites mainly in laminae III and IV; input is from two major types of boutons - 2/3 contain round clear vesicles and 1/3 contain flattened clear vesicles and these latter are located more proximally on the dendritic tree. PSDC cells have a more varied anatomy: the dorsal cells send their dendrites through lamina III into II where they receive a variety of contacts including the central element of the glomerular complexes; deeper neurones have dendrites in laminae V - VII and these receive large synaptic boutons, often on long-necked spines. Thus even though SCT and PSDC neurones occupy the same parts of the dorsal horn they constitute separate populations of cells differing in axonal projection, receptive field organization, light microscopical appearance and the ultrastructure of the synaptic contacts they receive.

INTRODUCTION

Two major ascending projections from the dorsal horn of the spinal cord in the cat are the spinocervical tract (SCT) and the post-synaptic dorsal column (PSDC) pathway. Both systems

convey information from cutaneous mechanoreceptors and both take origin in the same regions of the dorsal horn. Considerable knowledge is now available about their response properties and the anatomy of their neurones. It is, therefore, instructive to compare these two systems for the light they throw on organizational principles in the spinal cord.

The work to be described comes from a long series of experiments on the SCT [reviewed in 2 and 3] together with more recent work [13 and unpublished observations], and investigations on the PSDC neurones [1, 4, 6 and unpublished observations]. The experiments were performed on cats and the methods included extra- and intracellular recording from single neurones to study the cells' response properties, and the intracellular injection of horseradish peroxidase (HRP) into single neurones to allow their light and electron microscopical anatomy to be determined.

PHYSIOLOGY

The spinocervical tract

Physiologically, in comparison with the PSDC pathway, the SCT seems a relatively simple system. The vast majority, probably at least 95 %, of SCT neurones are excited by moving hairs on the coat of the animal. Indeed, about 30 % of SCT cells are excited exclusively by moving hairs and the input is via myelinated afferent fibres. About 70 % of SCT neurones are excited by heavy pressure and pinch of the skin (noxious stimulation) in addition to hair movement. This latter set of neurones is excited by activation of non-myelinated afferent fibres in

addition to myelinated ones and the nocireceptor input may largely be carried by these.

The above statements represent something of an oversimplification: the hair follicle inputs may be subdivided and noxious heat inputs may excite some SCT cells [5] as may muscle afferent fibres with thresholds to electrical stimulation greater than the Group I level [12]. However, the overriding impression one receives is that the SCT is geared towards sensing hair movement and noxious events at the skin. It is not excited by activation of other sensitive mechanoreceptors such as the slowly adapting Types I and II receptors or Pacinian and Krause corpuscles, nor does it receive input from sensitive thermoreceptors. It is exclusively concerned with input from hairy skin: there is no input from sensitive mechanoreceptors in glabrous skin.

The excitatory receptive fields of SCT neurones are also organized in a simple fashion (Fig. 1A, B). The fields are generally round or oval with their long axis in the long axis of the limb. The perimeter of the field is sharply defined and there is no subliminal fringe. Within the field there is no marked gradient of sensitivity; the periphery is nearly as sensitive as the centre. In those neurones responding to both hair movement and noxious mechanical stimulation there is no separation of the skin areas sensitive to the different stimuli: as far as one can determine the components of the field are coextensive. Inhibitory receptive fields are also simple, responding to hair movement or, more usually, noxious stimuli, and are generally separated from the excitatory field, often on the contralateral side of the body.

The post-synaptic dorsal column pathway

Much greater complexity is exhibited by PSDC neurones in terms of both the degree of convergence of their inputs and the organization of their receptive fields on the skin. Although about 80 % of PSDC cells are excited by hair movement, only some 2 % have an exclusive hair input and only 20 % respond to both hair movement and noxious mechanical stimulation of hairy skin. That is, only just over 20 % are similar to SCT cells.

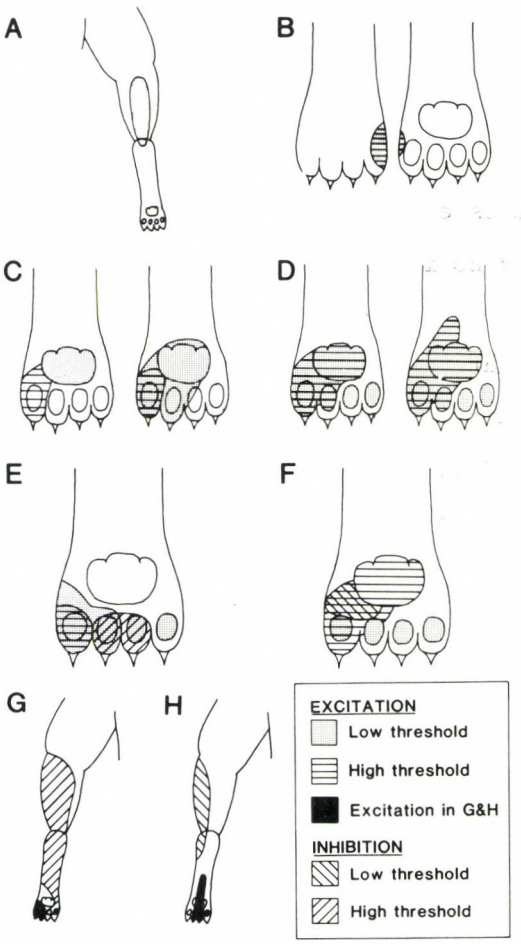

Fig. 1. Receptive fields of SCT and PSDC neurones. A,B: SCT receptive fields. C-H: PSDC receptive fields. Note the simple nature of the SCT fields compared with the PSDC ones

The other PSDC neurones show varying degrees of excitatory convergence from other receptors. Thus about 60 % receive an input from glabrous skin in addition to hairy skin and this glabrous skin input may arise in either sensitive mechanoreceptors and/or nocireceptive mechanoreceptors. A few PSDC cells are excited exclusively by Pacinian corpuscles or nocireceptors. Indeed all types of cutaneous receptors except the sensitive thermoreceptors (and possibly the slowly adapting Type II receptors) are capable of exciting some PSDC neurones. 15 - 30 % of PSDC neurones have excitatory receptive fields that demonstrate a marked subliminal fringe - the firing zone is surrounded by an area from which excitatory post-synaptic potentials may be evoked and the firing zone expands into this area following a variety of manoeuvres such as mechanical stimulation of the skin, electrical stimulation of cutaneous nerves or electrical stimulation of the cervical spinal cord.

Excitatory receptive fields of PSDC neurones are markedly different from those of SCT cells and are much more complex (Fig. 1C-F). Although similar in size, PSDC cells' fields are never as small as the smallest SCT fields. Furthermore, some

A further degree of complexity to the excitatory receptive fields of PSDC neurones is that some 20 % or so have fields made up of disparate parts, e.g. the low and high threshold components may be partly or completely separate from one another. It may be part or all of one of these components that makes up the subliminal fringe. Such complex fields are often of irregular shape.

The inhibitory receptive fields of PSDC neurones also differ from those of SCT cells. They fall into two categories (Fig. 1E-H), one in which the inhibitory field is small and

overlaps with or is adjacent to the excitatory field, and the other where the inhibitory field is large and proximal to the excitatory field.

ANATOMY

Location and density of cell bodies

Both SCT and PSDC neurones are located mainly in laminae III, IV and V of the dorsal horn [8, 9, 11, 19; and work from the author's laboratory reviewed in 2]. For the SCT about 25 % of cell bodies are in lamina III, 60 % in lamina IV and 10 % in lamina V. For the PSDC the figures are about 30 %, 50 % and 20 % respectively. In other words there is little difference between the two in the laminar location of the cells but the PSDC does have rather more cells in lamina V and these are aggregated in the medial part of the lamina.

The numbers of SCT cells in the cat's cord have been estimated on the basis of retrograde HRP studies, careful microelectrode grids of recording sites in the lumbosacral cord and sampling of the complete SCT system at cervical levels [7, 10]. The estimates provide figures of about 2200 neurones on each side of the cord and this total is made up of about 1100 for the forelimb representation and 750 for the hind limb representation with the remaining 350 representing the trunk and tail. That is, the forelimb to hindlimb ratio is only about 1.5:1. Similar studies have not been performed on the PSDC system but our subjective impression is that on the basis of electrophysiological experiments the number of cells in the lumbosacral enlargement is at least of the same order of magnitude.

Dendritic trees

SCT neurones have a wide range of dendritic tree anatomy. One common type of cell is the 'antenna type' neurone described by Réthelyi and Szentágothai [18] (Fig. 2A-C). All SCT cells have dendrites in lamina III and usually IV and all have well-developed dorsally directed dendrites and all have their greatest dendritic development in the rostro-caudal plane. An important fact is that very few SCT neurones have dendrites that enter lamina II: when they do they are usually the terminal parts of distal dendritic branches.

In contrast to the SCT, PSDC neurones fall into three fairly well-defined categories (Fig. 2D-F): the most dorsally

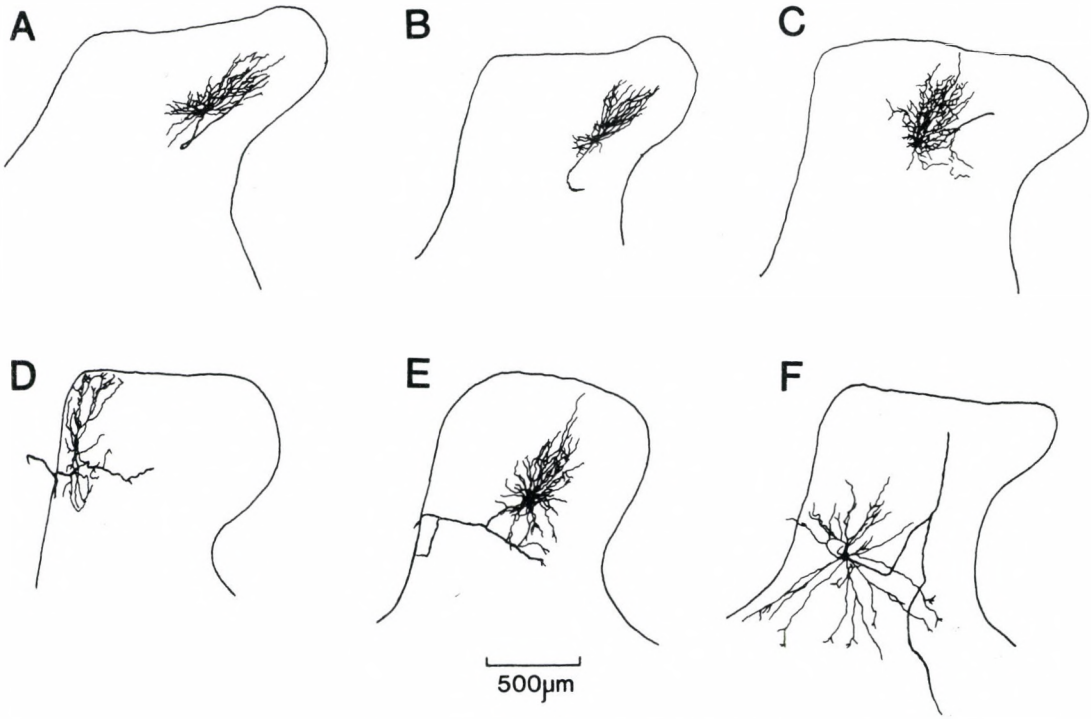

Fig. 2. Anatomy of SCT and PSDC neurones. A-C: SCT neurones. Many cells are of this typical 'antenna' type. The dendrites are almost totally excluded from lamina II. D-F: The three main classes of PSDC neurones. Note the dendrites in lamina II in D and the deep neurone in F with widely spreading dendrites

located neurones have trees contained within a more or less cylindrical volume, narrow medio-laterally and rostro-caudally, that ascends through lamina III into II and even into I; slightly deeper cells have dendritic trees similar to those of SCT neurones but not as well-developed rostro-caudally and also often extending into lamina II; cells in the medial parts of lamina V have transversely oriented dendritic trees that radiate widely from the cell body, ventrally as far as lamina VI and VII but not as far dorsally as lamina II. Obviously the dendrites of PSDC neurones cover a wider range, laminae II to VII, than those of SCT cells (laminae III-IV mainly) thus providing a greater opportunity for the cells to sample a wide range of inputs.

Ultrastructure of contacts upon SCT and PSDC neurones

Just as the physiology and light microscopical appearance of SCT neurones are straightforward, so the anatomical substrate of the input to these cells is also simple. There appear to be only two major types of synaptic boutons contacting SCT neurones (Fig. 3A, B). The most common type, about two thirds of all synapses, contains round clear vesicles and makes asymmetrical contacts. This type of bouton is similar to that formed by hair follicle afferent fibres [14] and it is known that these afferents make monosynaptic excitatory connexions with SCT neurones. Not all of these sorts of contacts can come from hair follicle afferents; there are other excitatory inputs too, probably from interneurones in the dorsal horn. An interesting observation is that while boutons of hair follicle afferent fibres often receive axo-axonic contacts [14] we have

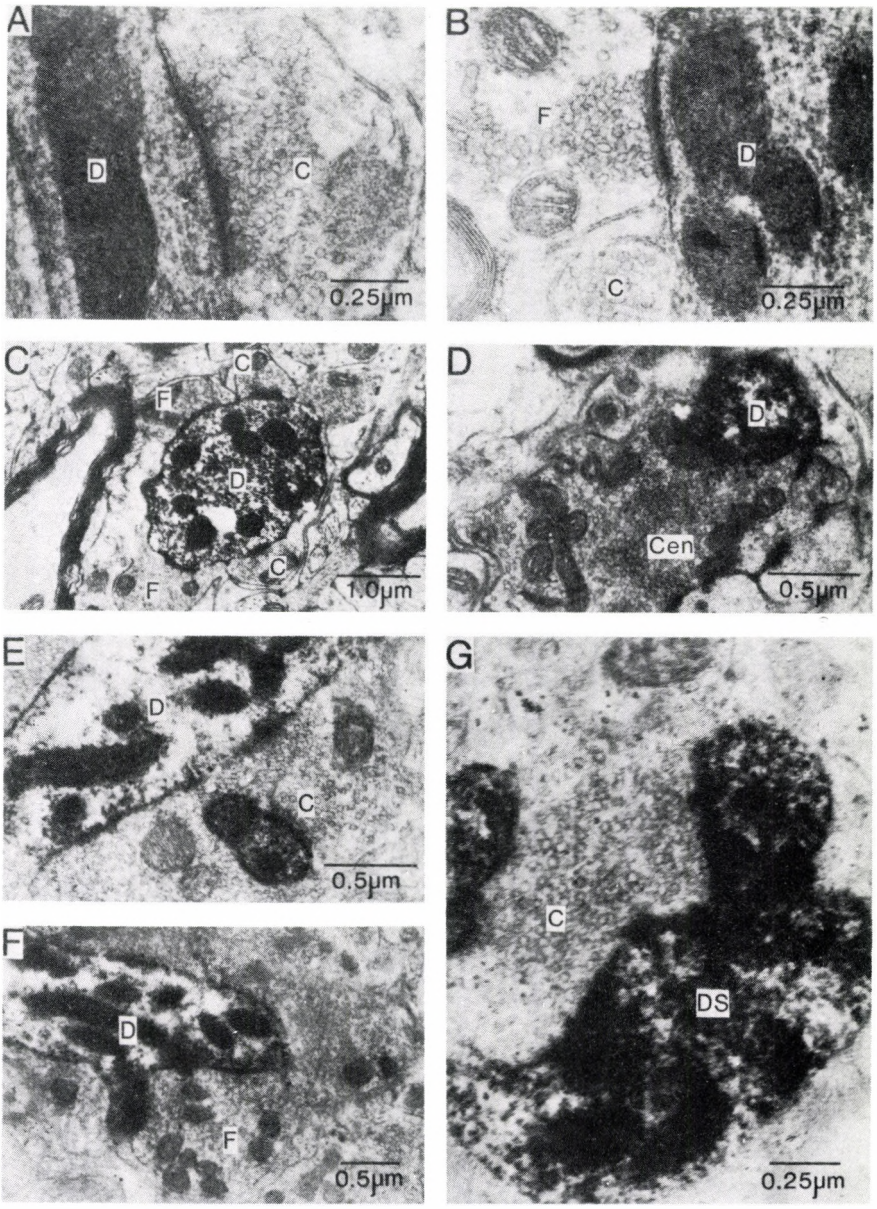

Fig. 3. Ultrastructure of contacts made upon SCT and PSDC neurones. A,B: SCT cells; C-G: PSDC cells. Note the major types of contacts with each cell category (A, B, C, E, F), the participation of a PSDC dendrite, in lamina II, in a glomerulus (D) and the large bouton contacting a PSDC dendritic spine of a deep neurone in lamina VI (G). Abbreviations: C, bouton containing round, clear vesicles; Cen, the central terminal of a glomerulus; D, HRP-stained dendrite; DS, HRP-stained dendritic spine; F, bouton containing flattened (irregular), clear vesicles

not seen axo-axonic contacts upon the similar boutons that contact SCT neurones. The remaining one third of boutons ending on SCT cells contain flattened clear vesicles. Although forming about one third of the total, they are preferentially localized to the proximal parts of the dendritic tree.

In contrast with SCT neurones, PSDC cells receive a greater variety of synaptic contacts, again reflecting their physiology and dendritic tree anatomy. Like SCT cells, PSDC neurones receive a majority of synaptic vesicles that contain round clear vesicles and also other boutons containing flattened (irregular) clear vesicles in about the same proportion as on SCT neurones (Fig. 3C,E,F). These types of contacts occur throughout the total extent of PSDC cells' dendritic range, from laminae II to VII. Within laminae II and III dendrites of PSDC neurones receive contacts from the central elements of the glomerular complexes [12, 15, 17] (Fig. 3D) which are now known to be, for the most part at least, of primary afferent fibre origin and indeed are the terminals of nonmyelinated and small myelinated axons (16). These observations show that the PSDC pathway provides an output from lamina II (and also of course from deeper laminae) to the dorsal column nuclei. Furthermore, one of the dendritic elements in the glomerular complexes of laminae II and III is now identified as belonging to PSDC neurones and the PSDC system receives input directly from nonmyelinated and/or small myelinated primary afferent fibres. The deeper dendrites of PSDC cells, in laminae V - VII, also receive a different type of synaptic contact (Fig. F): this is a large bouton, containing round clear vesicles, that makes asymmetrical contacts with the dendrites and especially with the heads of

long-necked spines which are a feature of the dendrites of deeper cells. At present the origin of these large boutons is unknown. In contrast to SCT neurones, the boutons containing round clear vesicles and ending upon PSDC cells often received axo-axonic contacts.

CONCLUSIONS

Two major output systems of the dorsal horn, the SCT and PSDC pathway, reflect in their anatomy at both light and electron microscopical levels, differences in their functional properties. In comparison with PSDC neurones, SCT cells have a simple anatomy and physiology. Perhaps of particular relevance is the fact that some PSDC neurones provide an output from lamina II (the substantia gelatinosa) whereas SCT neurones seem to be excluded almost entirely from this region of the cord.

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THE ROLE OF CATECHOLAMINES IN THE REGULATION OF GONADOTROPIN SECRETION

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Earlier neuropharmacological evidence suggested a role for the central catecholamine (CA) system in the regulation of gonadotropin secretion. To resolve whether dopamine (DA) or norepinephrine (NE) was the important CA involved in evoking the preovulatory release of LHRH and to evaluate the temporal sequence of changes in the CAs which occur prior to and during preovulatory-like gonadotropin surges, we evaluated turnover rates (T/R) of DA and NE in microdissected regions of the hypothalamus of proestrous rats. We also examined whether similar changes in T/R occur in ovariectomized (OVX), estrogen-(E_2)-treated rats and how progesterone (P) amplifies and advances the time of the LH surge in this rat model system. In these studies we used a radioenzymatic assay to measure CA content and α-MPT to block CA synthesis and provide information on the rate of CA efflux. This information was used to calculate T/R. In proestrous rats, a dramatic increase in NE T/R occurred prior to and during the LH surge whereas DA T/R initially increased and then markedly declined during the afternoon of proestrus. The NE T/R changes occurred in the medial preoptic nucleus (MPN), suprachiasmatic nucleus (SCN), arcuate nucles (AN) and median eminence (ME) whereas DA T/R changes were observed only in AN and ME. When phenobarbital was administered to block spontaneous proestrous LH surges it also completely blocked the increase in NE T/R which occur in ME and SCN but did not affect DA T/R. In OVX E_2-treated rats similar increases in MPN, SCN and ME NE T/R occurred during the time of the LH surge while DA T/R remained unchanged. The P advancement of the time of the LH surge (by 1 h) was accompained by an advancement of the time of increased NE T/R in the hypothalamus. In androgen-sterilized rats, neither spontaneous nor E_2-induced LH surges occurred. All microdissected brain regions in ASR contained less NE than controls but DA values in ME were comparable in both groups. No increase in NE T/R occurred between morning and afternoon in MPN or ME while DA T/R declined in both control and ASR between morning and afternoon. These observations suggest that NE rather than DA is the important CA responsible for triggering the preovulatory-like release of LHRH from

ME axon terminals. They further suggest that E_2 affects function of NE perikarya in the mid and hind-brain to increase the frequency of depolarization which accounts for the increase in NE secretion into the preoptico-suprachiasmatic, tuberoinfundibular system.

As early as 1947, Sawyer et al., [1] suggested that the central nervous catecholamine system (CA) and, in particular, norepinephrine (NE) was involved in the surge release of LH. This hypothesis was reinforced by the observations that chlorpromazine, an α-adrenergic receptor antagonist, when administered to rats before the presumptive time of the gonadotropin surge on proestrus, effectively blocked ovulation that night [2]. Reserpine, a depletor of CAs from presynaptic terminals also was effective in suppressing ovulation in cyclic rats. Further, both drugs induce pseudopregnancy [an index of prolactin (PRL) release], probably by blocking lactotroph DA receptors and/or depleting median eminence terminals of DA [3]. Since these early studies, numerous publications on the effects of a variety of pharmacological agents on LH surges in different animal models have been published and the results of these studies recently have been reviewed [4]. From this body of literature has emerged the concept that either norepinephrine (NE) or dopamine (DA) activates the discharge of median eminence LHRH and this event accounts for preovulatory LH surges. However, it has proven difficult to decipher how or where pharmacological agents act within the CNS and because of the inherent nonspecific actions of many of these drugs, considerable confusion on the role of the catechols in regulating the reproductive neuroendocrine axis has prevailed over the last 32 or more years since CAs were first implicated as modulators of gonadotropin secretion.

We recognize that the secretion of gonadotropic hormones depends upon a complex interplay of severial peripheral and central nervous processes. These include: (a) alterations in LHRH synthesis and releasibility during the reproductive cycle; (b) changes in the activity of hypothalamic catecholamine neurons and, (c) changes in responsiveness of the pituitary gonadotrophs to LHRH during the cycle. It is widely recognized that the increase plasma concentrations of estradiol (E_2) which occur between late diestrus and proestrous morning affect many of these regulatory processes to ultimately evoke preovulatory gonadotropin surges on proestrous afternoon in rats.

To learn more of how these systems interact, we have performed a series of indepth studies to examine further the changes in specific brain nuclei known to contain either cell bodies, axons and/or terminals of the LHRH system. As well, we have examined the turnover rates (an index of secretion) of NE and DA which occur in these discrete hypothalamic regions and have correlated such CA release with alterations in hypothalamic LHRH and with plasma concentration changes in LH, FSH, PRL, E_2 and progesterone (P) during proestrus and diestrous day 1 in 4-day cyclic rats.

1. CHANGES IN LHRH CONCENTRATIONS ON PROESTRUS AND DIESTROUS DAY 1

Using a modification of the micropunch technique [5] originally described by Palkovits [6] and a sensitive LHRH radioimmunoassay we examined how proestrous LHRH concentrations change during the day of proestrus within the

medial preoptic (MPN) and suprachiasmatic nuclei (SCN) (cell bodies), the retrochiasmatic area (RCA) and anterior hypothalamus (AHN) (axons) and within the median eminence (ME) (axon terminals).

In proestrous rats, ME-LHRH concentrations increase significantly between 0900 and 1200 h at a time when serum gonadotropin and progesterone concentrations are basal. During these hours serum E_2 levels are significantly elevated above the concentrations obtained on diestrous day 1 and they continue to rise significantly between 0900 and 1200 h proestrus. At the beginning of the proestrous LH surge (1200-1500 h), ME-LHRH and serum estradiol levels decrease and serum P concentrations increase. At 1800 h proestrus, ME-LHRH concentrations are not different from 1500 h values but serum levels of LH, FSH and P had increased above those values obtained at 1500 h. The same pattern of LHRH changes, that is, a significant increase between 0900 and 1200 h, a significant decrease between 1200-1500 h and no change between 1500-1800 h was seen in SCN, MPN and RCA. In contrast to the proestrous LHRH and serum gonadotropin fluctuations, no changes were observed in these hormones between morning and afternoon of diestrous day 1 [7].

These neuroendocrine (LHRH) events, which occur within the preoptico-suprachiasmatic-tuberoinfundibular system (PSTS), suggest that this system functions as an integrated unit whose function it is to synthesize, transport, store and ultimately release LHRH. The physiological importance for the increase in PSTS-LHRH on proestrous morning may be to make newly synthesized LHRH available for release that afternoon. We propose that this singular event of LHRH synthesis is insufficient in itself to account for the preovulatory release of LH and FSH. Rather,

activation of a second CNS system is required to provide the physiological "trigger" for the discharge of the newly synthesized LHRH and the data presented in the next series of experiments provides a clue to what this signal may be.

2. CHANGES IN CATECHOLAMINE TURNOVER RATES IN VARIOUS REGIONS OF THE PREOPTICO-SUPRACHIASMATIC TUBEROINFUNDIBULAR SYSTEM IN PROESTROUS AND DIESTROUS DAY 1 RATS

We have carefully examined the profiles of CA turnover rates (T/R) in microdissected areas of the hypothalamus after blocking tyrosine hydroxylase activity with α-methyl-p-tyrosine and using a micromodification of the CA radioenzymatic assay described previously [8,9] to measure CA content. As is shown in Figure 1, NE T/R were low in all brain regions examined between 0900-1100 h proestrus. During this interval, serum gonadotropin levels were basal. Between 1200-1400 h, ME-NE T/R increased significantly and they remained elevated between 1500-1700 h. During these intervals, LH, FSH and PRL proestrous surges begin and reach peak concentrations in blood. NE T/R in MPN, SCN and AN also were increased significantly at 1500-1700 h when compared to 0900-1100 h values.

MPN-DA T/R remained unchanged between morning and afternoon of proestrus. In contrast, AN and ME-DA T/R increased significantly between 1200-1400 h versus 0900-1100 h but between 1500-1700 h proestrous, the T/R in these areas were significantly depressed.

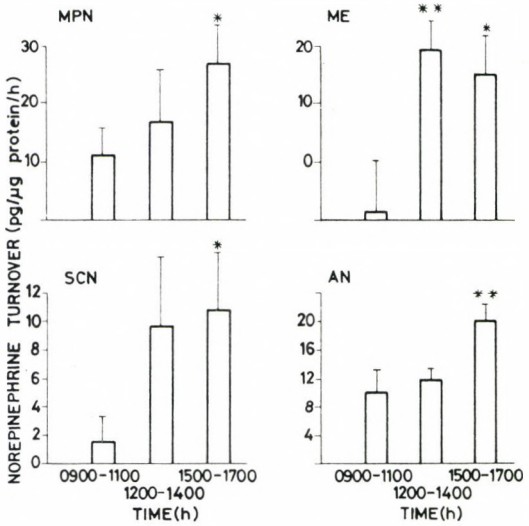

*Fig. 1. Norepinephrine (NE) turnover rates in discrete preoptic (MPN) and hypothalamic regions (SCN, AN and ME) during proestrus. NE turnover rates are low in all areas between 0900-1100 h proestrus. Between 1200-1400 h, NE turnover rates increase significantly in ME and remain elevated between 1500-1700 h. Also, during 1500-1700 h, NE turnover rates are significantly increased in SCN, AN and MPN. Abbreviations in this Figure and Figure 3: MPN, medial preoptic nucleus; SCN, suprachiasmatic nucleus; ME, median eminence; AN, arcuate nucleus. *-significantly different from 0900-1100 h values ($p<0.05$); **-significantlu different from 0900-1100 h values ($p<0.01$)*

Several interesting observations can be made regarding the changes in hypothalamic LHRH and CA T/R and the timing of preovulatory surge of gonadotropins in proestrous rats: (a) during the interval that LHRH is accumulating in the ME (0900-1200 h), both NE T/R in MPN, SCN, AN and ME and DA T/R in MPN, AN and ME are low and peripheral serum gonadotropin concentrations remain basal; (b) prior to and during the proestrous gonadotropin surge, ME-LHRH declines and it rises in portal blood and ME-NE and DA T/R greatly increase (1200-1400 h); (c) during the interval (1500-1700 h) that LH, FSH and PRL are still rising to peak serum concentrations, ME-NE turnover rates remain

elevated and increased NE turnover rates also now are evident in MPN, SCN and AN. During this same time period, DA turnover rates dramatically decline in ME and AN but not in MPN.

Particularly important to the initiation of preovulatory LH and FSH surges is the increased turnover rates of CAs which occur in ME. Clear temporal relationships exist between increased ME-CA turnovers, a decline in ME-LHRH and a rise in serum gonadotropins (1200-1400 h). Earlier studies by others have shown that third ventricle injections of NE produce LH release in ovariectomized steroid-primed rats [10]. Presumably this effect is expressed via the induced release of LHRH. We now provide evidence that the increased release of endogenous NE and perhaps DA into the ME, in close proximity with LHRH terminals, is correlated with the initiation of LH and FSH surges and a decline in ME-LHRH. Since these events do not occur on diestrous day 1, the changes in hormone patterns observed during proestrus are not part of an intrinsic diurnal rhythm. Rather, we suggest that they represent dynamic central nervous system events which ultimately are required for ovulation in the rat. Further, we propose that NE may act within the entire preoptico-suprachiasmatic system (PSTS) including the ME. The present results demonstrate NE turnover rates increased not only in MPN but also in ME, SCN, and AN between 1500-1700 h proestrus. Previous studies of serum LH and FSH profiles in our rat colony [11] show that during this interval of increased NE turnover, serum LH continues to rise to reach about 4000 ng/ml. Therefore it may be essential that NE release be maintained to affect the PSTS throughout this time interval for full expression of LH surges to occur.

We conclude, from these studies, that the increase in hypothalamic NE turnover rates, coupled with a decrease in ME-LHRH and an increase in serum gonadotropins, to mean that NE may be the physiological "trigger" which initiates the preovulatory surge of gonadotropins by evoking the release of newly accumulated LHRH in ME axon terminals.

3. EFFECTS OF ESTRADIOL AND PROGESTERONE ON HYPOTHALAMIC CATECHOLAMINE TURNOVER RATES IN OVARIECTOMIZED RATS

Estradiol (E_2) treatment of ovariectomized (OVX) rats results in daily LH surges and progesterone treatment of such E_2-treated OVX animals advances and amplifies peak plasma gonadotropin concentrations on the day that is is administered [12,13].

To examine the effects of E_2 and P on CA T/R in OVX rats we used an experimental paradigm which produces low physiological concentrations of these steroids (12). We observed that E_2-induced LH surges were accompanied by an increase in afternoon NE turnover rates in all brain areas examined (ME, AN, MPN and SCN). E_2P-induced gonadotropin surges were correlated with increased NE turnover rates during the afternoon in the AN and MPN. The NE patterns of increased neuronal acitivity from morning to afternoon were similar to those observed in animals treated with E_2 alone. In contrast, enhanced NE turnover rates were observed during the morning in the SCN and ME of E_2P-treated rats. NE turnover rates were significantly greater in E_2P versus E_2-treated rats between 1000-1200 h and they remained high during the afternoon. In contrast, there were no significant changes in turnover

rates of DA from moring to afternoon in E_2 or E_2P-treated rats in all brain areas examined. Similarly, when DA values were compared between E_2- and E_2P-treated rats, P treatment did not cause any significant change in the morning or in the afternoon. Thus, in such animals, E_2-treatment clearly increases NE-turnover rates in MPN, SCN, AN and ME and these changes occur concomitant with afternoon gonadotropin surges. This pattern of increased NE activity is identical to that observed by us in proestrus rats 8 although concentrations of plasma LH achieved in E_2-treated OVX rats are approximately 4-fold less that those obtained in normal proestrous rats. Perhaps the low physiological levels of E_2 used in these studies are insufficient to induce proestrous-like PSTS-LHRH concentration increases and thus only small amounts of LHRH are made available for release at the time of increased noradrenergic activity. Unlike the proestrous rat, LHRH does not change in PSTS between morning and afternoon in these E_2-treated OVX rats [12]. In contrast, P administration to E_2-treated OVX rats advanced and amplified the LH surge and also advanced the time of appearance of increased NE turnover rates (1000-1200 h versus 1500-1700 h) in SCN and ME but not MPN or AN. Earlier studies by us demonstrated that P also increases the accumulation of ME-LHRH between 0900-1200 h in E_2-treated rats so that additional LHRH is made available for release [12]. Together these data suggest that as a consequence of the increase in ME-LHRH concentrations and the advancement and increase in NE activity, LH surges in E_2P-treated rats are markedly amplified and temporally advanced when compared to those obtained in rats which received only E_2. Pituitary responsiveness to LHRH in E_2 and E_2P-treated OVX rats is almost identical.

4. EFFECTS OF PHENOBARBITAL OR NEONATAL ANDROGEN TREATMENT ON CATECHOLAMINE T/R

Phenobarbital

Phenobarbital (PB), when administered prior to the beginning of the preovulatory gonadotropin surge, effectively blocks LH, FSH and PRL release. In saline-treated proestrous rats, NE T/R increase markedly in the PSTS between morning and afternoon. When PB was injected at 1200 h it did not alter the elevated ME-NE turnover rates which occurred between 1200-1400 h. Further, the increased NE T/R noted in MPN between 1500-1700 h also were not affected by PB. In contrast, PB had a dramatic suppressive effect on NE release in both SCN and ME between 1500-1700 h.

While PB suppressed hypothalamic noradrenergic activity, neither the incertohypothalamic nor the tuberoinfundibular dopaminergic systems were affected by PB treatment of proestrous rats. These observations further support the concept that the increased release of NE which occurs in specific regions of the PSTS is essential for preovulatory gonadotropin surges to occur on proestrous afternoon [14].

Androgen-Sterilized Rat

Neonatally androgenized rats, on reaching adulthood, lack spontaneous preovulatory gonadotropin surges and after OVX they do not respond to the positive feedback effects of E_2 or P by having LH surges. We have examined CA T/R in ASR following OVX and E_2 or E_2P-treatment and compared such catecholaminergic activity to that obtained in control rats [15]. While LH surges occurred in E_2-treated control rats, no such LH release was observed in any of the E_2-treated ASR. In control and ASR there

were no significant differences in initial NE concentrations in MPN, AN or ME when morning and afternoon values were compared within each group. However, all microdissected brain regions in ASR contained less NE than controls but DA values in ME were comparable in both groups. Whether these reduced initial NE concentrations represent differential responsiveness of NE perikarya to E_2, or are due to a defect in the NE synthesis pathway or to a loss of neurons (or all of these) remains to be resolved. What is evident is that very little NE is available for release particularly from axon terminals within the ME and SCN.

In E_2-treated OVX controls, ME T/R increased significantly between morning and afternoon in ME, MPN, SCN but not in AN. In sharp contrast, no increase in NE T/R was observed between morning and afternoon in ME or MPN of ASR but SCN NE T/R were elevated in the afternoon. DA T/R in both control and ASR declined significantly between morning and afternoon in ME.

These data and those recently reported by us on the changes in NE T/R which occur in conjunction with LH surges in a variety of different animal models offer a new insight on how exposure of the developing brain to androgen (or indirectly to estrogen) may deleteriously alter the cyclic surge mechanism in the female rat. In the absence of any increase in NE release into the MPN and ME, the release of low NE concentrations into SCN may be inadequate to initiate the release of LHRH. Exactly how much of the PSTS must be activated by NE to induce LH surges remains to be determined.

In summary, by measuring the T/R of NE and DA in a variety of rat models and correlating such changes with the release of LH (or absence thereof) we believe we have provided a clearer

understanding of how newly synthesized LHRH may be released in the cyclic rat. Estrogen not only appears to be the "zeitgeber" for the increased synthesis of LHRH in the hypothalamic neurons on proestrous morning, but also it is essential for the increased release of NE during the early afternoon of proestrus. Is it possible that these sequential CNS processes (including increased NE neuronal activity) are genomically programmed events whose

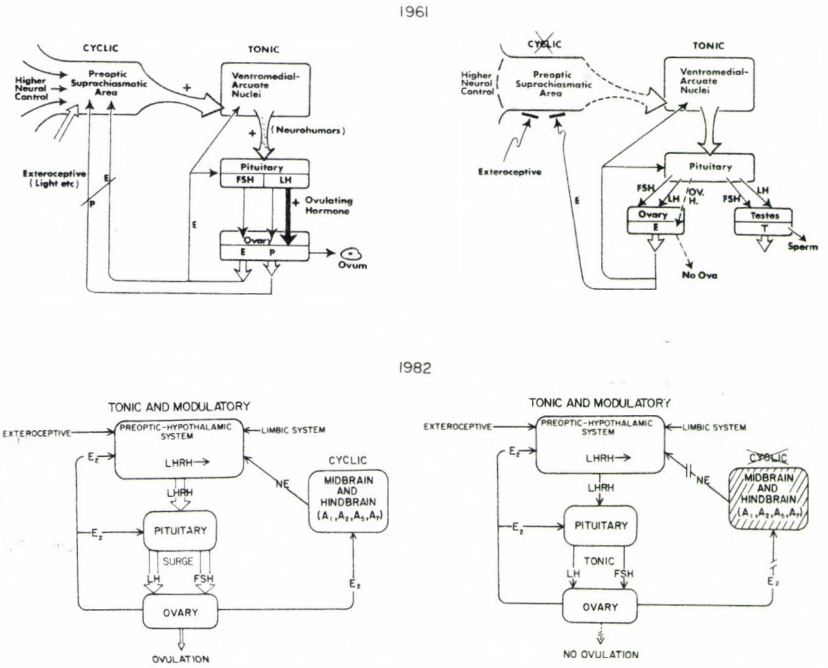

Fig. 2. In 1961 [18] we proposed that the cyclic center for the regulation of preovulatory gonadotropin surges resided in the preoptic brain. We further suggested that either androgen secretion from the neonatal male testes or androgen treatment of neonatal female rats deleteriously affected the preoptic-suprachiasmatic areas such that the cyclic surge center (MPN-SCN) was permanently damaged.

The present data on changes in noradrenergic neuronal activity in proestrous, in E_2-treated OVX rats and in neonatally androgenized rats have prompted us to reassign the site of initiation of the neural "trigger" to the mid- and/or hind-brain noradrenergic system. We further suggest that the major role of the PSTS is to synthesize, transport and store LHRH thus making it available for release from ME axon terminals when the noradrenergic neuronal activity increases

expression depends upon adequate concentrations and duration of exposure to endogenous estrogen? The PSTS remains competent to synthesize, transport and store LHRH in ME axon terminals and this hormone is readily releasable upon depolarization of the MPN in barbiturate-blocked proestrous rats [16] or even in ASR [17]. Further, pituitary responsiveness to LHRH is not altered in these animal preparations. When function of the noradrenergic system is temporarily (PB-treated) or permanently suppressed (ASR), LH surges do not occur. From these observations, we conclude that NE may serve as the neural trigger which releases ME-LHRH. If this conclusion proves to be correct, may we suggest that the center for cyclic gonadotropin surges be reassigned from the preoptic area to the site of location of the NE perikarya, namely, the mid- and hind-brain noradrenergic system (Fig. 2).

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INTERACTIONS OF SPINO-MEDULLAL ASCENDING NEURAL SYSTEM AND FOREBRAIN-HYPOTHALAMIC STRUCTURES IN REGULATING GONADOTROPIN RELEASE IN FEMALE RATS

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The role of noradrenergic neurons originating in the lower brain stem with preoptic projection in controlling the preovulatory release of gonadotropins was investigated in female rats.

Electrolytic lesions or diethyldithiocarbamate implantations in the ventrolateral part of the medulla oblongata (VLMO), but not in the dorsomedial part of the medulla oblongata, at 1100-1330 h on proestrus resulted in a blockade of the preovulatory release of LH, FSH and PRL as well as ovulation. Norepinephrine (NE) contents in the preoptic-anterior hypothalamic area at 1700-1800 h on proestrus were significantly reduced by VLMO lesions. Complete spinal transections at T3-T7, but not at T8-L5, of the spinal cord segments also blocked the preovulatory gonadotropin release and ovulation with significant reduction of NE contents in the preoptic-anterior hypothalamic area. Electrochemical stimulation of the suprachiasmatic part of the preoptic area (POSC) or NE injections into the third ventricle at 1400-1500 h on proestrus restored ovulation in animals with either VLMO lesions or complete spinal transections at T4-T5.

Some neurons in the VLMO were antidromically activated by electrical stimulations of the POSC or the bed nucleus of stria terminalis (BST) in ovariectomized estradiol-primed rats. Generation of action potential of some of these neurons was facilitated or suppressed by estradiol administered ionophoretically. In proestrus rats, the number of neurons in the POSC and BST which responded to VLMO stimulations decreased significantly after the injection of phenoxybenzamine. The injection of pimozide and methysergide did not affect unit responses.

These results suggest that noradrenergic neurons originating in the VLMO have stimulatory role in the induction of the preovulatory release of gonadotropins and that activities of these neurons are modulated by estradiol and spinal ascending neural input.

INTRODUCTION

Several researchers have demonstrated that the brain noradrenergic system plays an important role in the induction of the preovulatory release of gonadotropins [18,19]. Acute depression of hypothalamic norepinephrine (NE) by drugs which block the biosynthesis of NE [5,6] or treatments with α-adrenergic receptor blockers [13] resulted in the blockade of the gonadotropin release and ovulation. Histochemical fluorescence studies have shown that noradrenergic neurons are located in the lower brain stem [4,17] and surgical or pharmacological destructions of ascending noradrenergic fibers in the midbrain [8,10,12] or in the lateral hypothalamus [7] have been also shown to block the preovulatory gonadotropin release and ovulation with significant reduction of hypothalamic NE contents. The present study was designed to explore the candidates for the NE cell group which innervate the limbic-preoptic hypothalamic structures and are involved in control of the release of gonadotropins in the medulla oblongata. The effect of estradiol and spinal ascending neural input on the noradrenergic system was also investigated.

METHODS AND RESULTS

Expriment 1

The effect of electrolytic lesions and diethyldithiocarbamate (DDC; an inhibitor of dopamine-β-hydroxylase) implantations in the ventrolateral part of the medulla oblongata (VLMO) and the

dorsomedial part of the medulla oblongata (DMMO) on the preovulatory release of gonadotropins and ovulation was investigated in adult female Wistar rats. Operations were performed under ether anesthesia at 1100-1330 h on proestrus. Electrolytic lesions were placed by passing an anodal DC current (3 mA/5 sec) through a platinum electrode. Lesions were found as shown in Fig. 1. VLMO lesions, but not DMMO lesions, significantly blocked the preovulatory release of LH, FSH and PRL (Figs 2, 3) as well as subsequent ovulation (Table I). Similarly, DDC implantations through a stainless steel double cannula in the VLMO, but not in the DMMO, significantly blocked the preovulatory release of gonadotropins and ovulation. NE contents in the preoptic-anterior hypothalamic area at 1700-1800 h on proestrus were significantly reduced (Table II). In the midposterior hypothalamus, NE contents were not altered, though dopamine

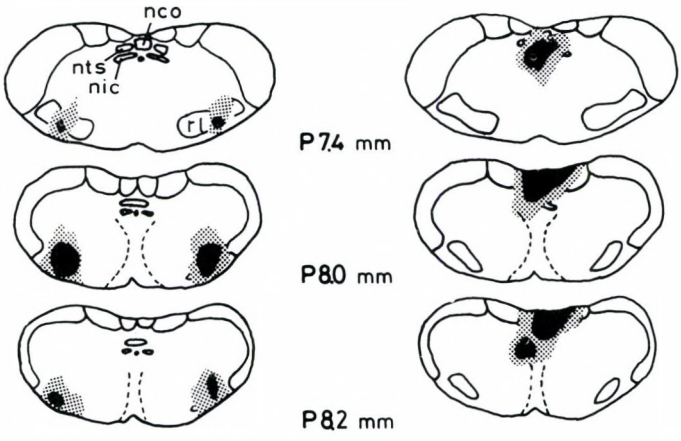

Fig. 1. Schematic illustrations of bilateral electrolytic lesions in the ventrolateral part of the medulla oblongata (VLMO; left panel) and the dorsomedial part of the medulla oblongata (DMMO; right panel). Solid and stippled areas indicate the common and total area, respectively, of lesions. nco, nucleus commissuralis; nts, nucleus tractus solitarii; nic, nucleus intercalatus; rl, nucleus reticularis lateralis

(DA) contents were significantly increased. Electrochemical stimulations (an anodal DC current, 100 μA/60 sec) of the suprachiasmatic part of the preoptic area (POSC) and NE (norepinephrine bitartrate, 40 μg/2 μl saline) injections into the third ventricle under ether anesthesia at 1400-1500 h on proestrus restored ovulation in all 7 animals and in 5 of 7 animals, respectively, with VLMO lesions.

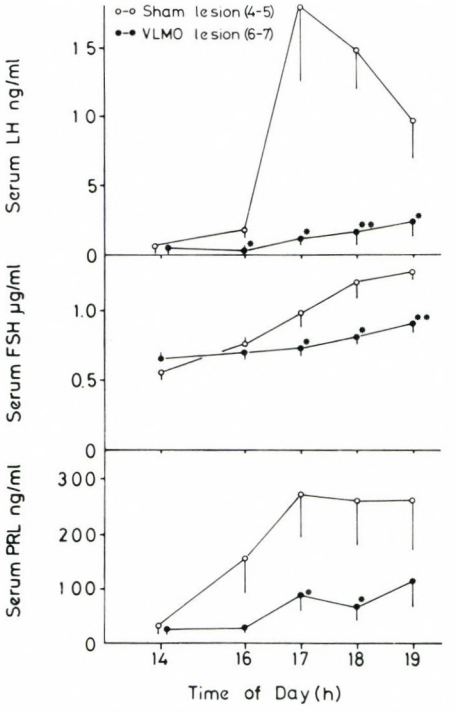

*Fig. 2. Effect of electrolytic lesions in the VLMO on the preovulatory release of LH, FSH and PRL. Each point and vertical line represents the mean and SE, respectively. The number of animals is given in parentheses. *p<0.05, **p<0.01 vs Sham lesion*

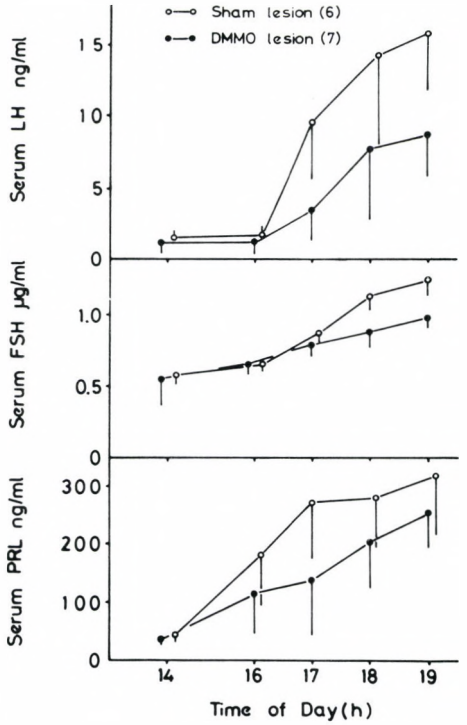

Fig. 3. Effect of electrolytic lesions in the DMMO on the preovulatory release of LH, FSH and PRL

Table I. Effect of electrolytic lesions in the VLMO or DMMO on ovulation in female rats

Operations	Number of animals	Incidence of ovulation	Number of ova in ovulating animals
A) Sham lesion	5	5/5	10.4 ± 1.1[a]
VLMO lesion	7	0/7*	-
B) Sham lesion	6	5/6	11.0 ± 0.5
DMMO lesion	7	6/7	9.3 ± 0.6

a: Mean ± SE.

* $p<0.005$ vs Sham lesion.

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Table II. Effect of electrolytic lesions in the VLMO on hypothalamic catecholamine concentrations (ng/g wet wt.)

Operations	Preoptic-anterior hypothalamic area		Midposterior hypothalamus	
	NE	DA	NE	DA
VLMO lesion (n=7)	1557.71 ± 78.28*[a]	350.29 ± 27.27	1792.43 ± 102.86	685.14 ± 31.97*
Sham lesion (n=7)	1927.14 ± 84.18	411.57 ± 53.47	1883.57 ± 127.77	542.57 ± 28.45

a: Mean ± SE.

* $p<0.01$ vs Sham lesion.

Expriment 2

Unit activity of neurons in the VLMO was recorded in ovariectomized estradiol-primed rats under urethane anesthesia. The units were antidromically driven by electrical stimulation (negative square wave pulses; intensity, 1.0-1.5 mA; duration, 0.1 msec; frequency, 0.6 Hz) of the POSC and the bed nucleus of

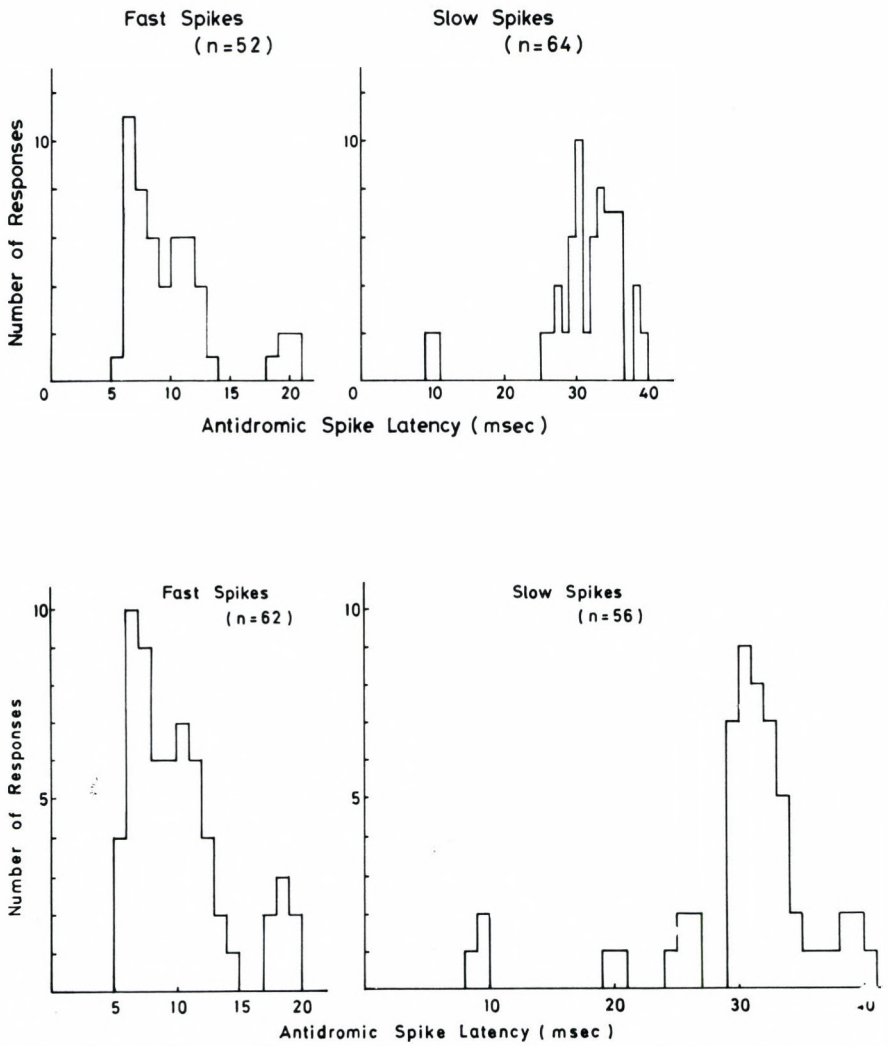

Fig. 4. Frequency distribution of antidromic spike latencies in the VLMO cells induced by POSC (upper panel) and BST (lower panel) stimulation in ovariectomized, estradiol-primed rats

stria terminalis (BST). Both in the POSC and BST, two types of antidromic spike potentials were distinguished on the basis of their spike configurations and antidromic spike latencies. Mean antidromic spike latency for one type (fast spikes) was 9.8 msec while the value for the other type (slow spikes) was 30.2 msec (Fig. 4). Fast spikes were characterized by sharp and smooth rising phase. Slow spikes had a noch in the rising phase. Ionophoretic injection of estradiol hemisuccinate was accomplished on 37 of antidromically identified cells, of which 21 showed slow responses and 16 responded with fast spikes. In cells with slow spikes, estradiol facilitated (n=9) or suppressed (n=3) their generation of action potentials. None of cells with fast responses changed their activity in response to estradiol.

Unit responses in the POSC and BST to the VLMO stimulation in proestrous and diestrous rats were examined by means of poststimulus time histograms and percentages of neurons responding in facilitatory or inhibitory manner were calculated. In proestrous rats, stimulation of the VLMO induced facilitatory and inhibitory responses in 38% and 16%, respectively, of POSC neurons. In diestrous rats, facilitatory responses were seen only in 8% and inhibitory responses were in 7%. In the BST, 30% of neurons showed facilitatory responses on proestrus, while only 8% neurons on diestrus. Inhibitory BST unit responses were seen in about 10% of neurons in either proestrous or diestrous rats. Unit responses in the POSC and BST to the VLMO stimulation were significantly different between proestrus and diestrus (Fig. 5).

Effects of receptor blockers of NE, DA and serotonine (5-HT) on the single unit responses of the POSC and BST neurons to the VLMO stimulations in proestrous rats were examined in

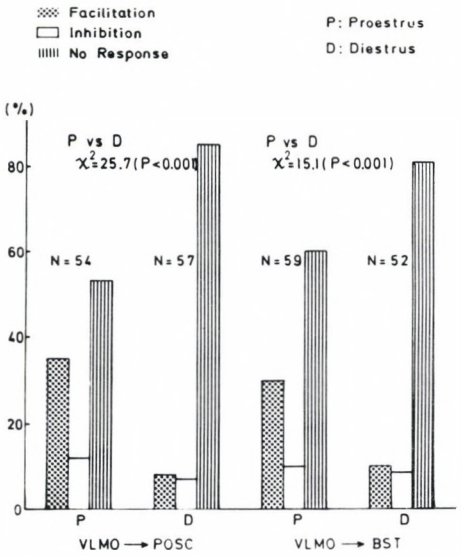

Fig. 5. Percentages of POSC and BST neurons responding to the VLMO stimulation in a facilitatory or inhibitory manner in proestrous (P) and diestrous (D) rats

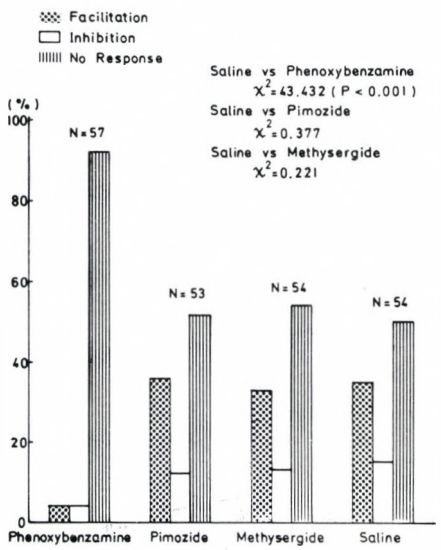

Fig. 6. Effect of adrenergic, d paminergic and serotonergic receptor blockers on response of POSC neurons to the VLMO stimulation in proestrous rats

order to know which of NE, DA or 5-HT are involved in the neural transmission from the VLMO to the POSC and BST. Receptor blockers used in this study were phenoxybenzamine hydrochloride (an α-adrenergic receptor blocker), pimozide (a dopaminergic receptor blocker) and methysergide hydrogenmaleinate (a serotonergic receptor blocker). Each blocker was injected at 1100 h on the day of experiment and saline was injected as controls. In the POSC, both facilitatory and inhibitory responses were seen only in about 5% of neurons after the injection of phenoxybenzamine. These rates were significantly different from those in saline injected rats. Percentages of facilitatory and inhibitory responses were not changed after the injection of pimozide or methysergide (Fig. 6). In the BST, about 3% of neurons were facilitated and 2% were inhibited after the injection of phenoxy-

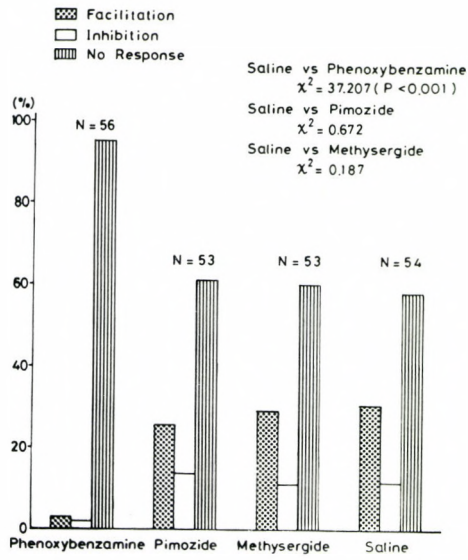

Fig. 7. Effect of adrenergic, doperminergic and serotonergic receptor blockers on response of BST neurons to the VLMO stimulation in proestrous rats

benzamine. No significant change was seen in the rates of facilitatory and inhibitory responses after the injection of pimozide of methysergide (Fig. 7).

Expriment 3

The effect of spinal transections on the preovulatory gonadotropin release and ovulation were examined in this experiments. The spinal cord was completely transected at various levels between T3-L5 of the spinal cord segments under ether anesthesia at 1000-1230 h on proestrus. Most animals showed withdrawal reflex by pinching a rear foot or tail at 1400 h, and only the animals showing this reflex were used in following experiments. As shown in Fig. 8, when the levels of transections were tentatively divided into T3-T7 and T8-L5, ovulation occurred in all animals with complete spinal transections (CST) at T8-L5. CST at T3-T7, however, significantly blocked ovulation. Serum concentrations of LH, FSH and PRL in animals with CST at T3-T7 were significantly lower than those in animals with CST at T8-L5. CST at T4-T7 significantly reduced NE, but not DA, contents in the preoptic-anterior hypothalamic area at 1700-1800 h on

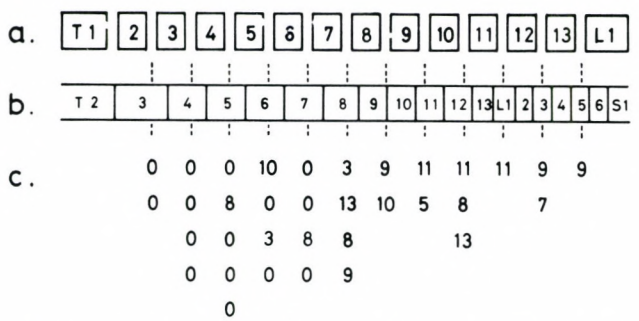

Fig. 8. Effect of complete spinal transections (CST) on spontaneous ovulation. a, the bodies of the vertebrae; b, the segments of the spinal cord. Dotted vertical lines indicate the levels of transections; c the number of ova in each animal with CST at corresponding levels

proestrus (Table III). In animals with CTS at L1-L5, neither NE nor DA contents were altered. Electrochemical stimulations of the POSC and NE injections into the third ventricle at 1400-1500 h on proestrus restored ovulation in animals with CST at T4-T5 (incidence of ovulation: 5/5 and 4/5, respectively). Estrous cyclicity following CST is shown in Fig. 9. Animals with CST at T4-T7 showed prolonged periods of diestrus (8-20 days). Thereafter, they resumed cycles, but not so regular as shown in animals with CST at L1-L5.

In ovariectomized rats, CST was performed at T4-T7 and L1-L5, and on the same day, estradiol benzoate (20 μg) was subcutaneously injected. Three days later, animals received an sc injection of 2 mg progesterone at 1200 h. As shown in Fig. 10, CST at T4-T7 significantly reduced the release of LH and FSH, but not of PRL.

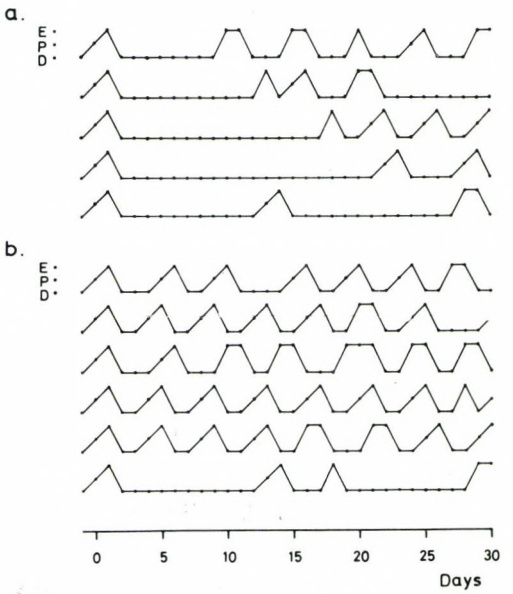

Fig. 9. Estrous cyclicity following CST at T4-T7 (a) and L1-L5 (b). Spinal transections were performed on the day of proestrus (day 0). E, P and D indicate the vaginal estrus, proestrus and diestrus, respectively

Table III. The effect of complete transections of the spinal cord on catecholamine concentrations in the preoptic-anterior hypothalamic area

Operations	No. of animals	NE[a]	DA[a]
Intact control	8	1907.13 ± 65.85[b]	389.99 ± 48.73
Transection at T4-T7	5	1364.40 ± 148.56[c]	466.60 ± 101.82
Transection at L -L5	5	1785.20 ± 87.37	300.80 ± 41.60

[a] ng/g wet tissue weight.

[b] Mean ± SE.

[c] $p<0.01$ vs. Intact control and $p<0.05$ vs. Transection at L1-L5.

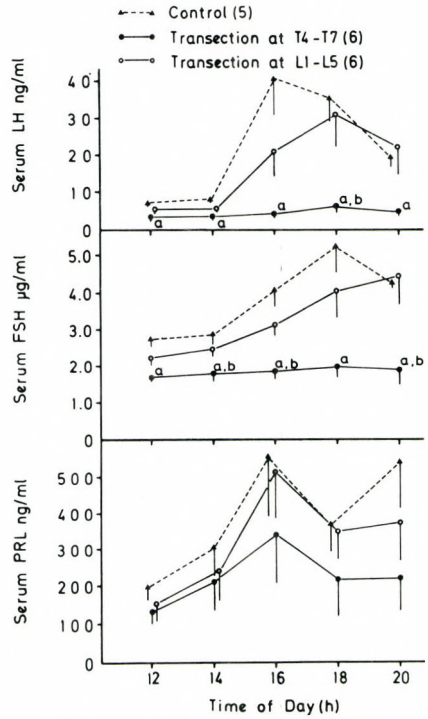

Fig 10. Effect of CST on the progesterone-induced release of LH. FSH and PRL in ovariectomized estradiol-primed rats. a, p<0.05 vs Control; b, p<0.05 vs Transection at L1-L5

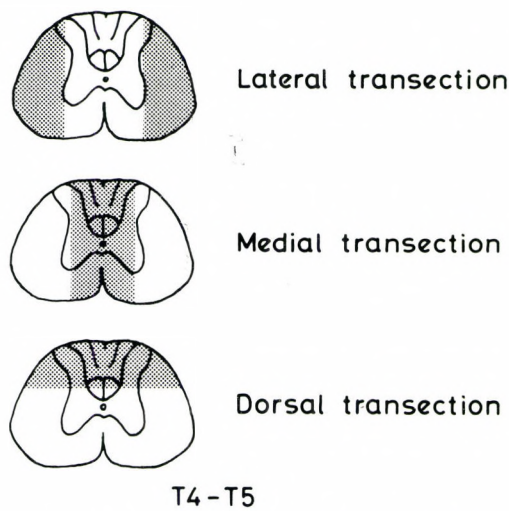

Fig. 11. Schematic illustration of selective transections of lateral medial and dorsal columns of the spinal cord at T4-T5. Stippled areas indicate the area of transection

Lateral, dorsal and medial columns of the spinal cord at T4-T5 were transected under ether anesthesia at 1000-1230 h in proestrous rats. The types of transections are schematically illustrated in Fig. 11. Transections of the lateral, but not dorsal and medial, columns of the spinal cord significantly reduced the preovulatory release of LH, FSH and PRL and blocked ovulation (Table IV). When the unilateral lesion of the VLMO and the unilateral transections of the lateral columns of the spinal cord were performed contralaterally, ovulation was blocked (2/8), but when they were performed ipsilaterally, ovulation occurred (5/6, $p<0.05$).

Table IV. The effect of selective transections of the spinal cord at T4-T5 on ovulation

Operations	No. of animals	Incidence of ovulation	No. of ova in ovulating animals
Lateral transection	10	1/10	7
Dorsal transection	9	7/9[b]	11.3 ± 0.6[a]
Medial transection	7	6/7[b]	10.8 ± 0.9

[a] Mean ± SE.

[b] $p<0.01$ vs. Lateral transection.

DISCUSSION

The present study showed that bilateral electrolytic lesions made in the VLMO blocked the preovulatory release of

LH, FSH and PRL as well as subsequent ovulation, while lesions in the DMMO failed to affect these processes. VLMO lesions were found in or closely adjacent to the lateral reticular nucleus where the A1 cell group is located [14]. Since VLMO lesions significantly reduced NE contents in the preoptic-anterior hypothalamic area, it was assumed that the blockade of the preovulatory release of gonadotropins was due to the disruption of normal functions of the A1 cell group. This was supported by subsequent findings that DDC implantations into the VLMO similarly resulted in the blockade of the preovulatory gonadotropin release and ovulation and that intraventricular NE injections restored ovulation in animals with VLMO lesions. Therefore, it was suggested that A1 cell group supply NE as one of the important factors in inducing the gonadotropin release, to the forebrain-hypothalamic areas.

DMMO lesions were found in or closely adjacent to the areas where the A2 cell group is located. Though it is not clear from the present data that the A2 cell group is linked or not to the forebrain-hypothalamic structures which are involved in the induction of the preovulatory gonadotropin release, it was shown that surges of gonadotropins and ovulation could occur without the ascending noradrenergic input form the A2 cell group. It has been shown that, in the midbrain, the ventral noradrenergic tract participates in the induction of the gonadotropin release [8,10,12], and that noradrenergic fibers running in the ventral noradrenergic tract to the hypothalamus originate mainly in the A1 cell group. Palkovits et al. [15] have reported that hypothalamic NE contents were significantly reduced by lesions of the A1 cell group, but not of the A2 cell group. These findings

support the physiological significance of the A1 cell group in the induction of the preovulatory gonadotropin release.

In the midposterior hypothalamus, VLMO lesions significantly increased DA contents. The mechanisms involved in the increase in DA contents is unclear, but it is possible that the increase in the midposterior hypothalamic DA contributed to the supression of the gonadotropin release. The inhibitory effect of DA on the gonadotropin release has been demonstrated [3,16].

From the electro-physiological studies, it is evident that some neurons in the VLMO send their axons directly to the POSC and BST and that some of them are sensitive to estradiol. Unit responses in the POSC and BST elicited by stimulations of the VLMO significantly decreased after the injeciton of phenoxybenzamine, but not of pimozide or methysergide, suggesting that NE is involved in the neural transmission form the VLMO to the POSC and BST. These unit responses were much more enhanced in proestrous rats than in diestrus rats. Thus, the noradrenergic projections from the VLMO appears to have some role in maintaining neural activity of the POSC and BST, and this system might be affected by ovarian steroids. The simultaneous localization of 3H-estradiol and dopamine-β-hydroxylase in neurons of the A1 cell group with a combined technique of thaw-mount autoradiography and immunohistochemistry [11] supports the direct action of estradiol on the A1 cell group.

When the spinal cord segments were tentatively divided into T3-T7 and T8-L5, CST at T3-T7 significantly blocked the preovulatory release of HL, FSH and PRL and subsequent ovulation. Although the mechanism by which spinal transections effect the gonadotropin release is not fully elucidated in the

present study, the reduction of NE contents in the preoptic-anterior hypothalamic area caused by CST at high thoracic levels might contribute to the blockade of the gonadotropin release. In support this, NE injections into the third ventricle restored ovulation in animals with CST at T4-T5. It therefore perimental conditions, the VLMO plays a stimulatory role in the induction of the preovulatory release of gonadotropins and ovulation and that the process is mediated by the ascending noradrenergic system which originates in the VLMO and projects to the limbic-preoptic hypothalamic structures. It was also suggested that the activity of this noradrenergic system is modulated by estradiol and the spinal ascending neural input and fluctuates during the estrus cycle.

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ROLE OF HYPOTHALAMIC CIRCUITRIES IN NEUROENDOCRINE REGULATIONS: HYPOTHALAMUS - ENDOCRINE PANCREAS INTERACTIONS, A NEW CONCEPT+

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Though there is no lack in proofs of hypothalamic involvement in regulating endocrine pancreas and related aspects of metabolism, the mechanisms thereof are still pending elucidation. There is no adequate tropic hormone in the anterior pituitary hence the conventional hypothalamic-hypophyseal route may only function as circuitous. This lends special appeal to a probable hypothalamic participation over purely neural route, one implicated in insulin secretion, for example via the vagus. This has long been considered possible but has not been explored. Summed up are results of many years of experimental research by the author and his group in hypothalamic-vagal neural connections. The following techniques were employed: karyometric and relevant histochemical studies of rat medullary vagal nuclei and hypothalamic nuclei under insulin deficiency; karyometric studies of transneuronal changes in dorsal vagal nuclei, as well as silver impregnation studies of preterminal axons and their terminal branches (Fink-Heimer technique) for degeneration together with ultrastructural studies of presynaptic profile degeneration in dorsal vagal nuclei of rats bearing electrolytic lesions placed in the hypothalamic areas, in wnich descending axons are presumed to originate. The results obtained indicate there exists a direct hypothalamic-vagal descending pathway, which originates in paraventricular nuclei and synapses on neurons of the dorsal vagal nuclei after partial crossing over in the lower brain stem. Possible mechanisms at hypothalamic and medullary levels in neuroendocrine regulations of pancreatic islets are discussed.

The two recent decades are marked with successful studies in the hypothalamic mechanisms regulating the hypophyseal tropic functions and their peripheral endocrine targets. However things were not quite as smooth in research of the pancreatic endocrine function. Though there is no lack in clinical or experimental proof of hypothalamic involvement in regulating this function

and related aspects of metabolism the mechanism thereof have yet to be fully elucidated. The difficulties were aggravated by the incapacity of the anterior pituitary to produce an adequate tropic hormone, which made the conventional hypothalamic-hypophyseal route possible only as a circuitous.

This has lent special appeal to a probable hypothalamic participation over a purely neural route, the peripheral constituent of which could have well been either the pancreatic autonomic nerves, the vagus or the splanchnic nerve. Considered here is a possible vagal contribution to such hypothalamic involvement in regulating endocrine pancreas.

Neuroendocrinologists have for some time now been centering their attention on the vagus. Stimulated subdiaghragmatically it causes a rise in radioimmune blood insulin [10,19,22,34,35, 80], an effect which also ensues on perfusion of acetylcholine or of other drugs, acting on cholinergic muscarine receptors in a similar way, while administration of an antagonist of these compounds, atropin, produced a reverse effect [9,33,45]. This facilitatory modulation of insulin release by the vagus was believed to stem from direct stimulation of pancreatic islets [58,63].

It was assumed, quite some time ago, that the hypothalamus can modulate pancreatic islet secretion as well as the metabolism via the autonomic nervous system [11,84], and then corroborated in experiments with ventromedial (VM) hypothalamic lesions which enhanced the pancreatic responsiveness to insulin secretory stimulation [43,47,60,72,84] and were followed by hyperinsulinemia, hyperphagia and obesity [60,84]. Moreover, this syndrome could be prevented by vagotomy [12,13,21,30,31,

37,62] or by removal of the pancreas from the usual neural influence [13,32].

Though idea grew more insistent, no morphological evidence was extant to prove a direct neural connection between hypothalamic neurons and medullary vagal nuclei, specifically the dorsal vagal nucleus (DVN) and the nucleus ambiguus (NA). Nor were there any data indicating that medullary vagal nuclei neurons were responsive to experimentally induced changes in pancreatic islet hormone release. We have devoted several years of research to elucidate whether such links and such responsiveness existed.

As we studied the responsiveness of the medullary vagal nuclei to insulin deficiency in alloxan-diabetic male rats we also explored, under identical experimental conditions, the neurons of the entire hypothalamus [2,3]. Neuron responsiveness was examined with the aid of the karyometric technique which affords quantitative results.

Only three hypothalamic nuclei, the supraoptic (SO), the VM and the arcuate (ARC), responded to insulin deficiency with significant changes. The SO response was however treated with caution, as its vasopressin-secreting cells are responsive to any fluctuations in osmotic equilibrium, which in diabetes may be considerable. Hence the changes identified in the SO may have well been secondary. For technical reasons we have only been able to study the DVN response in the medullary nuclei of the vagus. The karyometric technique envisages measurement of at least 200 neurons in each area whereas the NA is much too small a group of cells to meet this requirement. The DVN neurons responded with highly authentic changes.

The specificity of their response was determined histochemically [4]: studied were the distribution and the activity of acetylcholinesterase (AChE) in the DVN neurons of intact and alloxan-diabetic male rats. The enzyme activity of the neurons judged by the strength of their staining reactions to AChE. To obtain a quantitative assessment of this latter in the controls and the experimental animals, cells were counted on alum carmine counterstained sections, prepared from three standard hind brain levels. Cells with negligible to slight staining reactions were included in group 1, with moderate staining formed group 2, strongly positive stained cells made up group 3. The total number of cells in one animal was taken as 100%, the number of cells in each group was regarded as a percentage of the total number. The results were as follows. The number of moderate reaction nerve cells in alloxan-diabetic rats went down by 7% whereas the high AChE activity neurons increased by 6%. Thus in insulin deficiency, hence in hyperglycemia, the AChE became more active in the DVN neurons, an effect which could indirectly be indicative of an intensification of their physiologic activity. The AChE activity of the NA was high, but its distribution markedly, different from that in the DVN, is mostly along the periphery of motoneurons, and not evenly over the entire cytoplasm as in the DVN. As a result the NA neurons blended with the intensely stained neuropil and could not be differentiated into discrete entities for counting.

In our study of hypothalamic-vagal descending connections we proceeded from the premise that hypothalamic neurons which proved responsive to insulin deficiency could be the source. The VM seemed the most probable objective, as subdiaphragmatic

vagotomy removed the experimentally induced characteristic syndrome. Accordingly, we placed bilateral electrolytic lesions of the hypothalamic VM in rats and took karyometric transneuronal readings of changes in the DVN. In parallell, silver impregnation (Fink-Heimer technique) studies, using Szentágothai's coordinates we placed bilateral electrolytic lesions in the VM and examined the preterminal axons and their terminal branches in the DVN 1 to 5 days after placement [5]. Contrary to expectation the experiments yielded no results and we found neither degenerating axons nor transneuronal changes in the DVN. As ineffective proved lesions in the lateral hypothalamic area (LHA) which like the VM is involved in controlling feeding behaviour (hunger or feeding center) and which when stimulated displays a hyperresponsiveness of pancreatic insulin secretion to such chemical stimuli as glucose [38,56,73]. The LHA neurons recently identified as glucose-sensitive [55] and the VM neurons long identified as glucoreceptive [44], are traditionally regarded as generating parasympathetic and sympathetic responses respectively with regard to the control of insulin release and blood glucose regulation [for a review see 84].

In a series of experiments, wherein the lesion involved the paraventricular hypothalamus in the sagittal plane of the dorsomedial nucleus site, somewhat higher and posterior to it and anterior to the premamillary nucleus (hereafter referred to as AHDM) we have encountered karyometrically transneuronal changes which we expected in previous studies, while Fink-Heimer technique revealed degenerate axons and their preterminal branches (Fig. 1b). Degenerative axons were also observed in the nucleus of the solitary tract (NST), in the NA and in the reticular for-

mation. It followed, that descending hypothalamic axons, part of which, according to karyometry, terminated in the DVN, could safely be assumed to exist. Further, ultrastructural studies confirmed our assumption [6]; rats with AHDM lesions displayed terminal degeneration in the DVN 3 - 5 days after the lesions were placed, the degeneration was especially pronounced on the 4th day (Fig. 1a). It was seen as specific changes in the synaptic vesicles, the mitochondria and the cytoplasmic matrix of the presynaptic nerve terminals, with glial-cell phagocytosis occurring in the advanced stages. The degenerating presynaptic profiles were often observed in small clusters, separated by more or less considerable areas of intact neuropil. Clearly, descending hypothalamic axons synapsing on the DVN neurons did exist, but their origin remained unknown.

The following considerations pointed to the hypothalamic paraventricular nuclei (PV) as their possible origin. The hypothalamic-vagal route is a somewhat long projection character-

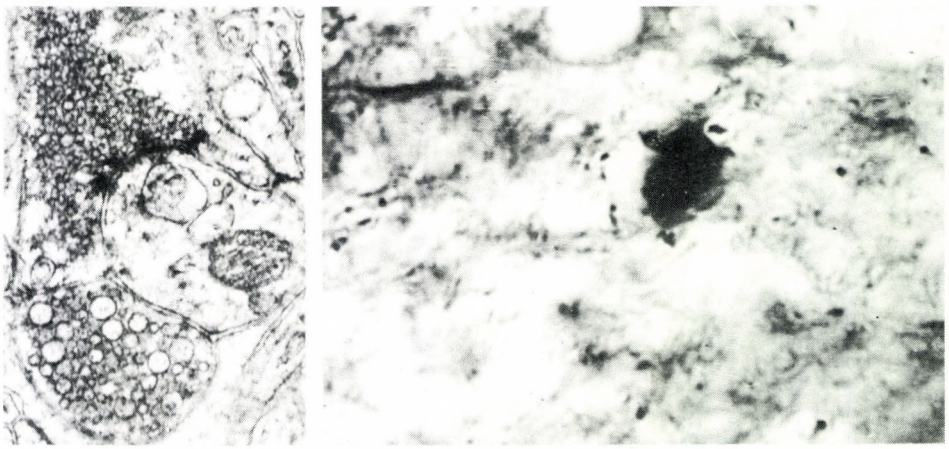

Fig. 1. Presynaptic profile degeneration (a) and Fink-Heimer degeneration of preterminal axons (b) within the dorsal vagal nuclei of the 4-day surviving rats with AHDM lesions; x 10.000 (a); x 2961 (b)

istic of large neuron axons, and the most proximate to the AHDM large-cell nucleus of the hypothalamus is the PV. It is on the same sagittal plane, on the same vertical level and, being its extension, is on the same line as the rostrocaudal AHDM stretch. Results of the recent neuroanatomical studies on axon projections using current tracing techniques were also in favour of such a possibility [15,18,28,50,54,65,68,71,74]. Traced in these studies were the hypothalamus (the PV included) and the extrahypothalamus neuron projections up to the vegetative centers of the lower brain stem (including the medullary dorsal vagal complex) and the spinal cord. Proceeding from this we have carried out an ultrastructural study of the DVN after placing uni- and bilateral lesions in the hypothalamic PV (Fig. 2). The medulla on the side of lesion and on the opposite side were studied after unilateral lesions were placed [7].

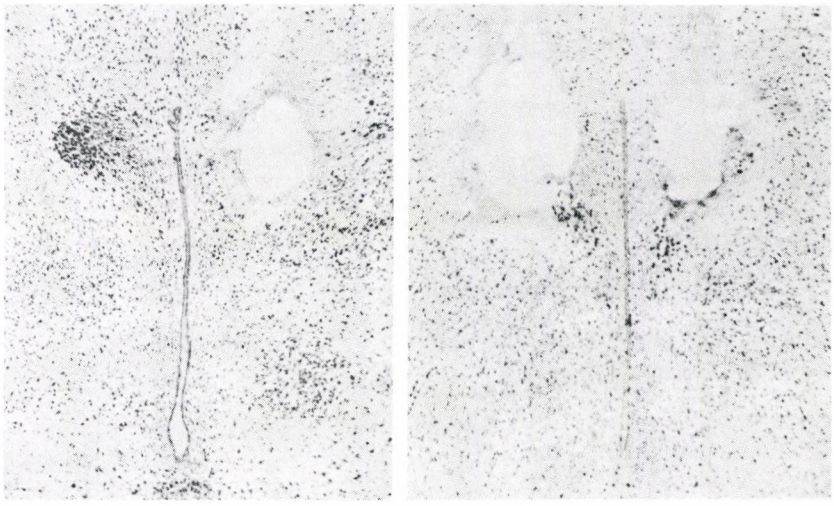

Fig.2. Electrolytic lesions (a, unilateral; b, bilateral) of the rat hypothalamic paraventricular nuclei; asterisks indicate lesion sites; x 56

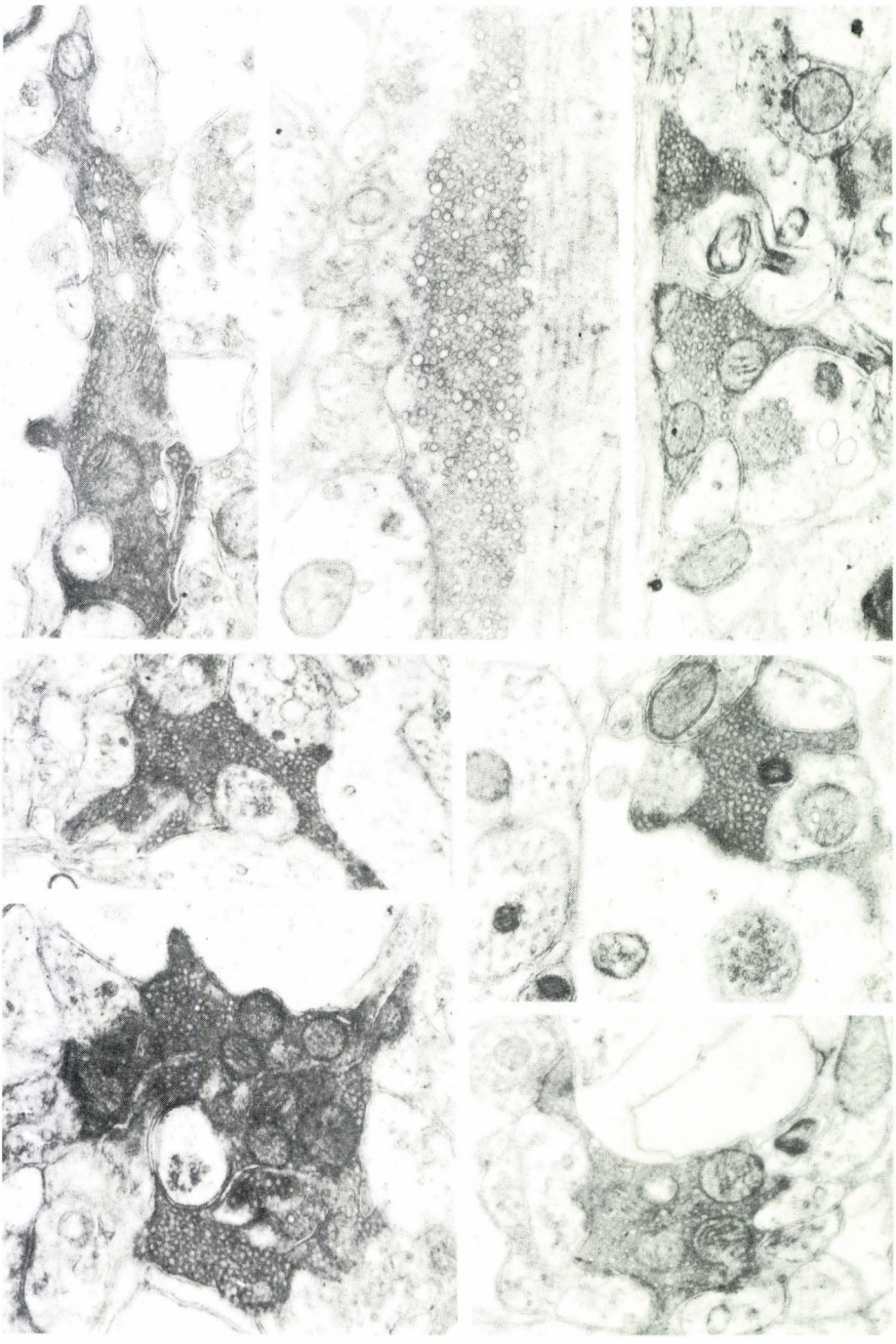

Fig. 3. Degenerating axon terminals within the dorsal vagal nuclei of the 3-, 4- and 5-day surviving rats with the hypothalamic paraventricular lesions; x 8.500

We observed in the DVN a terminal degeneration not unlike that found after AHDM lesions (Fig. 3). Following bilateral PV lesions these changes were pronounced. After the lesion was placed unilaterally the degenerating profiles were observed on the side of the lesion and on the opposite side. In latter case, however, the number of degenerating profiles was considerably smaller than in animals with bilateral lesions. We inferred that the descending hypothalamic axons we have described as synapsing on the DVN neurons, arise in the PV and partly intersect at some lower brain stem level with a similar system from the side opposite.

Thus, our results could be seen as a structural substantivation of the possibility of hypothalamic involvement in regulating endocrine pancreas over a purely neural route. But the unexpected identification of the PV as the source of a descending hypothalamic-vagal pathway, the flow of new knowledge on the PV cell composition and its vegetative neural connections as well as the recent information on insulin receptors in brain cells, all this imparted a new dimension to the problem.

The notion of a hypothalamic-vagal route in regulating endocrine pancreas and related aspects of metabolism, which may grow out of the above, presumes at least two interconnected levels of this regulation - the medullary and the hypothalamic, with the DVN being the main center at the medullary level. Its neurons, as mentioned earlier, are responsive to excess of blood glucose in induced insulin deficiency [3] when they are capable of intensifying their physiological activity [4]. It was recently demonstrated that the DVN neurons like glucoreceptor neurons of the VM are especially susceptible to the toxic effect

of goldthioglucose [61], which is considered further evidence of their responsiveness to blood glucose. It may therefore be assumed that blood-borne glucose stimulates the DVN and the VM glucoreceptor neuron activity on excess concentration in the blood induced by insulin deficiency. Cholinergic DVN neurons, which extend preganglionary fibres to autonomic neurons in the pancreas and also directly innervate this gland [61], are capable, in enhancing their activity, to facilitate insulin release. This will cause a reduction of the blood glucose, thus restoring the upset equilibrium in this functional system. A glucose feed-back control of this type is also possible when glucose does not act directly on the DVN motoneurons, but exercises its stimulative effect through the medial NST neurons, which too respond to the toxic effect of goldthioglucose [61].

The NST neurons may participate in regulating insulin secretion also as an important relay for vagal gustatory afferents synapsing on the neurons of the antero-lateral NST subdivision [8,36,64,79], and for parasympathetic visceral inputs, sent by the nodose ganglion neurons into its medio-caudal cell division [64]. Thus, at the medullary level insulin release may be enhanced via the simple vago-vagal reflexes [53]. Possibly, together with the DVN, the NA neurons also take part in the control of endocrine pancreas at the medullary level: the rostral half of the NA, having recently been shown to be the source of vagal efferent fibres, capable of facilitating insulin secretion [9].

However, glucose is not the only factor that can trigger cholinergic DVN neurons. The spectacular results obtained by

van Houten and Posner [29], indicating dendrite-associated insulin receptors in the area postrema (AP) neurons, located along the lateral border of the AP with the medial NST, suggest that blood-borne insulin, which readily penetrates the AP blood-brain barrier, to form with the insulin receptors a complex which is rapidly internalized within Golgi-related vacuolar elements of the AP neuronal dendrites and cell bodies. Significantly, the DVN areas, which Powley and Laughton [61] described as projecting to the pancreas appear to be precisely those where van Houten and Posner [29] have found evidence for AP neuron insulin receptors, suggesting a direct feed-back loop to the neurons which infuence pancreatic insulin secretion [see discussion in ref. 61].

As to the hypothalamic circuitries and their part in terms of the neural route of endocrine pancreas control under consideration especially prominent are the recently identified projections of the PV towards the DVN and the NST [15,50,65,68,70, 71,74,76,78], which our studies have proved to be monosynaptically connected with the DVN neurons [7]. The end of the seventies and the early of eighties witnessed a surge of neuroanatomical information, which also concerned the hypothalamic PV, long regarded a population of magnocellular neurosecretory cells transporting along their axons oxytocin and, to a lesser extent, vasopressin, to the neurohaemal areas of the posterior pituitary. In the PV some ten subnuclei had been distinguished, which were disparate in cell composition and in efferent connections [1, 28,40,54,76,77,78,83]. Recently has been obtained evidence which shed light upon the afferent connections of each of its cellular subdivisions [48]. The vast afferent connections of the PV [for

review see 86] testify to the importance of its role in peripheral and intracerebral homeostatic functions [75,77,87] making it a center capable of integrating much more afferent information than that at the medullary level. A special role, in this respect, belongs to its afferents, projecting from the NST and the pontine parabrachial nucleus, the relay nuclei important for gustatory and parasympathetic visceral input [26,27,39,48, 52,53,65,66,67]. Significantly, the amygdaloid nuclei (notably, the central nucleus, CE) essential in feeding behaviour, also receive gustatory afferents from the NST and parabrachial nucleus, and like PV the CE extends to them its projections [51,65] as well as establishes a monosynaptic connection with the DVN [27]. Similar reciprocal connections are operating between the PV and the CE [15,16,69,82]. However the functions of the PV in feeding behaviour are practically unexplored, as only few indications point to its involvement [23,41]. The direct nervous links between the PV and the gustatory and visceral afferents, as well as the DVN, may suggest a modulatory role of the PV in the gustatory-evoked cephalic phase reflex release of insulin secretion, but this kind of the PV involvement in regulating endocrine pancreas remains to be defined. Possibly, the signals over which the PV may be involved in regulating endocrine pancreas are not only gustatory inputs but also blood glucose and blood-borne insulin, as in the case of the DVN. Meanwhile the presence of insulin receptors in the axonal terminals of the median eminence zona externa is an indication in favour of PV involvement through an antidromous insulin stimulation of the PV neuron groups which, as recent studies show, innervate this zone [28,54,76,78,81,83,87].

Van Houten and Posner [29] associate the insulin receptors of the zona externa axonal terminals with the arcuate nucleus (ARC) neurons allowing for possible antidromous propagation of insulin "signals" via collateral axon projections to various brain areas, specifically over the amygdalopetal collaterals to the olfactory-related amygdaloid units. This assumption rests on electrophysiological evidence to the effect that insulin administered systemically evokes rapid electrical changes in these amygdaloid neurons [17]. Relevantly, the highest concentrations of insulin in the brain are centered in the olfactory bulb and the hypothalamus - both involved in regulating feeding behavior [24,55]. We have also provoked authentic responses to induced insulin deficiency from the ARC neurons [2]. We feel involvement of this nucleus as well as of the PV in control of endocrine pancreas over a purely neural route is quite tangible and supported by the presence of axonal NST projections to the basal hypothalamus [14,57,65] and those from the ACTH/B-endorphin neurons of the ARC to the caudal medulla [70], as revealed using axonal anterograde-retrograde transport techniques as well as histochemical and lesion-degeneration techniques.

It is not my intention to consider in this paper the vast information that had accumulated on the implication of the hypothalamic VM (satiety center) and LHA (hunger center) in regulating endocrine pancreas and related aspects of metabolism through physiological mechanisms of feeding. Detailed reviews on the subject will serve the purpose should such a need arise [20,59,60,84,85]. Suffice it to say that the VM and the LHA are believed to participate in these mechanisms over the autonomic nervous system (the vagus and the splanchnic nerve) but neural

mediation in this case is seen as effected via polysynaptic neural connections.

Considered too, is a possible regulation of the endocrine pancreas with the help of "releasing factors" secreted into the circulation from the hypothalamus [25,42,46,49], but their chemical nature has not as yet been defined and evidence for their precise localization and physiological effects is open to discussion.

It should be noted in conclusion that regulation of insulin secretion is obviously not limited to the mechanisms we have here discussed. Like some other endocrine pancreas hormones insulin is an essential factor in homeostasis, and obviously in the course of evolution many overlapping mechanisms have been drawn in to implicate in regulating these factors. In these terms the hypothalamic-vagal pathway we have considered is merely an element in the complex assembly of regulating systems which awaits a more profound exploration.

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PITUITARY AND HYPOTHALAMIC HORMONES AS PRECURSOR MOLECULES OF NEUROPEPTIDES

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The behaviorally active meiety of ACTH resides in only a few amino acid residues and is independent of its peripheral endocrine effects. Although pituitary hormones may reach the brain through the circulation and by retrograde transport through the portal vessel system, the recent discovery that ACTH as part of a big precursor molecule, pro-opiomelanocortin, is widely distributed throughout the brain suggests that "brain borne" ACTH may be the main supplier of behaviorally active neuropeptides. The biotransformation of pro-opiomelanocortin in the anterior pituitary differs from that in the intermediate/posterior lobe and the brain. Thus, the biotransformation of hormones at the site of release may determine their ultimate function i.e. as a hormone (pituitary) or as a neuropeptide (brain). Structure activity studies, and studies on the biotransformation of ACTH in brain synaptosomes corroborate the hypothesis that ACTH in the brain is a precursor of the second order of neuropeptides involved in adaptive behavior. A great number of studies in a variety of behavioral paradigms has shown that ACTH neuropeptides are involved in (visual) attention and motivation, vigilance and learning and maintenance behavior. Other pro-opiomelanocortin molecules such as β-endorphin undergo a similar processing. Structure activity studies and biotransformation studies of this hormone have revealed the generation of neuropeptides with neuroleptic-like and psychostimulant properties. The neurohypophyseal hormones vasopressin and oxytocin appear to function as precursor molecules of the second order of potent neuropeptides involved in memory processes. The same may hold for other "brain borne" hormones.

Classically, the main function of the pituitary hormones is the control of endocrine and metabolic processes. Developments during the past two decades, revealed the profound behavioral

effects of pituitary hormones which are dissociated from their peripheral influence and led to the concept that pituitary hormones may serve as precursor molecules of CNS active entities [13]. The discovery of the existence of a wide-spread distribution of several pituitary hormones in the brain formed a neuroanatomic basis for the neuropeptide concept.

Peptides with CNS activities were designated as neurogenic or neurotropic peptides [13] but for the sake of convenience the term "neuropeptide" was chosen [14,20]. The brain borne pituitary hormones such as ACTH, α-MSH, β-endorphin, vasopressin and oxytocin, may not only be active as such because certain fragments of these which have lost their peripheral endocrine activities exert powerful CNS effect. Pro-opiomelanocortin is a precursor protein of the first order of β-LPH, ACTH, β-endorphin, and α-MSH. Also, large precursors for the neurohypophyseal hormones have been found. The biologically active entities are all formed by a common biosynthetic mechanism from the so-called first order precursor. The cascade generation of neuropeptides from β-endorphin, ACTH, and the neurohypophyseal hormones will be the subject of this paper.

Two lines of research have been used to identify the various neuropeptides which affect CNS activity. One line of research is the structure activity study with synthetic peptide sequences of the pituitary hormone under investigation to determine the locus of action in the molecule using several behavioral bioassays. Another complementary line concerns the biotransformation of the various pituitary hormones to determine the possible fragments which might be generated and to assess their activity in the same behavioral bioassays.

ACTH

ACTH and related peptides delay extinction of active avoidance behavior [12]. These and a great number of other experiments [18] have revealed that ACTH and related peptides facilitate motivation, attention and concentration.

Structure activity studies

Structure activity studies performed on extinction of pole-jumping avoidance behavior showed that the behavioral activity of ACTH resided in the NH_2 terminal portion of the molecule. ACTH-(1-10) was as active as the whole molecule while ACTH-(11-24) possessed only slight activity [29]. Shortening ACTH-(1-10) from both ends (Table I) revealed that ACTH-(4-7) is the smallest sequence to have essentially the same effect on avoidance behavior and is as potent as ACTH-(1-24) [29].

Although ACTH-(4-7) contains full activity, other activity sites are present in the ACTH molecule. For example, ACTH-(7-10) is only slightly active in delaying extinction of pole-jumping avoidance behavior but the activity can be increased to the same level as that of ACTH-(4-7) by extending the C-terminal sequence to ACTH-(7-16) [29]. These findings concern avoidance behavior. Requirements for other CNS effects induced by ACTH may be different.

ACTH induces a stretching and yawning syndrome but only after icv administration. Ferrari et al. [26] found that in the dog ACTH was most effective while ACTH-(4-10) had marginal effects. Icv ACTH also induces excessive grooming in rodents and Gispen and associates [28,60,61] in a series of experiments designed to determinate the active core for this effect found

Table I. Amino acid sequence of ACTH and related fragments

	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
$ACTH_{1-24}$	H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-OH
$ACTH_{4-10}$	H-Met-Glu-His-Phe-Arg-Trp-Gly-OH
	0 ↑ D
Org 2766	H-Met-Glu-His-Phe-Lys-Phe-OH

that α- and β-MSH were as active as ACTH-(1-24). ACTH-(4-10) was inactive but ACTH-(4-7) did display grooming activity. The peptide had only 1/3 of the activity of ACTH-(1-24). Thus, excessive grooming needs a larger NH_2-terminal part of the ACTH-molecule than the effect on extinction of pole-jumping avoidance behavior.

Biotransformation

Little is known about the generation of ACTH fragments from the naturally occurring ACTH-(1-39) which is present in the anterior lobe of the pituitary as well as in the brain. The peptide is a biosynthetic intermediate for α-MSH and CLIP in the intermediate lobe and is not released as such but serves as a precursor of ACTH-(1-16) and ACTH-(17-39). These two peptides are generated from ACTH-(1-39) via endopeptidase activity acting in the sequence 15-18 (Table II). Subsequent carboxypeptidase activity followed by acetylation of the NH_2 terminal and amidation of the C-terminal amino acid generate α-MSH. It has been proposed that ACTH-(1-16) appears as a short-lived product during this process (39,40,48,49). Recently Wang and associates (unpublished findings) have been able to show that brain synaptic membrane fractions generate ACTH-(1-16) from ACTH-(1-39). The formation of smaller ACTH fragments in the brain has not been reported but is feasable since the brain is highly active in the proteolytic processing of ACTH [45].

Table II. Amino acid sequence of β-endorphin and various fragments

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
β-endorphin (β-LPH$_{61-91}$)	H-Tyr	Gly	Gly	Phe	Met	Thr	Ser	Glu	Lys	Ser	Gln	Thr	Pro	Leu	Val	Thr	Leu	Phe	Lys	Asn	Ala	Ile	
																						Val	23
																						Lys	24
																						Asn	25
																						Ala	26
																						His	27
																						Lys	28
																						Lys	29
																						Gly	30
																						Glu	31
																						OH	
γ-endorphin (β-LPH$_{61-77}$)	H-Tyr	Gly	Gly	Phe	Met	Thr	Ser	Glu	Lys	Ser	Gln	Thr	Pro	Leu	Val	Thr	Leu	OH					
α-endorphin (β-LPH$_{61-76}$)	H-Tyr	Gly	Gly	Phe	Met	Thr	Ser	Glu	Lys	Ser	Gln	Thr	Pro	Leu	Val	Thr	OH						
Met-enkephalin (β-LPH$_{61-65}$)	H-Tyr	Gly	Gly	Phe	Met	OH																	
Leu-enkephalin	H-Tyr	Gly	Gly	Phe	Leu	OH																	

β-ENDORPHIN

Opiates produce a variety of behavioral effects ranging from behavioral activation to immobility. Similar effects are found with β-endorphin. β-Endorphin also delays extinction of pole-jumping avoidance behavior and facilitates passive avoidance behavior. These effects cannot be blocked by specific opiate antagonists [18]. This suggests that the influence of endorphins on avoidance behavior is independent of opiate receptor sites. Structure activity studies reinforced the notion that β-endorphin has multiple CNS effects which are located in different parts of the molecule.

Structures activity studies

The β-endorphin fragment γ-endorphin facilitates extinction of pole-jumping avoidance behavior and attenutates passive avoidance behavior [22]. It also reduces acquisition of shuttle box avoidance behavior [36]. This effect is not dependent on opiate receptor activation since the removal of the NH_2 terminal amino acid residue tyrosine, which eliminates opiate-like activity, enhances the influence on active and passive avoidance behavior. The γ-type endorphins possess neuroleptic-like effects since they exert a positive grasping response [22], reduce electrical selfstimulation elicited from the ventral tegmental area and the nucleus accumbens at threshold currents [24,55] and attenuate apomorphine induced hypolocomotion [57]. Using extinction of pole-jumping avoidance behavior, two "grip tests" and apomorphine-induced hypolocomotion, it was shown that progressive shortening of the NH_2-terminus does not affect the

potency of γ-type endorphins unless the amino acid residue threonine^{7} is removed [23] (Table II). Thus, β-endorphin-(6-17) (DEγE) is the shortest sequence with full neuroleptic-like activities. The Met-enkephalin moiety which in itself has an effect opposite to that of γ-type endorphins [21] can be removed from γ-endorphin without loss of activity. More recent observations on active and passive avoidance behavior revealed that DEγE is markedly more potent that DTγE [27].

α-Endorphin and related peptides have an effect on extinction of active and retention of passive avoidance behavior and on electrical selfstimulation elicited from the ventral tegmental area opposite to that of γ-type endorphins [22,24,37,55]. In various aspects, α-type endorphins produce effects which resemble those of psychostimulants such as the amphetamines [17,56]. The α-type endorphins potentiate increased activity as induced by the administration of apomorphine and amphetamine [35,53].

Structure activity studies on extinction of pole-jumping avoidance behavior showed that removal of the NH_2 terminal amino acid residue tyrosine does not affect the potency but elimination of the whole enkephalin moiety markedly reduces the activity of α-endorphin in extinction. The active sequence seemed to be located in the β-endorphin-(2-9) sequence [30]. The same was found with regard to apomorphine induced stereotyped sniffing [53]. Thus, β-endorphin (2-9) is the most active sequence in this respect, while β-endorphin-(6-16), β-endorphin-(2-5) or β-endorphin (5-9) are less active or inactive (Table II).

Biotransformation

The formation of behaviorally active fragments of β-endorphin in the brain has been relatively well documented. Austen et al. [2] reported that striatal slices as well as brain membranes convert β-endorphin to γ-endorphin and α-endorphin, when the peptides were protected against loss of the NH_2-terminal tyrosine. Burbach et al [8] found that β-endorphin is converted by proteolytic enzymes associated with synaptic membranes into various fragments. It appeared that α-endorphin, γ-endorphin, β-endorphin-(2-17) (des-Tyr^1-γ-endorphin; DTγE) and β-endorphin-(2-16) (des-Tyr^1-α-endorphin; DTαE) were formed from β-endorphin (Table II). γ-Endorphin is formed by a single endopeptidase cleavage of β-endorphin. α-Endorphin is subsequently generated by action of a carboxypeptidase on γ-endorphin, while the des-tyrosine fragments of γ-, and α-endorphin involve an aminopeptidase activity [11]. Indeed, Hersh et al. [32] showed that a bovine brain enkephalin degrading aminopeptidase catalyses the hydrolysis of the NH_2-terminal tyrosine from Met-enkephalin as well as from α- and γ-endorphin. The accumulation of peptide fragments during conversion of β-endorphin by brain synaptic membranes is highly pH dependent since the formation of γ-type endorphins from β-endorphin is maximal at neutral pH; while α-type endorphins accumulate preferentially at a lower pH [8]. Major conversion products of DTγE by cleavage of internal bonds at pH 7.4 are β-endorphin-(5-17) and β-endorphin-(6-17) (DEγE) [11]. Schoemaker et al. [46] also found that DEγE is a principal metabolite of DTγE *in vitro*.

NEUROHYPOPHYSEAL HORMONES

Vasopressin increases resistance to extinction of active avoidance behavior [14] and facilitates passive avoidance behavior [1] while oxytocin facilitates extinction and attenuates passive avoidance behavior [4,49]. These memory effects of the neurohypophyseal hormones have been subject of intensive studies over the last two decades [19].

Structure activity studies

Several behavioral assays have been used to determine the active sites of the neurohypophyseal hormones on memory processes. One of these assays has been extinction of pole-jumping avoidance behavior [16]. To increase resistance to extinction, AVP-(1-9) is more potent than AVP-(1-8) (DGAVP) [59]. The behavioral effect on active avoidance behavior is present in the covalent ringstructure AVP-(1-6) but a second activity site may be present in the linear part of the molecule [16]. Alterations in the linear part of vasopressin (AVP-(7-9)) are less damaging than in the covalent structure of vasopressin [59]. The rate of extinction of pole-jumping avoidance behavior can be regarded as a measure of consolidation and these results were interpreted as indicating that the ringstructure of vasopressin is important in this respect. Retrograde amnesia can be used to measure retrieval processes. Vasopressin and related peptides attenuate retrograde amnesia. Several procedures are used to induce amnesia. Walter et al. [58] injected puromycin which caused amnesia for a maze learning task in mice. Vasopressin protects the animals against puromycin induced amnesia. However, the covalent ringstructures of vasopressin

and oxytocin are inactive [58]. The linear tripeptide OXT-(7-9) and the dipeptide Leu-Gly-NH_2 are more effective than vasopressin. These findings suggest that the active sequences for modulating consolidation and retrieval are differentially located in the neurohypophyseal hormones.

Passive avoidance behavior can be employed to measure influences of neuropeptides on consolidation as well as retrieval processes. Peptides are considered to influence consolidation processes if passive avoidance latency as measured 24 h after the learning trial is affected following injection immediately following the learning trial and retrieval when the peptides are given 1 h prior to the 24 h retention test. Using this paradigm, posttrial icv injected AVP-(1-9), AVP-(1-8), AVP-(1-7) and AVP-(1-6) facilitated passive avoidance behavior. However, OXT-(1-7) and OXT-(1-6) had the same effect. In fact, OXT-(1-7) is the most powerful peptide in this respect [38]. The linear parts of the two hormones are much less active. Facilitation of passive avoidance behavior following pre-retention administration was found with AVP-(1-9) and less pronounced with AVP-(1-8), AVP-(1-7), OXT-(1-6), OXT-(7-9) and OXT-(8-9). Attenuation of passive avoidance behavior was induced by posttrial injection of OXT-(1-9), OXT-(1-8) and vasotocin (AVT-(1-9)). Preretention administration of OXT-(1-9), OXT-(1-8), AVT-(1-9) and AVT-(1-8) attenuated passive avoidance performance.

The covalent ringstructures of vasopressin, oxytocin and vasotocin effectively facilitate consolidation of passive avoidance behavior. Although the linear part of oxytocin may be important for facilitation of retrieval processes, the results sofar obtained do not allow a firm conclusion in this respect.

To induce inhibition of memory consolidation as well as retrieval, seem to require practically the whole oxytocin or vasotocin molecule. Only the C-terminal glycinamide can be removed without appreciable loss of activity.

Biotransformation

Two main routes for the biotransformation of neurohypophyseal hormones in the brain have been identified, involving aminopeptidases cleaving N-terminal bonds and C-terminal cleaving enzymes producing C-terminal dipeptides and glycinamide [41,44]. Burbach et al. [9,10] found evidence for aminopeptidase and C-terminal cleaving peptidase activity acting on oxytocin in SPM preparations from rat limbic brain tissue. The aminopeptidase activity prevailed in these SPM fractions. The two types of oxytocin converting enzymes show some regional distribution. Compared to the activity in the parietal cortex, the aminopeptidase activity in SPM preparations of the medial basal hypothalamus, the nigrostriatal area and the region of the dorsal raphe nucleus were the highest [9,10]. These areas are densily innervated by neurohypophyseal hormone containing fibres [5,6,50]. In other studies on the fragmentation of vasopressin and oxytocin using high pressure liquid chromatography and amino acid analysis of the fragments, a number of peptide fragments was detected [7] which may have relevance for the memory effects of the neurohypophyseal hormones. Arg^8-vasopressin (AVP-(1-9)) was converted significantly more rapid than oxytocin (OXT-(1-9)). The SPM associated aminopeptidase cleaved the nonapeptides without prior reduction of the disulfide bridge. Major fragments of vasopressin found were [Cyt^6]-AVP-(2-9); [Cyt^6]-AVP-(3-9) and [Cyt^6]-AVP-(4-9). Those generated from OXT-(1-9) were [Cyt^6]-

OXT-(2-9); [Cyt^6]-OXT-(3-9); [Cyt^6]-OXT-(4-9) and [Cyt^6]-OXT-[5-9]. A number of these appeared to possess behavioral effects similar to those of the parent hormones. The [Cyt^6]-AVP-(4-9) and [Cyt^6]-OXT-(4-9) possess opposite effects on memory consolidation. This is remarkable because these fragments lack the Phe^3 and Ile^3 amino acid residues which characterize the covalent ringstructures of vasopressin and oxytocin respectively. This indicates that the original hypothesis which suggested that the ringstructures are important for memory consolidation has to be revised.

DISCUSSION

The structure-activity and biotransformation studies with ACTH and the neurohypophyseal hormones suggest the precursor role of these hormones in the generation of neuropeptides involved in motivation and attention and in learning and memory processes. Besides, β-endorphin appears to be a precursor of neuropeptides with neuroleptic-like and psychostimulant effects. In general, the behavioral bioassays with synthetic fragments of these hormones provided the information on the active fragments responsible for the CNS effect. In some cases, biotransformation studies suggested that such fragments could occur in the brain although the neuropeptides generated by ACTH in the brain affecting avoidance behavior or excessive grooming, have not been precisely determined. The opiate-like β-endorphin seems to be converted in the brain to neuroleptic-like and psychostimulant neuropeptides which are markedly different in

profile from the precursor and from each other. As yet, it is not known whether the psychostimulant neuropeptide β-endorphin-(2-9) is generated in brain tissue and if so whether β-, α- or γ-endorphin is the precursor of this fragment. The neurohypophyseal hormones form neuropeptides which may have potent and possibly more selective effects on consolidation and retrieval processes.

These findings can be extended to other brain borne neuropeptides. For example, the C-terminal part of Substance P [SP-(3-11)] contains the active core for the contraction of guinea pig ileum and SP-(6-11) for the binding in rat brain (31), cholecystokinin (CCK) may have opposite effects located in either NH_2-terminal or C-terminal part of the molecule (52) and the C-terminal tetrapeptide (CCK-5-8) is as potent as the whole molecule in affecting avoidance behavior [52]. Cyclo (His-Pro) a metabolic conversion product of TRH has in several assays a more potent effect in the CNS than its precursor [43]. It is conceivable that the generation of these neuropeptides takes place in or near the synaptic membrane upon release of the precursor peptide. Although several neuropeptides which are present in the brain may act as neurotransmitters, the various fragments may have a modulatory effect on neurotransmitter activity. The fragments as we have seen may have different, more potent or selective actions which make them highly suitable as homeostatic controllers of the neurons upon which the peptidergic pathways project. The presence of neuropeptides and transmitters in the same neuron [33] in addition suggests a local feedback modulation of the neuron's own activity. These modulatory influences might be excerted presynaptically as has

been shown for γ-type endorphins [53] or postsynaptically as seems to be the case with α-type endorphins [54]. The opposite effects of γ-type and α-type endorphins [22], the potent and selective effects of some fragments of vasopressin and oxytocin on memory processes which are in themselves opposite in character [7] etc., all point to an exquisite modulatory control of neurotransmitter activity. In addition, the beneficial influence of ACTH-(4-10) and related fragments on nerve regeneration [3,51] and of TRH on neurologic recovery after spinal trauma in cats [25] and the counteracting effect of SP on 6-hydroxydopamine induced inhibition of postnatal development in rats [34] point to a trophic effect of neuropeptides on nerve cells.

Characteristic of the action of the modulatory neuropeptides is the slow onset and the long duration. In view of this the peptide producing cells in the brain may be regarded as a separate division like the autonomic and the somatic nervous system. These cells synthetize peptides, amines and other substance. Such so-called "Amine Precursor Uptake of Decarboxylase (APUD) cells" seem to be derived from neuroendocrine programmed cells originating in the embryonic ectoblast [42]. The elucidation of the physiological control of this system over neurotransmission by the generation of multiple neuropeptides generated by the various brain-borne precursor hormones may revolutionize current views on CNS functioning.

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INTERACTION BETWEEN MOSSY FIBRE AND CLIMBING FIBRE RESPONSES IN PURKINJE CELLS

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A major problem in cerebellar physiology relates to the manner in which information from mossy fibres and climbing fibres is integrated in the efferent neurones of the cerebellar cortex, the Purkinje cells. Recent findings by Ekerot and Oscarsson [8] and by Ito, Sakurai and Tongroach [13] indicate that the important interaction between mossy fibres and climbing fibres takes place in the dendrites rather than in the Purkinje cell somata. It has been demonstrated that impulses in climbing fibres evoke not only the so-called "complex spikes" in the somata but also plateau-like depolarizations in the distal dendrites which may have durations of hundreds of ms. The plateau potentials are presumably produced by a voltage dependent calcium conductance increase which, under physiological conditions, is triggered off exclusively by the large synaptic potentials generated by climbing fibre impulses. The spread of the plateau potentials to the distal dendrites and their long duration would facilitate spatial and temporal interaction between the mossy fibre and climbing fibre inputs. The findings suggest two kinds of interaction.
(1) The duration of the plateau potentials in individual dendritic branches is modulated by the local mossy fibre/parallel fibre input. Thus, it might be postulated that dendritic branches act as independent integrators of mossy fibre and climbing fibre inputs.
(2) It has been suggested that interaction between mossy fibres and climbing fibres forms the basis of learning processes in the cerebellum mediated through plastic changes in the synapses between parallel fibres and Purkinje cell dendrites. The plateau potentials would influence parallel fibre synapses by increasing the intradendritic calcium which, in turn, would lastingly depress the sensitivity of the postsynaptic receptors of the parallel fibre synapses that are activated in conjunction with the climbing fibres.

The cerebellar cortex receives two main kinds of afferents, the mossy fibres and the climbing fibres. A major problem in

cerebellar physiology concerns the manner in which information from the mossy and climbing fibres is integrated in the efferent neurones of the cortex, the Purkinje cells.

Fig. 1A shows the basic circuit of the cerebellar cortex which has, in principle, remained unchanged in the vertebrate series since its first development in primitive fish [6,15,24]. The constancy of the circuit suggests that it has been a highly successful design. The basic circuit consists of the two afferent systems and a single efferent path (inhibitory interneurones have been omitted). The numbers in the figure give the degree of divergence (numbers within rectangles) and convergence (naked numbers) in the two afferent paths, as found in the commonly investigated mammals [5].

The mossy fibres originate from a variety of cell groups in the spinal cord and brain stem. These fibres activate the Purkinje cells disynaptically through the granule cells and their axons, the parallel fibres. The mossy fibre path is characterized by a high degree of divergence and an enormous convergence. About 100,000 of the 400,000 parallel fibres which pass through the dendritic tree make synaptic contacts with the distal dendrites, the spiny branchlets, of each Purkinje cell. The pattern of synaptic input formed by the large number of parallel fibres contacting a single Purkinje cell must be established, it seems, by some kind of motor learning [5].

The climbing fibres have an entirely different organization. They originate presumably from one source only, the inferior olive, and make monosynaptic contacts with the smooth dendrites of the Purkinje cells. There is little divergence, each olivary neurone innervates about 10 Purkinje cells, and

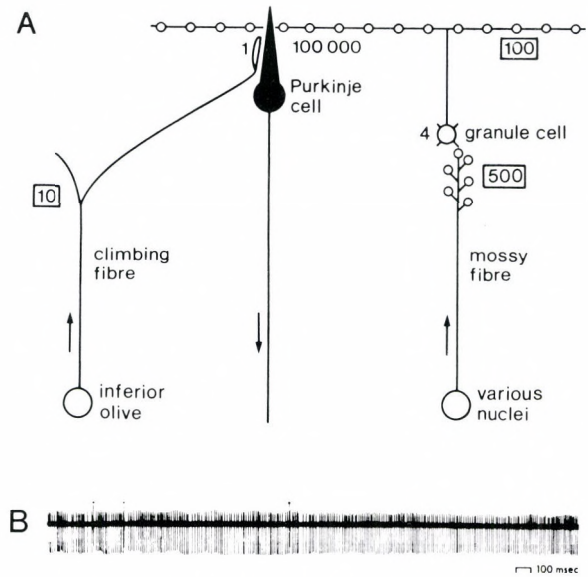

Fig 1. A. Basic circuit of cerebellar cortex showing excitatory connections between the two main afferent systems, the mossy fibres and climbing fibres, and the single efferent path, the Purkinje cells. Numbers give approximative degree of divergence (numbers within rectangles) and convergence (naked numbers) in the two afferent paths (from ref. 5). B. Extracellular recording from Purkinje cell showing typical background activity of "complex sipkes" (indicated by dots) and "simple spikes". (Modified from ref. 26)

there is no convergence, each Purkinje cell receives only one climbing fibre. The anatomical specificity is matched by a corresponding specificity in the information reaching each Purkinje cell from its climbing fibre. Each climbing fibre projecting to the anterior lobe has been shown to receive convergence of excitation from several spino-olivary and several cerebro-olivary paths, suggesting that an extremely complex integration occurs at the olivary level [2,23].

Activity in the mossy fibres is largely responsible for the "simple spike" activity, usually in the range of 20-100 Hz, which can be recorded from the Purkinje cell somata. The climbing

fibres have a low discharge rate of about 1 Hz and are responsible for the so-called "complex spikes" in the Purkinje cells. The complex spikes, because of their low rate of discharge, cannot be expected to influence appreciably the frequency coding of the Purkinje cells. This is illustrated in Fig. 1B, showing extracellular recording from a Purkinje cell. The few complex spikes, indicated by dots, hardly add to the high frequency simple spike activity. Moreover, the variability in latency of the climbing fibre responses evoked by stimulation of an afferent path to the inferior olive makes it unlikely that the climbing fibres signal exact timing of events [8]. The climbing fibres can, thus, be of little importance for the instantaneous control of ongoing movement which is executed by the output from the Purkinje cells. This control is presumably entirely dependent on the mossy fibre paths which carry precise information about peripheral events and activity in motor centres [7,18,22].

What is, then, the function of the climbing fibres? The unique one-to-one anatomical relationship between the climbing fibres and the Purkinje cells, together with the powerful synaptic responses generated in the Purkinje cells by impulses in climbing fibres, suggests a very specific function for this input. An explanation is offered by the recent discovery of Ekerot and Oscarsson [8,9] that impulses in climbing fibres evoke not only the complex spikes in the Purkinje cell somata, but also prolonged plateau-like depolarizations in the Purkinje cell dendrites. Such plateau potentials are, under physiological conditions, evoked exclusively by the large EPSPs generated by impulses in climbing fibres [3].

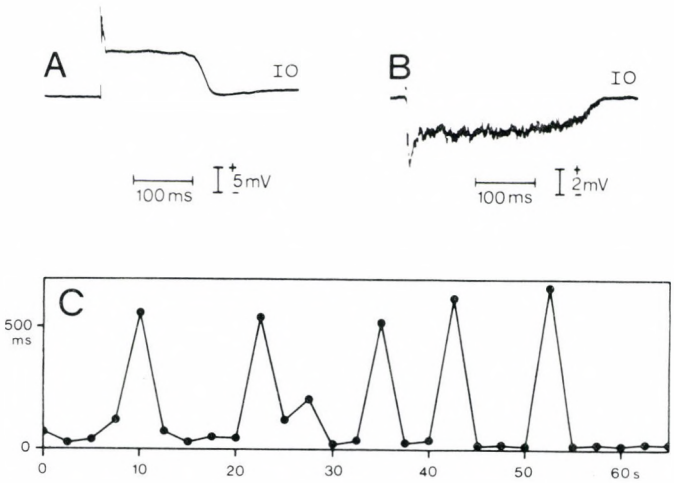

Fig. 2. Plateau potentials evoked in Purkinje cell dendrites by impulses in climbing fibres. A. Intracellular recording form proximal dendrite. B. Extracellular recording from distal dendrite. C. Variation in duration of consecutive plateau potentials evoked at intervals of 2.5 s and recorded extracellularly from distal dendrite. Ordinate, duration of response in ms. Abscissa, time of consecutive responses in s. The observations were made on cats under pentobarbitone anaesthesia

The plateau potentials have different characteristics in the coarse, proximal dendrites and in the thin, distal dendrites, as illustrated in Fig. 2. Intracellular recordings are readily obtained from the proximal dendrites and reveal responses consisting of an initial spike-like component followed by a plateau-like depolarization with a relatively constant duration of about 100 ms (Fig. 2A). Electrode penetrations are seldom successful in the distal dendrites. However, extracellular recordings demonstrate responses consisting of unitary negative potentials of long duration which presumably correspond to intradendritic depolarizations (Fig. 2B). The duration of the negative plateaus vary from about 20 ms to several hundreds of ms (Fig. 2C).

The plateau potentials in the Purkinje cells are probably generated by a calcium conductance increase [17] and are reminiscent of the prolonged plateau potentials generated by voltage dependent calcium currents in embryonic neurones [cf. 25]. In the adult stage of higher vertebrates, voltage dependent calcium conductances seem to be well developed in dendrites [cf. 14] and have been demonstrated in many regions of the nervous system, e.g. the neocortex, thalamus, hippocampus, inferior olive and cerebellum [for references see 10, 16]. However, plateau potentials of such long duration and constant amplitude as in embryonic neurones have only been observed in Purkinje cells. The typical plateau potentials in Purkinje cells can be found in recordings from cerebella not only of mammals, but also of reptiles and fish [for references see 9]. The plateau potentials thus represent a phylogenetically old mechanism presumably fundamental to the climbing fibre synapses.

The plateau potentials may provide an insight into the function of the climbing fibre input. The long duration of the plateaus, several hundreds of milliseconds, is of the right order to bridge the time gaps between the climbing fibre discharges, and would allow for a continuous interaction between the climbing fibre and mossy fibre inputs [9]. In the "learning" theories advanced by Marr [19], Albus [1] and Ito [11,12], the mossy and climbing fibres have entirely different tasks (see Fig. 3). Impulses in mossy fibres are largely responsible for the output from the Purkinje cells, which in turn modulates the execution of motor acts. The inferior olive evaluates the motor performance and errors are signalled through the climbing fibre to the Purkinje cell and result, according

to Albus [1], in a heterosynaptic suppression of those parallel fibre synapses which have just been active and therefore partly responsible for the misperformance. Repetition of these events increases the suppression of the transmission with, finally, elimination of the erroneous movement.

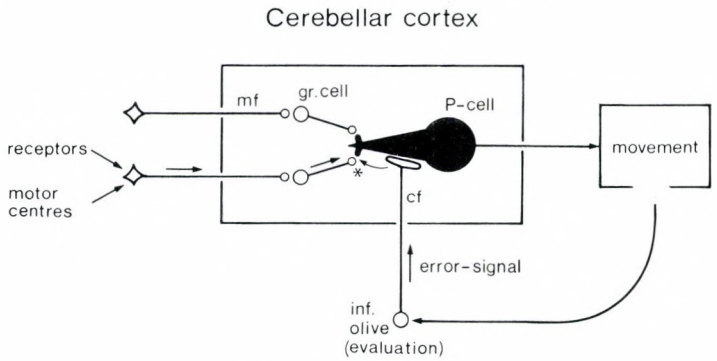

Fig. 3. The "Learning" theory of cerebellar cortex (1,11,12,19). Impulses in mossy fibres (mf) are largely responsible for the output from the Purkinje cells, which in turn modifies the execution of motor acts. The correctness of the motor acts is evaluated by the inferior olive and errors in motor performance are signalled through the climbing fibres (cf) to the Purkinje cells and result in heterosynaptic suppression of those parallel fibre synapses which have just been active and therefore partly responsible for the misperformance. Abbreviations: gr. cell, granule cell; P-cell, Purkinje cell. See text

These theories are supported by recent experimental findings by Ito and coworkers [13] who showed that simultaneous activation of climbing fibres and mossy fibres results in a long-lasting reduction in synaptic efficacy of the activated parallel fibre synapses. Since the climbing fibre synapses and the parallel fibre synapses are located in different regions of the Purkinje cell dendrites [6,21,24], there must exist a mechanism by which information about climbing fibre activity is transferred to the parallel fibre synapses. The climbing fibre evoked plateau

potentials, which are initiated at the climbing fibre synpases located on the smooth proximal dendrites and actively conducted out to the distal dendrites where the parallel fibre synapses are located, may constitute such a mechanism. The plateau potentials in the distal dendrites probably cause an increase in the intradendritic calcium concentration. The calcium ions might represent a second messanger responsible for reducing the sensitivity of the post-synaptic receptors for the transmitter in the parallel fibres, just as the intracellular calcium is supposed to desensitize acetylcholine receptors in muscle endplates [20].

Recent experimental findings [4] have revealed an additional facet to the climbing fibre/mossy fibre interaction. In this study, it was shown that the duration of the climbing fibre plateau potentials in Purkinje cell dendrites can be modulated by parallel fibre input. The results indicate that the duration of the plateau potentials probably depends on the membrane potential and it is suggested that changes in potential are mediated by the local parallel fibre activity. The marked variation in the duration of the plateau potentials in distal dendrites (Fig. 2C) might be a consequence of this interaction. If there exist local differences in parallel fibre input, then individual dendritic branches may act as independent integrators of mossy fibre and climbing fibre information.

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IMMUNOCYTOCHEMICAL AND AUTORADIOGRAPHIC METHODS TO DEMONSTRATE THE COEXISTENCE OF NEUROACTIVE SUBSTANCES: CEREBELLAR PURKINJE CELLS HAVE GLUTAMIC ACID DECARBOXYLASE, CYSTEINE SULFINIC ACID DECARBOXYLASE, AND MOTILIN IMMUNOREACTIVITY

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Evidence is presented from immunocytochemical experiments that Purkinje cells in the mammalian cerebellum of a number of species contain gamma aminobutyric acid (GABA), taurine and the peptide motilin. Purkinje neurons show immunoreactivity to antibodies against glutamic acid decarboxylase (GAD), the synthetic enzyme for GABA, cysteine sulfinic acid decarboxylase (CSADC ase), the synthetic enzyme for taurine and motilin. Single neurons have either unique localization for GAD or motilin or CSADC ase or two substances in combination providing evidence for coexistence of these neuroactive agents. Functional studies with iontophoresis of GABA, taurine, and motilin into rabbit lateral vestibular nucleus elicited strong depressant effect on neuronal firing. The interactions of motilin and GABA were additive and inhibitory. Thus, Purkinje neurons of the cerebellum use multiple chemical messengers in their interactions. These chemical messengers coexist in single neurons. Their effects as far as our studies indicate are inhibitory and consistent with previous demonstrations of the physiological actions of Purkinje cells.

COEXISTENCE OF NEUROACTIVE SUBSTANCES

This paper deals with the coexistence of neuroactive substances in single neurons of the mammalian central nervous system. In the strict sense, and for the present context, *coexistence* denotes the situation in which more than one neuroactive substance can be localized by cytochemical means in an individual neuron. Usually this has meant the combination of a traditional neurotransmitter, such as acetylcholine or nor-

epinephrine or an amino acid, together with a peptide, such as substance P or enkephalin. But this definition does not exclude the presence of more than one traditional neurotransmitter in the same cell or more than one neuroactive substance, such as peptides. The issue of the presence of multiple neuroactive substances in neurons is not limited to the mammalian central nervous system (CNS) - the peripheral nervous system of vertebrates and identified neurons in a number of invertebrates also have examples of this phenomenon [7,35,38] but they will not be reviewed here. True "coexistence" of neuroactive substances in single neurons as defined here must be distinguished from the looser definitions used by many investigators to denote the simultaneous *existence* of multiple neuroactive substances in *different* nerve cells in a particular geographic area of the brain. This can be illustrated by specific examples: Single serotonin neurons in the medulla and raphe nuclei have been shown to contain substance P also [10a,12,23]; Purkinje cells in the cerebellum have been found to have motilin and glutamic acid decarboxylase [15]. However, enkephalin, substance P, and glutamic acid decarboxylase immunoreactivity have been demonstrated in separate cell types in the neostriatum [37]; somatostatin and substance P have been distinguished in separate cell groups in primary afferent neurons [22]; and serotonin, tyrosine hydroxylase, and enkephalin exist simultaneously in separate cells elsewhere in the rat CNS [3]. Since it is clear that one geographic area or nucleus in the brain consists of many nerve cells that may be heterogeneous, it would be expected that these neurons could utilize a number of different chemicals in neural communication. Thus, the simultaneous existence of a number of

neuroactive compounds in a nerve cell group is not surprising. The phenomenon of true coexistence of multiple neuroactive substances in a single cell is, however, more interesting, and calls for careful investigation of the parameters of such coexistence, its function, the cytological substrates that allow for orderly synthesis, transport and release of the compounds, and, of course, its significance in the complexity of nerve circuits.

GABA AND MOTILIN AND CYSTEIN SULFINIC ACID DECARBOXYLASE IMMUNOREACTIVITY

GABA is a major inhibitory neurotransmitter in the mammalian CNS and deficits of GABA have been implicated in certain neurological and psychiatric disorders such as Huntington chorea, Parkinson disease, and schizophrenia. Considerable interest has been attached in recent years to the identification of GABA-contraining neurons, their possible inhibitory function, the identification of receptors, and the pharmacological manipulations of GABA-activated receptor sites. The cerebellum remains a major area in the CNS where the amino acid transmitters figure largely, and a considerable literature supports the identification of GABA as the inhibitory transmitter for many cerebellar neurons. Among the approaches that have been used to support the localization and role of GABA ara 1) auto-radiography following the uptake of ^{3}H-GABA [24]; 2) immuno-cytochemistry on tissue sections with antibodies directed and role of GABA are 1) autoradiography following the uptake of ^{3}H-GABA [24]; 2) immunocytochemistry on tissue sections with

antibodies directed against the enzyme GAD, responsible for the synthesis of GABA [2,26,27,44] or with anti-GAD antibody injected directly into the live tissue to trace GABA specific pathways [13]; 3) immunocytochemistry with antibodies directed against the enzyme GABA-transaminase (GABA-T), the major metabolic enzyme for GABA [2,14]; and 4) autoradiographic demonstrations of various receptor-GABA binding sites with ^{3}H-GABA analogues such as ^{3}H-muscimol [9,10,11]. Of the cerebellar neurons in the cortex, a majority (although not all) of the small interneurons - e.g., stellate, basket, and Golgi cells - are labeled by uptake of ^{3}H-GABA, anti-GAD antibody, GABA-T, and ^{3}H-muscimol binding, thus indicating that GABA is a reasonably consistent constituent. In the deep cerebellar nuclei, the largest projection neurons are never marked by these GABA methods, whereas a population of smaller neurons in [8,28] (see Fig. 1). The present studies, however, are concerned with the Purkinje cells, the intracortical projection neuron responsible for the major relay of information from the complex cerebellar cortical network to the deep nuclear cells and to the lateral vestibular nucleus.

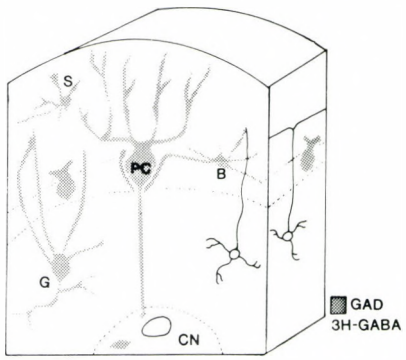

Fig. 1. Cerebellar cortical and nuclear neurons with GAD immunoactivity and uptake capacities for ^{3}H-GABA are indicated by stippling. These are stellate (S), basket (B), Purkinje cells (PC), Golgi cells (G) of the cortex, and some small neurons in the deep cerebellar nuclei (CN)

Since the basic organization, cytology, and connections of these cells are well known and have been the subject of major works [8,21,36,40], the details need not be repeated here. Only the pertinent facts will be drawn out to substantiate the case that coexistence of neuroactive substances occurs in Purkinje cells, and that this opens up a frontier for important advances in our understanding of the cerebellum.

Purkinje cells are demonstrated by a combination of pharmacological and physiological approaches to contain GABA and to have a postsynaptic inhibitory effect on Deiters neurons [30,31]. The presence of GABA was confirmed by microdissection techniques [32]. From these and subsequent findings it has been generally assumed that Purkinje cells are homogeneous, and that they all contain GABA and are inhibitory. Nevertheless, a critical analysis of the literature indicates that, in fact, whereas GABA could be localized to many cerebellar cortical structures reliably, the Purkinje cells remained the most inconsistent in this regard.

1. Autoradiography with ^{3}H-GABA uptake produced evidence for labeled cortical interneurons but did not reliably label Purkinje cells [8,24,42].

2. Autoradiography with ^{3}H-muscimol binding consistently labeled intracortical interneurons but not Purkinje cells [42].

3. Immunocytochemical studies with anti-GAD antibodies indicated that Purkinje cell somata are difficult to visualize unless colchicine is used in order to retard axoplasmic transport [41]. A recent study claims that all Purkinje cells can be labeled with the use of colchicine and a polyclonal antibody raised against a GAD preparation that is not the completely

purified enzyme. However, closer examination of this material indicates that, in fact, unlabeled Purkinje cells are present [33,34] and cells other than GAD immunoreactive ones may be labeled [17].

4. Other studies with anti-GABA-T also indicated that not all Purkinje cells exhibit immunoreactivity to this serum even with the aid of colchicine [2,9]. These reports were supported by other immunocytochemical studies in the literature indicating that labeling with anticyclic AMP [4,12] or with anti-cyclic GMP [12] was also patchy.

5. In vivo injections of anti-GAD antibodies combined with anterograde and retrograde transport as a means for tracing

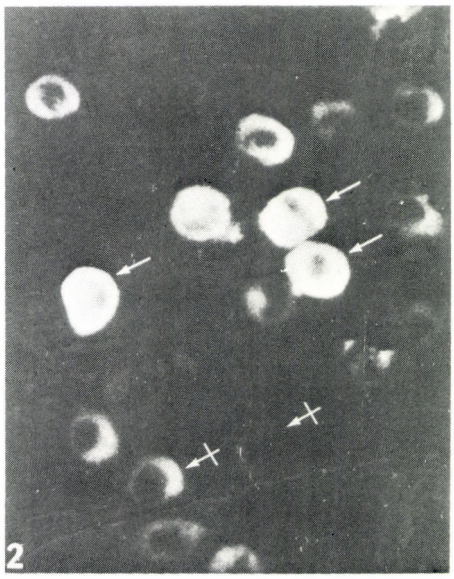

Fig. 2. Photomicrograph showing GAD-immunoreactive Purkinje cell somata, excluding nuclei, after an in vivo injection with anti-GAD antibodies into the cerebellar cortex. Some cells are intensely labeled (arrows), others lightly labeled (crossed arrows), and still others not reactive at all, indicating that not all Purkinje cells have the capacity to bind anti-GAD antibodies and that not all Purkinje cells contain GAD. Rat cerebellum, immunofluorescence, x 200

chemically specific pathways indicated that although a large number of Purkinje cells contain GABA, some Purkinje cells contain only minor amounts and some contain none (see Fig. 2). The question was raised then whether or not this patchy labeling of Purkinje cells reflects cyclic changes in GABA content and fluctuating levels of GAD [13].

The rising concern that other neuroactive substances are likely to be present in Purkinje cells has resulted in a search in our laboratory for candidates among the growing catalog of neuroactive peptides. One successful candidate for this role is motilin, a 22-amino acid polypeptide isolated from porcine gut, which stimulates enteric smooth muscle [5,6], has endocrine effects when administered systemically [19], has an excitatory effect on neurons of the cerebral cortex and spinal cord [39], and is inhibitory in the cerebellum [16]. Another is taurine, which is detected by antibodies against cysteine sulfinic acid decarboxylase, the synthetic enzyme for this amino acid transmitter [17].

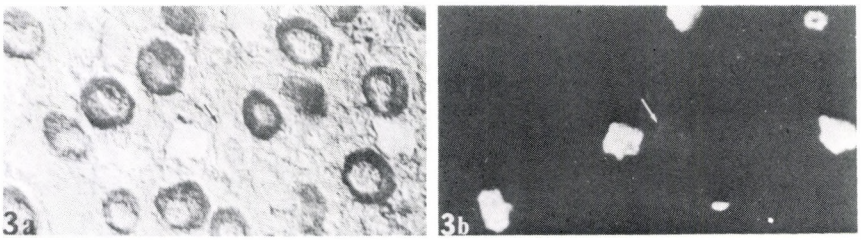

Fig. 3. Purkinje cell field rich in motilin immunoreactive cells. A pair of photomicrographs showing the same field of Purkinje cells in which 17 large neurons are labeled with motilin antibodies (upper) and five of the remaining Purkinje cells are labeled with GAD antibodies (lower). The arrow indicates a cell with low GAD immunoreactivity. Mouse cerebellum lobule V, vermis (a) immunoperoxidase and (b) immunofluorescence, x 200

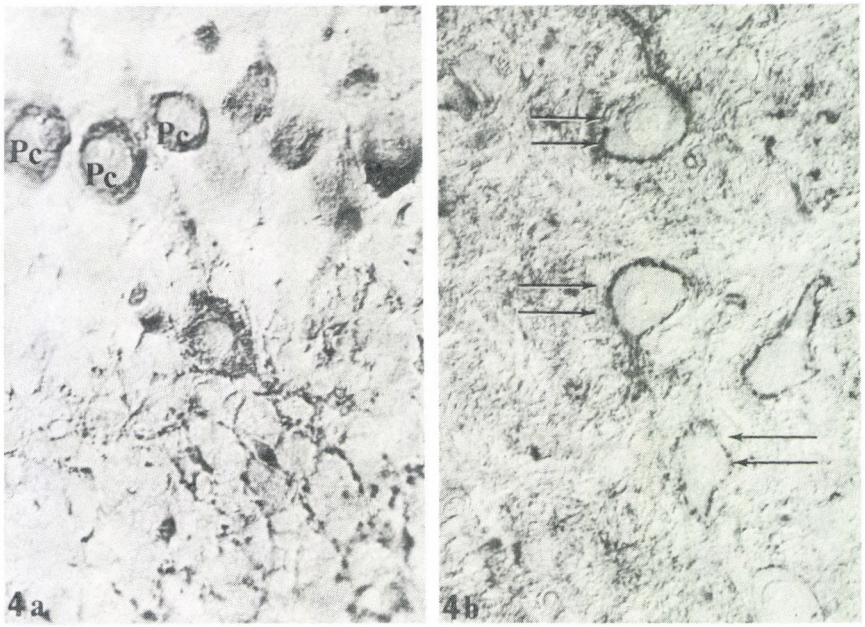

Fig. 4. a) Intense motilin immunoreactivity demonstrated in a row of Purkinje cells (PC) and more rarely, in the cell body, dendritic and axonal processes of a Golgi cell in the granular layer. Rat cerebellar cortex, Vibratome sections, peroxidase--antiperoxidase method (unpublished data, Chan-Palay and Nilaver), x 350
b) Motilin-immunoreactive terminals (arrows) on the somata and primary dendrites of large neurons in the dentate nucleus of the rat's cerebellum, x 350

Motilin-like immunoreactivity is detectable in peripheral nervous system [18] and in the brain of a number of species, in the hypothalamus and pituitary [25], and in the cerebellum [15,29]. In the cerebellum, motilin-like immunoreactivity can be found almost exclusively in Purkinje cells: approximately 60-70% of the Purkinje cells are motilin-positive and 30-40% are not. These motilin cells occur mainly in the lateral cerebellum and the flocculonodular lobe with numerous terminals in the deep cerebellar nuclei and Deiters nucleus (Figs 3-5), GAD immunoreactive Purkinje cells also form approximately 60-70% of the population, and 30-40% of Purkinje cells are not

GAD-positive. GAD Purkinje cells are found in the vermal and paravermal regions with a large representation in the flocculonodular lobe [15], and numerous terminals occur in the deep cerebellar nuclei and Deiters nucleus. None of these results is quantitatively altered by the administration of colchicine. A combined double labeling study with anti-GAD and anti-motilin antibodies indicates that not only do some Purkinje cells contair either GAD or motilin immunoreactivity exclusively, but also certain cells, approximately 10-20%, have both GAD and motilin [15]. These findings call for a reassessment of present assumptions that Purkinje cells are a homogeneous population in structure, connections, and function. The existence of chemical heterogeneity in Purkinje cells requires that this assessment take place at the single neuron level, and calls into question the present concept of how the cerebellum works.

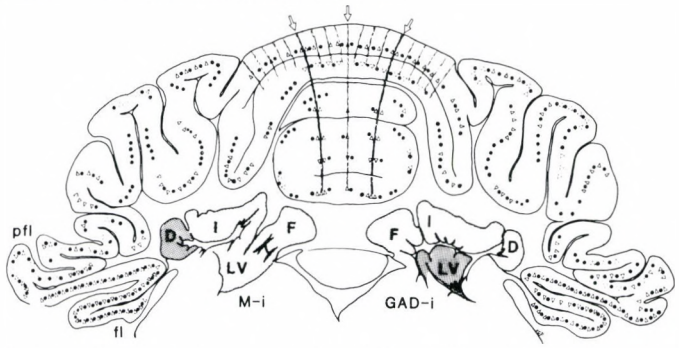

Fig. 5. Schematic drawing of the distribution of motilin--immunoreactive Purkinje cells (triangles) and GAD-immunoreactive Purkinje cells (black dots) in a coronal section of the cerebellum. Both cell types are more concentrated in the flocullus (fl) and dorsal and ventral paraflocculus (pfl) than elsewhere, and in the vermis they participate in the formation of the sagittal microzones (arrows). Motilin-immunoreactive terminal axon projections in the deep cerebellar nuclei - dentate (D), interpositus (I), fastigial (F), and lateral vestibular nuclei (LV) - are represented on the left, and a comparable representation for GAD-immunoreactive terminal axon projections is shown on the right. The intensity of immunoreactivity is indicated by the density of stippling in the nuclei. Arrows indicate microbands formed by motilin and GAD cells

In our recent studies [16] motilin, methionine-enkephalin (metenkephalin), leucine-enkephalin (leu-enkephalin), somatostatin, taurine, gamma-aminobutyric acid (GABA), and glycine were tested for their effects on neurons of the lateral vestibular nucleus of Deiters (LVN) in rabbits. Iontophoresis was carried out with multibarrelled micropipettes. All 4 peptides and 3 amino acids produced depression of neuron firing. No facilitatory responses were observed. The depressant action of each peptide when iontophoresed alone was dose-dependent and was rapid in onset and recovery. Their characteristic actions suggest the possibility of their independent roles as strong inhibitors, although the experimental paradigm does not allow conclusions about the individual potency of each peptide. When GABA was administered together with motilin, or metenkephalin, or somatostatin, the effects of the peptide and GABA were additive, producing depression greater than the application of either substance alone. When GABA was applied in conjunction with leu-enkephalin, more complex interactions were observed. At low iontophoretic currents, leu-enkephalin antagonized the action of GABA, producing a depression less than that of GABA alone and of considerably slower onset, thus suggesting an additional modulatory effect. These observations support the conclusion that all substances tested are chemical mediators in the LVN and leu-enkephalin may be a neuromodulator as well. Since recent immunocytochemical studies indicate that Purkinje cells in the cerebellar cortex are chemically heterogeneous and exhibit immunoreactivity for motilin, taurine, the enkephalins, and somatostatin, as well as for the GABA-synthesizing enzyme, glutamic acid decarboxylase, it is suggested that the Purkinje cell projections to vestibular

and cerebellar nuclei are multimodal in their chemical coding. The uniformly depressant action of the peptides and amino acids reported here is consistent with earlier observations that Purkinje cells exert an inhibitory influence on the vestibular and central cerebellar nuclei.

ISOLATED PURKINJE CELL PERIKARYA FROM DEVELOPING CEREBELLUM

Metabolically intact and viable perikarya from developing rat cerebellum at the sixth postnatal day were prepared according to previously described methods [1,20] with the collaboration of Dr. R. Balazs, London. The cells display normal ultrastructure and metabolic activity as a result largely of the gentle procedure employed in cell dissociation: a) low trypsin concentration combined with a trypsin inhibitor after a relatively short period of tissue digestion; b) the use of isotonic conditions and physiological pH throughout the procedures; and c) avoidance of high centrifugal forces. From these preparations several cell fractions could be obtained, one of which is of central interest to us, the "E" fraction consisting of the perikarya of large neurons, mainly, though not exclusively, of Purkinje cells. In this fraction, electron microscopic studies have shown good structural preservation. Plasma membranes are continuous, and mitochondria notably are intact. Viability of the cells is good, 80% exclude trypan blue, and plating efficiency of these cells in tissue culture is very high [1].

Preparations of the E or Purkinje cell fractions were layered onto slides, fixed with 4% formaldehyde and 0.1% glutaraldehyde in 0.12 M phosphate buffer, pH 7.3, for several

hours, and dried. The cells were then rinsed well in Tris buffer pH 7.6, allowed to react with a sequence of different antibodies, including anti-GAD antibody and anti-motilin antibody. Each set of Purkinje cells was exposed to a primary antibody, then the reaction was completed with peroxidase-conjugated goat anti-rabbit serum [43] and finally colorized with DAB and H_2O_2 (Fig. 6). All cells were counted and their immunoreactivity intensities were divided into three categories: high (++), medium (+), and absent (-).

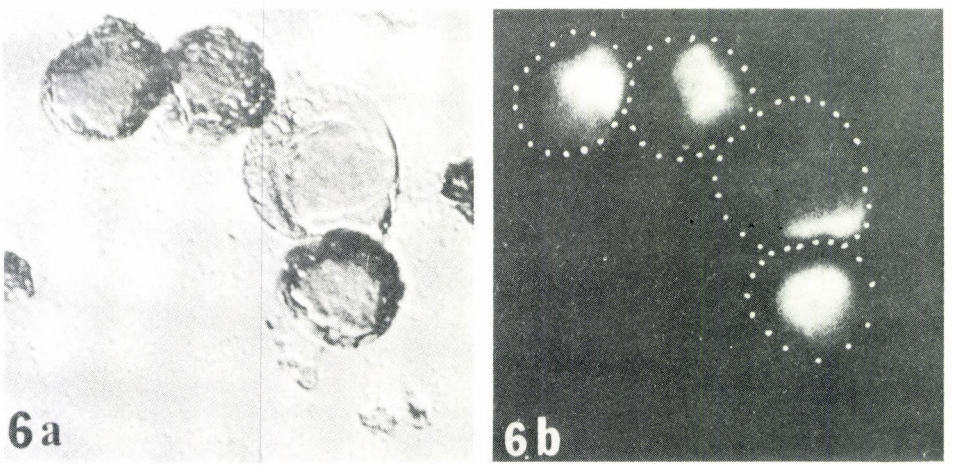

Fig. 6. Four isolated Purkinje cells from 6-day-old developing rat cerebellum after raction with anti-GAD antibody peroxidase (a) and anti-motilin antibody with FITC (b). Note that only three of the four cells have GAD, but all four have motilin, and three have both substances. × 350

GAD-immunoreacted preparations n = 19,716; 70.9% cells had (++) reactions; 18.1% cells had (+) reactions; 11.1% cells had (-) reactions.

Motilin-immunoreacted preparations n = 27,268; 68.3% cells had (++) reactions; 19.0% cells had (+) reactions; 12.4% has (-) reactions.

When the GAD-stained preparations were restained with anti-motilin antibody or vice versa, the numbers of stained cells were equivalent. Further observations indicate that many Purkinje cells contain both GAD and motilin immunoreactivities. These results indicate that 1) Purkinje cell fractions provide a suitable model for testing the coexistence of GAD with motilin and other neuroactive substances; 2) the quantitative estimates obtained from Purkinje cell fractions match those made on tissue sections; 3) at 6 days postnatally Purkinje cells already express the definitive neuromediator profile; and 4) the model will allow us to study selected Purkinje cells with known content of single or multiple neuromediators under various experimental conditions.

In summary, we present the Purkinje cell, the familiar giant cell of the cerebellar cortex, as a model in which the phenomenon of coexistence of neuroactive substances can be readily studied.

- What are the cytological features of a pure GAD-immunoreactive, GABA-containing Purkinje cell? What are its connections?
- What are the cytological features of a pure-motilin-immunoreactive Purkinje cell? What are its connections?
- What are the similarities and differences between these cells and what can be learned about their separate chemical and physiological functions?
- What are the ultrastructural correlates for dual neuroactive chemical content in the Purkinje cell?
- Does such a cell segregate the synthesis, transport, and release machinery for GABA and for motilin in different

populations of such cellular organelles, or do they coexist? Can these compartments be distinguished immunocytochemically at the ultrastructural level and are synaptic vesicles involved? (See Fig. 7.)

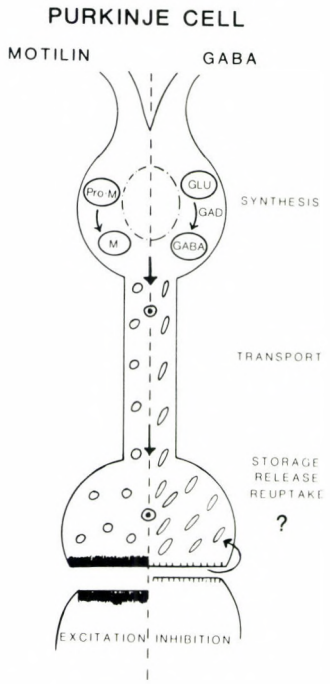

Fig. 7. Schematic diagram of Purkinje cell with motilin and GABA coexisting in its soma and processes. Separate mechanisms for the synthesis, transport, storage, and release of motilin and GABA are illustrated. A translation of promotilin (pro M) to motilin (M) is suggested for motilin on the left, whereas the synthesis of GABA from glutamate through GAD is shown on the right. The diagram raises these issues: What are the sizes and shapes of synaptic vesicles and the physiological functions associated with each neuroactive compound?

- Are the pure motilin-immunoreactive, pure GAD-immunoreactive, and dual motilin/GAD-immunoreactive cells related in a cyclic manner so that they are not separate cell types but a single type of neuron in different phases fixed in time by an immunocytochemical experiment? (See Fig. 8.)

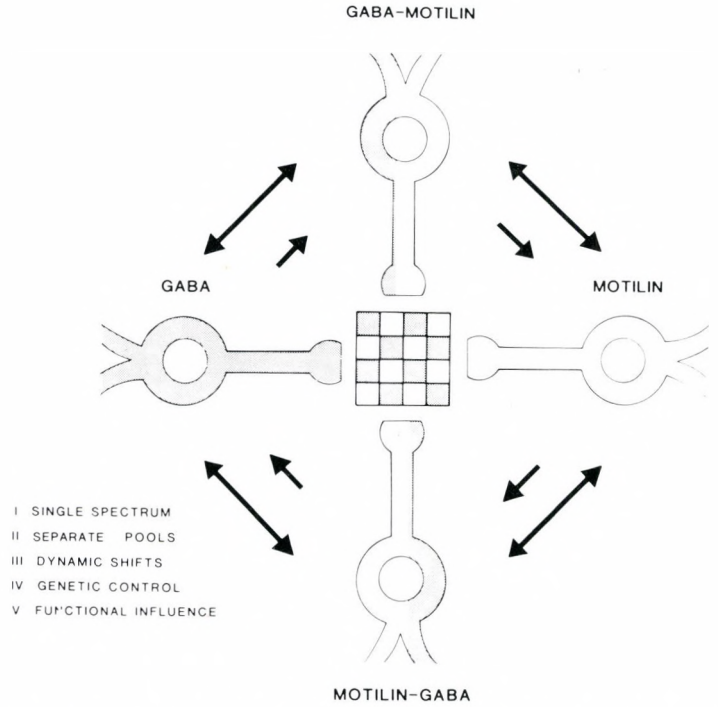

Fig. 8. Schematic diagram to illustrate the concept of dynamic interrelationships between motilin and GABA in a single neuron. A neuron with both substances in coexistence may have fluctuating levels of one or both substances depending upon parameters of rhythm, time, and physiologic demands for one or another mediator during specific types or phases of activity

- Motilin and GAD cells occur in small foci. Can one trace the origins of these groups of Purkinje cells to specific precursor cells very early in development and test these hypotheses of cyclicity and genetic regulation?
- At least 30% of all cerebellar Purkinje cells have neither GAD nor motilin immunoractivity. What other neuroactive substances might they contain?
- What neuromediative role does motilin have? Is it inhibitory, noninhibitory, "modulatory" or excitatory? Could it be a substance with no part to play in neuro-

transmission and neuromodulation, but perhaps with a trophic function?

These are a few of the many questions that are raised by the fact that Purkinje neurons (and likely other cerebellar neurons) are chemically heterogeneous, and have a chemical repertory for communication that is multimodal. The finding calls for a profound reassessment of how the cerebellum works.

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EVIDENCE FOR SYNAPTIC PLASTICITY IN THE CEREBELLAR CORTEX

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The learning machine model of the cerebellum by Marr and Albus contains a special type of synaptic plasticity. Experimental evidence for this synaptic plasticity has been meager, but very recently positive evidence has become available. Ito, Sakurai and Tongroach (7) demonstrated the occurence of a long-lasting depression in mossy fiber responsiveness of Purkinje cells subsequent to conjuctive stimulation of mossy fibers and climbing fibers. A similar long-lasting depression was shown to occurred in sensitivity of Purkinje cell dendrites to a putative neurotransmitter of parallel fibers, i.e., glutamate. Furthermore, Ito and Kano (6) produced a long-lasting depression in the molecular layer of the cerebellar cortex by simultaneous direct stimulation of parallel fibers and climbing fibers. These long-lasting depressions appear to represent a synaptic plasticity of the form proposed by Albus.

Any intricate electronic circuitry may have only a limited capability of information processing, if it contains no memory device. Therefore, when the neuronal circuitry of the cerebellum was dissected in detail in 1960's [2], it was natural that Marr [9] and Albus [1] introduced an assumption of synaptic plasticity as a memory device into their learning machine models of the cerebellum. This assumption implies that the transmission efficacy from a parallel fiber to a Purkinje cell is plastically modified when that parallel fiber activates the Purkinje cell conjointly with a climbing fiber. Validity of this assumption has been a matter of debate during the past decade, but recently positive

evidence has become available. Ito, Sakurai and Tongroach [7] demonstrated that conjunctive stimulation of vestibular mossy fiber afferents and climbing fibers induces a longlasting depression in responsiveness of flocculus Purkinje cells to the vestibular mossy fiber inputs. The site of plasticity is likely to be at the parallel fiber-Purkinje cell synapses involved in the vestibular mossy fiber-Purkinje cell pathway, because no evidence suggests its occurrence at other synapses. Furthermore, sensitivity of Purkinje cells to a putative neurotransmitter of parallel fibers, i.e., glutamate, was found to undergo a long-lasting depression when glutamate was applied to Purkinje cell dendrites in conjunction with climbing fiber stimulation. This observation suggests that the plasticity involves chemosensitivity of Purkinje cell postsynaptic membrane to parallel fiber neurotransmitter. Further evidence for the synaptic plasticity in the cerebellar cortex was obtained by Ito and Kano (1982) using direct electrical stimulation of parallel fibers and climbing fibers. This article introduces the results of this experiment.

In high decerebrate rabbits, superficial folia of the dorsal paraflocculus or the posterior vermis were exposed by partial craniotomy, and covered by mixture of mineral oil and vaseline or an agar layer. A bundle of parallel fibers was stimulated at 200-400 μm from the pial surface by passing 20-60 μA current pulses of 0.2 msec duration through a glass microelectrode. The evoked responses were recorded with another glass microelectrode inserted into the molecular layer at a distance of 0.7-1.0 mm along the longitudinal folial axis from the stimulating electrode. Climbing fibers were stimulated

directly at the inferior olive with bipolar metal electrode inserted dorsoventrally into the inferior olive.

The molecular layer stimulation evoked, along the beam of excited parallel fibers, field potentials consisted of two negative peaks, n_1 and n_2. n_1 represents conducting spike potentials of parallel fibers and n_2 synaptic currents and thereby evoked action currents in Purkinje and other cortical cells [2, 8]. This interpretation is supported by the fact that destruction of Purkinje and other cortical cells, except granule cells, with kainic acid resulted in a substantial reduction of the n_2 potential, while n_1 potential remains normal.

Climbing fibers were stimulated a 4Hz at which spontaneous discharge from Purkinje cells is not affected [7]. Conjunctive stimulation of climbing fibers and parallel fibers was performed

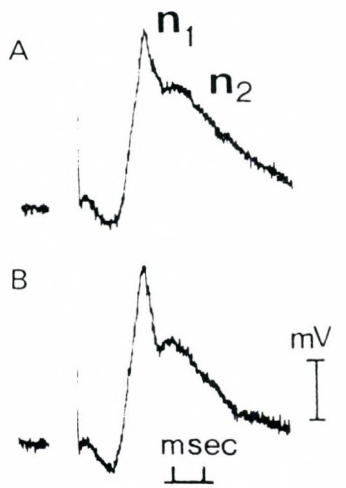

Fig. 1. Field potentials recorded from the molecular layer of rabbit cerebellum in response to direct stimulation of parallel fibers. Pen recorded potential curve averaged 30 times at a sweep rate of 2 Hz. n_1, n_2, two negative peaks. A, before and B after conjunctive stimulation of the parallel fibers with climbing fibers at 4 Hz for 60 sec. The time separation during the conjunction was 10 msec. Note the reduction of n_2 in B

with time separation of 5 to 12 msec between the climbing fiber stimulation and the parallel fiber stimulation. It was found that a trial of conjunctive stimulation for 30 to 120 sec induced an appreciable depression in the n_2 potential, without affecting the n_1 potential, as exemplified in Fig. 1. The depression was progressive after termination of the conjunctive stimulation, and reached a peak at 20 to 30 min. Thereafter, it turned to recover, but only a partial recovery was seen one hour after the conjunctive stimulation. Stimulation of climbing fibers or mossy fibers alone had no such a depressant effect on the n_2 potential.

During this experiment, it was recognized that the n_2 potential is highly vulnerable to local damage of the molecular layer. The n_2 potential rapidly diminished when bleeding or edema was caused by repeated insertion of a microelectrode into the molecular layer. The depressant effect of the conjunctive climbing fiber and mossy fiber stimulation was apparent only when the n_2 potential was sufficiently large, reflecting good experimental conditions of the molecular layer.

The above mentioned results by Ito and Kano [6] confirmed the previous results by Ito et al. [7] in a more direct manner and support the theoretical prediction by Marr [9] and Albus [1]. These results obtained by electrical stimulation are consistent with the inference based on the observations of Purkinje cell discharges during motor learning by alert animals [3,4,5]. These different lines of observations consistently suggest that the effect of conjunctive activation of climbing fibers and parallel fibers is depression at parallel fiber--Purkinje cell synapses in accordance with the assumption made by Albus [1].

Positive evidence thus afforded for the plasticity assumption encourages further investigation into molecular mechanisms of the synaptic plasticity in the cerebellar cortex. At the present time, two possible mechanisms are considered [7]; 1.) enhanced intradendritic Ca^{2+} concentration in Purkinje cells after activation through climbing fibers leads to desensitization of postsynaptic receptors in Purkinje cells to parallel fiber neurotransmitter, or 2.) climbing fibers liberate a certain substance (s) which, in cooperation which parallel fiber neurotransmitter, inactivates the postsynaptic receptors. It is an interesting future task to find out which is the case, or if a third mechanism accounts for the phenomenon.

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CEREBELLAR PATHWAYS IN THE BRAIN OF THE MORMYRID TELEOST FISH

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The hypertrophy of the mormyrid brain is due not only to the increased volume of the cerebellum but also to that of several other structures in the rhomb-, mes- and diencephalic brainstem. HRP injections in the different parts of the cerebellum - lobus caudalis, corpus cerebelli, valvula cerebelli - of the teleost fish *Gnathonemus petersii* (Mormyridae) reveal, besides common and specific sensory patterns, an important projection of the brainstem reticular formation towards the corpus and valvula cerebelli. According to the established connections, impulses of different sensory modalities are conveyed by specific pathways to certain cerebellar areas: common sensory and proprioceptive impulses to the lobus caudalis electrosensory and acousticolateral vestibular impulses to the valvula. The corpus cerebelli receives impulses only of this latter modality. These sensory impulses arrive in the cerebellum by second (common and proprioceptives) and third order neurons of the respective sensory pathways.The mesencephalic brainstem projection conveys impulses from the telencephalon and lobus caudalis whereas a part of the epithalamic projection conveys visual impulses towards the corpus and valvula cerebelli.

Through efferent connections, all the cerebellar structures considered control the mesencephalic reticular formation. The valvula, in addition, may control different sensory information at the bulbar-mesencephalic and telencephalic levels, whereas the lobus caudalis exerts a similar influence at the posterior lateral line lobe.

The extraordinary size of the mormyrid brain was discovered more than a hundred years ago by [5], who estimated that it was equivalent in importance to the human brain, if the brain/body weight ratios were compared. Indeed, for the average human, with a body weight of 70 kg and a brain weighing 1.35 kg, this ratio is 1:50 and a similar figure would be found for the teleost *Gnathonemus petersii*, in a specimen weighing 20 g. Compared to several other fish, the relative brain weight of the mormyrid is twice as great [1].

Since Erdl's discovery, it has been shown (Marcussen, 1864) that the huge size of the mormyrid brain is due to the extensive hypertrophy of the cerebellum, and in particular, the valvula cerebelli (Fig. 1). Several authors [4,10,18,23] pointed out later that the increased volume of other brain structures such as the acoustico-lateral area, the mesencephalon and the corpus cerebelli may also contribute to the very large size of the mormyrid brain. These observations led certain authors [4,9,16] to speculate on the functional aspect of this particular cerebellum and to relate it to the highly developed acoustico-lateral line system. The electrophysiological studies of Russel and Bell [22] have shown that some parts of the cerebellar valvula are functionally related to the electrosensory contingent of the acoustico-lateral system. However, only recent investigations, using different

tracer methods [2,3,11,13] have provided the necessary details for an understanding of its complexity and to explain the unusual development of this teleost cerebellum [13].

According to Nieuwenhuys and Nicholson [18], one can distinguish five regions in the mormyrid cerebellum: valvula, corpus cerebelli, lobus caudalis, lobus transitorius and eminentia granularis. We will deal here only with the first three, considering their basic structure, their cellular elements and extracerebellar connections.

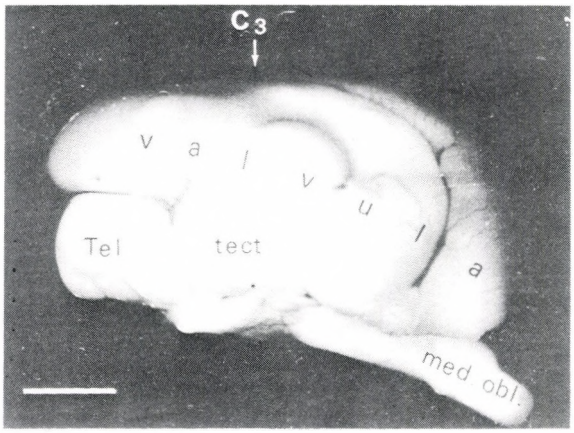

Fig. 1. Lateral view of the mormyrid teleost fish Gnathonemus petersii. Note the huge size of the cerebellar valvula (valvula) which covers almost entirely the dorsal and lateral brain surface. Tel, telencephalon; tect, optic tectum; med. obl., medulla oblongata. Bar, 4 mm. By courtesy, [12]

Among these, the corpus cerebelli (Fig. 2A) shows the most typical cerebellar structure with its molecular, ganglionic and granular layers bounded by the cerebellar white matter.

The valvula (Fig. 2B) is also constituted of three layers, although its general configuration is particular: the molecular layer is ridged, each ridge having a double ganglionic layer; the granular cells form a common layer at the base of the ridges

and do not penetrate the ridges. In this the mormyrid valvula is quite different, for instance, from the valvula of the trout (Fig. 2D) which has a classical tri-layered structure.

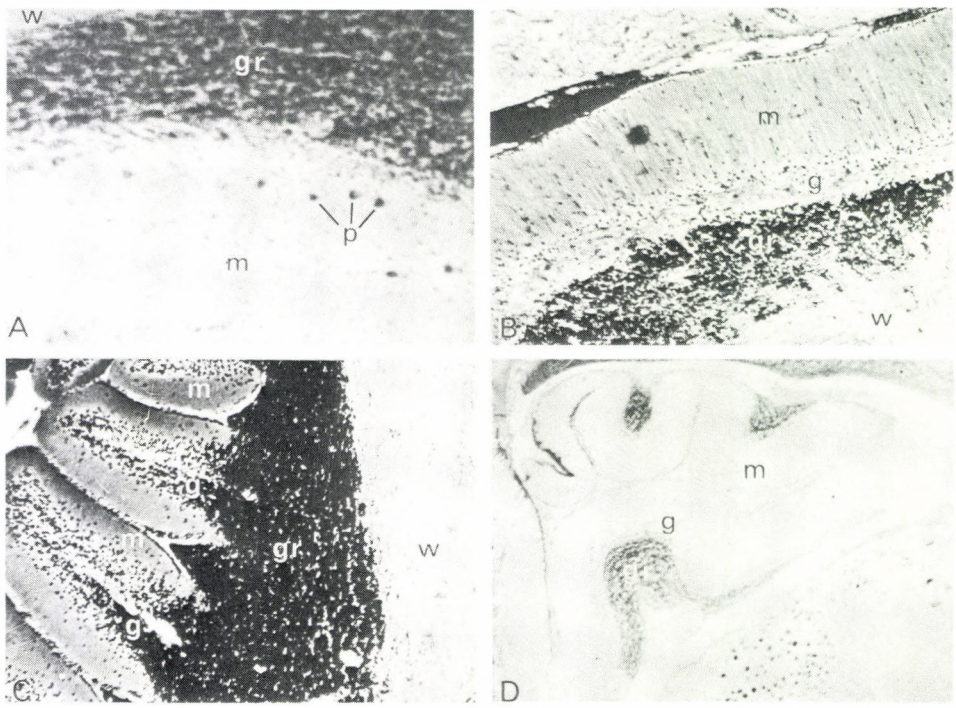

Fig. 2. Light microscopical photographs of different cerebellar regions: A, lobus caudalis; B, corpus cerebelli; C and D, valvula cerebelli. A, B, C, Gnathonemus petersii; D, trout. B and C, Bodian silver impregnation; A and D, cresyl violet stain. g, ganglionic layer; gr, granular layer; m, molecular layer; P, Purkinje and Purkinje-like cells; w, white matter. Bar,....

The caudal lobe also possesses the three basic cerebellar structures; however, the large Purkinje-like cells are not arranged in a single layer, but distributed irregularly in the molecular layer which is constituted by the mesh work of their dendritic processes.

According to Nieuwenhuys and Nicholson [19] the corpus cerebelli and the valvula (the only structures studied

histologically by these authors) have a similar basic cellular constitution. In the corpus cerebelli, the ganglionic layer contains three cell types: Purkinje, giant and fusiform. In the valvula "the ganglionic layer contains a single stratum of Purkinje cells and three other types of neurones: basal, vertical and central cells". In both, the dendrites of all these elements penetrate into the molecular layer. In both the axons of the granular cells reach the molecular zone where they bifurcate and form the parallel fibre system.

The existence of various cell types in the ganglionic layer raises a puzzling problem: which kind of cell constitutes the efferent pathway of the cerebellar cortex? It is still an unsolved question whether deep cerebellar nuclei are present in the cerebellum of the teleost. Kappers [14] and Pearson [21] assumed that Purkinje cells form the efferent pathway. According to Franz [9], associative giant cells (basal and giant cells of Nieuwenhuys) may link together several Purkinje cells and the axons of some of these form the cerebellar efferent pathway. Probably inspired by Franz's idea, Nieuwenhuys [20] proposed that the Purkinje cell axons contact the giant cells (termed eurydendroid cells on the basis of the relatively wide extent of their dendritic trees), the axons of which constitute the cerebellar output.

HRP injections into the mesencephalic brainstem partly confirm Nieuwenhuys' proposition: such an injection filled the basal cells in a certain number of valvular ridges (Fig. 3). The labelled dendritic trees of these cells at the base of the ridges allow a good identification of them as basal cells. Finger [6] came to a similar conclusion: he also found labelled

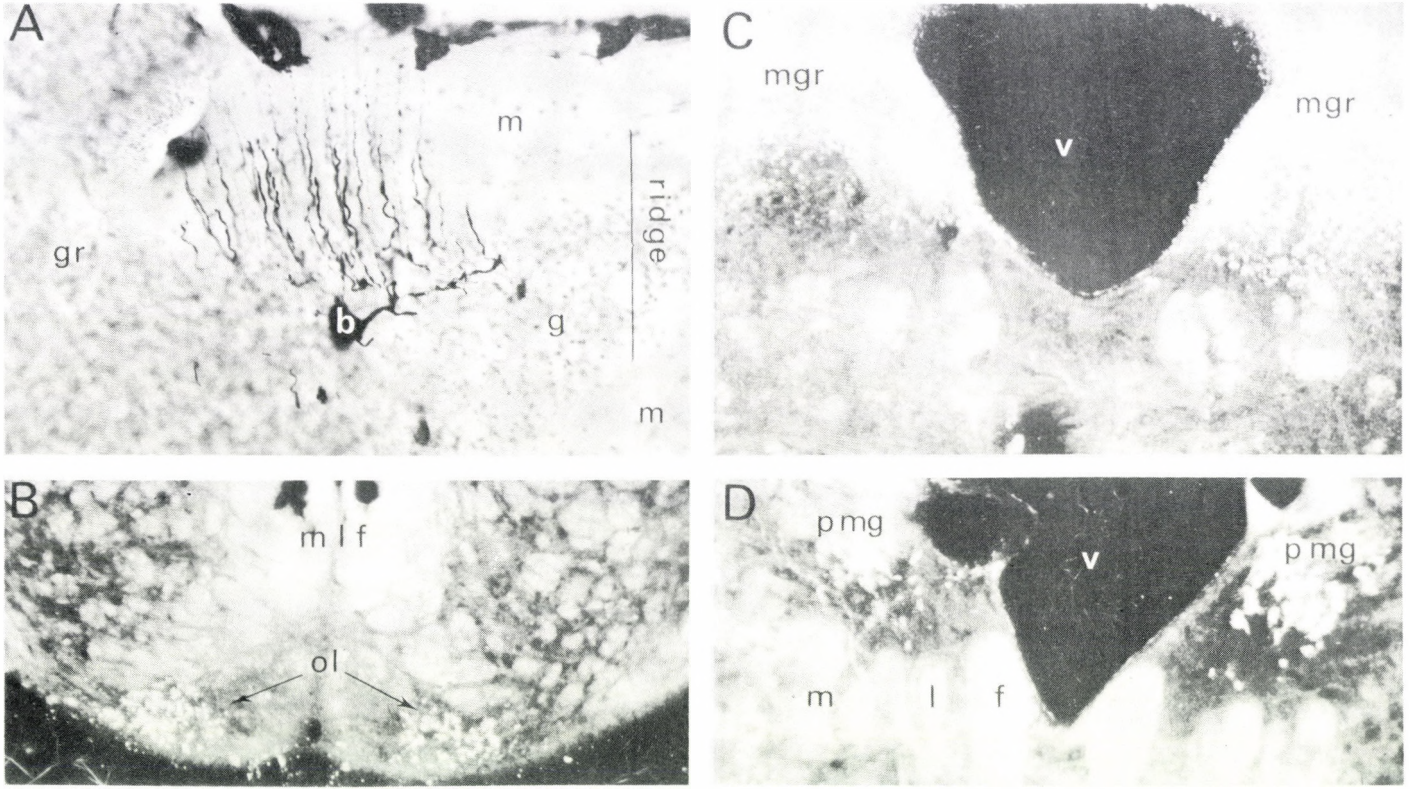

Fig. 3A. Labelled basal cell (b) of the valvula after HRP injection into the mesencephalic brainstem. Note the position of b at the base of the ridge. B, Labelling of the inferior olive, ol. C, mesencephalic granular cells, m gr, and D, cells of the posterior magnocellular nucleus, p mg, in the mesencephalic tegmentum after injection into the corpus cerebelli. Pollimyrus isidori. Light, A, and dark, B-D, field micrographs. m, molecular layer; g, ganglionic layer; gr, granular layer; mlf, medial longitudinal fasciculus;v, ventricle. Bar,....

large cells in the cerebellar cortex after mensencephalic injections, which he identified as "associative cells". However, basal cells seem not to be the only elements of the cerebellar cortex which, in mormyrids, contribute to the efferent pathway. With injection of HRP into certain nuclei of the torus semicircularis Finger et al. [8] found different kinds of labelled cells in the core of the cellular ridges. Although the dendritic tree was not labelled in these cases, the authors identify these cells as basal and, higher up in the ridge, as central cells. They claim that no Purkinje cells were labelled. It is interesting to note that neither is the caudal lobe efferent pathway constituted by Purkinje cell axons [3]. According to these findings one may conclude that the cerebellar efferent pathway takes its origin in different cell types of the cerebellar cortex, other than Purkinje cells.

While searching for connections in the mormyrid brain, our experimentation with HRP labelling revealed a certain number of particularities concerning the cerebellar afferents.

One of these conserns the spino-cerebellar connections. The mormyrid spinal cord exhibits a well developed dorsal column with long ascending fibres. Originating probably from the spinal ganglia, these fibres are relayed in the clearly distinct dorsal funicular nuclei - in which primary fibres also end - which in turn project to the caudal lobe of the cerebellum. In additions, the spinal cord contains in its ventro-lateral quadrant another long ascending fiber system which originates in large cells distributed along the spinal cord and projects to the caudal lobe.

Neither dorsal columns nor long ascending fibers have been demonstrated until now in any teleost fish.

A second particularity is the olivo-cerebellar pathway hitherto not demonstrated for any teleost brain. The inferior olive is not a thoroughly recognised structure of the teleost brain [14]. HRP injections into the cerebellar corpus or valvula disclose a strongly labelled cellular mass of spindle shaped cells at the caudal limit of the medulla just beneath the medial longitudinal fasciculus (Fig. 3B). Its location and its direct connection with the cerebellum show this cellular mass to be the homologue of the inferior olive, the caudal part of which projects to the valvula, while its cranial part projects to the corpus cerebelli.

The particularity of this olivo-cerebellar connection lies in the bilateral projection; in contrast to all known olivo--cerebellar pathways recognised as entirely crossed, mormyrids exhibit, in addition, uncrossed olivo-cerebellar fibers.

A third particularity of the mormyrid cerebellum is the large afferent projection from the mesencephalic brainstem. Any HRP injection into the cerebellar cortex results in a massive labelling of a large population of small granular-like cells (Fig. 3C) and that of several groups of large cells i.e. magnocellular nuclei (Fig. 3D) distributed throughout the mesencephalic tegmentum. The granular mass surrounds the major descending and ascending tracts in the mesencephalic brainstem and its posterior part extends over the ventral and ventro-lateral region of the brainstem, while its anterior limit reaches the ventricle and the valvular peduncle in the dorsal tegmentum. This projection is mainly ipsilateral.

No pontic structure has ever been mentioned in the brain of any non-mammalian vertebrate. However, the different characteristics

of the labelled elements - their location, cellular composition and projection areas - strongly suggest that they may represent a precursor of the pons. Although the bulk of the afferents to these tegmental structures has not yet been established, a telencephalic projection to the periventricular granular cells has already been traced (unpubl. obs.), a fact which again supports the above mentioned hypothesis.

Finally, a last particularity of the mormyrid cerebellum concerns the large projection from the diencephalon. The labelled structures are the so called thalamic nuclei, and two nuclei in the epithalamus, the pretectal nuclei and that of the posterior commissure. Similarly to the mesencephalic afferents, the diencephalic afferents towards the cerebellum are mainly ipsilateral.

So far as could be traced in the literature, projections from the diencephalon to the cerebellum have not been found. In higher vertebrates, the thalamus projects exclusively towards the telencephalon. Cerebellar connections through the superior peduncle contain - except the ventral spino-cerebellar tract - only efferent pathways to the mesencephalic tegmentum. Consequently, one may ask whether the "thalamic" denomination of these diencephalic structures is correct. Their connection with the cerebellum certainly does not justify this appellation. However, diencephalic projections to the cerebellum have also been described in other teleost fish [7].

In addition to these particularities of the mormyrid cerebellum, other cerebellar pathways have been previously established [10, 23] and traced [2,8,11,15,25] from specific nuclei of the ventral and dorsal mesencephalon: nucleus

preeminentialis, nucleus lateralis, mediodorsalis and medio-ventralis.

Fig. 4. Schematic representation of labelled structures in the brain of Gnathonemus petersii after HRP injection into the left valvula. Note in particular the substantia granularis (hatched areas). Labelled nuclei in the tegmentum are marked by small dots, in the dorsal mesencephalon by large dots. 1, nucleus pretectalis; 2, nucleus of the posterior commissure; 3, anterior magnocellular nucleus; 4, lateral magnocellular nucleus; 5, posterior magnocellular nucleus; 6, nucleus magnocellularis preeminentialis tegmenti; 7, X sensory nucleus; 8, nucleus sub-funicularis; valv, valvula; t. long., torus longitudinalis;

The cerebellar afferent connections (Fig. 5A) may be summarized by considering the constitution of the cerebellar peduncles. As in the classic description of the cerebellum in higher vertebrates, one may also distinguish three cerebellar peduncles in the mormyrid brain.

(a) The superior cerebellar peduncle contains spinal and a majority of bulbo-cerebellar fibers, projecting to the caudal lobe. These fibers convey cutaneous and proprioceptive impulses from the common sensory system.

(b) In the middle cerebellar peduncle fibers course from the mesencephalic reticular formation, the inferior olive and different nuclei of the torus semicircularis and project to the corpus cerebelli and the valvula. The fibers from the torus semicircularis convey specific sensory impulses, i.e. electro- and mechanosensory as well as auditory impulses.

(c) The superior peduncle contains fibers from the thalamus and epithalamus projecting to the corpus cerebelli as well as to the valvula. The fibers arising from the epithalamic pretectal nucleus convey visual impulses towards the cerebellum. Also, certain electrosensory impulses are conducted from the lateral nucleus via the postventral thalamic nucleus towards the cerebellum.

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tect., optic tectum; co.p.ch., postchiasmatic commissure; co.p., posterior commissure; C1, C2, C3, corpus cerebelli; nELa and nELp nucleus exterolateralis anterior and posterior; ped, valvula peduncle; nL, lateral nucleus; nVP, ventroposterior nucleus; nMD, mediodorsal nucleus; nMV, medioventral nucleus; n pv th postventral thalamic nucleus; co.ans, commissure ansulata; l inf, lobus inferior; EG, eminentia granularis; GI, ganglion isthmi; cc, crista cerebelli of anterior lateral line lobe; LLL, posterior lateral line lobe; ll, lemniscus lateralis; nP, nucleus preeminentialis; NX, vagus nerve; ol, olive; v, ventricle [11].

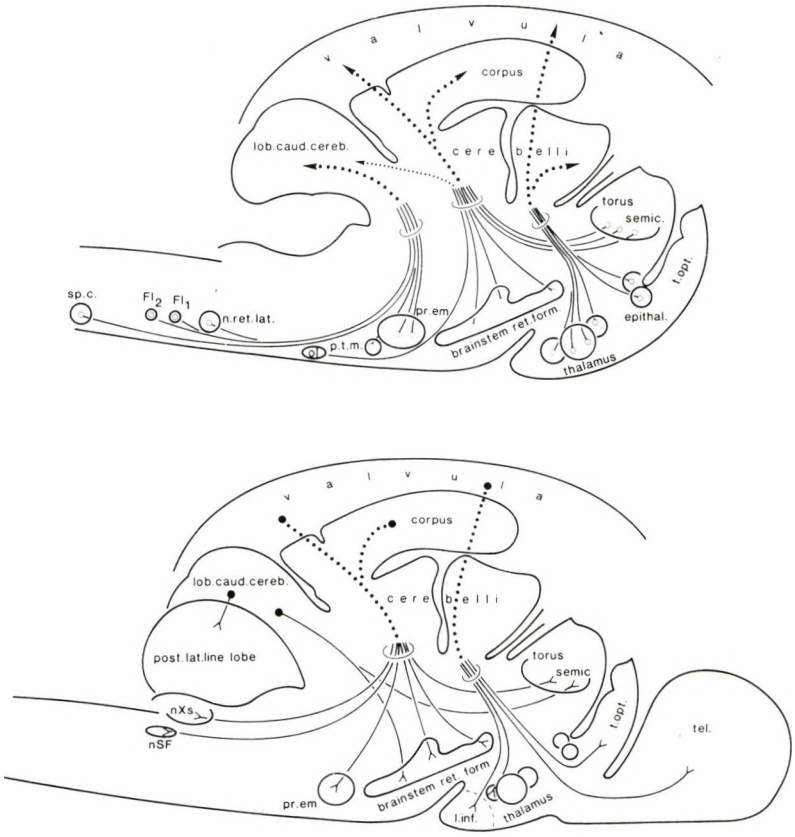

Fig. 5. Schematic representation of afferent (A) and efferent (B) connections in the mormyrid cerebellum Gnathonemus petersii. Note the bundling of afferent fibers in three cerebellar peduncles. sp.c., spinal cord; F12 F11, dorsal funicular nuclei; n.ret.lat., lateral reticular nucleus; p.tr., medial and lateral paratrigeminal nucleus; pr.em. nucleus preeminentialis; lob. caud. cereb., cerebellar caudal lobe; brainstem ret. form., brainstem reticular formation; torus semicirc., torus semicircularis; t.opt., optic tectum; epithal., epithalamus; post. lat. line lobe, posterior lateral line lobe; nXs, vagal sensory nucleus; nSF, nucleus subfunicularis; l. inf., lobus inferior; tel., telencephalon

The three peduncles also contain efferent fibers (Fig. 5B). In the superior cerebellar peduncle, efferent fibers are coursing towards the thalamus, the hypothalamus, the optic tectum and the telencephalon, whereas in the middle cerebellar peduncle efferent fibers are running towards the brainstem reticular formation,

the nucleus preeminentialis and towards certain sensory nuclei in the medulla. The efferent fibers in both originate in the corpus cerebelli or in the valvula. The inferior cerebellar peduncle may also contain some efferent fibers which originate in the caudal lobe and project to the nucleus preeminentialis, the mesencephalic reticular formation and the torus semicircularis. The lobus caudalis also projects directly to the posterior lateral line lobe.

The only available recent survey on pathways of the mormyrid cerebellum is that of Nieuwenhuys and Nicholson [18]. The schemes presented by these authors are entirely based on publications of the beginning of this century [9,10,23,24]. The findings reported presently allow to revise some of these fiber connections.

Neither the corpus cerebelli nor the valvula receive fibers from the spinal cord. The only detected spino-cerebellar tract ends in the caudal lobe and the bulk of the spinal impulses conducted by indirect pathways arrive also to the caudal lobe. Neverthless, the corpus cerebelli as well as the valvula may receive spinal impulses from the spinal cord via the inferior olive or the lateral reticular nucleus [3].

The other important revision concerns the large direct afferent valvular projection from the mesencephalic extrolateral nucleus. There is no such a direct projection: the electrosensory impulses from the extrolateral nucleus are relayed by several mesencephalic nuclei before reaching the valvula [11].

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NEURONAL MECHANISMS OF EXPERIENCE-DEPENDENT SELF-ORGANIZATION OF THE MAMMALIAN VISUAL CORTEX

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The analysis of cortical circuitry with morphological methods has revealed a phantastic degree of complexity of intracortical connectivity and for long this appeared far beyond the reach of physiological analysis. Thanks to the ingenious gift of John Szentágothai to extract basic principles of organization from what appears as an unresolvable puzzle, physiologists have now been provided with testable models of cortical wiring. Hence, during the past years we experienced a very fruitful convergence between morphological and physiological approaches and we now possess some insight into the general principles of cortical organization. One of the issues of these joint efforts is that intracortical pathways are highly specific and far from forming a meshwork of random connections. This in turn raises the challenging question of how nature solves the problem to specify the myriads of interneuronal contacts during development. This question applies of course to the development of nervous tissue in general but apart from the quantitative differences in complexity there is one feature which distinguishes cortical development from most other developmental processes. The formation and final determination of cortical connections occurs to

a considerable extent only postnatally and hence already under the influence of sensory experience.

This dependency of the developmental process on sensory experience is particularly well investigated and documented for the visual cortex of mammals. It is established that the functions of visual cortex develop normally only when visual experience is available during a critical period of early postnatal development. Conversely, the structural and functional organization of the visual cortex can be altered profoundly and irreversibly by manipulating early visual experience.

In the following paragraphs I shall illustrate with a few examples the phantastic malleability of the developing visual cortex and subsequently I shall discuss the mechanisms that are likely to mediate these experience dependent modifications. The conclusion will be that these processes are well adapted in order to specify and to optimize cortical connections according to functional criteria. This specification is thought to consist of an activity dependent selection process whereby particular subsets of excitatory connections increase their efficiency and consolidate while others weaken and eventually retract from their target. The criteria of selection appear to closely resemble those postulated by Hebb [11] for adaptive synaptic connections; Hebb assumed that afferents increase their gain and consolidate when the probability is high that they are active in temporal concordance with the postsynaptic cell while their gain decreases when this probability is low. In extension of these classical rules I shall discuss recent data which indicate that the Hebbian modifications are in turn gated by additional control systems. In order to induce Hebbian modifications retinal

signals must not only match the receptive field properties of the cells in striate cortex but must in addition be adequate in the more global context of polymodal and visuo-motor integration. Moreover, the retinal signals need to be processed by an awake brain and have to be attended to.

EVIDENCE FOR HEBBIAN MECHANISMS AT THE LEVEL OF BINOCULAR CONVERGENCE

By the time kittens open their eyes most neurones in visual cortex respond to stimulation of both eyes and with normal visual experience this condition is maintained [12, 13]. However, when signals from the two eyes are incongruent, either because one eye is occluded [40] or because the images on the two retinae are not in register - as is the case with strabismus [14], cyclotorsion [5, 8, 42], or anisometropia [4, 41] - cortical cells loose their binocular receptive fields. In the first case they stop responding to the deprived eye; in the other cases they segregate into two approximately equally large groups one responding exclusively to the ipsilateral and the other exclusively to the contralateral eye. During the critical period of early development these changes are fully reversible indicating that the efficacy of connections does not only decrease but can also increase as a function of retinal stimulation [4, 40]. Both processes depend on responses of the postsynaptic target cells. If the latter fail to respond to signals from either of the two eyes, changes in synaptic gain do not occur even if the pathways from one eye are much more active than those from the other. If, for example one eye is occluded light tight while the other is

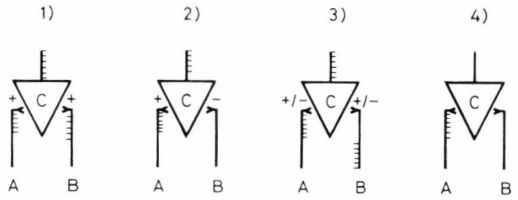
stimulated with flashes of diffuse light the ocular dominance of cortical cells does not change [34]. The reason is that the postsynaptic neurones in striate cortex cannot respond to changes in ambient illumination. Conversely, when one eye is occluded and the other exposed to contours of only a single orientation, differential gain changes occur only at junctions with those postsynaptic cells that are capable of responding to the signals conveyed by the open eye [25, 28]. For these cells the efficacy of afferents from the stimulated eye increases while that of afferents from the deprived eye decreases. Cells, by contrast, whose orientation preference does not correspond to the orientations seen by the stimulated eye cannot respond to activity from this eye and do not change their ocular dominance. Here, the afferents from the stimulated eye, even though they are much more active than those from the deprived eye do not increase their efficacy at the expense of the latter. The results of these and related experiments [26] made it possible to formulate three basic rules which have proven sufficient to account for the results of most if not all deprivation experiments published so far. These rules closely resemble those postulated by Hebb [11] for adaptive neuronal connections and can be summarized in the following way: 1) The gain of synaptic connections increases for afferent fibers if they are active in temporal contiguity with the postsynaptic target. 2) The gain decreases when the postsynaptic target is active while the presynaptic terminal is silent. 3) Irrespective of the amount of activation of presynaptic terminals differential gain changes do not occur when the postsynaptic cell is inactive (Fig. 1). Whether the required postsynaptic response has to consist of action potentials, or

whether the important parameter is the level of dendritic depolarization is still nuclear. As discussed below indirect evidence supports the latter assumption.

RULES FOR SYNAPTIC MODIFICATION

state of afferent pathway	active	inactive	active or inactive
state of postsynaptic element	active	active	inactive
change of synaptic gain	increase	decrease	no change

EXAMPLES



consolidation:	A and B	A	A or B	neither A nor B
disruption:	Ø	B	B or A	neither A nor B

Fig. 1. Rules for activity dependent modifications of neuronal connectivity in the developping visual cortex. Example 1: Afferences A and B are active simultaneously and in contingency with postsynaptic target. Example 2: Only afference A is active in contingency with C, afference B is inactive. Example 3: Both afferences A and B are active but not simultaneously; C is responding to both inputs. Example 4: C is inactive; the state of afferences A and B is irrelevant, changes in gain do not occur

EVIDENCE FOR HEBBIAN MECHANISMS IN EXPERIENCE-DEPENDENT MODIFICATION OF ORIENTATION SELECTIVITY

In three-week-old kittens reared without visual experience a substantial fraction of striate cortex cells possess a preference for stimulus orientation [6, 13, 27]. With increasing duration of visual deprivation the percentage of these cells de-

creases while it increases to nearly 100% with normal visual experience [6]. In the latter case cells with preferences for horizontal, vertical and oblique orientations are about equally frequent. When, however, then kitten experiences only contours of a single orientation the majority of cortical cells adopt preferences for this orientation [for a review of the extensive literature see 23]. Two recent studies, one based on single cell recording [26] and the other on deoxyglucose mapping of orientation columns [36], both indicate that such experience-dependent distortions in the distribution of orientation preferences could also result from Hebbian competition between converging excitatory pathways. The main finding of the deoxyglucose study was that restricting visual experience to a single orientation had relatively little influence on the development of the orientation column system within layer IV but produced massive distortions of the columnar system within non-granular layers. While cells responding to inexperienced orientations were still present within layer IV and were grouped within regularly spaced bands, activity from these cells was no longer relayed to cells in supra- and infragranular layers. By contrast, activity of layer IV cells whose orientation preference corresponded to the experienced orientations was now relayed not only to non-granular cells located above and below the active zone in layer IV but spread tangentially to adjacent cells (Fig. 2). In this respect the reorganization of the orientation column system after orientation deprivation closely resembles the reorganization of the ocular dominance columns after monocular deprivation [16]. Only the site of competition is different: In the case of monocular deprivation, competition occurs where afferents from

the two eyes converge onto the common cortical target cells which are located mainly in layer IV. In the case of orientation deprivation competition occurs most likely at the level where axons from orientation selective layer IV cells converge onto second order target cells that are located mainly in non-granular layers.

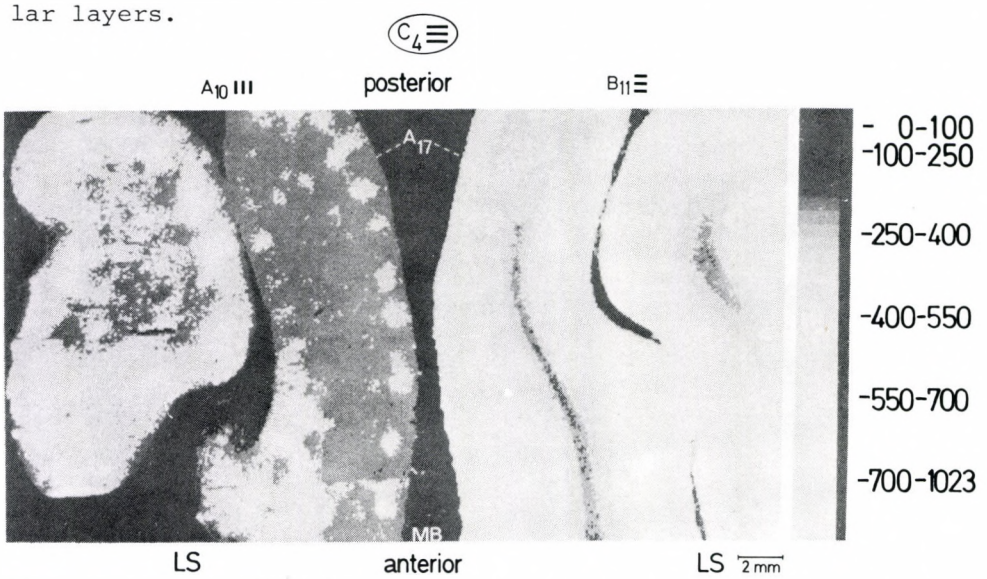


Fig. 2. Orientation columns in striate cortex of a kitten (C4) who had experienced only horizontally oriented contours. To demonstrate the topography of orientation columns the kitten was injected with 14C-deoxyglucose and then one hemisphere was stimulated with vertical (A 10) and the other with horizontal (B 11) contours. The autoradiographs A 10 and B 11 are from horizontal sections through the occipital pole of the two hemispheres. The plane of the sections is perpendicular to the lamination of area 17. The optical density of the autoradiographs has been quantified with an image processing system (scale on right margin), bright tones corresponding to high optical density. Comparison of the two hemispheres reveals considerably more activity in the hemisphere which, after application of the deoxyglucose pulse, has been stimulated with the same orientation as that experienced previously (B 11). On this side isolated columns are barely distinguishable because they have expanded and are confluent. By contrast, on the other side, zones of increased activity are well segregated from each other and confined essentially to the granular layer of area 17. Thus, cells which are responsive to orientations orthogonal to those which have been experienced previously are still grouped in regularly spaced clusters within layer IV but activity is no longer relayed to more superficial and deeper layers (from Singer et al., 1981)

A TELEOLOGICAL ARGUMENT

These few examples of cortical malleability raise the question why nature allows visual experience to interfer with the development of cortical functions, thus exposing the developmental process to the risk that transient and accidental disturbances of the uptake of visual signals may entrain severe and irreversible impairments of cortical functions. We do not know a definite answer to this question but I would like to propose the following teleological argument: Nature takes this risk because including sensory experience as additional guide-line for developmental processes allows to attain a degree of specificity in neuronal connections that could not be realized by genetic instructions alone. The following example illustrates this proposition. Animals with binocular vision have the problem to develop neurones which possess two corresponding receptive fields, one in each eye. This implies that the two receptive fields need to be precisely superimposed in visual space and need to have the same internal structure. Hence, the pathways connecting the two eyes with the binocular target cells in visual cortex have to originate from precisely corresponding retinal loci. However, which retinal loci will actually be corresponding in the mature system cannot be anticipated with any great precision by the time when the connections develop. The reason is that retinal correspondence depends on factors such as the size of the eyes, the position of the eyes in the orbit and the interocular distance, factors which are in turn dependent on epigenetic influences. Clearly, an economical and elegant solution to this problem would be to identify according to functional

criteria the pathways which originate from corresponding retinal loci. Per definition, afferents originating from corresponding retinal loci convey identical activity patterns when the animal is fixating a target with both eyes. The Hebbian modifications of cortical circuits in turn, have the effect to selectively stabilize connections which convey correlated activity and could therefore serve to optimize the correspondence of binocular connections. However, there is an important constraint. Selection according to function can only be successful when the Hebbian processes are gated. Selection should only occur when the kitten is actually fixating a target with both eyes and it must not occur in all the many other instances in which the eyes are not properly aligned. In the latter condition, Hebbian modifications would lead to competition between the afferents from the two eyes and cause the disruption of binocularity. Thus, teleological arguments lead to postulate a central control of experience dependent modifications, and in the second part of this review, I would like to present some experimental evidence in support of such gating processes.

EVIDENCE FOR A CENTRAL CONTROL OF LOCAL HEBBIAN MODIFICATIONS

Several recent studies indicate that, in order to induce Hebbian modifications, retinal signals do not only have to drive cortical cells - in which case it would suffice that they conform with the receptive field properties of striate cortex neurones - but they must in addition be adequate in the more global context of visuo-motor integration. Even when contour vision is unre-

stricted retinal signals may fail to induce modifications of cortical connectivity when the position or the motility of the eyes are interfered with and abnormal [35, 37]. Modifications of cortical functions also fail to occur when the kittens are paralyzed while they are exposed to visual stimuli [7, 9] and when cortical norepinephrine is depleted [18-20].

A first indication of one of the sources of such permissive signals came from experiments in which we tested the effect of abolishing the proprioceptive signals that are generated by the stretch receptors of the extraocular muscles (Buisseret and Singer, in prep.). We interrupted proprioception in dark-reared five-week-old kittens by severing bilaterally the ophthalmic branches of the trigeminal nerves. In addition we either sutured closed one eye or induced strabismus by resecting the lateral rectus muscle of one eye. Subsequently, the kittens were raised in normally lighted colony rooms and investigated with electrophysiological methods after they had passed the critical period. If modifications had occurred the large majority of cortical cells should have become monocular by that time and should respond only to the open eye in the monocularly deprived kittens and to either the ipsi- or the contralateral eye in the strabismic kittens. However, in both groups we found the majority of cells still binocular, which suggests that proprioceptive signals from extraocular muscles are involved in gating experience dependent modifications of cortical connectivity (Fig. 3). In addition to the non-occurrence of competitive interactions between afferents from the two eyes we also found a number of abnormalities in other receptive field properties. In many cells responses to optimally aligned light stimuli were sluggish and

only a small fraction of the neurones had developed orientation selective receptive fields.

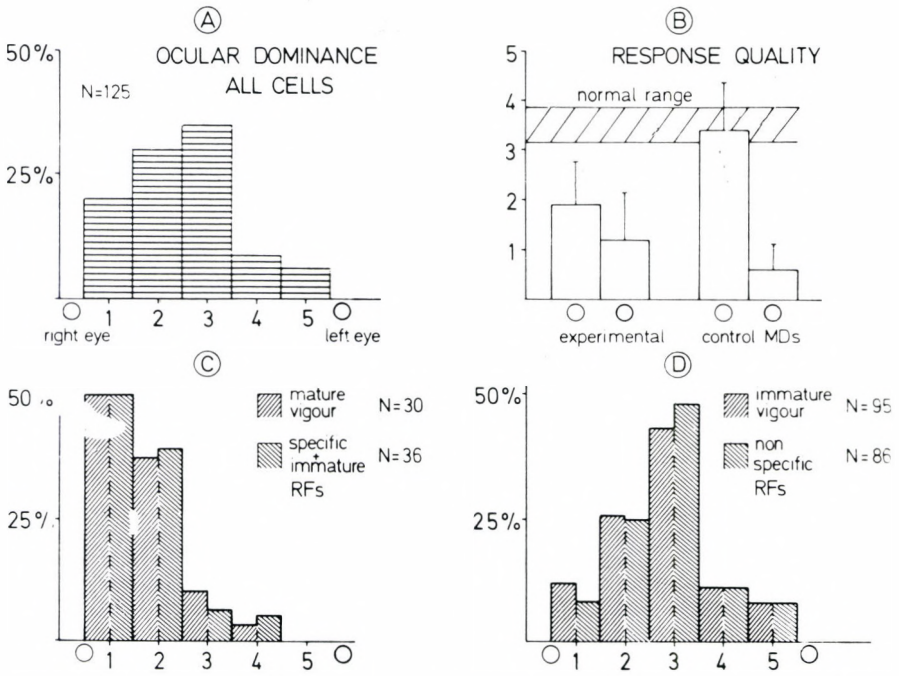


Fig. 3. Ocular dominance distributions of three kittens in which the left eye was closed by lid suture and in which proprioceptive signals from extraocular muscles were disrupted by bilateral section of the ophthalmic branch of the fifth cranial nerve. Classes 1 and 5 comprise monocular cells responding exclusively to either the right (deprived) or the left (normal) eye. Classes 2 and 4 refer to binocular cells in which one eye is dominant. Class 3 contains cells responding equally to both eyes. The ocular dominance histogram A reflects a moderate bias towards the open right eye but contrary to the condition after conventional monocular deprivation the majority of the neurones continue to respond to stimulation of both eyes. As indicated in B, the average indices for the vigour of responses to optimally aligned light stimuli are lower for the normal eye than in cats with conventional monocular deprivation or in totally normal cats (hatched range). As expected from the persistence of binocular cells the corresponding indices for responses from the deprived eye are higher in the experimental kittens than in the control MDs. In histogram (C) ocular dominance is plotted separately for cells responding vigorously to light and possessing normal or close to normal orientation selectivity; in histogram D ocular dominance is plotted of cells with immature response vigour and non-selective receptive fields. The ocular dominance distribution of the former is markedly biased towards the open eye while it remained virtually symmetrical for the latter

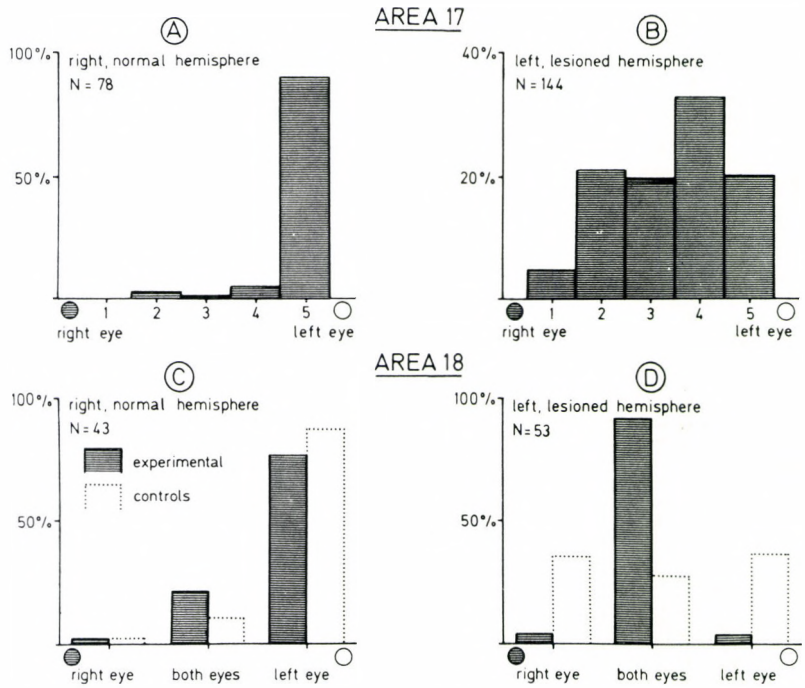


Fig. 4. The effect of unilateral thalamic lesions on developmental plasticity. Ocular dominance (OD) distributions in area 17 (A,B) and 18 (C,D) of the normal hemisphere (A,C) and of the hemisphere containing the lesion (B,D). Ocular dominance in the striate cortex was assessed from PSTH analysis of single unit receptive fields. In the hemisphere containing the lesion numerous cells have remained binocular despite monocular deprivation while in the other hemisphere ocular dominance had changed as expected from monocular deprivation. In area 18 ocular dominance was determined from single unit responses to electrical stimulation of the two optic nerves. To allow comparison with control data (columns drawn with dotted outlines) from monocularly deprived cats three classes were formed comprising cells responding to the right nerve alone, the left nerve alone, or both nerves. In area 18 the effects of conventional monocular deprivation are less pronounced in the hemisphere contralateral to the deprived eye than in the ipsilateral hemispheres (columns drawn with dotted outlines in D). However, in the hemisphere containing the lesion many more cells remained binocular than in the control cats, while in the normal hemisphere the shift in ocular dominance is similar to the shift in the corresponding hemisphere of the controls.

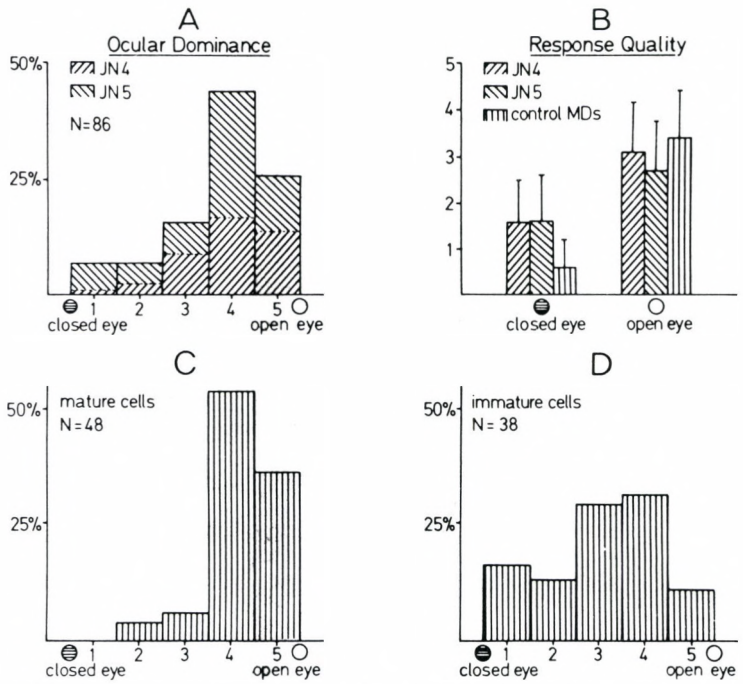


Fig. 5. Ocular dominance distributions (A,C,D) and average indices for the vigour of responses (B) of single cells recorded from striate cortex of two kittens which had 18 hrs of conditioning monocular light stimulation paired with electrical stimulation of medial thalamus prior to single unit analysis. Cells in ocular dominance classes 1 and 5 responded exclusively either to the ipsilateral closed eye or to the contralateral conditioned eye. Cells in class 3 reacted equally well to stimulation of either eye and cells in classes 2 and 4 responded more vigorously to one of the two eyes. The distribution in A summarizes the ocular dominance of all responsive cells in the two kittens 4 and 5. It shows a clear bias in favour of the conditioned eye although 67% of the cells are still binocular. The average vigour of responses from this eye has attained nearly the same level as responses from the normal eye in cats raised with conventional monocular deprivation, while the vigour of responses from the deprived eye is abnormally low but not yet as poor as that of responses from the deprived eye in conventional MDs (B). The distribution in C shows the ocular dominance of cells whose response vigour was ≥3 and whose orientation selectivity was in the normal range while the distribution in D summarizes the remaining cells in which either property was rated abnormal. This comparison reveals that the bias in the total sample of cells (A) is essentially caused by cells which have attained normal response properties [33]

EVIDENCE FOR A CONTROL OF CORTICAL MODIFICATIONS BY NON-SPECIFIC MODULATORY SYSTEMS

The kittens in which retinal signals failed to induce changes in cortical connectivity had in common a loss of attention to visual stimuli in comparison to normal kittens. In order to determine whether this was secondary to impaired maturation of visual cortex functioning or whether the deficit in visual attention was the cause of impaired maturation, a visual neglect syndrom was produced by placing lesions in the medial thalamus of kittens [31]. Subsequently, we determined whether this sensory neglect led to impairment of experience dependent consolidation of cortical functions. In five-week-old dark-reared kittens the splenium of the corpus callosum was split to allow access to the third ventricle and then a small lesion was placed in the intralaminar nuclear complex of one hemisphere. Simultaneously one eye was sutured closed to instigate ocular dominance changes and to use these changes as indicator for Hebbian modifications. As in adult cats [24] these lesions led to a contralateral sensory hemineglect. When visual stimuli were presented simultaneously in both hemifields the kittens would consistently neglect the stimulus in the hemifield contralateral to the lesion and orient towards the other target. Similarly, acoustic stimuli or somatic stimuli close to the midline elicited orienting responses that were nearly always directed towards the side contralateral to the normal hemisphere. After the kittens had grown up for at least three more months in normally lighted colony rooms the receptive fields of single cells were investigated in visual cortex of the two hemispheres. In areas 17 and 18 of the

normal hemispheres conditions were identical to those obtained with conventional monocular deprivation. By contrast, in the hemisphere containing the lesion the majority of the cells had remained binocular showing only a slight bias in the ocular dominance distribution towards the open eye (Fig. 4). Thus, although both hemispheres had received exactly the same signals from the open eye, these signals induced modifications only in the normal hemisphere and remained ineffective in the hemisphere which - because of the lesion - "attended" less to retinal stimulation. In this hemisphere also other parameters such as responsiveness to light and selectivity for stimulus orientation were abnormal indicating that retinal signals had not only failed to induce competitive suppression of the deprived afferents but had also failed to support the development of consolidation of normal receptive field properties.

Another significant abnormality of the hemisphere containing the lesion became apparent when during the experiment we tried to raise cortical excitability with electrical stimulation of the mesencephalic reticular formation. In normal animals this stimulus produces a large surface negative field potential over the visual cortex of both hemispheres and a massive facilitation of thalamic and cortical transmission [for review see 29, 30]. In the kittens with the thalamic lesion these effects were greatly attenuated in the hemisphere containing the lesion while they were fully developed in the other. Thus, the thalamic lesion has obiously affected modulatory systems known to control thalamic and cortical excitability as a function of arousal and perhaps also selective attention. This agrees with the behavioural evidence that the lesion has actually produced deficits in atten-

tion and supports the notion that modulatory systems might be involved in the control of cortical plasticity and act as a permissive gate.

This interpretation also predicts that electrical activation of the centrencephalic modulatory systems might facilitate experience dependent modifications of cortical functioning. It is well established that despite prolonged and intensive retinal stimulation modifications of cortical functions cannot be produced if the kittens are anesthetized and paralyzed [7, 9, 30]. Considering the previous results this is not surprising since these kittens are certainly not "attending" to retinal stimulation and, whether this is causally related or not, the modulatory systems controlling arousal and attention are certainly in a state of low activation. Thus, the prediction was that substititung facilitatory influences of modulatory systems by electrical stimulation of the mesencephalic reticular formation or of the intralaminar thalamic nuclei might allow experience dependent modifications despite anesthesia and paralysis.

Five-week-old dark-reared kittens were prepared as usual for acute electrophysiological experiments. They were anesthetized with nitrous oxide supplemented by barbiturates and paralyzed with a muscle relaxant. Several hours after surgery we started to stimulate one eye with a slowly moving star pattern, the other eye remained closed. As eypected, this procedure never led to changes in ocular dominance even when light stimulation was continued over two to three days. However, in 9 but of 10 kittens in which the light stimulus was paired with brief electrical stimulation of either the reticular formation or medial thalamus, clear changes in ocular dominance towards the open, stimulated eye be-

came apparent after one night of monocular conditioning [33]. These changes were seen at the level of evoked potentials elicited either with phase reversing gratings or with electrical stimulation of the optic nerves and they were also apparent at the level of single unit receptive fields (fig. 5). Moreover, there was an indication from both evoked potential and single unit analysis that the gain of excitatory transmission in the pathways from the conditioned eye has increased and that cortical cells had become more selective for constrast gradients and stimulus orientation. These results are in line with the issue of the lesion experiments and further corroborate the hypothesis that non-specific modulatory systems which increase cortical excitability facilitate experience-dependent modifications.

A VOLTAGE DEPENDENT THRESHOLD FOR HEBBIAN MODIVICATIONS?

The evidence presented so far indicates that in a variety of conditions, retinal signals fail to induce permanent modifications even though they are eliciting responses in cortical cells. Thus, temporal congruence between pre- and postsynaptic activity appears to be only a necessary but not a sufficient condition for the occurence of adaptive changes. Additional "now print"-signals appear to be required and these seem to be available only when retinal signals are attended to and identified as appropriate in a behavioural context. As the last experiment suggested, these permissive gating signals can be substituted by electrical stimulation of the mesencephalic reticular formation or of the intralaminar nuclear complex of the thalamus. We know the effects which stimulation of these structures have on

visual processes. It greatly facilitates the transmission of retinal signals through the lateral geniculate nucleus by blocking local inhibitory circuits [for a review see 29].Furthermore, it raises cortical excitability, enhancing dramatically transmission from granular to non-granular layers [for review see 30]. A predictable consequence of both effects is that the depolarization of cortical dendrites increases. Thus, it appeared conceivable that it is critical for the occurrence of adaptive changes that cortical dendrites be sufficiently depolarized. A dendritic process which is voltage-dependent and does have a high threshold is the activation of dendritic Ca^{++}-channels [22]. We hypothetized, therefore, that the final trigger signal for the occurrence of an adaptive change in response to retinal stimulation might be the influx of Ca^{++}-ions through activated, voltage-dependent Ca^{++}-channels. The prediction of this working hypothesis is that extracellular Ca^{++}-concentrations should transiently decrease with stimulation conditions which induce adaptive changes.

Preliminary results on stimulus induced changes of extracellular Ca^{++} concentrations are conform with this prediction. When light stimuli are coincident with electrical activation of the non-specific activating system - a condition sufficient to induce Hebbian modifications - sudden decreases of the extracellular Ca^{++} concentration can be observed. With light stimulation alone or with electrical stimulation of the modulatory projections alone - conditions which do not lead to adaptive changes - no changes of extracellular Ca^{++} were observed. Likewise, in adult cats in which modifications of striate cortex functions can no longer be induced, even contiguous stimulation

of the retina and of the modulatory projections failed to alter extracellular Ca^{++}-concentrations [10]. Although this covariance between Ca^{++}-changes and the occurrence of adaptive changes is no proof for an involvement of Ca^{++}-mechanism in experience dependent modifications of striate cortex functions, it is at least compatible with such an interpretation. The assumption that Ca^{++}-ions could serve as a messenger for the induction of changes in synaptic transmission and/or neuronal excitability is in agreement with the notion that the appearance of free Ca^{++}-ions in the cytosol is an important trigger signal for a large number of metabolic activities [for a review see 21].

CONCLUDING REMARKS

If Hebbian modifications of neuronal connectivity are a general feature of the developing visual cortex, it is appropriate to briefly speculate on their functional role in normal development. In general these processes have an associative function in that they selectively stabilize those connections between neurones that have been most often simultaneously active. This has different consequences at different levels of cortical processing.

As discussed above, at the level where afferents from the two eyes converge such a selection could assure that only those afferents become consolidated which come from corresponding retinal loci in the two eyes. In the domain of orientation selectivity Hebbian modifications could assure that second order cortical cells receive excitatory input only from those first order cells that share the same orientation preference. Two

considerations indicate that this selection problem is again not a trivial one: Second order cells with large receptive fields have to receive input from numerous first order cells that may be distributed over several hypercolumns [1, 2, 15]. Because first order cells sharing the same orientation preference are clustered within discrete regularly spaced columns this implies discontinuous sampling from clusters of first order cells which may be several millimeters apart. Moreover, because of the retinotopic organization of striate cortex, second order cells with elongated vertical receptive fields must receive input from first order cells along the longitudinal axis of striate cortex while second order cells with elongated horizontal fields must integrate input from first order cells along the medio-lateral axis. Because of the continuity of contours in the natural environment this extremely complex specification of connections can again be achieved by selectively consolidating connections which have a high probability of being activated simultaneously. This selection is aided by the strong inhibitory interactions between cells with differing orientation preferences [3] since this inhibition effectively prevents simultaneous firing of cells in columns with different orientation preferences. The finding that orientation selectivity of cortical neurones could remain immature even though retinal responses to oriented contours were readily available indicates that this selection process, too, is gated and probably occurring only when the pattern is sufficiently unambiguous. Some evidence is available that activity-dependent association may occur over rather large distances. When young animals are exposed selectively to regularly spaced contrasts, about one third of the cortical

neurones develop large, rather unconventional receptive fields with several, widely spaced excitatory regions. The spacing of these excitatory regions corresponds to the angular distance between the contrast borders of the periodic patterns which the animals had experienced previously [32]. We ignore the anatomical substrate of these large receptive fields. One of the candidates is of course the recurrent collateral of cortical neurones which can mediate horizontal interactions over considerable distances [39].

The functional role of Hebbian modifications at the level of the recurrent intracortical connection is still unclear. With normal experience the combinatory complexity of possible contingencies becomes so exceedingly large that it is impossible to predict the resulting pattern of differentially weighted interactions. Again it can be expected that cells become associated preferentially which have a high probability of responding simultaneously in the presence of particular feature combinations. Such preferential coupling would enhance and prolong by reverberation the responses of distinct cell assemblies to particular, frequently occurring patterns. This would distinguish cells of the assembly from other neurones which are not able to join or to form a cooperating ensemble. Hebbian modifications at this level of cortical processing could thus be a first step toward the formation of cooperative cell assemblies whose coherent and reverberating responses would signal the presence of a particular previously experienced combination of features. The formation by experience of such selectively coupled ensembles could in turn serve to facilitate the separation of figure elements from background elements.

In conclusion, during development activity-dependent selective tuning of neuronal connections appears to occur at various levels of cortical processing. Available data suggest that these processes could serve to adjust and to optimize connections according to functional criteria. This in turn could help to achieve a degree of selectivity of neuronal connections that could barely be attained by a priori genetic instructions alone. Moreover, the gated Hebbian modifications have the effect to associate neurones with particular functional properties. This could serve to develop ensembles of selectively coupled cells and this would be a first step towards the formation of neuronal codes which represent combinations and spatial relations of features rather than the mere presence of local contours.

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THE HORIZONTAL (TANGENTIAL) FIBRES SYSTEM OF LAMINA I OF THE CEREBRAL NEOCORTEX

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Lamina I has two principal components: a meshwork of fine unmyelinated fibres, the so-called horizontal fibres (h.f.); the profusely branching apical dendrites of all pyramidal cells. These dendrites are studded with spines on which the h.f.'s make excitatory synapses in a crossing--over manner. The h.f. system is produced by the fibres that ascend through the modules and bifurcate in lamina I, each branch extending for up to 5 mm from its origin. These ascending fibres are axons of Martinotti cells, and of corticocortical fibre inputs to the module, both ipsilateral and callosal. Electrophysiological investigations have shown that the h.f. system has a powerful excitatory action on the pyramidal cells.
There will be developed the hypothesis that the h.f. - pyramidal dendrite system of lamina I is a structure specially designed for the storage of memories. Conjunction of cartridge activation of pyramidal cell dendrites with a selection of impulses in h.f.'s could produce a synaptic potentiation encoding the experience that is dependent on that selection of h.f. impulses. On a later occasion these potentiated synapses could be effective in replaying the coded neuronal pattern and so retrieving the memory.

INTRODUCTION

It has been long recognized that lamina I of the neocortex was unique in that it had extremely few neurones in the adult. There are two main neural components: the profusely branching apical dendrites of the pyramidal cells; and the fine meshwork of nerve fibres that are mostly unmyelinated. Ramón y Cajal [31] described in great detail the neuronal and fibre structure

cf the Couche Plexiforme as he called lamina I. Unfortunately he mostly studied very young mammals, which had in addition a large population of neurones in lamina I, in contrast to the very low neuronal population in the adult. He suggested that the large nerve fibres in lamina I were axons of the numerous horizontal cells and that the small fibres were axons of the Martinotti cells of lamina VI that ascended to lamina I and bifurcated to form the horizontal or tangential fibres. Ramón y Cajal used both terms. Since that time the attribute 'tangential' has been usually adopted, I thing mistakenly. Strictly the word tangential means tangential to some curved surface of contact. Only the surface of lamina II would qualify for this role, which does not seem satisfactory, so I have preferred the other term of Ramón y Cajal, calling these fibres of lamina I, horizontal fibres. I believe that lamina I of the neocortex merits much more attention than it usually has been given. Following Marr [26] I have in recent years been developing the hypothesis that lamina I plays an essential role in the memory storage system of the cerebral cortex [30 , Chapter E8 11, 12, 13, 14].

IMPORTANT STRUCTURAL FEATURES OF LAMINA I (FIGS 1, 2, 3)

A. All pyramidal cells of the neocortex send their apical dendrites up to lamina I, where they branch profusely, often in an espalier-like manner, and are densely covered with spines. In lamina I the number of spine synapses on the dendritic branches of a single pyramidal cell ranges from 1000 of 3000 [40], which would be about 20% of the total excitatory input to that neurone.

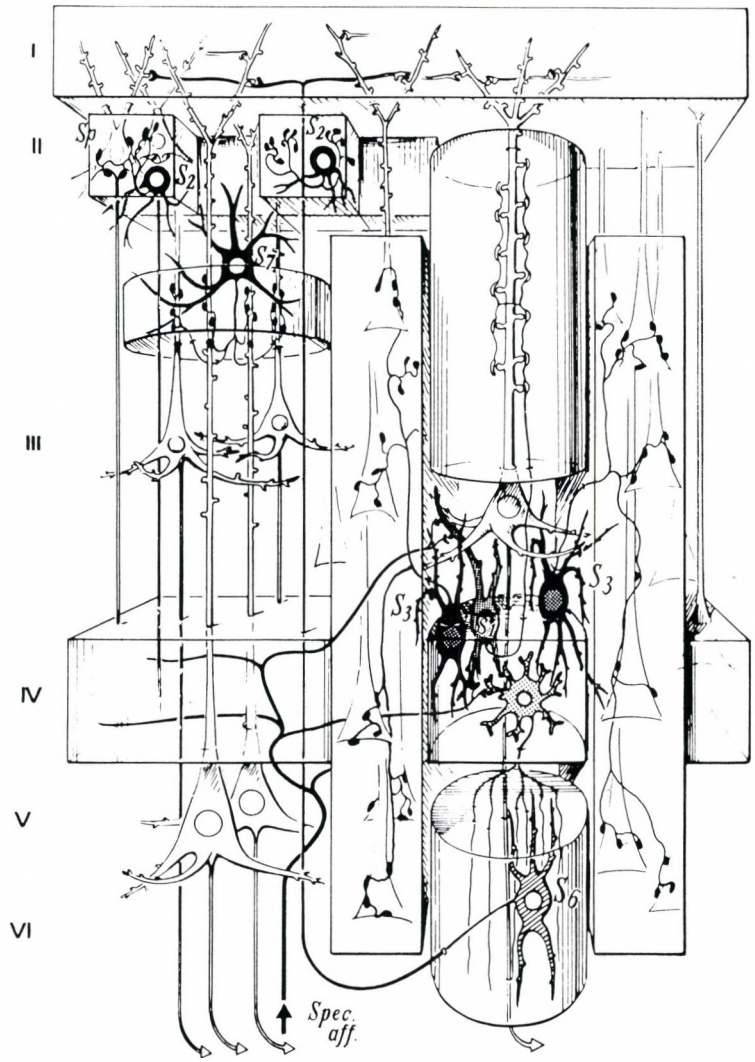

Fig. 1. Three dimensional construct showing cortical neurones of various types and the endings of a specific afferent fibre. There are two pyramidal cells in lamina V and three in lamina III, one being shown in detail in a column. In this column there are two inhibitory cells (S_3) with axons making basket-like synapses on shadowy pyramidal cells in two adjacent cortical slabs that are shown in perspective. In lamina II there are two stellate-pyramidal cells (Sp) and two small inhibitory cells (S_2). S_1 is a spiny stellate cell whose axon forms a cartridge synapse around the apical dendrite of a pyramidal cell. Just below S_1 is a neurogliform cell with a descending axon to the Martinotti cell (S_6) whose axon ascends to bifurcate in lamina I. Szentágothai, J. (personal communication)

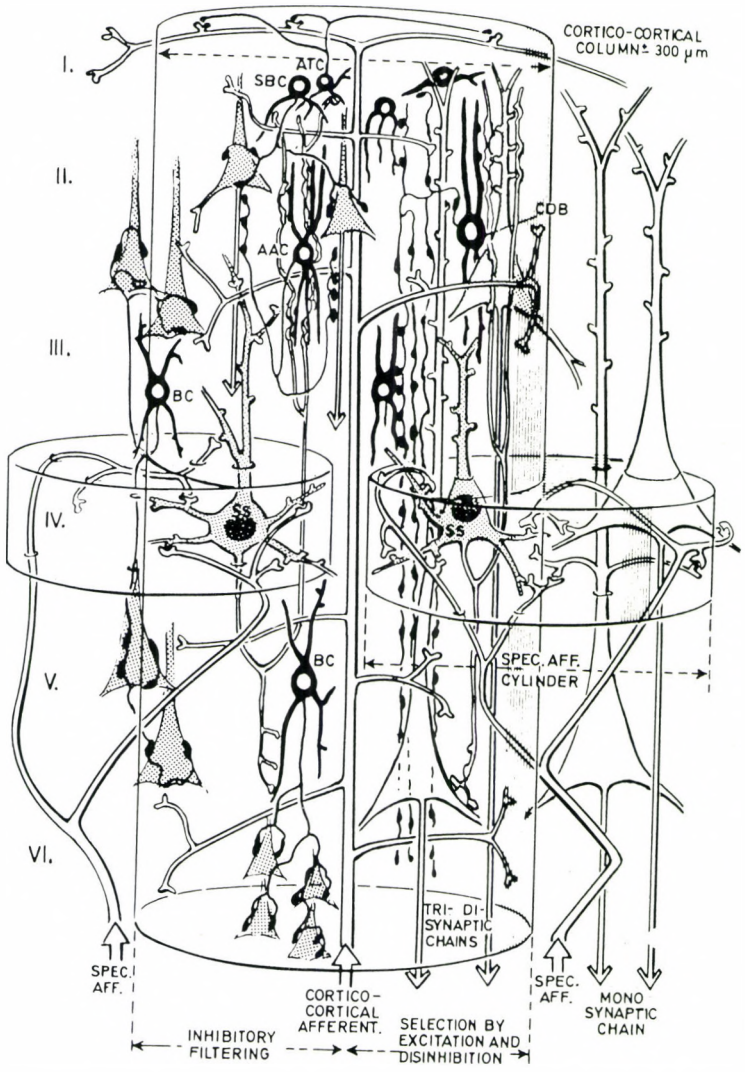

Fig. 2. Internal neurone connectivity in a cortico-cortical column or module, the vertical cylindrical space of about 300 *μm in the centre. The module is sharing part of its space with two flat discs in lamina IV in which specific afferents from thalamus (Spec. Aff.) arborize. The cortico-cortical afferent (indicated at bottom) terminates all over the cortico-cortical module, though with different densities of terminals. In lamina I, the horizontal spread of the cortico-cortical fibres extends far beyond the module. The selection of pyramidal cells for output is envisaged in the right half of the diagram over excitatory interneurons (Ss-spiny stellates) or over disinhibitory interneurons, the CDB (cellule à double bouquet), which is an inhibitory interneurone that acts specifically upon inhibitory interneurones which are shown in full black, the basket*

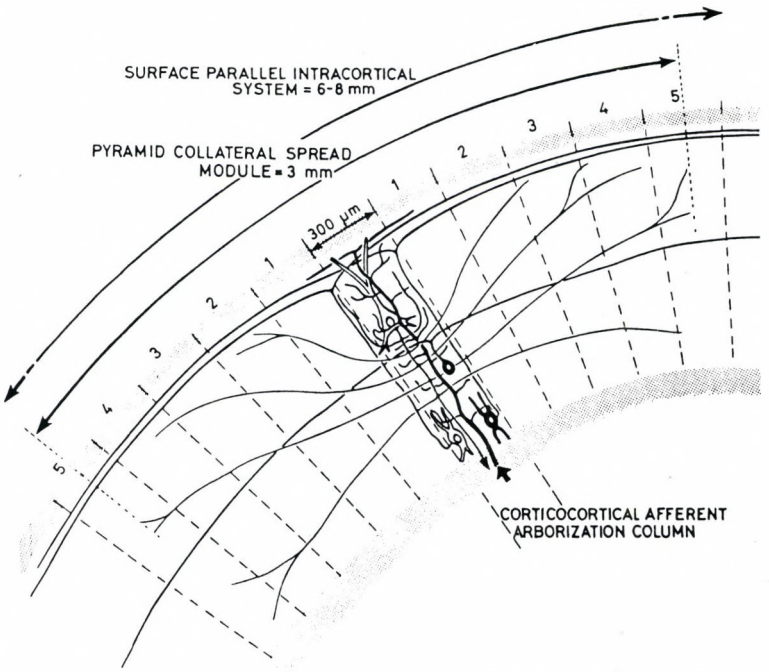

Fig. 3. Diagram illustrating long range intracortical connections. Two Martinotti cells are shown in lamina 6 with axons ascending to lamina I and bifurcating to form horizontal fibres that extend for 6-8 mm, even beyond the diagram. The thick fibre indicated by arrow is a cortico-cortical afferent that branches in the various laminae and then bifurcates in lamina I, but the full range of the horizontal fibres so formed is not indicated. Also shown is the wide extent of ramifications of axon collaterals from the pyramidal cell [41]

cells, BC, in the deeper laminae, the SBC (small basket cells) in lamina II, the axonal tuft cells, ATC, and a very specific axo-axonic cell, AAC, acting upon the initial segments of pyramidal cell axons (modified from [42])

B. The spine synapses are large, even the largest in the cortex, and of the typical asymmetric excitatory type [21, 22]. The spine is frequently of the same diameter as the parent dendrite (see Fig. 7, Plate 14, reference [22]).

C. Since, in the adult, the horizontal cells in lamina I are extremely rare [37], the immense fibre population must be axonal projections from neurones elsewhere. Already Ramón y Cajal (reference [31], p. 542 and Fig. 350, pp. 582, 583) had recognized that the Martinotti cells of lamina VI with their axons ascending to bifurcate in lamina I were important contributors to the horizontal fibre system of lamina I. By the technique of undercutting the cortex at various levels and allowing time for degeneration, Szentágothai [33 , 34] showed that a large fraction of the horizontal fibres had their cells of origin in the deepest lamina of the cortex, which is the location of the Martinotti cells. The very convincing results of Szentágothai are illustrated by Colonnier ([8], in Figs 1.5, 1.6, 1.7, 1.8). These experiments exclude the pyramidal cell axon collaterals as significant contributors to the horizontal fibre system ([39], cf. Fig. 3 below). The axons of Martinotti cells are still generally accepted as being perhaps the most important origin of horizontal fibres, but two other origins have been identified [37]. By the callosal degeneration technique [16, 23] it has been shown that there is a large callosal projection into lamina I, the callosal fibres presumably bifurcating there to form horizontal fibres. By a similar degeneration technique Szentágothai [37], and Jones and Powell [23] showed that the ipsilateral cortico-cortical fibres also projected to lamina I. It has been noted that the horizontal fibres

with an ipsilateral origin tend to be more superficial than those with a callosal origin [37 , 40].

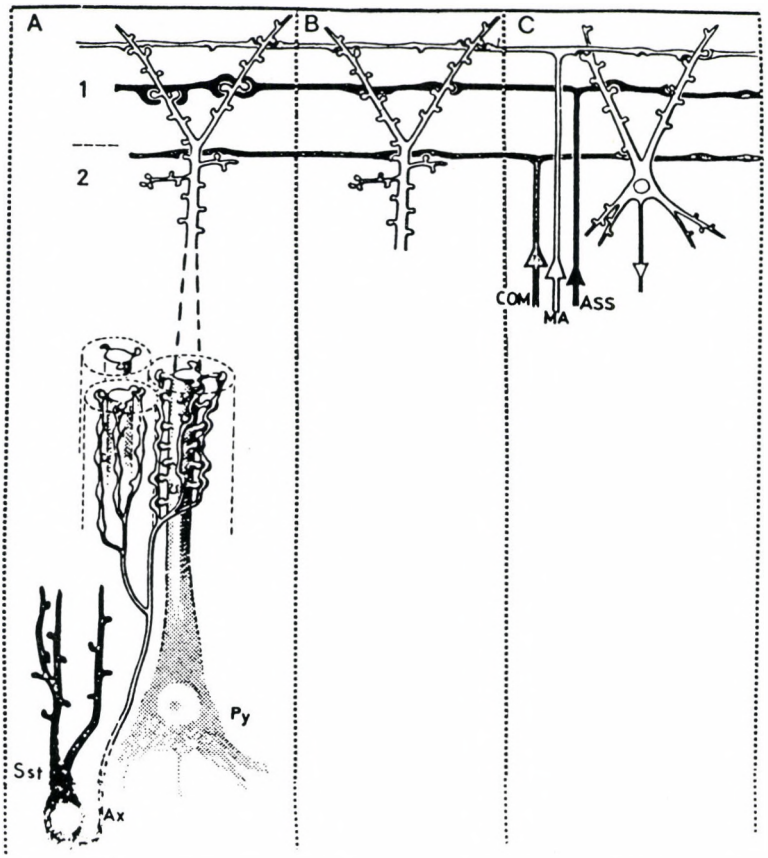

Fig. 4. Simplified diagram of connectivities in the neocortex that is constructed in order to show pathways and synapses in the proposed theory of cerebral learning. The diagram shows three modules, A,B,C. In lamina I and II there are horizontal fibres arising as bifurcating axons of commissural (COM) and association (ASS) fibres and also of Martinotti axons (MA) from module C. The horizontal fibres make synapses with the apical dendrites of the stellate pyramidal cell in module C and of pyramidal cells in modules A and B. Deeper there is shown a spiny stellate cell (Sst) with axon, AX, making cartridge synapses with the shafts of apical dendrites of pyramidal cells (Py). Due to conjunction potentiation the association fibre from module C has enlarged synapses on the apical dendrites of the pyramidal cell in module A (modified from [36])

D. Study of the degeneration of horizontal fibres after a vertical cut through the superficial laminae has shown that these fibres extend for 2.5 to 4.5 mm from their origin [33, 34, 37] (Figs 3, 4). A related finding is that a lesion of the cortex results in degeneration of horizontal fibres for 5 to 7 mm ([20], p. 9 ; [23], p. 61).

All these figures have to be doubled to give the total extent of the bifurcating fibres. A length of 5 to 14 mm would be in good agreement with the electrophysiological studies to be reviewed below. It has been assumed that the projection of the horizontal fibres from a focus of origin would be radially isotropic. However Fleischhauer and associates [15, 27] have presented evidence that this is not so, there being a strong preferential orientation to an axis about 50° from the sagittal plane in the lissencephalic brain of the rabbit.

E. Superimposed on the synaptic system of horizontal fibres to the spines of pyramidal cell dendrites there is an inhibitory system by the axonal tuft cells, ATC (Fig. 2) [39, 40] that lie in the upper zone of lamina II and send their axons into lamina I to form inhibitory synapses in close proximity to the excitatory spine synapses of the horizontal fibres. Such a cell is illustrated by Ramón y Cajal ([31] Fig. 345D), and, as Szentágothai [39] points out, by several later investigators. ACT's would seem to be numerous enough to exert an effective control on the excitatory system of horizontal fibres to pyramidal dendrites [14].

F. In its structural design lamina I would seem to be much simpler than any of the deeper cortical laminae. It is even simpler than the molecular layer of the cerebellar cortex, where

there is similarly the crossing-over system of parallel fibres to Purkyně cell dendrites and the stellate cell inhibitory control, but there is also the climbing fibre excitatory system. Another important difference is that the horizontal fibres do not have the strict parallel arrangemenet of the cerebellar parallel fibres, though as mentioned above, there does appear to be a preferential orientation.

G. It is generally agreed that for each module the horizontal fibres arise as bifurcations of fibres ascending from three origins, the Martinotti cells of that module and the fibre inputs to that module from cortico-cortical inputs either ipsilateral or callosal. Since these cortico-cortical inputs have a strong excitatory action on that module, the horizontal fibres arising from bifurcation in lamina I can operationally be considered also as projections from that module and not from the modules of origin of those cortico-cortical fibres. Some thalamo--cortical fibres ascend to lamina I [23], but it is doubtful if they make any substantial contribution to the horizontal fibres. However, by the powerful excitation of lamina IV stellate cells, thalamo-cortical inputs would indirectly excite Martinotti cells and so very effectively contribute to the horizontal fibre emission from that module.

H. Conclusions. In each module there ascends to lamina I at least one thousand fibres that bifurcate to generate the horizontal fibre system, the branches extending in opposite directions for 2.5 to 7 mm and each making hundreds of excitatory synapses on the spines of the pyramidal cell dendrites (cf. [11]). Each module would thus have in lamina I a potential excitatory zone extending in decrementing manner for many

millimetres. Doubtless the Martinotti cells have the dominant role, being excited by all inputs to the module, most notably the thalamo-cortical, but also by the cortico-cortical both

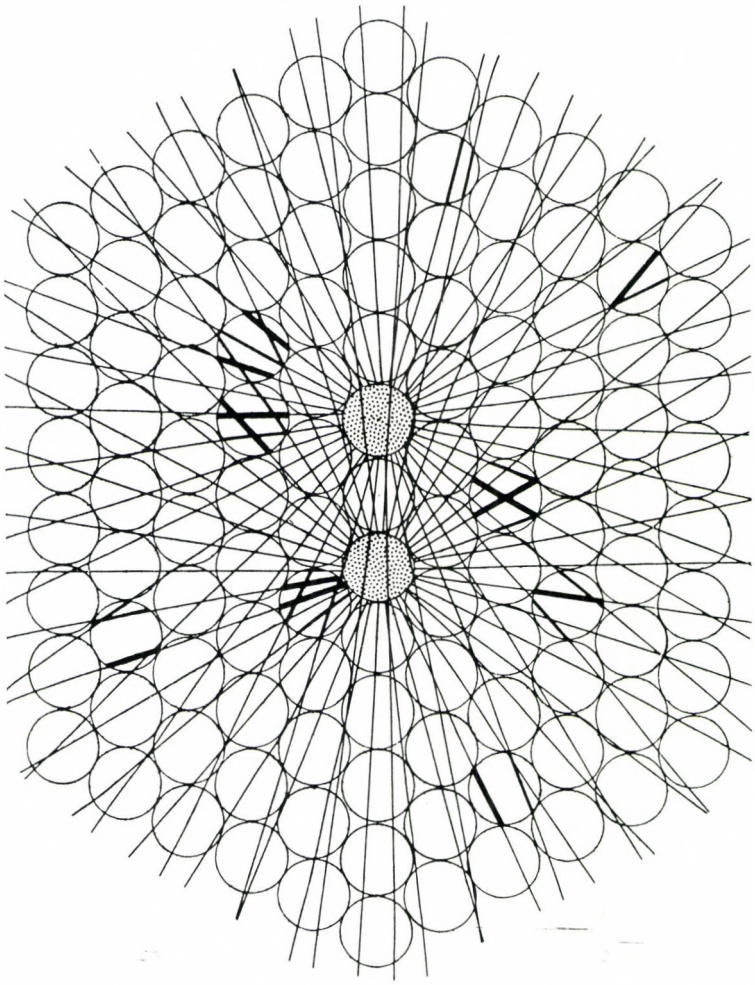

Fig. 5. Diagram of a large assemblage of modules as seen in plan, each outlined by a circle. From two modules there are seen 38 radiating horizontal fibres that would travel far beyond this illustrated zone. Each is of course bifurcated in lamina I (cf. Fig. 4), so the number of radiating fibres is twice the numbers of fibres of origin - association, callosal and Martinotti (cf. Fig. 4). In several modules there has been potentiation of the synapses made by the traversing horizontal fibres as shown in Fig. 4, and this is indicated by thickening of the lines. Further description in text [14]

ipsilateral and contralateral. In addition the fibres of these two latter inputs directly make a substantial contribution to the horizontal fibre population. It is important to recognize that the horizontal fibre system is reciprocal in operation [40]. Module A projecting to an adjacent module B in the same manner as B projects to A (cf. Fig. 5).

ELECTROPHYSIOLOGICAL INVESTIGATIONS ON LAMINA I

Adrian [1] discovered that weak electrical stimulation of the surface of the cerebral cortex set up a relatively slow surface negativity that spread decrementally for up to 4 mm from the stimulated focus, the superficial response. With stronger stimulation there were superimposed later complex potentials with deep negativity, the deep response. These findings have been confirmed in several later investigations, in particular by Burns [5, 6] who utilized the isolated slab technique to show that the superficial response was generated by local neuronal circuits of the cortex. With improved unipolar recording [4] the spread of the superficial response was found to extend to 10 mm from the stimulated focus. Since no trace of the superficial response was detectable when the recording electrode was thrust 1.5 to 2.0 mm below the surface, or when the superficial layers were killed by thermocoagulation, Adrian [1] proposed that the superficial response was generated by the nerve elements of the molecular layer (lamina I), suggesting the pyramidal cell dendrites for this role.

Later investigations showed that potentials of a comparable duration were produced by synaptic excitation of nerve cells,

and it was proposed [9] that the superficial response was due to the synaptic excitation of pyramidal cell dendrites by the crossing-over synapses made by the horizontal fibres. This interpretation was supported by impressive empirical evidence: the decrement in all directions from the stimulated focus corresponds to the surface spread of horizontal fibres; the time course of about 10 to 20 msec duration resembles that of excitatory postsynaptic potentials (EPSPs); the graded increase to a maximum with increasing stimulus strength corresponds to the excitation of the population of horizontal fibres; the latency of the superficial response and the conduction velocity of the onset (about 2 m/sec) correspond to the initiation by impulses in horizontal fibres. The depth profile confirms the generation in lamina I. Since that time, intracellular recording from pyramidal cells [24, 29] has demonstrated the synaptic potentials generating the superficial response and shown that these potentials are powerful enough to set up one or two impulse discharges in those cells. With stronger stimulation there may be a later IPSP, which could be interpreted as being generated by the axonal tuft cells.

The horizontal fibre-pyramidal dendritic system is likely to be the generator of the much slower DC shifts of many seconds duration that have often recorded from the surface of the cerebral cortex against an indifferent electrode. These excitatory responses have been induced by various procedures such as tactile and acoustic stimulation or by spontaneous movements of the animal or by arousal from sleep [7]. It would be expected that, with increased activity of the cortical modules, there would be increased impulse barrages in the horizontal fibres, their

synaptic excitation of the pyramidal cell dendrites producing the cortical DC potentials. It can further be suggested that other slow negative potentials recorded from the cerebral cortex are also produced in a considerable extent by the horizontal fibre pyramidal dendritic system, which is strategically located to give dipoles of surface negativity/deep positivity. As examples there are the contingent negative variation, the readiness potential and the N 140 potentials of attention (cf. [13], Chapter 4). The generation of impulse discharges by the horizontal fibre synapses may be surprising for synapses so remote from the somata of the pyramidal cells. However, there is evidence that impulses can be generated in the apical dendrites of hippocampal pyramidal cells [3]. Moreover it is now recognized that, with the motoneurones, synapses on dendrites remote from the soma generate surprisingly large EPSPs in the soma [17, 19]; and, as noted above, the synapses in lamina I are abnormally large [21, 22].

THE HYPOTHESIS RELATING MEMORY TO THE HORIZONTAL FIBRE - PYRAMIDAL DENDRITE SYSTEM

On analogy with the cerebellar learning hypothesis [10, 25] it has been proposed that synapses of horizontal fibres on pyramidal cell dendrites are potentiated if they are activated at about the same time (the conjunction) as the cartridge activation of that same apical dendrite, i.e. that there is a selection for potentiation on the criterion of approximate temporal conjunction [11, 12, 13, 14, 26]. In this analogy the horizontal fibres correspond to parallel fibres of the cerebellar cortex,

the pyramidal cells to the Purkyně cells and the cartridge synaptic complex [35 , 37 , 39 , 40] (Figs 1, 2, 4) to the climbing fibre. In parenthesis it should be mentioned that since that analogy was proposed, it has been discovered [18] that in the cerebellum the conjunction is associated with a prolonged (hours) depression of the parallel fibre synapses on the Purkyně cell dendrites [cf. 2] not a potentiation. However, since Purkyně cells are inhibitory in action, their depression is, by disinhibition, effectively an excitation of their target neurones in the cerebellar nuclei.

Thalamocortical fibres provide the most effective excitation of the focal spiny stellate cells that project as the cartridge complex on a minicolumn of pyramidal cells (Figs 1, S1; 2, Ss; 4, Sst). It is of particular significance that the focal spiny stellate cells will at the same time induce the disinhibitory action of the *cellule à double bouquet* (CDB in Fig. 2) on a minicolumn of pyramidal cells by means of the CDB inhibition of inhibitory cells [32, 43]. So by this collusive design the thalamo-cortical input to focal spiny stellate cells would be expected to set in train an intense pyramidal cell activation that could be a powerful potentiating signal to horizontal fibre synapses that were activated in temporal conjunction. It is important to recognize the selectivity of the potentiation. Of the 1000 or more horizontal fibre synapses on the dendrites of that pyramidal cell only the few excited in conjunction with the cartridge activation would be potentiated, as is indicated in Fig. 4. Of the synapses made by three types of horizontal fibres on the apical dendrites in three modules only one is shown potentiated by a previous conjunction.

It can be recognized that such enduring potentiations could form the basis for a permanently changed pattern of connectivities in the cortex, which can be regarded as a memory store. The elemental diagram of Fig. 4 gives almost no indication of the immense convergence of many hundreds of horizontal fibres that make 1000 to 3000 synapses on one pyramidal cell. There is thus a wealth of possibilities for selective potentiation of horizontal fibre synapses on a pyramidal cell which is only one unit amongst the more than a thousand pyramidal cells in a module.

It is generally believed that a memory is not focally stored, but rather that there is multiple representation so that sharply localized brain lesions result not in a specific memory loss but in a diffuse partial loss. It was conjectured ([13], Chapter 7) that '... any one cognitive memory would have a neural counterpart in the patterned performance of hundreds or even thousands of modules that owe their performance to the plastic changes induced in the learning process and that their replay participates in the recovery of the memory...'.

In Fig. 5 the modules are shown in plan as seen from the surface, each being enclosed by a circle of 300 μm in diameter. In this pattern two modules with cortico-cortical inputs are shown at about 600 μm apart with their outward radiating horizontal fibres. Around both are modules with a gradually diminishing activation by those horizontal fibres, as indicated by the progressive decrease in the density of horizontal fibre distribution. Potentiation of the horizontal fibre synapses to several modules, as illustrated in Fig. 4, is shown by the convention of a thickening of some horizontal fibres as they traverse these

modules. It can be seen that around the two excited modules there will be a constellation of modules with an augmented input to pyramidal cells from horizontal fibre synapses, and that, consequently, in the memory process these pyramidal cells could be excited to initiate via a modular discharge the development of a spatio-temporal pattern that could form the neural basis of the remembered experience. The augmented discharge of these pyramidal cells will have opened up new lines of modular communication that were hitherto ineffective. We might term the situation illustrated in Fig. 5 as 'modular jumping'. It could provide a very simplified model of the changes in modular patterns that are responsible for the storage and retrieval of a cognitive memory. It is to be noted that the horizontal fibre system in lamina I is purely excitatory.

In Figs 6 and 7, an attempt is made to diagram the conjectured development of modular patterns during a cognitive memory. In the lowest row of Fig. 6, there are five modules (A-E) that are serially connected by association fibres in the conventional cortico-cortical manner. For diagrammatic simplicity, only two pyramidal cells are shown for each module, a thousand-fold reduction. Further association fibre projections shown by a downward arrow from each module would contribute to the spatio--temporal patterns developed in response to thalamo-cortical and cortico-cortical inputs into modules A and B. Each connecting arrow for modules A and E would represent a bundle of up to 100 association fibres. The on-going activation is indicated by the punctate shading of the modules. Modules F and G and those sequential therefrom are inactive in the absence of inputs into F and G.

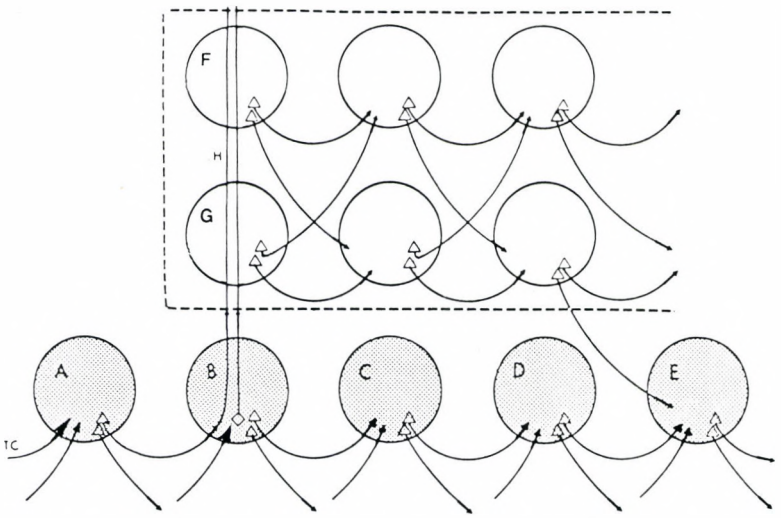

Fig. 6. Diagram of modular arrangement looked at from above with the modules separated so as to allow drawing of the cortico--cortical fibres. Modules A to E represent a basically connected sequence of modules (cf. Fig. 5). Only two pyramidal cells are shown for each module and input and output lines in part connect to modules out of the diagram. In A and B thalamo-cortical (TC) input lines are indicated by large arrow heads. Pyramidal cells project by cortico-cortical fibres to other modules (small arrow heads) that are activated, as indicated, by the punctate shading. From module B there are projecting upwards two horizontal fibres (H) as in Fig. 4, one being the continuation of a cortico-cortical fibre, the other the projection of a Martinotti cell (diamond). In the absence of inputs to modules F and G, the H fibre input is ineffective, as is shown by the empty modules, F and G and onwards in the box [14]

Figure 7 depicts the modular connectivities at the time of conjunction of the thalamo-cortical inputs to modules F and G with the thalamo-cortical inputs into modules A and B. These latter inputs excite the horizontal fibre (H) projections to modules F and G. When there is conjunction between on the one hand the cartridge activation of pyramidal cells by the thalamo--cortical inputs into modules F and G and on the other hand the horizontal fibre activation from module B, there is conjectured to be an enduring potentiation of the horizontal fibre synapses (Fig. 4) and this is shown by the convention of thickening in

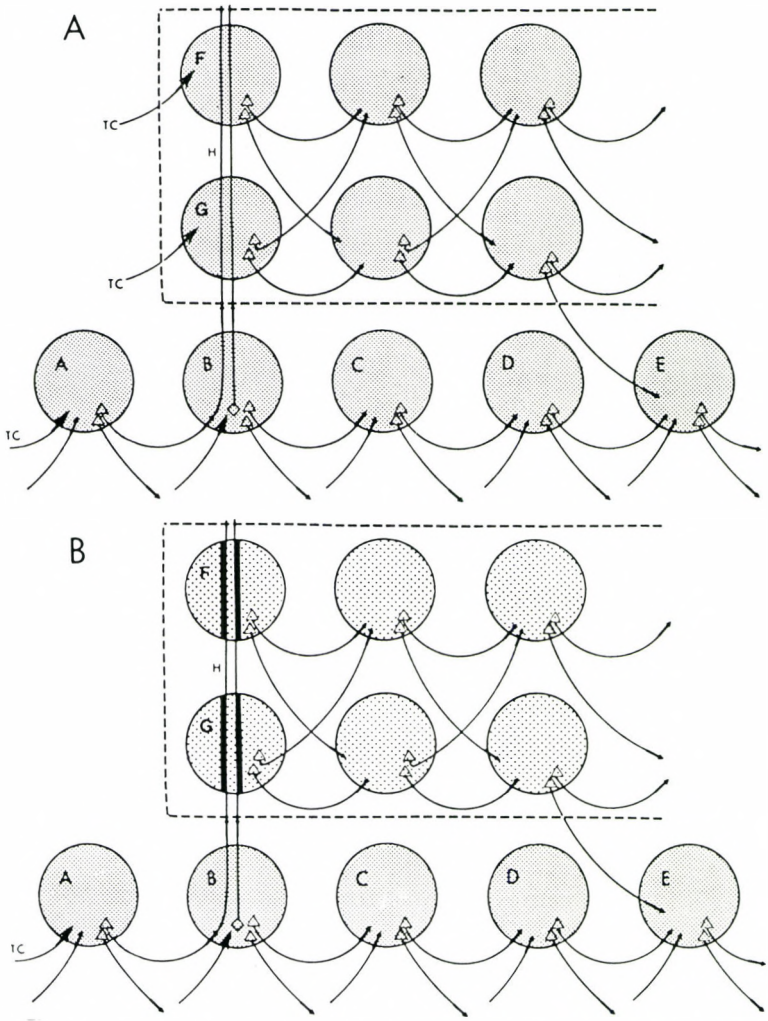

Fig. 7. As in Fig. 6, but in A there are thalamo-cortical inputs into modules F and G at the same time as the inputs into modules A and B with the activation of the H inputs to modules F and G. The spread of activation from modules F and G to the next modules in sequence is indicated by the punctate shading. The conjunction between the horizontal fibre input to modules F and G and the TC input into these modules is postulated to result in an enduring potentiation of the horizontal fibre synapses on the apical dendrites of some pyramidal cells (cf. Fig. 4), which is shown in Fig. 7B by thickening of the H fibres as in Fig. 5. As a consequence, even when there are no TC inputs into modules F and G, there will be discharge of impulses from their pyramidal cells in response to the inputs into modules A and B, as is indicated by the punctate shading. Thus by the connecting pattern of cortico-cortical fibres there can be activation of the modular pattern enclosed by the broken line, that otherwise (cf. Fig. 6) would not have been activated in the absence of the TC inputs into F and G [14]

Fig. 7B (cf. Fig. 5). As a consequence of this potentiation, even in the absence of thalamo-cortical inputs to modules F and G, the pyramidal cells of modules F and G would be excited to discharge by horizontal fibre activation from module B (cf. Fig. 4) as illustrated in Fig. 7B, which is in contrast with Fig. 6. There has been module jumping from module B to modules F and G with the consequent initiation of on-going spatio--temporal activation of the upper two rows of modules, as indicated by the weaker punctate shading. Thus the patterned development inside the box (outlined by broken lines) is a weak replica of that initiated in 7A by the normal thalamo-cortical input into F and G modules, but it now occurs in the absence of this input. This pattern is conjectured to be the neural correlate of the cognitive memory and the associated conscious experience. Thus Fig. 7B illustrates the retrieval of the memory laid down in the conjunction process of Fig. 7A. It also illustrates that memory retrieval is best secured by some related sensory input (the thalamo-cortical inputs to A and B) or by a deliberate mental effort to conjure up related signals, as when trying to remember a name.

It must be recognized that in Figs 6 and 7 there has been a tremendous diagrammatic simplification. There should be an amplification of at least 1000-fold in the pyramidal cell population and in their axons, the cortico-cortical fibres. Moreover, instead of the two output lines from each module there should be connectivities to up to 50 other modules. It would seem that with this transformation into many parallel modular connectivities the diagram could represent both the distributed system of Mountcastle [26] with modular elements in echeloned parallel and

serial arrangement, and the modular operation of superstructures suggested by Szentágothai [41, 42].

CONCLUSIONS

It is conjectured that the horizontal fibres act as key units in the storage and retrieval of memories in the cerebral cortex. The horizontal fibres of lamina I are an enormous fibre system that hitherto has had no defined role. Yet an important role is implied by the fact that all pyramidal cells send their apical dendrites up to lamina I, these dendrites being studded with spines for synapsing with horizontal fibres. This synaptic system provides about 20% of the excitatory synapses on a pyramidal cell. Hitherto the excitatory power of this system has been downgraded because of the remoteness from the soma with its axonal origin. With motoneurones there is some compensatory arrangemenet whereby synapses remotely placed on dendrites are as effective as those on the soma [17, 19]. Such an arrangement for cortical pyramidal cells would greatly augment the potential excitatory ability of the horizontal fibre system and thus give added plausibility to the hypothesis that the horizontal fibre synapses are the key units in the storage and retrieval of cognitive memories.

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SHORT-TERM REORGANIZATION OF THE CORTICAL NETWORK? - SOME QUESTIONS FROM VISUAL PSYCHOPHYSICS

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Research into visual system function requires the use of test stimuli as (would-be) neutral probes. Although the risk of transient 'fatigue' is well recognized, e.g. in relation to dark adaptation, we tend otherwise to assume that the system will respond reversibly to stimuli within normal physiological limits. It is on this assumption that gratings of near-parallel lines, for example, are commonly used to determine both physiological and psychophysical response characteristics.
This paper reviews evidence suggesting that certain classes of visual stimuli, including gratings in particular, can induce a short-term cooperative reorganization of the visual network that leaves it far from normal in its responsiveness to other inputs. It is suggested that the resulting abnormalities may help to shape our ideas as to the cooperative ensemble properties of cortical neuronal network.

INTRODUCTION

The beautiful anatomical work by Szentagothai and his colleagues, referred to elsewhere in this Volume, has elaborated a picture of cortical structure in which the anatomical possibilities of long-range cooperative couplings and re-entrant loops between elements are almost embarrassingly rich; yet physiological evidence of such cooperativity is so far almost completely lacking. The discovery by Hubel and Wiesel (1) and others of 'feature specific' sensory neurones has accustomed

most of us to think of the cerebral cortex as a network of 'hard-wired' elements 'tuned' to specific information-processing functions. Under prolonged stimulation such elements normally 'adapt' by giving a weaker response; but otherwise their characteristic functions are not usually expected to change. Sensory stimulation, however complex, is thought of as merely 'linghting up' a subset of the neuronal population, so that the form of the stimulus is represented by the profile of unit excitation, which is presumed to drop back to its earlier condition on removal of the stimulus. Of course, such phenomena as conditioning and memory require us to postulate that suitable stimuli can induce less reversible changes in some central structures; but by and large the sensory cortex is not supposed to suffer from such complications.

The aim of this paper is to question the foregoing assumption on the ground of some evidence from visual psychophysics, which suggests that even relatively peripheral levels of cortical circuitry can have their information-processing functions, and not merely their sensitivity, significantly altered by certain types of sensory input pattern.

THE COMPLEMENTARY AFTER-IMAGE

It has been known since the time of Purkinje (2) that after inspecting figures of near-parallel lines such as those in Fig. 1, a curious shimmering afterimage is visible for a second or so, made up of a host of wavy streamers, in rapid motion, oriented roughly at right angles to the lines of the original figure.

With a vertical grating, for example, wavy horizontal lines are seen, normally streaming to left and right, with a perceived wave-length of the order of five times that of the inducing grating (3 - 5). With the "ray" pattern of Fig. 1(a), the lines form rapidly rotating "rosettes" of wavy circles and spirals; with the "target" of Fig. 1(b) they form a chrysanthemum-like pattern of wavy radial streamers.

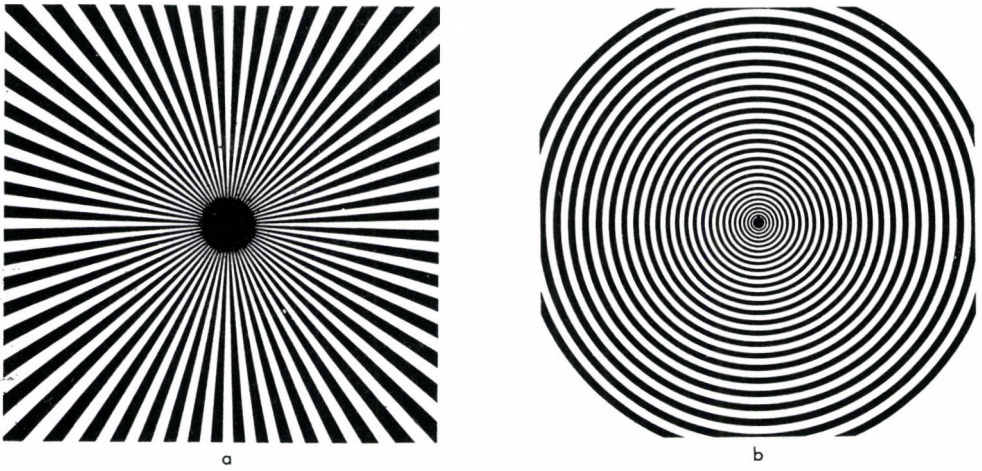

Fig. 1. Examples of stimuli which induce short-term reorganization of the visual network

Although with a single grating the wavy streamers are generally orthogonal to the lines of the pattern, with two gratings overlapping at an angle they run perpendicular to the mean orientation of the stimuli; and with other patterns, such as regularly spaced arrays of dots, they have a still more complex geometry. For this reason when I rediscovered the basic phenomenon (3) I suggested the term "complementary afterimage" (CAI) to distinguish it from the ordinary negative afterimage, whose contours are of course parallel rather than orthogonal to those of the original.

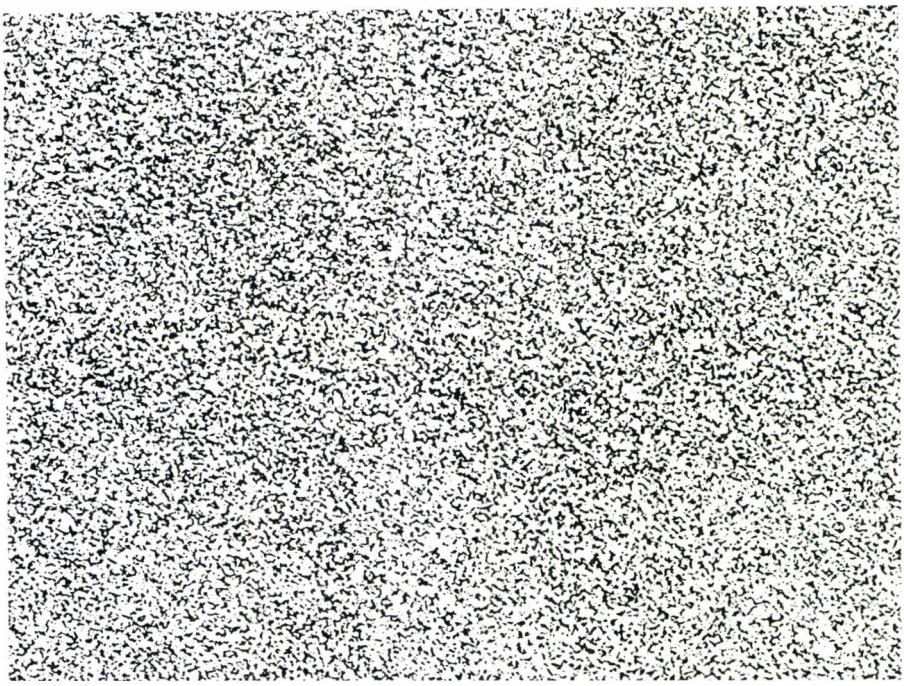

Fig. 2. Visual "static noise" used as a neutral test-stimulus to reveal anomalous reorganization of the visual system induced by patterns such as Fig. 1a or b

Much ingenuity has been expended in attempts to dismiss the CAI as an artefact of eye tremor (6), fluctuations of accomodation (7) and the like, apparently on the assumption that superposition of displayed images of the stimulus would give rise to Moire-type patterns of similar geometry. In fact, however, the form of the CAI is quite different from that of the corresponding Moire patterns; and in any case I found that it was visible after exposure of the inducing figure in a single flash lasting only a few microseconds, or with a stabilized retinal image even when homatropine was used to immobilize the lens [8, 9]. The CAI does not even require the presence of a negative afterimage of the stimulus, being actually more vivid if the inducing fig-

ure has been systematically moved during inspection so that no contours remained in one place on the retina [8].

WHAT DO GRATINGS DO TO THE VISUAL SYSTEM?

One might be tempted to suppose that the CAI reflects merely some transient disturbance of the visual system caused by the simultaneous removal of the many lines making up the inducing figure; and if this were all, it would still be a challenge to physiological theory. It turns out, however, that the visual system is set into a highly anomalous state for the whole time that the inducing figure is present. A dramatic way of demonstrating this [3, 4] is to superimpose on the figure a transparency of random visual noise (Fig. 2). (Readers might like to try this, using a sheet of glass as a mirror to superimpose the image of Fig. 2 optically on to Fig. 1.) Immediately, the grains of noise find themselves organized into an outline of the CAI appropriate to each figure, rather as iron filings are organized by the field of a magnet. Gently moving the transparency to and fro, one sees the induced complementary image (CI) moving with the noise [10]. At rest, the CI fades from view, but it can be reactivated at once by displacing the noise.

With dynamic noise (such as the 'snowstorm' on a detuned receiver) as a neutral test field, the CI can be rendered continuously visible [3]. It is now seen in the same rapid motion as the CAI, demonstrating that the latter reflects changes brought about in the visual network during presentation of the stimulus, and not a mere off-effect of its removal. The effect

is that of a dynamic 'ambiguous figure', the direction of the perceived streaming motion (along the contours of the CI) being spontaneously reversible and partly under voluntary control. Significantly, however, the motion is quite precisely locked to the succession of noise fields. If two or three successive fields are made identical, for example, the perceived streaming stops dead for a moment until the random sequence resumes [11]. This shows that the motion perceived is not due to some non-specific swirl of internal activity with its own momentum, but rather reflects a systematic bias in the local computational networks that stand ready to extract motion information from successive image-changes.

GRATINGS BIAS PERCEPTION OF PHI-MOTION

An early experiment by one of my students, M.E. Wilson [12], showed that this directional bias could be detected even with the simplest of motion stimuli. Wilson set up an ambivalent stimulus (Fig. 3) consisting of a pair of synchronously flashed lights A and B followed after approximately 1/10 sec by a third flash from a source C at the vertex of a right-angled isosceles triangle with A and B as base. The sources were embedded in a grating of black and white lines lying parallel to the direction AC.

Wilson found that in this context 'phi' motion was invariably perceived more strongly in the direction BC - i.e. across, rather than along, the grating lines. The presence of contours lying at a given orientation appears to pre-sensitize motion-

detecting correlators spanning those contours in a direction at right angles to them. Another of my students at that time, J.P. Wilson [5, 13] discovered that even a single black/white edge can be sufficient to induce a perceptually significant bias in favour of directions orthogonal to it.

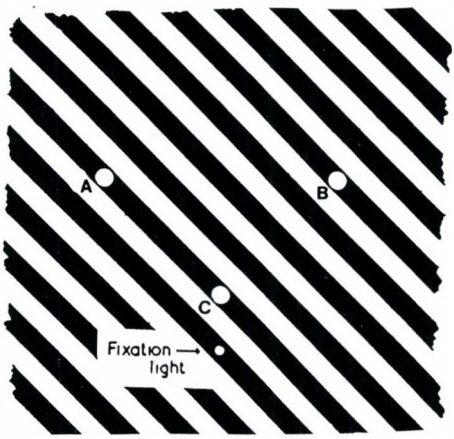

Fig. 3. Ambivalent phi-motion stimulus of the type used by M.E. Wilson [12]. Lamps A and B are flashed simultaneously, and lamp C at a suitable interval to induce apparent movement, which is seen predominantly in a direction perpendicular to the grating lines

It is not necessary to assume, as Georgeson [14] has recently suggested, that the interacitons responsible take the simple form of inhibition between contour- and motion-specific single units [see ref. 15]. As I shall argue below, the evidence suggests cooperative interactions on a larger sclae. What is clear, however, is that in the presence of gratings, or even single contours, the information-processing functions of the visual network can be significantly distorted over a relatively wide area, and in ways that are not obvious a priori.

TIME-COURSE OF INDUCTION

From the geometry of the CI, I was led in 1957 to postulate that in the visual network "direction of contour" was treated as a primitive variable, with directions at right angles being treated as competitive, and "directional satiation" resulting from prolonged exposure to near-parallel contours [3]. When Hubel and Wiesel [1] discovered visual cortical neurons specifically sensitive to contour-orientation, it was tempting to interpret the CI as due to selective adaptation of the subpopulation of such cells stimulated by the pattern [16]; but the physiological evidence is still inconclusive, and in any case the CI has some features (e.g. its wavy character) that would not be predictable directly from this assumption. It seems more profitable to treat it at this stage as a source of clues to the dynamics of the laterally coupled population of visual cells concerned.

To this end, it is important to know how the interaction between inducing and test stimuli manifested in the CI depends on their relative timing. In a series of experiments [17] using flash presentation of both inducing figures and test fields (random noise) I found that even briefly presented spatially-periodic patterns can induce in the visual network a highly abnormal transient physiological state, in which a randomly textured visual field evokes an anomalous non-randomly structured response. With flash presentation the anomalous excitability has a fairly well defined time-course, reaching a maximum for test signals delivered 80 - 90 msec after the inducing signal (Fig. 4(C)). With sufficiently short repetition periods the

overlap of induced activity from one presentation to the next gave rise to further anomalies of perception (see (A) and (B) in Fig. 4) that deserve separate investigation. It was clear, however, that such overlap is not essential for apparent movement to be seen in the noise at the critical phase.

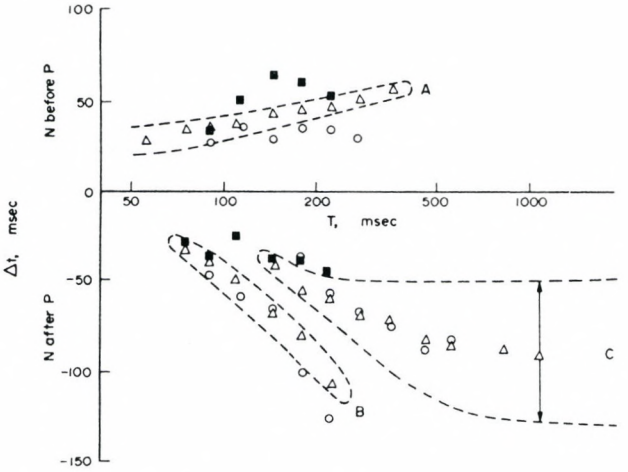

Fig. 4. Variation in critical interval for anomalous excitability, Δt, between flash-presentation of inducing pattern and test pattern, as a function of stimulus repetition period T. Δ, dynamic noise paired with grating, ray and target patterns (average over all three, for five subjects). 0, static noise paired with ray pattern (two subjects). , blank flash paired with ray pattern (two subjects). The approximate range over which each phase was visible is indicated (for the visual noise test pattern Δ) by dotted lines (from MacKay, ref. 17).

TEMPORAL ASPECTS OF INDUCTION OF "PHI-BIAS"

The critical time relations between inducing and test presentations in the foregoing experiments suggest analogous questions with regard to the directional bias found by M.E. Wilson [12]. In current investigations (unpublished), miniature light-emitting diodes (green), arranged as in Fig. 3, were flashed at

variable intervals in relation to a brief stroboscopic presentation of the (optically superimposed) striped grating. Preliminary results suggest that "phi-bias" is greatest when the strobed grating is presented 50-100 msec before the first diode flashes, broadly in line with the results of the preceding section.

ANATOMICAL LOCUS

In considering possible anatomical and physiological bases for this group of phenomena, it is helpful to know whether the effect "transfers" from one eye to the other - i.e. whether presentation of the inducing pattern to one eye causes a perceptual bias in the other. Evidence to date [3 - 5] indicates that any transfer of bias from the stimulated eye to the other represents only a small fraction of the full effect. Thus the great bulk of the changes in system characteristics brought about by exposure to gratings and the like must take place at a level in the visual system before information from left and right eyes is combined. The uniocular population of primary visual cortex offers a natural candidate; but where cooperativity is concerned one cannot rule out more peripheral structures (even including the retinal network) since there is no obvious reason to require that single units in the population concerned should show orientation-specificity.

On the other hand, both J.P. Wilson [5] and M.E. Wilson [12] have observed that CI induction is perceptually stronger with binocular viewing of the figure than with monocular - suggesting that the binocularly driven population may also be susceptible.

With stabilization of the retinal image of the inducing figure, MacKay et al. [9] found that dynamic noise presented to the same eye was reorganized in the form of the CI only for as long as the stabilized image remained visible. If the fading of stabilized images were due simply to retinal adaptation this would not help in the matter of localization; but if the fading turns out to have a more central basis [18] it might prove to be a significant clue, since whatever cooperative process is responsible for the perceptual bias of the CI must presumably be located at the same or a still more central level.

THEORETICAL IMPLICATIONS

My reason for injecting this piece of esoteric psychophysics into a Symposium on neurons and neuron assemblies will now be plain. The fact that at some levels of the visual network the presence of spatially repetitive figures of excitation can induce a highly anomalous cooperative state, grossly distorting the transfer characteristic, and capable of persisting for a second or more after removal of the stimulus, suggests some questions of wider relevance. For example, is there any reason to attribute this propensity to some special anatomical feature of the visual network? If not, what analogues might we expect to find in other cortical areas? Is it possible that by analysing cortical networks in terms of the "preferred stimuli" of single units or modules (however profitably) we are missing a whole complementary group of cooperative "ensemble properties" which may have their own functional significance? Granted that only a small class of

visual stimuli induce the anomalous effects here reviewed, is not their capacity to do so a sign that every stimulus component may have its repercussions throughout a relatively large region of the network? If so, does the recognition or classification of a stimulus depend on the specific spatio-temporal *mode* of cooperative activity it sets up in a distributed population, rather than merely on the specific constellation of units that it activates?

There are of course many other lines of evidence pointing to the existence of long-range interactions in the visual cortical population. Wilson and Singer [20], for example, have recently found that the ability to discriminate between double and single flash stimuli can be adversely affected by simultaneous presentation of a second, similar, stimulus at least 20 deg. of arc away. Foster [21] has also reported evidence of anomalous perception of the motion of grating stimuli.

Neurophysiologically, Hammond and I [22] have found both suppressive and excitatory interactions between stimuli presented respectively within and outside the classical receptive field of single units in cat Area 17. New multi-electrode recording techniques show particular promise in this connection. Toyama et al. [23, 24], for example, have found that under chemical stimulation about 10% of cells in area 17 of cat show delayed excitation or inhibition following excitation of cells at distances up to 200 - 300 μm. Since the duration of inhibition was found to be of the order of 80 msec, recurrent couplings between chains of such units could readily give rise to oscillatory activity at the frequencies subjectively observed in the CAI, and this would fit also with the critical induction time

of about 100 msec; but detailed model-making at this stage would seem to be premature. Methods such as that of Krüger [25], who has succeeded in recording simultaneously from an array of 30 microelectrodes over an area of 0.5 mm^2, can in principle allow relationships between the firings of widely separated cells to be computed and displayed. The picture revealed so far, however, is striking for the relative *absence* of close correlations, particularly over short time intervals [25].

To avoid information-overload with such a rich source of data, of course, one must have fairly specific questions to answer. The argument of this paper is that stimuli which give rise to short-term cooperative re-organization of the cortical network may be particularly appropriate for such studies; and that close interaction between psychophysics and neurophysiology in this area may provide one of the best ways of generating fruitful questions.

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INFLUENCE OF DEHYDRATION ON ACTIVITY CHANGES OF PINEAL GLAND AND SUBCOMMISSURAL ORGAN CELLS IN *RANA TEMPORARIA* L. IN ANNUAL CYCLE

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(Received 3 July 1981)

The karyometric analysis of the cells of the pineal gland and subcommissural organ in *Rana temporaria* L. was carried out under the influence of dehydration in the annual cycle. The dehydration stress was found to retard the activity of the pineal gland and at the same time to stimulate the activity of the subcommissural organ apart from the breeding period and the middle period of active life on land. The results showed that both the pineal gland and the subcommissural organ take part in the regulation of water balance in the species under study.

Introduction

Numerous experimental data suggest that the pineal gland (PG) affects activity and behaviour [34, 40], immunological reactions [19], the metabolism of neurotransmitters of the brain [46], the gonads [12, 35] and the function of the hypophysis [36].

Other reports suggest that the epithalamo-epiphyseal system including the subcommissural organ (SCO), takes part in the regulation of water and electrolyte metabolism [3, 6, 13, 26].

Although it is accepted that the SCO has a role in the regulation of salt and water balance [27], the role of PG in this respect is ambiguous [28], in spite of the possibility of an embryological and morphological correlation between the two organs [32]. Therefore, in the present study we have examined the influence of dehydration stress upon the activity of PG and SCO in *Rana temporaria* L. in the annual cycle.

Materials and methods

Experiments were carried out on 105 males 105 females of *Rana temporaria* L. in 7 characteristic periods of their life, accepted on the basis of studies done by Juszczyk [20]. In each period 15 female and 15 male frogs of similar length were caught in the surroundings of Kraków in their natural habitat. They were divided into three groups: control, experimental group I and experimental group II. The frogs in groups I and II were subjected to dehydration,

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those in group I until they had lost about 30% and group II until they had lost about 65% of their total amount of water together with their defensive reflexes. Dehydration was carried out at about 20 C° room temperature at 55–75% humidity. Similar values were found for the loss of water leading to the decline of defensive reflexes in this species of amphibian by Zamachowski [47].

The brains were fixed in formalin, neutralized with lithium carbonate, embedded in paraffin, serially sectioned at 5 μm and stained with Luxol Fast Blue, cresyl violet and carbol fuchsin [2]. In the sections the volume of cellular nuclei of PG and SCO was defined. Nuclear volume was considered a morphometric coefficient showing the functional activity of the cell [29]. Nuclear volumes were calculated after the long (L) and short (B) axis of each nucleus had been measured and after having included these values in Palkovits' formula [25]: $V = \pi/6\ 6LB^2$. In each frog 100 cell nuclei were examined. A total of 21 000 measurements were done and arithmetic means as well as standard deviations were calculated. In order to find whether the changes were significant statistically in the annual cycle, variation analysis was used.

Results

Pineal gland. Dehydration caused a decline of the nuclear volume in PG cells in both experimental groups (Table I, Fig. 1). The course of changes in the control group as well as in both experimental groups was significant statistically during the whole year (Table II).

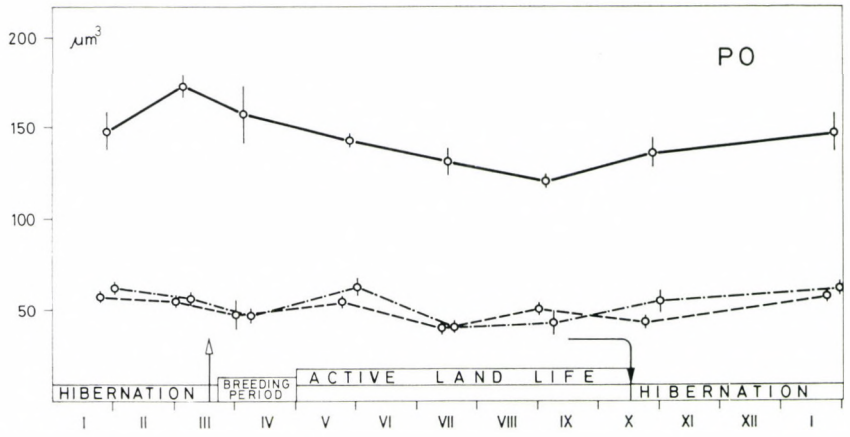

Fig. 1. Mean volume of cellular nuclei of the pineal gland in females of *Rana temporaria* L. under the influence of dehydration during the annual cycle. Continuous line — control, broken line — experimental group I, broken-spotted line — experimental group II, vertical lines — standard deviations, upward arrows — frogs' exit from their hibernation quarter, downward arrows — frogs' entering the hibernation quarters

Subcommissural organ. Dehydration stress caused a visible increase in volume of the cellular nuclei in this organ, apart from the breeding and the middle period of active life on land, periods when the obtained volumes decreased in comparison with the control values (Table I, Fig. 2). In the early and late periods of hibernation, in both the control and the experimental groups the mean nuclear volumes were similar. Their changes during the annual cycle were statistically significant in each group (Table II).

Table 1

The influence of dehydration on the changes of nuclear volume (in μm^3) of pineal gland (PG) and subcommissural organ (SCO) in *Rana temporaria* L. during the annual cycle

	Group	Sex	3rd decade of January	1st decade of March	3rd decade of March	3rd decade of May	2nd decade of July	1st decade of September	3rd decade of October
Pineal gland (PG)	Control	♀	147.2±10.58	172.8± 5.94	156.7±16.06	141.7± 2.56	131.0± 7.16	120.3± 1.98	135.9± 8.24
		♂	136.8±11.27	175.4±13.48	153.3± 6.84	149.0± 1.56	142.5± 8.35	101.5± 5.92	126.5± 6.13
	Group I	♀	57.5± 2.00	54.8± 1.56	47.5± 9.38	54.3± 1.83	39.8± 2.63	51.3± 2.80	55.2± 6.49
		♂	46.8± 3.48	43.5± 4.78	40.1± 2.28	49.0± 3.86	44.3± 4.20	52.2± 1.43	41.7± 2.20
	Group II	♀	62.1± 2.28	56.0± 2.57	47.3± 2.27	63.3± 4.47	40.6± 2.42	61.2± 6.07	55.6± 6.49
		♂	57.3± 4.29	53.5± 2.64	41.1± 4.10	53.3± 5.94	49.5± 1.53	55.8± 3.43	50.8± 2.20
Subcommissural organ (SCO)	Control	♀	63.5± 5.82	85.5±12.01	75.1± 7.06	55.7± 7.94	84.0± 5.98	38.0± 4.85	78.3± 4.37
		♂	68.8± 1.70	92.0±11.59	72.4± 4.08	69.9± 6.18	85.0± 9.53	48.1± 2.41	80.1± 4.24
	Group I	♀	78.0± 5.82	75.5±12.59	57.6± 6.92	86.8± 3.31	37.0± 4.57	68.3± 4.16	74.9± 6.48
		♂	73.5± 7.66	73.8± 7.31	59.6± 7.37	74.0± 9.25	39.3± 3.26	72.3± 2.41	95.2± 6.50
	Group II	♀	84.0± 7.32	88.5±10.52	60.7± 5.66	75.3±10.27	44.9± 3.02	84.0±17.43	77.1± 6.48
		♂	74.9± 5.32	82.3± 6.90	58.9± 3.24	71.6±15.88	43.6± 3.13	94.9±12.29	80.7± 4.24

Each phase contained 5 females and 5 males

Table II

Analysis of variance of mean nuclear volume of neurocytes of the pineal gland (PG) and subcommissural organ (SCO) in *Rana temporaria* L. after dehydration during the annual cycle

	Group	Sex	Source of variability	Degrees of freedom	Sum of square	Mean square	F_0
Pineal gland(PG)	Control	♀	Between all phases	6	9009.98	1501.66	20.34
			Within each phase	28	2067.17	73.82	
		♂	Between all phases	6	15961.73	2660.28	37.14
			Within each phase	28	2005.51	71.62	
	Group I	♀	Between all phases	6	1319.79	219.96	12.39
			Within each phase	28	496.79	17.74	
		♂	Between all phases	6	541.01	90.17	8.25
			Within each phase	28	306.11	10.93	
	Group II	♀	Between all phases	6	2138.77	356.46	20.46
			Within each phase	28	487.75	17.42	
		♂	Between all phases	6	867.13	144.52	10.45
			Within each phase	28	387.26	13.83	
Subcommissural organ (SCO)	Control	♀	Between all phases	6	8915.67	1485.94	32.21
			Within each phase	28	1291.70	46.13	
		♂	Between all phases	6	5983.36	997.22	23.40
			Within each phase	28	1192.81	42.60	
	Group I	♀	Between all phases	6	7950.44	1325.07	43.50
			Within each phase	28	852.78	30.45	
		♂	Between all phases	6	8892.58	1482.09	33.48
			Within each phase	28	1239.38	44.26	
	Group II	♀	Between all phases	6	7222.75	1203.79	12.48
			Within each phase	28	2699.11	96.39	
		♂	Between all phases	6	8467.19	1411.19	19.07
			Within each phase	28	2071.96	73.99	

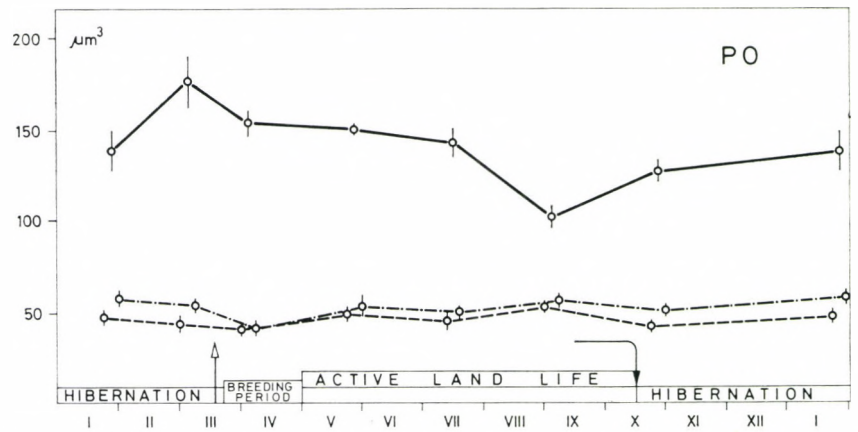

Fig. 2. Mean volume of cellular nuclei of pineal gland in males of *Rana temporaria* L. under the influence of dehydration during the annual cycle. Designations as in Fig. 1

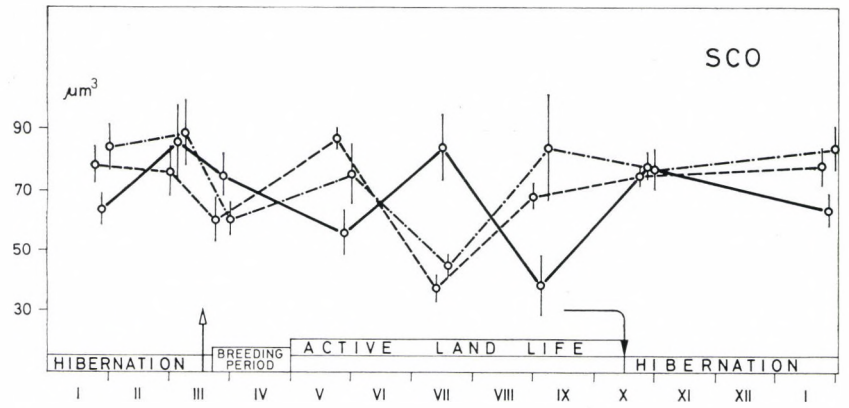

Fig. 3. Mean volume of cellular nuclei of the subcommissural organ in females of *Rana temporaria* L. under the influence of dehydration during the annual cycle. Designations as in Fig. 1

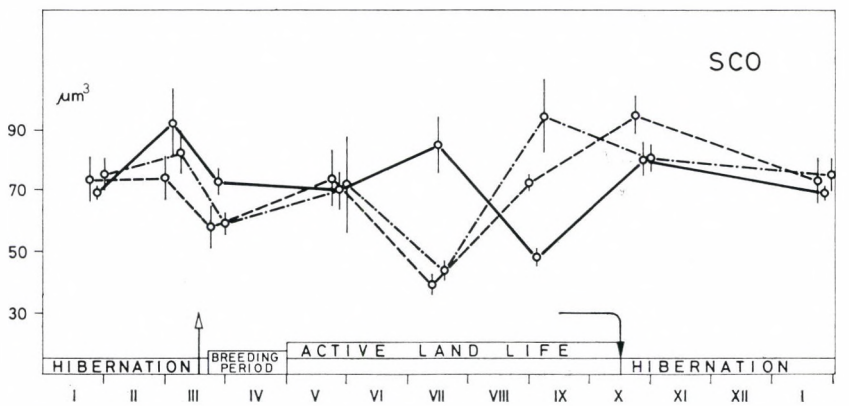

Fig. 4. Mean volume of cellular nuclei of the subcommissural organ in males of *Rana temporaria* L. under the influence of dehydration during the annual cycle. Designations as in Fig 1

Discussion

The results showed that dehydration has a considerable influence upon the activity of the cells of the PG — SCO complex in *Rana temporaria* L. during the annual cycle. The influence manifests itself with a decrease in nuclear volume in the PG and an increase in nuclear volume in the SCO.

In numerous experiments PG extracts were found to increase the production of aldosterone [3, 23, 38, 39], although several authors could not confirm this [22, 24, 30], nor could Coghlan et al. [4], Farrell [13], Keeler [21], Palkovits and Wetzig [32], Stalsberg [42] and Van der Wall et al. [45] observe changes in adrenal activity or aldosterone production after pinealectomy. In

contrast, Giacomelli [16] found karyometric changes in the zona glomerulosa after pinealectomy. Still, the exact karyometric studies of the rat's PG carried out by Palkovits [28] suggest that this organ is not directly involved in the regulation of salt and water balance.

The present study seems to point to the indirect influence of the PG upon the water balance in *Rana temporaria*, by a decline of pineal activity after dehydration. The decline of pineal activity may stimulate antidiuretic brain centres to intensive production of certain antidiuretic hormones and their increase would retain water in the amphibian organism [41].

This suggestion is based on the fact that the PG is generally credited with a retarding action on e. g. iodine accumulation by the thyroid gland [7, 8, 18], the function of islet cells [5] or of the gonads [37]. In addition, the suggestion seems to be supported by the increase after pinealectomy of neurosecretory activity of the supraoptic and paraventricular nuclei [9, 10]. Gilbert [17] was the first to observe the connection between the SCO and the changes in blood volume. Further studies then showed that the extract of the SCO had an influence upon sodium and water absorption [14] while after electrocoagulation of the organ aldosterone production decreased [31, 44]. The SCO is stimulated by a diet poor in sodium and potassium [15] and karyometric studies by Palkovits et al. [33] clearly showed that isoosmotic hypovolaemia could stimulate SCO activity.

The present results have confirmed the above data and suggest that dehydration affects the activity of SCO neurones in *Rana temporaria* L. throughout the year apart from the breeding period and the middle period of active life on land. The decline of neuronal activity in the mentioned periods is difficult to explain. It might be assumed that in these periods other antidiuretic brain centres are responsible for the regulation of water balance, probably the subfornical organ and the preoptic nucleus, areas which always have an influence on water balance [1, 11, 43].

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MORPHOLOGICAL STUDY OF THE KIDNEYS OF LITHIUM TREATED RATS

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(Received 2 September, 1981)

Short-term treatment of rats with low doses (2 meq/kg) of lithium carbonate led to renal damage which, however, appeared to be almost completely reversible even in nephrectomized animals. Renal damage caused by higher doses (6 meq/kg) was more explicit, but also appeared to be reversible to a certain degree even in the case of continuous lithium treatment. Regeneration was less expressed in nephrectomized animals. The collecting tubules and some segments of Henle's loop, as sites poor in mitochondria, display increased susceptibility to the damaging effect of lithium carbonate.

Introduction

The damaging effect on the kidney of lithium salts has been known almost since the discovery of their usefulness in psychiatry [7]. Papers on the subject are abundant [4, 8], but data regarding the structural changes, which are more pronounced in the distal tubule and collecting tubule, are scarce [2, 3, 5, 6]. Our animal experiments were aimed at clarifying any correlation between dosage and duration of lithium treatment and the site, character and severity of the structural changes.

Materials and methods

Sixteen 4–5 months old CFY rats of both sexes from our own breed weighing 250–300 g were used in the experiment. The animals were divided into three groups. One group was treated with 6 meq/kg, the other with 2 meq/kg doses of lithium carbonate once daily, orally through a gastric tube. The control animals received 1 ml/100 g physiological saline by the same route. Fluid intake was not restricted. One day after the first treatment, half of the animals in each experimental group were subjected to nephrectomy under ether anaesthesia. The other half of the high-dose group was nephrectomized on day 5, and of low-dose group, on day 12. On day 5 and 12, the nephrectomized rats of the high-dose group, on day 12 and 23, those of the low-dose group and on day 23, one control rat were killed by decapitation and the kidneys were removed for histological processing. One rat of the high-dose group, 3 rats of the low-dose group and one control rat died due to complications of physical harms caused by the gastric tube.

Examination of the kidneys was thus performed on days 1, 5 and 12 in the group treated with daily doses of 6 meq/kg lithium carbonate, on days 1, 12 and 23 in the 2 meq/kg group and on days 1 and 23 in the control group. The removed kidneys were cut into halves, one half was fixed in 10% formaldehyde, embedded in paraffin and stained with haematoxylin-eosin. The other half was fixed in 4% glutaraldehyde or formaldehyde, and 1 mm^3 blocks excised from them were embedded in resin (Durcupan). 1 μm semi-thin sections cut with an LKB ultramicrotome were stained with toluidine blue and examined light-microscopically.

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Table I

	Lithium treated rats				Lithium treated rats								Control group			
	6 meq/kg				2 meq/kg											
Serial number of rats	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Day 1	N	N			N	N	N	N					N	N		
Day 5	K	K	N	N												
Day 12			K	D	K	K	K	K	N	N	N	N				
Day 23									K	D	D	D			K	D

The course of lithium treatment, day of nephrectomy (N) and that of death: killed (K) or died (D)

Results

In the rats treated with 6 meq/kg lithium carbonate, severe necrosis of the epithelial cells in the collecting tubules and in some segments of Henle's loop was detectable after the first day of treatment. The cells of the convoluted tubules showed fine vacuolization only. The glomeruli were intact (Fig. 2). On day 5, significant regeneration of the damaged epithelium could be observed in the unoperated animals (Fig. 3*a*). Signs of regeneration could also be detected in the kidneys of the nephrectomized animals, but they were much slighter than in the unoperated group (Fig. 3*b*). On day 12, regeneration in the kidney of the nephrectomized animal was more expressed than on day 5, but a great number of necrotic or damaged cells were still visible (Fig. 4). Vacuolization did not increase appreciably and the glomeruli were unchanged.

In the low-dose group degeneration or necrosis of the epithelium in the collecting tubules and in some segments of Henle's loop was also observed on the first post-treatment day, but the changes were slighter than in the high-dose group (Fig. 5). Vacuolization of cells of the convoluted tubuli was very slight and did not increase on continuation of treatment. On day 12, regeneration was almost complete in the unoperated animals (Fig. 6*a*). Signs of regeneration were also present in the nephrectomized animals, but smaller in degree than in the unoperated ones. (Fig. 6*b*). On day 23, regeneration in the nephrectomized rat reached the degree observed in the unoperated animals on day 12 (Fig. 7). In the glomeruli, in contrast to the earlier negative finding (Fig. 8*a*, day 12), at some sites proliferation of the mesangial matrix was suspected (Fig. 8*b*).

Discussion

The results indicated that renal damage caused by lithium treatment develops after 24 h. Epithelial damage and necrosis can be detected in the collecting tubules and in some segments of Henle's loop already after the administra-

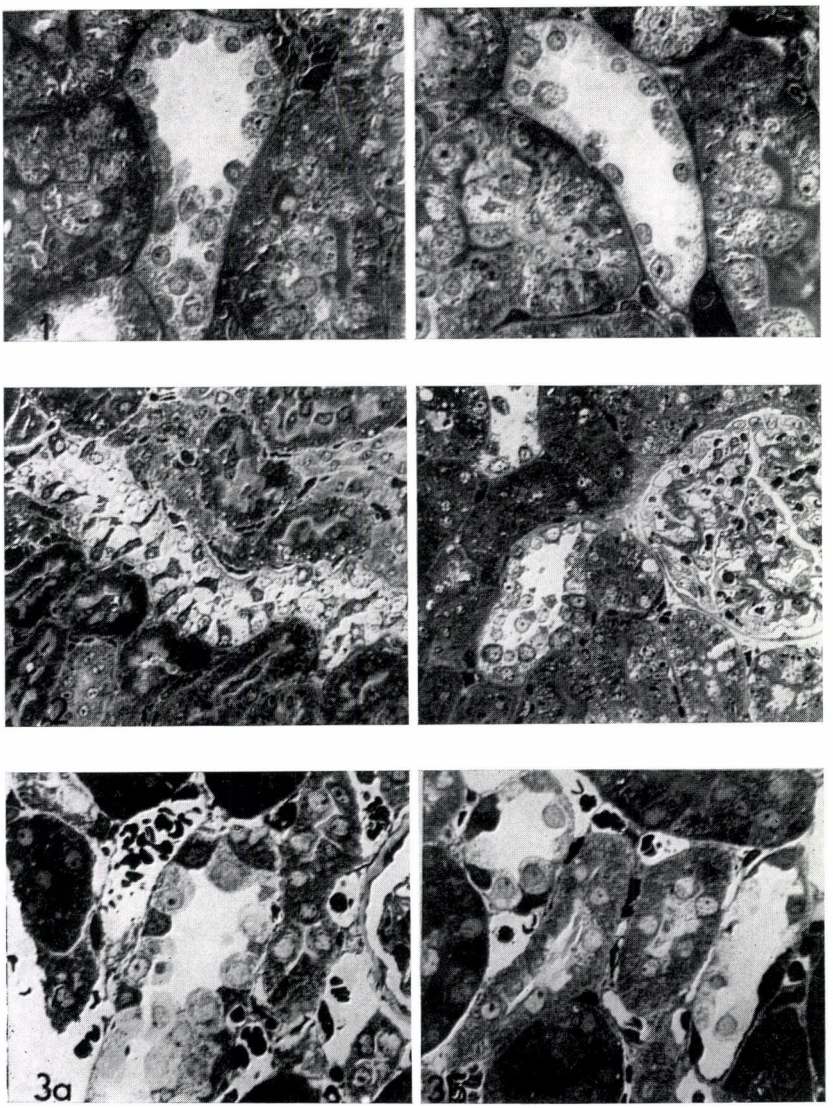

Fig .1. Semi-thin section of kidney of NaCl treated control rat on day 23. Some cells of the epithelium lining the collecting tubules and Henle's loop show darker staining. A few detached epithelial cells in the lumen of the collecting tubules. × 800

Fig. 2. Semi-thin section of kidney of rat treated with 6 meq/kg lithium carbonate. One day after treatment. Degeneration or necrosis of epithelial cells of the collecting tubules and Henle's × 500

Fig. 3. Semi-thin section of kidney of rats treated with 6 meq/kg lithium carbonate. Day 5. *a* Considerable regeneration of epithelium of the collecting tubules in non-operated animal. The epithelial lining of the tubules is almost complete. *b* Although signs of regeneration occur in the kidney of the nephrectomized animal, this is not complete. × 800

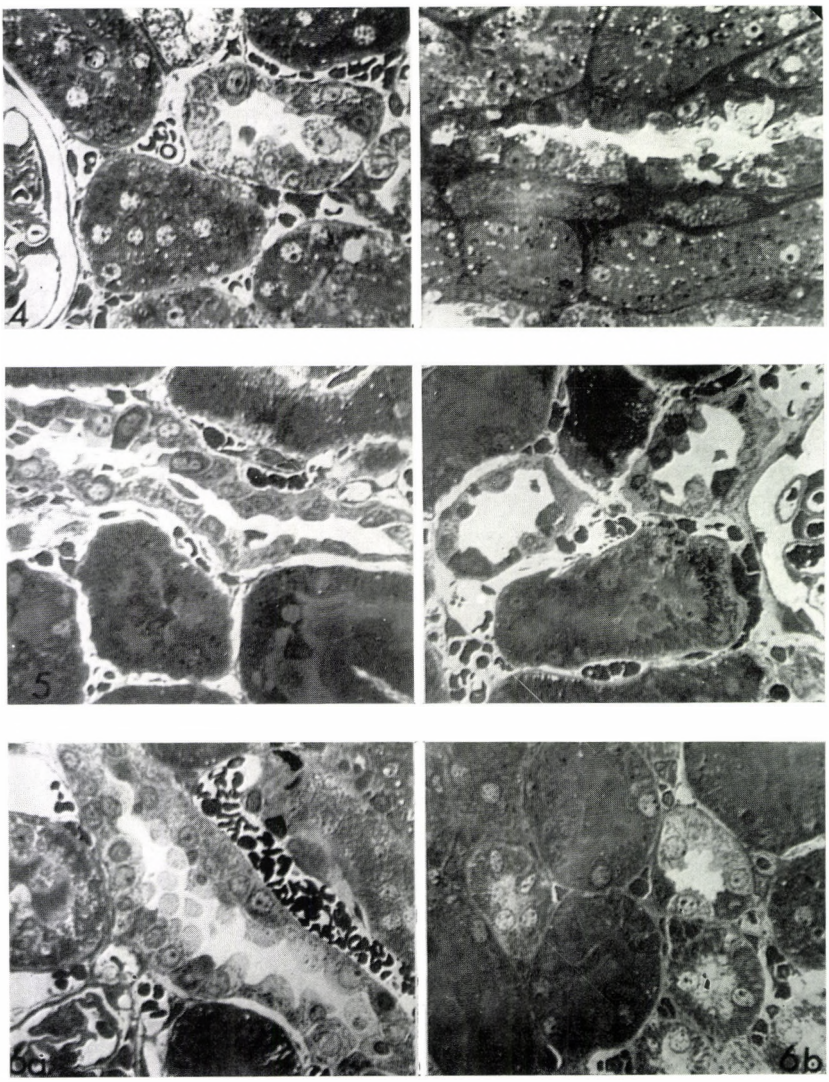

Fig. 4. Semi-thin section of kidney of nephrectomized rat treated with 6 meq/kg lithium carbonate. Day 12. Regeneration of the epithelium lining the collecting tubules and segments of Henle's loop is more complete than in the nephrectomized animal on day 5 (Fig. 3*b*), but a considerable number of damaged or necrotic cells is still visible. Vacuolization of the epithelial cells of the convoluted tubules did not increase appreciably. × 800

Fig. 5. Semi-thin section of kidney of rat treated with 2 mEq/kg lithium carbonate, one day after treatment. Degeneration or necrosis of the epithelium of collecting tubules and some segments of Henle's loop is visible. The changes are less severe than in the 6 meq/kg group (Fig. 2). × 800

Fig. 6. Semi-thin section of kidney of rats treated with 2 meq/kg lithium carbonate. Day 12. *a* Regeneration of lining epithelium of collecting tubules is almost complete in the non-operated animal. *b* Regeneration in the kidney of nephrectomized animal lags behind that in the non-operated rats, but is still significant. × 800

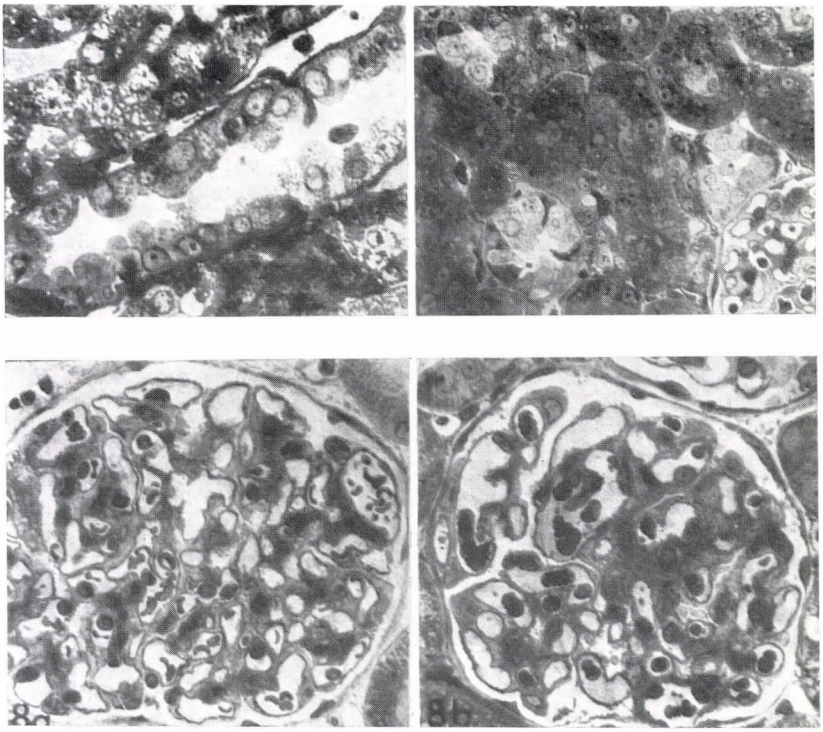

Fig. 7. Semi-thin section of kidney of rat treated with 2 meq/kg lithium carbonate. Day 23 Regeneration of the epithelium lining the collecting tubules reaches the degree seen on day 12 in non-operated animal (Fig. 6*a*). It is almost complete. × 800 and × 500

Fig. 8. Semi-thin section of kidney of rats treated with 2 meq/kg lithium carbonate. Day 12 and day 23. *a* No pathologic change on the glomerulus on day 12. *b* On day 23, proliferation of mesangial matrix can be suspected. × 800

tion of a single dose of 2 meq/kg lithium carbonate. Regeneration is almost complete on the 12th day. In the nephrectomized animals, regeneration on day 12 did not reach the level observed in the unoperated animals, but by day 23 it was almost complete.

In the kidneys of the rats treated with 6 meq/kg, necrosis of the epithelium of the collecting tubules and that of some segments of Henle's loop was more significant. In spite of this, regeneration on day 5 was pronounced in the unoperated animals, but slight in the nephrectomized ones. On day 12, the degree of regeneration was slighter than in the 2 meq/kg group, but it was still considerable.

It may therefore be assumed that the renal damage accompanying lithium treatment develops at sites where the epithelium shows increased susceptibility. It was conspicuous that epithelial damage or necrosis should develop at these sites where the cells are poor in mitochondria, thus in the col-

lecting tubules and some segments of Henle's loop. Therefore, a correlation may be suspected between the increased vulnerability of these areas and their paucity in mitochondria.

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ULTRASTRUCTURAL CONCEQUENCES OF REPERFUSION OF THE ISCHAEMIC MYOCARDIUM

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Ultrastructural changes following ischaemic myocardial injury have been investigated in dogs. After 15 to 120 min global normothermic ischaemia the hearts were reperfused in a special heart-lung model for 60 min and then subjected to electron microscopic examination. The degree of morphological alterations and the duration of ischaemia were approximately interrelated. The ischaemic hearts showed characteristic changes such as mitochondrial, sarcolemmal and myofibrillar defects (swelling, disruption of cristae, loss of matrical density and contractile bands, myofibrillar rupture and lysis, blurred Z-lines). The hypercontractions are no artifacts but consequences of irreversible cellular injury and the observed stiffness during reperfusion is a good marker of the myocardial damage suffered

The influence of ischaemia on post-ischaemic myocardial function is well established and known to result in pump-failure [2, 3, 5, 6, 7, 9, 12, 17]. Since structure, metabolism and function are closely interrelated, ultrastructural investigation provides information on the effects of pathological influences. If possible, ischaemic damage to the heart is considered and post-ischamic recovery time is protracted, in such cases the critical point of ischaemia-tolerance must have already been exceeded and structural changes have to be reckoned with [2, 19].

There has been some controversy about the degenerative ultrastructural changes after ischaemia and reperfusion. Some authors [15] regard contraction banding a common change in tissues obtained by biopsy and consider these contractile bands artifacts, while other authors [21] believe that the number of contractile bands increases with the severity of ischaemia.

In our earlier work [22] unprotected and preserved myocardial ultrastructure was analysed in prepump and post-reperfused states. Because distinct morphological changes had been observed by electron microscopy, additional investigations were undertaken to study the ultrastructural sequence of myocardial injury after prolonged ischaemia.

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Materials and methods

A special in situ isolated heart-lung model was surgically prepared in dogs as described previously [22] (Fig. 1). In addition, a heart-lung preparation was used with normal coronary and partial pulmonary circulation; retrograde pumping only served to stabilize blood pressure.

Normally functioning hearts were exposed to rapid general ischaemia: the pump was stopped, all blood was drained to the reservoir, and breathing was suspended. The heart after some powerless contractions stopped beating. In 45 cases normothermic ischaemic hearts were reperfused after 15, 30, 45, 60, 90, and 120 min ischaemia.

In the applied system the resting ischaemic hearts were reperfused retrogradely. In most cases fibrillation appeared in a short time; it could easily be suspended by a single electroshock. Exceptionally, after 15 to 30 min ischaemia the hearts started spontaneously; after 90 to 120 min ischaemia they showed reduced contractility, dilatation or no contractions.

During and after 60 min normothermic reperfusion the hearts were examined, the ECG and haemodynamics and the external signs of myocardial damage were recorded. Thereafter specimens were obtained from the subepicardial, midmyocardial and subendocardial layers an subjected to electron microscopic examination.

To avoid possible artifacts, i.e. the zonal supercontraction (so-called „minor" banding) consisting of an outer zone tissue damage immediately below the excision, the marginal zones of the tissue blocks were removed after embedding and the central parts were only evaluated.

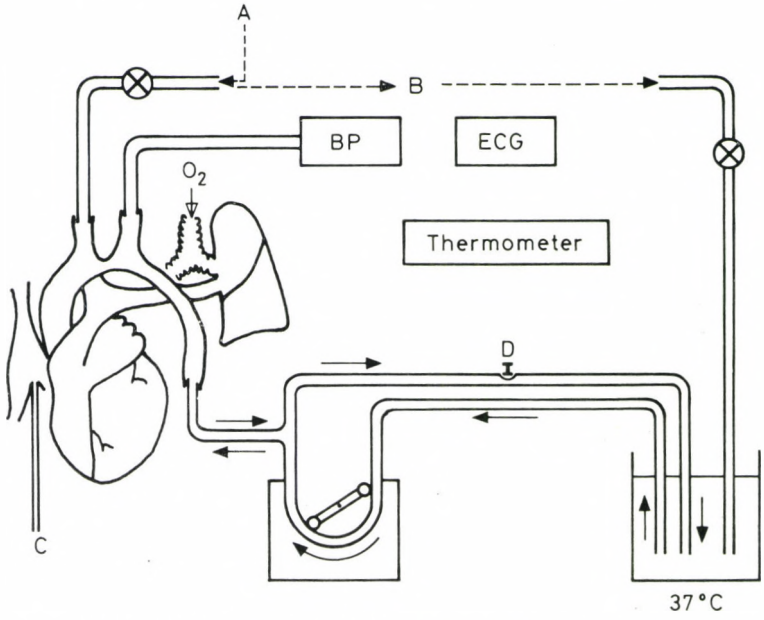


Fig. 1. The experimental model. A and B = functioning during surgical phase only; C = drainage and blood samples (if necessary); D = blood pressure regulating valve; → = flow dircetion during experimental phase

Results

Observations in vivo

In our model the nonworking ischaemic heart was always easily restarted except after 120 min anoxia. After normothermic reperfusion the coronaries filled up and immediately irregular fibrillation activity appeared; it became

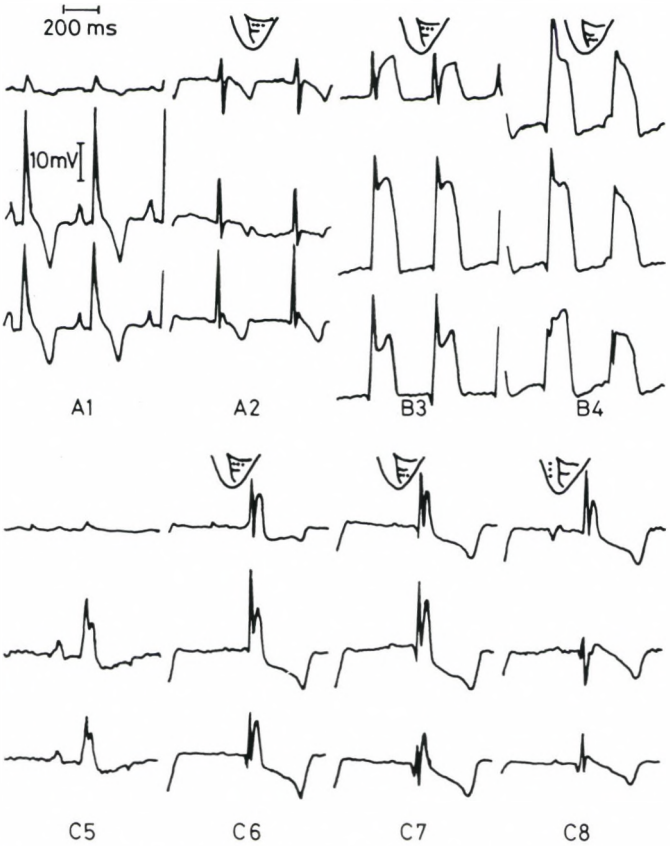


Fig. 2. ECG from normal, regional and global ischaemic hearts. A = normal heart (1 = standard lead, 2 = epicardial mapping s. symbols). B = typical regional ischaemia (3 and 4 = = maps), after ligature of LAD severe ST elevation, high positive T. Diversity of ischaemic signs, often arrhythmia. C = global ischaemic sequences (5 = standard lead, 6, 7 and 8 = = maps), no or only transient arrythmia. Slight ST elevation. T wave remains negative. QRS broadening is typical. Uniformity of ischaemic signs predominates

stronger in inverse proportion to the duration of ischaemia. Defibrillation was successful except in one case. The ECG showed slight ischaemic signs (Fig. 2).

After 90–120 min ischaemia the contractions became reduced, dilatation or an absence of contractions with stiffness of the left ventricular wall was observed.

In the early reperfusion period more and more subepicardial petechial haemorrhages appeared especially after 60 to 120 min anoxia. At the end of the 60 min reperfusion period transmyocardial haemorrhages appeared. In such cases the myocardium displayed signs of severe tissue damage [22].

Table I

Classification of myocardial injury after normothermic ischaemia plus reperfusion

Normothermic ischaemic time min	Grade	Mitochondrium	Cell membrane	Nucleus	Sarcomere	
					concentration	Z-line
0	0	Normal	Normal	Normal	Normal	Normal
15	1	Swollen				
30	1	Oedematous disruption	Blebs		Slight or moderate bands and elongation	
45	2	Clarification of matrix		Margination and clumping of chromatin		Many blurred and moth-eaten Z-lines
	2	Disruption of cristae				
60	3	Amorph densities Ca-deposits	Irregular tubular system		Severe bands and myofibrillar rupture and lysis	
90	3	Loss of integrity of mitochondrial membranes	Myelin figures	Early digestion		
120				Collection of sarcomeres to a thick belt (stone-heart)		

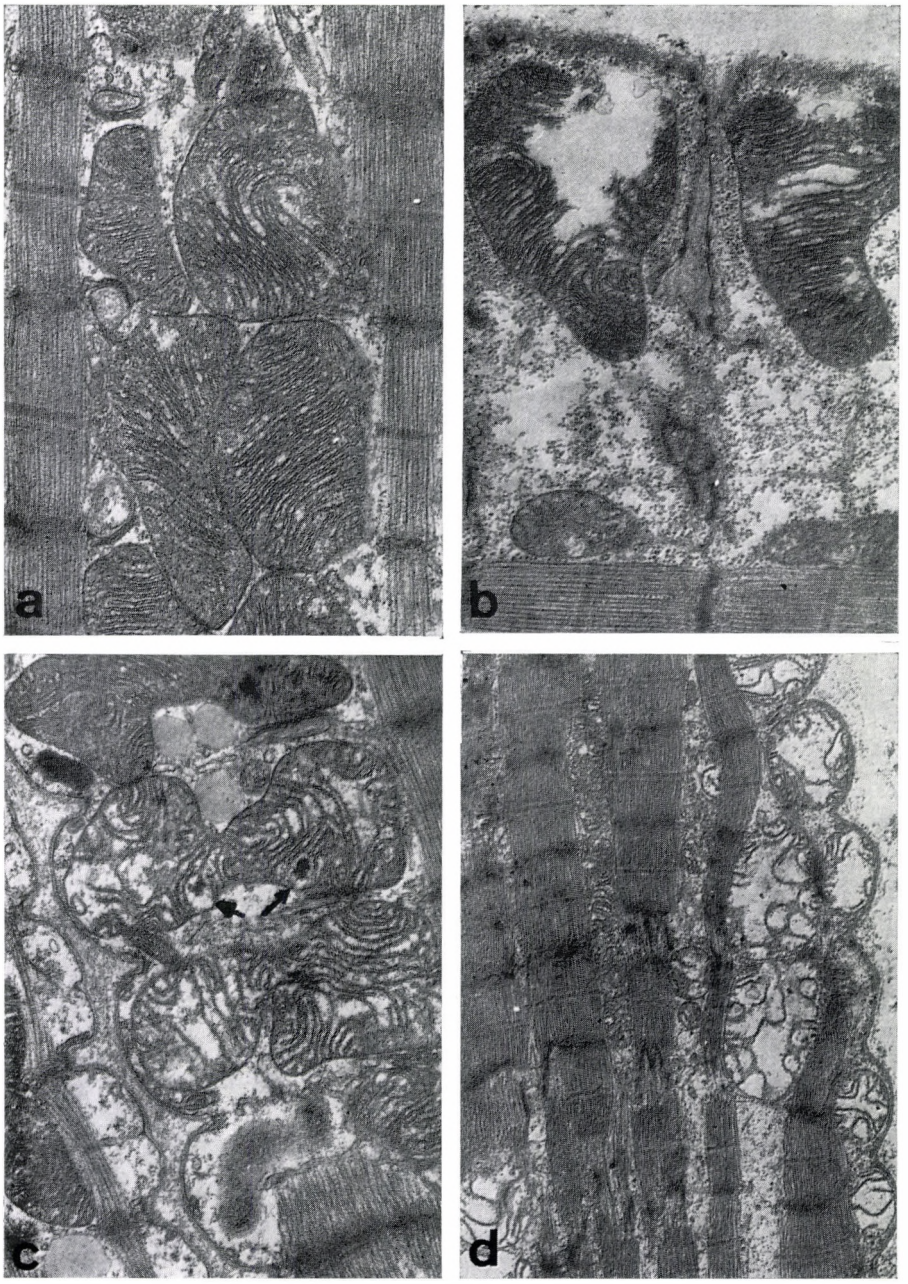


Fig. 3. EM of global ischaemic hearts after reperfusion; irreversible changes in mitochondria. a = swelling (grade 1); b = oedematous disruption (grade 1—2); c = Ca-deposits (arrows) and disruption of cristae (grade 2—3); d = loss of cristae (grade 3)

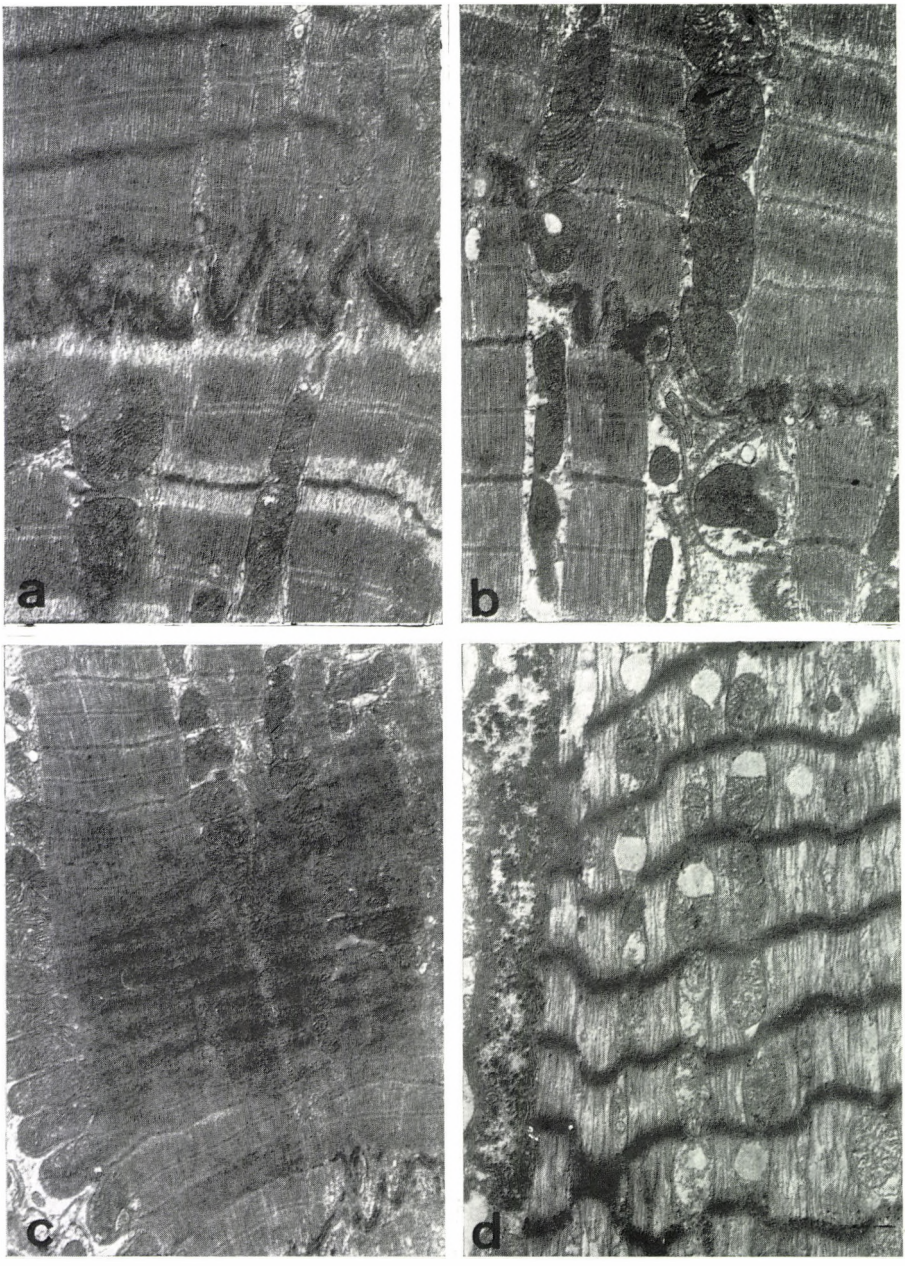


Fig. 4. Changes of contractile system. a = sarcomere shortening and elongation (grade 1—2); b = irreversibly shortened A bands (arrows); c = hypercontraction area (grade 3); d = irregularly contracted bands containing 5—8 sarcomeres (stone-heart)

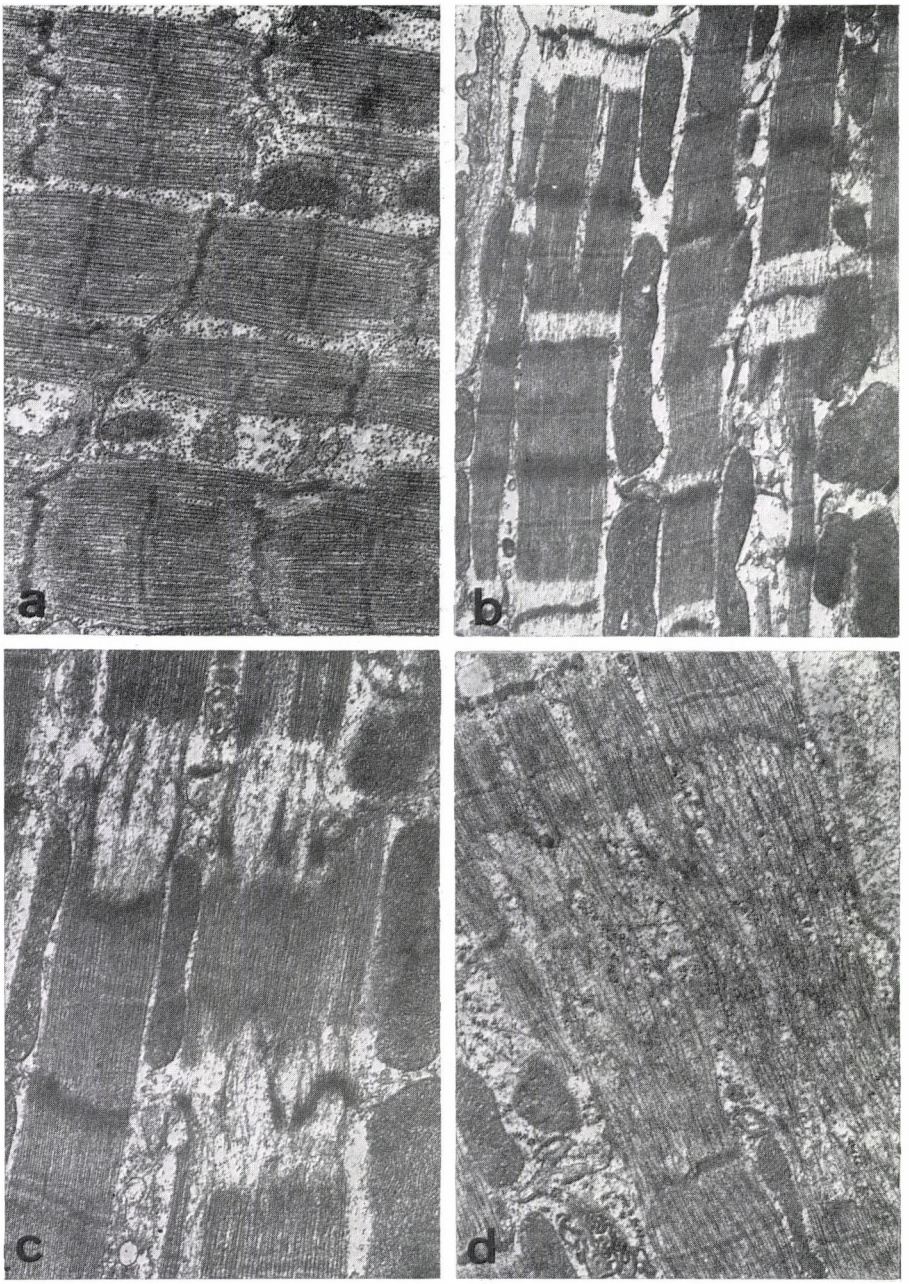


Fig. 5. Z-line anomalies. a = irregular Z-lines (grade 1); b = rigor-streched zones along several Z-lines (grade 2); c = disrupted Z-lines (grade 2); d = totally disintegrated myofibrillar structure in 3–5 sarcomeres (grade 3)

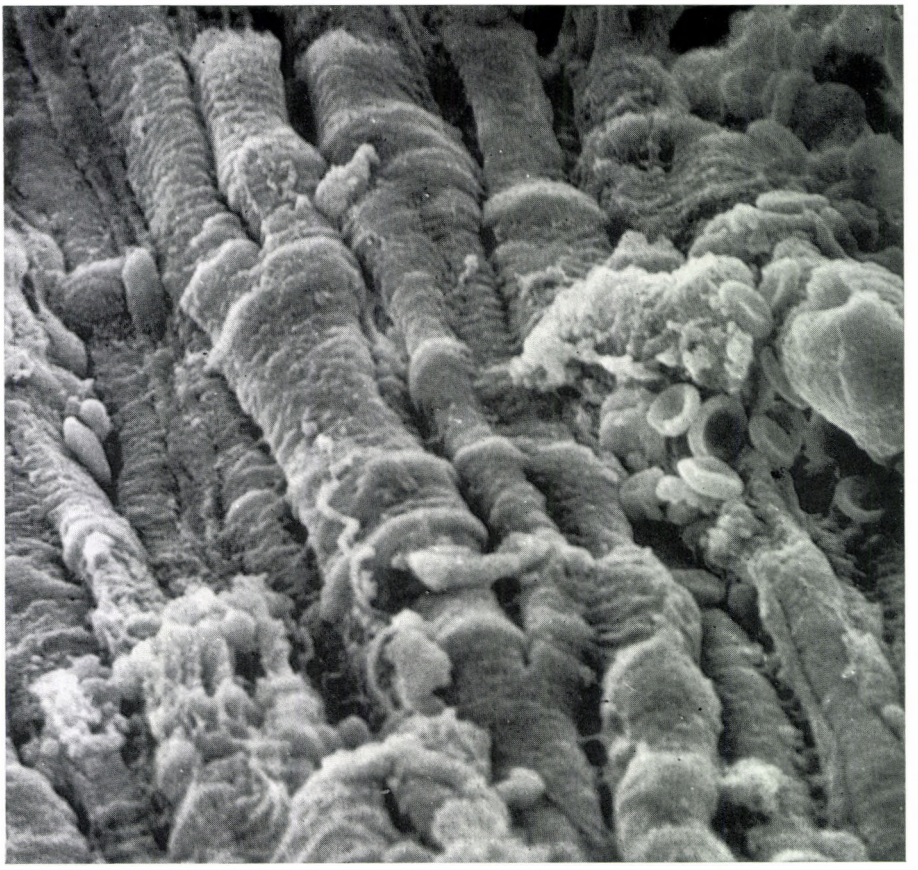


Fig. 6. Scanning EM. Side by side hypercontraction fibres and exravasal erythrocytes (usual-picture of haemorrhagic areas)

Ultrastructure

Reperfusion of the global ischaemic hearts caused ultrastructural changes in the energy-system and in contractile elements; signs characteristic of ischaemia were found in every case.

According to the severity of myocardial changes, the ischaemic injuries could be classified as described in Table I. It was evident that slight alterations prevailed after shorter ischaemia and severe ones after longer ischaemic periods.

Ischaemic hearts without reperfusion did not show such severe damage of the ultrastructure. In arrested hearts the mitochondria and cell membranes were swollen and showed slighter changes almost identical to those seen in Table I. The myofibrils were almost normal in the early phases. After reperfusion and functioning the changes were more severe owing to the appearance of previously hidden ischaemic injuries.

Figures 3, 4 and 5 demonstrate the mitochondrial and sarcomere injuries of different degrees. The pathologic changes accompanying irreversible mitochondrial injury included swelling, disruption of cristae, loss of density and evidence of mitochondrial fragility. Amorphous densities seemed to be characteristic of irreversible cell injury, they presumably contained slightly elevated amounts of calcium. The sarcomere changes are secondary to the lack of energy; the number of contractile bands, myofibrillar rupture and lysis, and blurred Z-lines were increasing with the severity of the mitochondrial and membrane damage.

Scanning electron micrographs revealed the described changes clearly. The energy defect induced by ischaemia increased the permeability of different membrane structures and so the reperfusion produced oedema, tissue haemorrhages, mitochondrial injuries and irregular contractions of the sarcomeres. The extensive haemorrhages, the widened contractile bands and other changes of the sarcomeres are clearly seen in Fig. 6.

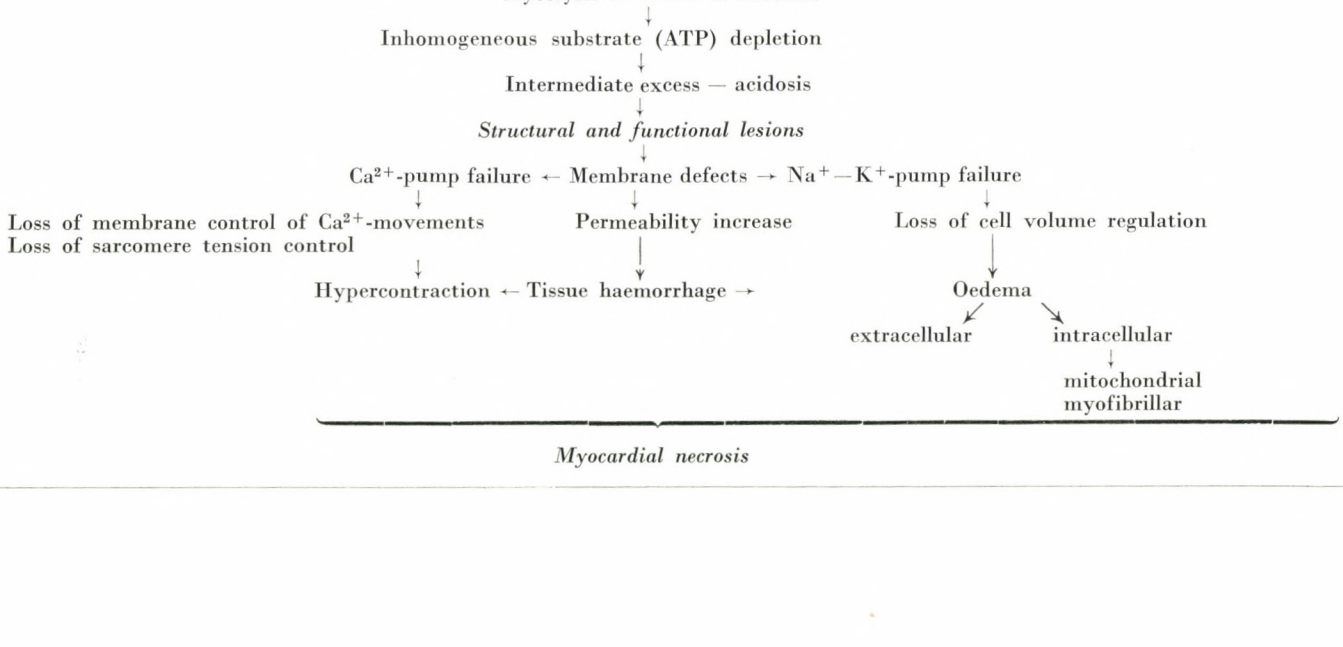
Discussion

The purpose of the experiments was to produce ischaemic changes in hearts and to determine quantitatively the consequences of global ischaemia by electron microscopy. The results revealed that the degree of produced ultrastructural alterations approximately correlated with the duration of myocardial ischaemia time, although the changes varied in distribution and extent [18, 23].

In the experiments, some parts of the dog myocardium suffered irreversible damage after 30 min of normothermic ischaemia. The affected elements underwent necrosis even if they had been reperfused with arterial blood. Moreover they showed quantitatively and qualitatively characteristic ultrastructural changes such as mitochondrial, sarcolemmal and myofibrillar defects.

According to the majority of authors, the decrease of ATP plays an important role in the development of the described ischaemic damage [7, 8, 10, 11, 13, 19, 20]. Extensive membrane defects result after the failure of the Na^+—K^+-pump and the sarcoplasmic reticulum Ca^{2+}-pump. For contraction and relaxation 80–85% of the available energy is essential, and about 15% is needed to maintain the Ca^{2+}-pump function and 2–3% for the Na^+—K^+-pump. In lack of energy, the Na^+—K^+-pump failure leads to a loss of cell volume regulation and subsequently to extensive oedema formation. Ca^{2+}-pump failure induces a loss of membrane control of Ca^{2+}-movement and sarcomere tension. Since ischaemia causes a rapid decrease of the cellular energy level, the consequences manifest themselves with the observed pathologic functions during reperfusion and with the structural changes revealed by electron microscopy (Table I).

Table II


Decrease in cardiac cellular energy level
Glycolysis inhibited or blocked
Inhomogeneous substrate (ATP) depletion
Intermediate excess — acidosis
Structural and functional lesions
Ca2+-pump failure ← Membrane defects → Na+—K+-pump failure
Loss of membrane control of Ca2+-movements
Loss of sarcomere tension control
Permeability increase
Loss of cell volume regulation
Hypercontraction ← Tissue haemorrhage →
Oedema
extracellular
intracellular
mitochondrial
myofibrillar
Myocardial necrosis

Thus, extensive membrane defects increase the permeability, which means that reperfusion after a period of ischaemia aggravates the ischaemic injury by unmasking the damages induced previously [9].

According to Jennings [7], Lowe et al. [13] and Sink et al. [20], the ischaemic contracture and ATP depletion are interconnected events and the latter would be the cause of the increased stiffness of the ischaemic and reperfused myocardium. The high calcium and low ATP levels impair the relaxation, i.e. they increase the cardiac rigor and lead ultimately to the "stone-heart" syndrome [2, 4, 6, 8, 10, 14].

Our results demonstrate that hypercontractions occurring after reperfusion of ischaemic hearts are not artifacts but indications of irreversible cellular injury. The observed stiffness during reperfusion is a good marker of the myocardial damage and suggests that a myocardium irreversibly injured by severe ischaemia always becomes stiff and at the same time other signs of irreversibility also appear. With accumulation of contractions other progressive morphological changes occur including swelling and severe injury to the mitochondrial elements.

According to the findings, the earliest ischaemic heart damage is a loss of cell membrane function which implies distinct functional and morphological changes (Table II).

In this context our finding of hypercontractions demands further investigations since it is assumed to result from an intracellular accumulation of calcium caused by the declining ATP level due to the lack of oxygen.

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ELECTRON MICROSCOPIC STRUCTURE OF THE HUMAN ROUND WINDOW MEMBRANE

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The fine structure of the human round window membrane has been investigated. It was found to consist of three layers: the tympanic cavity layer, the middle connective tissue layer, and the scala tympani layer. The middle connective tissue layer is considered the most important part of the round window membrane as it permits the movements of the inner ear fluid caused by the movements of the stapedial foot plate. The strength and elasticity of the membrane is guaranteed by three-dimensionally arranged collagen and elastic fibres and elastic networks in the middle connective tissue layer. The rupture of the round window membrane seems to be due to the diminished number and elasticity of the elastic elements in the middle connective tissue layer of the round window membrane.

The overall structure of the fenestra rotunda had already been described in 1772 by Antonio Scarpa, the well-known anatomist of Modena [18]. In 1841 Weber had suggested that the fenestra cochleae is a "yielding area", which allows fluid waves to pass through the canalis spiralis cochleae (cit. by Harty [7]). The physical properties of the round window membrane were investigated by Kobrak [10], who gave a quantitative comparison between meatal and fenestral sound conduction. The bioacoustical importance of the round window membrane has been proved in practice by the surgical procedure of Garcia–Ibánez [4], designated "sonoinversion".

Despite the importance of this tiny part of the boundary between the middle and inner ear, the only study describing its fine structure in humans was that of Hattori and Yuge [8]. The other authors publishing observations on the ultrastructure of the round window membrane have used experimental animals (guinea-pigs, cats) for their investigations. Our attention has been focussed on this topic by the increasing number of papers reporting on rupture of the round window membrane.

Materials and methods

The material used in this study was provided by the Department of Forensic Medicine, Semmelweis University Medical School. A total of 19 human round window membranes from embryos, newborns, children and adults were fixed in situ by injecting 3% glutaraldehyde in

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0.1 M cacodylate buffer, pH 7.3 into the tympanic cavity. The pyramids were removed at autopsy and the round window membrane was cut out under a surgical microscope. The isolated membranes were postfixed in phosphate buffered osmium tetroxide (pH 7.3), dehydrated in graded ethanol and embedded in Durkupan ACM (Fluka). Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Philips EM 300 electron microscope.

For light microscopy, 142 round window membranes were fixed in 4% formaldehyde, then dehydrated and embedded in paraffin wax. Sections were stained with aldehyde-fuchsin or azan.

Results

The entire cross section of the round window membrane of a fetus from the seventh month of pregnancy will be demonstrated in a series of electron

Fig 1. Tympanic cavity layer of the round window membrane of a seven months old fetus. The layer consists of epithelial cells provided with microvilli, some irregularly shaped fibroblasts, mainly cross-sectioned collagen fibres, a few cross-sectioned elastic fibres and a capillary. × 6600

micrographs. The secondary typanic membrane consists of three layers as follows:

1. *The layer which borders the typanic cavity* and which can be considered a part of the tympanic cavity mucosa (Figs 1 and 2). The epithelial cells are provided with microvilli. At least two epithelial cell types can be distinguished: one having a rather electron-translucent, the other an electron-dense cytoplasm. Both cell types contain numerous dark granules and mitochondria. At many sites desmosomes can be found between the epithelial cells. The cells lie on an uninterrupted basement membrane. Under the epithelial cells and the basal lamina it is a loose connective tissue-layer which consists mostly of collagen

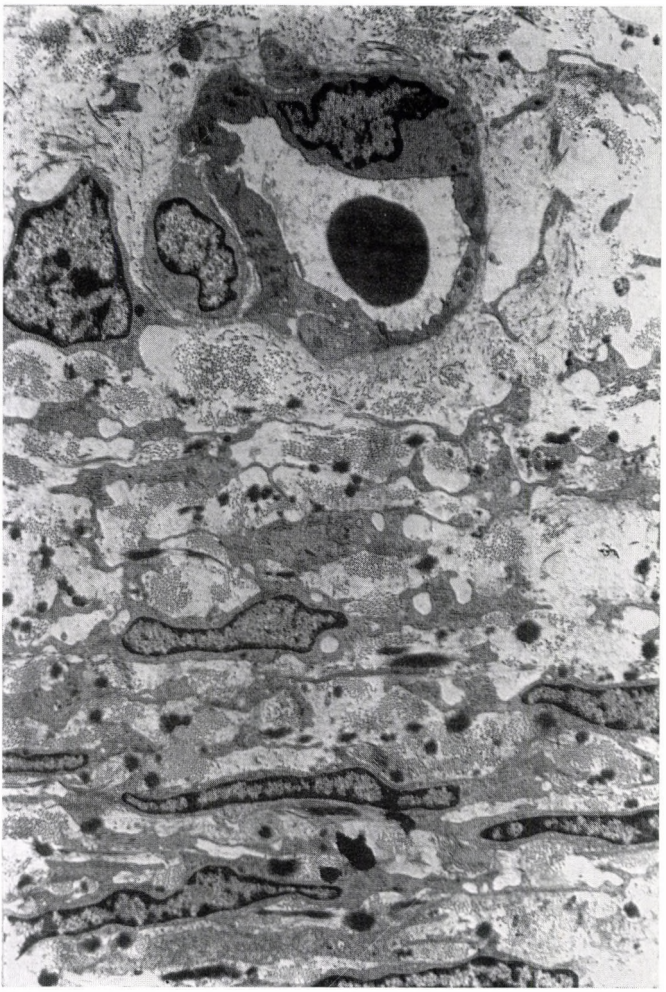

Fig. 2. Transition zone between the tunica propria mucosae and the middle connective tissue layer. Increased number of elastic fibres and densely arranged fibroblasts in the middle layer. (Seven months old fetus). × 6600

fibres in addition to some fibroblasts and blood capillaries. The collagen bundles are mostly cross-sectioned, but one can see some longitudinally and obliquely sectioned collagen fibres, too. A few cross-sectioned elastic fibres can also be observed.

2. *The middle connective tissue layer.* In the transition zone between the tunica propria mucosae and the middle connective tissue layer of the round window membrane the fibroblasts increase in number, they have an elongated appearance with many sail-like processes which form compartments containing fibrous components. Most of the elastic and collagen fibres are cross-sectioned, but some of them are cut obliquely (Fig. 2). Practically the same structure is

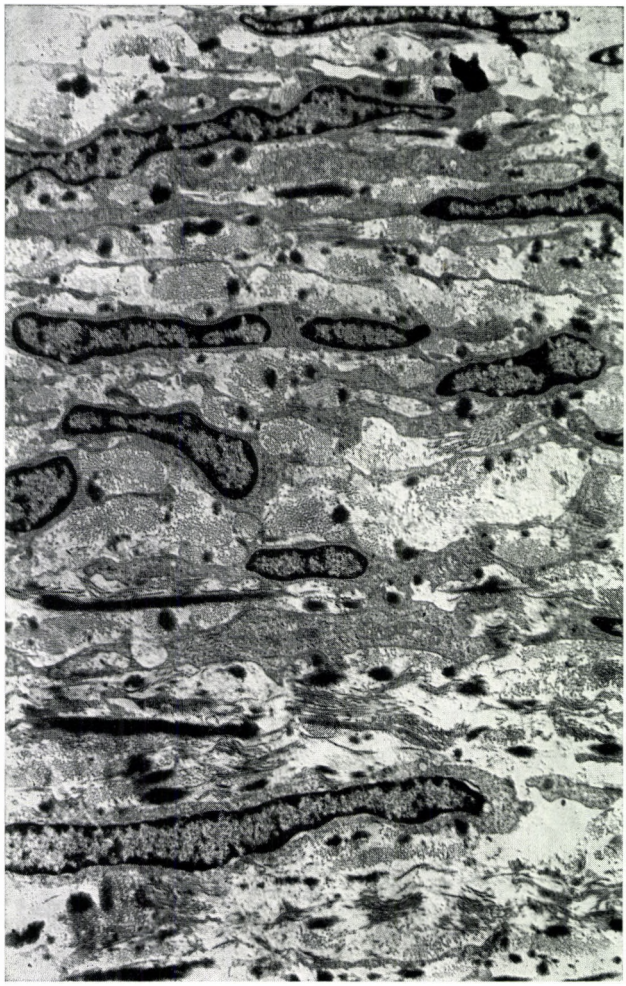

Fig. 3. Longitudinally, obliquely and cross-sectioned connective tissue fibres in the middle layer. (Seven months old fetus). × 6600

seen in Figs 3 and 4 which show the middle connective tissue layer near the scala tympani. The only difference is the increased number of obliquely sectioned fibres and the appearance of longitudinally sectioned collagen and elastic fibres. The longitudinally sectioned connective tissue fibres together with the cross-sectioned ones are more numerous in the immediate vicinity of the scala tympani layer (Fig. 4).

3. *The third layer of the round window membrane facing the scala tympani* is one cell thick; the cells show very long, thin cytoplasmic processes in the section. Some endoplasmic reticulum can be found in these cells which lie on a

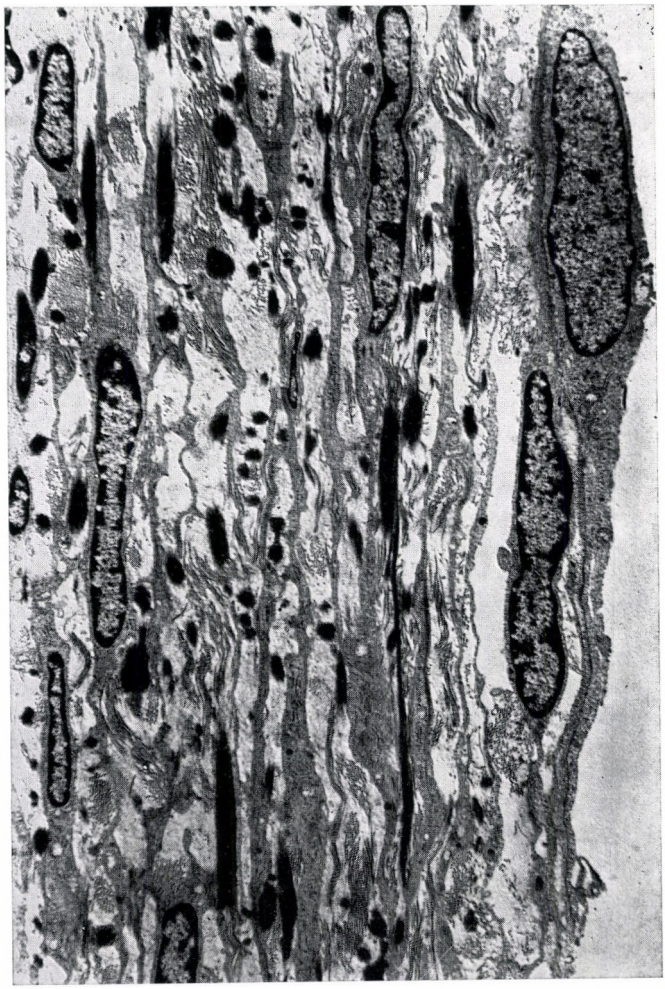

Fig. 4. The middle connective tissue layer beneath the scala tympani layer. For detailed description see text. (Seven months old fetus). × 6600

disrupted basement membrane. Occassionally, capillaries covered by the thin cytoplasmic processes of the cells of the scala tympani layer can be found.

The arrangement of the connective tissue in the middle layer of the round window membrane is complicated and it is almost impossible to follow exactly the direction of the fibres in electron-microscopic preparations. We can see side by side longitudinally, obliquely and cross-sectioned, straight, arcuate and wavy fibres as well (Fig. 5). In suitable oriented preparations a network composed of branching elastic fibres can be found (Fig. 6). The elastic fibres seem to be anchored at least partially to the collagen bundles (Fig. 7).

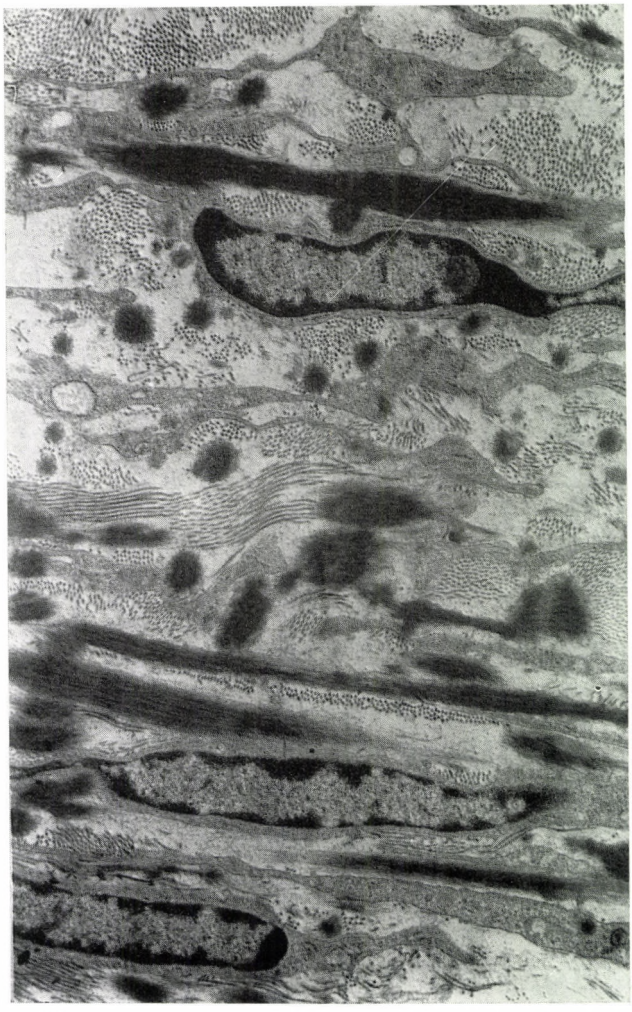

Fig. 5. Part of middle connective tissue layer under high power. Between the fibroblasts longitudinally, obliquely and cross-sectioned connective tissue fibres. (Seven months old fetus). × 13 600

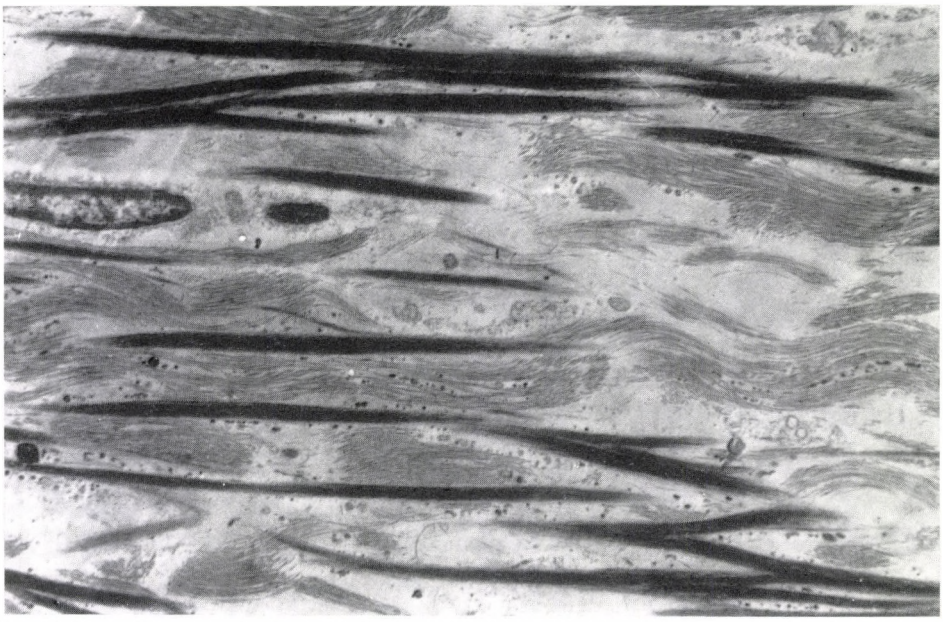

Fig. 6. Branching elastic fibres and elastic networks in the middle connective tissue layer. × 5000

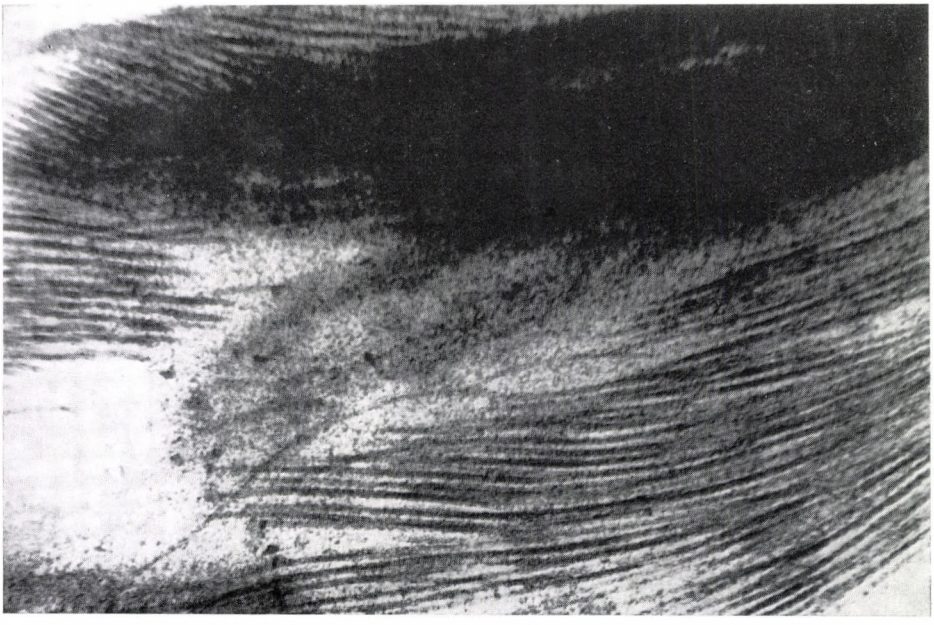

Fig. 7. Elastic fibres anchored to collagen bundles. × 40 000

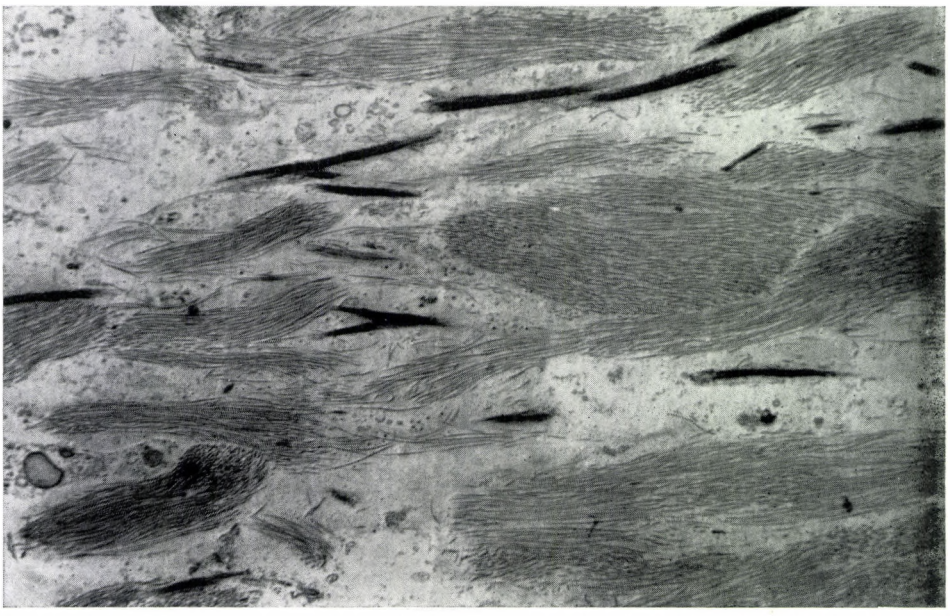

Fig. 8. Middle connective tissue layer of membrana rotunda of an adult. Diminished number of elastic fibres. The layer consists chiefly of collagen bundles. × 4000

In advanced age the cellularity of the round window membrane diminishes, the middle connective tissue layer is composed chiefly of collagen bundles interspersed with some elastic fibres (Fig. 8).

In flat sectioned material stained with aldehyde-fuchsin and investigated under light microscope one can find at the periphery of the round window membrane an elastic ring the fibres of which seem to be continuous at many sites with the longitudinally or transversally arranged elastic fibres (Fig. 9).

Discussion

The function of the round window membrane is not quite clear. Beside its generally accepted role in the transmission of acoustic energy to the inner ear, some authors attribute to it further functions. According to Richardson et al. [17] "the round window membrane may be bifunctional, not merely a membrane to prohibit the escape of fluid and dissipate fluid movement, but may also be capable of secretion and/or absorption". This conception was accepted by Miriszlai and co-workers [11]. It has been shown that different drugs (chloramphenicol, local anaesthetics) applied to the round window membrane caused decreased cochlear function [3, 12]. On the basis of electron-microscopical investigations of the round window membrane of the cat

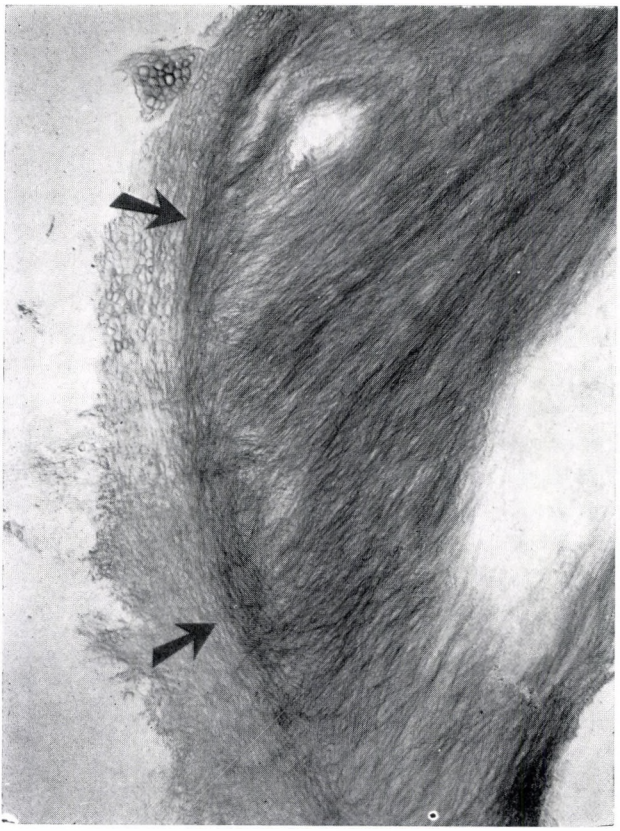

Fig. 9. Flat section of round window membrane embedded in paraffin wax and investigated under the light microscope. At the margin of the membrane (arrows) the elastic fibres run circularly, elsewhere they are arranged transversally. Aldehyde-fuchsin stain. × 95

Bellucci et al. [2] arrived at the conclusion that the ultrastructure of the membrana tympani secundaria does not indicate the presence of an active transport mechanism. The most probable mode of transfer of topically applied substances is be free diffusion after alteration of the cell junctions (desmosomes, tight junctions).

Our electron-microscopical findings indicate that the most important role of the round window membrane is to permit the movement of inner ear fluid caused by the movements of the basis stapedis. This role is guaranteed by the middle connective tissue layer of the round window membrane which is strong and elastic enough to fulfil the mechanical requirements. Since the collagen fibres do not stretch their three-dimensional arrangement in the middle layer gives a certain motility to the round window membrane. The presence of elastic fibres and elastic networks in the middle layer makes it possible for the collagen fibres to return to their original position. The membrana tympani

secundaria not only gives a certain protection to the labyrinth, but has an important acoustical role, too. The surgical procedure of Garcia–Ibánez [4] is based on this recognition. He conducted the sound waves through the membrana rotunda to the inner ear.

The supposed secretory and absorptive function of the round window membrane can be neglected in humans. The few capillaries found in the tunica propria mucosae and under the cells of the scala tympani layer are probably nutrient vessels of the membrane. But we agree with Bellucci et al. [2] in that topically applied substances may pass through the round window membrane from the middle ear into the inner ear by diffusion after altering the epithelial cell junctions.

As to ruptures of the round window membrane, it seems to be due to a diminished number and elasticity of the elastic fibres of the middle connective tissue layer. This question, however, requires further investigations.

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FINE STRUCTURE OF PERISINUSOIDAL CELLS IN DEVELOPING HUMAN AND MOUSE LIVER

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The fine structure of hepatic perisinusoidal cells was studied in human fetuses and in mouse embryos and fetuses. The perisinusoidal cells differed in structure from the sinusoidal lining cells, and occasionally mitosis of perisinusoidal cells was observed suggesting that these cells are independent and self-proliferating at the stage of development studied. Unlike perisinusoidal cells of the normal mature liver, those of the developing liver contained only sparse and small lipid droplets. They possessed a well developed rough endoplasmic reticulum and Golgi complex, and many microtubules. Collagen fibrils appeared in close proximity to perisinusoidal cells. In perisinusoidal cells of the mouse liver development of the rough endoplasmic reticulum and Golgi complex, accumulation of microtubules and increase of collagen fibrils progressed with the time of gestation. The thick cytoplasmic processes of the perisinusoidal cells frequently encircled the sinusoids, and 5–6 nm thick filaments appeared at the cell periphery. Perisinusoidal cells and hepatocytes were frequently linked by junctional elements. These findings strongly suggest that the perisinusoidal cells are responsible for the production of type III collagen. Also they may indicate that these cells reinforce the sinusoides, influence the sinusoidal blood flow by contractile activity and participate in the maintenance of the parenchymal organisation in the developing liver.

Introduction

The existence of persinusoidal (PS) cells [14, 28, 31], also termed as fat-storing cells [5] or lipocytes [2], was observed in the liver of fishes, amphibians, reptiles, birds and mammals, including man [30]. They are located in the spaces of Disse, and contain fat droplets which give a vitamin A fluorescence [2, 4, 18, 25]. The PS cells were described by Kupffer in 1876 [28], and were rediscovered by Ito and Nemoto [5] in 1952 under the light microscope but interest has been focused on them only later, when electron-microscopic observations led to the assumption that these cells produced the reticulum fibres (type III collagen) of the hepatic lobule and were responsible for intralobular fibrogenesis under pathological circumstances [2, 14, 22]. This view has been supported by the close resemblance of some of their structural features to fibroblasts, by their close topographic relationship with reticulum fibres, and by association

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of an increased formation of type III collagen with accumulation of PS cells in pathological conditions [1, 2, 7, 9, 10, 13, 14, 17, 18, 22].

The PS cells have chiefly been studied in adult liver, and only scanty, incomplete information has been available on their structure during embryonic and fetal life [30], although their electron microscopic study in the developing liver could contribute to the better knowledge of their nature. If the reticulum fibres of the liver lobule are in fact produced by PS cells, this activity should —under physiological circumstances—be most pronounced during hepatic development, when increase in the hepatocyte population must be accompanied by a parallel formation of the type III collagenous stroma. In this case it may be expected that the collagen producing activity of the PS cells is also reflected by their structure.

Observations on the fine structure of PS cells in human fetal liver as well as in embryonic and fetal mouse liver are described in this paper. Furthermore, shortly we summarize the main structural features of the developing liver, the tissular environment of PS cells, by pointing out the differences between that and adult liver.

Materials and methods

Human liver. Livers of three fresh human fetuses, obtained from legal abortions at 8 weeks of gestation, were used.

Mouse liver. White mice were used. The morning of vaginal plug detection after one overnight mating period was counted as Day 0 of gestation. For preparation of the 13-and 15-day-old embryos and of the 17-and 20-day-old fetuses the mother was anesthetized with Nembutal, the abdomen was opened and the embryo/fetus was removed from the uterus. Two mothers were used from each described day of pregnancy and two embryos/fetuses were obtained from each mother.

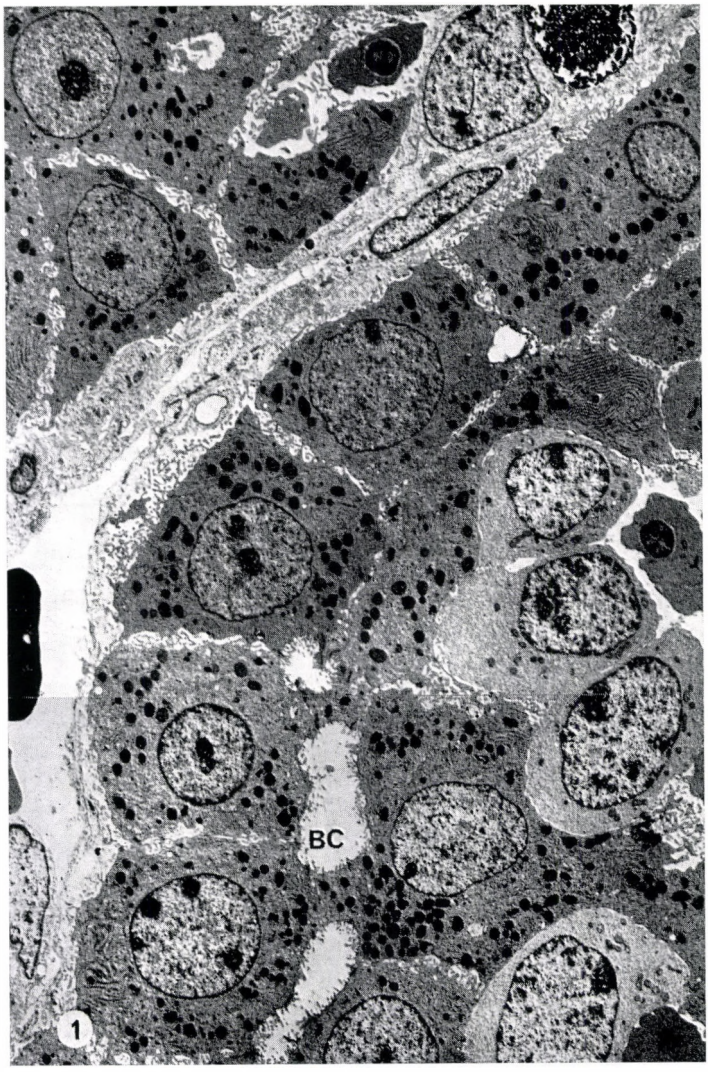
Part of the liver was immersed and dissected in fresh 5% glutaraldehyde diluted in equal parts of Millonig buffer at pH 7.2 and distilled water. Fixation was carried out at room temperature for 2 h followed by a wash in buffer and postfixation in buffered 1% osmium tetroxide solution at 4 °C. Ethanol dehydration preceded embedding in Araldite. One μm thick sections were stained with toluidine blue for examination by light microscopy. Thin sections were double-stained with uranyl acetate and lead citrate for examination in electron microscope.

Results

Main structural properties of the embryonic and fetal liver

The sinusoids of both the developing human and mouse liver were lined by endothelial cells, but infrequently Kupffer cells, containing a phagocytized material, mostly erythroblast fragments, were also present among the lining cells (Figs 1, 2).

In the eight-week-old human fetuses part of the sinusoidal circumference was surrounded by hepatocytes giving rise to a narrow space of Disse between them and endothelial cells (Fig 1). In other places the endothelial cells and the hepatocytes were separated by a wide space, filled by hemopoietic cells (Fig. 2).



Figs 1—6. Livers of 8-week-old human fetuses

Fig. 1. A Kupffer cell(top)containing phagocytized material is present among the sinusoidal lining endothelial cells. The space of Disse communicates with the wide intercellular space lined by the microvilli of the hepatocytes. Note hemopoietic cells between the hepatocytes. The dilated bile canaliculi (BC) are surrounded by more than two hepatocytes. × 3240

The hepatocytes formed goups or two-cell thick plates, separated by many hemopoietic cells, which often compressed the hepatocytes, whose surface was smooth in the area of contact. There were rather wide intercellular spaces between neighbouring hepatocytes, which possessed microvilli on the surfaces facing the intercellular spaces and the space of Disse. There were only few, dilat-

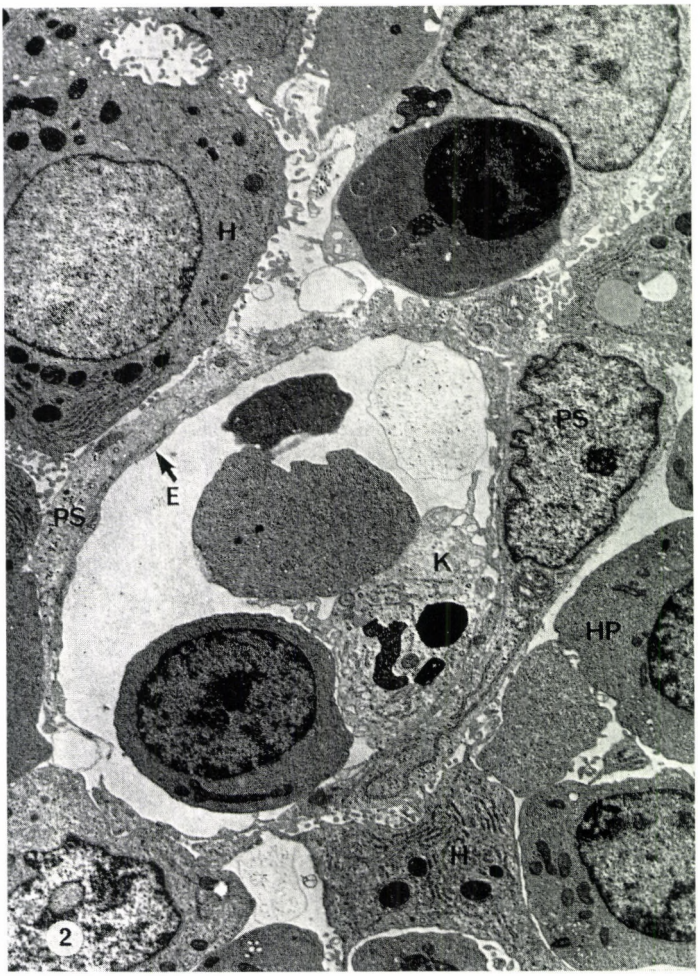


Fig. 2. The sinusoid is lined by endothelial cells (E) and by a Kupffer cell (K) showing erythrophagocytosis. Beneath the sinusoidal lining there is a PS cell (PS), whose thick processes encircle almost the entire sinusoid, and are in contact with sinusoidal lining cells, hemopoietic cells (HP), and hepatocytes (H). There is no lipid inside the PS cell. × 5700

ed bile canaliculi, surrounded by three or more hepatocytes. At the canalicular margin the membranes of adjacent hepatocytes were linked by junctional complexes, mainly zonulae occludentes, but junctional elements were exeedingly rare in areas remote from the canaliculi. The intercellular space was thus continuous between many hepatocytes and communicated widely also with the space of Disse (Fig. 1).

The hepatocytes had one or two large nuclei. Their cytoplasm contained many mitochondria, moderately developed rough endoplasmic reticulum (RER) and Golgi complex, scanty smooth endoplasmic reticulum (SER), minimal

amount of glycogen, a few peribiliary dense bodies, several microtubules, and many filaments located near the cell membrane.

In the embryonic mouse liver the spaces between the sinusoids were filled mainly by hemopoietic cells, among which a few compressed hepatocytes could be seen. Perisinusoidal spaces bordered by hepatocytes and sinusoidal lining cells appeared only seldom (Fig. 7). Contact between hepatocytes was limited to short surface area. Bile canaliculi were rare, and intercellular junctions hardly occurred in sites away from the canalicular margin.

In the 17-day-old mouse fetuses the hemopoietic cells were still numerous beneath the sinusoidal lining, but the space of Disse was more conspicuous than in the embryonic liver. The hepatocytes formed small groups between the hemopoietic cells, their surfaces of contact were smooth and became more extensive than before. Bile canaliculi, surrounded by several hepatocytes, were more numerous than at the earlier stage of gestation, but intercellular junctions were still rare in places distant from the canaliculi. At 20 days of gestation, the hemopoietic cells were less numerous than earlier and rather large part of the sinusoidal circumference was surrounded by the space of Disse. The hepatocytes formed groups or more than one cell thick plates, however, hemopoietic cells were still present between them. Flattened membrane areas of neighbouring hepatocytes were in contact along extensive abutments, the junctional complexes became more numerous.

The hepatocytes of mouse embryos contained few organelles, and very little if any glycogen at 15 days of gestation. In the fetal mouse liver the mitochondria and RER cisternae became more numerous, the Golgi complex was more developed, the glycogen content increased and filled a large part of the cytoplasm by 20 days of gestation.

Ultrastructure of PS cells in eight-week-old human fetuses

The PS cells localized beneath the sinusoidal lining cells. They possessed thick elongated cytoplasmic processes often surrounding the sinusoid in a ring-like manner (Fig. 2). Some PS cells or their processes also appeared between hepatocytes and hemopoietic cells. The surfaces of the PS cells were in close contact with the sinusoidal lining cells and hemopoietic cells along extensive abutments (Figs 2, 3), but there were no intercellular junctions between them. The PS-cell surface adjacent to hepatocytes were in contact with short villous or flattened surface portions of the latter (Figs 2, 3, 6). Moreover, in places the PS cells and hepatocytes were found to be attached by junctional elements which were characterized by parallel and straight arrangement of the opposing cell membrane portions, electron opaque material in the 9–11 nm wide intercellular space, and condensation of the subjacent cytoplasm (Fig. 5). On the cell membrane of PS cells frequently occurred dense cytoplasmic plaques, and at-

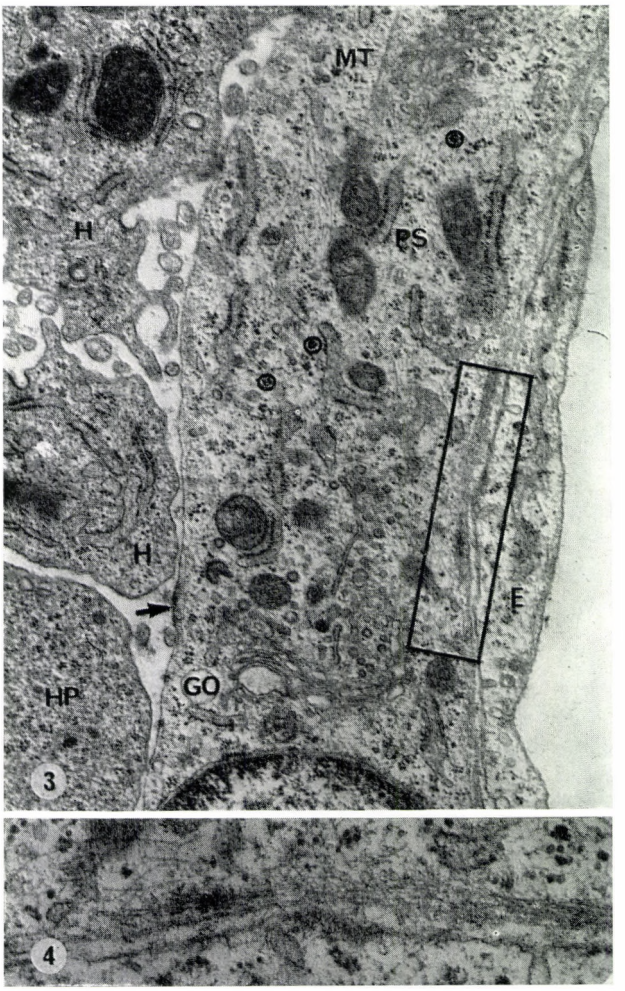


Fig. 3. Detail of a PS cell (PS). Note contact with an endothelial cell (E), a hemopoietic cell (HP), and with hepatocytes (H). Note cytoplasmic plaque (arrow) on the PS cell membrane, and bundles of parallel filaments displaying irregular densities at the cell periphery (squared). The cytoplasm contains dispersed filaments, well developed Golgi complex (Go) and RER filled with a flocculant material, microtubule (MT), small mitochondria, polyribosomes, and glycogen particles (encircled). × 26 000

Fig. 4. Filaments in the squared area of the PS cell at higher magnification. × 55 200

tachment of fibrillar or flocculant extracellular material to the opposing membrane sites (Fig. 3). Single collagen fibrils were very infrequently present in the space of Disse, always near to PS cells (Fig. 5). In places a discontinuous basement membrane-like material could be seen between PS cell and endothelial cell (Fig. 6).

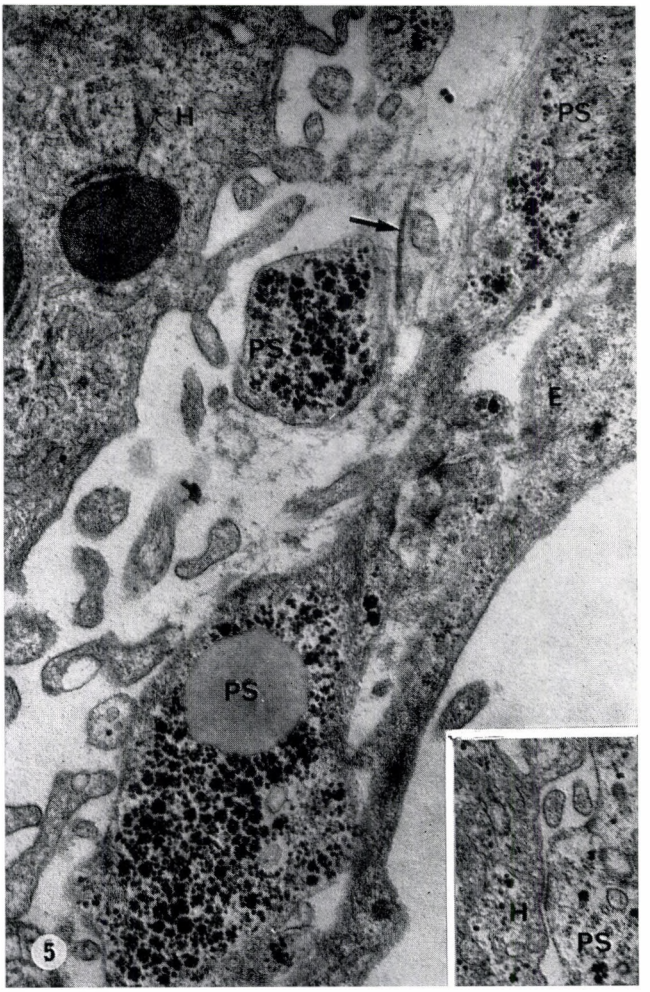


Fig. 5. Glycogen containing PS cell processes (PS) are located in the space of Disse. Note lipid droplet, intracytoplasmic filaments and collagen fibril (arrow) near the PS cell. E: endothelial cell, H: hepatocyte. × 30 000 Inset: Cell abutment with a primitive inercellular junctional complex between a hepatocyte (H) and a PS cell (PS). × 30 800

The nuclei of the PS cells were oval-shaped, the nuclear membrane was slightly undulated (Fig. 2). Nucleoli could often be seen in the nuclei. The cytoplasm included a few surface vesicles subjacent to the cell membrane. Only part of the PS cells contained one or two small lipid droplets, not delimited by membrane, in the cell body or in a process (Fig. 5). The RER was rather well developed (Fig. 3), the ribosomes frequently formed curved chains on its surface; some cisternae were slightly dilated and filled by a flocculant material (Fig. 3). The free ribosomes frequently formed polyribosomes. There was no

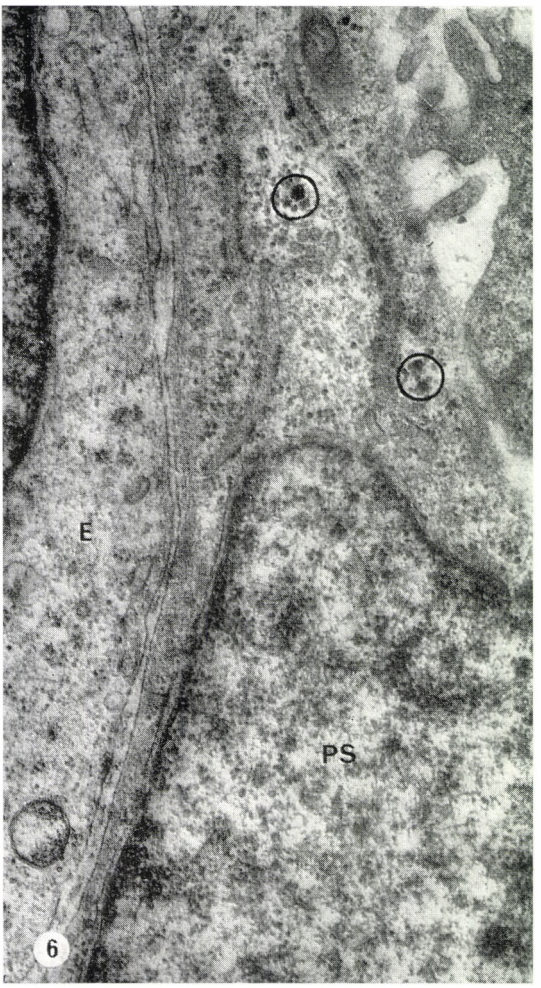


Fig. 6. Discontinuous basement membrane-like material in the narrow space between PS cell (PS) and endothelial cell (E). There are a few glycogen granules (encircled) in the cytoplasm of the PS cell. ×26 000

indication of a SER. The Golgi complex was well developed (Fig. 3). The mitochondria were small and sparse (Fig. 3). Several microtubules and fairly numerous filaments were present. Seven to ten nm thick filaments were dispersed in the cytoplasm. At the cell periphery 5–6 nm thick filaments formed thin fascicles with irregular densites and arranged parallelly with the cell membrane (Figs 3–5). Occasionally a lysosome-like dense body and a ciliary rootlet occurred. Nearly all PS cells contained alpha glycogen particles, which were numerous in part of them (Figs 3, 5, 6).

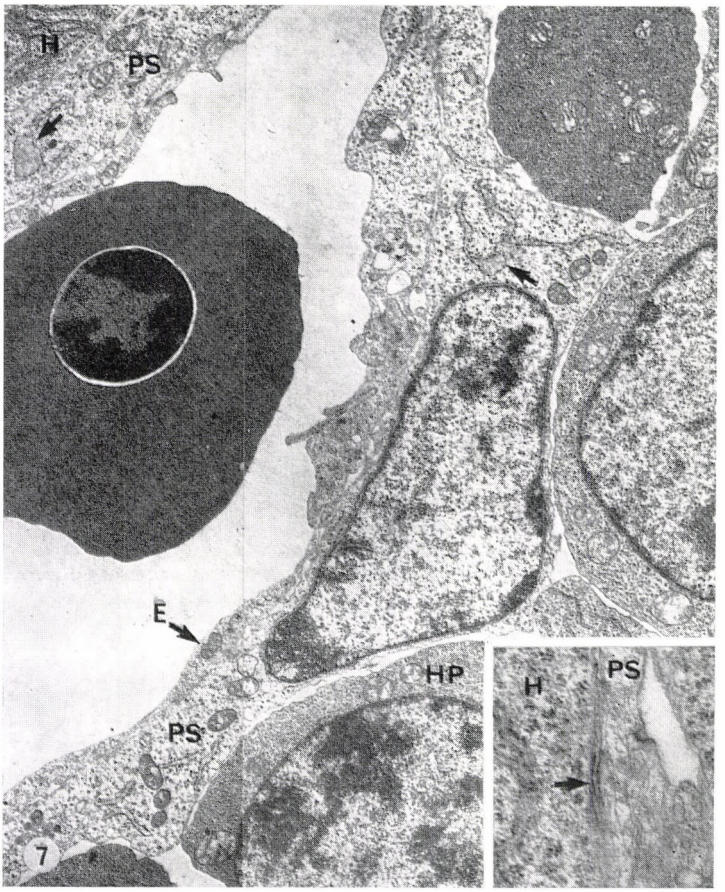


Fig. 7. Liver of a 13-day-old mouse embryo. Beneath the sinusoidal endothelial lining (E) a PS cell (PS) is seen whose thick processes encircle the sinusoid. The PS cell possesses a rather well developed RER with dilated cisternae (arrows) enclosing a flocculant material, and a few small mitochondria. Note the close contact of the PS cell with endothelial cells, hemopoietic cells (HP) and hepatocytes (H) × 6900
Inset: Junctional complex between a PS cell process (PS) and a hepatocyte (H), in mouse liver at 13 days of gestation. Dispersed filaments in the PS cell process × 35 000

Ultrastructure of PS cells in embryonic and fetal mouse liver

The localization, shape and relation of PS cells to sinusoidal lining cells and hemopoietic cells was similar in the mouse embryos and fetuses to that seen in the eight-week-old human fetuses (Fig. 7).

At 13 and 15 days of gestation, when hemopoietic cells predominated around the sinusoides, the PS cells were in contact with only a few hepatocytes, but junctional elements could in places be seen on the abutting surfaces (Fig. 7). A few dark cytoplasmic plaques were also present on the PS cell membranes.

In places a basement membrane-like material could be seen between PS cells and endothelial cells. Rarely a single collagen fibril appeared in interspaces between PS cells and hepatocytes or hemopoietic cells.

The PS cells of the embryonic rat liver had usually round or oval-shaped nuclei (Fig. 7); the nucleolus was discernible in many of them. Some surface vesicles could be seen in the peripheral cytoplasm. One to three minute lipid droplets were present only in about half of the PS cells. The RER comprised several cisternae some of which were dilated and filled with a flocculant material (Fig. 7). There were numerous free ribosomes. The Golgi complex consisted of a few flat saccules and vesicles. Scanty small mitochondria a few 7–10 nm thick filaments (Fig. 7) and microtubules, and occassional dense bodies were also present.

At 17 days of gestation, abutments between PS cells and hepatocytes were more frequent and more extensive than in the embryonic liver. The abutting surfaces were smooth and were attached by junctional elements in several places. A few collagen fibrils appeared near to several PS cells. Lipid droplets could be seen in more PS cells than previously. Also, sometimes, they were more numerous and larger (Fig. 8). The RER and Golgi complex were better developed than in the embryonic PS cells. The RER cisternae were dilated and enclosed a flocculant material (Fig. 8). A quantitative increase of microtubules was also noted. Occassionally mitosis of PS cells was observed (Fig. 8).

At 20 days of gestation the PS cells were in contact with hepatocytes along extensive abutments, and the contacting membranes were in several places linked by junctional elements. Near to the PS-cell surfaces facing either sinusoidal lining cells or hemopoietic cells and hepatocytes, thin fascicles formed by a few collagenfibrils in parallel array, made appearance (Fig. 9). A discontinuous basement membrane-like material appeared more frequently than at the earlier stage along the sinusoidal surface of PS cells.

The cytoplasmic processes of the PS cells frequently became thicker. The majority of the PS cells contained 4–5 lipid droplets which were larger than at the earlier gestational age, yet occupied only a minor part of the cytoplasm (Fig. 9). The RER was well developed and co nsisted of cavernous cisternae filled by a flocculant material (Figs 9, 10). The free ribosomes were numerous, the Golgi complex was prominent. Many microtubules, and 7–10 nm thick filaments (Fig. 10) were dispersed in the cytoplasm, and few 5–6 nm thick filaments formed a parallel array subjacent to the cell membrane. Infrequently a small dense body, and exceptionally a multivesicular body was also present.

No glycogen was detected in the PS cells of the embryonic and fetal mouse liver.

Structural differences between sinusoidal lining cells and PS cells

Structural differences between PS cells and sinusoidal endothelial cells

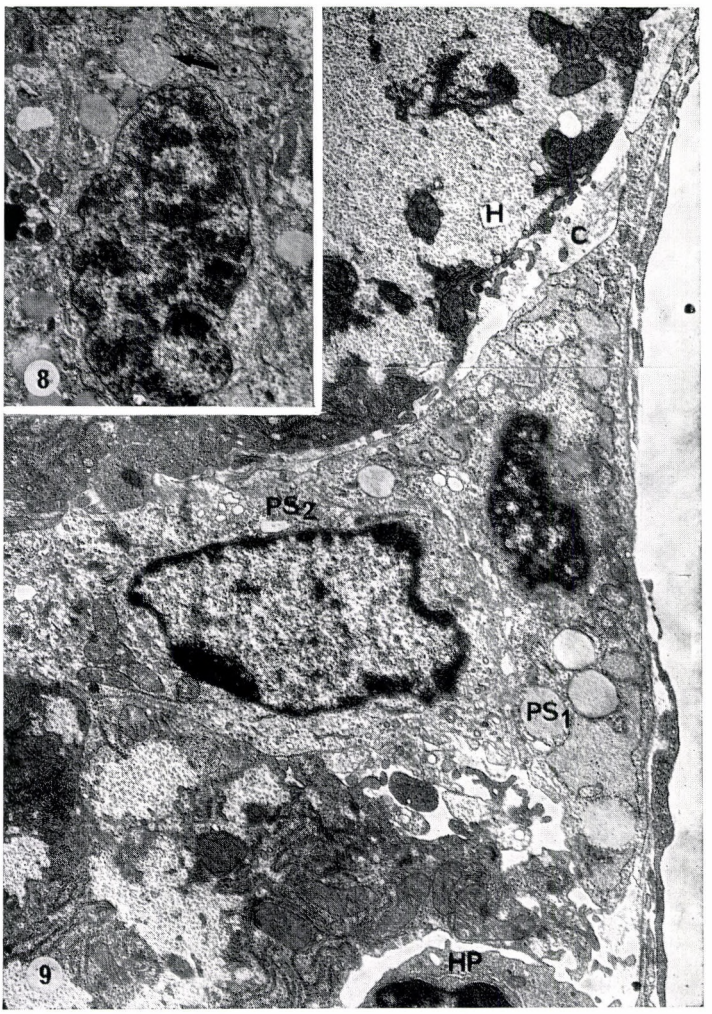


Fig. 8. Mouse liver at 17 days of gestation. Mitotic PS cell, containing several small lipid droplets, and dilated RER (arrow). × 8000

Fig. 9. Two PS cells (PS_1, PS_2) in mouse liver at 20 days of gestation. The cytoplasm of PS_1 contains a few small lipid droplets and markedly dilated RER-cisternae filled with a flocculant material. Note collagen fibrils (C) between PS_1 and hepatocyte (H).
HP: hemopoietic cell. × 8400

were demonstrable in the embryonic rat liver already at 13 days of gestation. The cytoplam of the PS cells was more abundant, extended more numerous and thicker processes and the RER was better developed than that of the endothelial cells (Fig. 7). Part of the PS cells also contained small lipid droplets not delimited by membrane. The structural differences were even more pronounced in both human and mouse fetuses as the PS cells contained more numerous di-

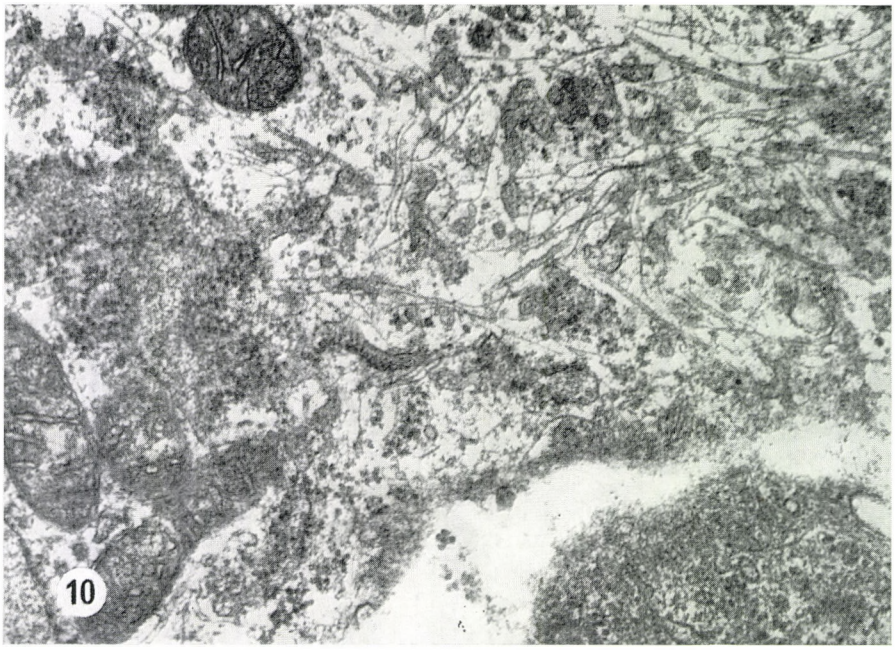


Fig. 10. Cytoplasmic detail of a PS cell in mouse liver at 20 days of gestation. Polyribosomes on the surface of RER-cisterna; many microtubules and dispersed filaments are present. × 39 600

lated RER cisternae filled with a flocculant material (Figs 3, 8–10), many microtubules and cytoplasmic filaments (Figs 3–5, 10) and often small lipid droplets (Figs 5, 8, 9). Kupfer cells were sparse and always contained phagocytized material (Figs 1, 2) which did not occur in PS cells.

Discussion

Both in the embryonic and fetal liver there were well discernible ultrastructural differences between the PS cells and sinusoidal lining cells. A rare occurrence of dividing PS cells was also noted. These observations agree with those of Naito and Wisse [15], and indicate that the PS cells are independent and self-proliferating at the stage of development studied.

Two functions have been attributed to the hepatic PS cells. One of these is the production of extracellular connective tissue components, collagen fibrils in particular. This is supported by the observations that in experimental liver injury induced by CCl_4 [9, 10, 14], ethionine [10] and alcohol [13] or in human alcoholic hepatitis [17], chronic hepatitis [24] and hypervitaminosis A [4, 8] the increase of intracellular collagen fibrils has been associated with an accumu-

lation of PS cells and transitional cells having morphological characteristics of PS cells and fibroblaste [9, 10]. In fish liver, where the space of Disse contains no collagen fibrils but a type IV collagen-like substance, vitamin A treatment resulted in an increase of the latter substance, dilatation of the RER cisternae of PS cells and accumulation of a flocculant material in them which has suggested the responsibility of PS cells for the production of this basement-membrane like substance [25].

Secretion of collagen in fibroblasts involves cooperation of the RER, Golgi complex and microtubules. The procollagen is assembled in the dilated RER wich enclose a flocculant material, then passes through the Golgi complex before leaving the cell [16, 19]. The transcellular moving of the procollagen is influenced by the microtubules. Colchicine, which disrupts microtubules, may delay the secretion of procollagen [11, 19] and induces accumulation of collagen fibrils in chick embryo fibroblasts [3]. Treatment with colchicine accounted for reduction of fibrosis in experimental liver cirrhosis [11, 21] and in several cases of human chronic active liver disease [11, 12].

In the embryonic and fetal liver the PS cells were more similar to fibroblasts than to the fat storing cells of the adult liver. They stored only little fat. The PS cells of eight-week-old human fetuses contained a well developed RER and Golgi complex, and several microtubules. In mouse liver, development of the RER and Golgi complex, and the increase of microtubules proceeded with the time of gestation. The more developed the RER, the wider were its cisternae, which enclosed a flocculant substace. The collagen fibrils always appeared near to PS cells and never appeared in absence of them. Similarly, appearance of a basement membrane-like material in the perisinusoidal space also was associated with the presence of a PS cell. In mouse liver, the collagen fibrils tended to become more numerous, and the basement membrane-like material was more prominent at the later stage of development when the organelles of the PS cells also were better developed. These observations strongly suggest that the PS cells are responsible for the production of type III and perhaps also type IV collagen in the liver lobule.

The other function ascribed to the PS cells is a role in the storage and release of vitamin A [2, 4, 18, 28]. The PS cells of the normal adult mammalian liver enclose large lipid droplets which store vitamin A, while those of the liver of mouse embryos and of eight-week human fetuses contained little lipid, if any. In the fetal mouse liver the fat storing PS cells tended to increase in number at the 17th and 20th day of of gestation, and the lipid droplets tended to become more numerous and larger, though the lipid content was still considerably lower as compared to the adult. The lipid droplets did in all probability contain vitamin A at 17 and 20 days of gestation, to judge from evidence of the presence of considerable amounts of vitamin A in the liver of rat fetuses between 16 and 20 days of gestation [23]. Wake [28] has presumed on studying the PS cells of

vitamin A-treated adult rats that multivesicular bodies played a role in the formation of the vitamin A-containing lipid droplets. We observed an exceptional appearance of multivesicular bodies in the PS cells of 20-day-old mice fetuses only and never saw lipid droplets with structural signs indicating their formation in multivesicular bodies. This suggests that multivesicular bodies do —at least in man and mouse — not participate in the formation of the lipid droplets of PS cells under physiological conditions.

Presence of junctional elements between PS cells and hepatocytes was frequently observed during the prenatal development of both human and mouse liver. Such junctions are in all probability temporary, since they have not been found in the adult mammalian liver [1, 25]. On the other hand many desmosomal junctions have been shown between PS cells and hepatocytes in the normal liver of the crucian Crassius crassius [25] which, however, differs in structure from the adult mammalian liver in that the bile canaliculi are exclusively intracellular and a wide, continuous intercellular space extends between hepatocytes, which are only linked by scanty junctional complexes [25]. In the immature human and mouse liver the hepatocytes are much less firmly joined than in the adult liver, being separated from one another by hemopoietic cells and wide intercellular spaces which allow little surface contact between adjoining cells; in addition bile canaliculi are sparse and intercellular junctions are rare between hepatocytes. This suggests that, by analogy of the crucian liver [25], the temporary junctions between PS cells and hepatocytes in the immature mammalian liver play a role in the maintenance of the parenchymal organisation.

There was always a very close juxtaposition of the sinusoidal lining cells and the PS cells; the thick cytoplasmic processes of the latter frequently encircled the entire sinusoid or its greater part. This suggests that the PS cells may serve as a support of the sinusoidal lining [7]. The surface cytoplasmic matrix the PS cell processes contained thin bundles of filaments. which were, like the actin filaments, 5–6 nm thick. This might indicate a contractile function of the PS cells, through which they could influence the sinusoidal blood flow.

Observations on the glycogen content of PS cells have been contradictory. Considerable amounts of glycogen were demonstrated within PS cells of adult human and rabbit liver by light microscopic histochemistry [6, 27], but electron microscopy revealed only rarely a small amount of glycogen in the PS cells of the adult liver [2, 4, 14, 22, 28, 30]. We have ourselves failed to demonstrate glycogen in the hepatic PS cells of children, adult men, rats, dogs, pigs [1] and 1–28 days old piglets [20]. Against this, Tanuma et al. [26] detected many beta glycogen particles in the PS cells of 67-day-old kittens. In the present study numerous alpha glycogen particles were found in the PS cells of eight-week-old human fetuses, when very little, if any glycogen was present in the hepatocytes. On the contrary, glycogen was absent altogether in the PS

cells of mouse fetuses even if a large amount of glycogen was accumulated in the hepatocytes. It appears that the glycogen content of the PS cells is related neither to the fetal stage itself nor to the hepatocellular glycogen content. The factors influencing the glycogen content of PS cells, and its functional importtance requires further study.

The designations "fat storing cell" and "lipocyte" only refer to one of the characteristic properties of the PS cells. Storage of vitamin A containing lipid is the main feature of PS cells in the normal adult mammalian liver. However, both during hepatic development and in hepatic injury [9, 10, 14, 17], structural changes take place in PS cells: the fat storing property is superseded by other features. This suggests that the PS cells are capable of various activities, and the predominance of one or another activity depends on the actual functional requirements.

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MORPHOLOGICAL INVESTIGATIONS OF MIXED PITUITARY ADENOMAS

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Thirteen pituitary adenomas were investigated by light and electron microscopy and in tissue culture. In all adenomas the production of growth hormone and prolactin was proved by endocrine symptoms of the patients and by laboratory tests. Histopathological findings were compared with culture characteristics. In 3 cases the adenoma consisted of three kinds of cell population, in 7 cases of two kinds, and in other 3 cases of one kind. In cases where 3 kinds of cell population were found, beside the granulated cells star-shaped forms without secretion granules could be observed. They probably corresponded to follicular cells. In three cases one single cell population was found. In these cases two different hormones might have been produced by one type of cell. These cells could be considered stem-like cellular elements or differentiated committed cells.

Introduction

A single pituitary adenoma secreting both growth hormone (GH) and prolactin (PRL) has repeatedly been produced in rats by several investigators [7, 13, 24]. Previous studies of spontaneously occurring human tumours have also supported the existence of double secreting adenomas [3, 9, 16, 23, 27].

We found 13 adenomas producing GH and PRL. In all cases hormone production was proved by endocrine symptoms of the patients and laboratory tests. Serum GH and PRL hormone levels were high in all patients. They had no hyperthyroidism and none of them received relevant therapy which could have accounted for the elevated serum PRL level. Our aim was to find out whether two kinds of cell population were present or whether the same cell type found ultrastructurally could also be observed in the tissue cultures.

Materials and methods

The tumours were removed by transsphenoidal approach. For light microscopy, tumour tissues were fixed in 4% buffered formalin and embedded in paraffin. Sections were stained by haemotoxylin and eosin, Mallory and PAS techniques.

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For electron microscopy tumour tissues were fixed in 4% glutaraldehyde in 0.2 M phosphate buffer at 4 °C, washed in the same buffer, postfixed in 2% osmium tetroxide in phosphate buffer, dehydrated in graded ethanol and embedded in Durcupan ACM (Fluka AG Buchs, Switzerland). Semithin sections cut with Reichert OmU 2 ultramicrotome were stained with toluidin blue. Ultrathin sections were stained with uranyl acetate and lead citrate [19] and investigated with a JEM 100 B electron microscope.

Tumour specimens were explanted immediately after surgery. From the biopsies cell suspensions were prepared with a 4:1 mixture of TC-199 and fetal calf serum. Cells were inoculated into Leighton tubes on coverslips in a density of $3–5 \times 10^5$ cells/ml. Nutrient fluid was renewed every other day. Cell growth was controlled by phase contrast microscopy and in samples stained with May–Grünwald–Giemsa. Histopathological findings were compared with culture results.

Observations

I. *At light microscopic level* tumours were divided into the following groups:
   2 chromophobe adenomas
   5 mixed adenomas [18]
   6 acidophil adenomas

II. *For ultrastructural evaluation* we used the classification by Horváth and Kovács [11]:

(*A*) *In three cases*, three cell types could be observed.

(*a*) They contained densely or sparsely granulated, presumably growth hormone producing cells (GH cells). The cytoplasm of the densely granulated cells possessed well developed rough surfaced endoplasmic reticulum (RER), Golgi complex, centrally located round nuclei and secretory granules 200–400 nm in diameter. Sparsely granulated cells displayed granules 100-200 nm in diameter. Their nuclei were irregular in shape. RER and Golgi complex were more developed than in the densely granulated cells. These cells contained fibrous bodies consisting of microfilaments. They were surrounded by tubular smooth surfaced endoplasmic reticulum (SER) while sometimes secretory granules or centrioles were embedded into the mass of filaments.

(*b*) The second type was sparsely granulated prolactin producing cells (PRL cells) with extensive Golgi zone and elongated RER arranged in parallel or produced concentric so-called "Nebenkern" formations. The nuclei were polygonal. The secretory granules 150—500 nm in diameter were not numerous and showed so-called "misplaced exocytosis", that is on the lateral cell membranes a granule extrusion into the intercellular space, distant from the perivascular spaces (Figs 1*a* and *b*).

(*c*) Star-shaped cells without secretory granules were intermingled with the granulated cells. Their cytoplasm was rich in ribosomes and contained short RER and few organelles. Sometimes three or four cells formed follicles with desmosomal contacts and microvilli at the luminal surface. Occasionally cells with secretory granules also took part in follicle formation (Figs 2 and 3)

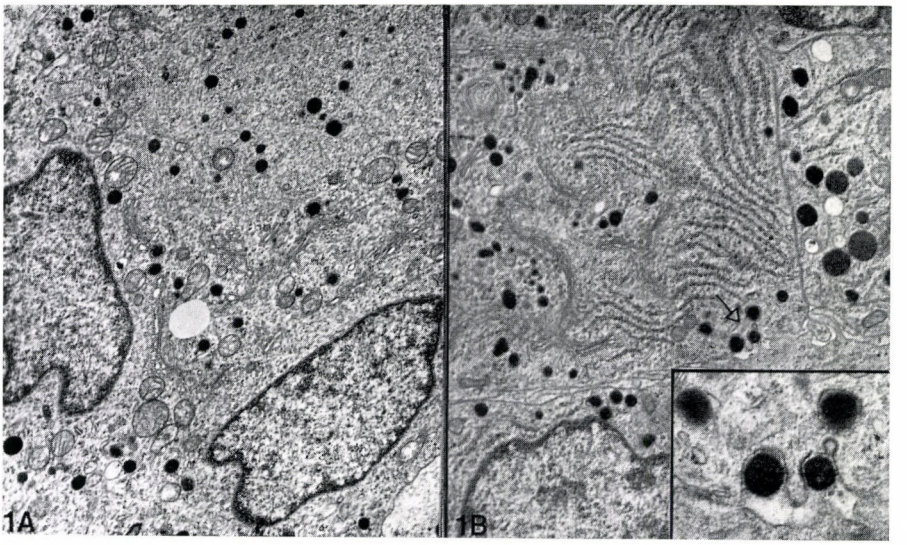


Fig. 1a Adenoma containing sparsely granulated GH cells with fibrous bodies, *b* Sparsely granulated prolactin cells with misplaced exocytosis. × 3000. Inset: high-power picture of misplaced exocytosis. × 15 000

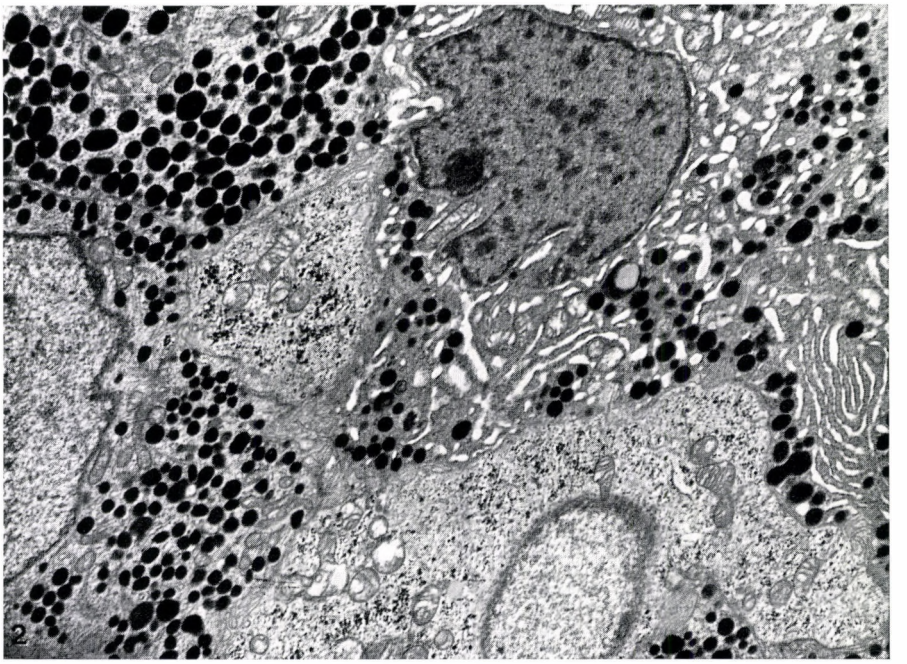


Fig. 2. Adenoma composed of densely granulated GH cells, sparsely granulated PRL cells and star-shaped follicular cells. × 4000

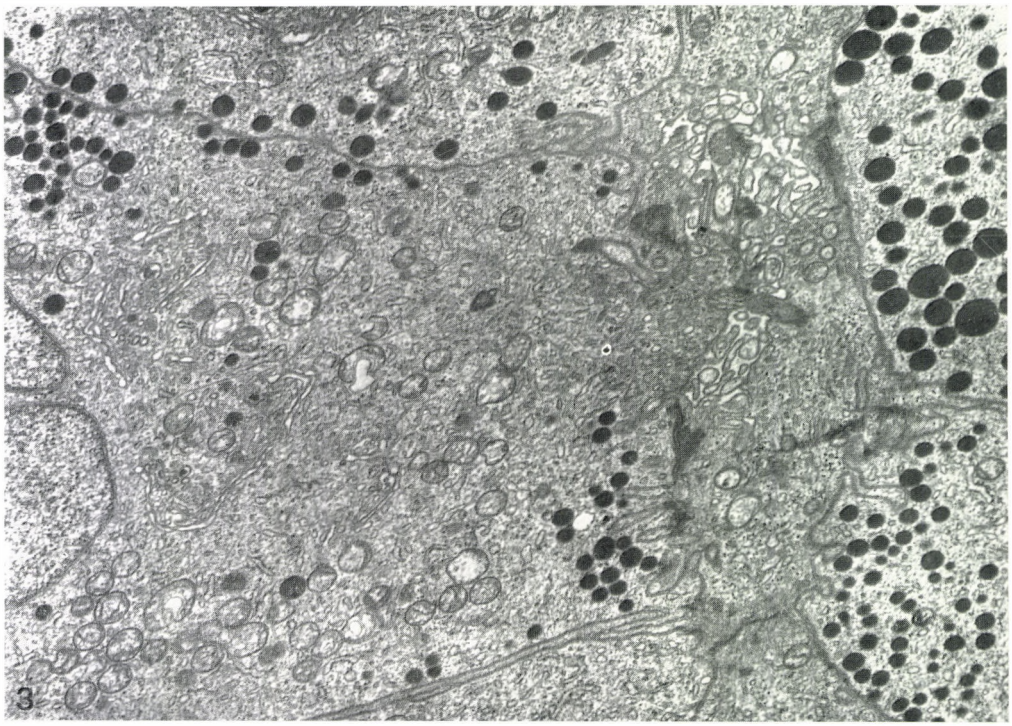


Fig. 3. Follicular cell with follicle and desmosomes on the surface. × 5000

(*B*) *In seven cases* two cell types were found,
  (*a*) densely or sparsely granulated GH cells, and
  (*b*) sparsely granulated PRL cells (Fig. 4).

(*C*) *In three cases* one cell population could only be seen.
  (*a*) In one case the cells showed characteristics of sparsely granulated PRL cells.
  (*b*) In the other two cases the cells forming the adenoma displayed fine structural features of both sparsely granulated GH and PRL cells. Misplaced exocytosis, fibrous bodies and multiple centrioles were sometimes revealed within the same cell. The cells were irregular with uneven surface. They showed small, dense granules, short RER and a hardly developed Golgi complex, as well as slightly swollen mitochondria (Figs 5 and 6).

III. On cultivation, individual biopsies showed various features. Differences could be observed immediately after explantation. Culture characteristics coincided with electron microscopic histology. Accordingly, pituitary adenomas consisting of sparsely granulated prolactin cells developed either confluent monolayers, or arranged into follicle-like cell islets.

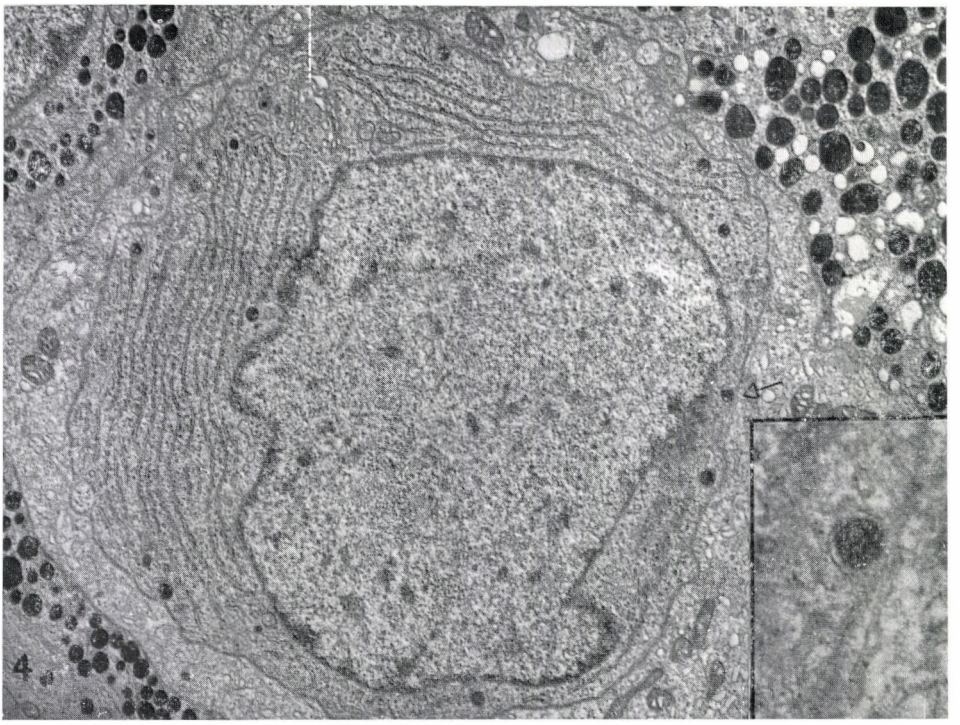


Fig. 4. Adenoma consisting of densely granulated GH cells and sparsely granulated PRL cells. × 4000 Inset: high power picture of misplaced exocytosis. × 15 000

Densely and sparsely granulated cells with putative growth hormone production developed elongated migratory shapes. They were not connected with each other but grew individually. In cell bodies of sparsely granulated cells, circumscribed formations could be found which by their diverse density differed from the overall consistence of the cytoplasmic matter. These structures could be identified ultrastructurally with the fibrous bodies. In densely granulated cells these formations could not be demonstrated. In cultures of some tumours large star-shaped cells occurred which showed no similarities either with prolactin cells or with GH cells. Ultrastructurally, these cells did not contain any secretory granules and it was supposed that they might correspond to the follicular cells [5] (Figs 7*a*, *b* and *c*).

Discussion

Many clinical and pathological observations and experimental data have supported the fact that a single pituitary adenoma produced a number of different hormones. The most common combination is a hypersecretion of

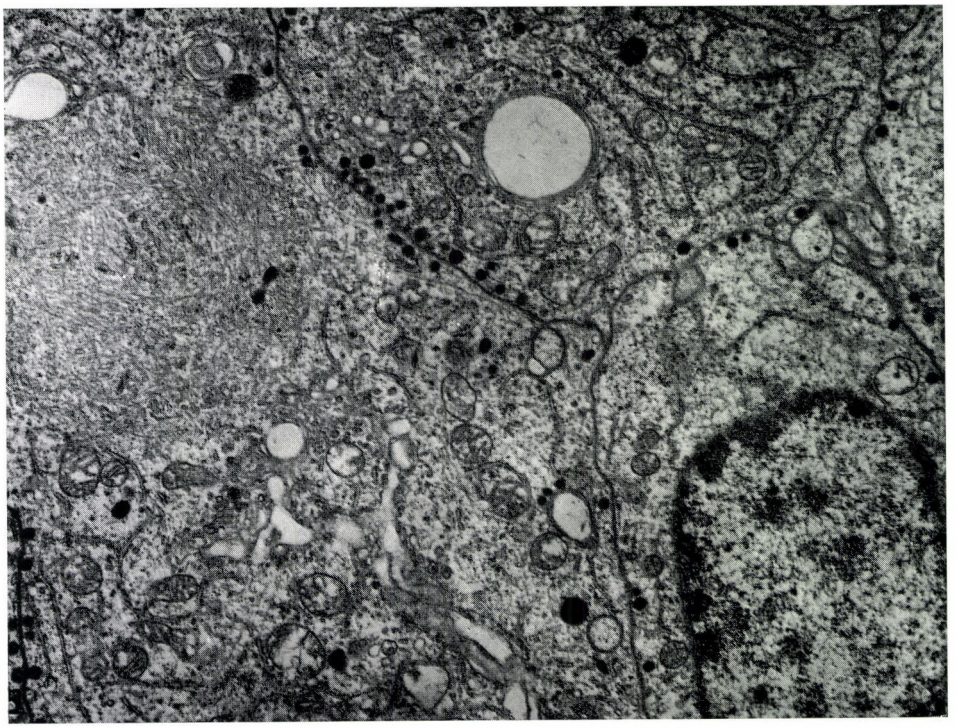


Fig. 5. Adenoma cells showing stem-like features and resembling sparsely granulated GH and PRL cells. In the cytoplasm a fibrous body can be seen. × 4000

GH and PRL [3, 9, 13, 24, 27]. A combined secretion of other pituitary hormones is less frequent. The occurrence of pituitary adenomas secreting thyrotropin (TSH) and PRL [4, 26], GH and adrenocorticotropin (ACTH) [16] or more than two hormones [17, 21, 22] has also been reported. These multiple hormone secreting adenomas often do not show any clinical sign of hormone hypersecretion and high hormone levels can be found only in laboratory tests. The adenomas may contain one or more cell types.

In acromegaly with hyperprolactinaemia the elevated serum PRL level may be the consequence either of an adenoma consisting of GH and PRL cells or of a GH secreting pituitary tumour with suprasellar extension. This may cause the disturbance of hypothalamic function leading to hyperprolactinaemia [12]. According to Horváth and Kovács [11] both GH and PRL cells are acidophilic and have ultrastructural similarities. Corenblum et al. [3] reported on six pituitary adenomas with hypersecretion of both GH and PRL; they contained two distinct cell types without any intermediate cell form. These tumours were considered to be real mixed adenomas. On the other hand, Saeger [20] is of the opinion that the designation mixed adenomas should not be used as

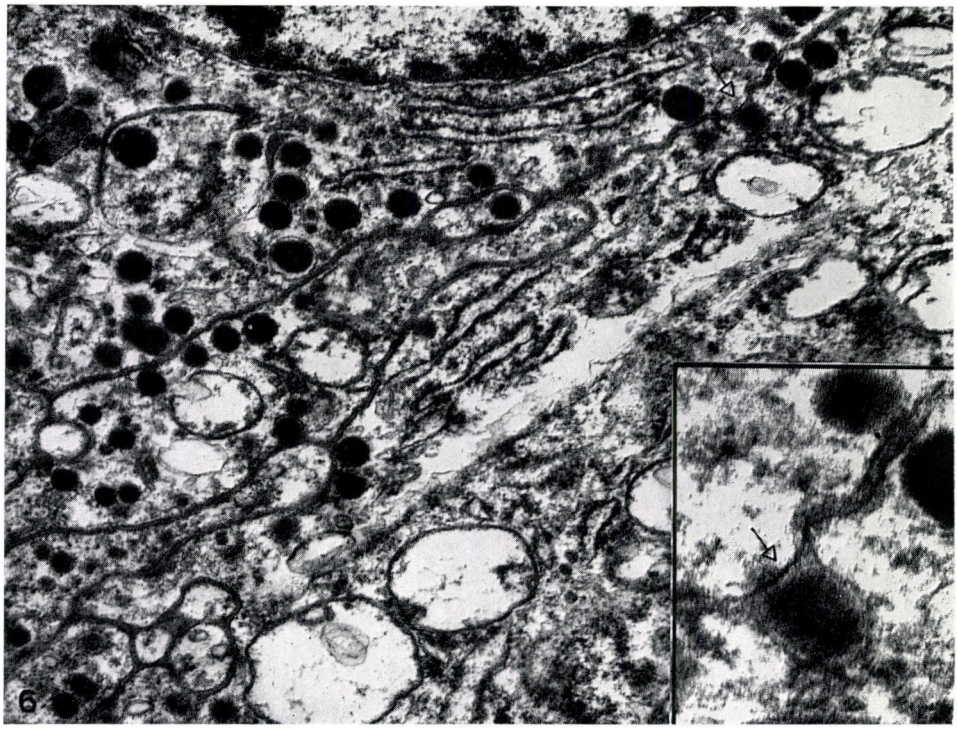
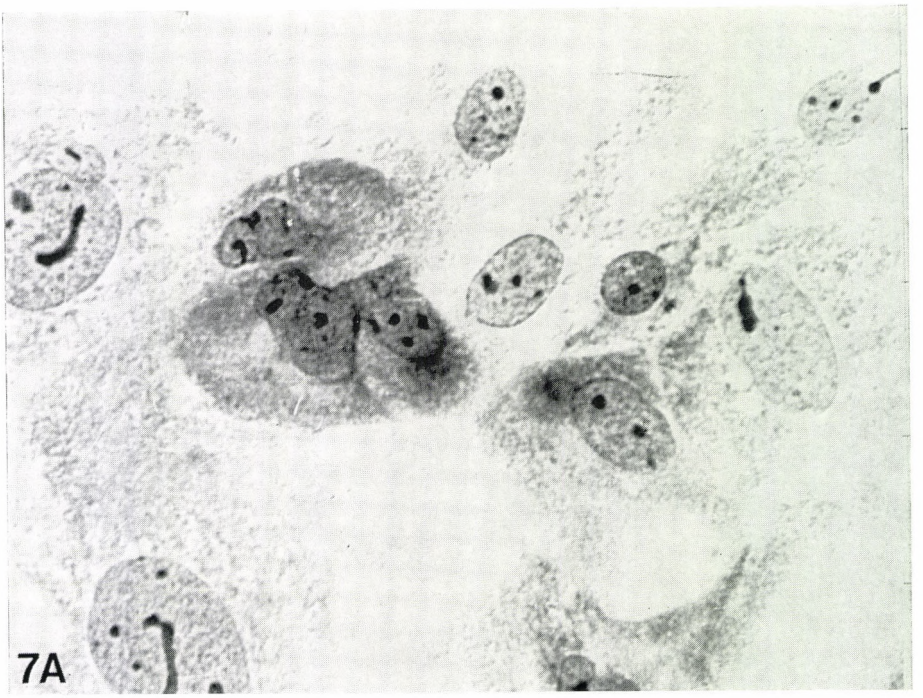


Fig. 6. Other part of the adenoma shown in Fig. 5, with misplaced exocytosis. × 9000. Inset: high power picture of misplaced exocytosis. × 36 000

long as the origin of the different cell types has not been proved. For pituitary adenomas containing several identifiable adenohypophyseal cells Martinez and Barthe [15] used the term heterogeneous pituitary adenoma.

In our material we found 10 cases composed of two or more cell types; these could be distinguished ultrastructurally and in tissue cultures as well. Since GH and PRL producing cells in culture reveal divergent characteristics, it may be assumed that a particular pituitary adenoma is consisting of one or two kinds of cell population [8].

In tumours and their cultures containing three cell types we observed star-shaped forms intermingled with GH and PRL cells. Their ultrastructural features were reminiscent of the follicular cells described by Farquhar [5]. Although their role is not defined it has been assumed that they had a supportive function or they might be involved in intercellular transport [1, 6, 25]. Experimental data pointed to the fact that in the rat pituitary they differentiated into granulated cells forming a "renewal cell system" [28]. According to Horváth et al. [10] the follicular cells do not take part in hormone production and the follicular structures are not permanent components of the human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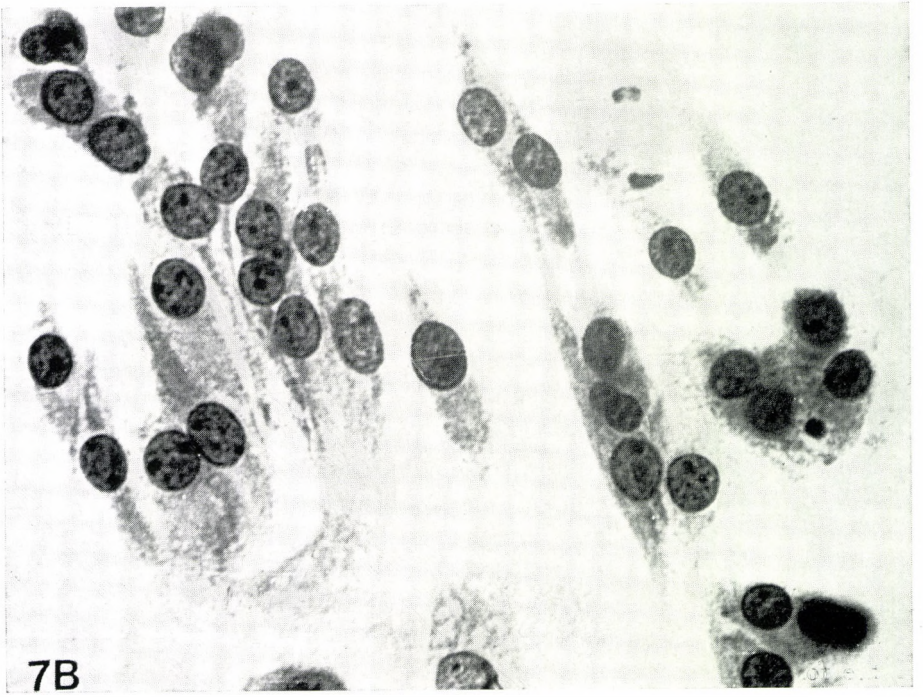
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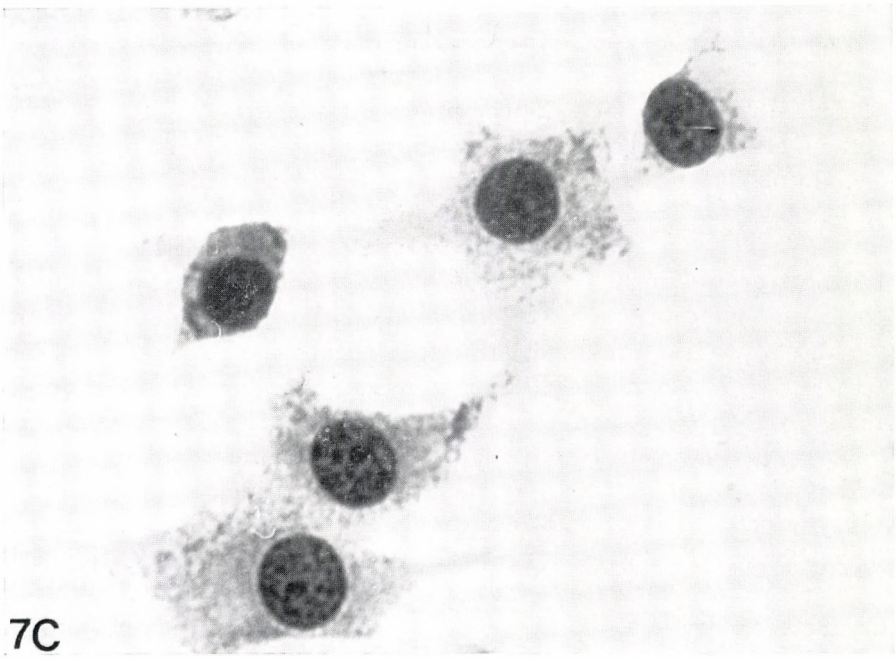

Fig. 7. Various cell populations of a mixed-type pituitary adenoma after one week of explantation. *a* Confluent monolayer of sparsely granulated prolactin cells. May-Grünwald-Giemsa staining. × 250. *b* Individually growing elongated cells without fibrotic bodies. They could ultrastructurally be identified as densely granulated GH cells. May–Grünwald–Giemsa staining, × 250. *c* Star-shaped cells showing ultrastructurally no granulation. May–Grünwald–Giemsa staining. × 400

anterior pituitary lobe. They originate from the glandular epithelium around one or more ruptured adenohypophyseal cells undergoing destruction. The phenomenon is presumably not common in the normal resting adenohypophysis and occurs more frequently in cases of altered function or in adenomas. Bergland and Torack [1] described follicular cells in a single ACTH adenoma. Landolt [14] demonstrated them in two GH adenomas. From our investigations it could not be defined whether they were remainders of normal cellular elements or could be considered proper tumour cells.

In two cases, adenoma cells showed fine structural characteristics of both sparsely granulated adenomatous GH and PRL cells, e.g. in the cytoplasm of some cells fibrous bodies and on the lateral cell membranes extrusion of secretory granules – the so-called misplaced exocytosis – were to be seen. These adenomas resembled the acidophilic stem cell adenomas described by Horváth et al. [12]. In our cases both patients showed high serum GH and PRL levels and had endocrine symptoms. One of them showed a combination of acromegaly with galactorrhoea and the other acromegaly with decreased libido.

In a third case the adenoma cells showed the characteristics of sparsely granulated PRL cells. In the serum of this patient a high level of PRL and GH was found and it was assumed that both hormones were produced by the prolactin cells. In one case out of 29 GH producing adenomas Landolt [14] found symptoms of acromegaly and galactorrhoea and supposed that the same tumour cell produced GH and PRL, since ultrastructurally only a single cell type could be observed. Furth and Clifton [7] induced pituitary adenomas in rats where the cells had multiple hormone secreting activity. These adenomas presumably were consisting of one cell type only. Zimmerman et al. [27] suggested that some normal pituitary cells might contain both GH and PRL granules. Ito et al. [13] when investigating experimental rat PRL adenomas found that there was a close connection between the production of GH and PRL hormones, and that the neoplastic acidophils had the capacity to secrete PRL, GH and occasionally ACTH as well. McCormick et al. [16] described a case of acidophilic adenoma of the pituitary with coexisting acromegaly and Cushing's syndrome. They thought that ACTH was produced also by the acidophilic adenoma cells. Berthezene et al. [2] reported an adenoma with amenorrhoea galactorrhoea syndrome where the recurrent tumour caused Cushing's syndrome three years after operation. Hypothalamic damage and multifunctional activity of tumour cells had been suggested.

From our investigations it appears that adenomas producing two kinds of hormones—GH and PRL— might consist of two cell types, which could be distinguished both ultrastructurally and by tissue culture. They presumably correspond to real mixed adenomas [3]. We cannot exclude that these cells derive from a common progenitor cell and subsequently differentiate, although we could not find immature cells. We observed follicular cells in association with granulated cells in three cases but could not decide what their functional role was.

In three cases one cell population was found which produced two hormones. These cells with multiple hormone secreting capacity could be stem-like cells or differentiated, committed cells. The adenomas composed of stem-like cells could correspond to acidophilic stem cell adenomas [12].

It thus seems that there are some pituitary adenomas which are difficult to classify. Adenomatous cells while differentiated to a certain extent may retain their capacity for producing several hormones simultaneously or alter periodically their hormone secreting ability.

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SIMULTANEOUS LOCALIZATION OF TWO DIFFERENT TISSUE ANTIGENS BASED ON THE SILVER INTENSIFIED PAP-DAB AND ON THE TRADITIONAL PAP-DAB METHOD

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A new immunohistological method was elaborated for the light-microscopic detection of two tissue antigens in the same section. One of the antigens was visualized by the silver-gold intensified peroxidase-antiperoxidase complex (PAP)-diaminobenzidine (DAB) method resulting in a black coloured end-product. The other antigen was labelled by the brown reaction-product of the traditional PAP-DAB method. Both reaction-products are stable, electron-dense and they can clearly be distinguished from each other.

Introduction

The discovery of the coexistence of different peptide hormones and transmitters in single neurones [6], as well as the demand for studies regarding possible interactions between peptide hormone synthesizing structures and neurotransmitter containing pathways have urged a simultaneous visualization of more than one antigenic substance in a single section. The pioneer method of Nakane [9] was followed by a more sensitive procedure based on the unlabelled antibody peroxidase-antiperoxidase PAP method [10] of Vandesande and Dierickx [12]. The major difficulties of these immunohistochemical double staining mehods, such as crystal formation, solubilization in organic solvents, and auto-oxidation arise from the chemical nature of the end-products of the different substrates used. 3,3-diaminobenzidine (DAB) is one of the best substrates for this purpose. In the present work, we aimed at elaborating an immunohistochemical double staining method, which eliminates the disadvantages mentioned above, resulting in stable, easily distinguishable electron-dense deposits. In our method we used the same 3,3'-diaminobenzidine substrate for the detection of both antigens, utilizing the brown colour of the end-product of 3,3'-diaminobenzidine and the black colour of its silver-gold intensified form. Silver intensification of the end-product of 3,3'-diaminobenzidine has recently been introduced by Gallyas et al. [5] and demonstrated at the light microscopic and ultrastructural level [8].

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Materials and methods

Adult female rats were anaesthetized with hexobarbital and perfused through the ascending aorta with phosphate buffered saline (PBS 0.05 M, pH 7.8) followed by picric acid formaldehyde solution [13]. The brain and the pituitary gland were removed, dehydrated in graded alcohols and embedded in polyethylene glycol (PEG, Polywachs 1000, Chemishe Werke, Hüls). From the brain in the coronal plane and from the anterior pituitary gland 10 μm thick sections were cut and processed for the double labelling immunohistochemical technique according to the following steps.

1. Detection of the first antigen with the aid of the conventional PAP method and DAB substrate. The antigenic sites appear brown in colour.

2. Silver-gold intensification of the end-product of DAB labelling the first antigen. As a consequence of this procedure, the brown colour of the end-product turns to black.

3. Visualization of the second antigen in the same section, using the traditional PAP–DAB sequence.

ad 1. The immune-staining procedure, as it is applied in our laboratory has been described easlier [2].

ad 2. The sections were washed in PBS for 30 min and transferred into a 10% solution of thioglycolic acid for 6–12 h. Preincubation in thioglycolic acid solution is necessary to suppress the argyrophilia of some tissue elements, preventing non-specific silver deposition in unlabelled structures [4]. Following brief washing of the sections in sodium acetate-acetic acid buffer (0.15 M pH 7.4) they were treated with the physical developer of Gallyas [3]. The end-product of DAB catalyzes the interaction of silver ions and formaldehyde molecules to produce metallic silver grains in structures labelled with DAB. The physical development is controlled under the light microscope and is continued until all structures labelled with the brown end-product have turned to black. This incubation usually takes 5–10 min. Silver intensification is stopped by placing the sections into a 1% acetic acid solution for 5 min. Thereafter, the sections are washed in sodium acetate-acetic acid buffer solution for 10 min and transferred into 0.2% gold chloride solution $H(AuCl_4) \times 4H_2O$ for 5 s to replace the metallic silver with metallic gold. Following this gold toning procedure [1] the sections are immersed into the sodium acetate–acetic acid buffer solution for 10 min, and then into a 3% solution of sodium thiosulphate to wash out the silver. After detection of the first antigen, the antibodies used were eluted according to the method of Vandesande and Dierickx [12] or in some cases antibody elution steps were omitted [7, 11].

ad 3. see ad 1.

Results

In our model study, somatostatin and luteinizing hormone releasing hormone (LH–RH)-containing nerve fibres were visualized simultaneously in the median eminence of the rat, and growth hormone (GH) and follicle-stimulating hormone (FSH)-containing cells were detected in the same sections of the anterior pituitary gland.

Fig. 1. LH–RH-containing nerve fibres around the tubero-infundibular sulcus of the median eminence. PAP method, DAB substrate. Scale: 100 μm. Figs 1—4

Fig. 2. Somatostatin fibres in the middle part of the median eminence. Traditional PAP-DAB method

Fig. 3. Silver-gold intensified somatostatin-containing nerve fibres in the median eminence. Intensive black colour of the fibres represents gold deposition on the end-product of DAB

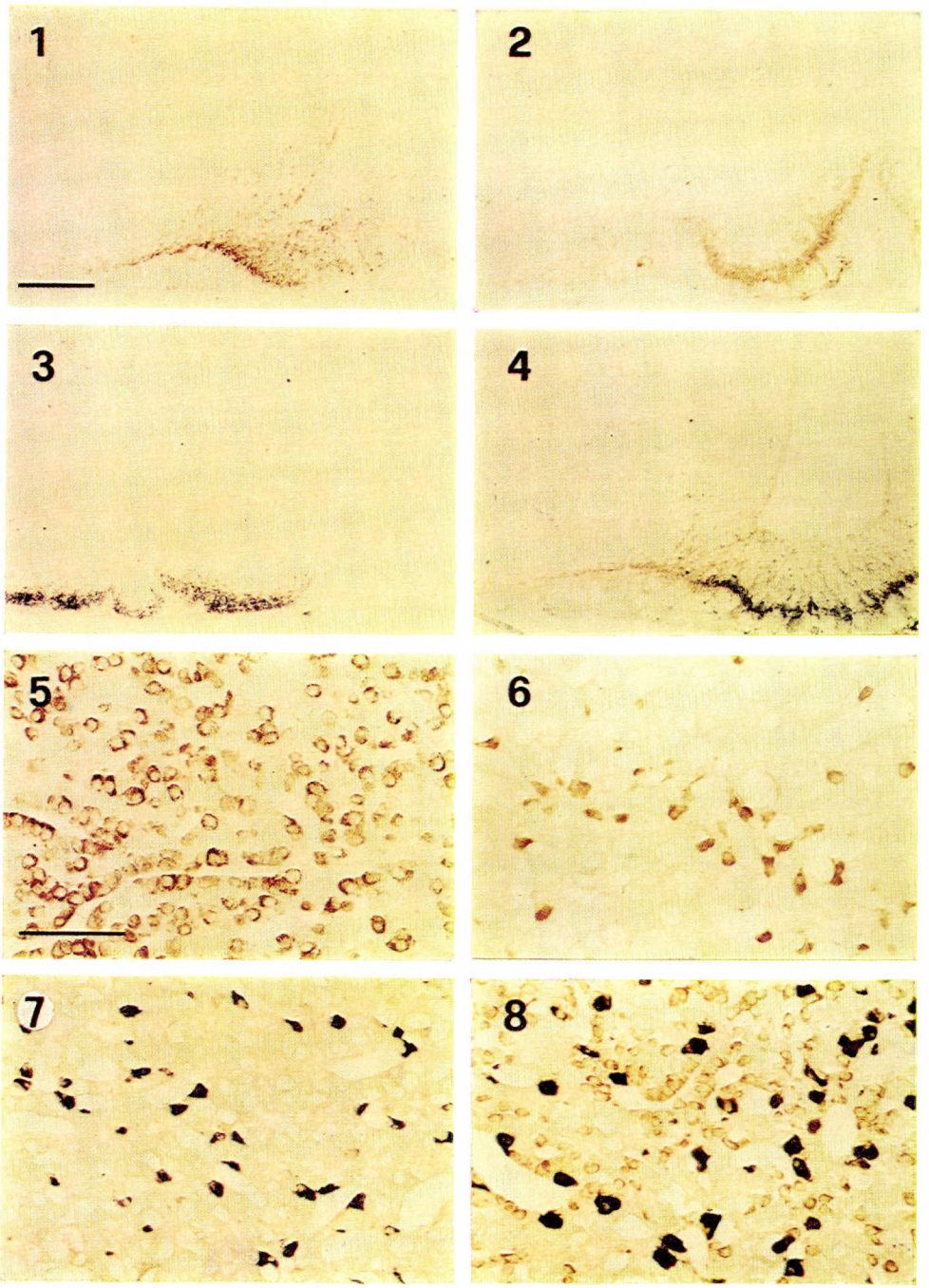
Fig. 4. Simultaneous localization of somatostatin-(black)- and LH–RH-(brown)-containing nerve fibres in the median eminence. Distinct colours indicate the two hormones and the overlapping two neuronal systems

Fig. 5. GH-synthesizing cells in the anterior pituitary gland. PAP-DAB method. Scale: 50 μm. Figs 5–8

Fig. 6. FSH-cells detected by PAP-DAB method

Fig. 7. FSH-cells detected by silver-intensified PAP–DAB method

Fig. 8. Simultaneously localized silver-gold intensified FSH-cells (black) and unintensified GH-cells (brown) in the section of the anterior pituitary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LH–RH-containing nerve fibres occupy the lateral part of the median eminence, especially its external zone (Fig. 1), while labelled somatostatin fibres are arranged in the middle part of the median eminence (Fig. 2). In both cases, the hormone content of the nerve fibres was detected with the traditional PAP-DAB method. Immunostained and silver-gold intensified somatostatin fibres appear in black colour (Fig. 3). The simultaneously localized somatostatin and LH–RH containing fibres are demonstrated in Fig. 4. Somatostatin was detected first with the silver-gold intensified PAP-DAB method, then the LH–RH containing nerve fibres were visualized by the traditional PAP-DAB method. The black somatostatin and the brown LH–RH fibres are seen in their characteristic position in the median eminence and are easily distinguished from each other.

In the anterior pituitary gland, GH- (Fig. 5) and FSH-containing cells (Fig. 6) were detected and labelled by the classical PAP-DAB method. Figure 7 represents immuno-labelled silver-gold intensified FSH-cells. The simultaneous labelling of the two cell populations of the anterior pituitary gland is demonstrated in Fig. 8.

Discussion

Our observations indicate that

1. The natural and the silver-gold intensified forms of the end-products of DAB can be distinguished on the basis of their different colour.

2. The physical developer of Gallyas does not destroy the antigenic sites of the tissue; they remain detectable following the silver intensification procedure.

3. The metallic gold deposited onto the end-product of DAB increases the masking effect of DAB upon the antigen and catalytic sites of the first sequence of immunoreagents, therefore before applying the second sequence of antibodies the step of antibody removal is unnecessary.

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EFFECT OF EXPERIMENTAL PATELLAR LUXATION ON THE KNEE JOINT IN THE RAT

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In 16 male Wistar albino rats the patella was fixed to the lateral aspect of the lateral condylus of the femur. Animals with luxated knee joint and controls were sacrificed 7–9 months after surgery. Operated knee joints were processed for histology and subjected to morphometric analysis in comparison to the controls.-

It was found that (1) the artificial disturbance of the femoro-patellar junction resulted in a severe destruction of articular cartilages of the tibio-femoral and femoro-patellar junction; (2) the destructions progressed with time; (3) there was no substantial difference between the changes ofthe medial and lateral articular surfaces of the femur and tibia; (4) changes comprised focal cartilage necrosis, widening of cartilaginous area under the tide-mark, proliferation of subchondral bone and formaton of osteophytes; (5) the fact that a change of the statics of femoro-patellar junction leads to panarthrosis, moreover that articular capsule, menisci, epi-, meta-, and diaphyseal bone areas are also involved, indicate the complexity of the question. The findings indicated that changes of the femoro-patellar junction play a role in the degenerative processes of the knee joint. It is concluded that early recognition and treatment of femoro-patellar alterations may help in the prevention of knee panarthrosis.

Introduction

Of all human joints the knee joint is the most liable to denegerative changes [16, 17, 20]. Over a certain age these are detectable in almost everybody [10]. While the cause of primary arthrosis is unclear, that of secondary arthrosis can now be explained. Exogenous (mainly traumatic) or endogenous (dysplasia of joint constituents) factors induce progressive changes not only in the articular cartilage but also in the articular capsule and subchondral bone that lead gradually to a disintegration and total destruction of the joint.

According to the mechanical theory, a disturbance in the balance of loading and the capacity of a joint (injury, malformation, overloading, malnutrition, drugs, etc.) causes degenerative changes. According to the enzyme theory, the increase in lysosomal enzymes is essential in the pathogenesis. The knee joint has extensively been studied from this respect. Although the joint comprises two articulations, the tibio-femoral and the femoro-patellar, interest has

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mainly been focussed on the tibio-femoral articulation as indicated by a number of experiments in dogs and rabbits [3, 4, 5, 8, 18, 22, 28, 30, 31, 33]. In these experiments one of the knee joint constituents was injured (patellectomy, meniscotomy, transection of the cruciate ligaments); the result was a degeneration of the tibio-femoral joint.

Earlier views on the pathogenesis of human knee joint arthrosis were to some extent modified by recent studies of the femoro-patellar joint [1, 2, 13, 15, 24, 27]. These refer to its role in development of the so-called panarthrosis of the knee. In the light of these findings the observations of Walton [39, 40] reporting on "spontaneous" arthrosis of the tibio-femoral joint in STR/ORT mice having a congenital medial luxation of the patella, are particularly remarkable. The work of Williams et al. [41] has shown that the rat is a suitable model of arthrosis. On these grounds we have put forward the hypothesis that in the rat knee, patellar luxation would induce arthrosis in addition to the affected area also in the tibio-femoral articulation. The present paper describes morphological changes of the cartilages of the femoro-patellar and tibio-femoral joints in the menisci, articular capsule and bones, induced by the disturbed biological balance of the femoro-patellar joint.

Materials and methods

For the experiments 22 male Wistar rats (initial weight 100 g) were used. In 16 rats under ether anaesthesia an incision was made at the medial side of the knee, the medial retinaculum was transected, the patella displaced and fixed by the lateral retinaculum with Dexon suture to the lateral condylus of the femur. Five months after surgery an X-ray picture was made of the knee (Fig. 1). Ten and three rats of the operated and control groups, respectively, were killed by exsanguination 7 months after surgery while 6 operated and 3 control animals were sacrificed 9 months after surgery. The knee joint was removed together with the distal portion of the femur and the proximal portion of the tibia, halved in the midsagittal plane, fixed and decalcified in Susa-solution. Sections were stained with the haematoxylin-chromotrop, Goldner and Azan methods. Glycosaminoglycans (GAGs) of the ground substance were demonstrated with the alcian-blue-PAS reaction. Thechanges of tibio-femoral and femoro-patellar articular surfaces, the capsule, the epiphyseal and transitional cartilages and of the bones were carefully examined. The degree of cartilage destruction was estimated by the method of Mankin [25] after staining with haematoxylin-chromotrop or Azan. Accordingly, various numbers of points denote the state of the cartilage with regard to general structure, cellular enrichment, staining of its ground substance and continuity of the tide-mark. The summary of points ranging from 4 to 14 reflects semiquantitatively the severity of the changes seen in one section. Measurements with eye-piece micrometer were also included (Fig 2). The thickness of the articular cartilage, within this the thickness of the calcification zone under the tide-mark and the thickness of subchondral bone in the femur and tibia were measured at 7 points. The thickness of the epiphyseal cartilage was measured at 6-points, while that of the bone trabeculae and osteoid at 5–5 points in every section. The thickness of the cartilage and bone at the medial meniscus was measured at six points. Corresponding values were pooled and the means were calculated. Mean values were informative concerning the degree of the change. This was also indicated by the values obtained for the calcification cartilage and subchondral bone. The thickness of bone trabeculae in the bone and the progress of ossification in the meniscus were indicative of the role played by the bone and meniscus in development of the changes.

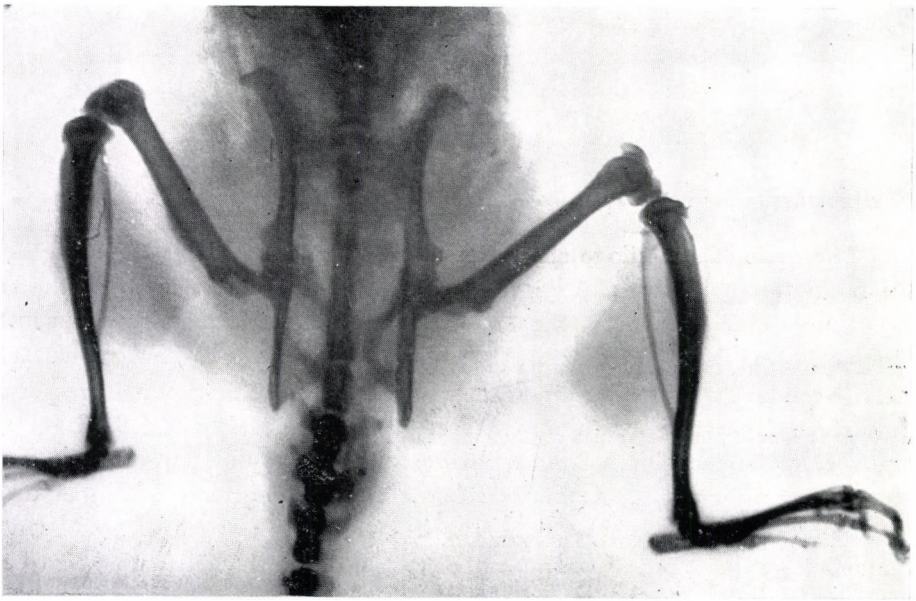


Fig. 1. X-ray picture 5 months after surgery indicating the displaced position of the patella

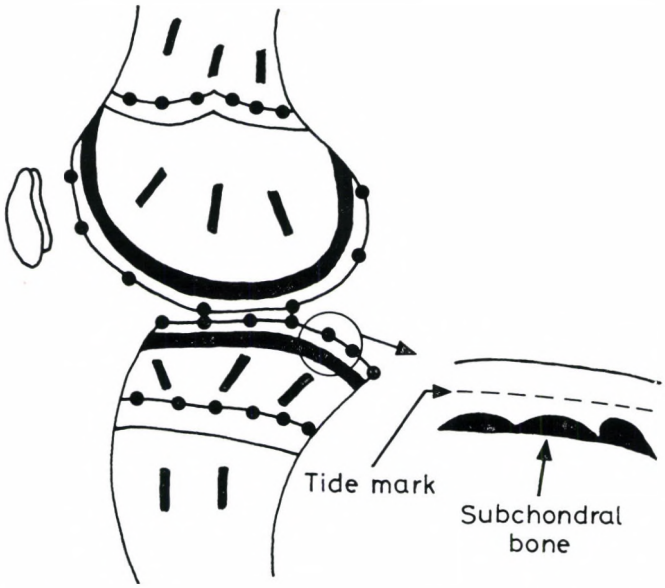


Fig. 2. Points of morphometric measurements on the knee joint complex

Results

Seven months after operation (Figs 3, 4, 5)

Proximal articular cartilage of the tibia

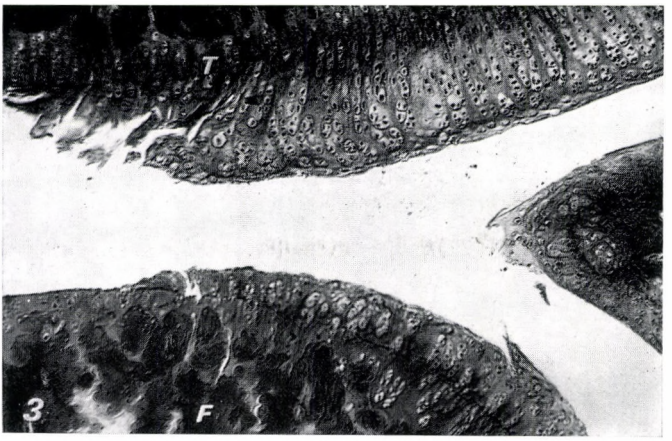
The articular cartilage shows an altered structure. Its thickness varies; at some places it is very thin or absent, at others it is thickened. At the thin parts the superficial zone is mostly missing. The middle zone is regular in the thin part, while it is irregular in the thick part; the cells are larger, clustered and the cell orientations are diverging (Fig. 4). In the thicker cartilage portion the surface layer is voluminous with an increased number of cells. Over this zone without a clear-cut border a fibrillary layer is found. The inner zone also differs from the normal. At the thin part a few chondrocytes are seen under the tide-mark which continue in the thickened subchondral bone. In the widened part an "empty" zone under the tide-mark with some scattered, swollen chondrocytes extend to the subchondral bone (Figs 4, 5). Even the intact territories show a widening of the area under the tide-mark. In the articular cartilage often fissures and pseudocysts are present, penetrating till the subchondrial bone (Fig. 7). Osteophyte formation also occurred.

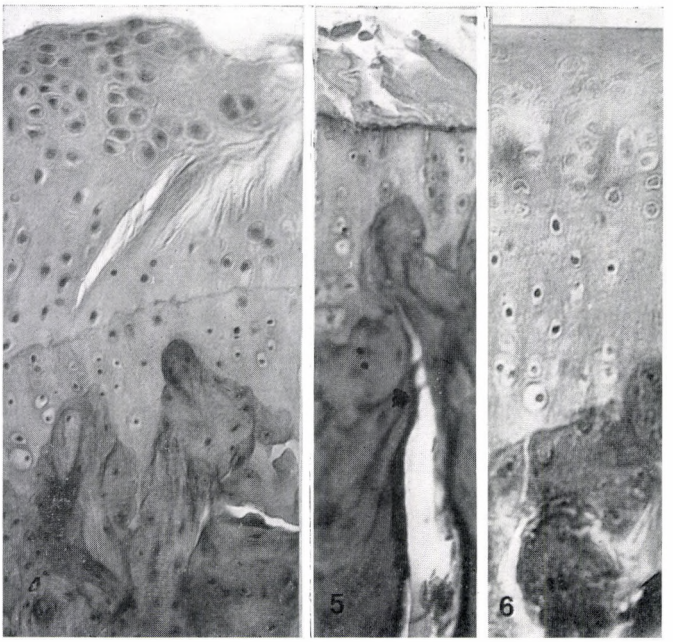
Distal articular cartilage of the femur (Figs 3, 6)

The articular cartilage has thicker and thinner parts. The structure of the cartilage is better preserved in the thicker parts, the position of the tide-mark is higher and may even be double whereby the deep zone becomes wide (Figs 6, 8). The penetration of subchondral bone is moderate, the bone-cartilage boundary is not everywhere discernible (Fig. 9). Towards the medial edge of the articular surface a gradual necrosis of the cartilage can be seen extending till the subchondral bone. In severe cases ulceration of the cartilage with formation of pseudocysts extending into the joint cleft and filled with fibrocartilage occurs. At the lateral edge of the articular surface ulceration, pseudocysts with cartilage islands are found. On the border of the subhormonal bone there are capillaries, the bone lamina is thick. Near the patella, osteophytes can be observed.

Patellar articular surface of the femur

At the area opposite to the patella, the zonation is preserved, the cartilage is partly thickened due to a widening of the territory under the tide-mark (Fig. 10).





Seven months after operation.

Fig. 3. Knee joint. Articular cartilages of the tibia (T) and femur (F) and the ossified medial meniscus. Goldner's stain. × 50

Fig. 4. Articular cartilage of the tibia. In the widened area, proliferation of cells and subchondral bone. Haematoxylin-chromotrop. × 130

Fig. 5. Articular cartilage of the tibia. Fibrillation, subchondral bone proliferation and scarcity of cells in the zone below the tide-mark. Haemotoxylin-chromotrop. × 130

Fig. 6. Articular cartilage of the femur. Even in the intact area widened zone below the tide-mark. Goldner's stain. × 180

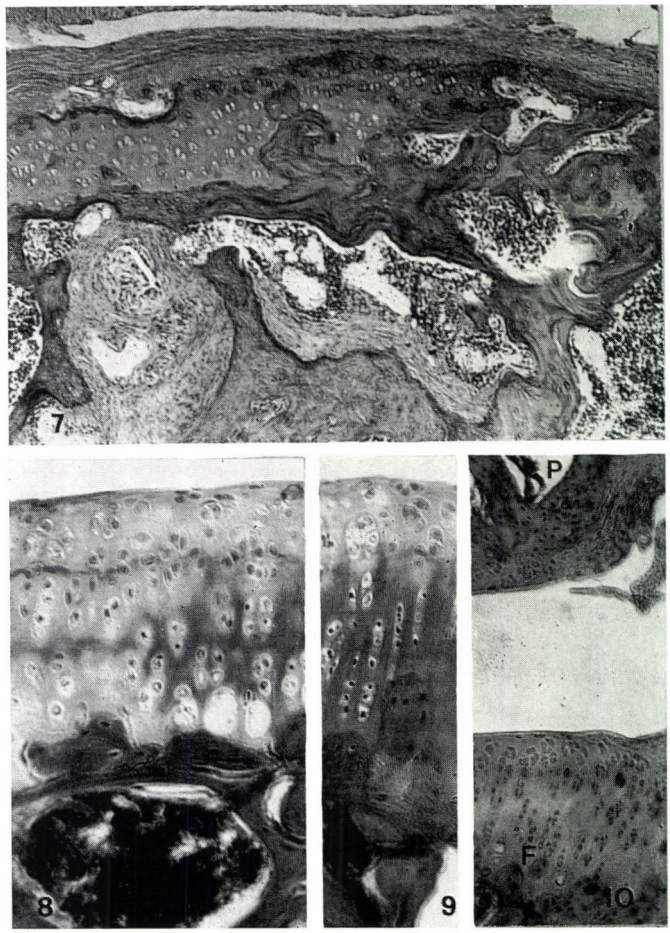


Fig. 7. Seven months after operation. Articular cartilage of the tibia. Different changes. Deep fissure filled with connective tissue. Fibrillation and pseudocysts. Haematoxylin-chromotrop. × 60

Fig. 8. Articular cartilage of the femur. Double tide-mark. Goldner's stain, × 130

Fig. 9. Articular cartilage of femur. Subchondral bone penetrating into cartilage, indistinct bone-cartilage border. Goldner's stain. × 130

Fig. 10. Articular surfaces of femur and patella. The articular surface of the patella is severely damaged, in the femoral articular cartilage the zone below the tide-mark is widened with a proliferation of subchondral bone. Haematoxylin-chromotrop. × 50

Articular surface of the patella

A minor part of the articular cartilage is intact with normal zonation and regular tide-mark, but the major part of the cartilage is destroyed covered by connective tissue or in some places by fibrocartilage (Fig. 10).

Menisci

In the rat there is a difference between the two menisci; the medial one consists of hyalin, the lateral one of fibrous cartilage (Fig. 11). Postoperative changes affect mainly the medial meniscus. One part of the surface cartilage undergoes necrosis or fibrillation and the central cartilaginous part ossifies (Fig. 12). In some cases the lateral meniscus also suffers similar changes.

Articular capsule

The fibrous capsule, particularly the synovial layer becomes thick, its epithelium is flattened, partly destroyed. In the sections numerous vessel profiles are seen with thick vascular walls. Proliferation of the fibrous and adipose synovial layers (Fig. 15) and in the fibrous capsule chondroid metaplasia occur (Fig. 16).

Epiphyseal cartilage of the tibia

The cartilage is in various stages preceding its final occlusion. The zonation is mostly preserved. The decrease of chondrogenetic activity is indicated by a narrowing of the proliferation zone, a widening of the intercellular matrix and the presence of a homogeneous substance (Fig. 17). The increased invasion of blood vessels and the appearance of chondroclasts are indicative of cartilage destruction.

Epiphyseal cartilage of the femur

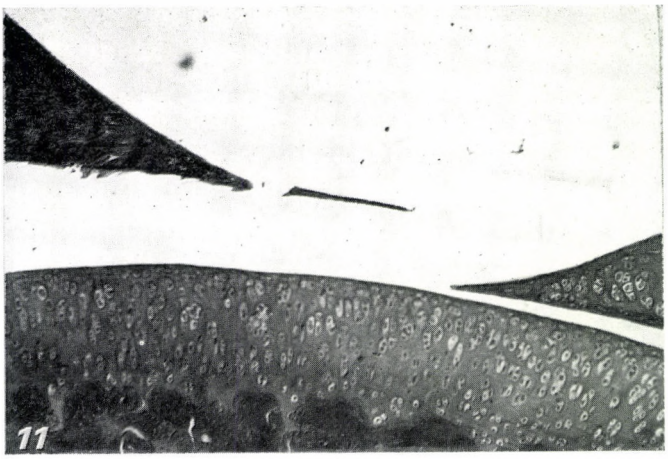
Occlusion of the cartilage is in an advanced stage, its zonation is disintegrating. A few cell layers constitute the irregular proliferation zone with widened intercellular substance. The maturation zone is also widened.

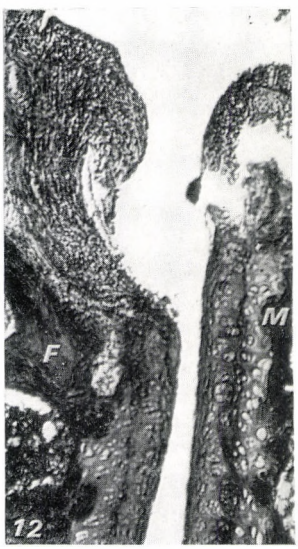
Transitional cartilage

This is a hyaline cartilage interposed between the epiphyseal and articular cartilages. In most cases it became fibrous in the tibia. In the femur the surface of this cartilage was also often fibrous.

Meta- and diaphyseal bones

The spongy bone trabeculae are thin with osteoid at the edges. The marrow cavity reaches to the bone-cartilage boundary. The periostal side of the cortical bone is irregular; near the epiphyseal cartilage Howship lacunae con-





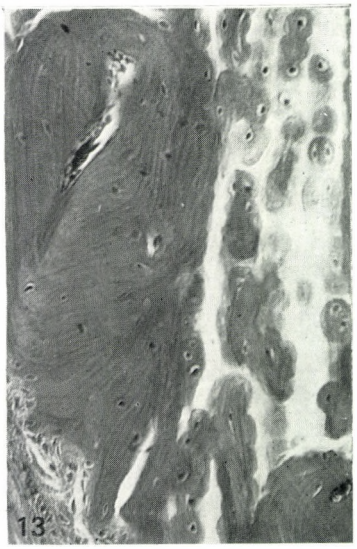


Fig. 11. Tibial articular cartilage and menisci. The medial meniscus is composed of hyalin cartilage, while the lateral meniscus is composed of fibrocartilage. Goldner's stain. × 50

Fig. 12. Articular cartilage of femur and the medial meniscus. The surface cartilage is severely damaged, and a part of meniscus is ossified. Goldner's stain, × 50

Fig. 13. Portion of tibial diaphysis. Fragmented cortical bone, numerous Howship-lacunae. Goldner's stain, × 130

taining osteoclasts are often observed. In the cortical bone a "fragmentation" process is characteristic (Fig. 13). These changes may occur in both the femur and tibia.

According to Mankin's method the tibia and femur yielded mean values of 8 and 8.3, respectively.

Nine months after operation

These changes were in an advanced stage.

Proximal articular cartilage of the tibia (Fig. 18)

The edges of the cartilage surface show fibrillation and ulceration, the latter penetrating down to the subchondral bone. The ulcer is occassionally filled with fibrocartilage. Towards the centre of the cartilage surface fibrillation decreases and layering of the cartilage is seen. The tide-mark is situated higher than normal and proliferation of subchondral bone into the cartilage can be observed. Laterally, deep fissures reaching to the subchondral bone, disorganization of cartilage structure, marked thickening of subchondral bone and osteophyte formation are found.

Distal articular cartilage of the femur (Fig. 19)

Zonation of the cartilage is preserved only in the centre of the articular cartilage but even here the superficial and middle zones are narrow and contain swollen chondrocytes. At the edges of the articular surface deep fissures are observed reaching to the bone; they are filled with connective tissue. The subchondral bone is thick and contains pseudocysts. Osteophyte formation occurs at several sites.

Menisci

Most of the medial meniscus is ossified and partly its destruction has begun. In the remaining hyalin cartilage fibrillation and ulceration occur. The lateral meniscus is composed of fibrocartilage and connective tissue.

Joint capsule

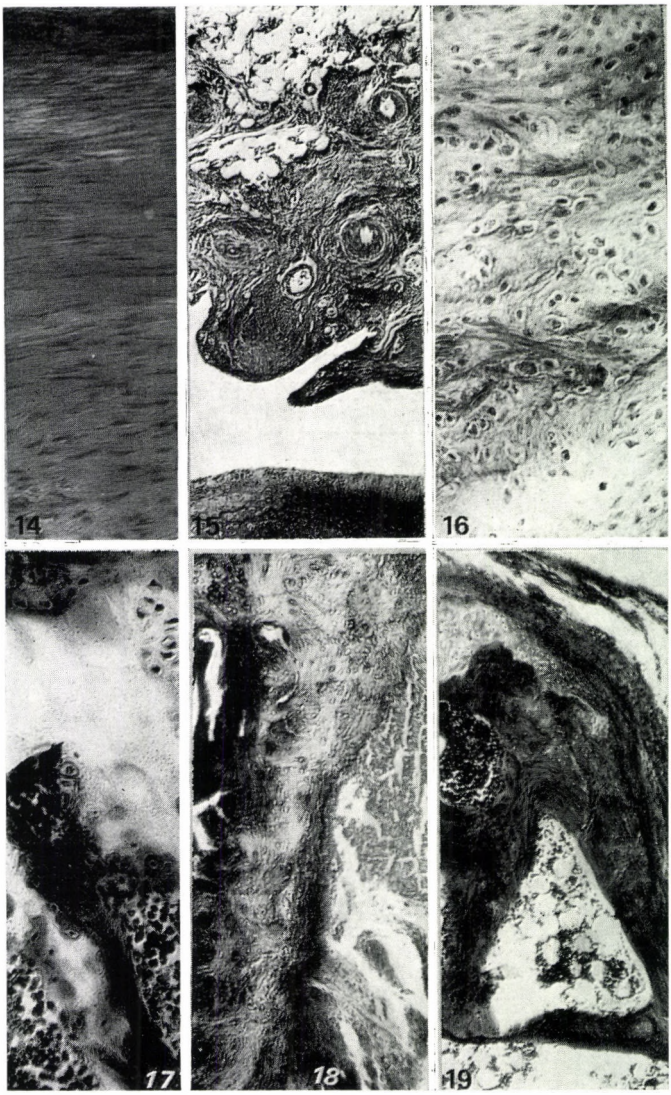
The capsule is significantly thickened, inside with an increased extent of chondroid metaplasia and subsynovial fibrosis.

Ephyseal cartilage of the tibia

The cartilage is occluding. Mainly the cells of the proliferation zone decrease in number, the columnar organization is disappearing. The hypertrophic zone is still visible. At the border of the metaphysis chondroclasts appear.

Epiphyseal cartilage of the femur

This cartilage is in a stage just prior to occlusion. Zonation can be recognized only in patches. The presence of chondroclasts indicates advanced destruction.



Seven months after operation.

Fig. 14. Thickened tunica fibrosa of articular capsule. Goldner's stain, × 130

Fig. 15. Articular capsule. Subsynovial fibrous and adipose layers are well discernible. Flattened synovial epithelium thickened vascular walls. Goldner's stain × 50

Fig. 16. Articular capsule with chondroid metaplasia. Goldner's stain. × 150

Fig. 17. Epiphysis cartilage of tibia. Intercellular territories are widened and filled with amorphous substance. Goldner's stain, ×150

Fig. 18. Articular cartilage of tibia 9 months after operation in advanced stage of damage, fibrillation and ulceration. Goldner's stain, × 150

Fig. 19. Articular cartilage of femur 9 months after operation. Severe damage indicated by fibrillation and deep fissure formation. Goldner's stain. × 150

Transitional cartilage

It has partly preserved its hyaline character, but shows partly a fibrous structure. In some cases it is missing.

Dia- and metaphyseal bone

Both femur and tibia contain in the central trabeculae non-staining, amorphous areas, the remnants of cartilage ground substance, often with an osteoid rim. Osteoid is increased also at the edges of other trabeculae. At the periostal edge of cortical bone, Howship lacunae are present with osteoclasts.

Table I

		1	2	3	4	5	6	7	8
1.	SD	170.0 38.0	75.5 1.1	2.23 44.7%	188.0 24.0	85.0 64.0	2.22 45.0%	94.5 11.0	125.0 28.0
2.	SD P<	134.0 46.0 NS	60.0 20.0 NS	2.23 44.8%	108.0 39.0 0.025	58.5 17.0 NS	1.84 54.16%	258.0 118.0 NS	161.0 53.0 NS
3.	SD P<	128.0 45.0 NS	48.0 18.0 NS	2.67 37.4%	99.0 17.0 0.005	69.0 22.0 NS	1.43 69.7%	224.0 95.0 0.05	218.5 33.0 0.025

		9	10	11	12	13	14	15	16	17
1.	SD	170.0 41.0	146.0 38.0	3.8 0.98	4.2 1.5	59.6 5.1	59.6 13.0	50.4 25.0	170.0 27.0	0.3 337.0%
2.	SD P<	165.0 48.0 NS	181.0 23.7 NS	18.0 5.7 0.025	16.0 4.4 0.005	50.3 17.0 0.05	52.0 6.5 NS	186.0 102.0 NS	118.0 59.7 NS	1.57 63.4%
3.	SD P<	136.0 42.6 NS	140.5 51.0 NS	12.0 1.9 0,0025	14.0 1.7 0.005	49.0 6.6 0.05	55.0 13.9 NS	132.0 72.0 NS	126.5 26.9 NS	1.04 95.8%

1. Control
2. Displaced patella, 7 months
3. Displaced patella, 9 months

1 = total thickness of tibial articular cartilage (μ)
2 = thickness of calcification zone of tibial articular cartilage (μ)
3 = numerical and percentual ratios of 1 and 2
4 = total thickness of femoral articular cartilage (μ)
5 = thickness of calcification zone of femoral articular cartilage (μ)
6 = numerical and percentual ratios of 4 and 5
7 = thickness of tibial subchondral bone (μ)
8 = thickness of femoral subchondral bone (μ)
9 = thickness of tibial epiphyseal cartilage (μ)
10 = thickness of femoral epiphyseal cartilage (μ)
11 = thickness of tibial osteoid (μ)
12 = thickness of femoral osteoid (μ)
13 = thickness of tibial trabeculae (μ)
14 = thickness of femoral trabeculae (μ)
15 = thickness of ossified meniscus portion (μ)
16 = thickness of cartilagenous meniscus portion (μ)
17 = numerical and percentual ratios of 15 and 16

Table II

Comparison of medial and lateral surfaces

	Medial surface	Lateral surface
Total thickness of tibial articular cartilage (μ)	150.0	114.5
	SD 49.0	SD 27.0
Thickness of calcification zone in tibial articular cartilage (μ)	65.0	53.0
	SD 27.0	SD 11.0
Total thickness of femoral articular cartilage (μ)	99.0	122.0
	SD 20.0	SD 49.0
Thickness of calcification zone in femoral articular cartilage (μ)	53.0	63.0
	SD 13.0	SD 19.0
Thickness of tibial subchondral bone (μ)	228.0	303.0
	SD 133.0	SD 101.0
Thickness of femoral subchondral bone (μ)	201.5	194.0
	SD 109.0	SD 100.0

According to Mankin's method the tibia and femur yield 9.5 and 9.7 mean values, respectively.

Morphometric results obtained by measurements with an eye-piece micrometer are summarized in Table I. The thickness of the articular cartilages of both femur and tibia is decreased, inside the calcified cartilage-zone under the tide-mark is, however, comparatively wide. The subchondral bone is substantially thickened, the osteoid is wide, whereas the width of the trabeculae is decreased. In the medial meniscus the shift of the cartilage (bone ratio in favour of the latter was a remarkable finding). Table II shows the comparison of the medial and lateral articular surfaces. In the case of the tibia the destruction of cartilage, widening of the calcified layer and thickening of the subchondral bone are less pronounced on the lateral side, while the opposite situation was found in the case of the femur.

Discussion

Our findings corroborate and complement the results of Walton [39, 40] who carried out his studies in mice. It seems that the lateral experimental luxation of the patella causes a degeneration of the articular cartilages in the tibiofemoral and femoro-patellar joints. Walton [39] has, however, described degeneration after the medial luxation of the patella only in the cartilage covering the medial condyli of the tibia and femur. Moreover in his studies differences between the changes of the tibial and femoral articular cartilages are hardly dealt with, and no mention is made of changes of the femoro-patellar joint. Comparing the changes of the articular cartilages of the tibia and femur it appears that while on the tibia fibrillation and bone proliferation are predominant, on the femoral cartilage destruction and proliferation of cartilage are more conspicuous with a more pronounced tide-mark. Cartilages on the femoro-

patellar surface are also severely damaged. These findings argue for a strong influence on the tibio-femoral joint of changes in the mechanical balance of the femoro-patellar joint. Similar conclusions are reached from the comparison of damages to the medial and lateral parts of the tibio-femoral joint. In the femur the patellar luxation resulted in a severe lesion of the cartilage covering the medial condyli. In the lateral part of the atricular surface of the tibia fibrillation and cartilage destruction were the predominant features. Progession with time of the changes was verified by histological findings supported by morphometric measurements made in animals left to survive for prolonged periods. Most of the semiquantitative data were not significant due to the high scattering of values brough about either by the marked changes in focal disease [26] or by methodological factors. The differences were, however, conspicuous, their tendencies clear-cut and in agreement with the results of Vignon et al. [38] in human material.

Lateral luxation of the patella affects also some structures other than the articular cartilages. We regard the changes of the tibio-femoral and femoro-patellar articular cartilages as primary in contrast to those of the menisci and articular capsule which are thought to be secondary and those occurring in epiphyseal cartilages and diaphyseal bone to be tertiary.

The secondary changes are characterized by a partial ossification of the meniscus, widening of the tunica fibrosa of the articular capsule (involving chondroid metaplasia), and a proliferation of the subsynovial fibrous and adipose layers.

The tertiary changes were the disintegrated zonation of the epiphyseal cartilage, widening of its intercellular substances, accumulation of an amorphous substance and its penetration into the new spongiosa, accumulation of osteoid at the edges of the spongious bone, thinning of bone trabeculae, and Howship lacunae with osteoclasts at the periostal edge of cortical bone. These point to the effect of the altered mechanical balance on the menisci, capsule, epiphyseal cartilage and epi-, meta-, and diaphyseal bone besides the articular cartilages. Some relevant points need further attention.

(*a*) Changes of the articular capsule manifest themselves with inflammatory cellular infiltration, fibrosis, capsular thickening, chondroid metaplasia, increased collagen snythesis and calcification, etc. Generally the detaching cartilage is thought to induce a synovial reaction [21]. Surface cartilages and more often detached pieces of the medial meniscus may act in this way. On the other hand, post-traumatic synovitis [36] and sterile synovitis [12] are known to bring about cartilage degeneration. In other words, there exists an interaction between articular cartilage and capsule, as indicated by the synovitis following cartilage destruction: pannus grows onto the cartilage leading to its absorption. This is the basis of Dettmer's view [9] that cartilage and capsule are acting as a functional unit which manifests in the control of lactate-hyaluronic acid synt-

hesis. In arthritis, the destruction of cartilage reduces lactate production which leads to the reduction of hyaluronic acid synthesis in the synovial epithelial cells. This affects proteoglycan aggregation in the cartilage. The water content increases whereby the physico-chemical properties of the cartilage alter. Furthermore, lesions of the synovial blood vessels affect substrate transport and nutrition of the cartilage [16, 17].

(*b*) The role of subchondral bone is not unequivocal. According to Collins [6] cartilage destruction causes two kinds of change in the subchondral bone, an irregular advance of ossification in the calcified zone of cartilage and a cellular reaction in the marrow cavity. As compared to rheumatoid arthritis, in arthrosis the inflammatory reaction is less intense. The subchondral bone appears to have a buffer role in distributing the force of passive loading, this allows to assume its primary role in the pathogenesis of arthrosis, as the injury of the bone (for example microfractures) lead to a damage to the cartilage by bone remodeling, dimensional changes and the decrease of elasticity [16, 32]. Another view is that subchondral bone changes are associated secondarily with the degeneration of articular cartilage [6, 14, 34]. In our material the proliferation of subchondral bone was consistently observed, but this was also true for cartilage destruction. The enhanced bone and osteophyte formation may be interpreted as an adaptation phenomenon indicating the degree of cartilage destruction and the reparatory capacity of the organism.This latter is supported by the vascular proliferation caused by synovitis. This "extremely enhanced ossification" is served by the appearance of osteoid in the epi- and metaphyses and by the increased bone destruction, suggesting that in addition to the articular cartilages, the capsule, the menisci and the subchondral, epi-, meta-, and diaphyseal bones are all the constituents of a unit that has the common task of ensuring the statical balance of the joint. The damage to any of these constituents may indirectly affect the articular cartilage. With increasing time more and more constituents may be involved, aggrevating the clinical picture.

(*c*) Our present studies concentrated on the role of the mechanical factor which is only part of the pathomechanism. In his review Mohr [29] distinguished between the mechanical and enzyme digestion theories. Synthesis of these theories seems to be justified on the asbis of several studies. Accordingly, a mechanical injury leads to the damage of chondrocytes as a result of which catabolic enzymes are activated. This brings about a breakdown of proteoglycans and collagen fibres and finally leads to advanced cartilage destruction [7, 9, 11, 16, 17, 19, 23, 25, 35, 37]. It is reasonable to suppose that proteoglycans and collagenous fibres, these two macromolecular components of the cartilage ground substance, are essential in ensuring the normal loading capacity and the function of cartilage and the regulation of cartilage and bone calcification, playing thereby an important role in the pathomechanism at the molecular level.

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EFFECT OF TRITON X-100 ON CYTOCHEMICAL AND ULTRASTRUCTURAL PATTERN OF CHROMATIN

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The action of Triton X-100 on chromatin was observed in normal rat liver, thymus and ascites hepatoma cells. For DNA cytochemistry and thin-section electron microscopy the cells were treated with the permeabilizing 0.05% concentration of Triton 0.5–1% Triton treatment was applied to rat thymus nuclei spread on electron microscopic supports.

Triton caused a compactness of chromatin in stained nuclei, in ultrathin sections and nuclear spreads. The most prominent feature of the tritonized higher order fibre is an increased regularity of its structure. In the nuclei stained for DNA with toluidine blue, Triton caused a sharp increase of optical density in the comparable zone of the spectral maximum and a shift to the shorter wave lengths. Cells treated with Triton exhibit an exaggerated anisotropic staining reaction.

The cytochemical and cytophysical changes induced by Triton X-100 are explained by a polymerization caused by an increased regularity of the chromatin fibre structure.

Introduction

Triton X-100 is a nonionic detergent widely applied in cell research. It binds apolar domains of amphiphiles by dissolving them, and does not bind hydrophilic proteins [6]. Triton is used in chromatin studies mainly for purification and lysis of nuclei and for permeabilization of cells [4]. Treatment of rat liver nuclei with 2% Triton was shown to remove 95% of phospholipids and about 10% polypeptides originating from the nuclear envelope while the histone content remained unchanged [1].

In the work reported below Triton X-100 is shown to alter the cytochemical and ultrastructural features of chromatin.

Materials and methods

The influence of Triton on chromatin was investigated in two model situations: 1. Treatment of intact Zajdela hepatoma cells with 0.05% Triton (permeabilizing concentration (4)) diluted in 120 mM NaCl, 20 mM Na-phosphate buffer pH 7.2. and 5 mM 2-mercaptoethanol at 22 °C for 10 min. Smears of normal rat liver and thymus were treated likewise. 2. Treatment of

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isolated rat thymus nuclei with 0.5—1% Triton (lytic concentration) diluted in Hanks' solution at 0 °C for 2 min.

In the first case ascites hepatoma cells washed in cold buffered saline were treated with Triton,monitored by phase contrast microscopy, smeared on slides for DNA histochemistry, and fixed for thin-section microscopy.

DNA histochemistry

The cells were immediately fixed on slides with ethanol-acetone mixture (1:1), dried, hydrolysed with 5 n HCl at 22 °C for 60 min, and stained either with Schiff reagent or with toluidine blue 0.05% pH 4.0 [2] The latter was purchased from Fisher Scientific C°.

Cytophotometry was carried out with digital scanning integrating cytospectrophotometer CITO-2 (USSR) with a slit 0.44 μm. In each case the optical density of 120 cells was assayed (this number of cells has been proven to be sufficient in preliminary experiments; and variation coefficient never exceeded 8%) at 550 nm for the Schiff-stained specimens and at 570 nm for the specimens stained with toluidine blue. The entire spectra were taken at every 10 nm of 6 cells in each case. Cytophotometry was carried out in 5 experiments.

Electron microscopy

Thin sections. Glutaraldehyde was added to the suspensions of the control and Triton-treated cells to 2.5% final concentration. The cells recovered after centrifugation pelleting were fixed with the same fixative at 4°C for 2 h, washed, postfixed in 1% osmium tetraoxide, dehydrated in ethanol series, acetone and epon-acetone. Finally the cells were embedded in epon. Thin sections were preparated with glass knives in LKB ultramicrotome, mounted on-formvar-coated grids and double-stained, with uranyl acetate and lead citrate.

Whole mounts. The crude fraction of rat thymus nuclei was recovered after mild homogenization of the tissue in Hanks' solution with 5 mM 2-mercaptoethanol and 1 mM phenylmethyl-sulphonylfluoride. The nuclei were then disrupted by tight homogenization in the same solution and allowed to adsorb to the positively charged electron microscopic supports. They were the treated with 0.5—1% Triton in Hanks' solution at 0°C for 2 min, fixed in 2% formaldehyde, washed, stained with 2% aqueous uranyl acetate and rotary shadowed with tungsten oxide at an angle of 7°. Electron microscopy was carried out with EMV-100L and JEM-100S, ight microscopy with MBI-6 apparatus.

Results

Triton-treated hepatoma cells visualized by phase contrast microscopy had a distinct and rough nuclear structure as compared to the control cells (Fig. 1.). The same cells stained for DNA had nuclei of coarse structure and enlarged roundish nucleoli. The same changes were shown by the liver and thymus cell nuclei in smears.

In ultrathin sections some compact of chromatin and its margination resulting in lightening of the rest of the nucleus could be seen in hepatoma cells after Triton treatment (Fig. 2). The same observations have been reported by Ērenpreiss et al. [3].

Triton did not cause any change of the average DNA content as shown by the quantitative Feulgen test performed in 5 experiments. Nuclei of the cells stained for DNA with toluidine blue after Triton treatment acquired a deep-violet colour in comparison with the blue nuclei of control cells. When examined between crossed polarizers the toluidine blue stained hepatoma cell nuclei which were isotropic in the controls, became anisotropic and the slightly aniso-

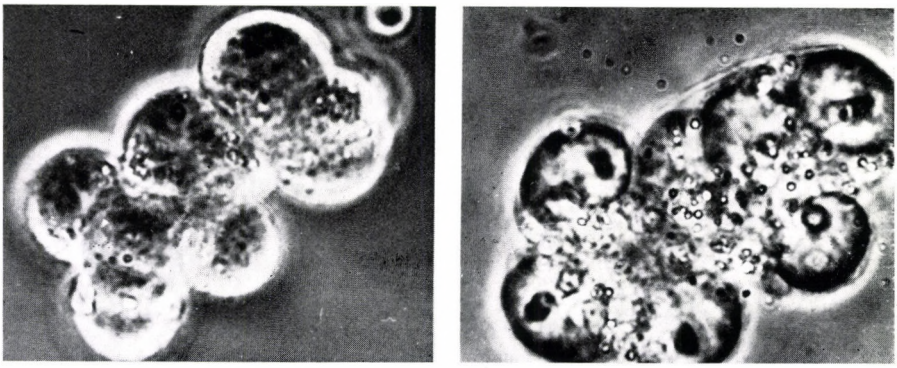

Fig. 1. Phase contrast micrographs of Zajdela ascites hepatoma cells. × 1150. *a* control cells treated with buffered saline; *b* cells treated with 0.05% Triton X-100 in buffered saline

tropic hepatocytes became intensely anisotropic (Fig. 3), and the birefringent nuclei of thymocytes still brighter.

The absorption spectrum of control hepatoma nuclei stained with toluidine blue had one prominent peak at 570 nm and a slight one at 630 nm (Fig. 4). After Triton treatment the maximum remained at 560– 570 nm (Fig. 4) while the absorbance in this zone increased by about 35% per nucleus (Fig. 5), besides there appeared a slight shift towards shorter wawe lengths with a small shoulder at 510—530 nm.

Investigation of rat thymus nuclei spreads on electron microscopic supports and treated with Triton (or with Hanks' solution alone for the controls)

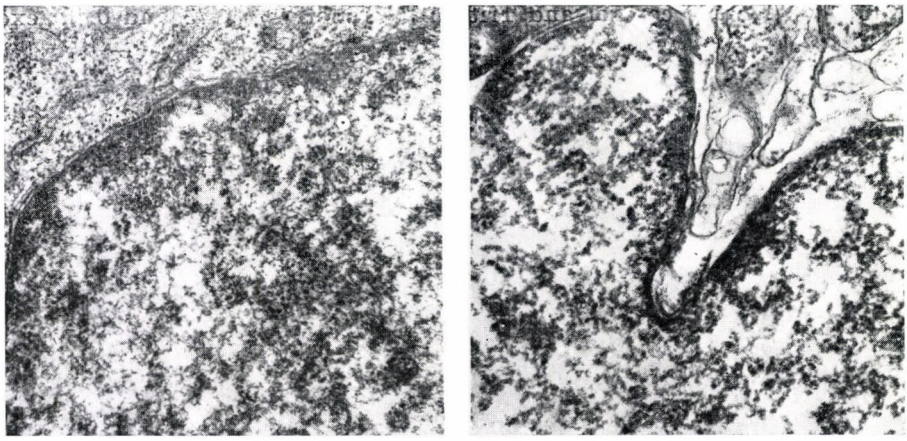

Fig. 2. Electron micrographs of Zajdela ascites hepatoma cells in thin sections. × 35 000. *a* a fragment of control cell; *b* Triton treated cell

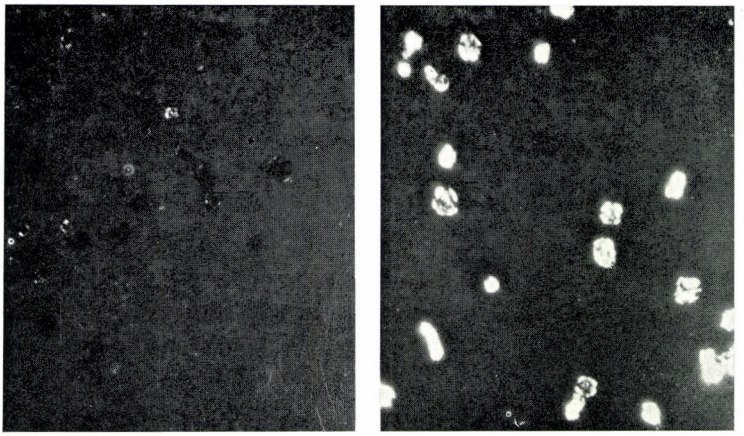

Fig. 3. Rat liver imprints stained for DNA with toulidine blue and photographed in polarized light. × 300. *a* control cells; *b* Triton-treated cells with exaggerated birefringence

allowed to monitor the action of the detergent on individual chromatin fibres. A chromatin fibre in these conditions is composed of knobs about 30 nm in diameter arranged in tandem which in Triton-treated specimens looked more like disks (Fig. 6). They appeared to be settled tighter and more orderly than in control specimens. We have made measurements of the interspaces between the centres of 50 clearly distinct knobs. The arithmetical average for the control and Triton were 27.36 $\pm$ 0.63 nm and 27.72 $\pm$ 0.68 nm and thus the modal values 31.5 nm and 27.5 nm respectively. The results of these measurements are also presented in histogram form in Fig. 7. The difference in distribution of the values could be substantiated by calculating asymmetry and excess coefficients. These for the control and Triton: asymmetry — 0.73 and 0.45; excess

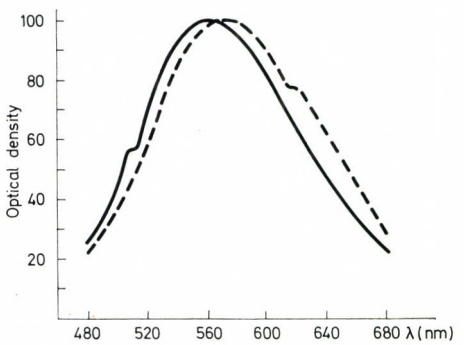

Fig. 4. Absorption spectra of Zajdela ascites hepatoma cells stained with toluidine blue for DNA. Discontinuous line: control; continuous line: cells treated with 0.05% Triton. Every curve represents the average of 6 cells, normalized to their own maximum.

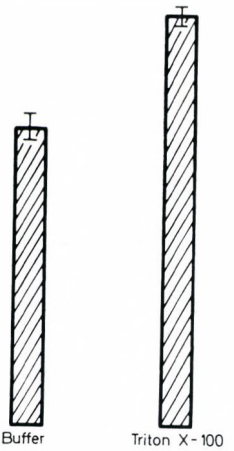

Fig. 5. Increase in absorbance at 570 nm in toluidine blue stained nuclei after Triton-treatment; cytophotometric results of 5 experiments

—0.69 and +2.58 respectively. An exaggerated positive excess value for the Triton specimen means a highly regular apposition of knobs in a chromatin fibre unlike the control one (a negative excess).

Thus, Triton X-100 caused: 1. A substantial increase in optical density of the cell nuclei stained for DNA with toluidine blue per a DNA unit; 2. a metachromatic shift towards short wavelength of the absorption curve in toluidine blue-stained nuclei; 3. an exaggerated anisotropic toluidine blue staining reaction; 4. compactness of chromatin observed at all levels; 5. a change of the chromatin fibre superstructure as shown by a more regular arrangement of the knobs.

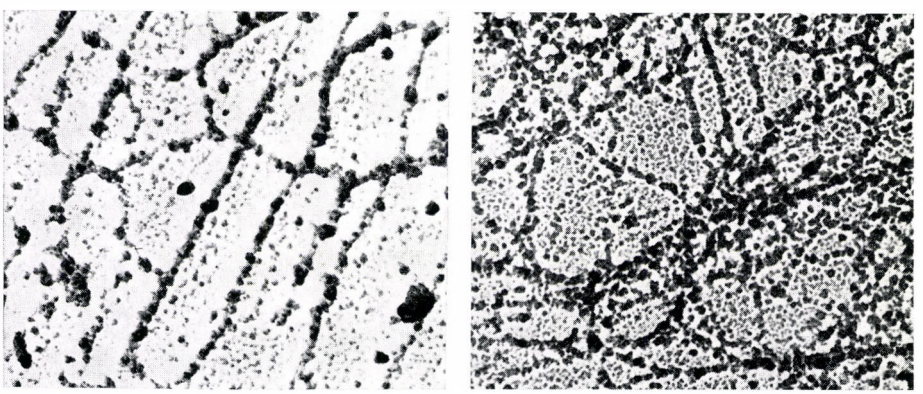

Fig. 6. Fragments of rat thymus nuclear spreads. × 33 000. *a* control spread; *b* Triton-treated spread (0.5% Triton X-100)

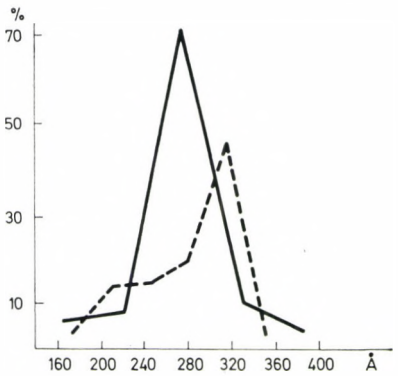

Fig. 7. Histogram of interspaces between the centres of chromatin fibre knobs. Continuous line: Triton-treated chromatin; discontinuous line: control chromatin

Discussion

The change of the chromatin fibres may have resulted from a decrease of surface tension caused by Triton. This, however, has no connection with the compactness of chromatin observed in whole cells. The simplest explanation for the increase of DNA stainability with the basic dye of pH 4.0 would be an enhanced accessibility of DNA phosphates under Triton action. Such a release of free phosphates would result in electrostatic repulsion and hydration of these residues, leading to relaxation of the chromatin structure. We have, however, observed just the contrary, a compactness of chromatin.

The clue, probably, lies in a reconstruction of the chromatin fibre induced by Triton. The increased regularity of the high order structure of tritonized chromatin may stimulate polymerization of the planar toluidine blue molecules without a substantial release of new binding sites, thus resulting in development of new high energy bonds [9]. Polymerization may be the cause of the increased absorbance at 570—560 nm, a zone of toluidine blue β—metachromasia due to dimers and oligomers of the dye molecules and the same may cause the appearance of some γ— metachromasia at 510–530 nm due to the dye polymers (Michaelis 1947, quoted by Pearse [8]). The anisotropy, i.e. birefringence, of the chromatin as a result of Triton treatment, which means a parallel orientation of the dye molecules in respect to polymeric substrate [5], confirms this assumption. The mechanism of changes caused by Triton X-100 in chromatin and their reversibility remain to be elucidated. The two possible ways of action may be that: 1. Triton binds to some apolar residues in the chromatin; 2. Triton, solubilizing nuclear membrane components and changing ion balance in the nucleus, influences the chromatin structure in an indirect manner. The role of nonorganic ion balance in the chromatin structure and function has recently been stressed [7].

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