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Distribution of Acetyl Cholinesterase in the Hippocampal Region of the Guinea Pig

I. Entorhinal Area, Parasubiculum, and Presubiculum*

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Summary. 1. The distribution of acetyl cholinesterase has been examined in the guinea pig in three areas belonging to the hippocampal region, viz., the entorhinal area, the parasubiculum, and the presubiculum.

2. Formaldehyde-fixed serial frozen sections and unfixed cryostat sections were subjected to a modification of the Koelle thiocholine method. Ethopropazine was used to suppress non-specific cholinesterase. One series of sections was processed according to the copper ferricyanide method of Karnovsky and Roots (1964).

3. In each area the obtained staining displayed a specific pattern of stratification, ceasing at the transition to neighbouring areas. The distribution in the guinea pig has been compared with that in the rat, previously described.

4. The deep part of the cortex was similar with regard to AChE in the three areas, comprising a well-stained layer IV, a paler layer V, and a moderately dark layer VI.

5. In the entorhinal area two subfields were discernible. One termed pars medialis has a very distinct stratification of precipitate; in the other, pars lateralis, a different and much less conspicuous stratification is seen. In pars medialis relative absence of enzyme in layer III and abundance in layer IV are the most striking features. The similarity with the rat is considerable.

The parasubiculum consists of a very narrow strip of cortex, smaller than in the rat. Its layers I-III constitute a wedgeshaped field very rich in AChE. The presubiculum at most levels is several times wider than the parasubiculum and much wider than in the rat. Histochemically, it differs from the presubiculum of the rat, particularly by layer III being much poorer in AChE than its surroundings.

Key-Words: Hippocampal region—Guinea pig—Acetylcholinesterase—Stratification.

The present paper is the first in a series intending to present a detailed description of the distribution of acetyl cholinesterase (AChE) in the hippocampal region of the guinea pig. The work was motivated by the observation that chemoarchitectonic patterns in the hippocampal region and elsewhere have proved to correlate significantly with other tissue characteristics such as chemoarchitectonics of other types, cyto- and fibroarchitectonics, and distribution of afferents. The present study is analogous to a previous one on the rat by Storm-Mathisen and Blackstad (1964) in which references to earlier papers on AChE in the hippocampal region are found. A recent study including electron microscopy was carried out by Shute and Lewis (1966). Storm-Mathisen (1970) checked the validity of the histochemical AChE picture with biochemical means.

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In our description we shall not hesitate to go into considerable detail, because it has become clear that even structural minutiae with time regularly become useful as a correlate in further anatomical, biochemical or electrophysiological work.

It has been deemed appropriate to deal first with the multilayered posterior part of the hippocampal region. The subiculum, hippocampus proper and the dentate area will be described in subsequent articles.

Material and Methods

15 young adult guinea pigs of either sex and weighing about 500–900 gm were used for histochemical staining. Most of the animals were subjected to the following procedure. Under combined ether-Nembutal anaesthesia the animals were perfused through the abdominal or thoracic aorta, first for a few minutes with Ringer solution and then for about 30 minutes with 4% formaldehyde (neutralized with $CaCO_3$) at 0–4° C. After perfusion the brain was removed and kept in the same fixative until sectioning, i.e., for $^1/_2$–2 hours. It was then divided in the midline and washed briefly in distilled water. Serial sections of 50 μ thickness were cut on the freezing microtome in the horizontal, frontal and sagittal planes, and in other, special planes. One of these was intermediate between the frontal and the horizontal plane and thus approximately perpendicular to the posterior aspect of the hemisphere and to a major part of the hippocampal region. This plane was chosen in order to get a more normal cross section of parts that are cut obliquely in conventional planes. Of the frozen sections, every fifth was preserved and was subjected to the histochemical procedure for acetyl cholinesterase immediately after sectioning.

Other animals were killed by decapitation under brief ether anaesthesia. The brain was quickly removed and immediately frozen and cut at −12° C in a Dittes-Duspiva cryostat. The section thickness was 40 μ and usually all sections were used. After cutting they were mounted on slides by thawing, dried at room temperature for one to two hours, and then transferred without fixation to the incubating medium. Except for a slight decrease in staining contrast, no differences have been observed between the distribution of AChE activity in the fixed frozen sections and the unfixed cryostat sections. The use of unfixed material lead to a reduction of incubation time of no more than about 25%.

For comparison with the histochemical material, other brains were subjected to silver impregnation or thionin staining. Silver impregnation was carried out on frozen serial sections of 50 μ thickness according to Nauta (1950), and thionin was used on celloidin embedded material, sectioned at 30 and 100 μ. The planes of sectioning were as for the histochemical material.

The Histochemical Procedure. In order to demonstrate acetyl cholinesterase, a modification of the Koelle copper thiocholine method was used, essentially as done by Storm-Mathisen and Blackstad (1964) except for the inhibitor[1].

After cutting, the sections were collected in cold 25% aqueous sodium sulphate and were passed through three further changes of this solution. They were then incubated at 37° C in a medium containing 4 mM acetylthiocholine (AThCh), 2 mM copper sulphate, 10 mM glycine, 50 mM acetate buffer at pH 5 and 0.2 mM ethopropazine[2] (Lysivane, N-diethylaminoethylphenothiazine), a selective inhibitor of unspecific cholinesterase (ChE) recommended by Gordon (1948), Bayliss and Todrick (1956), and Lewis (1961). A detailed consideration of biochemical investigations of incubation media and properties of inhibitors is considered beyond the scope of the present paper. Optimal staining contrast between areas of different enzyme activity was obtained after incubating for 75 minutes (test sections

1 In addition, a single series of fixed, frozen sections was processed essentially according to the copper ferricyanide method of Karnovsky and Roots (1964). This resulted in the same staining pattern as the one obtained with the Koelle copper thiocholine method.

2 Kindly supplied by Pharma Rhodia, A/S Scandia Rhodia, Topstykket, Birkerød, Denmark.

being examined every 15 minutes). Control sections without substrate were always run and were constantly found to be blank.

After incubation, sections were rinsed in 7 changes of distilled water and then treated for one minute with 1.25% sodium sulphide in 0.1 N hydrochloric acid, the final pH being 7.8 (attempts to bring the pH nearer neutrality caused a precipitate to form in the solution). To render the precipitate in the sections darker and more stable, the cupric sulphide generally was converted to silver sulphide by transferring the sections, after 7 further changes of distilled water, to 1% silver nitrate for one minute as suggested by Storm-Mathisen and Blackstad (1964). The sections were dehydrated in ethanol up to 96%, cleared in xylene-creosote-phenol solution and mounted in Dammar resin. After dehydration the sections were often left overnight in terpineol and mounted the next day under a dissecting microscope to avoid folds. Every second section of several series was counterstained with thionin before dehydration.

Results

Consideration of the Staining. The precipitate had a finer grain than observed in the study by Storm-Mathisen and Blackstad (1964), the sections from which were available for comparison. Needle-like crystals were lacking but a distinct granularity could be discerned. In the intricate neuropil it is in general hard to distinguish clearly the nature of the stained irregular structures, whether they are axonal or dendritic. Details will be given where possible. In several places cell bodies stood out clearly as areas rich in precipitate or, conversely, as areas devoid of or very poor in precipitate. On the whole, it is the purpose of this paper to describe the topography of the precipitate caused by AChE as seen with the low powers of the microscope and not the distribution at the cytological level. In part this is motivated by the facts that the freezing causes damage to many structures and that the use of thick sections reduces the visibility of fine detail. A further motivation is that treatment of cytological problems would require a much lengthier description, and also involve cytochemical problems that are better referred to later publications. For the purposes of this study our material was entirely adequate, and since minor defects in the material was no limiting factor, they will not be further discussed at this place.

General Comment on the Pattern of Cortical Staining. Comparison of a number of sections from approximately the same level, taken from one or more animals, reveals certain differences between sections. In single sections staining in a given zone may be either reduced or enhanced. In many cases, the reason for such variations may be seen, being for example, displacement of neuropil by a blood vessel or variations in the thickness of the sections. In other places, the sections may have been covered by folds, have adhered to the glass, etc., with uneven staining as a result. All of these variations, examples of which occur in nearly every section and photomicrograph, are in the present context considered insignificant and superimposed on the true, fundamental pattern. By the study of a large number of sections, we have tried subjectively to sort these phenomena out and will describe the average pattern only. The possibility of transient local variations in enzyme content is not denied, but its demonstration is beyond the scope and possibilities of this study.

No true quantification was attempted in this study, and the amount of AChE-dependent precipitate will merely be described in general subjective terms. However, the photomicrographs provide an impression of the relative staining intensities.

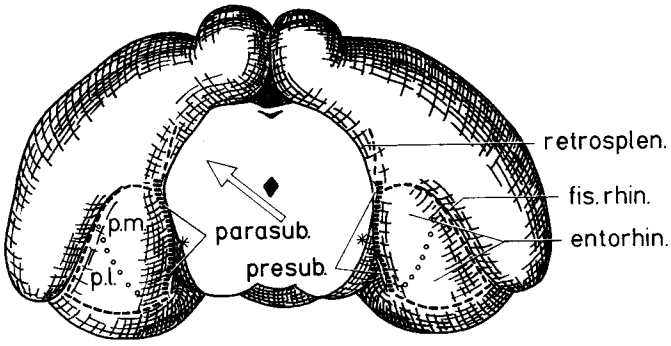


Fig. 1. Posterior aspect of guinea pig brain, transected at the midbrain. Drawing based on photograph. The direction of view is shown with the upper open arrow in Fig. 2 and is approximately as in Fig. 450 of Cajal (1911) reproduced by Blackstad (1956, Fig. 1). The entire *entorhinal area* is visible (delimited by broken line), but its top to bottom extent appears reduced due to foreshortening caused by the slope of the posterior surface of the hemisphere (about 45° with respect to the dorsal surface of the hemisphere). The line of open circles across the entorhinal area indicates the boundary between the medial and lateral subfields of the area. The slender *parasubiculum* (covered with horizontal bars) lies at the medio-caudal edge of the hemisphere. Its upper and lower ends are situated sufficiently far posteriorly to be fully visible whereas the middle portion (at asterisk) lies more on the medial aspect and is partially hidden. The *presubiculum* is largely situated on the medial aspect of the hemisphere (cp. Fig. 2) so that only its uppermost and basal portions are partially visible and appearing much foreshortened. Total removal of the brain stem would have allowed no more than a tangential view of the other parts of the hippocampal region which, together with a part of the retrosplenial cortex, surround the brain stem (cp. Figs. 2 and 3). The open arrow indicates the direction of view in Fig. 2

Abbreviations for all Figures: a.d. area dentata (f.d. and hilus f.d.); *alv.* alveus; *am.* amygdaloid complex; *am.l.* lateral amygdaloid nucleus; *c.a.* anterior commissure; *c.c.* corpus callosum; *coll.ant.* anterior colliculus; *entorhin.* entorhinal area; *f.d.* fascia dentata (*f.d.'*, upper, "septal" end, *f.d."*, temporal end, homologous to limbus Giacomini); *fis.hip.* hippocampal fissure; *fis.rhin.* rhinal fissure; *hip.* hippocampus; *hip., CA1* hippocampus, subfield CA1; *hip., CA3* hippocampus, subfield CA3 (extraventricular aspect); *ne.* neocortex dorsal to entorhinal area, parasubiculum and presubiculum; *parasub.* parasubiculum; *p.c.* cerebral peduncle; *p.l.* pars lateralis of entorhinal area; *p.m.* pars medialis of entorhinal area; *prepyr.* prepyriform cortex; *presub.* presubiculum; *psalt.dors.* psalterium dorsale; *psalt.ventr.* psalterium ventrale (ventral hippocampal commissure); *retrosplen.* retrosplenial cortex; *sub.* subiculum; *t.o.* optic tract; *v.l.* lateral ventricle (unobliterated portions)

The terminology for cortical areas, subfields and layers employed here is the one used by Blackstad (1956) and Storm-Mathisen and Blackstad (1964). Note that "medial" will signify the direction towards the subiculum, "lateral" the direction towards the rhinal fissure, irrespective of the inappropriateness of the terms caused by the sagittal position of some of the areas at certain levels (see arrow labelled "medial" and "lateral" in Fig. 4).

Outlines of the Studied Region. The three cortical areas dealt with here occupy in the guinea pig parts of the postero-medial aspect of the hemisphere (Figs. 1–4), much as in the rat, cat, and rabbit. The reader is referred to the text to Figs. 1–3 for a description of the main topographic features and to Figs. 4–6 for details. The lateral border of the entorhinal area runs very close

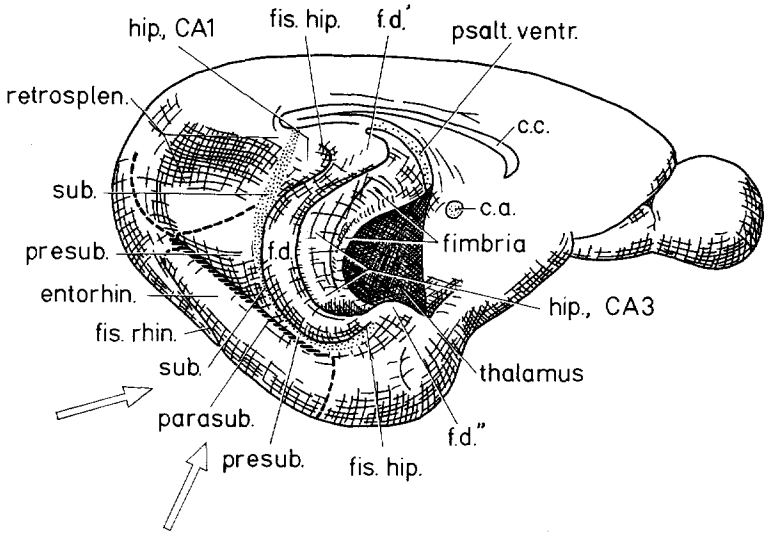


Fig. 2. Mediobasal aspect of left cerebral hemisphere of guinea pig viewed as indicated with open arrow in Fig. 1, i.e., at an angle of 45° with the sagittal and horizontal planes. Drawing based on photograph. The fascia dentata (dentate gyrus) represents a conspicuous landmark which can be followed from the temporal tip below, in a curve towards the upper rostral portion situated behind the septum. The hippocampal fissure delimits the fascia dentata (dentate gyrus) except at the extreme temporal and splenial ends. The posterior bank of the fissure is occupied by the subiculum except dorsally (at *hip., CA1*) where an increasing proportion of the hippocampal subfield CA1 appears between the depth of the fissure and the subiculum. The subiculum forms the anterior boundary of retrosplenial cortex dorsally and of the presubiculum at lower levels. At the extreme temporal end of the hippocampal region, the subiculum is adjacent to the ill-defined cortex at the transition from the entorhinal area to the amygdaloid region. The delimitation of the subiculum here is tentative. The wedgeshaped presubiculum is largely located on the medial aspect of the occipitotemporal portion of the hemisphere (cp. Figs. 1 and 3). The upper side is joined to retrosplenial cortex and reaches farthest dorsally on the subicular side, i.e., anteriorly. The parasubiculum rides on the mediocaudal ridge of the hemisphere (cp. Figs. 1 and 3) and is slender at all dorsobasal levels. In this illustration the entorhinal area is seen nearly tangentially. The upper and lower open arrows indicate the directions of view in Figs. 1 and 3 respectively

to and nearly parallel with the rhinal fissure, which is much deeper and extends farther dorsally in the guinea pig than in the rat. Dorsally, the fissure ceases shortly before it reaches the medial aspect of the hemisphere, at a point about one mm from the side of the posterior colliculus. This point also roughly marks the dorsal level of the entorhinal area and of the parasubiculum which here are succeeded by neocortex. The adjacent presubiculum is replaced dorsally by the retrosplenial cortex. Basally (anteriorly) the three areas under study end approximately at a common level and are succeeded by prepyriform cortex and amygdaloid structures. The medial (anterior) edge of our field of study is represented by the anterior border of the presubiculum (*d*, Figs. 4 and 5), which lies on the medial aspect of the hemisphere, close to the hippocampal fissure. The presubiculum-subiculum border becomes visible on the posterior aspect of the hemisphere only at the most ventral levels.

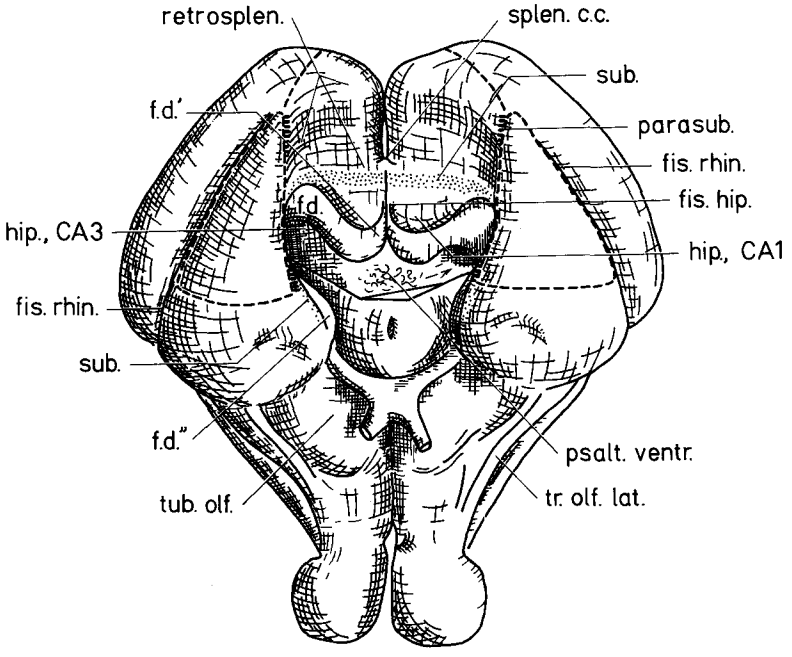


Fig. 3. Basal aspect of guinea pig hemisphere. The direction of view differs 20–25° from the vertical direction as indicated with lower open arrow in Fig. 2. Drawing partly based on photograph. The 50–55° tilting of the brain as compared to Fig. 1 exposes more of the base of the brain to view than visible in Fig. 1, including the amygdaloid and prepyriform regions, whereas the dorsolateral parts of the hemispheres are less visible. Despite the removal of the brain stem caudal to the mammillary level, the presubiculum remains nearly completely hidden because of its largely sagittal orientation. The parasubiculum is partially visible as in Fig. 1. Retrosplenial cortex and subicular and hippocampal structures in front of it under the corpus callosum are seen

The Entorhinal Area. It is characteristic of this and of the other areas that the staining is not diffuse, but occurs with a laminar pattern, typical of each area and ceasing abruptly at the borders of the area. Therefore, it seems appropriate to describe first the layers and thereafter the transitions to the neighbouring areas.

Within the entorhinal area two different portions, each with a specific pattern, are recognizable, one constituting the major, dorsomedial part of the area and here called pars medialis (*p.m.*, Figs. 1 and 5d–i), and another the ventro-lateral part of the area and here called pars lateralis (*p.l.*, Figs.1 and 5f–i). Pars medialis will be described first.

In all planes of sectioning pars medialis is a very conspicuous part of the cerebral cortex, first and foremost because of a thick zone poor in precipitate (*II* and *III*, Fig. 4; *15*, Fig. 6b, c, g), which contrasts with its darker surroundings. Comparison with thionin preparations shows that the major inner part of it is congruous with layer III of Lorente de Nó (1933), i.e., the layer of pyramidal cells, his layer IIIa, and the cell-poor zone beneath it, his layer IIIb (identical

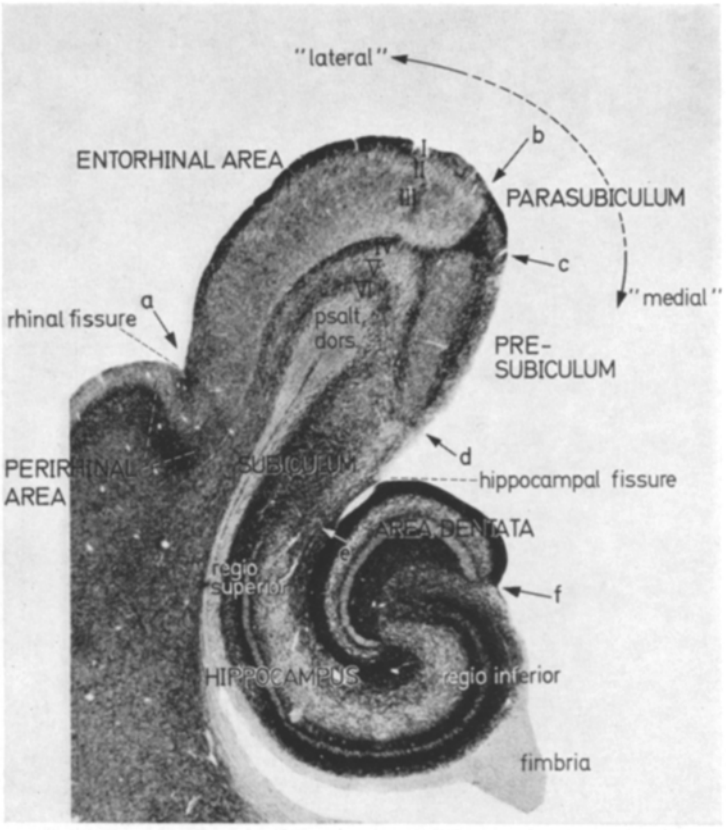


Fig. 4. Photomicrograph of horizontal 50 μ section through cerebral hemisphere of adult guinea pig showing the general interrelations of areas constituting the hippocampal region. Arrows labelled *a* to *f* indicate interareal boundaries. Curved double-headed arrow labelled "lateral" and "medial" symbolize the definition of the terms lateral and medial as used for the purposes of the present paper. Section incubated for AChE, inhibited with ethopropazine, and darkened with sodium sulphide and silver nitrate. ×18

with layer IV of Cajal, 1901, 1911). The latter contains the basal dendrites of the lowermost pyramids and corresponds to lamina dissecans of M. Rose and other authors. The staining of the outer third to half of layer III (*1*, Fig. 5f, g, i) is slightly less pale than the rest of the layer (*2*, Fig. 5f, g, i), displaying a reciprocity with the cell bodies in so far as these are more numerous in the inner half of IIIa than in the outer half (the ratio being approximately 3:2). Very few cells in layer III have well-stained cell bodies; the precipitate occurs in the form of diffuse strands and accumulations between the cell bodies. There is no sharp limit between outer, less pale and inner, more pale parts of layer IIIa. Layer IIIb of Lorente de Nó is the palest portion and there is possibly an identifiable degree of abruptness in the transition from IIIa (pale) to IIIb (very pale). Sagittal sections show clearly that layer II and particularly layer III (a and b) are much thicker dorsolaterally than at middle and basal levels of the

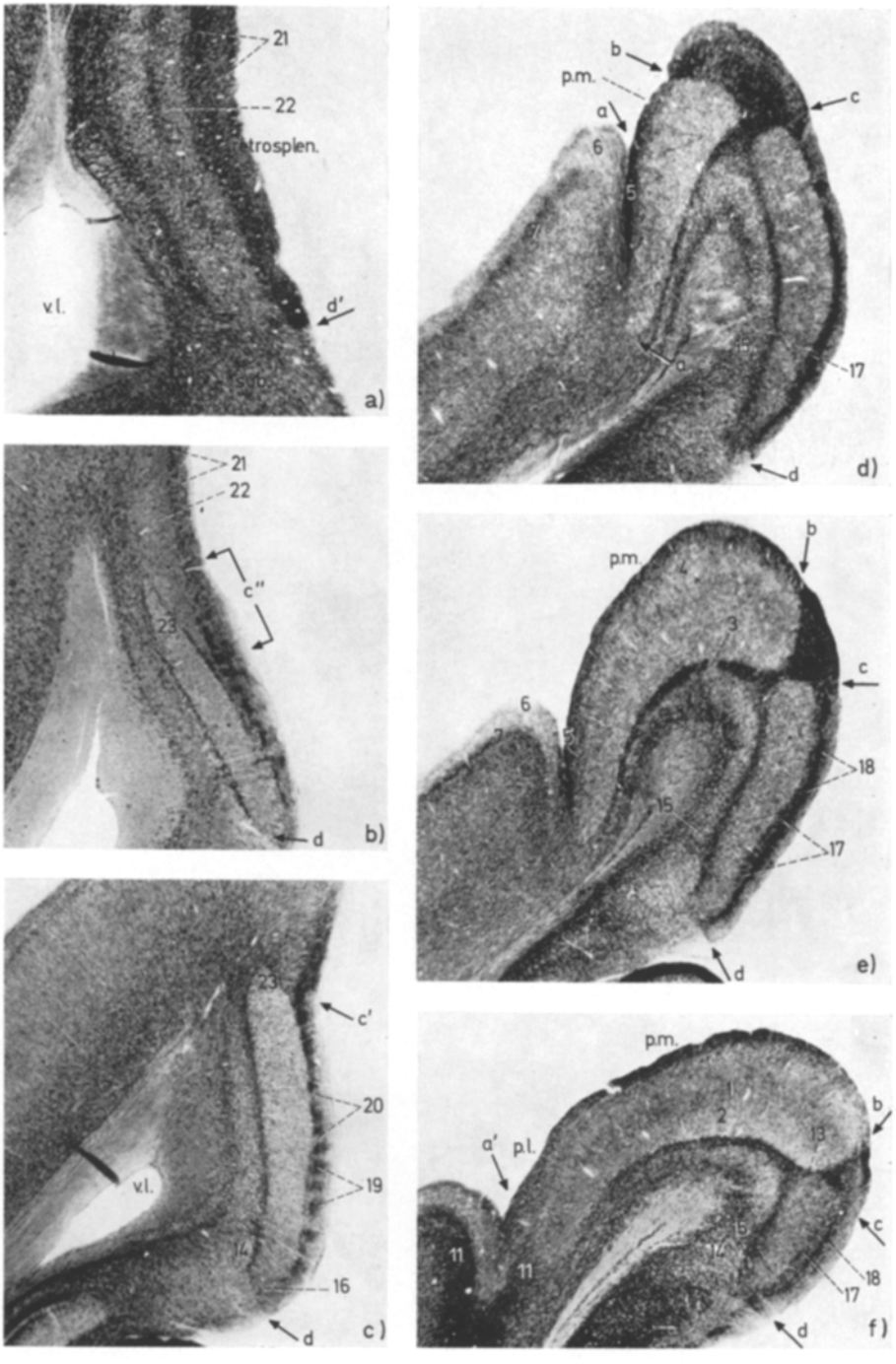


Fig. 5 a–f (Leg. see p. 469)

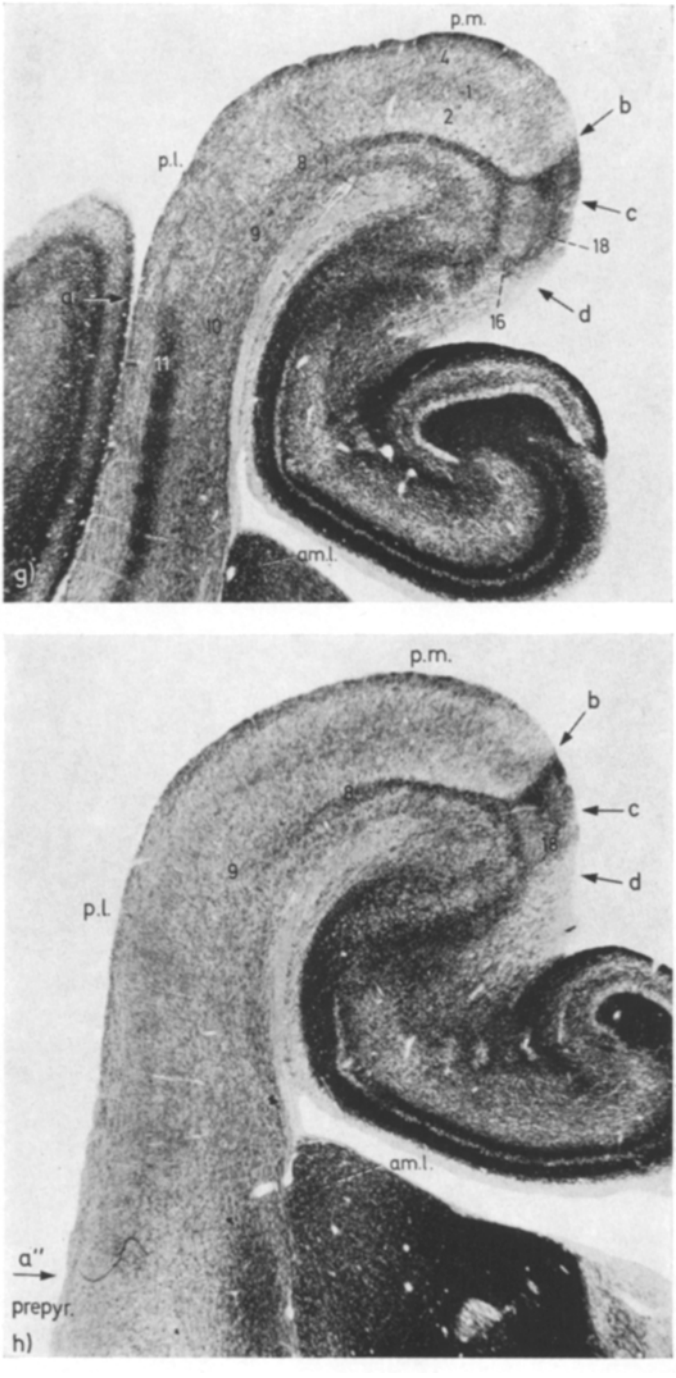


Fig. 5g and h (Leg. see p. 469)

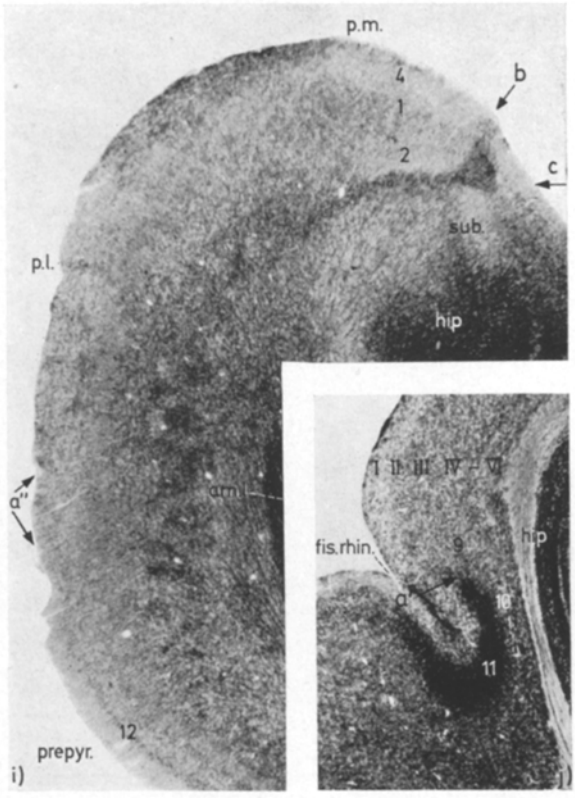


Fig. 5i and j

Fig. 5a–j. Photomicrographs of 40 μ horizontal sections selected from three different animals and showing a sequence of dorsobasal levels. The sections were incubated for AChE, inhibited with ethopropazine and darkened with sodium sulphide and silver nitrate. Lettered arrows indicate the boundaries between the following areas: *a* neocortex (area 36?) and entorhinal area (pars medialis, *p.m.*); *a'*, perirhinal area and entorhinal area (pars lateralis, *p.l.*); *a''*, prepyriform cortex and entorhinal area (pars lateralis, *p.l.*); *b*, entorhinal area (pars medialis, *p.m.*) and parasubiculum; *c* parasubiculum and presubiculum (subiculum in Fig. 5i); *c'*, retrosplenial cortex and presubiculum; *d*, presubiculum and subiculum; *d'*, retrosplenial cortex and subiculum. Top of pictures represents caudal direction. a Level dorsal to presubiculum; subiculum present along retrosplenial cortex. b Dorsalmost level of presubiculum. c Section through most inferior posterior corner of retrosplenial cortex. d Section through upper expanded end of parasubiculum and upper part of rhinal fissure. The detail of the relief varies between the animals. A slight bulging of the surface corresponding to the parasubiculum is common at this and lower levels. e Middle level, slightly above that of Fig. 4. f Uppermost end of pars lateralis and of the perirhinal area; level slightly below that of Fig. 4 as visible inter alia from the reduced width of the presubiculum. g Longitudinal section through perirhinal area and rhinal fissure, a heel of neocortex being included (left). h Section immediately basal to the horizontal course of the rhinal fissure; lower end of presubiculum visible. i Level below the presubiculum. The hippocampal fissure (which is more shallow in h than at higher levels) is now absent. j Section approximately perpendicular to the posterior aspect of the hemisphere (i.e., intermediate between frontal and horizontal to better show the rhinal fissure and the transition from entorhinal area (pars lateralis, *p.l.*) to perirhinal cortex. a–j ×18

entorhinal area (Fig. 6d, e). The relative thickness of the zones of layer III presenting different shades of staining as just mentioned, is the same here as at more basal levels, but the overall density of precipitate is lower.

The deep limit of layer III is absolutely sharp and represents the superficial border of a distinct, narrow zone (*IV*, Fig. 4; *16*, Fig. 6b; *21*, Fig. 7c; *22*, Fig. 7d) with a high density of precipitate. Counterstained sections show that it contains many horizontally oriented cells as characteristic of layer IV of Lorente de Nó (1933). Also the inner limit of layer IV is sharp. In occasional horizontal sections pale gaps are seen (*3*, Fig. 5e) in layer IV. They are few and often incomplete, and rather than indicating a fragmentation of layer IV into islands would suggest a moderate degree of fenestration of the layer. Perusal of sagittal series showed no true insulation or fenestration, but a moderate unevenness of staining at some places (Fig. 6c, d) mainly due to clumping of unstained cell bodies in the otherwise wellstained neuropil. Sections with, as well as without, thionin counterstaining clearly show that at least the major proportion of the precipitate lies in the interstices between the cell bodies, the vast majority of which are unstained. A few distinctly AChE-positive cells occur, the ratio of positive to negative cells possibly being of the order of 1:100. AChE-positive cells are not more numerous in layer IV than in the neighbouring layers.

Below the dark layer IV come, first a zone poor in precipitate and then one bordering on the white matter with a low to medium density of precipitate. These zones have been labelled layers V and VI respectively (Fig. 4), although criteria for exact definition of the borders of layers V and VI are not available in our material or from other sources.

Superficial to layer III is layer II, with conspicuous large stellate cells (*II*, Fig. 4; *4*, Fig. 5e, g, i). It is very poor in AChE and a little paler than the outer part of layer III. The large stellate cells themselves are definitely unstained, and the neuropil contains no more precipitate than in layer III.

The staining intensity of layer I is medium to heavy superficially, and greater at dorsal than at basal levels (Fig. 6b, e), but decreasing gradually toward layer II. The inner part of layer I contains nearly as little precipitate as layer III. It is hardly possible to recognize two distinct sublayers within layer I. In some sections from certain animals, the predominance of AChE in the superficial part of layer I is more pronounced than usual, but such variations are without recognizable topographical consistency, and the pattern just described in layer I is, as a rule, present at all dorsobasal levels and from the medial to the lateral limit of pars medialis.

The above description of the general pattern of enzyme distribution in layers I-VI of pars medialis has to be supplemented with an account of the changes that the layers undergo on the transition to non-entorhinal cortex and to pars lateralis.

Medially, the entorhinal area is adjacent to the parasubiculum (at *b*, Figs. 4 and 5). The interareal border is absolutely sharp at the level of layers II and III, where enzyme-rich tissue in the parasubiculum (*19*, Fig. 6b) contrasts with the enzyme-poor entorhinal tissue (*15*, *18*, *20*, Fig. 6b). Further details are included in the description of the parasubiculum. None of the hippocampal layers as seen in AChE preparations change their appearance significantly on approaching the

parasubiculum. Layer I tends to become somewhat paler. The medial border of the entorhinal area is distinct in the described manner at all dorsobasal levels, including the ventralmost levels where only the last remnant of pars medialis is seen in horizontal sections (Fig. 5i).

The *dorsal* end of the entorhinal area (which is occupied by pars medialis alone; Fig. 5d, e) abuts on cerebral cortex (isocortex) with fairly evenly distributed AChE activity (*ne.*, Fig. 6a–c, e); sagittal sections show particularly well the extremely sharp upper limit of layers II and III (*3*, Fig. 6b–g). The degree of staining of the neocortical tissue above this limit (Figs. 5b, c; 6c–g) is approximately as in the entorhinal layers IV and VI and no clear-cut border between these two layers and the adjacent neocortex can be seen therefore. The light staining of layer V of the entorhinal area ceases fairly suddenly at the upper end of area 28, exactly at the same level as layers III-II (*3*, Fig. 6c). In the dorsolateral part of area 28 (still belonging to pars medialis) the paleness characteristic of layer V cannot be traced so far dorsally in sagittal sections, but ceases a few hundred microns more basally. However, these features are difficult to evaluate due to the oblique plane of sectioning with regard to this point. Before layer V ceases dorsally, there may be a tendency to appearance of indistinct darker "bridges" passing through the layer from IV to VI and splitting it incompletely into rounded or elongated, pale areas (vaguely suggested in Fig. 6c).

The *lateral* border of pars medialis (Fig. 1; arrow *a*, Figs. 4 and 5d, e) constitutes the dorsal part of the lateral border of the entorhinal area. It is similar to the dorsal border inasmuch as layer III stops absolutely abruptly just medial to the bottom of the rhinal fissure (Figs. 5d, e; 6f, g). The light layer V is also replaced by darker tissue. The darkness characteristic of the superficial part of layer I of the entorhinal area (*5*, Fig. 5d, e) stops in the cleft of the rhinal fissure; layer I of the adjacent cortex (*6*, Fig. 5d, e) is very pale (except immediately above layer II (*7*, Fig. 5d, e). These criteria of transition from entorhinal to non-entorhinal cortex give a more precise delimitation than the cruder criterion represented by the rhinal fissure. More ventrally (Fig. 5f–i), pars lateralis takes over the role of carrying the lateral edge of the entorhinal area. See below for a description of this part of the border.

Transition from Pars Medialis to Pars Lateralis. The difference between pars lateralis of the entorhinal area (*p. l.*, Fig. 5f–i) and pars medialis is considerable. On passing laterally from pars medialis, the distinct stratification of the latter becomes lost, the most striking feature being that layer III turns darker, first superficially and last close to layer IV. Layer II undergoes little change with regard to degree of staining. Layer IV becomes paler and thicker (*8*, Fig. 5g–i) before becoming undiscernible in the darkening surroundings. Similarly, the level of layer V darkens and reaches the common level of staining (*9*, Fig. 5g, h) and is indistinguishable in pars lateralis.

The lateral border of pars lateralis constitutes a significant ventral part of the lateral border of the entorhinal area (Fig. 1). Here, layers III-VI of the entorhinal area (*9*, Fig. 5g, j) are diffusely stained, with a medium intensity similar to that of the deeper part of the adjacent cortex (*10*, Fig. 5g, j). Therefore no sharp limit is seen at the lateral border of the entorhinal area at this laminar level. By contrast, at the level of layer II a zone of much greater

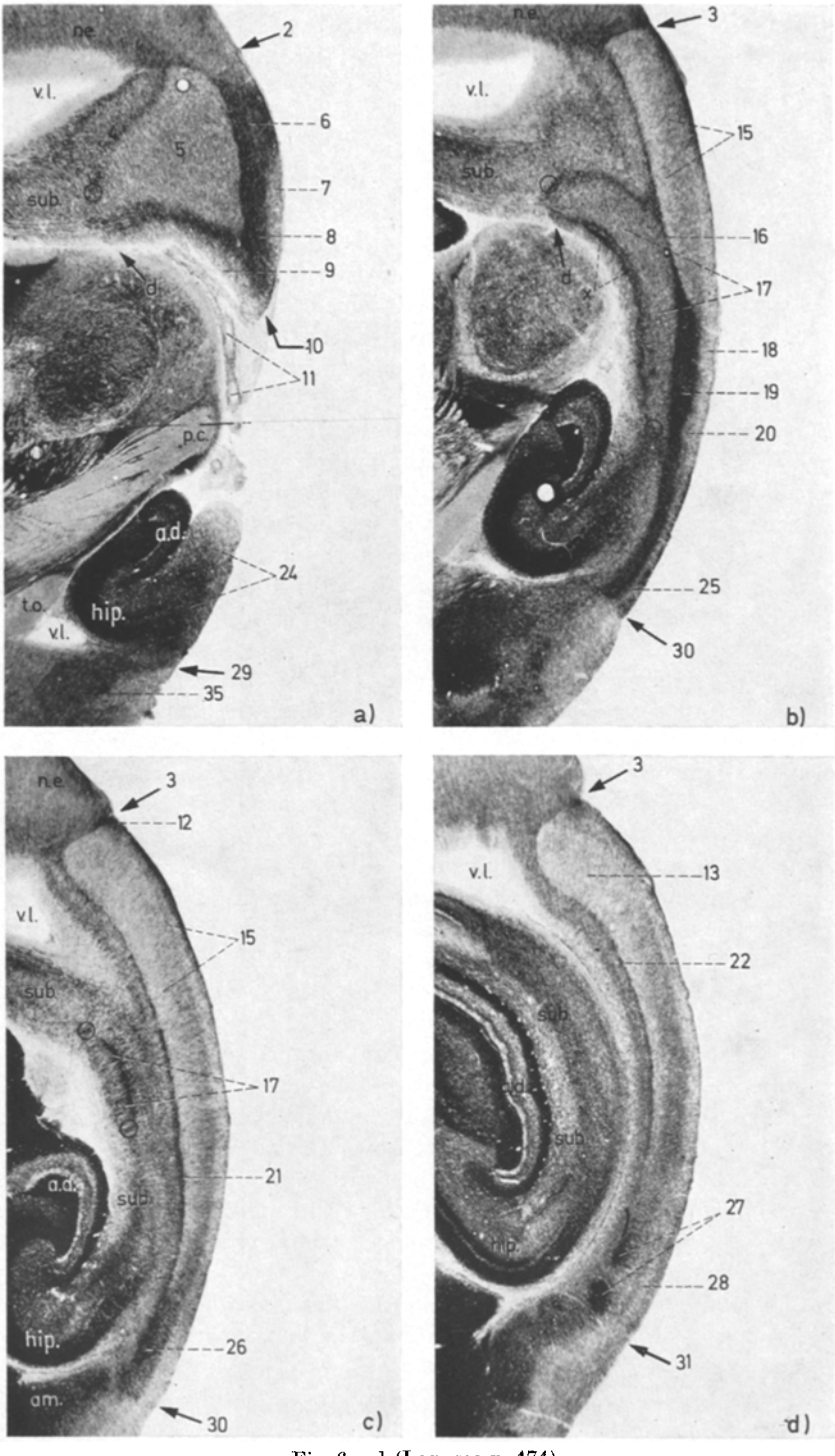
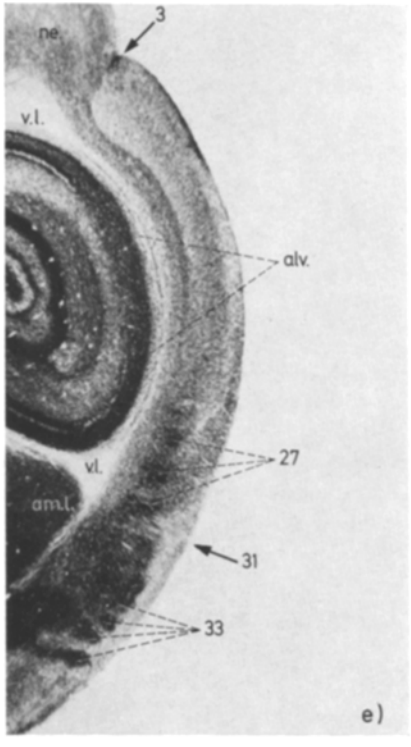
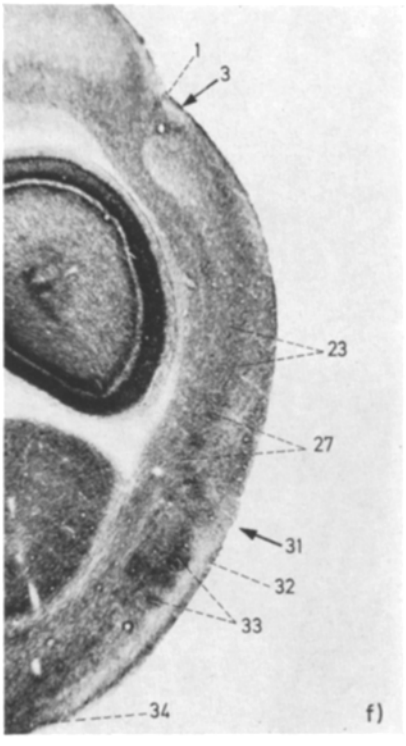


Fig. 6a–d (Leg. see p. 474)





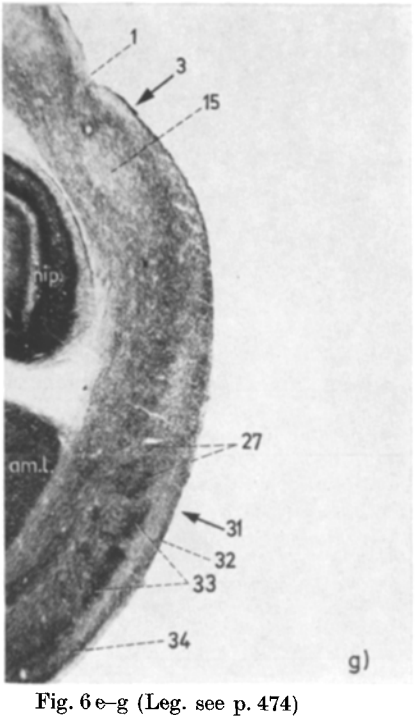


Fig. 6 e–g (Leg. see p. 474)

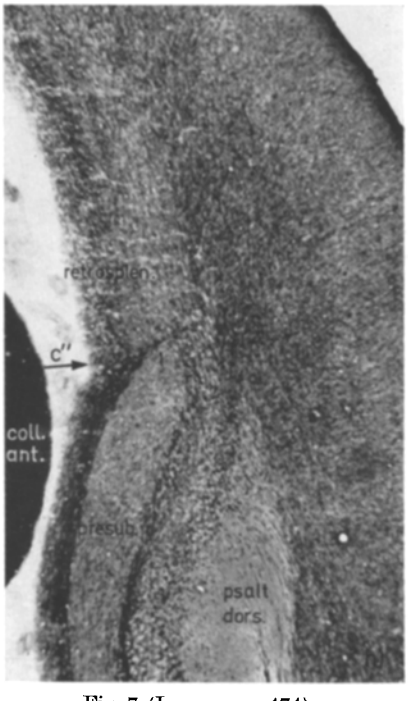


Fig. 7 (Leg. see p. 474)

enzyme activity appears, which at middle dorsobasal levels of the pars lateralis extends U-shaped around the rhinal fissure (*11*, Fig. 5j). Toward the upper end of pars lateralis it lies mostly on the nonentorhinal side of the fissure, and at the upper end of pars lateralis exclusively so. Conversely, close to the basal end of pars lateralis, the cortex containing this dark layer is found exclusively on the entorhinal bank of the rhinal fissure.

The AChE-rich cortex just described has the position shown by Brodmann (1911, Fig. 107) as characteristic of his perirhinal area, area 35, in a lower mammal, the rabbit. Therefore, it is concluded here that the zone just described belongs to the perirhinal area and that the arrow *a'* in Figs. 5f, g, and j separates area 28 (pars lateralis) and area 35. In thionin sections the transition between pars lateralis of the entorhinal area and the perirhinal area is sharp in all layers, but AChE preparations display a distinct change only at the level of the U-shaped dark tissue. Superficial to the latter, layer I can be traced essentially unchanged from area 28 to 35. The light layer II of area 28 abuts on the U. The tissue beneath the U is continuous with layers IV–VI of area 28 and is stained like these. In AChE preparations layer III of the entorhinal area also is similarly stained, but comparison with thionin preparations shows that layer III tapers and ceases exactly at the boundary to area perirhinalis (*a'*, Fig. 5g, j).

The *basal* end of the entorhinal area is in contact with the amygdaloid region medially and the prepyriform area laterally. It is modified and characterized by the appearance of rounded tissue areas below layer II somewhat richer in acetyl cholinesterase than layer II (*27*, Fig. 6e–g). This pattern contrasts with that of the adjacent prepyriform cortex in which rounded darker patches also occur (*33*, Fig. 6e–g), but in layer II. Therefore, the delimitation of entorhinal from prepyriform cortex can be done with fair accuracy in frontal and sagittal sections, although the AChE picture of the two areas is not strikingly different where they meet (*31*, Fig. 6d–g). Horizontal sections are not suitable for an evaluation of these features since deeper layers seen in the sections belong to more basal levels than layer I in the same sections (Fig. 5h, i). In layer I the different appearance of prepyriform cortex and pars lateralis allows the recognition of the

Fig. 6a–g. Photomicrographs of sagittal sections, 40 μ thick and separated by the following intervals (exclusive of the thickness of the sections shown here): a and b 560 μ; b and c 560 μ; c and d 720 μ; d and e 640 μ; e and f 440 μ; f and g 220 μ. a is the most medial, g the most lateral section. *The entorhinal area* is present in all figures except in a, its upper limit being at *3*; its light layer III of pars medialis *15*; layer IV *21* and *22*; and its lower limit at *31*. *The parasubiculum* is included in a, b and c; *6*, *8* and *19* being the wider part of the enzyme-rich layer II-III; *16* being the narrow stalk-like part of the latter (*13* of Fig. 5f), and *30* representing the lower limit of the parasubiculum towards the amygdaloid region. *The presubiculum* is present in a, b and c; *5* and *1* being its wide, enzyme-poor layer III, *4* its layer IV, and *9* layer I. The limit between the presubiculum and the subiculum is seen at *10*. For explanation of other symbols, see text to Fig. 1. a and g are montages of neighbouring sections. a–g ×9

Fig. 7. Photomicrograph of section approximately parallel to the posterior aspect of the hemisphere and accordingly intermediate between frontal and horizontal. This plane of sectioning is roughly perpendicular to the boundary between the presubiculum and the retrosplenial cortex, shown here. Cp. Figs. 2 and 5b. ×24

boundary between these areas also in horizontal sections. The conspicuous tangential, AChE-containing fibres in the middle of layer I of the prepyriform cortex (*12*, Fig. 5i; *34*, Fig. 6f, g) dwindle towards the entorhinal area (*32*, Fig. 6f, g) within which the staining of layer I is more even. The modified entorhinal cortex just described belongs at least largely to pars lateralis, forming its basal wider end. Whether the medialmost part of the modified entorhinal cortex (*27*, *28*, Fig. 6d) constitutes a ventral narrow tip of pars medialis or represents a strip of pars lateralis separating the lower tip of pars medialis from the amygdaloid region has not been settled. The zone of transition from the modified entorhinal cortex to the amygdaloid region (*31*, Fig. 6d) is narrow.

The Parasubiculum. This term is applied to an area, wedgeshaped in cross section, very rich in AChE and placed medial to the entorhinal area (Fig. 4). The superficial part of it is triangular and consists of a molecular layer (layer I), resting on a deeper zone, here called layer II. The deep corner of the triangle continues, as it were, as a short stem, or stalk (*13*, Fig. 5f), which soon reaches the narrow, enzyme-rich stratum that runs uninterrupted from the entorhinal area, there being its layer IV (*IV*, Fig. 4), through the parasubiculum and into the presubiculum (asterisk, Fig. 4). Accordingly, it will be called layer IV also in the latter two areas. The stalk-like structure has its maximal length at dorsal and middle levels; at the ventral end of the hippocampal region it is nearly absent, the triangle proper reaching layer IV (Fig. 5h). It may be called layer III of the parasubiculum as it separates layer III of the entorhinal area from layer III of the presubiculum. Layers V and VI of the entorhinal area, like layer IV, seem to continue unchanged through the parasubiculum and need no further description. Further details may, however, be given concerning the superficial portion of the parasubiculum. Along most of the dorsobasal extent of the parasubiculum the I-II triangle displays a characteristic pattern, best seen in sections perpendicular to the long axis of the parasubiculum. The outer part of layer I is more heavily stained than the inner part and also more than the molecular layer of the adjacent entorhinal and presubicular cortex (Figs. 4, 5d–h, 6a). However, a mediolateral difference exists in layer I: Close to area 28, nearly the whole depth of layer I displays heavy staining, but toward the presubiculum the dark zone tapers, moves into a subpial position and may virtually disappear (Figs. 4 and 5f, h). These details like many others are obscured in occasional sections.

Layer II is darker than the inner part of layer I but not so dark as the outer part. The deeper portions of layer II are the darkest ones.

At the basal tip of the parasubiculum the I-II triangle is paler and the described division into three zones of different staining intensity less accentuated. On the other hand, the central part of layer II is paler than its surroundings, except at the side facing the presubiculum. Altogether the parasubiculum is at basal levels darker towards the entorhinal area, lighter toward the presubiculum. The heavy staining of layer I-II is conspicuous down to the lower tip of the parasubiculum where it ceases suddenly (at *30*, Fig. 6b, c) and is succeeded by the medial part of the amygdaloid region.

Toward the dorsalmost end of the parasubiculum, the enzyme activity increases somewhat, and the tripartition of the I-II triangle is indistinct. The parasubiculum, defined as the dark substance of layer I-II, continues a few hundred microns

beyond the level of the upper border of the entorhinal area but not quite as far as the upper end of the presubiculum. It merges with the neocortex immediately dorsal to the entorhinal area (Fig. 5d, c). This tissue is much darker than layer III of the entorhinal area, but less dark than layers I-II of the parasubiculum. The staining fades gradually, and no absolutely sharp dorsal and dorsolateral limit of the parasubiculum can be defined using the AChE pattern as a criterion.

The size of the parasubiculum is greatest at the dorsal end. The smaller cross section seen at middle and low levels remains fairly constant down to the inferior end.

The Presubiculum. A characteristic laminar pattern is found in the presubiculum and is present essentially unchanged at all dorsobasal levels, merely quantitative variations being seen (Fig. 5a–i). The pattern ceases abruptly toward the neighbouring retrosplenial cortex, parasubiculum and subiculum, all of which have an entirely different histochemical stratification.

In the wide part of the area seen at middle dorsobasal levels the pattern is as follows. The innermost layers are, as mentioned, similar to and continuous with those of the entorhinal area and the parasubiculum (*VI-IV*, Fig. 4) and are accordingly labelled layers VI, V, and IV from within. Layer VI, adjacent to the white matter, has a medium degree of staining, layer V is quite pale, and layer IV (asterisk, Fig. 4) is very well stained. However, the latter is not so dark as in the entorhinal area, and its borders toward layers V and III are a little blurred. Layer V widens toward the subiculum (*14*, Fig. 5c, f) and one might well classify this tissue as subicular substance wedged into the presubicular cortex. A "bridge" of moderately active tissue (*15*, Fig. 5d–f) separates this region from the rest of the presubicular layer V. This bridge is increasingly difficult to identify at higher and lower levels. The reader is referred to the next paper in this series for further discussion of these details.

Superficial to layer IV comes a deep, very pale zone here called layer III.

Apart from the peculiarities just described for layer V, layers VI to III show no change of appearance along the distance from the parasubiculum to the subiculum, with one exception: an increased darkness in layer III at the border to the subiculum (*16*, Fig. 5c, g; ○ Fig. 6a–c).

The picture is more complex at the transition from layer III to the cell-poor molecular layer (layer I). Here a thin zone of cells which on the whole are a little smaller than those of layer III is recognizable in some of the thionin stained sections. Often it is, however, impossible to define a distinct inner limit for this zone. In the AChE preparations, a thin zone relatively poor in precipitate is seen at this level (*17*, Fig. 5d–f; *x*, Fig. 6b) and is provisionally labelled layer II. It is delimited inwards by a zone rich in enzyme and either equally thin or a little thicker. Superficial to layer II there is an intensely stained zone (*18*, Fig. 5e–g) occupying approximately the inner half of layer I. The more superficial portion of the layer is paler.

The abundant precipitate above and below layer II is not sharply delimited from it, often invading the layer from one or both sides and rendering it indistinct. On the other hand, the inner, thin dark zone is hardly distinguishable in many sections, particularly at basal levels.

One important supplement to the description of layer II and the adjoining dark zones must be given, viz., that the latter have interruptions (*20*, Fig. 5c) of varying length, producing a sequence of stained fragments or islands (*19*, Fig. 5b, c) themselves of variable length. When the small cells of layer II are seen to be grouped into islands, the precipitate is largely associated with these, surrounding and partially invading each island; in particular, abundant precipitate is assembled on the superficial side of each island (*19*, Fig. 5c). The islands are seen not only in horizontal sections, which might have been compatible with the idea that they represent vertical, longitudinal zones or columns, but also in frontal sections. Sections tangential to layer II confirm that islands, of varying size and shape, are really present. However, they touch extensively and in part fuse, creating a somewhat reticulated appearance of the stained levels of layers I-II. The AChE preparations do not clearly show fibre bundles in the gaps between the islands. The insularity is most clearly seen in the dorsal larger part of the presubiculum and is regularly much less pronounced, or absent, in the basal end of the area. As the thin inner dark zone at the II/III border is also sparse or lacking basally, the presubiculum accordingly is simpler here than dorsally: one medium dark zone corresponding to the inner part of layer I runs from the parasubiculum to the subiculum (*18*, Fig. 5h).

Delimitation from Neighbouring Areas. Against the parasubiculum the delimitation is very clear-cut: layer III in the presubiculum abuts on the dark layers II-III of the parasubiculum. The transition between the dark, most superficial part of the parasubicular layer I and the paler, superficial part of layer I of the presubiculum is fairly abrupt (*c*, Figs.4 and 5d–h).

Dorsomedially, the presubiculum is replaced by retrosplenial cortex (Figs. 2, 3), which has a quite different pattern of stratification in AChE preparations. Both deep and superficial levels of layer I are dark in retrosplenial cortex (*21*, Fig. 5a, b) in contrast to layer I of the presubiculum of which the deep portion only is dark. The darkness of layer I extends into layer II in the presubiculum but not in the retrosplenial cortex (*22*, Fig. 5a, b). On the whole, the stratification seen at the deeper levels of the presubiculum (layers VI-IV) is absent in the retrosplenial cortex.

The transition between the presubiculum and the retrosplenial cortex is sharp although not forming a straight line extending from pia to white matter; e.g., layer III of the presubiculum continues a little in under the corresponding layer of the retrosplenial cortex (at *23*, Fig. 5b, c; *1*, Fig. 7). The presubiculum-retrosplenial boundary is not far from horizontal (Fig. 2) and is located at a part of the hemisphere where the surface has a nearly sagittal orientation (Figs. 1, 3). The study of this boundary by means of horizontal or sagittal sections is, therefore, not convenient. Frontal sections are more suitable, and a plane intermediate between frontal and horizontal, parallel to the posterior aspect of the hemispheres (Fig. 7) demonstrates the sharpness of the boundary particularly well.

The subiculum, which forms the anterior border of the presubiculum (*d*, Fig. 5b–h), reaches farther dorsally than it (Figs. 2, 5a) and therefore forms the anterior border of some retrosplenial cortex as well (Fig. 2; *d'*, Fig. 5a). Retrosplenial cortex has an equally sharp transition to the subiculum (*d'*, Fig. 5a) as the presubiculum, but should of course not be mistaken for the latter on this basis.

Discussion

This study was confined to a topographic mapping of the distribution of AChE and provided no data immediately shedding light on the functional role of acetyl cholinesterase or acetyl choline. A discussion of such problems is, therefore, omitted here. It is encouraging to note, however, that a close correspondence has been found between histochemical and biochemical determinations of the relative content of AChE in the layers of the hippocampal region (Storm-Mathisen, 1970). The degree of staining of the various layers of incubated sections was recorded photometrically, and AChE content of layers dissected from freeze-dried sections was determined by biochemical radio- and fluorometric methods.

In the following paragraphs, the distribution of AChE in the guinea pig will be discussed with particular emphasis on the relation to the distribution in the rat (Storm-Mathisen and Blackstad, 1964).

The entorhinal area could easily be identified in our sections because it is very characteristic in thionin and other stains. Moreover, its identification in AChE preparations of the guinea pig was easy on the basis of similar preparations in the rat because the most conspicuous histochemical features (primarily present in pars medialis) are shared by the two species. The light layer III and the darker layer IV of pars medialis are recognized in both species. There is in both a paler staining below than within layer IV, and also a darker staining in layers I and II than in layer III.

Closer inspection reveals more details and certain differences between the two species. Whereas layer I was slightly paler than layer II in the rat, it was definitely darker than layer II in the guinea pig (particularly in its outer part). However, it is important that in both species the large stellate cells of layer II were seen to be negative. The appearance of layer I-II in the rat and the guinea pig could well be explained by a quantitative rather than a qualitative species difference, certain AChE-positive dendritic or axonal structures being more abundant or containing more enzyme in the guinea pig than in the rat. It seems likely that the high enzyme activity in the outer part of layer I is associated with axons rather than dendrites, in view of the low number of AChE-rich cells in layers I-III. No conjecture is permissible concerning the nature of such axons except that a contralateral origin is improbable because stained fibres are nearly completely absent from the cortical commissures. A description of the termination of commissural fibres in the guinea pig hippocampal region is as yet not available.

Layer III of pars medialis is essentially identical in the rat and the guinea pig. In both, the vast majority of cells, including the pyramidal cells, are without a demonstrable content of AChE. The same is true about the majority of dendrites and axons. In both species it may safely be stated that the dense plexus of afferents illustrated in layer III both by Cajal and Lorente de Nó cannot be associated with acetyl cholinesterase or belong to a cholinergic tract.

The perforant path (the entorhino-hippocampal and -dentate pathway) has been shown to degenerate after lesions of pars medialis and pars lateralis of the entorhinal area (Nafstad, 1967, and references therein). The absence of AChE from the preterminal fibre bundles and the terminal area of this tract is not surprising in view of the paucity of cells rich in AChE in the entorhinal area. AChE pre-

parations accordingly yield no information on the origin of the perforant path and do not permit to exclude any entorhinal layer from being a source of the tract.

Layer IV is conspicuously positive when compared with the adjacent layers, and it would be of much interest to know what structures the acetyl cholinesterase is associated with. Since the "deep pyramids" according to Lorente de Nó (1933) predominate numerically in this layer, followed by the horizontal (fusiform, spindleshaped) cells, and since the vast majority of cells in layer IV are AChE-negative, it can safely be stated that the precipitate is not associated with the cell bodies of these two types of cell. Moreover, both have abundant dendrites ramified in this layer and it is unlikely that these are responsible for the cholinesterase. The same holds true for the axons of the horizontal cells which have been observed to ramify within the confines of the layer (Lorente de Nó, 1933). The AChE-rich cell bodies seen in layer IV are so few that their dendrites or axons also seem an improbable source of the great amount of enzyme in the layer. Neither Cajal nor Lorente de Nó described abundantly ramified dendrites in layer IV belonging to perikarya in other layers. Therefore, it seems the most plausible hypothesis that the acetyl cholinesterase is associated with extrinsic axons ramified in layer IV, being located either presynaptically in the axons and their terminals or postsynaptically. Such axons could be identical with the cingulum fibres described in silver preparations approximately at this level by White (1959). It is also possible that the observed enzyme activity could correspond to a different contingent of fibres terminating within layer IV, seen in silver preparations after lesions in the CA 3 part of the hippocampus (Hjorth-Simonsen, 1971). The abundance of AChE in the neuropil between the cell bodies suggests that any AChE containing axonal plexus would make contacts not only with somata but also, or exclusively, with dendrites. Whether one or both major types of cell in layer IV are in case impinged on by AChE containing terminals has to be decided in future studies with better techniques. Activation of afferents to layer IV would presumably lead to specific local effects that could be recorded electrophysiologically. It would seem worth while to test experimentally whether such effects, if present, depend on acetyl choline as a transmitter or not. It is interesting that the structural specificity of layer IV was seen by Lorente de Nó to be present from the mouse to the monkey. Mellgren and Blackstad (1967) observed that the layer differed markedly from the neighbouring layers with regard to content of dehydrogenases.

Layers V-VI were too uniformly stained in the rat to allow a subdivision into distinct strata. Still, a tendency to somewhat higher enzyme activity was observable at deeper levels than subjacent to layer IV (see, e.g., Figs. 4 and 5 of Storm-Mathisen and Blackstad, 1964). A steeper gradient of staining is seen in the guinea pig in layers V-VI but it has the same direction; thus the concordance between the two species is considerable.

By a comparison of *pars lateralis* in the two species under consideration, a loss of the conspicuous laminated pattern of pars medialis is seen in both but to a slightly different degree. In the rat, layer IV is sufficiently well stained in pars lateralis to contrast with the other layers, particularly III and V, which themselves are slightly paler than I-II and VI. In the guinea pig, layer IV is definitely paler in pars lateralis than in pars medialis, and differs little from the

other layers, so that a consistent pattern of stratification is difficult to define. These quantitative differences between the rat and the guinea pig are rather subtle and pars lateralis seems to be fundamentally identical in the two species, as far as distribution of AChE is concerned.

The Parasubiculum and the Presubiculum. The structure designated the *parasubiculum* in this paper differs so much from the region given the same name in the rat that the homology between the two could be doubted. It is so peculiar with regard to its shape that one might even ask whether it is reasonable to give it the status of a cortical field. However, a study of the neighbouring fields seems to settle the problem. The delimination of the entorhinal area is easy. Both in the rat and in the guinea pig the cessation of the pale layer III is an unequivocal criterion of the beginning of a new cortical field, which has to be the parasubiculum. The *presubiculum* of the guinea pig as defined in the present paper is at several levels relatively much wider than the presubiculum of the rat, but homology of the two could hardly be questioned. The delimitation of the presubiculum against the subiculum shares important histochemical features in the two species, particularly the sudden drop in AChE in layer I when the subiculum is entered; in addition, the replacement of the complex multilayered type of cortex in the presubiculum with the simpler pattern of stratification in the subiculum defines this boundary beyond doubt. Since the piece of cortex extending from the subiculum nearly to the entorhinal area (i.e., to the parasubiculum) is uniform in appearance, all of it undoubtedly represents presubiculum. However, it differs significantly from the presubiculum in the rat with regard to the amount and distribution of acetyl cholinesterase. In the rat, the outer half of the cortex, lamina principalis externa of Rose, is heavily stained, with the exception of layer II. Thus, layers I and III are very dark. In the guinea pig, the part of lamina principalis externa showing the best staining is represented by narrow zones superficially, whereas the deeper parts, here labelled III, are much paler. These differences between rat and guinea pig are sufficiently surprising to invite investigations designed to check the interpretation of layers advocated here. Such investigations should include mapping of the distribution of afferents.

The present study indicates that at least most of the cell bodies, dendrites and axons in the three areas examined are not associated with high concentrations of cholinergic synapses. A mapping of noradrenaline-containing terminals in the hippocampal region of the rat and the guinea pig (Blackstad, Fuxe and Hökfelt, 1967) showed only few terminals in the same areas. On the whole we are still without any positive evidence concerning the nature of the neurotransmitters in these areas. The distinctness of the layers and the marked histochemical differences between many of them make it reasonable to believe, however, that a search for transmitters and associated metabolic factors may be a practicable possibility within this part of the cortex.

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