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MODULAR ORGANISATION OF THE RODENT CEREBRAL CORTEX

by

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A thesis presented in candidature for the degree of Doctor of Philosophy in the Faculty of Science.

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ABBREVIATIONS

Hippocampal formation

AD area dentata

CA1) subfields of hippocampus
CA3) (Cornu Ammonis)

DF dentate fascia

DF (IB inner blade
(OB outer blade

E entorhinal cortex (area 28)

F fimbria

G stratum granulosum

H hippocampus

HF hippocampal fissure

P parasubiculum

PR presubiculum

PS postsubiculum

R retrosplenial cortex

RS rhinal sulcus

S subiculum

SL stratum lacunosum

SLu stratum lucidum

SM stratum moleculare

SO stratum oriens

SP stratum pyramidale

SR stratum radiatum

X innermost part of stratum moleculare in dentate fascia

Y layer immediately beneath granule cells in the hilus

Somatosensory cortex

FP forepaw area

HD head area

HP hindpaw area

LL lower lip area

WB whisker barrel area

Cerebellum

CB cerebellum

GL granular layer

ML molecular layer

Cerebellum (cont.)

PCL Purkinje cell layer

WM white matter

Orientation

A anterior

D dorsal

L lateral

M medial

P posterior

V ventral

Superior colliculus

SC superior colliculus

SGI stratum griseum intermediale

SGS stratum griseum superficiale

SOP stratum opticum

SZ stratum zonale

IC inferior colliculus

ABBREVIATIONS (contd)

Olfactory bulb

EPL external plexiform layer

LF lamina fibrosa (olfactory nerve layer)

OB olfactory bulb

C caudoputamen

Biochemical

AMP adenosine monophosphate

^{14}C2DG ^{14}C 2-deoxyglucose

CAMP cyclic 3',5'-adenosine monophosphate

CGMP cyclic 3',5'-guanosine monophosphate

GABA gamma amino butyric acid

GAD glutamic acid decarboxylase

NADPH-DH nicotinamide adenine dinucleotide phosphate (reduced form) - dehydrogenase

NOTE

Unless otherwise indicated all sections shown from Fig.15 onwards demonstrate the postmortem levels of phosphorylase a. These sections have all been stained with acidified iodine. No counterstain has been used. The variety of colours in the section occurs as a result of the different environments produced by glycogen molecules with different unbranched chain lengths.

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SUMMARY

Postmortem phosphorylase *a* activity has been used in muscle as a histochemical marker of metabolic changes produced by electrical stimulation. Therefore it seemed worth investigating the possibility that, in the brain, postmortem levels of phosphorylase *a* might be linked to functional activity. In the present study, it was found that in the forebrains of mice, within 10 sec. of decapitation, the phosphorylase *a* levels increased to a maximum. Thereafter the levels of phosphorylase *a* fell gradually and at varying rates in different parts of the brain. When 5 min was allowed to elapse between decapitation and freezing the brain, the phosphorylase *a* levels were relatively stable. It was such steady-state levels of phosphorylase *a* that were studied for the remainder of the project.

The first section of the Results presents evidence that consistent differences in the postmortem levels of phosphorylase *a* could be produced by altering the functional state of the brain prior to sacrifice. To do this, sections were taken from the brains of mice which had been transiently anaesthetised with ethyl chloride vapour immediately before decapitation ("unanaesthetised" mice) and from mice which had been anaesthetised with sodium pentobarbitone for at least 10 min prior to death. In sections from the "unanaesthetised" mice there were relatively high levels of phosphorylase *a* in layers I, IV and Vb of the somatosensory cortex. In surface parallel sections through layer IV the staining was patchy; the hollows of the whisker barrels were strongly stained whereas the barrel walls were relatively pale.

By contrast, the staining in sections from the anaesthetised group was more variable and in most cases the levels of phosphorylase *a* were higher in the outer three layers of the somatosensory cortex than in the inner three. In sections from three of these mice the phosphorylase *a* activity in coronal sections was organised into bands of high staining

that were oriented at right angles to the surface of the cortex. The bands extended from layer I to layer V. These represent cylinders of high phosphorylase a activity each of which has a diameter of about 250μm. These cylinders were not in register with the whisker barrels but are arranged at random with respect to them. This provides evidence for the existence of two separate overlapping systems of modules in the mouse neocortex.

Evidence of a columnar organisation was also observed in the primary and secondary visual and auditory areas and in the motor cortex. There was also evidence of a modular organisation in the superior colliculus and striatum and in many parts of the hippocampal region, including parts where there has not been any previous description of modules.

In studying a penicillin induced epileptiform focus no evidence was found of any significant changes in phosphorylase a activity associated with either the primary or the secondary foci. This finding was in marked contrast to the results obtained in an experiment where an epileptiform focus was studied with the ^{14}C-2-deoxyglucose technique.

Phosphorylase a levels were found to be related to the architectonic organisation of the brain. Thus when surface parallel sections are cut through layer IV of the neocortex, the three primary sensory areas can be distinguished because of their relatively high levels of phosphorylase a activity. As the postmortem phosphorylase a levels are altered by tissue damage, such as that produced by inserting a glass microelectrode, the phosphorylase method allows microelectrode tracks to be studied in relation to the known architectonic organisation.

An upper limit to the number of modules in the mouse neocortex and hippocampal formation was estimated at 3000 columns in the neocortex and 800 modules in the hippocampal formation.

GENERAL INTRODUCTION

The implicit aim of modern neuroscience is to provide an explanation of man's consciousness in terms of biological mechanisms. Already a comprehensive theory[57] has been proposed that is an attempt to produce a theoretical framework on which future studies of human consciousness can be developed. This theory is based on the vertical organisation of neurones within the cerebral neocortex which form functionally distinct groups or "columns" of cells stretching through all six layers of the cortex.[158] These columns of cells have basically the same intrinsic organisation in all parts of the neocortex; they are functionally differentiated by the source of their dominant input and the target of their output. Similar groupings of cells (modules) also occur in other parts of the brain concerned with information processing: both laminar structures such as the superior colliculus[259] and in cell nuclei such as the ventrobasal[232] and reticular[222] nuclei of the thalamus and in the striatum.[95]

Although this general principle of a widespread modular organisation is becoming established for the mammalian brain, much work is still necessary before a detailed knowledge of the modular organisation of the various brain structures will emerge. In the neocortex there are two separate systems of columns;[223] one determined by the specific thalamic input and the other by cortico-cortical inputs. However the spatial relationship between these two systems is unknown.[116] In the allocortex and transitional or periallocortex of the hippocampal formation there has been comparatively little evidence found of a modular organisation. This is partly due to the complicated convoluted nature of the hippocampal formation but also because of the small size of the cellular fields within it and the correspondingly small modules of which they may be composed.

Towe[228] has shown that it is impracticable to demonstrate the small

columns found in the rodent cortex by electrophysiological techniques. Therefore in the present study no electrical recordings were made in relation to the modular organisation. Instead a histochemical technique has been developed which reflects the brain's functional activity.[240] It is based on an enzyme which acts as a marker of intermediate-term changes in metabolic activity. Histochemical techniques as well as having a higher spatial resolution than electrophysiological recording methods also have the advantage of allowing a direct comparison of the modules with the architectonic organisation of the cortex.

It is usually impractical to study human or even primate brains and other mammals are used instead. Lorente de No in his classical studies of the cellular architecture of the cerebral cortex[141,144] stated that the neocortex of the mouse and man are basically the same, both in the cell types present and the form of the afferent input. In the present investigation results were mainly obtained from mice and rat brains. The smooth unfolded nature of the neocortex in these brains meant that it was relatively easy to flatten the cortex and cut sections which passed through all the neocortical areas. In constructing a 3-D image of the modular organisation of the cortex, it is simpler to have tangential or surface parallel sections as well as sections cut at right angles to the cortex, rather than working from sections of the latter orientation alone. A major goal of the present study was to examine the cortical modules in both surface parallel sections and in sections cut at right angles to the cortex.

The principal marker of functional activity in use at present is ^{14}C labelled deoxyglucose.[214] However the spatial resolution of this technique is limited[119] and individual cells cannot be distinguished, so an alternative method has been developed that does not involve autoradiography. This is based on the enzyme glycogen phosphorylase which exists in two histochemically distinguishable forms.[77] The relative proportions

of the two forms are determined by the protein phosphorylation system. This is a universally occurring regulatory system[38] which mediates both short term and intermediate term metabolic changes. It was anticipated that these latter changes would be sufficiently stable to survive the processes occurring immediately after decapitation and so even the post-mortem levels of the active form of phosphorylase would reflect some aspect of the brain's functional activity. This was important because flattening the brain to allow surface parallel sections to be cut, prohibits the study of in vivo levels of phosphorylase a.

The results are divided into three sections. The first is related to the columnar organisation of the mouse neocortex. It deals with questions concerning the size and number of columns and the spatial relationship between the columns defined by the thalamic afferents and those defined by the corticocortical afferents. The second section is related to the modular organisation of other regions of the rodent brain. It is primarily concerned with parts of the limbic lobe, superior colliculus and caudoputamen, all of which are involved in information processing functions. The third section is primarily concerned with the use of phosphorylase a as a marker of functional activity especially in relation to studying the spread of an epileptiform focus.

Preliminary accounts of this work were presented as a communication to the E.E.G. Society,[237] in two communications to the Physiological Society[238,239] and as a paper submitted to "Brain Research".[240]

LITERATURE REVIEW

1. Hierarchy of Organisational Levels

a. Introduction

Human problem solving involves breaking the problem down into a number of simpler units which are then sequentially solved.[162] In line with this the problem of understanding the brain has been broken down into successive levels of systems, each of which involves the interaction of a limited number of components.[29] For example, in clinical work the brain is described in terms of a limited number of neurological systems concerned with such aspects as arousal, the emotions or the various senses.

In studying the brain in terms of systems of interacting components it is important to split up such studies into a hierarchy of different organisational levels. As Rose[197] has pointed out, failure to recognise that there are different organisational levels has led to considerable confusion especially in attempts to link the disciplines of psychology and physiology. Thus vain attempts have been made to explain psychological states in terms of biochemical or physiological mechanisms. Such attempts were largely futile because the most they could achieve was a description of the same brain state at two different levels - something quite different from an explanation. A simple example of this would be the finding of increased noradrenalin levels being associated with the state of being in love. It would be equally wrong to say that the state of being in love was caused by changes in brain chemicals such as noradrenalin, as it would be to say that being in love caused the changes in noradrenalin. Explanations can only be offered within a level, not between levels.

Ideally each level should be composed of a reasonably small number of components (100,000) and there should be a continuous series of steps between the levels of molecules and the whole brain.[197] At the time Rose

published his diagram showing the different hierarchical levels (1975) there was no intermediate level between simple circuits such as the tendon-jerk reflex and the neurological systems. Consequently there was a sharp division between neuroscientists working at the neurological level and those working at the level of reflex arcs. However following the 4th Intensive Study Program of the Neurosciences Research Program of 1977, which was partly directed towards this problem, Mountcastle[158] published a paper on the neocortex outlining a theory of distributed systems forming a level of organisation intermediate between the levels of reflex arcs and neurological systems.

This theory was based on his classical discovery[157] of vertical groupings of cells in the cat somatosensory cortex which had permitted him to formulate a theory for the arrangement of the neocortex into physiological columns.[158] In his 1977 paper[158] he extended this theory to suggest that most anatomical structures of the brain involved in information processing had an analogous organisation. In his view the individual structures were composed of sets of replicated modules each of which had the same basic structure and performed the same type of transformation on a common afferent input. Furthermore the set of modules or columns composing a structure may itself be fractionated into subsets by different linkages to similarly segregated subsets in other structures. This fractionation would mean that closely linked subsets of modules in different and often widely separated structures would form precisely connected but distributed systems each serving a distributed function. The neurological systems composed of these sets of distributed modules are not restricted to a small number of structures but rather are composed of groups of modules in a multitude of anatomical structures. Thus the difficulties which arise from assigning specific functions to individual areas of the brain have been avoided without having to propose that the majority of the brain's connections are randomly arranged as suggested by Lashley.[135]

Until the advent of the electron microscope in the 1950s and the subsequent clear description of different types of synapses and their ultrastructural components[41] there was no clearly formulated organisational level between biochemical molecules and individual neurones. By the time of the 4th N.R.P. Intensive Study Program Heuser[97] and his colleagues had obtained evidence for vesicle fusion and membrane recycling which has allowed the formation of molecular models describing the mechanism of action potential induced neurotransmitter release.[104] In freeze fracture studies especially it is possible to identify individual membrane particles which apparently correspond to the ion translocation pores.[98] The introduction of modern neuroanatomical techniques in the early 1970s especially those based on immunocytochemistry and the cellular transport of specific molecules such as horse-radish peroxidase (HRP) and radioactive amino acids[119] has also meant that the examination of individual neurones can proceed in terms of the organisation of specific enzymes and metabolites such as neurotransmitters.

As a result it is now possible to construct a hierarchical table of the various organisational levels in the brain (Table I) where each level is logically linked to the next. Any such table is of course to some extent arbitrary and it would have been possible to split some of the levels into two separate levels. For example , the neurone could have been considered as being composed of twenty or more dendritic branches forming a level immediately above the cytological level of synapses. However it was felt that further subdivisions of this sort would not be helpful and so the table was restricted to 9 levels.

Table I

Hierarchy of organisational levels in the brain

Level of hierarchy	Basic units	Type of organisation	Example of function
social psychology	people	community	village fair
neurology	(100) systems	brain	conscious decision
architectonics	(10,000) primary repertoires composed of sets of modules	system	novelty detection
anatomy	(1,000) simple circuits	set of specifically interconnected modules	cross-correlation of inputs
morphology	(1,000) neurones	simple circuit	tendon-jerk reflex
cytology	(10,000) synapses	neurone	integrating input to produce a specific frequency of A.P.s
ultrastructure	(10,000) molecular assemblies	synapse	release of neurotransmitters
biochemistry	(1,000) complex molecules	molecular assembly	change in distribution of intracellular messenger
molecular biology	(100) simple metabolites	complex molecule	conformational change

This represents a rather arbitrary classification of the various organisational levels at which neuroscience is studied and emphasises its essential unity. At each level the complicated interaction of units forming a system becomes the basic unit of the succeeding level. Collectively the neurosciences attempt to provide an explanation of brain function in terms of the interaction of small biological molecules. The number of units comprising a system varies from between 20 to perhaps

40,000 and a figure giving the rough order of magnitude of units in each system is indicated by the numbers in brackets.

b. Biochemical levels

To start at the lowest level simple precursor molecules such as amino acids or hexose sugars are formed into enzymes or polysaccharides of 100 or more subunits by the synthetic machinery of the cell [219] which follows the instructions stored in the sequence of nucleotide bases of DNA or the specificity of the active clefts in enzymes. During synthesis these complex molecules may be assigned specific cell compartments, incorporated into the fast axonal transport system, [82] or inserted into the membrane of the rough endoplasmic reticulum prior to the formation of membrane vesicles which will ultimately fuse with the plasmalemma. [255] Within the plasma membrane [212] these molecules will arrange themselves to some extent spontaneously into loose aggregations of perhaps a hundred molecules, or more tightly knit units such as membrane channels, ion pumps or neurotransmitter receptor complexes which control membrane potential, ion concentration gradients and the level of intracellular messengers respectively. During morphological development [249] these molecular complexes will be formed into specific structures such as dendritic spines or presynaptic swellings where there is a particularly close association between the plasmalemma and the underlying cytoplasmic machinery. In the case of presynaptic terminals there may be a particularly high turnover of cytoplasmic membrane vesicles fusing with the plasmalemma, [97] although this has not been proved.

c. Morphological levels

Membrane junctions whether of the conventional synaptic type [41a] or of dendro-dendritic [193] or dendro-somatic variety, make up virtually the whole surface of the neurone and so the neurone can be considered as being a collection of between 10 and 40,000 [41a] synapses which are maintained in a viable condition by the underlying cytoplasmic

machinery and surrounding glial cells. The neurone functions as a unit which integrates a variety of analog neuromodulatory, neurohormonal, neurotransmitter or electrotonic inputs[57] and converts them to a digital output signal in the form of an ordered sequence of action potentials.[90] Some neurones are organised into simple reflex circuits[74] of perhaps 1,000 cells such as the one underlying the tendon jerk reflex or the neuronal pathway within the hippocampal region.[181]

d. Modular levels

Simple circuits have also been described in the neocortex and it has been suggested[193] that the bundles of apical dendrites found in the neocortex[172,65] (Fig. 1) may form the basis of cortical units of the type described by Hubel and Wiesel[106] in the striate cortex as responding to only one discrete orientation and termed minicolumns.[107] Each of these minicolumns will form reentrant loops with other sub-cortical and cortical areas because there are reciprocal connections with many thalamic nuclei[116] as well as other cortical regions.[193,172] A number of both anatomists and physiologists[108,107] have provided evidence that these simple circuits composed of cortical minicolumns and their system of reentrant loops are organised into sets or macrocolumns[107] because of a common afferent input in the form of either a bundle of cortico-cortical fibres[223] or a bundle of thalamic fibres.[116] This column and a limited number of other specifically connected columns or modules in other areas of the brain will form the basic unit[57] of the neurological systems[48] which have been described in the brain.

e. Relative specificity within different levels

Each organisational level is composed of basic units which are organised into a number of systems. For each level the basic units are formed by the systems of the lower level and the systems form the basic units of the next level above. For example, at the morphological level, the function of a simple circuit composed of individual neurones can best

be understood as part of a system such as the ∝ motor system of the spinal cord[74] whereas the structure and mode of operation of the individual neurones can best be described by treating the neurone as a specifically ordered system of synapses.[90,180] Thus the different levels form a continuum with no one level being separate from its neighbours.

There is considerable variation in the number of basic units making up the various systems with numbers ranging from a few tens up to 40,000. A rough estimate of these is given in the table. This variation is thought to reflect the comparatively high degree of degeneracy exhibited by neural systems at certain levels of organisation.[57] Human neurones in the neocortex may require about 100 excitatory synapses acting simultaneously before they will fire[180] but as each cell may have about 40,000 synapses on its dendrites,[41a] there is a large number of different patterns of active synapses which could give rise to the same output pattern. It follows that there is a high degree of degeneracy among the patterns of active synapses giving rise to the neuronal output.

Similarly each neurological system may be composed of tens of thousands of modular sets. Many of the sets are relatively unspecialised but the large number of modular sets is thought to be essential for the high degree of plasticity and adaptability displayed by neurological systems.[57] Thus the destruction of even a large number of modular sets has comparatively little long term effect on the function of that system.[135] However at the level of the whole brain which is composed of a relatively small number of systems, some of which are highly specialised, the functions of any one of these systems cannot be taken over by the others. One example of this is long term memory formation mediated by the temporal lobe. Bilateral removal of the hippocampus and the surrounding temporal lobe prevents the formation of any new long term memories.[169]

2. Architectonic Organisation of the Cerebral Cortex

a. Historical introduction

For the early anatomists the human cerebral cortex appeared as a relatively homogeneous soft jelly-like substance with little evidence of any structural organisation despite its complicated convolutions. The first hint of a laminar arrangement in the cortex came in 1776 when Gennari[74] studied a frozen brain and described a white line in the middle of the cortex that was most prominent in the occipital lobe. An alternative means of studying the cortex was by cutting thin sections. By using sections of fresh cortex held up to the light between two glass plates,[25] Baillarger in 1840 was able to describe six layers in the cortex. He did not know their significance, but thought that they resembled a voltaic pile. However the detailed description of cortical structure required the use of chemical fixatives and dyes which initially were mainly alcohol (introduced by Reil in 1809)[25] and carmine dyes.[25] Using such methods Meynert produced the first detailed histological examination of cortical organisation in 1872. He described five layers in the precentral cortex and eight in the occipital cortex. Each of these layers was composed of discrete aggregations of different types of cell bodies. He also showed the bundles of vertical myelinated fibres that are typical of the neocortex.

Meynert did not restrict himself to studying the neocortex but attempted to find where the cerebrum terminated.[36] He discovered that the edge of the cerebrum formed a ring on the medial surface of the brain and that this ring was roughly centred on the interventricular foramen (Foramen of Monro). Broca examined this ring in many different mammals and found it present in all of them. He termed it the limbic lobe because it formed the limbus or border of the cerebral hemispheres. Ontogenetically the cerebrum is also divided into two basic types termed isocortex and allocortex by O. Vogt The allocortex fails to cleave

completely from the mantle layer during development. The allocortex does not correspond to the limbic lobe but forms a subdivision of it. An alternative means of subdividing the limbic lobe was provided by comparison with the cortex or pallium of amphibia and reptiles. In these two classes there are three cortical areas and by analogy there are taken to be three mammalian cortical areas termed the archicortex, paleocortex and neocortex. The neocortex is included in Vogt's isocortex.

Evidence that the cortex could be divided up into functionally discrete areas came from clinical and physiological experiments in the last quarter of the 19th century.[74] In 1870 Hughlings Jackson concluded that there was an area of cortex which controlled isolated movements following his investigation of patients with focal epilepsy. In the same year Fritsch and Hitzig provided experimental verification by demonstrating that electrical stimulation of the frontal cortex of various mammals produced movement of the extremities on the opposite side. Three years later Ferrier confirmed the importance of these areas by ablation studies. Clinical studies also showed that other localised areas had a specific function such as the speech areas of Broca and Wernicke.

At the same time anatomists were studying the cellular structure of the cortex partly using reduced silver techniques for neurofibrils, but largely through the use of Golgi's technique based on osmium tetroxide/potassium dichromate fixation followed by immersion in silver nitrate. This stained individual cells in their entirely and as only about one in a hundred[167] cells were stained at random it was possible to study the different cell types in great detail. Golgi, his pupil Martinotti and Cajal described four basic types of cell in the neocortex.

i. Pyramidal cells with descending axons passing into the white matter. (Fig. 1)

ii. Cells with short axons ramifying in the proximity of the cell body

and often within the same zone as the dendritic plexus.

iii. Cells with ascending axons ramifying in one or several cortical layers.

iv. Cells with horizontal axons.

However since then there have been dozens of cell types described with differences in the position of the soma, axonal arborisation, dendritic arborisation and density of dendritic spines.[144,267]

Because of the clinical and physiological demonstration of relatively discrete cortical regions with particular functions and Cajal's description of cortical areas with very different arrangements of cells,[181] anatomists were encouraged to make a careful study of the cortical regions with a view to subdividing them into areas each with a specific structure.[144] Thus in 1903 Campbell, Brodmann and Vogt all gave communications on the parcellation of the cerebral cortex. Campbell distinguished 20 areas, Brodmann about 50 and later von Economo described nearly a hundred areas while the Vogts described about 200.[144]

These parcellations of the cortex depended upon differences in the cellular (cytoarchitectonic) and fibrillar (myeloarchitectonic) structure of each region. Brodmann based his cytoarchitectonic studies on the six-layered diagram of cortical structure prepared by Bevan Lewis.[144] The Vogts prepared a myeloarchitectonic equivalent to this diagram and so the cortex was divided up on the grounds of alterations in the form and number of the cells making up the different layers and sublayers and also on variations in the source and distribution of myelinated afferents and efferents.

b. Neocortical organisation

Between the years 1911 and 1914 four authors - Isenschmid, De Vries, M. Rose and Droogleever Fortuyn - published cytoarchitectonic maps of the mouse cerebral cortex.[268] These studies did show a large degree of variation, because although all were an attempt to find

homologous areas to those found in the human brain, there were no physiological or pathway degeneration studies available and so the parcellations were largely based on guesswork. Despite this some of the areal boundaries were clear enough to be observed in all the studies and in two of the papers there is the first hint of a modular subdivision of a single neocortical field. De Vries described one of his fields (H^2) as being composed of small subfields in layer IV each of which had a quadrangular or elliptical shape. Droogleever Fortuyn confirmed this observation for the mouse and also made observations on the rat and guinea pig brains where he was also able to describe similar structures.[268]

The first person to study this area in depth was Lorente de No who in 1922[141] published a paper on his Golgi studies of this area. He referred to it as auditory cortex following Rose's nomenclature[196] and stated that it was characterised by having an enormous layer of granule cells in layer IV which were disposed in the form of glomeruli. Like the olfactory glomeruli[181] these glomeruli each received afferent fibres with dense arborisations confined within the cell boundaries. They also had specific intracortical stellate cells with dendritic and axonal arborisations specifically related to the glomeruli (Fig. 9) and altogether he described 7 different cells which are specifically related to them.

Partly on this work but also on work involving Golgi studies of the entorhinal cortex[142] Lorente de No reached a number of important conclusions regarding the organisation of the cerebral cortex. Although there was great variation in the detailed cellular and fibre arrangements from species to species and from one cortical area to another, he found that the arrangement of the plexi of dendritic and axonal branches remained constant throughout the neocortex and in different mammalian species. Thus the mouse cortex can be used as a rather simplified model or "skeleton" of the human neocortex. Although the

cells and afferent plexi are smaller, their basic arrangement is the same as in man.

From the functional point of view Lorente de No thought of the neocortex as a system of elementary units, each composed of a vertical chain of neurones spanning all six layers. Thus he thought of the neocortex as being composed of an enormous number of elementary units, some of which would be arranged side by side while others would be overlapping.[144] Each elementary unit would have a series of axonal and dendritic plexi, where the synapses between intracortical elements and from afferent fibres are established. The bodies of the cells which form similar links in the intracortical chains are grouped together to form the horizontal layers. Any change in the constitution of the intracortical chains must produce a variation in the density of the plexi and in the number of cells in each layer. These changes formed the basis of the architectonic maps.

With the introduction of silver impregnation methods for studying anterograde and retrograde degeneration produced by specific lesions, it became possible to study cytoarchitectonic organisation in relation to the terminal fields of thalamo-cortical fibres and the fields of origin of cortico-thalamic fibres. Using information gained in this way, as well as traditional cytoarchitectonic criteria, Krieg in 1946[132] produced the only comprehensive cytoarchitectonic division of the rat neocortex ever to have been published. However within three years an additional criterion was introduced for defining an architectonic area by Rose and Woolsey.[195] They defined the primary auditory cortex of the cat by three separate criteria: cytoarchitectonic, the retrograde degeneration pattern observed following destruction of the thalamic nucleus and by the evoked potential map produced by the stimulation of cochlear nerve fibres. By doing so they showed for the first time that cortical areas defined by anatomical and physiological criteria coincided.

The first study of the sensory areas of the mouse neocortex using the evoked potential method was carried out by T.A. Woolsey in 1967.[265] As well as the visual and auditory areas, two somatotopic representations were found which in general were similar to those found in other mammals.[263] The representation of the face and particularly the mystacial vibrissae was greatly expanded in SmI. This area corresponded to that where there had been a description of clouds and columns among the layer IV granule cells. By correlating the appearance of layer IV Nissl stained sections cut in planes perpendicular and tangential to the plane of layer IV, it was shown that the granule cells form cylindrical multicellular cytoarchitectonic units which because of their shape, were called "barrels".[268] This was an important step forward because the previous studies had only been two dimensional. Woolsey started to build up a 3-D picture which could be correlated with the evoked potential map.

Electrophysiological studies of the rat cortex have also been made, using the evoked potential method to study the sensory areas and electrical stimulation to study the motor cortex.[244,245] The discovery of barrels in the mouse cortex led to their description in the rat as well.[87] Using the barrels as markers a cytoarchitectonic field was defined. This field was confined to the primary somatosensory cortex (SmI) and contains at least 220 barrels.[246] Within it there is a special region - the posteromedial barrel subfield (PMBSF) - where barrels are arranged in rows and are larger than elsewhere. However in contrast to other barrels in the rat barrel field and the mouse barrels, the PMBSF barrels in the rat are filled with small neurones nearly throughout the thickness of layer IV.[266] In studying the barrels Welker[246] introduced a significant methodological innovation by flattening the hemispheres during processing so that the organisation of layer IV could be seen through the full extent of SmI in a single section. This study confirmed the proposal

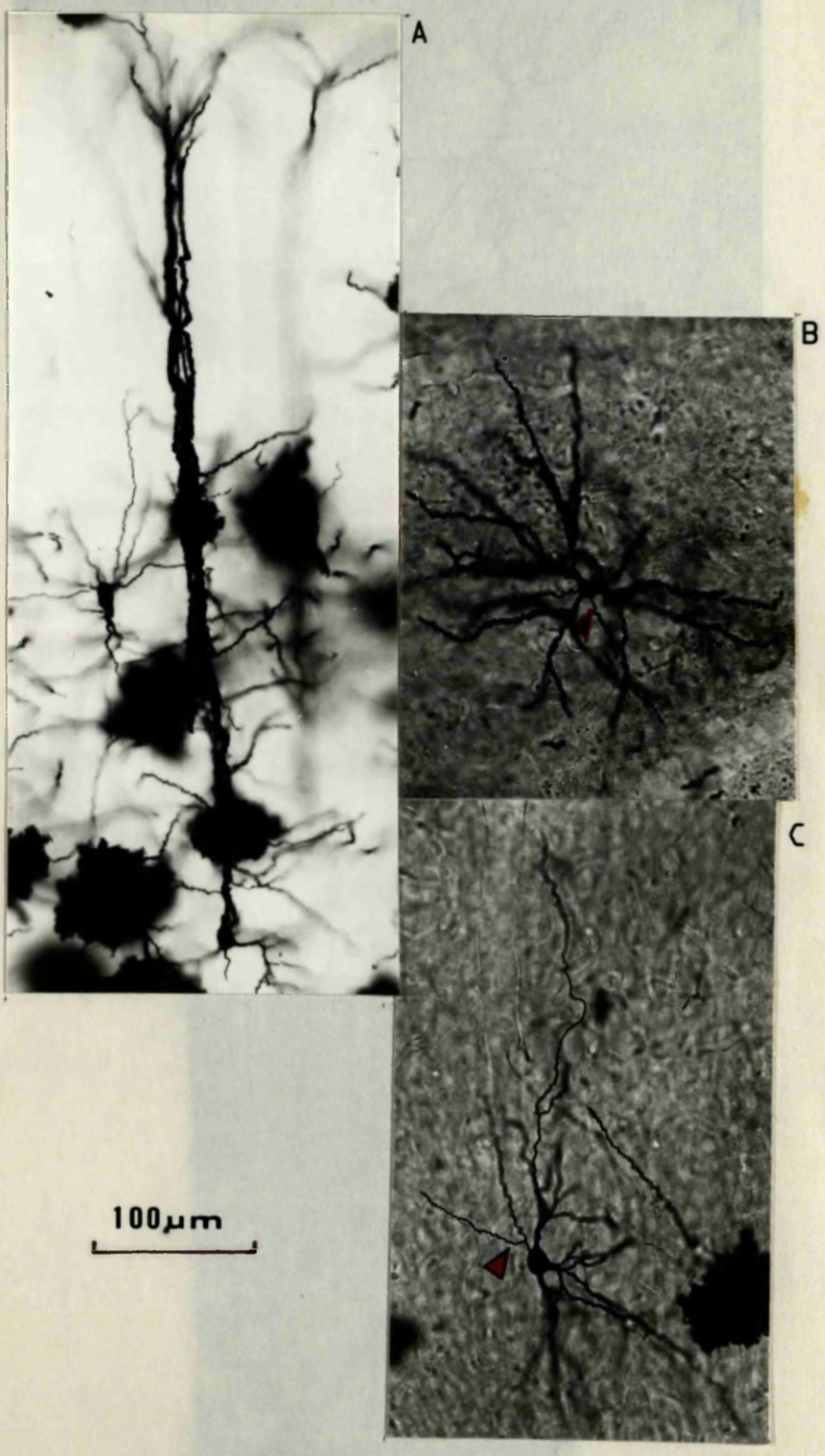
put forward on anatomical grounds[268] that there is a one to one relationship between the barrels and sinus hairs. The large whisking vibrissae project to the large cell dense barrels of the PMBSF and small non-whisking sinus hairs project to smaller barrels that have relatively cell sparse hollows.[244] Barrels are also found in areas receiving projections from the hindpaw and forepaw even although these do not have any sinus hairs. The barrel subgroups and other somatotopic areas are separated by narrow barrel-free zones. All areas with a primary somatosensory input have a higher density of granule cells than the surrounding cortex. As a result of this pattern of contrasting cell densities, an outline of the rat's body is delineated within layer IV (Fig. 2). This is termed the sensory ratunculus.[244]

Using modern neuroanatomical methods based on the anterograde transport of radioactive amino acids and the retrograde transport of horseradish peroxidase (HRP), an updated architectonic map of the mouse cortex has been prepared[34,35] (Fig.3). Specific thalamocortical fibres ascend to all neocortical fields except perhaps 13, 25 and 35. The specific thalamic projections to any particular field in the mouse (except perhaps Sm2) originate in only one thalamic nucleus.[35] Therefore it is now possible to define the architectonic areas in terms of their specific thalamic input in a precise unequivocal way.

Specific thalamic fibres terminate[72] mainly in layer IV and lower III and it is often in layer IV that the architectonic differences are most striking. Nevertheless differences also occur in other layers and these reflect variations in the targets of the efferent cells in the different architectonic areas. The significance of the layers is that they contain the cell bodies of efferent cells with a common target. Thus in the rat somatosensory cortex fibres which descend to the midbrain, pons, medulla and spinal cord arise exclusively from the largest pyramidal cells, the somata of which are found in the deep part of layer V (layer Vb).[258] Corticostriatal fibres arise mainly in layer Va and those

Fig.1 Example of cell types observed in rat neocortex with the Golgi-Kopsch staining procedure. A - Dendritic bundle formed by the apical dendrites of five pyramidal cells only one of which is fully impregnated. B - Layer IV spiny stellate cell with partially impregnated descending axon (marked by arrow). C - Layer V aspinous interneurone with ascending axon. D - Two layer III pyramidal cells. Notice how the apical and basal dendrites are confined to a cylinder of tissue with a diameter of 250 μm. E - Layer III bipolar cell similar to those described in the visual cortex by Valverde[230] and thought to contain the inhibitory transmitter GABA. Notice how its dendrites are confined to a narrow cylinder of cortex with a diameter of about 20 μm.

Fig.2 A and B Diagram of some of the major architectonic areas in the rat cerebral cortex. This is based on a surface reconstruction of the hippocampal region, imagined unfolded, published by Swanson and Cowan[220] (2 B) and a partially modified version of Krieg's[132] architectonic map of the neocortex provided by Welker[244] in the somatosensory cortex. Some of the barrels found in the somatosensory cortex are also indicated.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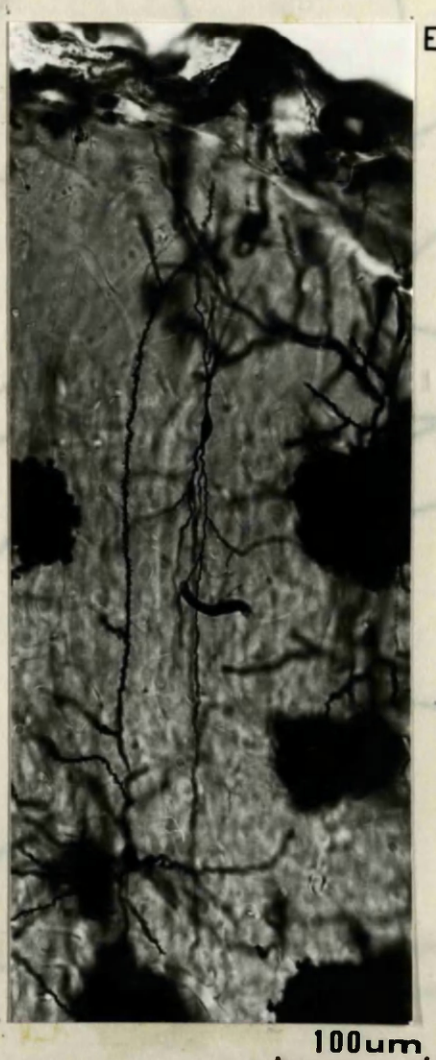
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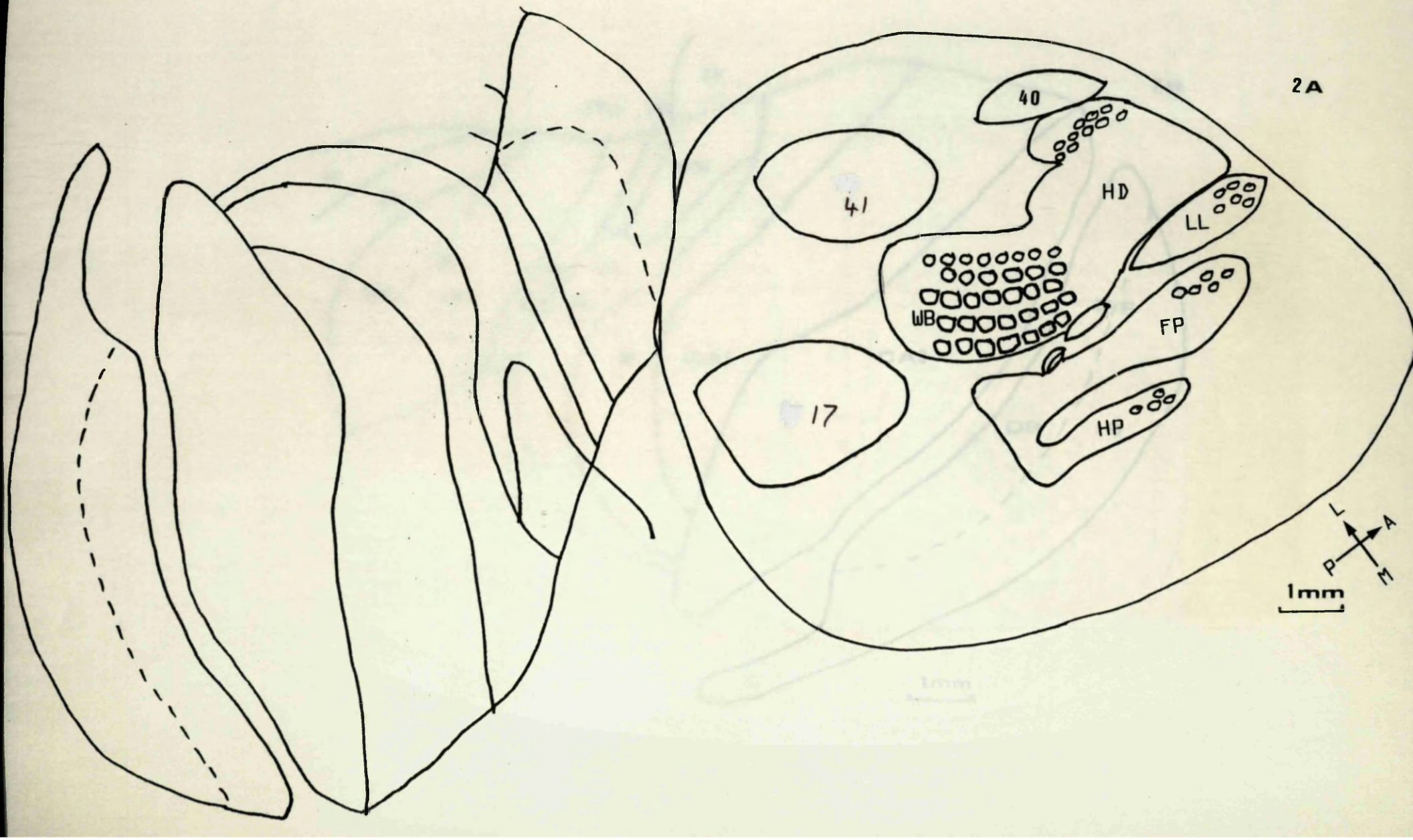
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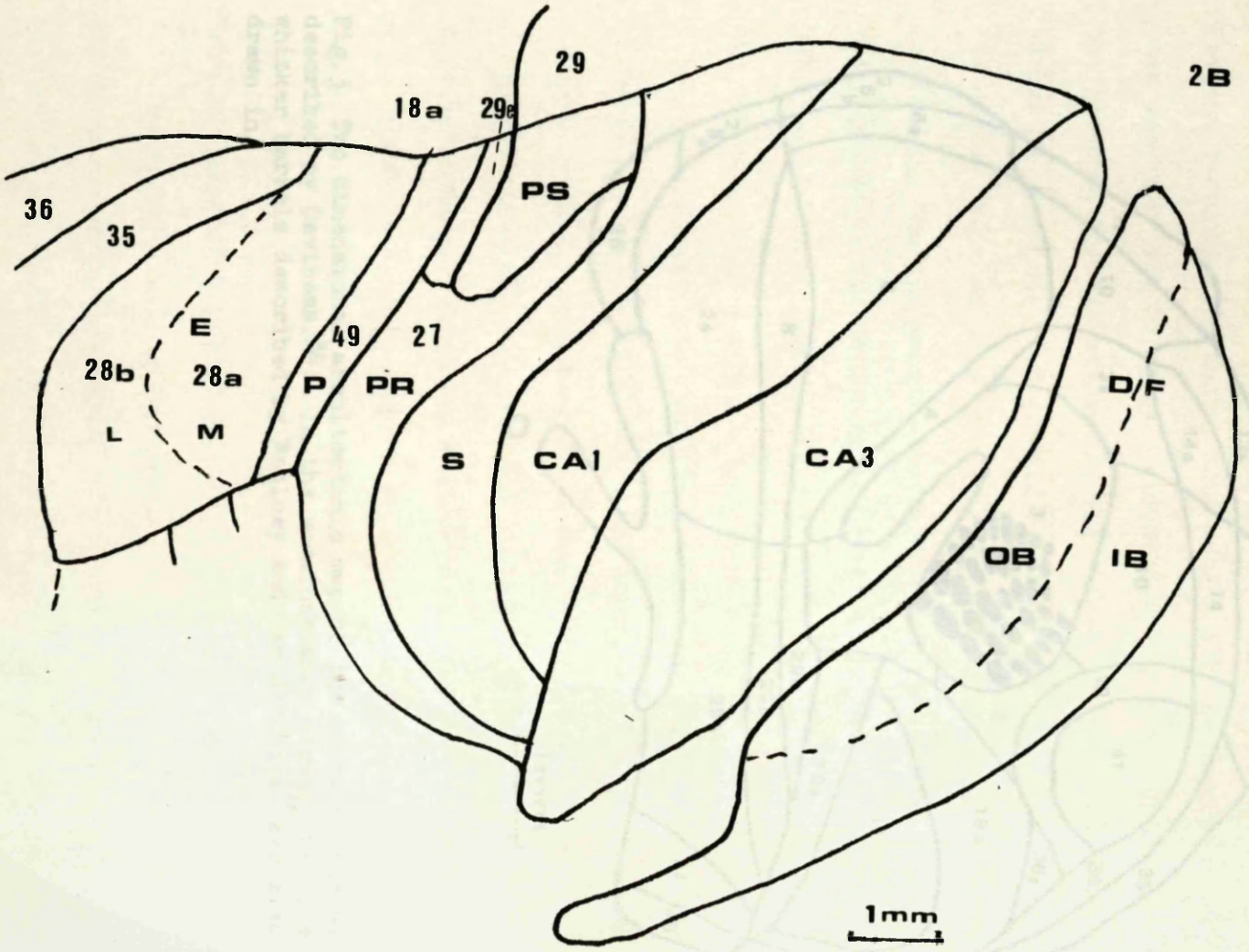
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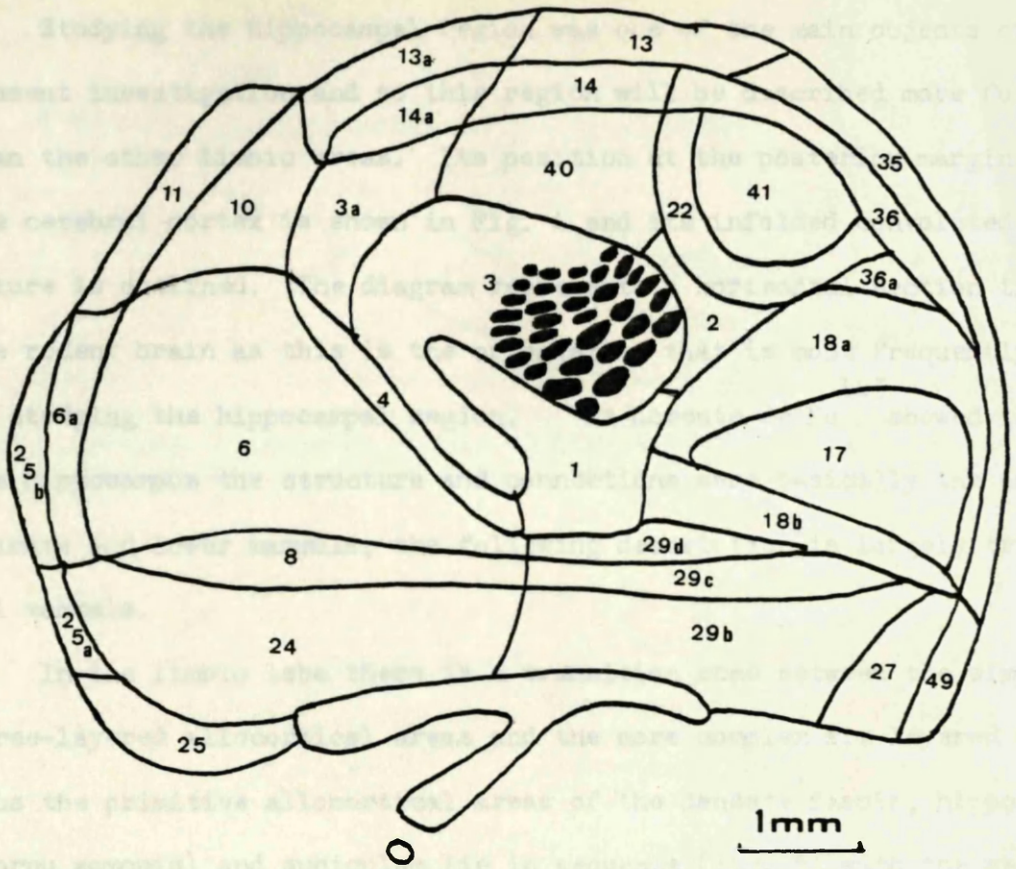


Fig. 3 Two dimensional architectonic map of the mouse neocortex as described by Caviness.[34] In the somatosensory cortex the five rows of whisker barrels described by Woolsey and Van der Loos[26] have also been drawn in.

terminating in the diencephalon mainly in layer VI.[258] The supragranular layers II and III give rise to cortico-cortical fibres.[116]

c. Anatomy of the hippocampal region

Studying the hippocampal region was one of the main objects of the present investigation and so this region will be described more fully than the other limbic areas. Its position at the posterior margin of the cerebral cortex is shown in Fig. 4 and its infolded convoluted nature is outlined. The diagram represents a horizontal section through the rodent brain as this is the orientation that is most frequently used in studying the hippocampal region. As Lorente de No[143] showed that in the hippocampus the structure and connections were basically the same in primate and lower mammals, the following description is largely true for all mammals.

In the limbic lobe there is a transition zone between the simple three-layered allocortical areas and the more complex six-layered cortex.[36] Thus the primitive allocortical areas of the dentate fascia, hippocampus (cornu ammonis) and subiculum lie in sequence (Fig. 5) with the periallocortical areas of the presubiculum (area 27), postsubiculum (area 48), area retrosplenialis e (area 29e), parasubiculum (area 49) and the entorhinal cortex which, as its name implies, lies immediately ventral to the rhinal sulcus. (For a definition of the relationship between the various terms used to describe the hippocampal region see Table 2).

The primary cell of the dentate fascia is granular while that of the hippocampus is pyramidal. (It is thought that because of growth distortion and specific cell migration, the normal structure of the cortex has been altered so that the primary cortical granule cells lie perched on the primary cortical pyramidal cells.)[36] The granular cells of the dentate fascia exist in a single layer with the apical dendrites oriented toward the pial surface within the hippocampal fissure. Beneath them lie layers of polymorphic cells which form the hilus of the dentate

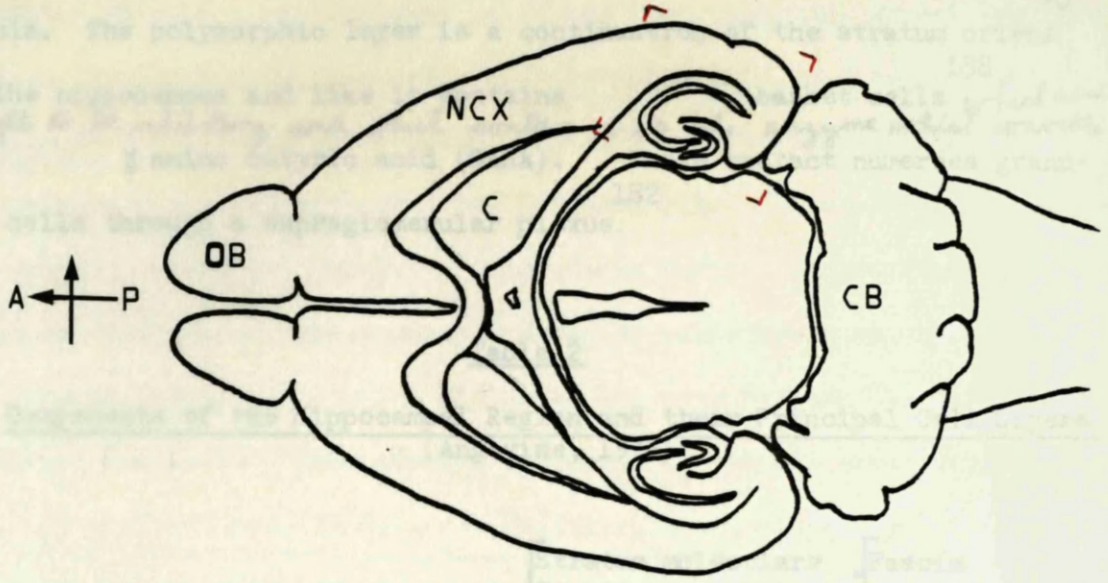


Fig.4 Horizontal section of mouse brain showing location (rectangle) of high power diagram of hippocampal region shown in Fig.5.

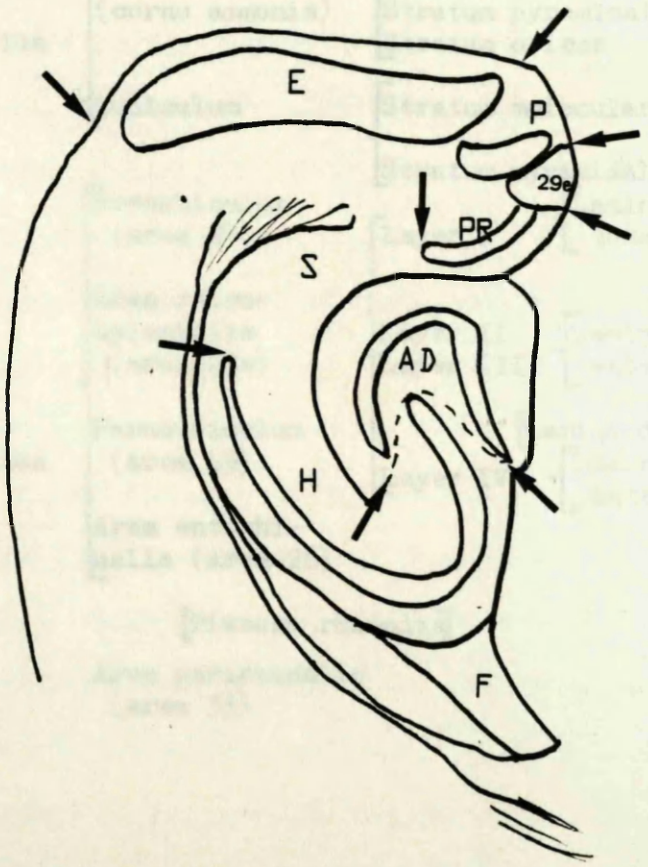


Fig.5 Diagram of the hippocampal region in the rat, based on horizontal silver-impregnated sections. The arrows mark the interareal limits of the various architectonic areas which are labelled by the abbreviations used throughout the text.

fascia. The polymorphic layer is a continuation of the stratum oriens of the hippocampus and like it contains basket cells which are thought to be inhibitory and which contain GAD the enzyme which converts glutamate to γ amino butyric acid (GABA). These contact numerous granular cells through a supraglomerular plexus.

Table 2

Components of the Hippocampal Region and their Principal Cell Layers
(Angevine, 1975)

- Hippocampal region
  - Hippocampal formation
    - Area dentata
      - Stratum moleculare
      - Stratum granulosum
      - Hilus fasciae dentatae
    - Hippocampus (cornu ammonis)
      - Stratum moleculare
      - Stratum lacunosum
      - Stratum radiatum
      - Stratum pyramidale
      - Stratum oriens
    - Subiculum
      - Stratum moleculare
      - Stratum pyramidale
  - Retrohippocampal formation
    - Presubiculum (area 27)
      - Layer I
        - Lamina zonalis
    - Area retrosplenialis (area 29e)
      - Layer II
        - Lamina principalis externa
      - Layer III
    - Parasubiculum (area 49)
      - Lamina dissecans
      - Layer IV
        - Lamina principalis interna
    - Area entorhinalis (area 28)

[Fissura rhinalis]

Area perirhinalis (area 35)

The hilus of the dentate fascia contains a considerable number of pyramidal cells comprising area CA4 of Lorente de No. Pyramidal cells stream out of the hilus and are packed together to form the pyramidal cell layer of the hippocampus. In the area CA3 adjacent to the hilus the

pyramidal cells are particularly large and their proximal apical dendrites receive terminals from the mossy fibre output of the dentate fascia (Fig. 6 B) which forms the stratum lucidum.[147] A small transitional zone CA2 separates the CA3 area from the CA1 area where the pyramidal cells are smaller and do not receive a mossy fibre input.[143] Instead they receive an input from the CA3 cells via the Schaffer collaterals which arise in the stratum oriens beneath the CA3 layer and pass between the CA3 cell bodies to terminate in the stratum lacunosum of the CA1 area (Fig. 6 B). Like the mossy fibres the Schaffer collaterals travel throughout the whole length of their target area in a direction at right angles to the apical dendrites.[6] Indeed most of the afferent fibres to the dentate fascia and hippocampus travel in this direction[211] so that the main input to the hippocampal formation which comes via the perforant path from the entorhinal cortex terminates at right angles to the apical dendrites[139] in the outer two thirds of the stratum moleculare of the dentate fascia and in the stratum moleculare of the CA1 and CA3 areas.[181] Similarly the commissural and crossed association fibres to the CA1 area produce en passant synapses as they travel at right angles to the proximal apical dendrites in the stratum radiatum.[20] Next to the field CA1 is the subiculum which is the main target for the output of the CA1 field.[63,19,220] These fibres are thought to ascend vertically[143] to terminate among the apical dendrites of the pyramidal cells in the lower layers of the subiculum. Near the CA1 area the subiculum starts out as a simple structure (prosubiculum of L. de No)[143] but becomes increasingly complex as it nears the periallocortex and can be divided into five layers.

The structure of the periallocortical region is much more complex than that of the allocortical portion, having five or six layers.[36] Adjacent to the subiculum lies the presubiculum but this has been subdivided into two areas on the basis of afferent fibre termination[220] and the presence of cell clustering.[194] Thus the dorsal regions of the pre-

subiculum in the cat are characterised by clusters of cells in layer II and this part has been termed the postsubiculum.[194] Between it and the parasubiculum lies a small triangular area (area 29e) about which very little is known.[20] The parasubiculum only appeared relatively recently in phylogenetic development[36] and may have arisen in conjunction with the orbitofrontal convexity of the frontal lobe.

Adjacent to the parasubiculum lies the entorhinal area which provides the main input to the dentate fascia.[181] Like all of the areas in the hippocampal region it has been subdivided into a number of subareas varying from 4 to 16.[36] The entorhinal cortex receives input from association cortex linked to olfactory, somatosensory, auditory and visual input. There is some evidence that the different modalities are kept separated[140] so that at least some parts of the hippocampus will receive highly processed input of a single modality. However the entorhinal cortex also receives multimodal inputs from the cingulate[253] and temporal lobe[17] which will mediate higher order functions.

The rodent hippocampal formation is usually studied in sections cut in a horizontal plane because this orientation allows most of the areas to be sectioned simultaneously.[153] Coronal and sagittal sections are also used and series of sections of all three orientations have been used[253,220] to produce diagrams portraying the surface of the hippocampal region imagined unfolded. These diagrams are basically the same although two were based on reduced silver stains[253,20] while the third was based mainly on a stain for the Nissl substance.[220] Swanson and Cowan[220] did not recognise the area retrosplenialis e (29e) and White[20,253] did not differentiate between the pre- and postsubiculum. Apart from this there is general agreement and their diagrams are summarised in Fig. 2.

This figure (Fig. 2) includes the tip of the cortical region forming the postero-medial edge of the limbic lobe. This region is the retrosplenial cortex which again has been subdivided into a number[34] of areas

(29a-e) and is found between the neocortex and the remnant of the hippocampus that lies above the corpus callosum and is known as the indusium griseum.[36] Rostral to this is the orbito-frontal cortex and the septal area, while the olfactory tubercle, amygdaloid areas and the pre-pyriform cortex (or in the rat for simplicity pyriform cortex) form the ventral part of the limbic lobe.[36]

On the basis of differences in neuronal morphology and the existence of afferent pathways, L. de No[143] divided the hippocampal formation into 14 subfields. He stated that the subdivision of cortical regions such as the hippocampus depended on the number of types of intracortical cell and afferent fibres that were considered. The subdivision would end up with groups of a few cells forming the physiological units[142] discussed in connection with the neocortex, i.e., groups of cells included within the arborisation of a fibre, be it an afferent or an intracortical fibre. However for simplicity Blackstad[20] grouped some of these subdivisions together to form the five fields shown in Fig. 5.

L. de No[143] also discovered that the two main systems of afferents coming from the entorhinal cortex and cingulate cortex arrived in parallel, implying that the hippocampal formation could be split up into a pile of segments each with a similar 2-D structure (Fig. 6 A). L. de No considered that the afferent fibres to the hippocampus - as with those to other parts of the nervous system - would not be completely segregated in their areas of termination but would exhibit a degree of "partially shifted overlap".[142] Although the afferent fibres were overlapping the input would be focused physiologically partly because of the increased synaptic density in the centre of the overlapping fields, but also possibly from inhibitory interneurones providing lateral inhibition. Thus it is possible for there to be a point to point projection provided the nerve cells have a high threshold and only discharge when a high number of their excitatory synapses are active.

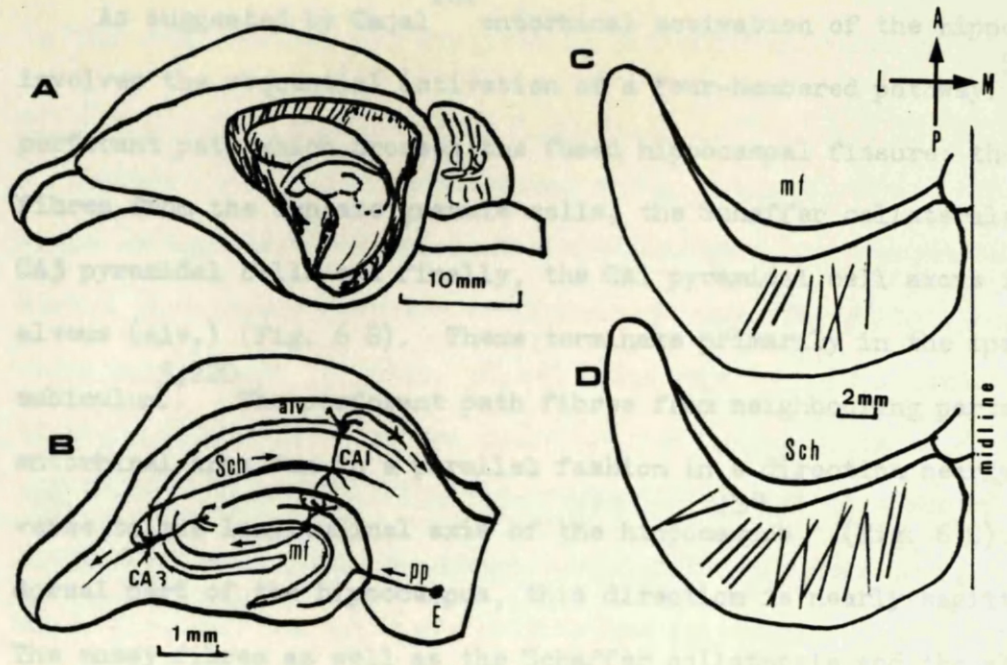


Fig.6 Anatomy of the hippocampal formation. A - Lateral view of the rabbit brain with the parietal and temporal neocortex removed to expose the hippocampal formation. The indicated section of the hippocampal formation is enlarged in B to show the main neuronal elements. In C and D each line represents a full experiment with electrical recording or stimulation from a series of tracks. C gives the direction of mossy fibres and D that of the Schaffer collaterals. 56

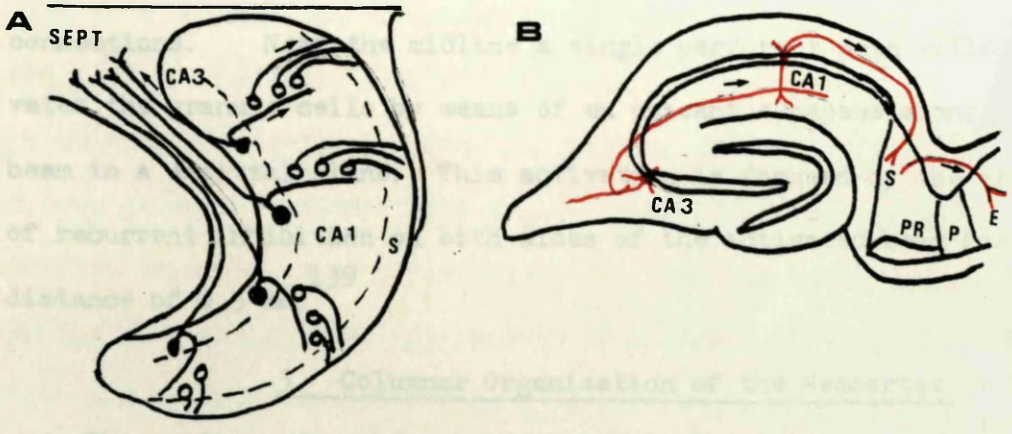


Fig.7 Distribution of CA3 and CA1 axons. A - Diagram of a dorsal view of the hippocampal formation showing the distribution and orientation of axons of CA3 cells (large filled circles) and CA1 cells (open circles). The dashed lines represent the CA3-CA1 border and the border between CA1 and the subiculum. B - Diagrammatic transverse section of the hippocampal region, showing an approximation of the histological relationship of CA3 and CA1 cells and axons. 6,56

d. Lamellar organisation of hippocampal formation

As suggested by Cajal[181] entorhinal activation of the hippocampus involves the sequential activation of a four-membered pathway:[5,6] the perforant path which crosses the fused hippocampal fissure; the mossy fibres from the dentate granule cells; the Schaffer collaterals of the CA3 pyramidal cells and finally, the CA1 pyramidal cell axons in the alveus (alv.) (Fig. 6 B). These terminate primarily in the ipsilateral subiculum.[5,220] The perforant path fibres from neighbouring parts of the entorhinal area run in a parallel fashion in a direction nearly transverse to the longitudinal axis of the hippocampus[139] (Fig. 6 C). In the dorsal part of the hippocampus, this direction is nearly sagittal. The mossy fibres as well as the Schaffer collaterals and the alvear fibres were all found to run in the same direction (Fig. 6 D). Thus a point source of entorhinal activity projects its impulses through the four-membered pathway along a slice, or lamella, of hippocampal tissue oriented normally to the alvear surface (Figs. 6 & 7). Confirmation of this organisation came when it was shown that this four-membered neuronal loop was maintained intact when 300 - 400 μm slices were maintained in an incubation chamber.[211] By means of this lamellar organisation small strips of the hippocampus are thought to operate as quasi-independent units, although some cross-talk is possible because of longitudinal connections. Near the midline a single perforant path volley activates the granule cells by means of en passant synapses along a narrow beam in a sagittal plane. This activation is focused by the presence of recurrent inhibition on both sides of the activated beam for a distance of 0.5 mm.[139]

3. Columnar Organisation of the Neocortex

The evidence for the columnar organisation of the neocortex has been dealt with in a number of recent review articles[223,116,158,55] and so will only be briefly dealt with here. Only historical and morphological aspects

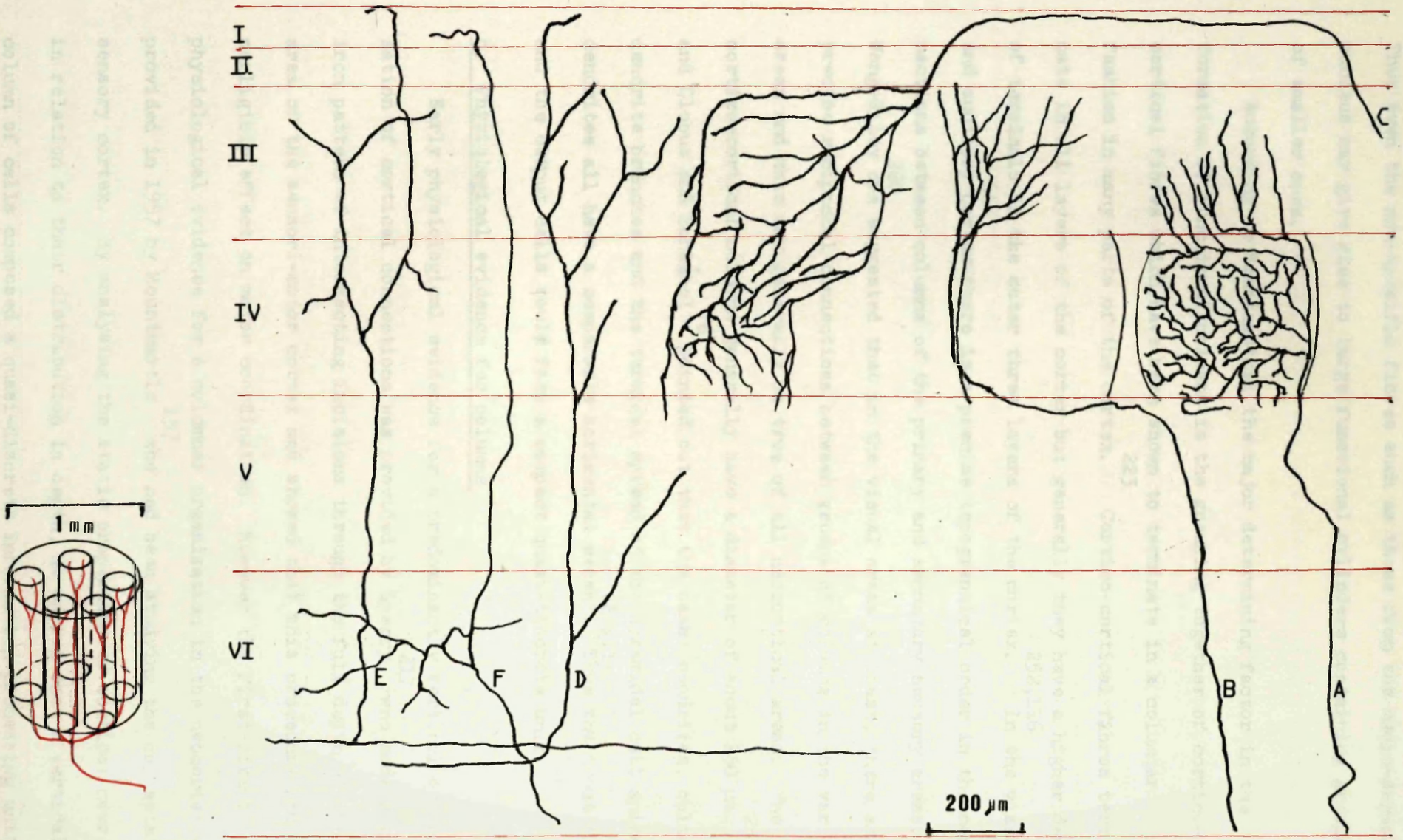
will be described. The more recent evidence for a columnar organisation in the mouse cortex will be given in the section I discussion.

a. Relationship of columns to bundles of afferent fibres

Lorente de No[142] in his classical study of the entorhinal cortex, which he considered was basically the same as other neocortical areas, stated that it was composed of elementary units formed by a narrow vertical cylinder of cells. These units were each constituted of an afferent fibre, the efferent cells that would carry the impulses further and the intracortical cells which regulate the efferent cell discharge. As each afferent fibre generates a ramification with roughly the shape of a cylinder, the units are cylindrical in shape and each one contains every element of the cortex and is responsible for carrying out all the elementary functions. Lorente de No considered that adjacent cylinders could overlap almost entirely when they belonged to two adjacent afferent fibres, but even in the case of extreme overlapping there would be some differences between the two cylinders, i.e., some neurones or neurone chains that were in connection with only one of the afferent fibres.

The different types of afferent fibres giving rise to cortical cylinders were not specified although one type which obviously gave rise to a cylindrical organisation was the specific thalamic afferents (Fig. 8).[141] Later on Scheibel and Scheibel[199] described what they presumed to be nonspecific fibres from the thalamic reticular formation. These fibres generate a number of parallel pathways after dividing into four or five branches in or below layer VI. They run mainly perpendicular to the cortical surface and produce considerable divergence. Each fibre produced an arborisation within a large area of roughly cylindrical shape with a diameter of 1 mm or more. These fibres were seen in the rat and mouse brains and the branches resembled the nonspecific fibres described by Lorente de No in the mouse neocortex[144] (Fig. 8 fibre E).

Fig.8 Afferent fibres to the mouse barrel cortex as illustrated by Lorente de No.[141] A - Specific thalamic afferent with a glomerular arborisation terminating largely within a single barrel in layer IV but also in the base of layer III. B - Afferent which terminates in layer III and avoids coming into contact with the neuropil of the barrel hollows. C - Afferent which ascends to layer I before descending to form a terminal plexus in layers III and IV. D - Fibre possibly of cortico-cortical origin that terminates mainly in the upper three layers of the cortex. E - Fibre which ascends to layer I and which may correspond to the non-specific thalamic fibres described by Scheibel and Scheibel.[199] F - Afferent fibre which terminates mainly in layer VI but which also ascends to layer I. Fibres C - F are apparently not specifically related to the layer IV barrels. At the left hand side the small diagram represents the cylindrical organisation of the non-specific thalamic afferents described by the Scheibels. Thus the axon branches appear to be organised in small cylinders of tissue arranged to form a larger cylinder.


I
II
III
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V
VI
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C
D
E
F
1 mm
200 µm

Thus even the non-specific fibres such as those from the medio-dorsal nucleus may give rise to large functional cylinders containing groups of smaller ones.

According to Szentagothai the major determining factor in the formation of functional columns is the grouping together of cortico-cortical fibres which have been shown to terminate in a columnar fashion in many parts of the cortex.[223] Cortico-cortical fibres terminate in all layers of the cortex but generally they have a higher density of terminals in the outer three layers of the cortex.[252,116,156,260] In the visual and auditory cortex[64] there is a precise topographical order in the connections between columns of the primary and secondary sensory areas. Wong-Riley[260] has suggested that in the visual areas at least, there are precise reciprocal connections between groups of columns in the various areas and this may generally be true of all neocortical areas. The cortico-cortical columns generally have a diameter of about 250 µm[252,223] and Globus and Scheibel[76] pointed out that the basal dendrites, oblique dendrite branches and the terminal spread of the pyramidal cell apical dendrites all have a comparable horizontal extent.(Fig.1) Thus the input fibres and the output cells could form a compact quasi-discrete unit.[116]

b. Physiological evidence for columns

Early physiological evidence for a predominantly vertical organisation of cortical connections was provided by Sperry[217] who made a grid-iron pattern of intersecting incisions through the full depth of the arm area of the sensori-motor cortex and showed that this procedure had a negligible effect on motor coordination. However the first direct physiological evidence for a columnar organisation in the neocortex was provided in 1957 by Mountcastle[157] who had been studying the cat somato-sensory cortex. By analysing the static properties of cortical neurones, in relation to their distribution in depth, he showed that a vertical column of cells composed a quasi-discrete information-processing unit,

which had only limited lateral spread. When microelectrode penetrations were made normal to the cortical surface all the neurones encountered were of the same modality type and had almost identical peripheral receptive field centres. Furthermore the latencies of the responses of cells in the different cortical layers to stimulation of the peripheral field centrewere within 2 to 4 msec. of each other: the shortest latencies occurred in layer IV where the thalamic afferents terminate.

Initially Mountcastle did not define the shape of the columns very precisely. He did later state[159] that in the medio-lateral direction the columns were sharply demarcated by abrupt changes in the submodality being processed, but he emphasised that this strict demarcation would only hold true for the primary evoked activity of short latency. He also produced evidence that activation of one column produced inhibition in immediately surrounding columns.[159] Since then other workers have obtained electrophysiological evidence of columns in the visual,[107,106] motor[12] and auditory[113] areas of the cortex.

A columnar organisation was demonstrated in the barrel cortex of the mouse by Durham and Woolsey[53] in 1977. By removing various combinations of whiskers in freely behaving mice and studying alterations in the radioactive 2-deoxyglucose (^{14}C2DG) uptake, they showed that the barrels were part of a column of activity stretching through all six layers of the cortex. Confirmation of this arrangement was obtained in electrophysiological studies of the barrel cortex of the neonatal[10] and adult rat.[209] Most recorded units in a vertical penetration were activated by the deflection of a particular vibrissa. In layer IV 85% of the cells responded to one vibrissa only[209] and this corresponds with the number of cells which have been shown to have dendrites confined to the interior of one barrel[166] (see below). Simons[209] also showed that a functional hierarchy exists within the whisker barrel cortex of rats that is based, at least in part, on a sequential processing of information that proceeds from the barrels of layer IV to the superficial and deep cortical layers.

c. Morphological basis of columns

Jones[115] has pointed out that there are at least two cell types the dendrites of which are located in layer IV and which have an axonal arborisation terminating in a vertical cylinder above and below layer IV respectively. Lorente de No[141] described examples of such cells in the mouse barrel cortex (Fig. 9 cells C and D and Fig. 10 cell X) and these may form the morphological basis of the vertical spread of electrical activity from layer IV into the adjacent layers.

In a study of the mouse visual cortex Valverde[230] described five classes of non-pyramidal cells with intracortical axons. All five classes were composed of cells the axons of which terminated within a vertical cylinder of tissue having a diameter of 200 - 300 μm. Some of these cells will be excitatory[59] but other studies[171] indicate that most are probably inhibitory and that at least some contain the inhibitory transmitter GABA.[59] Such cells could provide the pericolumnar inhibition postulated[159] to act in focusing the cortical input into discrete functional columns.

The barrel cortex is the cortical area where the most detailed studies of cellular morphology have been made.[266] The whisker barrels are in some respects similar to the glomeruli of the olfactory bulb, being composed of hollow cylinders of cells surrounding an area of specific thalamic termination.[125] However they are more complicated as they involve more cell types. Woolsey[166] made a detailed Golgi study of the cells within layer IV of the barrel cortex and described eight cell types. These are divided into two cell classes - class I had a modest complement of dendritic spines and an axon that descended with respect to the pia: class II had sparse spines and thick, smooth varicose dendrites and an ascending thick axon. There were equal numbers of class I and class II cell types. Within each class cells could be partitioned in the same proportion: 85% of the cells had their processes restricted

to a single barrel and 15% of the neurones had their processes ramifying in more than one barrel. Of the cells with dendrites restricted to one barrel some had radial dendritic patterns (type C in Fig. 9) and some had eccentric dendritic patterns (type B in Fig. 9). Of the other cells, type Y (Fig. 10) had its cell body within the barrel whereas type D (Fig. 9) had its body in the spaces or septa between barrels. As there are about 2,000 neurones per barrel then each barrel contains the following numbers of neurones:

IC	480	IB	360	IY	100	ID	100
IIC	480	IIB	360	IIY	100	IID	100

Thus the ratio of cell types is 24:18:5:5 and there is the possibility that each barrel and hence each column, could be subdivided into 20 subgroups. In studying the motor cortex of cats Asanuma[11] found that neurones in layers II and III only have synaptic contacts with neurones that are within a distance of 0.5 mm. The connections between neurones in this volume was highly specific with on average only one in 30 neurones making contact with a given cell. This implies that any one macro-column of the motor cortex may be formed by a group of smaller columns. Evidence for this was also obtained in the cat visual cortex where Hubel and Wiesel[107] found evidence that the orientation columns or slabs could be subdivided into about 18 mini-columns.

Roney and Scheibel[193] have suggested that groups of closely apposed apical dendrites are related to mini-columns. This idea was first put forward by Peters and Walsch[172] who along with Fleischhauer et al[65] were the first to show bundles of apical dendrites in the neocortex. In the rat[172] the apical dendrites are clustered together in groups of varying number (4 -14) to form a bundle with a diameter of about 30 µm (Fig. 1). The dendrites within a bundle course along very closely together[193] (usually from 1 µm apart to direct conjunction). It is likely that they share a common afferent input[172] and so they are thought to form a

Fig.9 Four cells the processes of which are thought to be in register with the layer IV barrels. Cells A and C have descending axons and a symmetrical dendritic tree. The apical dendrite of cell A ascends towards layer I and its axon descends into the white matter underlying layer VI. The dendrites of cell C are confined to a single barrel within layer IV and its axon does not leave the cortical grey matter. Cell B is similar to A except that its soma is located in the barrel walls and its dendrites all leave from one side to terminate within a single barrel. Cell D has symmetrical dendrites and an ascending axon. All cells are copies of those illustrated by Lorente de No.[141] The dendrites of all cells in Figs. 9, 10 and 13 are shown with spines to differentiate them from the axons even although some of the actual cells would have aspinous dendrites.

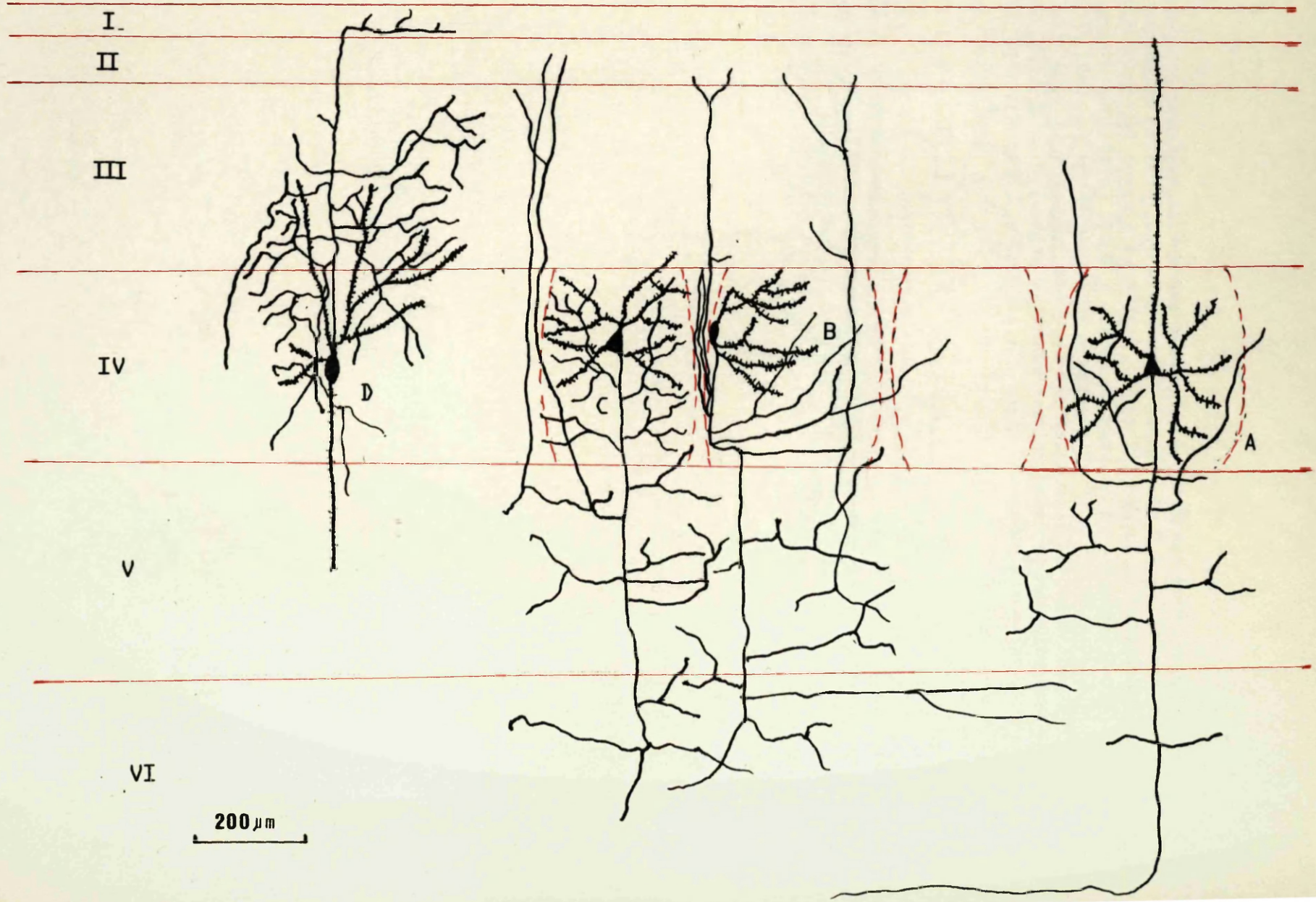
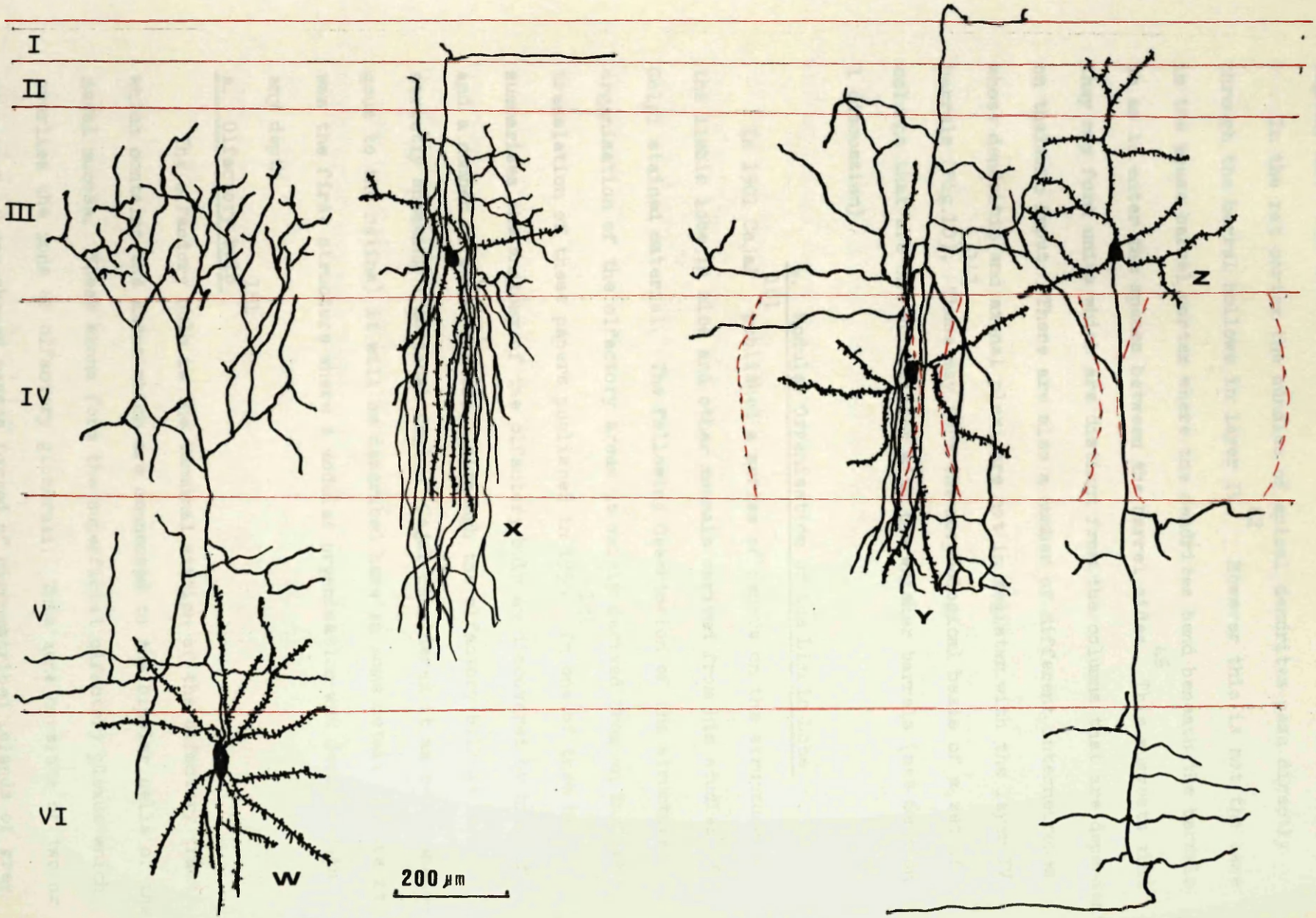

I
II
III
IV
V
VI
D
C
B
A
200 µm

Fig.10 Four cells whose processes are not in register with the layer IV barrels. Cell W is a layer VI stellate cell with an ascending axon that terminates in layers III to V. Cell X is a layer III cell with an axon which ascends to terminate in layers I - III before descending to form a 100 μm wide cylinder of terminals in layer IV and V. Cell Y is a layer IV stellate cell the dendrites of which terminate in more than one barrel and which has an axon which terminates primarily in layers III and Va. All cells are copies of those illustrated by Lorente de No.[10]


I
II
III
IV
V
VI
W
X
Y
Z
200 µm

tightly knit functional unit.

In the rat cortex the bundles of apical dendrites pass directly through the barrel hollows in layer IV.[62] However this is not the case in the mouse barrel cortex where the dendrites bend beneath the barrels so as to enter the spaces between the barrel sides.[46] This suggests that they may form units which are distinct from the columns that are dependent on thalamic input. There are also a number of different interneurones whose dendritic and axonal plexi are not in register with the layer IV barrels (Fig.10).[141] These may form the morphological basis of a set of columns that are out of register with the whisker barrels (see Section 1 discussion).

4. Modular Organisation of the Limbic Lobe

In 1901 Cajal[181] published a series of papers on the structure of the limbic lobe in mice and other mammals derived from his studies of Golgi stained material. The following description of the structural organisation of the olfactory areas is mainly derived from an English translation of these papers published in 1955.[181] In one of them he summarises the anatomy of the olfactory bulb as discovered by himself and a number of other workers. Although the olfactory bulb is not strictly speaking a cortical region (Cajal considered it as being analogous to the retina) it will be described here in some detail because it was the first structure where a modular organisation was described in any depth.

a. Olfactory bulb[181]

The olfactory bulb is the terminal station of the olfactory tract which contains the axons which are connected to the bipolar cells of the nasal mucosa. These axons form the superficial olfactory plexus which overlies the zone of olfactory glomeruli. This zone consists of two or more rows of egg-shaped masses formed of circumscribed islands of grey matter. The glomeruli are formed by the aggregation of cell bodies which

form a hollow structure similar to the bowl of a goblet (Fig. 11). Within this hollow a group of olfactory fibres terminate in dense ramifications on the dendritic tufts of the tufted and mitral cells whose cell bodies are found in the two underlying layers. This structure, composed of a dense interdigitating plexus of axonal and dendritic terminals, is also seen in lower vertebrates and will permit a high degree of security in synaptic transmission. Cajal also emphasised the other conclusion that can be drawn: namely, that in this system at least, synaptic transmission does not really occur between individual cells but rather between small bundles of axons and small groups of cells.

The walls of the glomeruli are composed of the cell bodies of small stellate cells whose dendrites arborise within one or more glomeruli. The axons of these cells travel laterally and branch to terminate either within or between the glomeruli. Thus as well as the groups of elements forming a strongly connected vertical pathway there is also a significant lateral pathway which allows interaction between the parallel throughput channels. Cajal also provided evidence for a lateral interaction mediated by the axons of tufted cells passing via the anterior commissure from the contralateral side.

b. Olfactory tubercle[181]

An alternative modular arrangement consists of islands of cells separated by cell poor zones. Following the observations of Calleja Cajal described such an organisation in the olfactory tubercle of several mammals including the mouse. The clusters of cells may occur mainly in the second layer which is composed of small and medium pyramids but sometimes the islands of cells extend into the superficial layer and also occur in the third layer. These islands of Calleja receive a dense plexus of terminal fibres of unknown origin. Again this area is not strictly part of the cerebral cortex because although laminated it is in some ways more closely related to the striatum than the prepiriform

cortex.[92b] By contrast dopaminergic fibres produce a dense plexus of fibres surrounding the islands of Calleja while the islands themselves only receive a sparse innervation of dopaminergic fibres.[61]

c. Pyriform lobe[181]

Hammerberg also described islands of cells separated by bundles of white matter in the human hippocampal gyrus (pyriform lobe of animals). In layer II of the internal regions of the gyrus the well defined islands form two planes. The superficial one contains islands of small pyramids while the deeper has islands of large pyramids. The terminal plexi of axons from intracortical cells of both more superficial and deeper layers are confined to the discrete clusters of cells so that the input is precisely correlated with the cellular organisation. In small mammals the pyriform lobe has a similar arrangement except that the islands of cells are not so obvious and appear rather as condensations of cells than as discrete clusters.

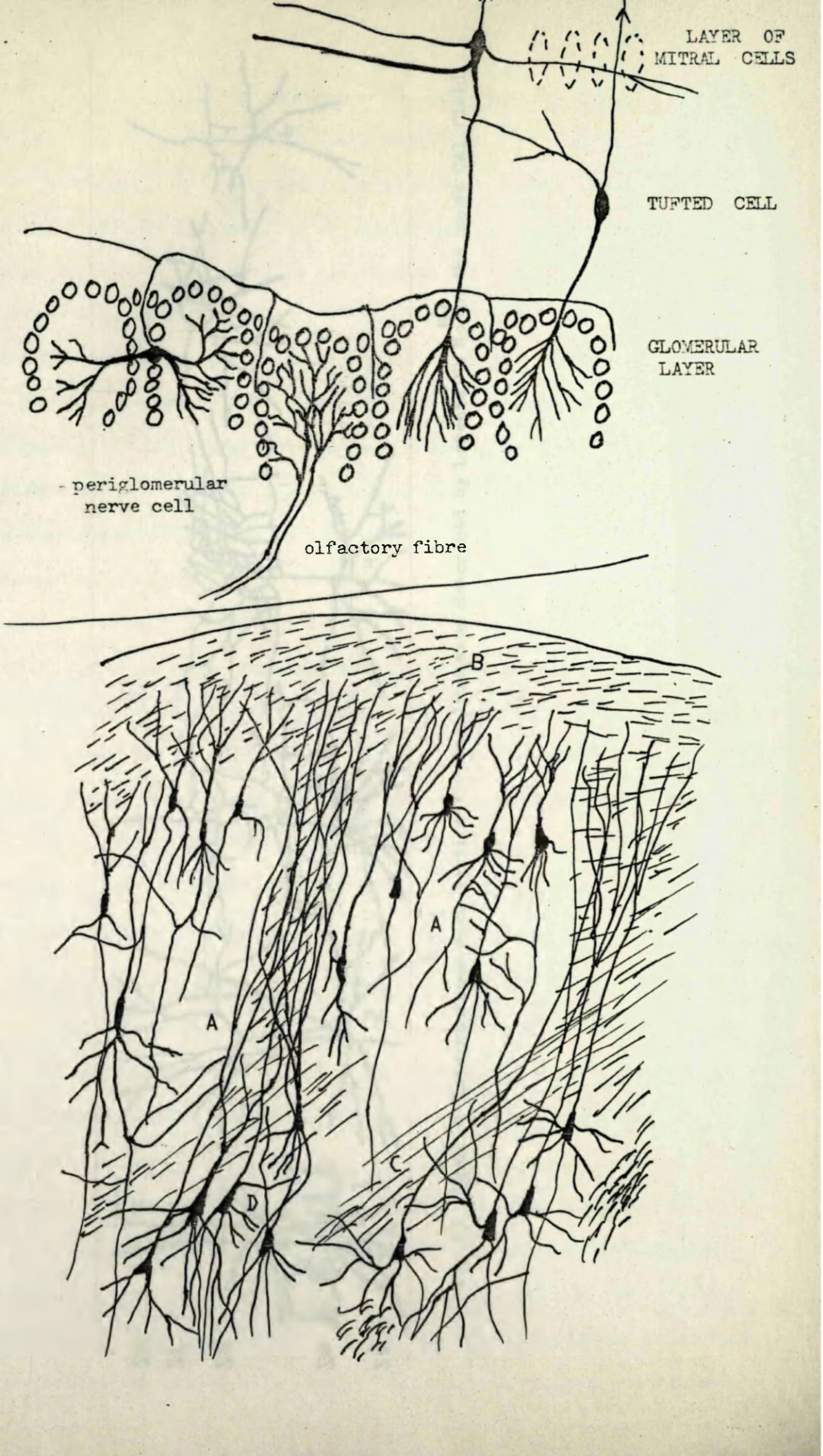
d. Hippocampal region

Cell clusters associated with vertically ascending fibres have been described in the entorhinal cortex,[20] postsubiculum[194] and subiculum.[153] There is also evidence for a columnar organisation in the cingulate cortex[18] that is dependent on vertically ascending fibres. The evidence for this modular organisation will be described in greater detail in the Section II discussion.

Therefore in many fields of the limbic lobe in both humans and lower mammals a general organisational principle seems to be the aggregation of cells into more or less discrete clusters each of which has specific axonal arborisations associated with it. These may be of both external and local origin. The local interneurones provide both a vertical and lateral transmission of information but the external afferents associated with the cell clusters are primarily oriented in a vertical fashion. The main area where this organisational principle is least in evidence is the hippocampal formation both in the hippocampus and dentate fascia. Nevertheless although the major afferent and intracortical pathways in the hippocampal formation are arranged horizontally with respect to the layer of cell bodies, there are vertically ascending afferent fibres in the hippocampus[143] and vertically ascending intracortical afferents in the dentate fascia.[182] These vertically ascending fibres could give rise to functional modules even although there is no evidence of cell clusters.

Fig.11 Glomerular layer of the olfactory bulb as described by Cajal (1956).

Fig.12 Diagram of human subiculum as described by Cajal.
A - Island of small pyramids.
B - Mass of superficial white matter.
C - Bundle of fibres that descends to deep white matter.
D - Medium and large pyramids the dendrites of which rise through the septa of white matter.


LAYER OF
MITRAL CELLS
TUFTED CELL
GLOMERULAR
LAYER
periglomerular
nerve cell
olfactory fibre
B
A
A
C
D

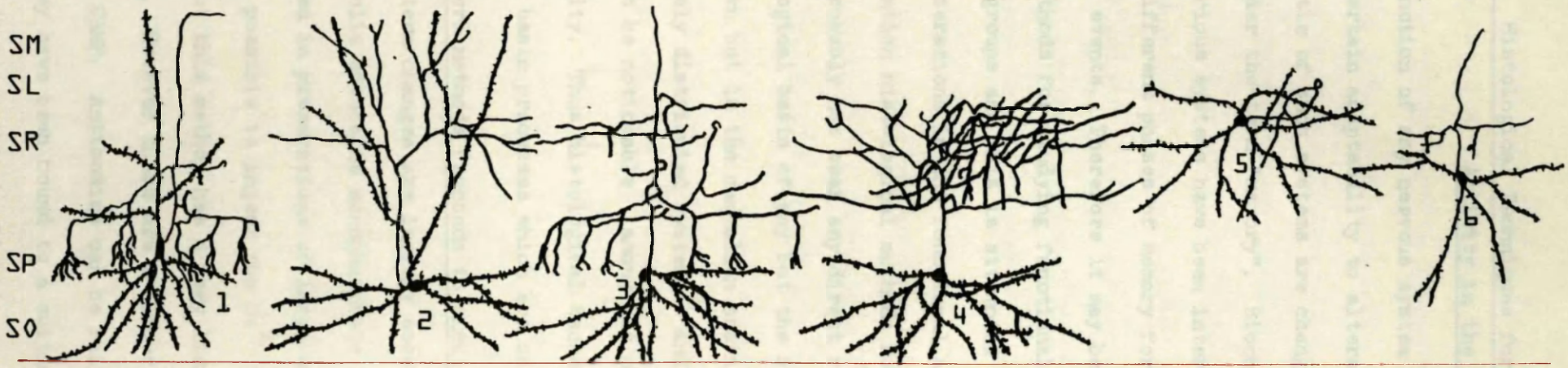


Fig.13 Types of interneurones with vertically ascending axon described by Lorente de No [141] in the mouse CA1 field.

5. Histological Techniques for Studying Functional Activity in the Brain

The function of any nervous system however simple or complex is to provide a certain adaptability to alterations in the environment. Characteristic of such systems are changes which are loosely gathered together under the term "memory". Biochemical studies of memory formation in various systems have been interpreted as showing[75,198] that there are three different phases of memory formation associated with various biochemical events. Therefore it may be useful to divide the various possible methods for studying functional activity in the brain into three comparable groups and this is attempted in the following paragraphs. Although alterations in the functional activity of the brain result in memory formation histological methods for demonstrating functional activity will probably not bear any direct relation to memory formation. The morphological basis of any but the simplest[137] forms of memory formation is not known but in the mammalian brain memory formation is thought to involve widely distributed systems[57] and the small changes involved would probably not be noticeable among the more general alterations in metabolic activity. Thus histological techniques will demonstrate alterations in numerous basic processes which are only indirectly related to memory.

a. Short-term methods (seconds to minutes)

Short term changes are largely associated with the second messengers Ca^{2+} and cyclic adenosine monophosphate (CAMP).[85] Calcium levels have been observed in preparations of large cells such as the squid axon[28] where it is possible to inject the Ca^{2+} sensitive luminescent protein aequorin, but this method has a very limited applicability for assessing Ca^{2+} levels. However there are a number of immunocytochemical methods relating to CAMP. Antibodies can be raised against cyclic nucleotides provided they have been bound to a suitable protein molecule beforehand.[218] These antibodies can then be linked to peroxidase enzymes or fluorescent

molecules to provide methods for the demonstration of cyclic nucleotide levels. However such methods have limited use partly because of the difficulty of fixing cyclic nucleotide molecules, but also because the antisera tend to be rather nonspecific and there is the danger of cross reactivity with other molecules such as ATP. Adenylate cyclase activity has been demonstrated histochemically in nervous tissue[184] but as the cyclase enzyme is generally only active in the presence of a receptor complex with a bound neurotransmitter or neuromodulator molecule and these would normally dissociate from the receptor in the incubation medium, such a method is probably of little use in studying short term changes in functional activity.

Of potentially greater interest is the development of immunocytochemical methods for studying the cellular distribution and levels of the regulatory and catalytic subunits of soluble CAMP-dependent protein kinases. These kinases are activated by CAMP binding to the regulatory subunits.[85] This results in the dissociation of the holoenzyme to yield a dimeric CAMP-regulatory subunit and two free catalytic subunits.[16] Antisera have been raised[218] against the holoenzyme and the separate regulatory and catalytic subunits. Therefore the degree of activity of the CAMP-dependent kinase can be assessed.

b. Intermediate-term methods (minutes to hours)

The most widely used technique for studying functional activity is the 2-deoxyglucose method[214] which makes use of the regional changes in glucose consumption that occur during central nervous activity.[213] ^{14}C -2-deoxy-D-glucose is a labelled structural analogue of glucose that was first introduced by Sokoloff and his associates[122,214] in their studies on regional changes in cerebral blood flow. Since its introduction there have been a large number of studies using the method to study various anatomical pathways and structures within the brain.[88,213,175]

The deoxyglucose molecules are transported across the blood-brain

barrier by the same carrier as glucose and are then phosphorylated to deoxyglucose-6-phosphate.[214] The rate of breakdown of this molecule in the brain is negligible and so the deoxyglucose is effectively trapped in the cells in the phosphorylated form which cannot diffuse across cell membranes.

The major disadvantage of the deoxyglucose technique is its limited resolution.[119] As with all autoradiographic and bioluminescent techniques radiation from a point source is emitted as a sphere so that the image of a point source is formed as a circle on the photographic emulsion. The diameter of the circle depends on the radius of the sphere and so high resolution can only be obtained with a low energy tritiated source. Using ^{3}H -2-deoxyglucose individual neurones have been detected[186,54] but even with a tritiated source the resolution is limited by the highly diffusible nature of the deoxyglucose. Therefore according to Jones and Hartman[119] "the identification of active synapses may ultimately depend upon the introduction of a glucose analogue that is converted into a larger molecule that may then be held in the tissue by aldehyde or other fixatives." No such compound has been reported.

Although ^{14}C glucose is converted into labelled glycoproteins and other macromolecules that could be fixed by aldehydes, increased glucose utilisation appears to be associated with a decreased incorporation of glucose into macromolecules.[203] So far the best experimental results using ^{14}C glucose have been achieved using short experimental periods of under 10 min.[91] Even shorter experimental times can be used with inert tracers used in cerebral blood flow studies such as ^{14}C-antipyrine.[187] However such methods have much lower resolution than the deoxyglucose technique. Metabolites such as ATP or nicotinamide adenine dinucleotide (NADH) can also be used as markers of functional activity. Kogure and Alonso[127] have developed a technique for the bioluminescent demonstration of ATP using an extract from fireflies' tails, while Welsh[247] and his colleagues are able to demonstrate NADH by the fluorescence of

this compound at a particular wavelength. However at the moment both these techniques have a low resolution.

Another technique, the basis of which is not understood, is the use of basic dyes such as cresyl violet or gallocyanin to stain the Nissl substance of cell bodies. Under abnormal conditions such as the induction of an epileptiform focus the intensity of staining in the cell body increases.[44] This is not due to an increase in messenger ribonucleic acid (mRNA) as this species of RNA only forms about 2% of the cellular RNA. One possible explanation is that there is an increase in the phosphorylation level of ribosomal proteins as this has been shown to occur during increased activity in some cellular preparations.[190] Increasing the number of negative groups on the proteins would give them a much higher affinity for the basic dyes.

One further method which has apparently not previously been used to study intermediate-term changes in the brain's activity is the histochemical demonstration of phosphorylase a levels. The basis of this method will be discussed in detail in the final part of this literature review.

c. Long-term methods (hours to days)

There are a large number of histochemical methods which have been used in studying long term changes in neurones and only a few will be mentioned. One of the most widely used histochemical markers is cytochrome oxidase which has been used to study functional deafferentation in a number of sensory systems including the cat visual system[261] and the mouse somatosensory system.[262] Studying the increases in the amounts of certain enzymes during development has been used to map certain pathways in the brain. For example, by studying the time course of appearance of succinate dehydrogenase activity in neonatal rats, Killackey and Belford[126] succeeded in mapping the maturation of the trigeminal pathways from periphery to cortex. Direct measurement of protein

synthesis by the use of radioactive amino acids can be used in similar mapping studies. In studying the immature monkey visual system with radioactive leucine Kennedy[124] and his colleagues found that unlike glucose, the rate of protein synthesis does not appear to reflect short term changes in functional activity. Protein synthesis is apparently altered only indirectly and after a long period (more than 3 hr) of visual deprivation.

The original concept of the cortical column was of an ephemeral collection of cells functionally connected in a vertical direction.[157] The transience of the sturcture usually precludes its demonstration by long-term functional methods unless its lifetime can be prolonged artificially by a process such as functional or structural deafferentation of adjacent columns.[261]

6. Phosphorylase a as a Marker of Functional Activity in Nervous Tissue

Histochemical method for phosphorylase

In the intact cell phosphorylase is one of the three enzymes responsible for breaking down glycogen to glucose-1-phosphate.[219] As glycogen is a highly branched structure there are many terminal residues for phosphorylase to act upon and so produce a rapid supply of glucose-1-phosphate when required. However as phosphorylase is a very specific enzyme it can only cleave the α 1, 4 linkages to within four residues of a branch point. Thereafter a transferase and glucosidase are required to produce an unbranched chain for the phosphorylase to act on.

In the histochemical medium, where the reaction is reversed by having a low pH and a high glucose-1-phosphate concentration,[152] phosphorylase by itself could only produce long unbranched chains. Furthermore phosphorylase can only add further residues to a previously existing polymer: it cannot join two individual residues together.[219] Therefore in the tissue being studied, for any new glycogen to be synthesised there must be present,[168] precursor polyglucose chains with a

length exceeding four residues as well as a branching enzyme. Of 17 published variations on Takeuchi's original method[225] for demonstrating phosphorylase activity most include glycogen in the incubation medium.[112] However in his study of infarcted cardiac muscle Meijer[152] showed that exogenous glycogen could not act as a glucosyl acceptor in anoxic glycogen depleted tissues. When brain tissue is removed for microscopical study then even with the rapid fixation required by tissue to be examined under the electron microscope, most neurones are depleted of their glycogen before adequate fixation is achieved.[173]

These findings were confirmed by Ibrahim[112] in the brain. He found that in the rat neocortex no phosphorylase activity could be demonstrated in ischaemic hypoxic tissue after the external carotid had been ligated. Both he and other workers[204,69] did find some phosphorylase in neurones as well as glial cells in the brain, but the staining was not consistent. Some pyramidal cells in the hippocampus were strongly stained whereas other adjacent cell bodies showed no phosphorylase activity at all.[112] This may have been due to more extensive glycogen depletion in some cells compared to others.[151] It was not due to an absence of the enzyme (Fig.72). Therefore in the present study Meijer's[152] incubation medium was used as it contains the polyglucose primer dextran which does act as a glucosyl acceptor in anoxic glycogen-depleted tissues but which is not itself stained by iodine.

Apart from the introduction of dextran the two main changes in the incubation media used over the last 30 years have been the addition of ethylenediamine tetraacetate (EDTA) and sodium fluoride. Sodium fluoride, known to inhibit the phosphorylase phosphatase which catalyses the inactivation of phosphorylase *a*, was introduced by Guha and Wegmann in 1959.[168] EDTA, which inhibits the activity of phosphorylase kinase also has a direct effect on stabilising the enzyme in the incubation medium.[102] Thus the presence of these two compounds in the incubation medium prohibits the interconversion of phosphorylase *a* and *b*. Usually the

activity of the phosphorylase is monitored by staining the glycogen synthesised in the incubation medium but it is also possible to measure the phosphate released when the glucose-1-phosphate is added to the precursor molecule. This phosphate can be precipitated by iron and viewed

with an electron microscope. However this method has several disadvantages, one of which is that EDTA cannot be used in the incubation medium as it chelates out the iron ions. Therefore in the present study Meijer's incubation medium was used as this contained dextran, sodium fluoride and EDTA. It has recently been reported[159a] in "Nature" that the presence or absence of a phosphate group on specific amino acid residues can be detected immunohistochemically. Therefore it may be possible in future to study the phosphorylation state of phosphorylase by immunohistochemical procedures which are usually more sensitive than classical histochemical methods.

METHODS

1. Animal Material

Material was obtained from a total of 113 animals for use in the present study. This collection was composed of 2 baboons, 4 cats, 5 kittens, 1 rabbit, 46 rats and 55 mice. These animals, apart from the rats and mice, had been used in other physiological studies. The baboons were small imported males which were being used in cerebral blood flow experiments at the Wellcome Surgical Institute, Garscube. The cats were farm animals which were being used for teaching purposes and in an experiment on knee-joint distension. The kittens were also farm-bred and had had their eyes removed just after being given an over-dose of anaesthetic. In the case of the rabbit, abdominal organs were being removed for histological fixation and so a period of 20 mins elapsed before a start could be made on removing the brain. The rats were all Wistar albino rats bred in the Animal House at the Institute of Physiology, Glasgow University. The rats weighed between 170 and 230g. The mice were all of inbred Animal House stock and were of three different strains. They weighed between 25 and 40g. Originally Parkes (albino) mice were used, but as these mice often had defective vision and hearing when mature, latterly only CBA and C3HHE mice were used. Of the 55 mice 4 were Parkes, 3 were CBA and the rest were of the C3HHE strain.

2. Anaesthesia

The baboons, cats, kittens and rabbit were all anaesthetised with commercial forms of sodium pentobarbitone and were killed either by an overdose of the anaesthetic or in the case of two cats by injecting concentrated $MgSO_4$ solution into a venous catheter. Of the rats, ten were killed by a blow on the spine followed by decapitation without the use of any anaesthetic, while three mice were killed by decapitation

using a pair of large scissors. This procedure was considered to be completely painless but was kept to a minimum because of the danger of a misdirected blow damaging the brain. Therefore 21 mice and 6 rats were decapitated after being rendered insensible with ethyl chloride vapour. Of the remaining mice one was anaesthetised with a mixture of N_2O and halothane, two with ether, three with urethane and 25 with sodium pentobarbitone (Sagatal). Of the rats 9 were anaesthetised with sodium pentobarbitone and 21 with urethane. Most of the anaesthetised animals were brought to a level of anaesthesia where the forepaw withdrawal reflex was abolished. For 200g. rats this involved a dose of about 1.5 ml of 25% urethane made up in distilled water. For 30g. mice it usually required an initial dose of 0.3 ml of Sagatal diluted to 6mg ml^{-1}.

3. Surgical Procedures

Surgical manipulations were only carried out on the 13 anaesthetised rats used in the study of an artificially induced epileptiform focus. These animals were laid supine and a tracheotomy was performed to permit the withdrawal of fluid from the throat. Fluid often accumulated during the experiment as some animals had a mild throat infection. With the use of a scalpel a midline incision, 1.5 cm long, was made in the skin of the throat from the hyoid arches towards the sternum and the underlying sternohyoid muscles were then separated and the trachea exposed by blunt dissection. Two short lengths of cotton thread were passed under the trachea using sharp curved forceps. The trachea was opened by making a small incision with the points of a fine pair of scissors between adjacent cartilaginous rings. A 3 cm long piece of thin plastic tubing with a diameter of 2.5 mm was inserted and held in position by tying it firmly in place by means of the two threads.

Next the rat was laid prone on a water heated tablet at 37°C and the head firmly held in a stereotaxic apparatus composed of two blunt

rods for insertion into the external auditory meatus and a screw clamp to grip the snout. A midline incision 3 cm long was made from between the eyes to the base of the skull. The connective tissue overlying the skull was scraped away using a spatula and the skin flaps retracted with small clamps. The connective tissue around the anterior end of the temporal ridge was dissected away. Then the temporal muscle was separated from the skull just below the coronal suture and a small block of the muscle removed carefully so as to avoid cutting a blood vessel at the back of the eye.

Using a Kaltenback and Voight dental drill that had a small spherical burr head a circular channel with a diameter of 3 mm was drilled in the skull. When the channel had been drilled down to within a few hundred μm of the inner surface of the skull, the island of bone was removed by forceps. In most cases the bone was removed over the intersection of the coronal suture and the temporal ridge as this point lies almost directly over the primary somatosensory forepaw area. However on some occasions other holes were drilled in positions determined by reference to the maps of the rat brain provided by Welker[244] and Hall and Lindholm.[87] Using a Zeiss binocular dissecting microscope to provide a magnification of x25 or x40 the dura mater was punctured with the tip of a surgeon's triangular needle and deflected from most of the exposure to reveal the arachnoid mater. Constant irrigation with either 0.9% sodium chloride or paraffin oil, both at 37°C, was required to keep the exposed area moist. If a long experiment was planned then a paraffin pool was made by attaching the flaps of skin to a metal ring suspended about 1 cm above the skull.

Two curved No.5 needles were inserted subcutaneously into one or both forepaws between the outer digits to allow electrical stimulation. The stimuli were rectangular voltage pulses of 0.1 msec duration and with an amplitude of between 10 and 50 volts.

4. Electrophysiological Techniques

a. Apparatus

Cerebral cortical mass potentials were recorded by means of chlorided silver ball electrodes, 0.3 mm in diameter, on the cortical surface and saline filled glass microelectrodes at depth. All recordings were taken with respect to an indifferent chlorided silver ball electrode on the exposed skull. The potential difference from the one or more recording channels was amplified x 1000 by Digitimer Neurolog NL 103 preamplifiers. The output was led to a Tektronix dual beam oscilloscope with either 2 or 8 recording channels. Photographic records were taken with a Nihon Kohden PC-2A camera using Kodak high contrast RAR estar base film. Stimulation to the rat forepaw was from a Devices isolated stimulator Mk. IV. A Devices digitimer was used to synchronise this stimulation and the recording equipment.

b. Penicillin and extracellular dye application

In many cases the penicillin was applied by soaking a small (1 mm^2) piece of filter paper in a concentrated (300,000 units ml^{-1}) solution of sodium benzyl penicillin and placing it on the exposed cortex. In a few experiments the penicillin was kept relatively localised by applying it via a penicillin filled glass microelectrode. The microelectrode was filled with a mixture of penicillin at a concentration of 500,000 units ml^{-1} and 2% Pontamine Sky Blue dissolved in 0.5 M sodium acetate. Recording electrodes were filled with the same solution minus the penicillin. The extracellular dye and penicillin-dye mixture were ejected from the glass microelectrodes using D.C. currents of between 0.5 and 4 uA. A D.C.-current source was used to provide a driving voltage of up to 200 volts to the iontophoresis adapter of a Transdyne General Corporation Neuroprobe Amplifier 1600. A chlorided silver wire was used to connect the head stage of this amplifier to the dye solution.

The current was applied with respect to earth and the electrophoretic current was monitored during the period of dye ejection.

c. Electrodes

All microelectrodes were made from borosilicate glass capillaries ("Kwik-Fil", Clark Electromedical Instruments, 2GC 150F-10) and pulled in a laboratory-made vertical puller. This had been constructed to allow two separate extensions of the microelectrode. For both recording and penicillin application purposes the tips of the microelectrodes had to be broken back to specific outside diameters (O.D.). To do this a polished metal bar was placed with its end within the focal plane of the low power lens of a microscope. The microelectrode was mounted on the microscope stage and advanced to within a few μm of the metal bar. Then the bench was given a sharp tap. The resultant vibrations removed the tip of the electrode. By altering the distance of separation and the size of the bang a tip size of 2 μm could be regularly produced and these electrodes were then filled and after checking that their resistance was below 20 MΩ they could be used for recording. For the penicillin electrode a tip diameter of 4 - 6 μm was used. This allowed a larger current to be passed during penicillin application and meant that the electrode was less likely to get clogged. The electrode always became clogged unless the dye solution had been carefully filtered immediately before the electrode was filled. Millipore type RA filters with a mesh diameter of 1.2 μm were used. With the penicillin electrodes a current of 2 - 4 μA was passed for 2 minutes in order to produce a small epileptogenic focus and leave a blue dye-spot. With the recording electrodes a current of 1 - 2 μA was passed for from 2 to 5 min.

5. Visual Stimulation

The first mouse of the group was immobilised by strapping it to a wooden block. Its right eye was occluded and then it was left in the

dark for five minutes. Subsequently a slide projector was used to flash a pattern of black and white vertical lines on to a screen placed at a distance of 30 cm. The pattern was 15 cm long and so the angle subtended at the eye was approximately 30°. This pattern was flashed on and off manually for a period of five minutes. There were 31 black lines separated by white lines of the same width. Therefore each line subtended an angle of $\frac{1}{2}^{\circ}$.

The second mouse was anaesthetised with 0.15 ml of 25% urethane. The right eye was occluded and the other stimulated by black and white vertical lines each subtending an angle of about $3/4^{\circ}$ at the eye. Stimulation was achieved by illuminating a photographic print at a frequency of 3 Hz by a stroboscope for a period of 5 minutes. The third mouse was also anaesthetised with urethane before having its right eye occluded and being stimulated for 7 minutes by a pattern of vertical lines each of which subtended an angle of 3° at the eye. A fourth mouse anaesthetised with urethane and left in the dark for 15 minutes served as a control. In each case coronal sections were cut through the visual cortex of both sides of the brain.

Of the four adult cats two had one eye occluded and the others served as controls. The eye of one cat was closed by putting a couple of stitches through its eyelid fifty minutes before it was killed, while the eye of the other cat was covered with a patch for ten minutes prior to death. After the animals had been killed blocks of brain were removed from the primary visual cortex as defined by Hubel and Wiesel.[105] Some of these blocks, which came from both hemispheres, were flattened to permit surface parallel sections to be cut, while other blocks were sectioned in a coronal plane. All blocks were frozen between 15 and 30 min. after the administration of an overdose. Blocks were also cut from the visual cortex of the kittens which ranged in age from 6 days to 61 days and these served as other controls. Blocks were also cut from the parietal cortex of the kittens.

6. Vibrissae Removal

In one mouse 3 whiskers were cut off at their base in the central row of the right-hand muzzle after the mouse had been rendered insensible with ethyl chloride vapour. It was then left in a darkened shallow box with an assortment of objects for it to investigate for 40 mins. before being killed. The second mouse had all but three of its whiskers cut off at their bases and was again placed in a darkened box for 40 mins. before decapitation. Subsequently surface parallel sections were cut through the somatosensory cortex of both sides of the brain.

7. Phosphorylase Histochemistry

a. Removal of brain

The usual procedure in the case of mice and rats was to decapitate the animal, cut away the skin lying over the top of the neck and head and then insert pointed scissors into the foramen magnum so that the roof of the skull could be split along the midline. Next, rongeurs were used to crush the skull between the two eye sockets and the scissors again inserted to cut along the midline of the skull. Care was taken not to cut too far in from either end and so, although the cerebellum and olfactory bulbs were damaged, the cerebral hemispheres were usually removed intact. Splitting the skull also usually resulted in the dura mater being torn along the midline. If this had not occurred then the dura was cut with fine scissors before the skull was levered open and the brain eased out with a spatula. The brain was generally removed within about 2 mins. Following this it was cut in half. One half was placed in a beaker of cold saline, cooled by iced water or by pouring liquid N_2 into it. The other half was cut immediately into blocks for sectioning in various orientations. The blocks of brain were from 2 to 4 mm thick and were placed on squares of damp filter paper.

For surface parallel sections however, the cortical areas had to be dissected away from the underlying structures. To achieve this a sharp scalpel and a small spatula were used. Disposable Swann Morton BS2982 scalpel blades were used as these had a curved end ideal for cutting small brains. This blade was inserted along the base of the exposed corpus callosum to cut away the cortex from the caudate nucleus and adjoining diencephalon. The cut was curved round to end in the olfactory cortex and the subcortical structures were eased away with the spatula. Thereafter the cortex was placed surface down on a square of stainless steel foil. There it was flattened out and the hippocampus partially unfolded and flapped over at the posterior end. This meant that the cortex over a large part of its surface was flat against the stainless steel. Subsequently, a square of damp filter paper was pressed on to the exposed face of the cortex and the sandwich so formed was placed into a metal basket for quenching in isopentane.

A similar procedure was used in dealing with baboon and cat brains. To remove the brain from these animals the skin and fascia were reflected back from the top of the head and then the top of the skull was removed with rongeurs. After slitting the dura the cerebral hemispheres were cut free and lifted out so that blocks could be cut from a number of different areas. When surface parallel sections were to be cut the curved surface of the gyrus had to be flattened and to allow this the underlying white matter had to be cut away so that the block of cortex could be flattened on stainless steel foil.

b. Freezing blocks of brain

The isopentane was cooled to -150°C by liquid nitrogen. Such a steep temperatue gradient between the block of brain and the quenching fluid meant that any blocks over a few mm in size would crack on freezing. Thus nearly all the large blocks from the baboon brains and even some small blocks of mice brain cracked during freezing. No

cracking occurred when thin blocks about 2 mm wide were cut from mice brains. Cutting such thin blocks required a sharp thin razor blade and an unmounted Wilkinson's stainless steel blade that had been broken in half was found to be best. Cooling the brain beforehand made it more rigid and consequently much less likely to be deformed and damaged while it was being cut into blocks. Freezing in isopentane cooled to about -50°C did not cause any cracking in even relatively large blocks of brain. However the much slower rate of cooling associated with this temperature would have led to the formation of larger ice crystals. Thus the colder isopentane was nearly always used.

An alternative means of freezing the blocks was to mount the blocks in a drop of mouting medium on a metal chuck and then immerse the chuck into liquid N_2 up to a level just below that of the tissue. The tissue is frozen by being in contact with the rapidly cooling metal. However, although this can be a very effective method for freezing blocks of tissue, it was only used in a few experiments because each block has to be lowered into the liquid N_2 individually and so it takes longer to freeze a number of blocks than if they had all been placed in one metal basket for immersion in isopentane. The rate of freezing was slower than in isopentane and consequently there was serious ice crystal formation which led to cracked sections when these were being cut at 24 μm.

c. Freezing the brain in situ

After being anaesthetised with either urethane or Sagatal the mouse or rat had its head fixed in a horizontal position by placing it in a stereotaxic head-holder. A midline incision was made along the top of the skull and the skin and connective tissue reflected back. A plastic filter funnel with its end cut short was placed on top of the exposed skull and liquid nitrogen poured directly on to the skull for about 30 sec. until the whole head was frozen. The head was then cut off and immersed in liquid nitrogen to complete the freezing process. Once the

head was completely frozen it was placed on a cork mat and the brain was removed using a small sharp chisel. In removing the brain it usually broke into at least two fragments and these pieces were mounted directly on to the chuck for sectioning. Sections were dropped directly into cold acetone and left there for a few minutes to become lightly fixed and partially dehydrated. The sections were then lifted out on coverslips and dried on to them before being placed in the incubating medium.

d. Mounting blocks

Blocks of brain were stored in liquid N_2 until they were to be cut. They were then removed and placed in the cryostat for their temperature to equilibrate. The square of stainless steel was removed if it was still adhering to any of the blocks. The blocks of brain were mounted by putting a few drops of Cryotec mounting medium diluted 1:1 with water on to the surface of a metal chuck and immediately pressing the block of brain on to the chuck. The block was held by its square of filter paper so that it would not be damaged by the forceps. In the case of coronal or sagittal blocks a solid metal cylindrical chuck was used. However for cutting surface parallel sections the block of brain had to be accurately aligned to be in the same plane as the cutting edge of the knife. To permit this an adjustable chuck had to be made. It was constructed by welding a sawn-off hollow chuck onto a metal shaft that terminated in a metal sphere. By loosening the clamp of the cryostat holding the sphere the surface of the chuck could be made to move a limited distance in any direction.

e. Cutting and incubating sections

The relatively thick sections (24 μm) used to study the architectonic patterns meant that the sections were much more likely to become cracked during cutting. Thus if there had been the least thawing out of the block during mounting large ice crystals formed. These made the block become brittle and liable to shatter during cutting so that a

multitude of cracks appeared that were parallel to the knife edge. This effect was lessened when thinner (12 μm) sections were cut, but after being incubated, sections of this thickness showed less than half the activity of the thicker sections and were usually too pale to be of much use.

The position of the guide-plate in relation to the knife edge was a critical factor in obtaining a reasonable quality of section and great care had to be taken to ensure that the edge of the plate was parallel with the knife and at an optimal distance away. If the guide-plate was too near the edge of the knife then compression or tearing of the tissue occurred, while if it was too far away, the section rode over the top of the plate, instead of passing between it and the knife. Even if this did not occur, having the guide-plate too far away increased the susceptibility of the sections to crack during cutting. Guide-plate position also had an effect on the number of bubbles formed when the section was picked up on the coverslip. However even with optimal placing of the guide-plate, bubbles were still formed.

After being picked up on coverslips the sections were left in racks to air dry. On some occasions the sections were blown dry in a stream of warm air from a hair-drier but, as this procedure did not appear to make any difference to the localisation or level of activity of phosphorylase, it was not usually employed. Sections were usually incubated within a few hours of being cut but occasionally they were left overnight. For overnight storage the sections were thoroughly dried before being placed in a sealed plastic container partly filled with silica gel and left in the cryostat at a temperature of -20°C. No noticeable decrease in phosphorylase activity was evident when the sections were incubated and stained the next morning.

The sections were placed in Columbia jars and incubated at 37°C, usually for a period of 90 min, in Meijer's[152] incubation medium for

phosphorylase. In the brain, glycogen phosphorylase exists partly in the inactive b form, which can be allosterically activated by the presence of adenosine-5-monophosphate (AMP). By addition of AMP to the incubation medium, it is possible to demonstrate total phosphorylase activity, while without it only the active form (phosphorylase a) is demonstrated. The composition of the incubation medium is given below:

Incubation medium for phosphorylase

disodium glucose-1-phosphate	100 mg
disodium ethylene diamine tetraacetate (EDTA)	50 mg
sodium fluoride	40 mg
acetate buffer 10mM pH5.6	25 ml
dextran (average molecular weight 40,000)	2 g
when total phosphorylase activity is desired	
disodium adenosine-5-phosphate	20 mg

f. Staining and mounting sections

After incubation the sections are gently washed by placing them for a few minutes in ordinary tap water. After the incubation period the unfixed tissue is very delicate and easily detached from the coverslip. Therefore any more vigorous washing has to be avoided. The sections are then stained by placing them in a dilute solution of acidified iodine. This is made up by adding a few drops of 7 M sulphuric acid to a solution of Lugol's iodine diluted 1:10 with water. The acid stabilises the glycogen-I_2 complex and prevents fading occurring for up to three months.[121] The sections are left in the solution for between 1 and 5 min. depending on the amount of glycogen synthesised in the section. After a short rinse in water the sections are allowed to drain and the back of the coverslips are polished dry on a tissue. They are mounted in an aqueous mounting medium consisting of one part Lugol's iodine and five parts glycerol. This has to be mixed up at least an hour earlier to allow all the air bubbles to rise to the

surface. It is important to clean and polish the back of the coverslip before mounting because this cannot be done afterwards without the risk of damaging the section. The aqueous mounting medium does not bind the coverslip to the slide and so care must be taken to ensure that the coverslip is not dragged across the slide.

8. Tissue Fixation

a. Choice of fixative

Phosphorylase was reported to be very sensitive to fixation by even mild fixatives such as absolute alcohol or acetone.[226] Nevertheless it was felt that some attempt should be made to improve the localisation of the reaction product by fixing the tissue prior to incubation. Initially sections of brain were placed in a variety of fixatives at different concentrations for periods ranging from 10 sec. to 5 min. with the aim of finding a possible fixative to allow perfusion of the brain. The fixatives used were glutaraldehyde, paraformaldehyde and a carbohydrate fixative formed of a mixture of sodium periodate and lysine. The periodate anion is a mild oxidising agent which oxidises carbohydrate moieties to aldehyde groups. These groups are then cross-linked by condensation reactions involving the divalent amino acid lysine.[149] Even very low concentrations of glutaraldehyde (0.001%) were found to inactivate the phosphorylase but a reasonable level of phosphorylase activity was found after fixation for 10 sec. in a mixture of 0.1% paraformaldehyde, 0.005 M periodate and 0.075 M lysine. As the permeability of fixatives such as paraformaldehyde through the brain during perfusion is limited, higher concentrations of fixative may be used in the perfusate than would be advisable for solutions used directly on sections. Therefore for the perfusion of the rodent and baboon brains perfusates containing up to 2% paraformaldehyde, 0.01 M periodate and 0.075 M lysine were prepared.

b. Preparation of fixative solution

The fixative was prepared in the manner described by McLean and Nakane.[149] A 0.2 M solution of lysine - HCl was made up in distilled water and 0.1 M dibasic sodium phosphate (Na_2HPO_4) was added until the pH was 7.4. The solution was then diluted to 0.1 M lysine with 0.1 M sodium phosphate buffer, pH 7.4. The paraformaldehyde was made up separately into a 4 or 8% solution by dissolving the powder in hot distilled water to which a few drops of 1 M sodium hydroxide had been added. Care was taken not to let the temperature of the water exceed 60°C because beyond this temperature the formaldehyde becomes oxidised to produce formic acid. Just before use, 3 parts of the lysine - phosphate buffer were combined with 1 part paraformaldehyde solution and solid sodium periodate was added. All solutions had to be made up freshly each day. For perfusing mice brains, 360 ml of solution was made up and twice this amount for the rats. A different solution was used for perfusing the baboon brain. This consisted of a 1% paraformaldehyde solution made up in a solution of the incubation medium for phosphorylase a minus the glucose-1-phosphate and dextran. All solutions were cooled down prior to perfusion by immersion in a mixture of water and ice. Cooling the brain with cold perfusate retards autolytic breakdown and made the cutting of coronal blocks easier.

c. Perfusion

Transcardiac perfusion was used as this is the simplest and most rapid method of perfusion. For a successful perfusion it is important to clear out the blood as quickly as possible and to do this the needle should enter the aorta while the heart is still beating. A high flow rate is also desirable and so the needle and plastic tubing should be of relatively wide bore to minimise the resistance to flow. For the mice a needle with an internal diameter of about 1 mm was used, while for rats a larger needle with a diameter of about $1\frac{1}{2}$ mm was used. The

shaft of the needle was cut back to a length of 3 cm. to minimise the resistance to flow and the tip of the needle was rounded off so that it would not tear the aorta.

After being anaesthetised the animal was laid out supine on a plastic tray and its legs were stretched out with tape. Then using a scalpel a deep, midline incision was made from throat to abdomen. The skin over the thorax was dissected away and reflected back and a transverse cut made that passed through the abdominal muscles immediately below the thorax. The diaphragm was carefully cut through and then using a strong pair of scissors the sternum was cut in half along the midline. If necessary the pericardium was cut to expose the beating heart. Then the right atrium was cut open to let the blood and perfusate flow out and a small nick made in the left ventricle. Immediately this had been done the tap on the bottle of the perfusate was turned on and the needle containing the cold perfusate was pushed into the left ventricle. The tip of the needle was gently manipulated until the top 5 mm. were in the aorta. Once in place the needle was anchored in position by holding it and the ventricular muscle firmly between the jaws of a clamp.

As the descending aorta was not usually clamped off, the success of the perfusion could be gauged by watching the colour change of the liver as the blood was washed out. In order to ensure an adequate perfusion pressure the bottle was placed $1\frac{1}{2}$ metres above the preparation. To achieve as rapid fixation as possible the blood was not washed out with saline - instead the fixative was brought directly into contact with the blood. This did not cause the blood to clot, presumably as the precursor proteins became denatured before they could start polymerising. After the perfusion the brain was removed in the same way as those that had not been fixed. Although the brain was only lightly fixed, it was not soft enough to be properly flattened. This was especially true of the hippocampal formation where the various areas

are sharply curved and so surface parallel sections could not be cut. As it was important to be able to compare surface parallel sections with those of other orientations taken from brains which had not been fixed, only a small number of brains were perfused.

9. Deoxyglucose Experiment

As only one experiment was performed only a brief outline of the experimental protocol will be given. For a detailed discussion of the method as it is used for tracing anatomical pathways reference may be made to a number of review articles.[213,175,88] An adult rat was anaesthetised with a halothane, nitrous oxide, oxygen mixture (2%: 73%: 25%) and was maintained on this mixture until the end of the experiment. A canula was inserted into its right femoral vein and a craniotomy performed, as described previously, to expose a small area of cortex over the forepaw area of the primary somatosensory cortex. The syringe attached to the femoral canula was filled with 0.5 ml of ^{14}C 2-deoxyglucose (^{14}C 2-DG) with a specific activity of 100 μ Ci. After this a tiny ($2m^2$) piece of filter paper soaked in concentrated K penicillin G was placed on the exposed cortex and one minute later the deoxyglucose was injected into the blood. The piece of filter paper was left in place for 10 mins. and then removed. The rat was killed 30 mins. after the injection of the ^{14}C 2-DG and perfused transcardially with a 3.3% formalin solution for 4 minutes. Then the brain was removed and each side of the cortex dissected off and partially flattened without cutting the brain in half. The remaining block containing the thalamus was also frozen so that horizontal sections could be cut. All sections were cut on a cryostat at 20 μm and picked up on coverslips. After being dried the sections were stuck to cardboard and placed in a sealed cassete along with a sheet of X-ray film. After five days the sheets of film were processed.

The epileptiform focus was large enough to be studied directly by eye. However to study two of the sections through the primary focus

more clearly use was made of the Image Enhancer facilities available at Cold Spring Harbor. The grain density of two autoradiographs was analysed microscopically. The grain density values were assigned an arbitrary colour code and after about 20 mins. a colour image of the autoradiograph was built up on a television screen. The colour code was altered to achieve the maximum contrast between the epileptiform focus and the surrounding cortex. As this was a qualitative neuro-anatomical study, no attempt was made to obtain values of the glucose uptake for the various areas.

10. Golgi Methods

The golgi studies of the rat brain were also performed largely at Cold Spring Harbor under the direction of the neuroanatomists in charge of the course and as standard techniques were used only a brief outline of the methods will be given. Two different Golgi methods were used. The first was the Golgi-Kopsch which has been described by Colonnier[41]

In this method the brain is perfused transcardially with a freshly prepared solution containing 1% glutaraldehyde and 4% paraformaldehyde in phosphate buffer. After about 800 ml. of fixative has passed through the brain the brain is cut into slices and placed in a mixture of fixative and 4% potassium dichromate resting on glass wool for the next 24 hours. Then the brain is rinsed and left in a solution of 0.75% silver nitrate for five days, dehydrated and embedded in celloidin. Embedding in celloidin must be done as quickly as possible (1 - 2 days) otherwise the silver salts start to be leached out of the block of tissue. The blocks are stored in butanol before being sectioned at 100 - 150 μm. The sections are mounted in permount or some other resin, under weighted number 0 coverslips. The Golgi-Kopsch method gives beautiful clear staining of individual neuronal cell bodies and dendrites but axons are seldom impregnated.

To study the axons in the hippocampal formation the Golgi-Cox method was used by following the procedure described by Van der Loos.[231] This method also has the advantage that counterstains can be used and this makes it much easier to determine the exact location of stained cells. The blocks of brain are immersed in a solution of mercury salts for three or more weeks, embedded in celloidin and stored in 70% alcohol. Sections are cut at 100 - 150 µm and after processing are counterstained with thionin and mounted under weighted coverslips.

11. Photography

Sections were photographed using the camera associated with a Leitz Orthoplan microscope and microphotometer. The microphotometer could be adjusted to measure the light arriving from the whole field or a small circle in the middle of the field and automatically altered the time for which the negative was exposed. For black and white photography fine grain Ilford Pan F film was used and it was developed in Kodak's Microphen developer. For colour photography Kodak EPY 135-36 Tungsten film was used and the transparencies were developed commercially. Initially, because the iodine staining was known to fade, every section was photographed on black and white film. However this was found to be impractical considering the large number of sections used. Therefore latterly only selected sections were photographed in black and white. Prints (14 x 10 cm.) were made from the best of these negatives for a more detailed examination. Usually Kodachrome high contrast grade 4 paper was used to emphasise the difference in activity between the patches of high and areas of low phosphorylase activity. Latterly Kodachrome II resin paper was used as the processing time for this paper is much shorter. The best sections were also photographed using colour film.

12. Drawing Sections

Free-hand drawings were never used as it would not have been possible to obtain sufficient accuracy. All drawings were made over the image projected by a photographic enlarger. In some cases the brain section was placed directly in the enlarger and an image produced that was magnified by about x16. This was used quite frequently for the large surface parallel sections of mouse and rat brain, because it allowed immediate comparison of the section with a diagram of the architectonic areas that had been prepared previously. The diagram was drawn at the same magnification and so the image of the section could be projected directly onto the diagram. There were usually enough characteristic features in sections passing through layer IV to allow these and the other sections to be fairly accurately orientated with respect to the diagram. At least three well spaced points were required to provide an accurate orientation and these were usually provided by the posterior and medial aspects of the rhinal sulcus and the occurrence of characteristic clusters of barrels in the somatosensory cortex. As all the primary somatosensory areas were usually more highly stained than the surrounding cortex, any of them could serve as additional useful landmarks in studying the section in relation to its architectonic organisation. Thus the enlarger provided an important supplementary method of studying the architectonic organisation of the brain.

For drawing coronal sections and any section where a higher magnification was required, photographic negatives or colour transparencies were used to obtain a high quality image when drawings were to be made. As photographic prints were made from negatives of the best sections, most measurements of the size of the bands or patches of phosphorylase were made from the prints rather than by direct observation down the microscope. When preliminary measurements were to be made using the microscope a calibrated graticule was placed in the right eyepiece.

SECTION I NEOCORTICAL COLUMNS

Introduction

Many recent anatomical [116,79] and physiological [107,158] studies of the mammalian neocortex have provided evidence that it is organised into groups of vertically arranged cells; such groups are called "columns". However Bousfield [23] has suggested that the rabbit cortex does not have a columnar organisation and so questioned the assumption that the columnar hypothesis is applicable to the cerebral cortex of all mammals. Woolsey [53] obtained evidence of a columnar organisation in the mouse barrel cortex using the (^{14}C) 2-deoxyglucose technique and White and De Amicis [252] obtained anatomical evidence of a columnar organisation based on the cortico-cortical efferents of the barrel cortex. Therefore there is strong evidence that at least the sensori-motor areas of the mouse cortex do have a columnar organisation. The results of the present work are based on the histochemical demonstration of the active form of phosphorylase (phosphorylase a). The reasons for the choice of this approach are outlined below.

According to Szentagothai [223] there are two fundamental types of columns produced by the modular termination of specific thalamic afferents and of cortico-cortical afferents. So far, although the two sets of columns are thought to develop independently of each other,[79] their spatial relationship, i.e., whether they are in or out of register, is not certain.[116] An obvious place for examining this problem is in the barrel cortex of the mouse where it has been shown that the thalamic afferents[125] associated with a single whisker terminate[226] within a single barrel-like cluster of cells in layer IV and give rise to a functional column.[210,53] As each anatomical barrel is the morphological manifestation in layer IV of a physiologically defined thalamic column,[226] any technique allowing the simultaneous demonstration of cortico-cortical columns and

the cellular barrels will provide information on the spatial relations of the two sets of columns. One approach to studying cortico-cortical columns is by using a microscopical technique to demonstrate cortical functional activity under conditions when the cortico-cortical input is dominant.

It is not known if, in the mouse, parts of the neocortex other than the sensori-motor cortex also contain columns. Drager[49,51] studied the mouse visual cortex with electrophysiological techniques but although she did find clustering of orientation selective cells she could not find evidence of a columnar organisation. This was not surprising because Towe[228] has argued on theoretical grounds that the small columns that might be expected to exist in the rodent cortex are too small to be convincingly demonstrated by electrophysiological methods. Consequently only microscopical methods were used in the present study.

At present, the main microscopical methods, of which the author is aware, for demonstrating the functional activity of the brain are based on the uptake of radioactive metabolites such as 2-deoxyglucose[214] or the bioluminescent demonstration of adenosine triphosphate (ATP) levels.[127] The resolution of these methods is limited because the radiation is emitted as a cone which forms the image of a point as a comparatively large fuzzy circle on the photographic emulsion. The diameter of the circle depends on the height of the cone and so relatively high resolution can only be obtained by using low energy β particles from a tritiated source. However as these particles can only travel through about 1 μm of tissue, a tritiated source is not very suitable for cytoarchitectonic studies.[268] Woolsey has shown that morphological structures such as the whisker barrels can only be convincingly described in sections with a thickness of greater than 10 μm. As some of the barrels in the mouse cortex are only 100 μm in diameter, studying the columnar organisation of the mouse cortex in relation to

the barrels requires a microscopical technique of high spatial resolution that can be used with thick sections.

The procedure chosen was a histochemical method based on the active form of phosphorylase. In muscle the histochemical demonstration of phosphorylase a levels has been used in conjunction with the glycogen depletion method to study the fatigue produced in single motor units by repetitive electrical stimulation.[134,32] In the brain phosphorylase histochemistry has been used to study chronic changes in metabolism such as those produced by stab wound[111] or prolonged ischaemic hypoxia.[112] However phosphorylase has not previously been used as a marker of more physiological changes in the brain. Therefore a primary aim of the present study was to obtain evidence that the postmortem levels of phosphorylase a could be used as a marker of physiological activity.

Phosphorylase a levels were compared in sections taken from mice anaesthetised with sodium pentobarbitone and from mice killed directly without the use of an anaesthetic or after being transiently stupefied with ethyl chloride vapour. The relative intensity of staining of the cortical layers was studied in a number of neocortical areas to look for differences between the two groups of animals.

In alert mice there is a strong thalamic input to layer IV of the neocortex and so it was expected that, if phosphorylase a levels reflected functional activity, then the staining pattern in sections cut from the brains of unanaesthetised mice and incubated to demonstrate phosphorylase a activity would reflect the thalamic input. On the other hand Angel[8] has shown that in animals anaesthetised with sodium pentobarbitone the thalamic input is depressed. Therefore it was expected that the input from other cortical areas via cortico-cortical fibres might be dominant and that this would be reflected by phosphorylase a levels. If this were the case then the demonstration of columns in the barrel cortex of pentobarbitone anaesthetised mice would indicate the

relationship between thalamic and cortico-cortical columns.

Using the (^{14}C) 2-deoxyglucose technique Sokoloff[123] has provided evidence of a columnar organisation in most parts of the neocortex of unanaesthetised monkeys, while the ATP bioluminescence technique has been used to show a columnar organisation in most parts of the neocortex of anaesthetised animals.[127] If a columnar organisation does exist in the mouse cortex outside the sensori-motor cortex, then it could be expected that a marker of functional activity would provide evidence of its existence when sections from the brains of either anaesthetised or unanaesthetised animals were examined.

RESULTS

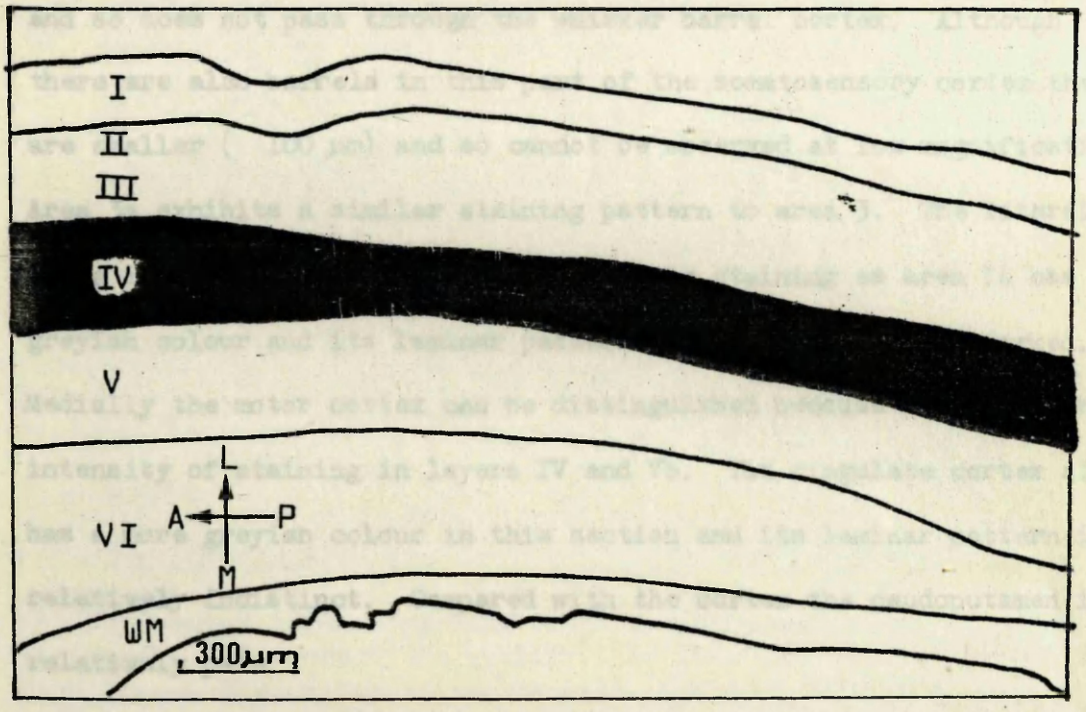
Total phosphorylase activity

In the brain phosphorylase exists in two interchangeable forms: active phosphorylase a and the inactive phosphorylase b. The b form can be activated allosterically by relatively high concentrations of adenosine monophosphate (AMP).[168] Therefore the addition of AMP to the incubation medium[152] permits the demonstration of total phosphorylase activity, i.e. the sum of the phosphorylase a and b forms. This indicates the maximum possible level of phosphorylase a which could occur in the brain, as the allosterically activated b form has a similar level of activity to the a form that has been covalently modified by phosphorylation.

Total phosphorylase levels are shown in a horizontal section through the mouse sensori-motor cortex in Fig.14. All layers have moderately high levels of activity but the layers do not all show the same intensity of staining. Layer IV has the highest activity followed by Vb. Other layers have similar levels of activity. This section was only incubated for 30 min. whereas all the sections incubated in the medium for demonstrating phosphorylase a activity were incubated for



Fig.14 Horizontal section through mouse sensori-motor cortex incubated to demonstrate total phosphorylase activity. All layers exhibit high levels of activity but they do not have equal activity. Layer IV has the highest activity followed by Vb. Other layers have similar activity. Iodine stained in this and all subsequent figs except where otherwise specified.



90 min. The shorter incubation time was to prevent too high an intensity of staining as this would have obscured the differences between the various layers. As a result, the absolute intensity of staining is actually lower than in some sections incubated for phosphorylase a, even although there is a higher level of enzyme activity.

Unanaesthetised mice and mice transiently anaesthetised with ethyl chloride

Sections taken from the transiently anaesthetised and unanaesthetised mice were basically the same and so no distinction will be made between them. Both the unanaesthetised and the mice treated with ethyl chloride vapour will be referred to as "unanaesthetised" mice in future. The levels of phosphorylase a are much lower than in sections stained for total phosphorylase activity. Despite this layers I, IV and Vb can be distinguished because they have a higher activity than the adjacent layers. This is shown in Fig.15 which is a coronal section representative of sections from all of the 24 mice in this combined group of animals. This section passes through the anterior commissure (AC) and so does not pass through the whisker barrel cortex. Although there are also barrels in this part of the somatosensory cortex they are smaller (100 μm) and so cannot be observed at low magnification. Area 3a exhibits a similar staining pattern to area 3. The lateral edge of 3a is marked by an abrupt change in the staining as area 14 has a greyish colour and its laminar pattern is much less clearly marked. Medially the motor cortex can be distinguished because of the lower intensity of staining in layers IV and Vb. The cingulate cortex also has a more greyish colour in this section and its laminar pattern is relatively indistinct. Compared with the cortex the caudoputamen is relatively pale.



Fig.15 Coronal section passing through the anterior somatosensory cortex at the level of the anterior commissure (AC). The outer part of layer I, layer IV and layer Vb have higher activity than adjacent layers in the primary somatosensory cortex (area 3). The caudoputamen (P) is pale and has a few irregular darker patches. (Unanaesthetised mouse)



Fig.16 Coronal section passing slightly posterior to where the anterior commissure (AC) crosses the midline. In the sensori-motor cortex there are high levels of activity in layers I-III compared to layers IV-VI which are very pale. Few architectonic boundaries can be distinguished and the caudo-putamen (P) has high levels of activity. (Pentobarbitone anaesthetised mouse)

Pentobarbitone anaesthetised mice

In sections taken from animals which had been anaesthetised with sodium pentobarbitone there was considerable diversity in the staining pattern within the group. Of the 19 mice in this group 10 show a high level of staining in layers I - III as compared with layers IV - VI (see Fig.16). In this section as in sections from the other 9 mice, the boundaries between most architectonic areas are not visible because layers IV and Vb have very low activity. Only the boundary with 14 is clearly demarcated because of the greyish colour and pale layer II of area 14. Another difference between this section and those taken from the "unanaesthetised" group is that the caudoputamen (P) is relatively strongly stained. Sections from three other brains were similar to those from the urethane anaesthetised mice (see later). Sections from the remaining six mice in this group showed a modulation of staining intensity over a large extent of the neocortex. In surface parallel sections these modules are seen as a mosaic of irregular dark patches set against a pale background (Fig.22A). Each patch has a diameter of about 250 μm. In coronal sections the different laminae when stained are still evident; layers IV and Vb are darkest; the other layers are relatively paler. The results described in more detail below come mainly from three of these six mice whose brains showed a modular organisation particularly clearly. Sections from these three brains will be compared with those from "unanaesthetised" brains and differences noted in some of the main architectonic areas.

"Unanaesthetised" Mice

Somatosensory cortex

Most of the primary somatosensory cortex is composed of a field of 200 cellular barrels[266] whose cell dense walls appear as pale rings in surface parallel or tangential sections through layer IV (Fig.17). Relatively high levels of phosphorylase a occur both in cell bodies and

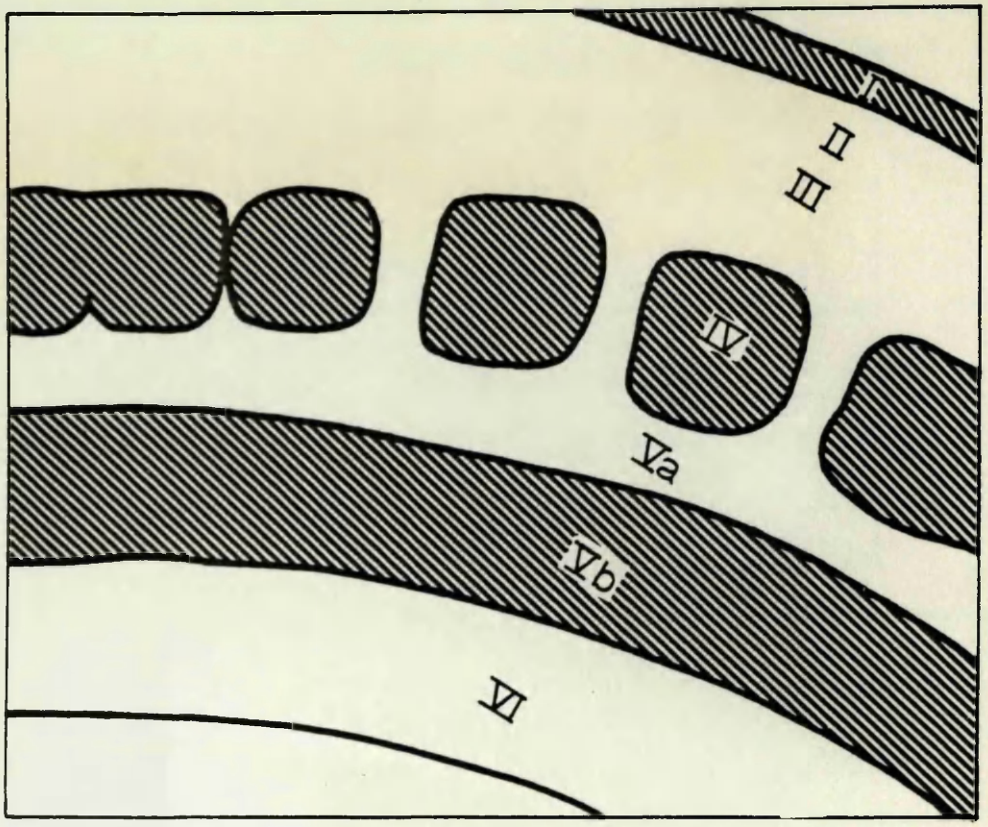


Fig.18 Coronal section through posterior somatosensory cortex showing the higher activity in layers I and Vb and the dark patches corresponding to the barrel cores in between the paler cell walls in layer IV. (Unanaesthetised mouse) This mouse had been perfused with fixative prior to decapitation.

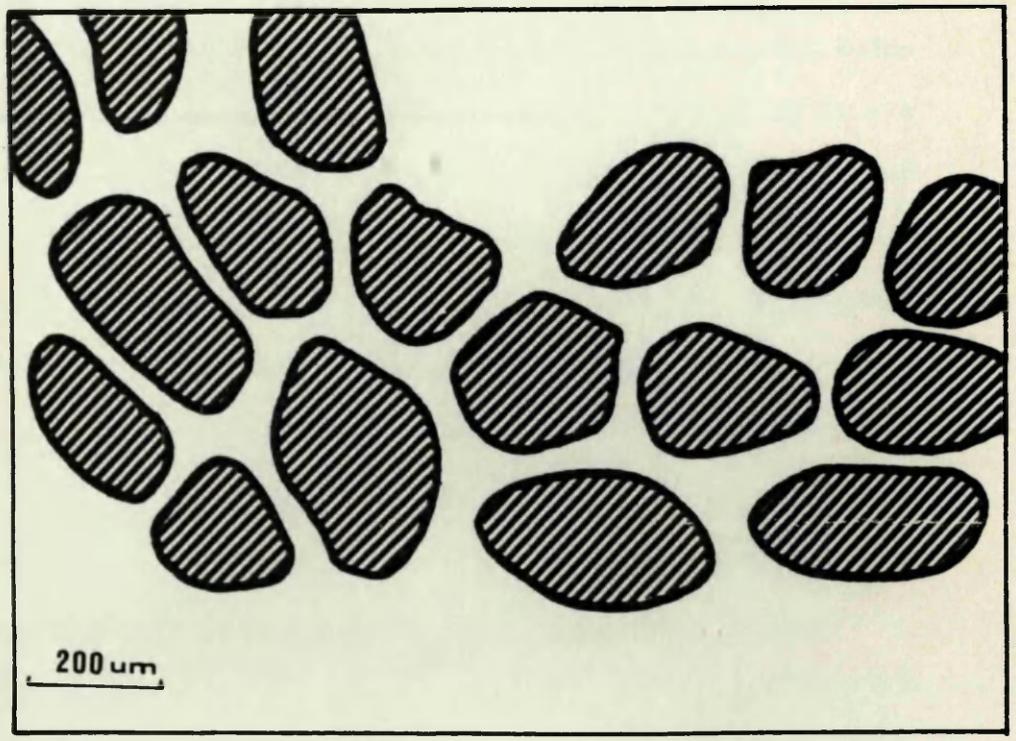
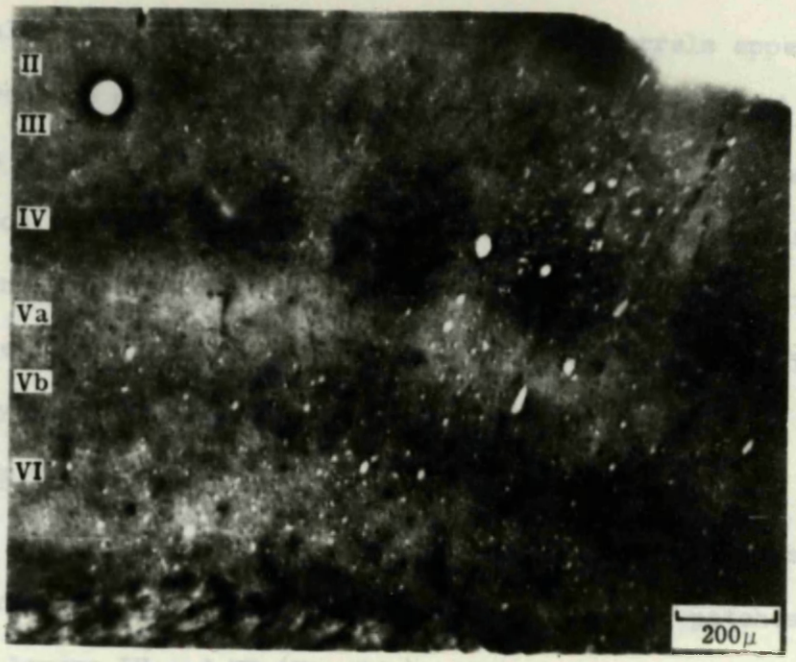
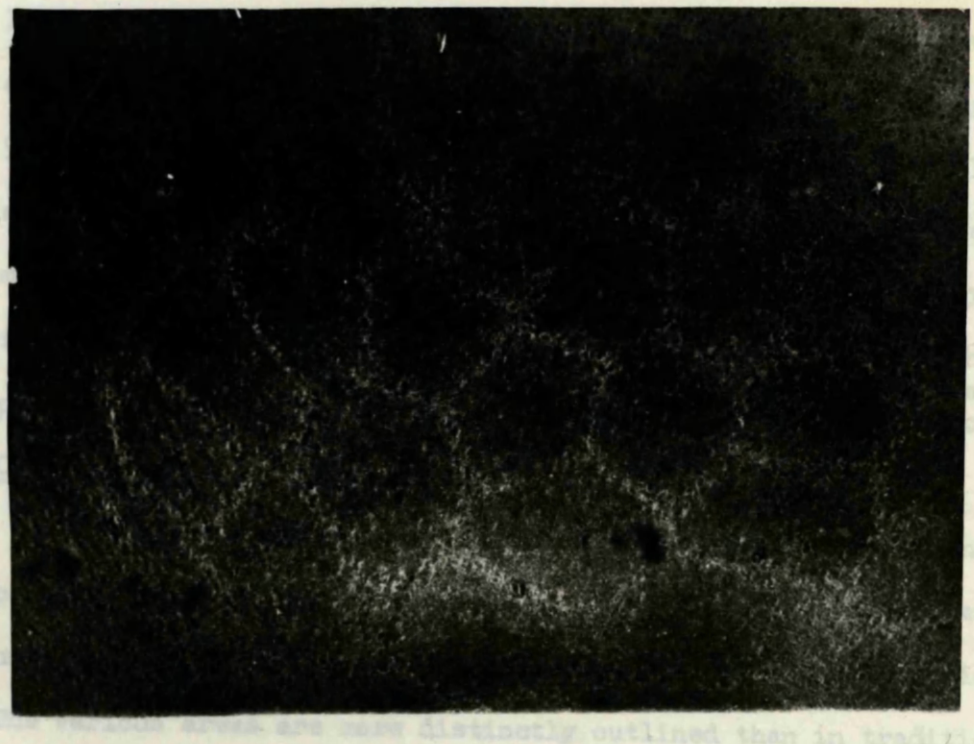


Fig.17 Tangential section through layer IV of the barrel cortex showing the pale barrel walls containing a higher density of cells and surrounding the darker barrel core. (Unanaesthetised mouse)


II
III
IV
Va
Vb
VI
200μ



in the part of the neuropil corresponding to the hollows of the barrels where the thalamic afferents terminate. The neuropil of the barrel walls is only weakly stained and so the barrels appear as pale rings around a darker core. In coronal sections (Fig.18) the barrels appear as patches of higher activity located in layer IV and separated from each other by pale strips indicating the barrel walls. There is no consistent evidence for an extension of the patchy staining pattern of the layer IV barrels for more than a short distance into the other layers of the cortex.

Visual cortex

In two of the 22 mice the phosphorylase a levels in coronal sections through the visual cortex exhibit two rows of patches of high activity in layers IV and VI (Fig.19). The patches in layer IV are the more prominent and they sometimes extend into layer V. The two rows of patches are in register with each other. This gives the impression that the visual cortex is organised into bands of alternating high and low activity each of which has a width of about 300 μm. The width is comparable to that of the whisker barrels whose diameters range from 90 to 330 μm. The spatial extent of the visual areas 17 and 18 as well as other architectonic areas was obtained from the cytoarchitectonic map published by Caviness.[34] Assuming the bands are roughly cylindrical in form it is estimated that there are about 45 cylinders of high phosphorylase activity in the visual areas 17 and 18 (see/discussion). General

Cortical architectonics

The major architectonic divisions can be distinguished in surface parallel sections through layer IV where the thalamic afferents terminate and differences between architectonic areas are most obvious. The various areas are more distinctly outlined than in traditional cytoarchitectonic studies where sections are stained to show the Nissl substance present in cell bodies. This is illustrated in Fig.21 which

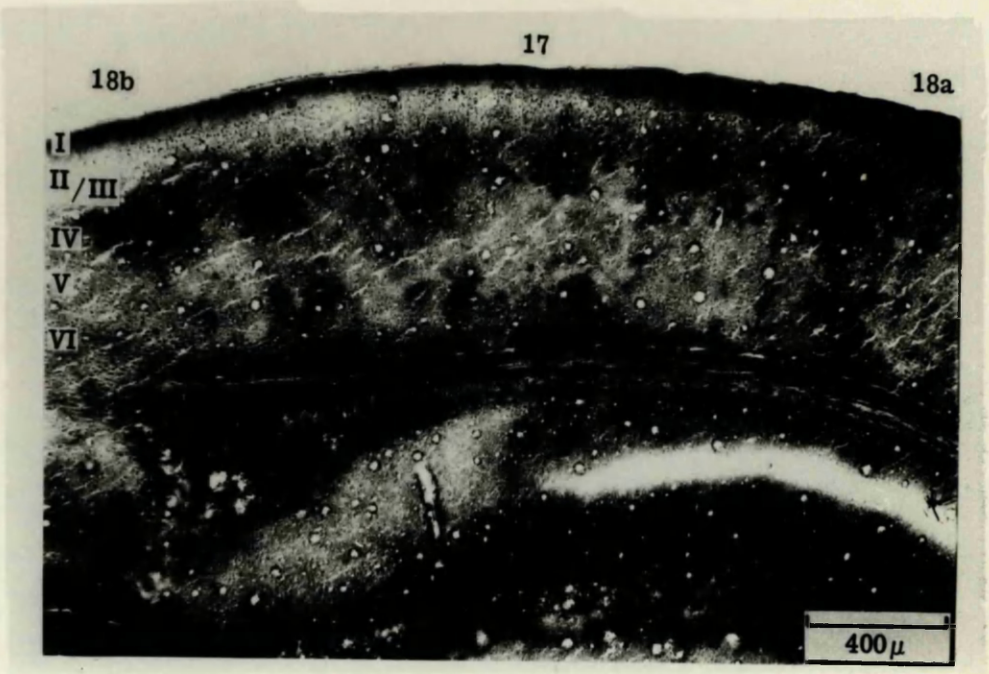


Fig.19 Coronal section through the visual cortex showing bands of higher activity which are most evident in layers IV and upper VI.
(Unanaesthetised mouse)

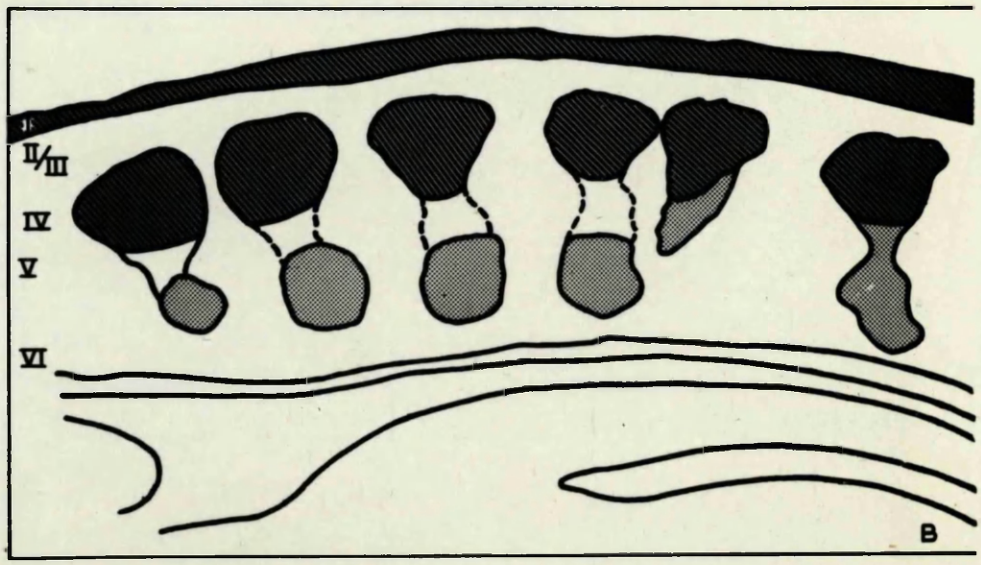


Fig. 20 Coronal section through the mouse whisker barrel cortex. The higher intensity of staining within the hollows is indicated by the arrow-heads. Sections were cut from unfixed brain. (Unanaesthetised mouse)

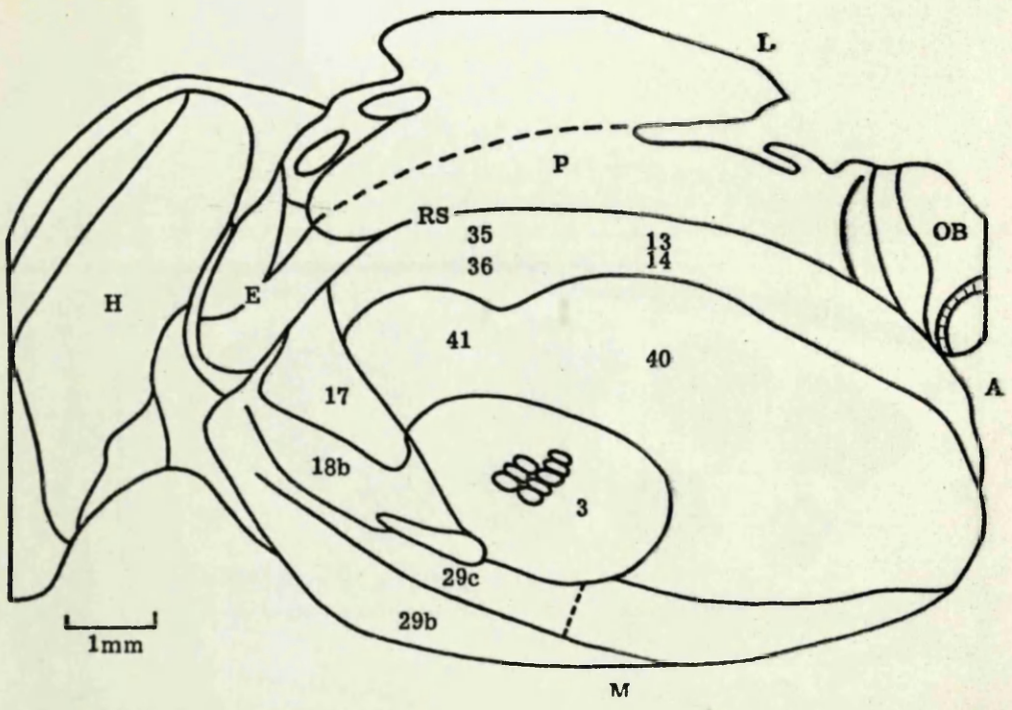


Fig. 21 Surface parallel section passing largely through layer IV of the mouse cerebral cortex. Some of the main architectonic areas are outlined in the adjacent diagram. Within the somatosensory cortex some of the barrels are evident as pale rings. (Unanaesthetised mouse)


II
IV
Vb
200 µm



is a surface parallel section cut at a depth of 600 μm and passing mainly through layer IV. The primary auditory cortex (area 41) has a pale brown colour different from the purple of the primary visual area (area 17). The primary somatosensory cortex (area 3) is characterised by its more orange colour as well as by the cellular barrels. These are more clearly observed at the higher magnification shown in Fig.17 The greyish colour of areas 13 and 14 which was noted in describing the coronal section is also distinctive.

Thus the different regions can be distinguished because of the large variations in both the colour and the intensity of the iodine staining. The colour of the iodine in the section is determined by the unbranched chain length of the glycogen with which it is associated.[221] In the different regions of the brain the colour varies from pale brown to deep purple. This implies that different architectonic regions often have different levels of active phosphorylase and of the associated branching enzyme which determines the unbranched chain length of the synthesised glycogen.[224] Although useful in identifying the different architectonic regions, the significance, if any, of the variations in chain length is not known and so the colour variations will only be described briefly.

Pentobarbitone Anaesthetised Mice

Sensori-motor cortex

In the posterior part of the barrel field where the whisker afferents terminate, individual barrels can be histologically distinguished in layer IV. This area allows the direct comparison of the phosphorylase modules with the modules of thalamic input. In contrast to the dark patches in the "unanaesthetised" brain, the dark patches of high phosphorylase a activity in these brains are not in register with the barrels (Fig. 23); they are arranged at random with respect to the barrels, some straddling the septal areas and others largely or

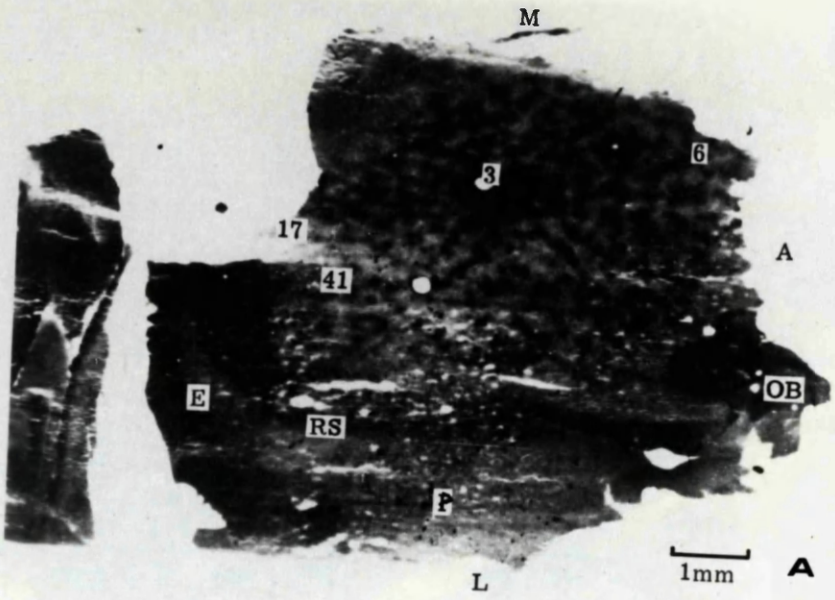


Fig.22A Surface parallel section through layer IV of the mouse cerebral cortex. (Anaesthetised mouse). Discrete patches of higher activity are seen in the frontal, parietal and temporal regions. In this section parts of the retrosplenial and visual cortex are missing.

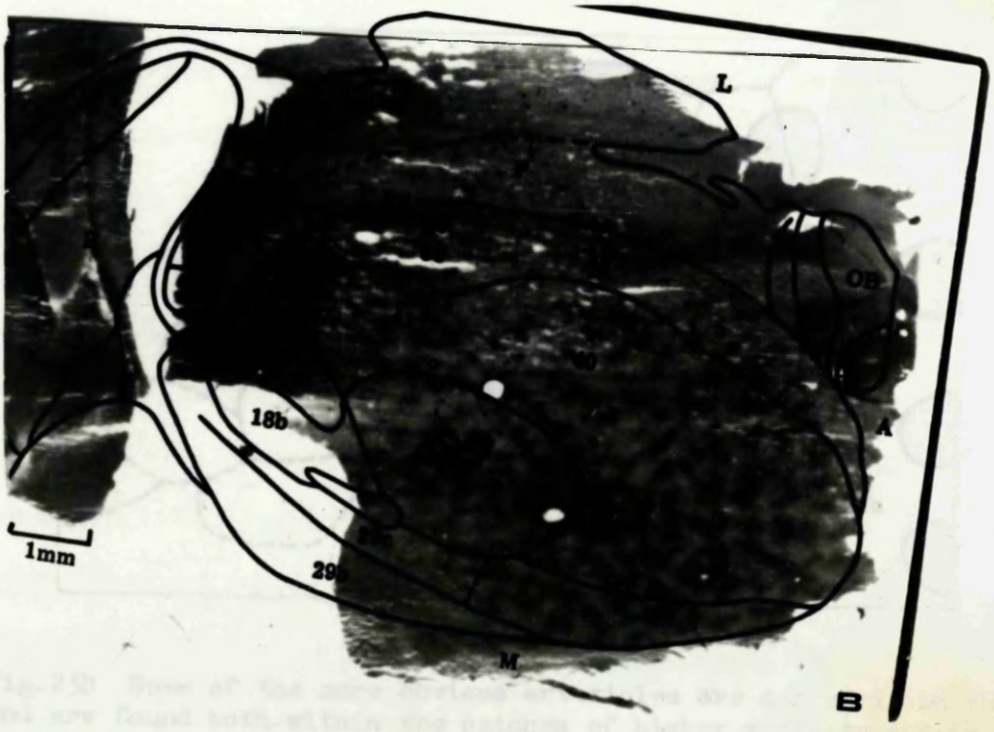


Fig.22B Same section which has been inverted so that the diagram in Fig.21 could be superimposed for comparison.

partly confined to the central part of the barrel. This is histochemical evidence for the existence of two separate overlapping modular subdivisions of the cerebral cortex.

In coronal sections the modules appear as alternating dark and light bands each with a width of about 250 μm (Fig. 24). Again in contrast to the patches of the "unanaesthetised" brains which are restricted to layer IV, these bands extend from layer I down to the base of layer V. It is difficult to discern the barrels in coronal sections even when Harris's Haematoxylin is used as a counterstain. When it is possible to see the barrels they are found to be arranged at random with respect to the bands of high phosphorylase a activity. Taken together the surface parallel and coronal sections show that the phosphorylase a activity is arranged in irregular cylinders of high activity which are out of phase with the thalamic modules. There are about 100 of these cylinders in the somatosensory areas 3, 1, 3a, 40 and 40a.

Auditory cortex

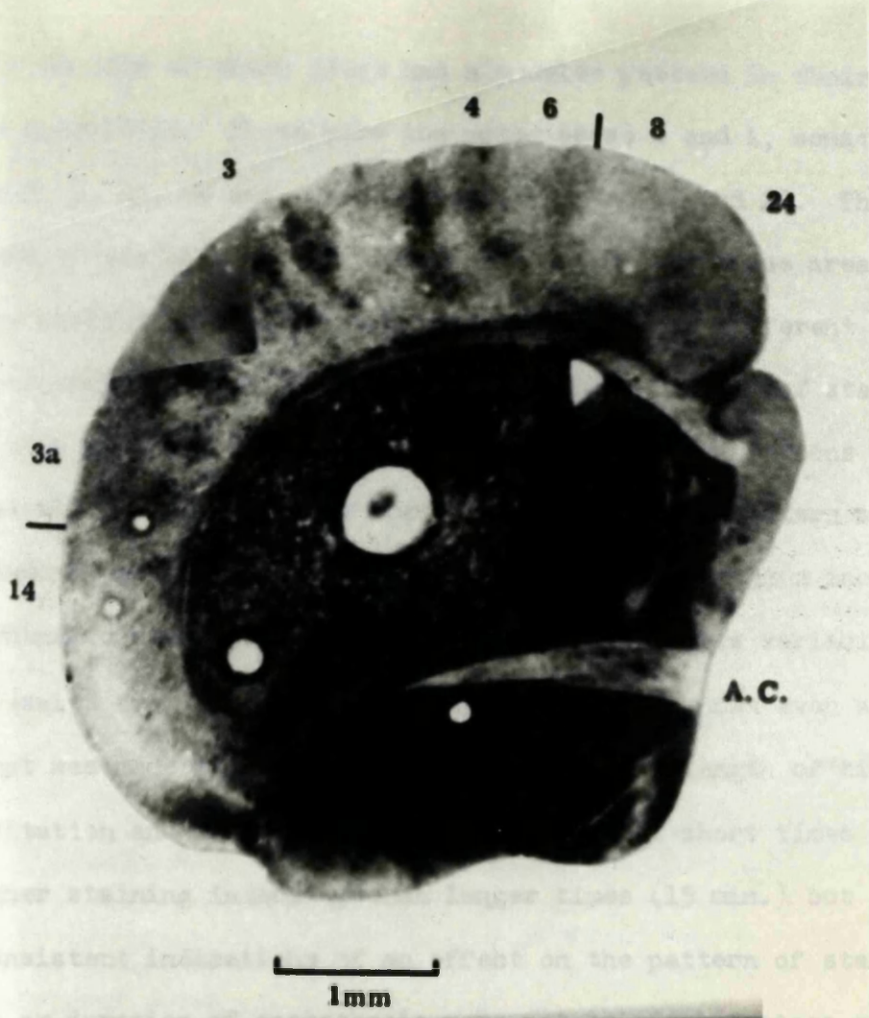
The modules in the auditory areas 41 and 22 were similar to those in the sensori-motor cortex being between 250 and 350 μm in diameter. In coronal sections (Fig. 25) the modules appear as bands of high activity extending from layer II to the base of layer V. Layer IV of these cylinders is more intensely stained than the other layers. There were about 40 of these cylinders in the auditory areas 41 and 22. The bands in area 22 have a width of about 250 μm and are narrower than those in area 41 which are about 350 μm wide.

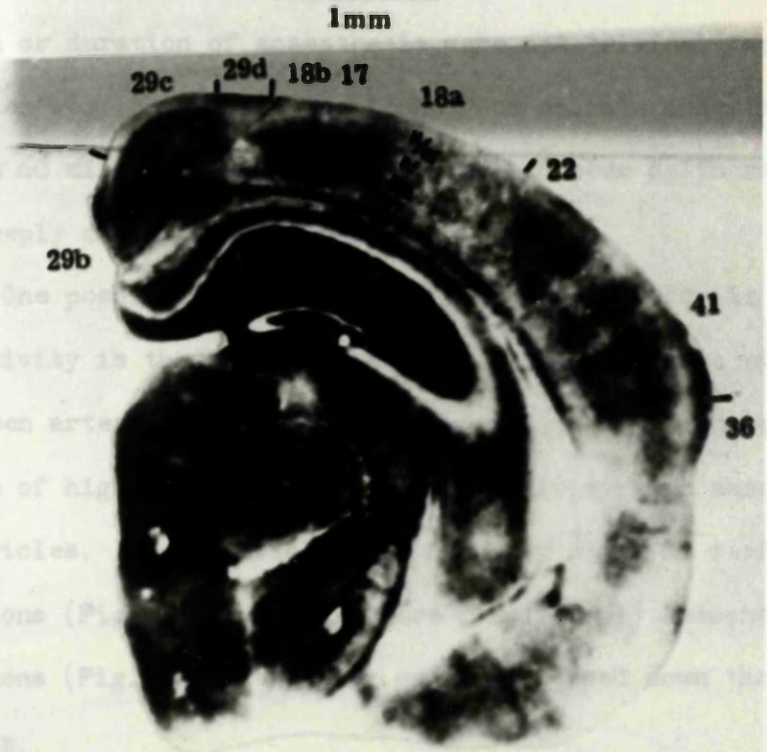
Other cortical areas

In sections from the brains of this group it was very difficult to make out the borders of the different architectonic regions. However, by superimposing photographic images over a diagram of a surface parallel section with the various regions drawn in (Fig.22B), it was possible to

Fig.24 Coronal section through the sensori-motor cortex of a mouse that had been anaesthetised with sodium pentobarbitone and then perfused with cold incubating medium and paraformaldehyde. Bands of high activity each of about 250 μm are seen throughout the extent of the sensori-motor cortex but not in adjacent regions.

Fig.25 Coronal section through auditory and visual areas of the same brain as shown in Fig.24. Bands of high activity are only seen in the auditory areas 41 and 22. These are between 250 and 350 μm in diameter with those in area 22 being smaller than the ones in area 41. (Anaesthetised mouse)


4
6
8
3
24
3a
14
A.C.
1mm


29c
29d
18b
17
18a
22
29b
41
36

obtain an idea of which areas had a modular pattern in their phosphorylase a activity. These were the motor areas 6 and 4, somatosensory areas 3, 1, 3a, 40 and 40a and auditory areas 41 and 22. The modular pattern of staining is uniformly distributed over these areas and there are no obvious borders between the modules in the different areas as seen in surface parallel sections. A modular pattern of staining has only been observed once in the cingulate and visual regions and so far not at all in the olfactory cortex. However, this pattern of staining is commonly seen in many parts of the hippocampal region including the entorhinal cortex (see later). The reasons for this variability among the results from the anaesthetised mice are not known even although an attempt was made to discover them. Varying the length of time between decapitation and freezing the brain showed that short times (5 min.) gave a higher staining intensity than longer times (15 min.) but there were no consistent indications of an effect on the pattern of staining. The depth or duration of anaesthesia were not critical factors either; modules were observed in sections from animals anaesthetised for between 5 and 60 min. and from animals which had been deliberately more lightly or deeply anaesthetised than usual.

One possible cause for a modular organisation in the phosphorylase a activity is that the modules were formed in areas either around or between arterioles. This was found not to be the case as both the areas of high and low phosphorylase activity were associated with arterioles. The arterioles are not very clear in surface parallel sections (Fig. 23 B) but are more convincingly demonstrated in coronal sections (Fig. 26) where they can be followed down through the different layers.

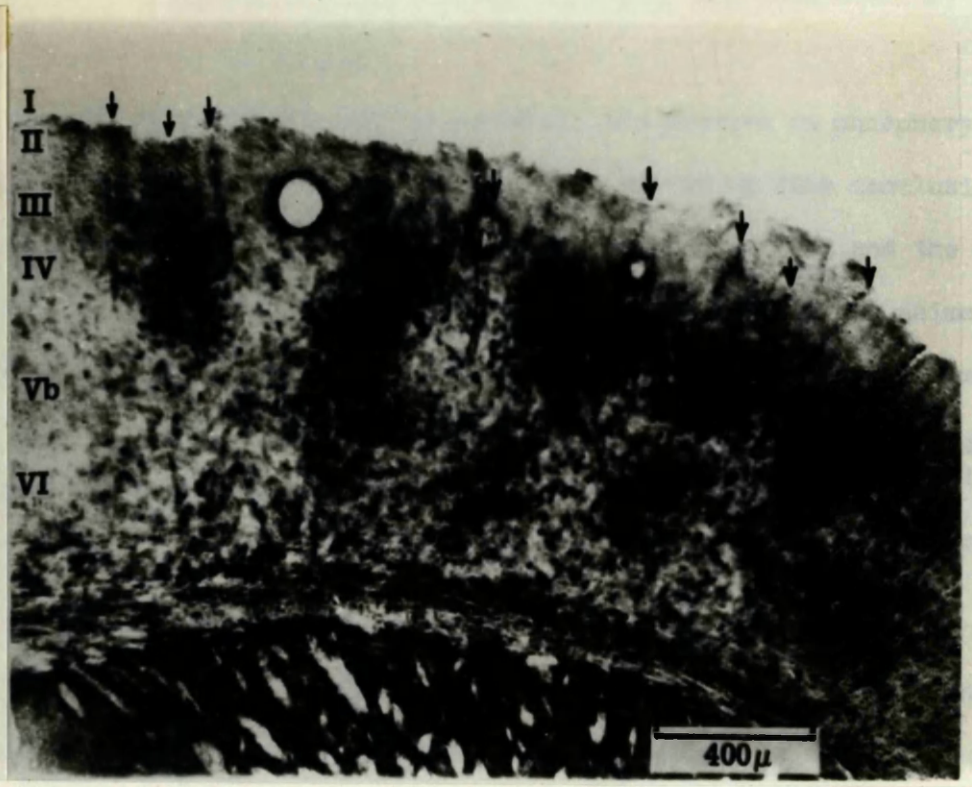
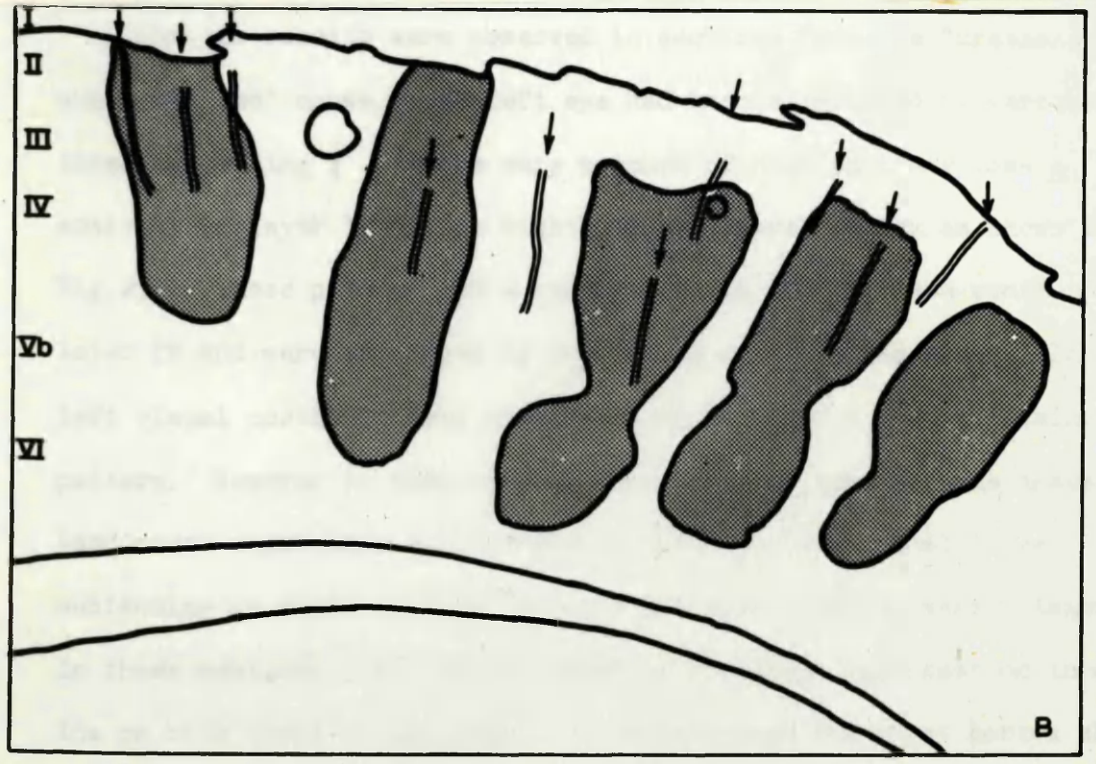


Fig.26 Coronal section through somatosensory cortex. Both the bands of high and the intervening areas of low phosphorylase activity are associated with arterioles. These are marked by arrows. (Pentobarbitone anaesthetised mouse)



Preliminary Experiments on Visual and Somatosensory Cortex

Mouse visual stimulation

Only 3 mice were used in studying the changes in phosphorylase a activity produced by visual stimulation and so no firm conclusions can be reached. Of the three mice one was unanaesthetised and the other two were anaesthetised with urethane. The three experimental animals each had its right eye occluded by a patch and its left eye was stimulated

* Most of the mouse primary visual cortex has a monocular input and only a small lateral part of area 17 has a binocular input.[236, 51] Therefore area 17 in the cortex ipsilateral to the stimulated eye can be used as a control.

some coronal sections through the right primary visual cortex. The area 17 patches were mainly located in layer IV but a few extended down to the base of layer V. The patches had a width of about 200 μm but because of a relatively high background phosphorylase activity they were indistinct and ill defined. Patches were not observed in the left visual cortex.

Similar results were observed in sections from the "urethane anaesthetised" mouse whose left eye had been stimulated by vertical lines subtending $\frac{3}{4}^{o}$.. There were patches of high phosphorylase a activity in layer IV of the right primary visual cortex as shown in Fig.27 . These patches had a width of about 200 μm, were confined to layer IV and were separated by pale zones of a similar width. In the left visual cortical areas there were no signs of a modular staining pattern. However in sections from the brain of the urethane anaesthetised mouse whose left eye had been stimulated by vertical lines subtending an angle of 3° at the eye different results were obtained. In these sections there was evidence of a modular organisation in area 18a on both sides of the brain. A section from the right cortex showing an example of the patches is shown in Fig.28 . In area 18a there were indistinct patches of higher phosphorylase a activity in layer IV and

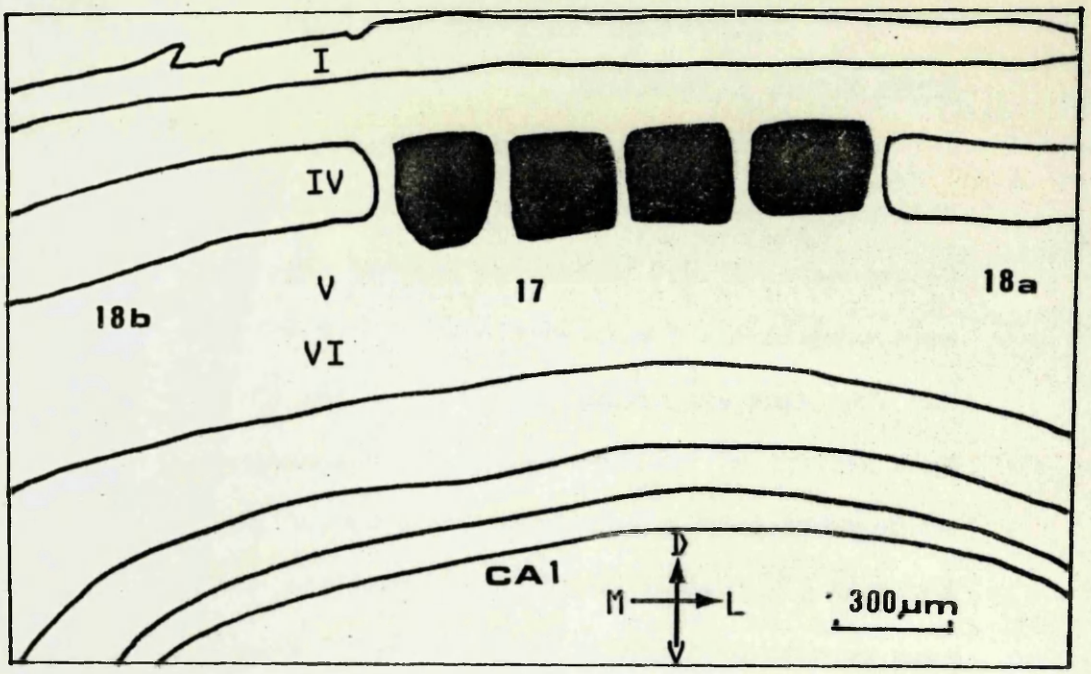


Fig. 27 Coronal section through the right visual cortex of urethane anaesthetised mouse the left eye of which had been stimulated by vertical lines subtending $\frac{3}{4}^{o}$.

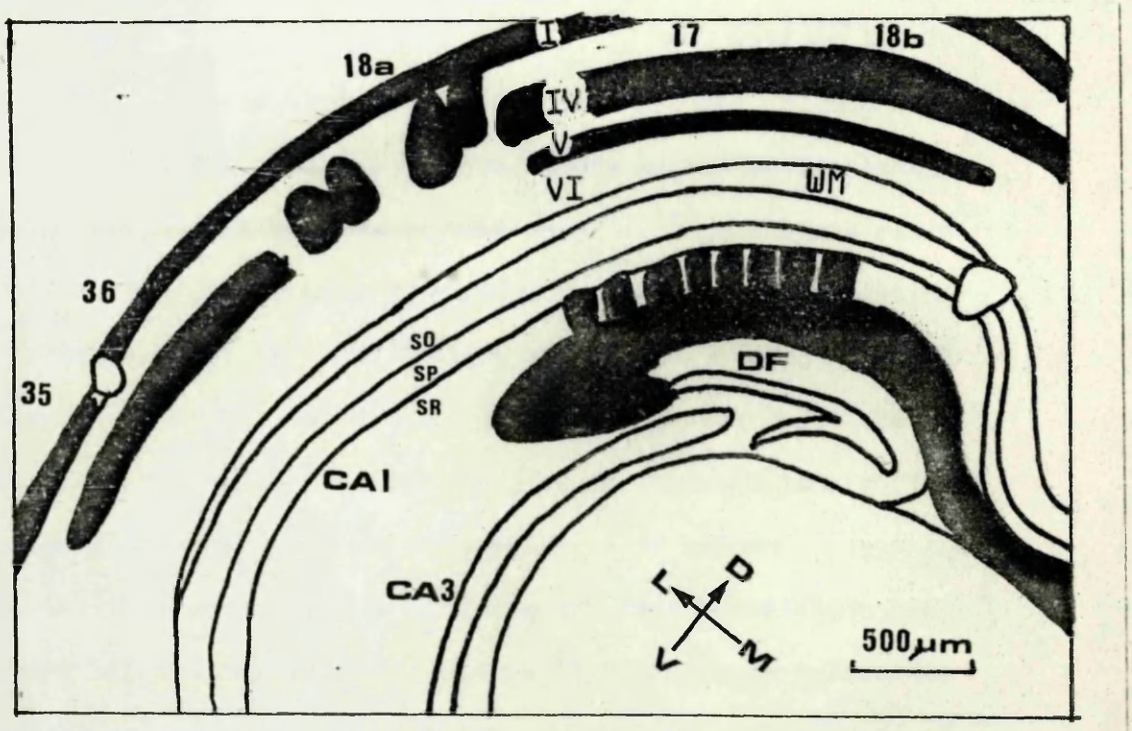
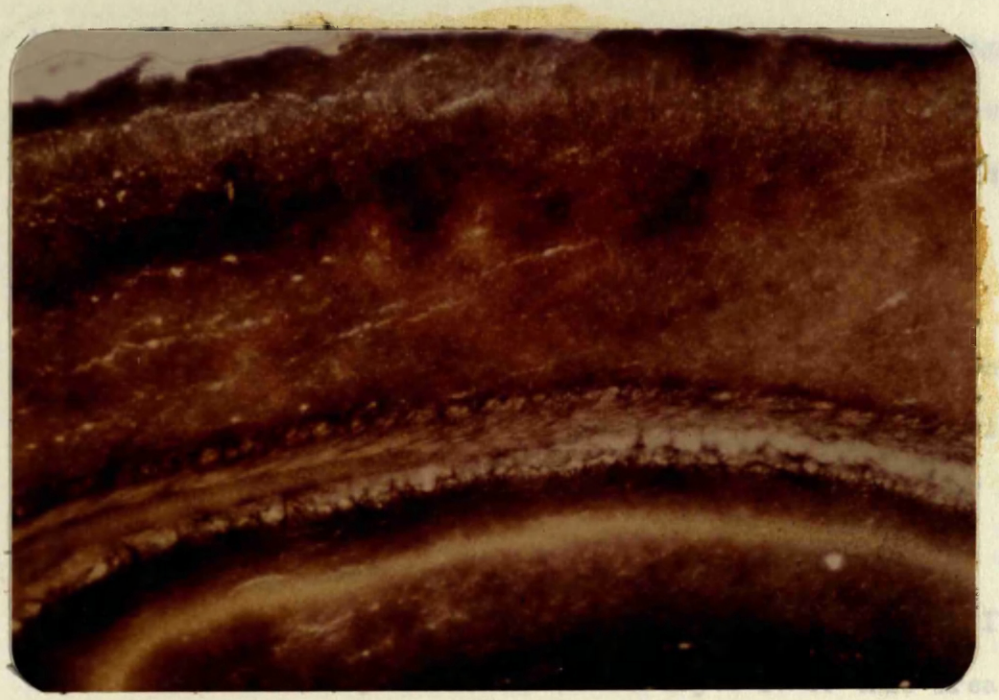
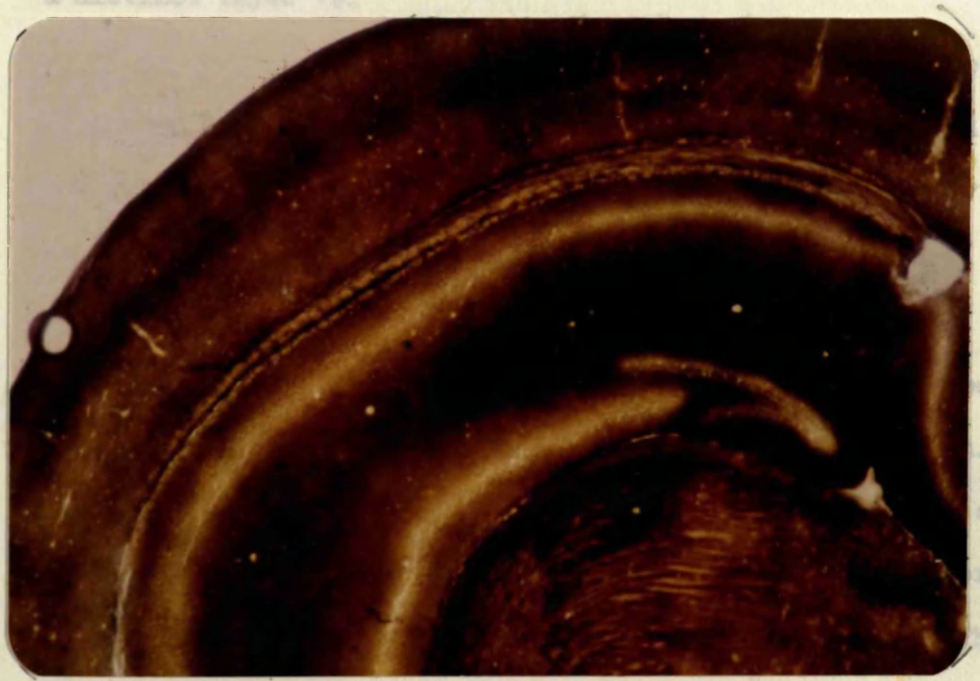


Fig. 28 Coronal section through the right visual cortex of urethane anaesthetised mouse the left eye of which had been stimulated by vertical lines subtending 3°. Note also the faint patches of higher phosphorylase activity in SR of the CA1 area of the hippocampus.





the base of layer III. The patches were about 200 μm wide but were occasionally fused together to give larger patches. No patches of higher staining were observed in area 17 of either side. Fig. 28 also illustrates the faint patches of higher phosphorylase a activity which are seen in the stratum radiatum (SR) of the CA1 area in sections from "urethane anaesthetised" mice.

Control urethane anaesthetised mice

In sections taken from the 4 mice which had been anaesthetised with urethane there are high levels of phosphorylase a activity in all layers of the cortex as shown in Fig. 29 . This figure is of a section passing through the primary (area 3) and secondary (area 40) somatosensory areas. In the primary somatosensory area layers I, IV and Vb are the most intensely stained. Within layer IV of this area there are patches of high staining intensity separated by pale bands. These patches are indicated on the diagram and correspond to the whisker barrels described previously. Area 40 can be distinguished because of the relatively lower staining intensity of layer IV and the absence of a distinct layer Vb.

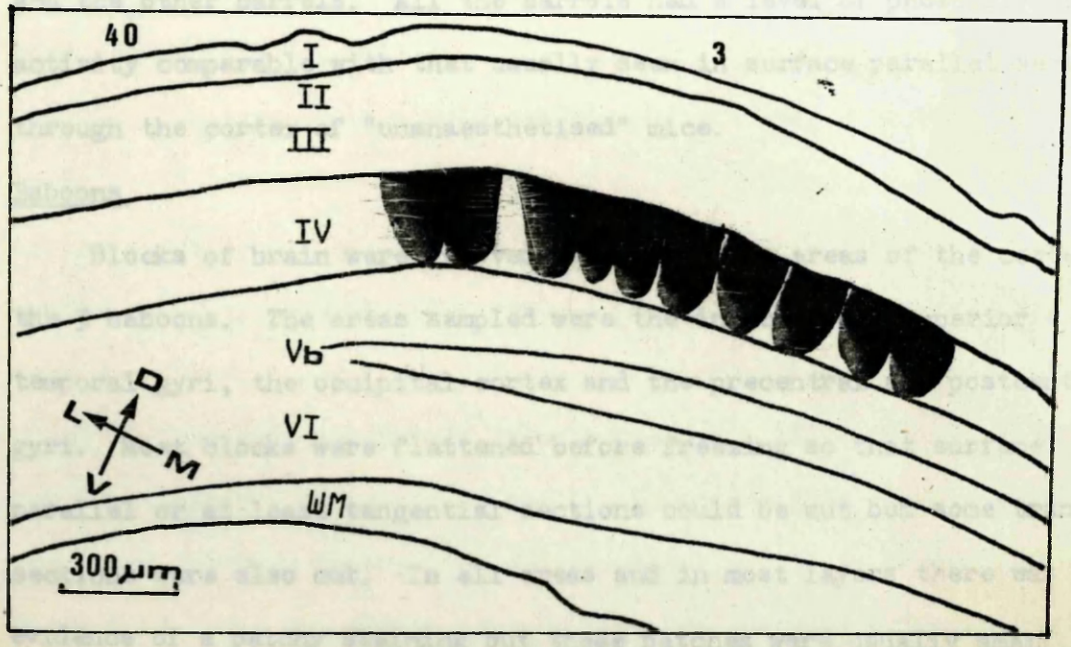
The visual cortex was sectioned in one unstimulated mouse. The intensity of staining was again highest in layers I, IV and Vb. There was no indication of a modular organisation in any of the visual areas on either side of the brain.

Cats and kittens

No evidence of a columnar organisation was found in sections from 2 cats which had had one eye closed or covered with a patch. In surface parallel sections there were small patches with a diameter of about 60 μm. These were also seen in coronal sections and so the phosphorylase staining was in the form of small spheres not cylinders. There were fewer patches in layers II and VI but otherwise they were fairly evenly distributed. In a few coronal sections through the visual cortex from



Fig. 29 Coronal section through somatosensory cortex of a urethane anaesthetised mouse showing the higher staining intensity in the barrel hollows.



one of the control cats the patches had coalesced into irregular bands with a width of 300-400 μm. However these bands were very vague and indistinct.

The brains of 4 out of the 5 kittens provided no evidence of a modular organisation when sections were cut through area 17 and incubated to demonstrate phosphorylase a activity. However there was an indication of a possible modular organisation in sections from area 17 of the 41 day old kitten. An irregular patchy appearance occurred in layers II - IV of the cortex. These patches were irregular in shape and had very indistinct outlines. They were about 300 μm in diameter and did not seem to be arranged in rows. No coronal sections were cut and so it is not known whether the patches represent cylinders of high phosphorylase a activity or not.

Mouse whisker removal

Groups of whiskers were removed from the right muzzle of 2 mice and after 30 min. the mice were decapitated and surface parallel sections were cut through the somatosensory region of the cortex on both sides of the brain. There was no obvious difference between the barrels receiving a primary input from the whiskers that had been cut and the other barrels. All the barrels had a level of phosphorylase a activity comparable with that usually seen in surface parallel sections through the cortex of "unanaesthetised" mice.

Baboons

Blocks of brain were removed from different areas of the cortex in the 3 baboons. The areas sampled were the inferior and superior temporal gyri, the occipital cortex and the precentral and postcentral gyri. Most blocks were flattened before freezing so that surface parallel or at least tangential sections could be cut but some transverse sections were also cut. In all areas and in most layers there was evidence of a patchy staining but these patches were usually small,

being only 60-100 μm in diameter. These patches appeared both in coronal and surface parallel sections and so represent spots of high phosphorylase activity that are more spherical than cylindrical in shape.

Larger patches of high phosphorylase activity were only seen in the two primary sensory areas among the cortical regions sampled. In area 17 large patches with a diameter of about 250 μm were observed in layers II and III (see Fig. 30). The patches were clearest in layer III but even here they were indistinct and ill-defined. Further blocks would need to be sectioned in order to obtain a clearer picture of their arrangement. No clear pattern of rows could be observed because although the patches of high activity were often joined by areas of moderate activity the areas of moderate activity did not have any marked preferential alignment. In one place the dark patches coalesced to form a large dark area with a maximum diameter of about 2 mm. In layers IV and V no large patches were observed, instead the smaller patches found in all areas of the cortex were present as indicated in Fig.31 which is a surface parallel section through layer IV. Layer I was relatively dark and fairly homogeneously stained. No transverse sections were cut through the visual cortex and so the large patches have not yet been observed in this orientation.

Some preliminary evidence of large patches was also obtained in layer IV of the primary somatosensory cortex. These were bigger than in the visual cortex being about 300-400 μm in diameter and were formed by the close association of a group of small patches. The small patches were similar to those found in other parts of the cortex and occurred singly in the relatively pale areas between the large dark patches of the somatosensory cortex. The small patches are seen more clearly in a transverse section through the somatosensory cortex as shown in Fig. 32 . There is a dense collection of small patches throughout layer IV where



Fig. 30 Surface parallel section through layer III of area 17 of baboon cortex. Large patches with a diameter of about 250 µm are observed.



Fig. 31 Surface parallel section through layer IV of area 17 of baboon cortex. Only small patches of phosphorylase activity with a diameter of about 100 µm are observed.

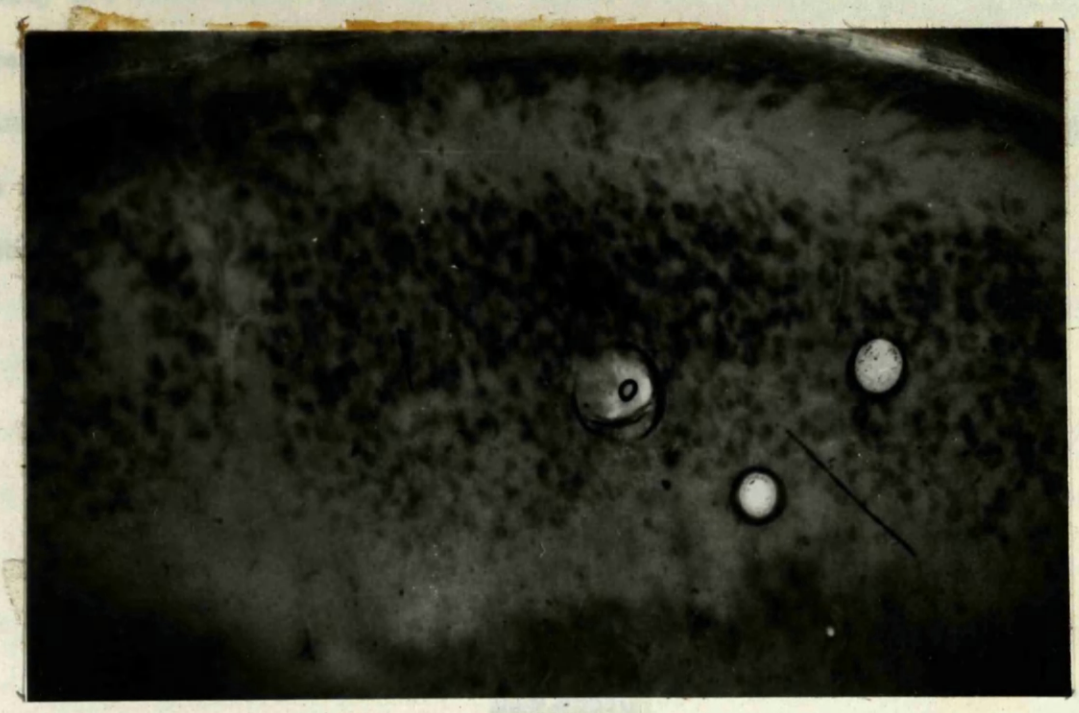
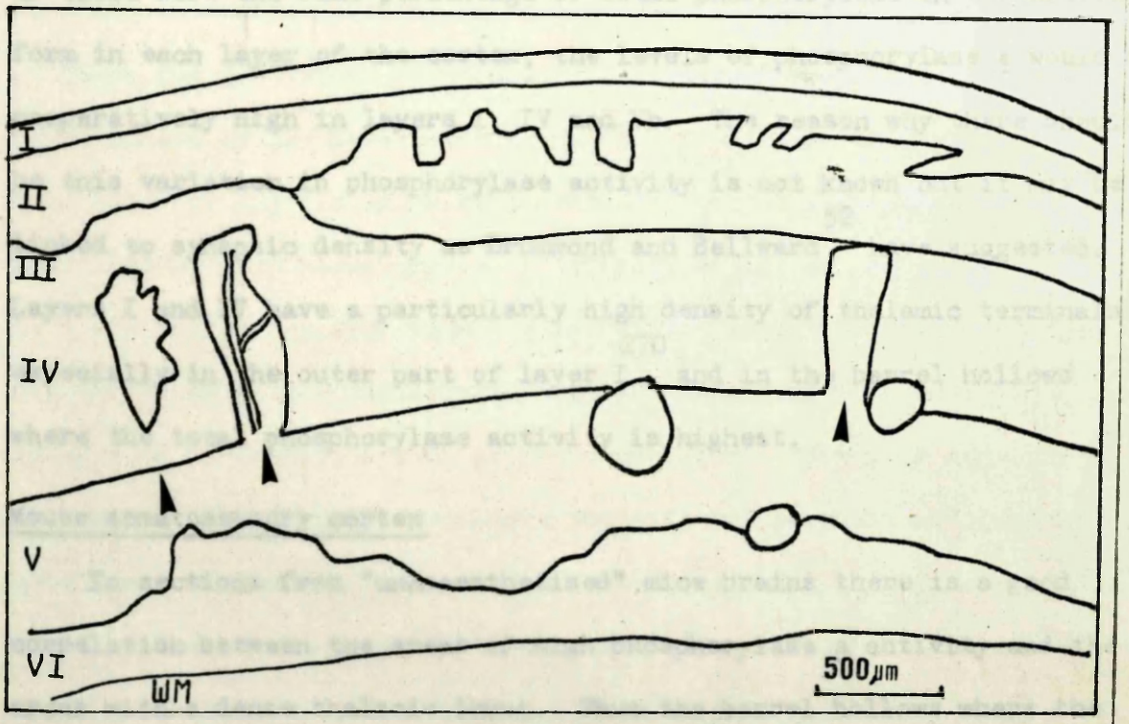


Fig. 32 Transverse section through baboon primary somatosensory cortex. Small patches are observed in all layers except layer I. In layer IV the patches are sometimes collected together to form bands of more densely aggregated patches.



they are relatively homogeneously distributed. Occasionally there are pale bands in layer IV which have relatively few small patches as indicated by the arrows in Fig. 32 . These isolate bands of tissue with a high density of patches that may correspond to the large patches observed in tangential sections.

DISCUSSION

Total phosphorylase

The comparatively high level of total phosphorylase observed in layers I, IV and Vb of mouse neocortex was also seen in a histochemical study of total phosphorylase activity in the sensori-motor cortex of the rat.[204] This laminar variation in total enzyme levels implies that, even if there were the same percentage of total phosphorylase in the active form in each layer of the cortex, the levels of phosphorylase a would be comparatively high in layers I, IV and Vb. The reason why there should be this variation in phosphorylase activity is not known but it may be linked to synaptic density as Drummond and Bellward[52] have suggested. Layers I and IV have a particularly high density of thalamic terminals especially in the outer part of layer I[270] and in the barrel hollows where the total phosphorylase activity is highest.

Mouse somatosensory cortex

In sections from "unanaesthetised" mice brains there is a good correlation between the areas of high phosphorylase a activity and the areas with a dense thalamic input. Thus the barrel hollows where the

thalamic afferents terminate have high levels of phosphorylase a activity[125] as does layer Ia which also has a thalamic input.[270] Furthermore the phosphorylase a levels in layer IV are higher in area 3 than in areas 6, 40 or 29c (Fig. 21) and these latter three areas have a lower density of thalamic terminals in layer IV than area 3.[72] In contrast in sections from most "pentobarbitone anaesthetised" mice layer IV is relatively pale while the top three layers are more intensely stained. Cortico-cortical afferents terminate primarily in the upper three layers of the cortex[252] and so the phosphorylase a activity may be more closely correlated with cortico-cortical afferents than thalamic afferents in the sections from "pentobarbitone anaesthetised" mice.

In sections from control unanaesthetised mice there was no evidence of a columnar organisation in the ^{14}C2DG levels[53] in the layers adjacent to layer IV of the barrel cortex. Similarly in the present work, the phosphorylase a activity in sections from "unanaesthetised" mice showed no evidence of discrete areas of functional activity extending through the various layers of the cortex. In contrast, the brains of six "anaesthetised" mice have discrete cylinders of high postmortem phosphorylase a activity extending through 4 or 5 layers of the cortex, providing strong evidence for a columnar organisation. Evidence of a similar arrangement of cylinders in the somatosensory cortex of anaesthetised rats was obtained[127] by the bioluminescent demonstration of ATP levels. The cylinders of high phosphorylase a activity of the six "anaesthetised" mice are not in register with the barrels. Therefore these cylinders are unrelated to the thalamic afferents. Experiments investigating the effects of anaesthetics such as pentobarbitone[8] have been interpreted as showing that during anaesthesia the somatosensory modulation system inhibits the specific thalamic input to the primary sensory areas of the cortex. Thus a non-thalamic input may be dominant during anaesthesia and this is reflected in the postmortem phosphorylase a levels.

Even within the specialised barrel neuropil, only 20% of the synapses are formed by thalamic afferents.[251,250,96] The other major input to the cortex is from corticocortical fibres.[24] As there are few callosal fibres terminating in the barrel cortex,[270] a large proportion of the input to the barrel cortex must be from ipsilateral corticocortical fibres. Such fibres arising from the mouse barrel cortex have been shown[252] to terminate in columns with a diameter of about 250 μm. Szentagothai[223] has suggested that generally within the neocortex corticocortical fibres terminate in 250 μm wide columns in an array that is out of register with the thalamic afferents. Therefore the cylinders of high phosphorylase a activity in the somatosensory cortex and perhaps elsewhere may correspond to the columnar terminal fields of corticocortical afferents.

There are significant callosal connections between the barrel areas on each side of the brain and these are homotopically arranged.[174] However the callosal afferents are not in register with the thalamic afferents as the callosal terminals have been shown anatomically[252] to occur both in the barrels and in the septa between.

Morphological studies support this hypothesis because Lorente de No[141] described a number of cell types with both ascending and descending axonal arborisations which were not in register with the layer IV barrels (see Literature Review). He also described at least two types of afferent fibres, the terminal fields of which were not in register with the layer IV barrels. Furthermore Detzer[46] has shown that the bundles of apical dendrites ascending from layer V bend to avoid the barrel hollows thus suggesting that the output columns may not be in register with the input columns.

The hypothesis of two overlapping sets of columns is consistent with the results of degeneration studies in the rat.[1] Large lesions placed in the upper three layers of the barrel cortex produce a uniform degeneration of fibres within layer V. If there were only one set of columns

in the barrel cortex then there should be a patchy degeneration in layer V that would be in register with the barrels. However if there are two sets of overlapping columns in the barrel cortex, each with its vertically arranged intracortical connections, then the layer V degeneration produced by a layer III lesion should be fairly uniform, as observed.

As phosphorylase a levels in the brain are related to functional activity, it is necessary to be cautious about assuming that a physiological marker would be able to demonstrate columns which have only been defined on anatomical grounds. However evidence from rodent[53] and monkey[70] somatosensory cortex and cat visual cortex[108] show that at least in some cases physiologically and anatomically defined columns are the same.

Mouse visual cortex

In the visual cortex of a few "unanaesthetised" mice there were rows of patches of high phosphorylase a activity in layers IV and VI. As the patches were in register, this area of cortex appears to have a columnar organisation. This is in accord with results from alert unstimulated monkeys where the levels of ^{14}C2DG in the primary visual cortex indicate a clear columnar organisation.[93,123] In the monkey these bands of high and low activity may represent orientation columns[106] because these have been demonstrated in the monkey[108] and cat[136a] with the ^{14}C2DG technique after 45 min. of the appropriate stimulation. By using electrical recording techniques in conjunction with the ^{14}C2DG method Schoppman and Stryker[201] proved that the bands of altered 2DG activity do correspond to the orientation columns in the cat.

In the mouse Drager[49] found that about 45% of the cells in the primary visual cortex are orientation sensitive but there was little evidence of these cells being organised into columns. This was not surprising because Towe[228] has demonstrated on theoretical grounds, that the small columns found in the mouse cortex could not be demonstrated by

electrophysiological techniques alone, as their spatial resolution is not high enough. This was confirmed in electrical studies of the rat[209] and mouse barrel cortex,[210] where even when great care was taken to make perpendicular electrode penetrations, the electrode passed from one column to another and there was an apparent shift in receptive field location with depth. Thus the clustering of orientation selective cells observed by Drager[49] may in fact represent columns and the bands of high phosphorylase a activity observed in the mouse cortex may be related to line orientation.

The bands of high phosphorylase a activity which were found largely in parts of the visual cortex with a monocular input cannot correspond to ocular dominance columns. Most of the rodent primary visual cortex has a monocular input[236,51] and even in the small lateral area which receives a binocular input Drager[50] was unable to find any evidence of ocular dominance columns.

If as in the barrel cortex of "unanaesthetised" mice the patches of high phosphorylase a activity correspond to the thalamic input then, as the thalamic input to areas 18a and b is from the lateral thalamic nuclei and not the lateral geniculate,[35] the finding of modules in area 18 implies that these thalamic nuclei and not just the primary sensory relay nuclei may give rise to afferents which terminate in a columnar fashion.

Both Lorente de No[144] and Mountcastle[159] suggested that the afferent input forming physiological columns would be focused by pericolumnar inhibition. In rodent cortex there is no evidence[171,230] of inhibitory basket cells similar to those found in the primate but there are other cells with a vertical axonal arborisation that are thought to be inhibitory. In the mouse visual cortex Valverde[230] described 5 main classes of non-pyramidal cell. All of these had axon arborisations arranged in a predominantly vertical fashion in a cylinder of tissue having a diameter of 200 - 300 μm. In the rat[171] there are four types of smooth multipolar

stellate cell all with axon arborisations arranged in a vertical cylinder with a diameter of about 200 μm. These cells apparently correspond to glutamic acid decarboxylase (GAD) containing cells identified in the rat visual cortex by Ribak.[59] As these cells contain GABA they could provide the basis of an effective pericolumnar inhibition. Thus morphological findings support the contention that the mouse visual cortex also has a columnar organisation.

No evidence of a columnar organisation was observed in sections from the visual cortex of "pentobarbitone anaesthetised" mice. However this does not imply that there are no corticocortical columns in the mouse visual cortex. Indeed Montero[156] has shown that there is a columnar organisation in the corticocortical connections of the rat visual areas. Therefore it is likely that the corticocortical connections of the mouse are arranged on a column to column basis as they are in the primate brain.[260]

Mouse auditory and motor cortex

In three of the "anaesthetised" mice cylinders of high phosphorylase staining were observed in the motor and auditory areas. These cylinders were very similar to the ones found in the somatosensory cortex and may also correspond to corticocortical columns. There is physiological evidence for a columnar organisation of the motor cortex in cats, monkeys[12] and rats[40] as well as direct anatomical evidence for columns produced by corticocortical fibres in the mouse motor cortex.[252] Similarly in the auditory cortex there is physiological evidence for a columnar organisation in higher mammals[113] and anatomical evidence from the rat for callosal afferents terminating in discrete strips.[257,37] In the monkey auditory cortex Fitzpatrick[64] showed that a column of cells in the primary auditory area was specifically connected with a number of other columns in the secondary auditory areas. He concluded that this was a general property of corticocortical connections in the auditory cortex. If this is so then it implies that in the mouse auditory cortex the corticocortical connections may also be arranged on a column to column basis.

Temporal duration of the metabolic cylinders

The fact that vertical bands of phosphorylase a activity were only seen in sections from a few of the brains implies that they are related to transient functional structures. Mountcastle[157] emphasised that columns produced by primary sensory input under normal circumstances would have a short duration - perhaps of less than 40 msec. Most of the bands of phosphorylase a activity were not related to primary sensory input as they occurred in sections taken from brains of animals which had not received any specific sensory stimulation. The metabolic changes were also of comparatively long duration as they were stable enough to survive the processes associated with death. The lack of any specific sensory input suggests that they were produced by internal processes. The fact that the vertical bands were of high contrast in sections from some brains, only vaguely outlined in others and not present in most, implies that the underlying processes were cyclical in nature.

Electrical recording from the scalp of both man and animals has provided evidence that there are a number of processes in the brain which exhibit a cyclical form.[159] Also nocturnal mammals such as the rat show cyclic variations between rest and activity with a period of 60 to 90 min.[159] Thus there are cyclical processes in the brain that might be indirectly related to systems producing a columnar organisation in the cortex. The state of arousal is thought to be related to input from the reticular formation which arrives at the cortex via non-specific thalamic afferents.[159] Scheibel and Scheibel[199] showed that the branches of these fibres terminated in narrow vertical cylinders and they suggested that they might be correlated with the cortical columnar organisation. As these fibres serve an energising function[159] they might be able to selectively alter one system of columns with respect to other systems in a way that would be registered by postmortem phosphorylase a activity. In the sections from "anaesthetised" mice especially the widespread occurrence of the

cylinders of high phosphorylase a activity implies that some generalised system is involved.

Preliminary experiments involving sensory stimulation

It was suggested above that the bands of high phosphorylase a activity seen in the visual cortex might be related to orientation columns. The obvious way to test this hypothesis was to stimulate one eye of the mouse with a pattern of vertical lines and then to study the phosphorylase a activity in sections from the contralateral cortex. Sections from the ipsilateral cortex could be used as a control as the optic pathways in the mouse are almost completely crossed. The three mice studied in this way did show evidence of a modular organisation in layer IV of the visual cortex thus providing additional evidence of a columnar organisation in the mouse visual cortex. As the modules in area 17 were only observed in the contralateral cortex the results from the first two mice were consistent with the hypothesis that the bands of phosphorylase a are related to line orientation. In the third mouse evidence of modules was observed in 18a from both sides of the cortex. However as this area

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receives input from both retinae this result was also consistent with the above hypothesis. However further experiments will be required before any firm conclusions can be drawn.

No evidence for a columnar organisation was observed in sections from the two cats which had had patches placed over one eye. No evidence of a columnar organisation was observed in sections from the visual cortex but this may have been because the open eye was not specifically stimulated. There was some evidence of a columnar organisation in sections through the visual cortex of one control cat and one of the 5 control kittens where indistinct bands of phosphorylase a activity were observed. The absence of any clearer evidence of the columnar organisation known

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to exist in the cat cortex may have been due to the depth of anaesthesia and general condition of the animals. The three cats were deeply

anaesthetised and their general body functions had been disrupted by a day of experimental manipulation by undergraduate students. Similarly, the kittens had been killed by an overdose of anaesthetic and so their brain activity in the minutes before death would have been distinctly abnormal.

No noticeable alteration in phosphorylase a activity was found in sections from the barrel cortex of the two mice which had had some of their whiskers cut off. This was probably due to the experimental design. After some of the whiskers had been removed the mouse was placed in a covered tray containing a variety of objects for it to explore. However the mice were intimidated by the strange environment and sat huddled in a corner. As the whiskers were not used in exploration the efferent input from the ventrobasal thalamus would not be markedly different in the barrel cortex on the two sides of the brain. A more effective experimental protocol would have been to manipulate a single vibrissa manually as this has been shown to produce an increase in deoxyglucose uptake[88] in the corresponding whisker barrel.

Baboon cortex

In tangential sections through the macaque primary visual cortex cytochrome oxidase staining is arranged in an array of dark oval patches with a size of about 150 x 200 μm. These patches are most obvious in layers II and III and are generally aligned in rows spaced about 350 μm apart. A similar pattern of patches is observed in tangential sections stained for glutamic acid decarboxylase (GAD)[94] the enzyme responsible for synthesising GABA. Also in monkeys which have had both eyes stimulated with lines of all orientations a similar array of patches is seen in autoradiographs used to detect the level of ^{14}C2DG uptake in layers II and III. The histochemical and ^{14}C2DG patches are all in register[94] and so the supragranular layers of the monkey cortex are organised into cylinders of tissue with a diameter of about 300 μm that contain a particularly high density of inhibitory GABA-ergic cells and

are primarily responsible for processing information about lines of all orientation from one eye. As distinct phosphorylase a patches were only observed in area 17 of the baboon cortical areas studied in the present investigation, these patches are thought to correspond to those described above.

Both thalamic [157,70] and corticocortical [71] columns have been described in the primate somatosensory cortex but there has been no report of discrete patches in sections stained for cytochrome oxidase activity. Therefore the lack of discrete patches in sections incubated to demonstrate phosphorylase a activity is not surprising. The baboons were killed by an overdose of anaesthetic. Therefore any pattern of functional columns could well have been erased by the time the blocks of brain were frozen. The significance of the small patches of phosphorylase a activity observed in all areas of the cortex, especially in layer IV, is not known but they may represent the terminal fields of axons from small intracortical neurones such as the small basket cells.[223]

SECTION II MODULAR ORGANISATION OF LIMBIC AREAS

Introduction

Kornhuber[129] has proposed that the neural control of the input into long term memory stores in the brain is largely mediated by the limbic system. The evidence for this comes mainly from clinical studies where a correlation has been found between partial loss of memory and the destruction of certain structures. Thus bilateral removal or damage to some cortical structures such as the cingulate gyrus[170] or prefrontal cortex[165] are associated with partial amnestic syndromes. Complete anterograde amnesia (inability to lay down any new memory traces) has only been specifically produced by the bilateral removal of the hippocampus and adjacent areas of the hippocampal gyrus.[169] Therefore it is now widely accepted[163] that the hippocampal formation has a fundamental role in the selective registration of information. Animal experiments have confirmed the role of the hippocampus in memory formation.[163] They indicate that the hippocampus is simultaneously involved in the selection and organisation of the different afferent inputs to be used in memory trace formation and in the selection of an appropriate response to the sensory input to the brain, whether this is a shift of attention or of a goal-directed purposive nature.[43]

Eccles[56] has pointed out that these functions would require a finesse of connectivity far beyond that of the lamellar organisation described by Andersen.[5,6] The existence of such a high degree of specific connectivity in the form of interconnected modules has been postulated to occur in all the higher brain centres involved in information processing.[158] As the hippocampus is involved in processing all the information arriving at the cerebral cortex[43] then for Mountcastle's theory to be correct the hippocampal region must have a modular organisation.

There is a growing body of evidence that the cerebral cortex is arranged in arrays of specifically interconnected modules or columns in both primary sensory[64,260] and association areas.[79,71] Some of these association areas provide the afferent input to the entorhinal cortex and other parts of the hippocampal region.[129,17] At least some of these inputs are strictly segregated[140] and may give rise to a columnar organisation. A number of anatomical studies have provided evidence that at least parts of the hippocampal region have a modular organisation (see Literature Review). Consequently sections were cut at various orientations through the hippocampal region of rats and mice in order to look for evidence of a modular organisation. It was anticipated that if the hippocampal formation does have a modular organisation then this would be reflected by the phosphorylase a activity as it was in the neocortex.

During mammalian evolution the structure of the hippocampus has been strictly conserved[9] and although there are some differences between species such as the rat and rabbit,[163] or monkey,[143] recent allometric studies have shown that the hippocampus of the mouse is just a smaller version of the rat's.[248] Therefore the results obtained from 28 mice and 19 rats will be described together because the organisation and specificity of connections should be the same even if there are a few minor differences.

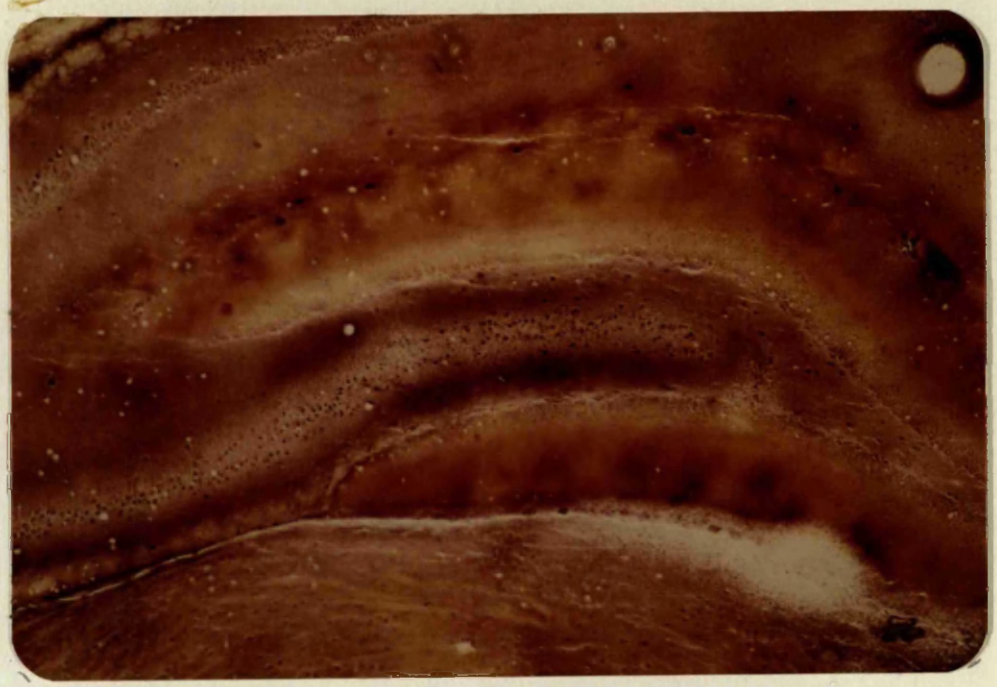
The results from the neocortex showed that giving the animal sodium pentobarbitone altered the postmortem phosphorylase a activity to provide evidence of a columnar organisation not seen in sections taken from the brains of "unanaesthetised" mice. Therefore in studying the hippocampal formation both anaesthetised and unanaesthetised animals were used. The study group was composed of 15 unanaesthetised mice, 13 unanaesthetised rats, 6 urethane anaesthetised rats, 9 Sagatal anaesthetised mice and 4 urethane anaesthetised mice. The evidence of a modular organisation observed in sections from these brains is summarised in the diagram shown in Fig.79.

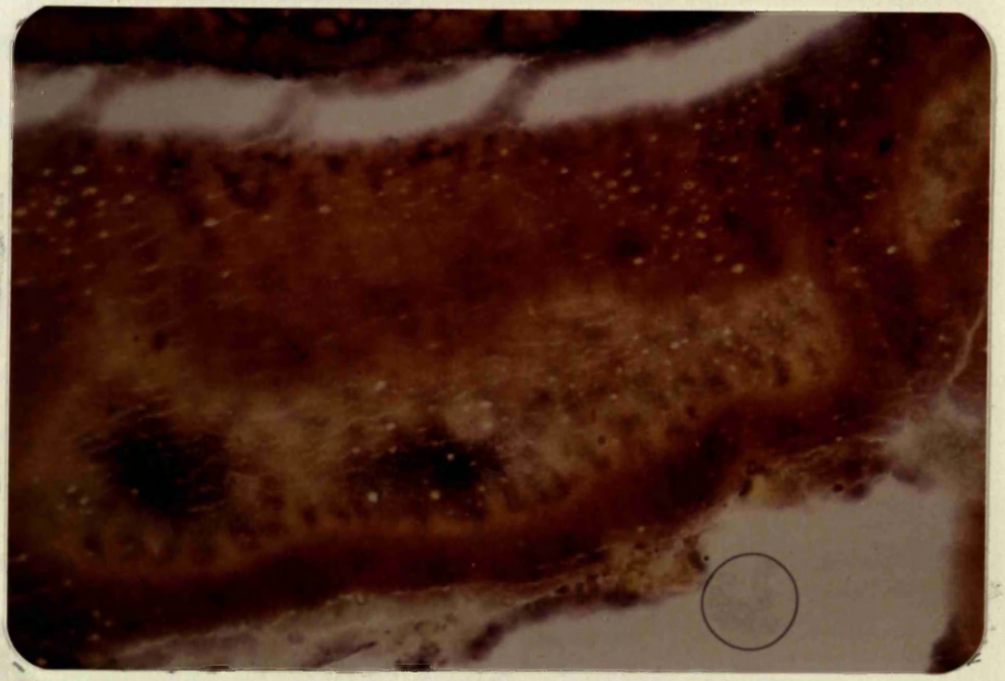
RESULTS

General differences in the intensity of staining of the various layers in the different areas of the hippocampal region were not studied, as the main object of the investigation was to look for evidence of a modular organisation, not to compare sections from "anaesthetised" and "unanaesthetised" brains. There were differences in the modular organisation observed in sections from "anaesthetised" and "unanaesthetised" brains but on the whole these were more in the clarity with which the modules were observed rather than in their absence from any one group. The exception to this was that patches were seen in layer III of the entorhinal cortex in sections from "unanaesthetised" mice but not in those from "anaesthetised" mice. Therefore in the following paragraphs each architectonic area will be discussed in turn and evidence of its modular organisation presented, using the best illustrations irrespective of whether they come from anaesthetised or "unanaesthetised" animals.

Dentate Fascia

The hippocampus is known to be organised into lamellae arranged roughly at right angles to the long axis.[5,6] When sections were cut parallel to the long axis there were indications of discrete subdivisions which may be related to these lamellae. In Fig. 34 which is a surface parallel section through the ventral blade of the dentate fascia, there is a series of patches of high phosphorylase a activity in the granule cell layer. The dark patches are wider than the intervening pale areas and in some instances they nearly fuse. The distance between the centre of each patch is about 80 µm. In the part of the section grazing through the middle of the granule cell layer the patches are seen face on and are broader than they are long. Thus the phosphorylase activity is arranged in patches or modules and not strips. There is evidence of these patches in coronal sections as well (see Fig. 52) but they are not as clearly defined as in the surface parallel sections.





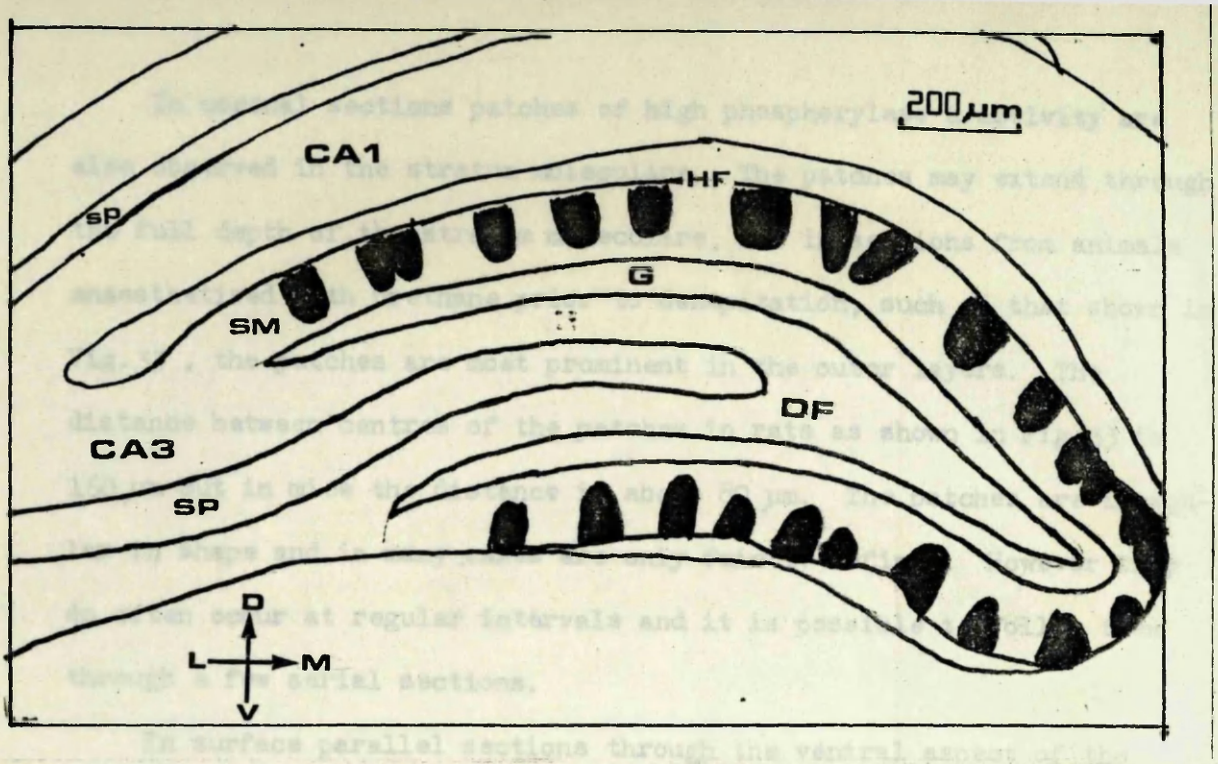


Fig. 33 Coronal section through the hippocampus demonstrating the patches observed in the stratum moleculare of the dentate fascia. Individual cell bodies in the granule and pyramidal cell layers appear as small purple dots and some of their apical dendrites appear as fine purple-brown lines. The section was cut at a level corresponding to Craigie's plate A21. (Urethane anaesthetised rat)

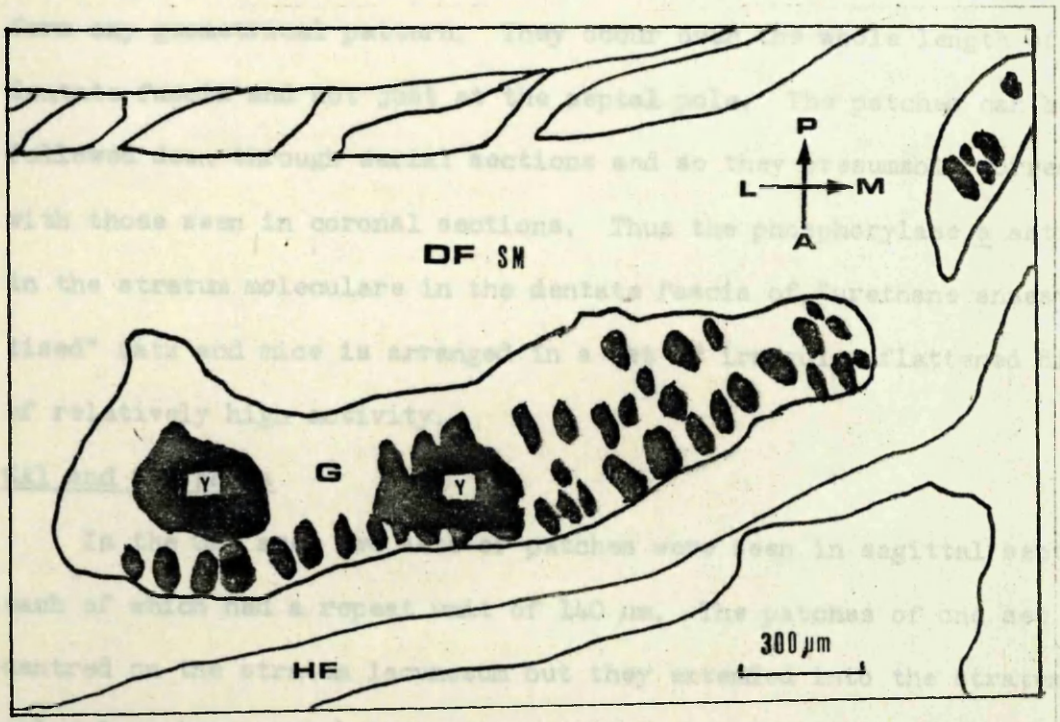


Fig. 34 Surface parallel section through the partially unfolded and flattened ventral blade of the dentate fascia. This is the septal end of the dentate fascia and so there is no purple band (X) lying above the granule cell layer. Elongated purple patches are seen in the granule cell layer (G). (Unanaesthetised rat)

In coronal sections patches of high phosphorylase a activity are also observed in the stratum moleculare. The patches may extend through the full depth of the stratum moleculare, but in sections from animals anaesthetised with urethane prior to decapitation, such as that shown in Fig. 33 , the patches are most prominent in the outer layers. The distance between centres of the patches in rats as shown in Fig. 33 is 160 μm but in mice the distance is about 80 μm. The patches are irregular in shape and in many cases are only faintly defined. However they do often occur at regular intervals and it is possible to follow them through a few serial sections.

In surface parallel sections through the ventral aspect of the dentate fascia of brains taken from "urethane anaesthetised" rats the patches have a roughly ovoid shape (see Fig. 35). The patches have diameters ranging from 80 to 250 μm. They are irregularly arranged and like the patches in the neocortex of "anaesthetised" mice they do not form any geometrical pattern. They occur over the whole length of the dentate fascia and not just at the septal pole. The patches can be followed down through serial sections and so they presumably correspond with those seen in coronal sections. Thus the phosphorylase a activity in the stratum moleculare in the dentate fascia of "urethane anaesthetised" rats and mice is arranged in a set of irregular flattened discs of relatively high activity.

CA1 and CA3 areas

In the CA1 area two sets of patches were seen in sagittal sections each of which had a repeat unit of 140 μm. The patches of one set were centred on the stratum lacunosum but they extended into the stratum moleculare (str. mol.) and radiatum. This is illustrated in Fig. 36 which is a sagittal section through the dorsal hippocampus. The patches of high activity are about 120 μm in width and are separated by pale strips with a width of about 20 μm. The row of eight patches extend

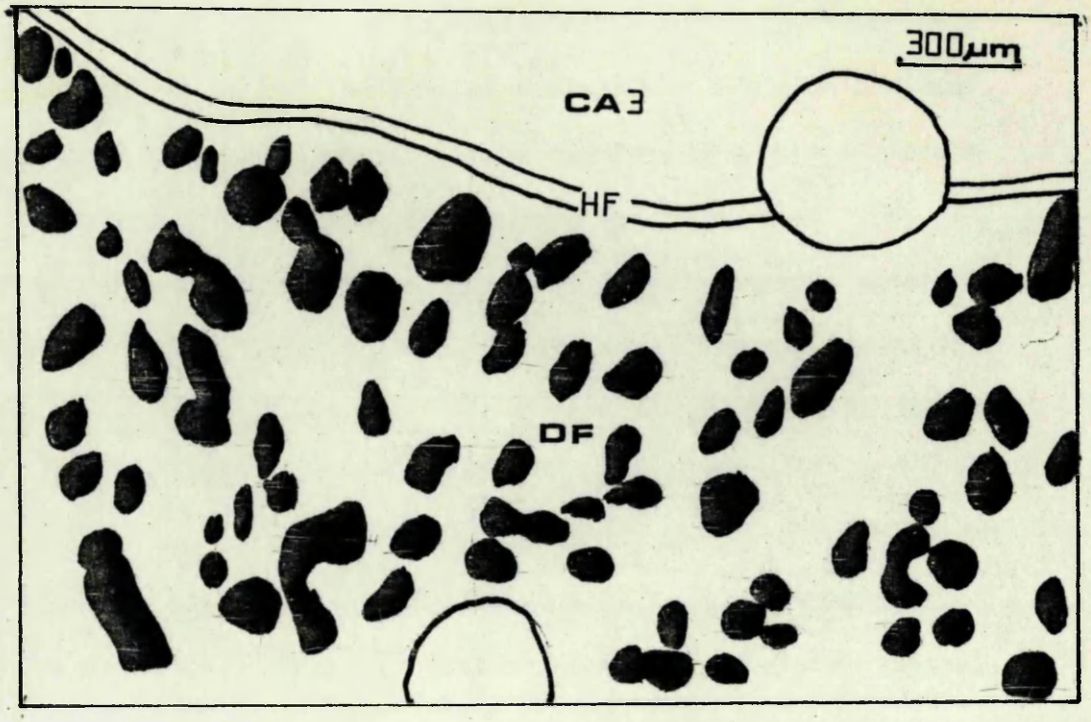


Fig. 35 Surface parallel section through the ventral aspect of the stratum moleculare of the dentate fascia showing ovoid patches. (Urethane anaesthetised rat)

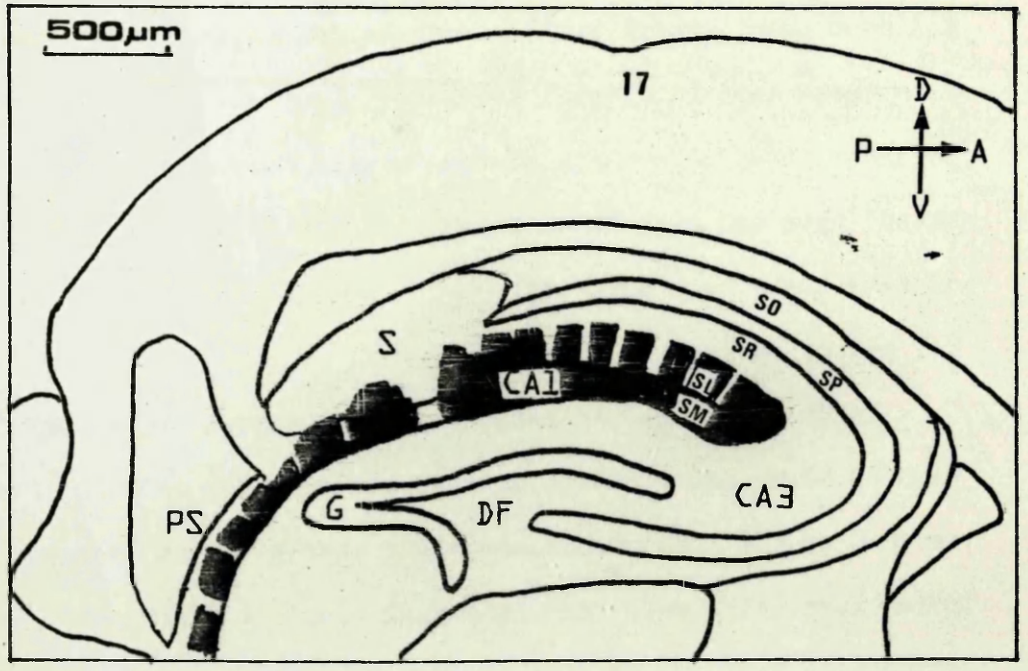
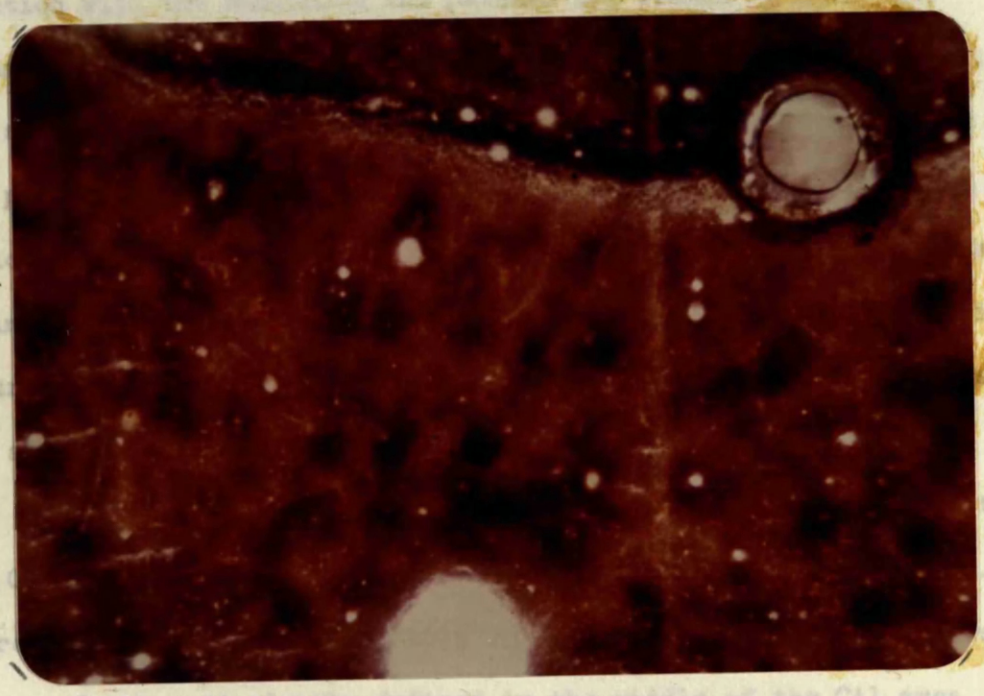


Fig. 36 Sagittal section through the septal pole of the hippocampus showing the patches in the CA1 area centred on the stratum lacunosum (SL). (Unanaesthetised mouse)





from the edge of the str. mol. overlying the CA3 area into the near part of the subiculum (the prosubiculum of Lorente de No). At the junction with the subiculum the patches are within 30 μm of the pyramidal cell layer but at the CA3 end there is a greater depth to the stratum radiatum (str. rad.) and the patches are about 150 μm away. The patches have ragged edges but are regularly spaced. They can be followed for one or two sections cut on either side but in any one brain they have only been observed in one part of the CA1. This is in contrast to the dentate fascia patches which occurred in all parts of the area in any one brain.

The other set of patches were also only found in a small part of the CA1 area. This set of patches was restricted to the str. rad. A representative section is shown in Fig. 37 . The patches have irregular edges and are most clearly defined in the middle of the CA1 area. At the subicular and CA3 ends they are either less distinct or not present. The distance between the centres of each patch is 140 μm. The patches vary in width from about 70 μm to 120 μm and are separated by pale areas of 20 to 70 μm. The patches are only 150 μm high at most and so do not extend through the full extent of the str. rad. Faint patches are also seen in str. rad. of the CA1 area in coronal sections (see Fig. 28). The rest of the str. rad and stratum lacumosum (str. lac.) are homogeneously stained. Sagittal sections of the septal pole of the hippocampus are in a plane roughly at right angles to the long axis and so are in the plane of the lamellae described by Andersen.[6] The two sets of patches have not been observed together.

Coronal sections passing through the posterior hippocampus are in a plane approximately parallel to the long axis and so cut the lamellae approximately at right angles. In a few of these sections a regular arrangement of dark patches was seen in the stratum moleculare (str. mol.) of the CAI area. This is shown in Fig. 38 which is a coronal

section from a "pentobarbitone anaesthetised" mouse. The patches are again rather irregular in shape but occur at a reasonably regular interval of about 300 μm. Each patch is between 150 and 200 μm wide. The dark patches are separated by pale areas of 100 to 150 μm. The patches are often tapered being narrower at the hippocampal fissure and broader where they merge into the dark str. lac. No surface parallel sections were cut through the str. mol. of the CA1 area of a "pentobarbitone anaesthetised" mouse and so the patches have not yet been studied in three dimensions.

In the mice and rats studied so far there has been no evidence of a modular organisation in the CA3 area. Within any one layer the phosphorylase levels have always been fairly uniform.

In an attempt to investigate the anatomical basis for the modular organisation in the CA1 area, a study was made of sections taken from a rat brain that had been impregnated according to a modified Golgi-Cox procedure and then counterstained with thionin. In a few of the sections from these brains, axons were observed running vertically through the CA1 pyramidal cell layer. These axons were only partially impregnated and so could not be followed back to their origin. They apparently arose in the stratum oriens and terminated just beneath the stratum lacunosum. Two of these fibres which were in close proximity to each other are illustrated in Fig. 39. Both are partially out of focus but are shown in full in the diagram opposite. A few pyramidal cells are shown nearby to emphasise that the axons are running parallel to the apical dendrites and would be in a position to form many synapses with one dendrite. No estimate was made of the density of these vertically ascending fibres because the Golgi-Cox procedure only stains from about 1 in 100 to 1 in a 1000 cells and perhaps even fewer axons.

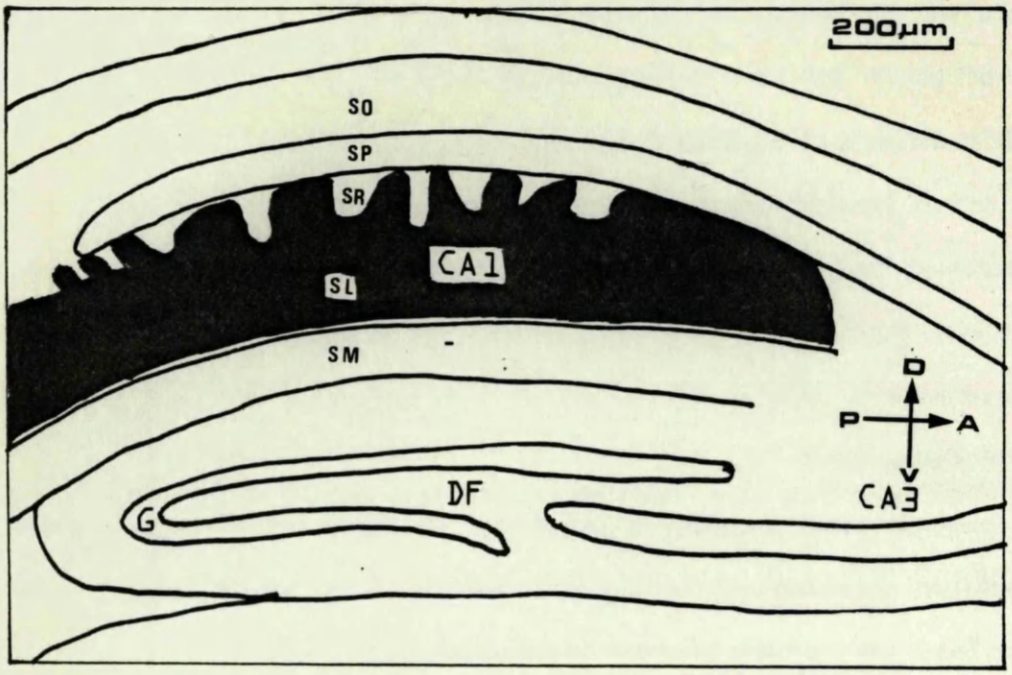


Fig.37 Sagittal section through hippocampus showing patches in stratum radiatum (SR). (Unanaesthetised mouse)

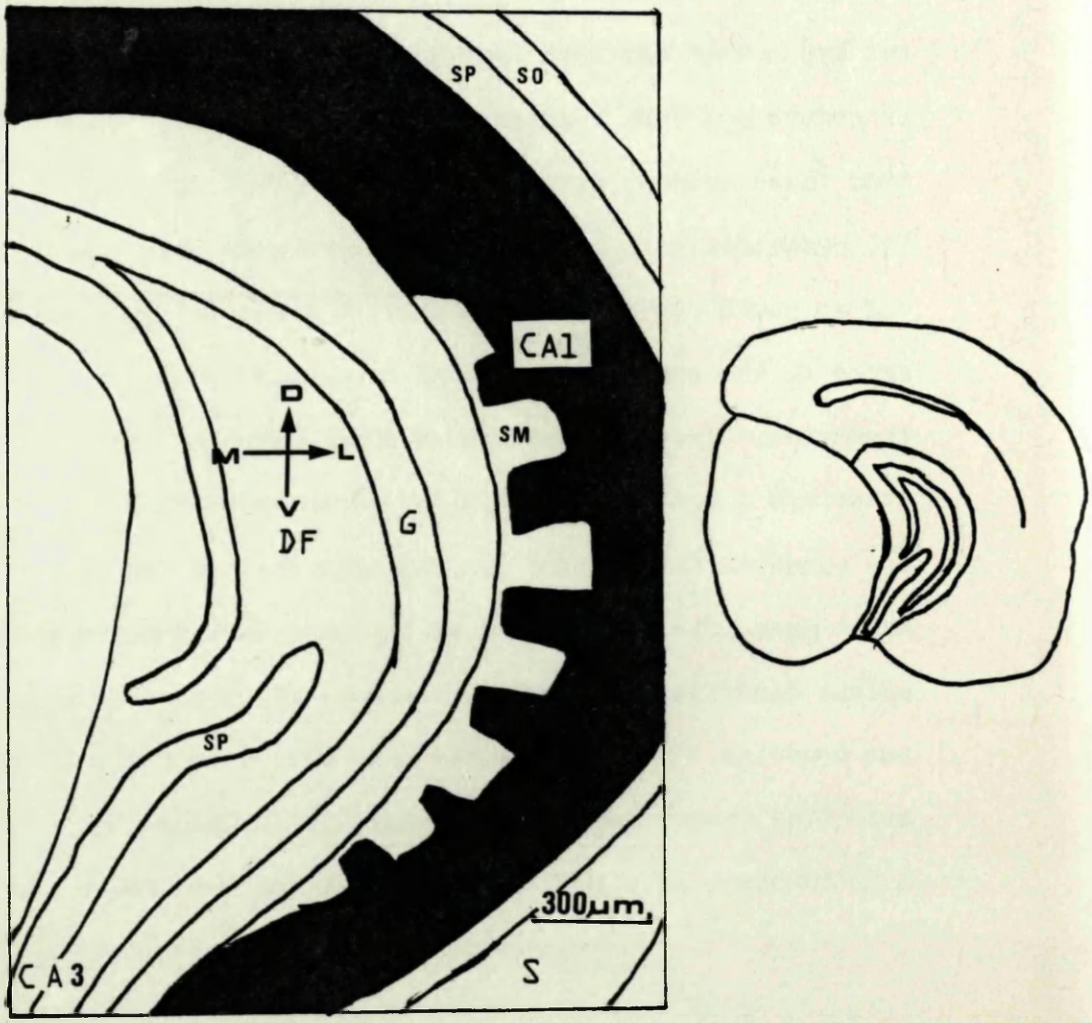
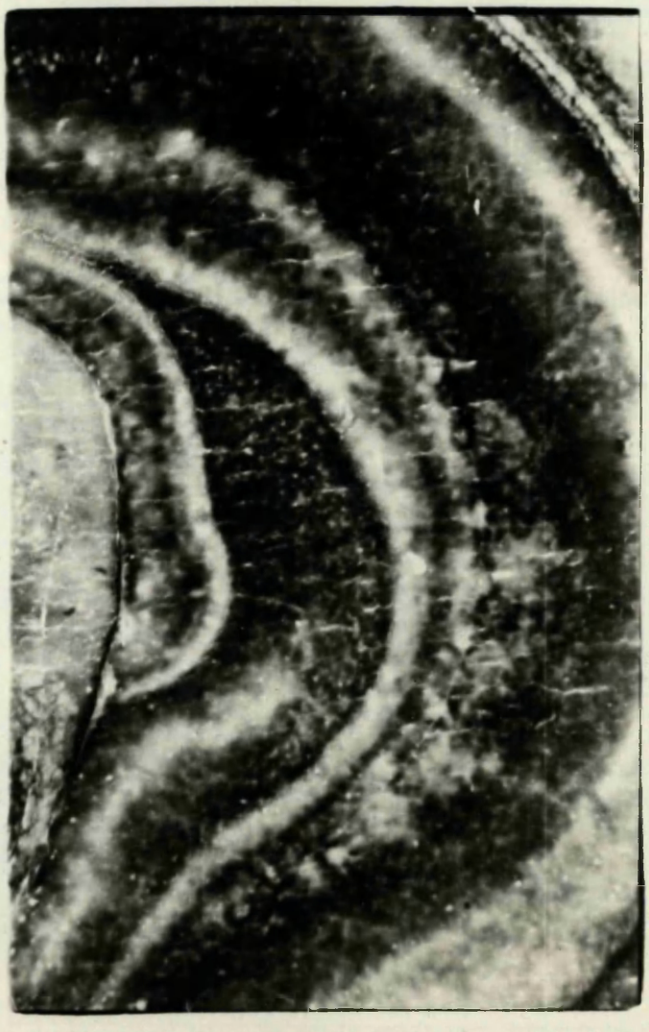


Fig.38 Coronal section through hippocampus showing patches in stratum moleculare (SM). Small diagram on right shows the position of the section. (Pentobarbitone anaesthetised mouse)





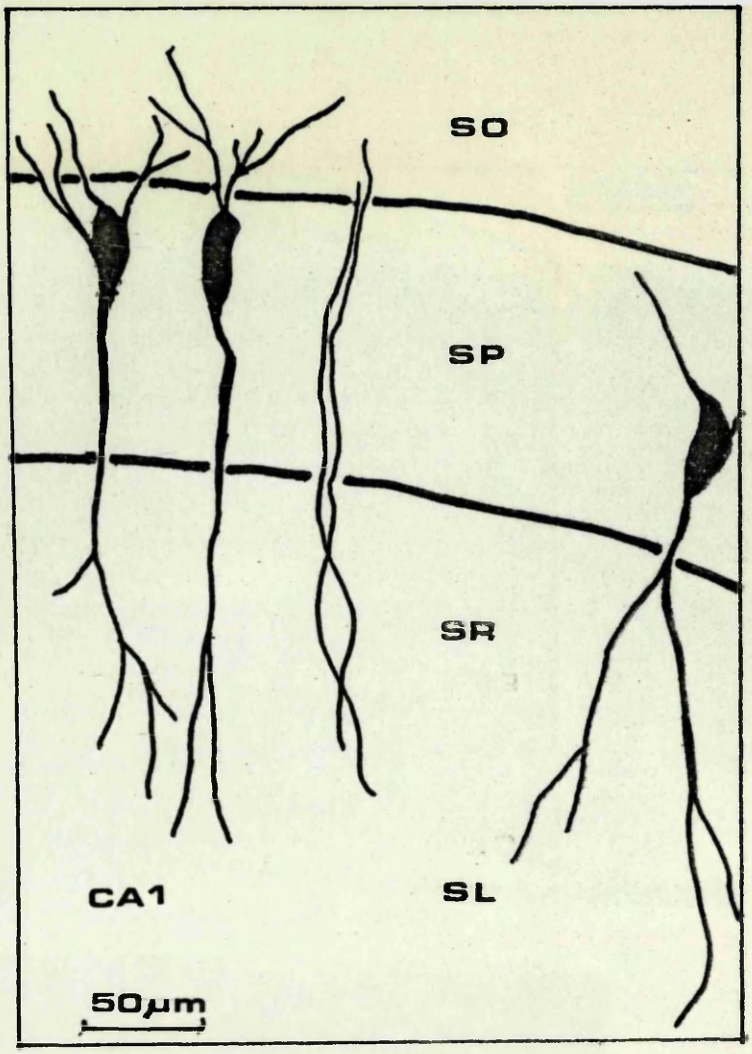


Fig.39 Coronal section through the CAI area of the hippocampus which had been impregnated by the Golgi-Cox procedure and counterstained with thionin.

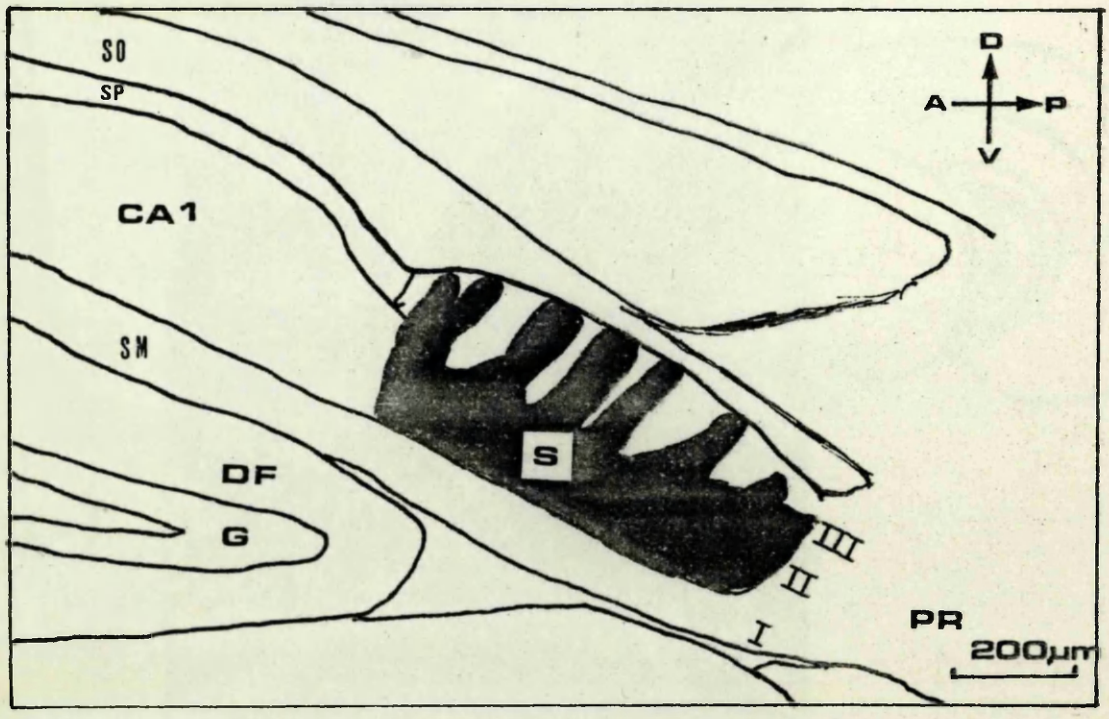
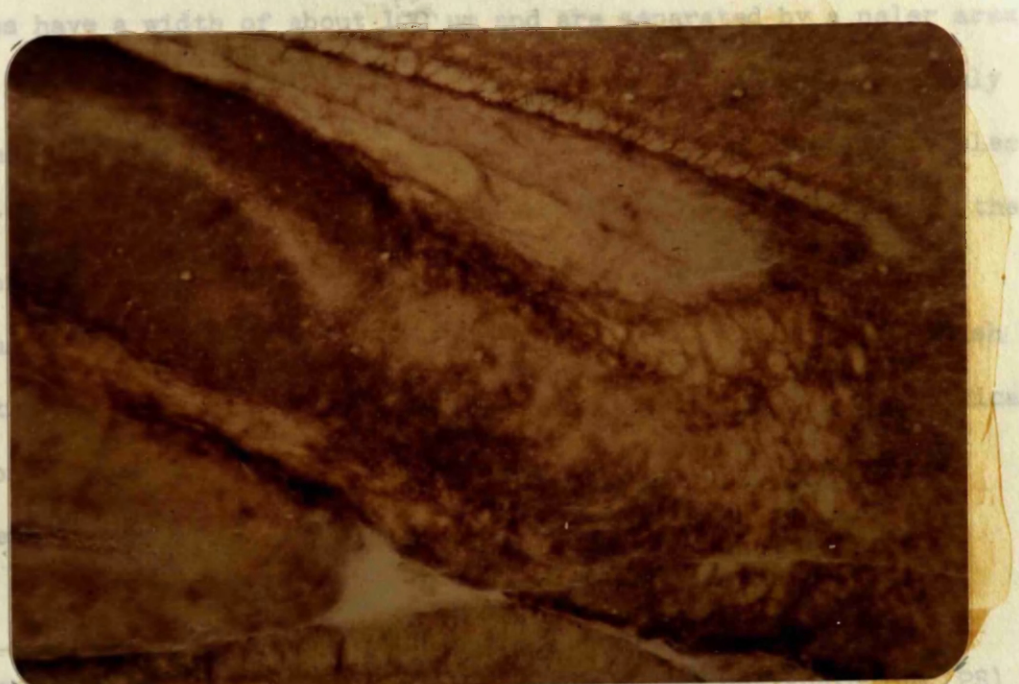


Fig.40 Sagittal section through the subiculum showing patches in layer III. (Unanaesthetised mouse)





Subiculum

Patches of high phosphorylase activity are also seen in the subiculum (S). Patches have been observed both in layer III and in layer II but as in the CAI area the patches have not been seen together in sections from the same brain. An example of the patches observed in layer III is shown in Fig.40 which is a sagittal section from an "unanaesthetised" mouse. The patches are irregular in shape but have a high phosphorylase activity compared to the pale intervening areas. The patches, of which there are seven in this section, start just beyond the end of the layer of the CAI pyramidal cells and continue to near the border with the presubiculum. The patches are about 300 µm high and fuse together as they come near layer II, the layer of small pyramids. The patches are never clearly delineated and in this section appear rather faint because the I_2 has been allowed to fade before being photographed. Layer II is dark and homogeneously stained.

In sections from other brains dark patches are observed in layer II. This is shown in Fig.41 which is again a sagittal section from an "unanaesthetised" mouse. Only two patches are seen in this section. The patches have a width of about 100 µm and are separated by a paler area of about 40 µm. As the patches are restricted to layer II they are only 100 µm in depth. The patches fuse with the equally dark stratum moleculare. In this section there is also an indication of the patches that were previously described as occurring in the stratum radiatum and stratum lacunosum. In this section they are difficult to distinguish from the relatively high background activity. There is also an indication of wide patches in area 17 the primary visual cortex. These patches are about 200 µm wide and are centred on layer IV.

Post- para- and presubiculum

Clearly defined patches are often seen in the postsubiculum (PS)

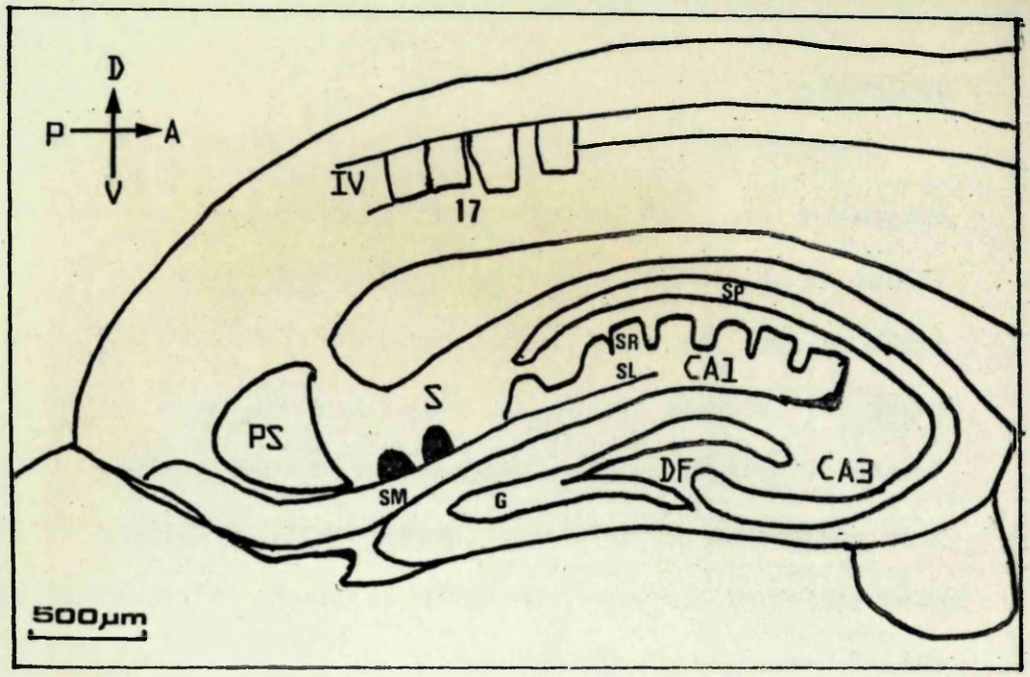


Fig.41 Sagittal section through the hippocampal formation showing patches in layer II of the subiculum and in the CAI area. (Unanaesthetised mouse)

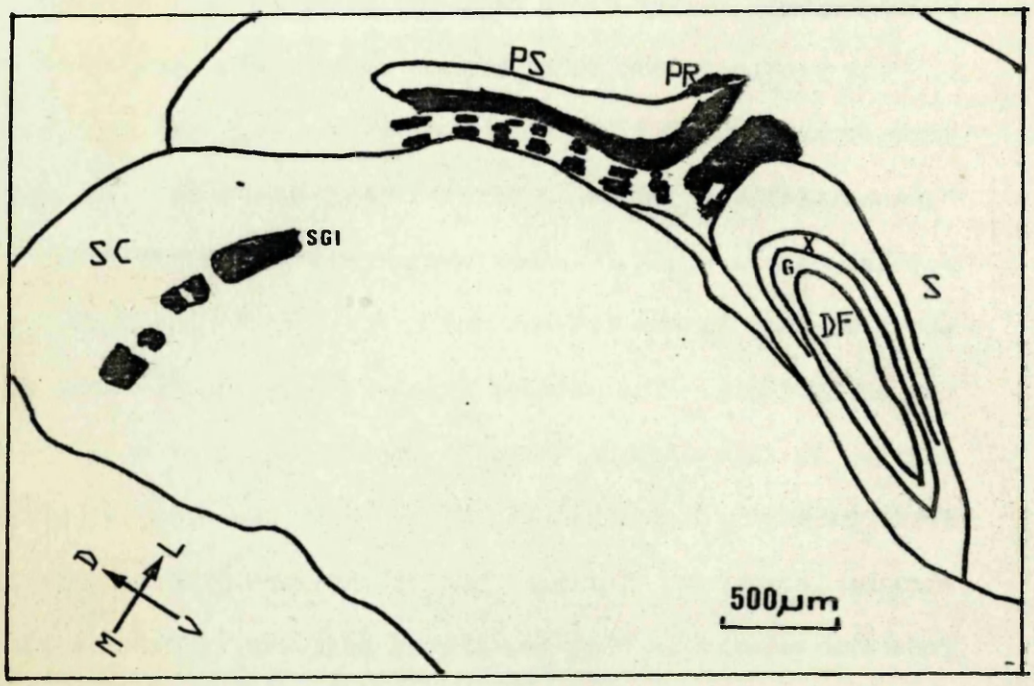
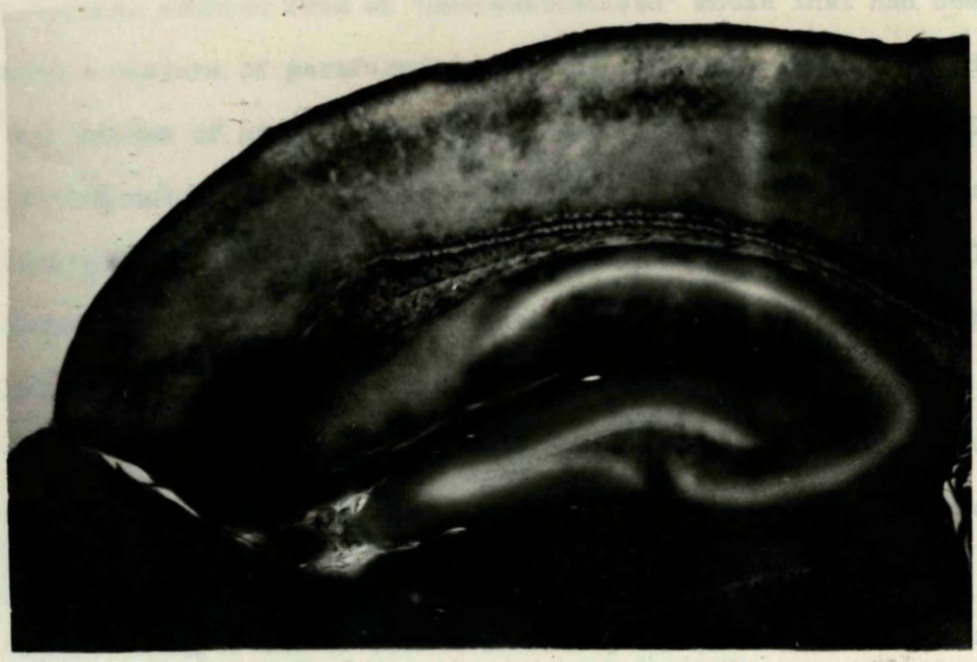
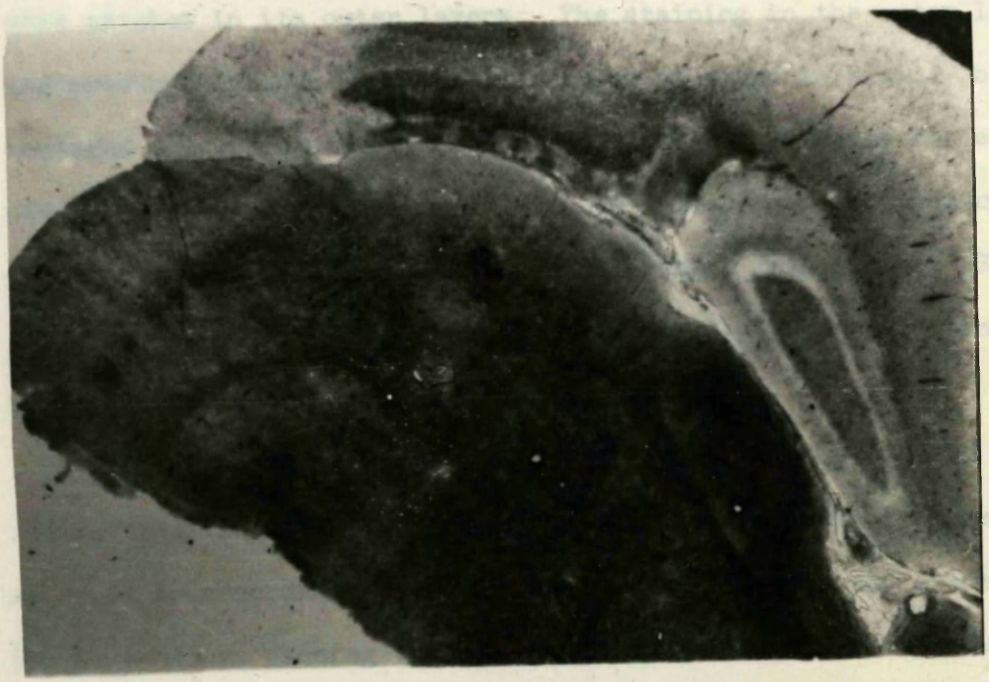


Fig.42 Coronal section through the postsubiculum showing patches in outer layers. (Perfusion fixed, unanaesthetised mouse brain)





especially in coronal sections such as that shown in Fig.42. This is a coronal section from an "unanaesthetised" mouse that had been perfused with a mixture of paraformaldehyde and periodate-lysine. It demonstrates the patches of high phosphorylase occurring in layers I and II of the postsubiculum. Layer I can be subdivided into three sublayers. The dark patches in layer I can also be subdivided into three sublayers according to the intensity of their staining relative to the paler intervening areas. This laminar appearance of the patches is seen more clearly at higher power as shown in Fig.43 which is again a coronal section but from a different "unanaesthetised" perfusion-fixed mouse brain. The patches are darkest in the outer sublayer, palest in the middle sublayer and of medium intensity in the innermost part of layer I. In layer II the patches are not very clearly distinguished. The distance between the middle of successive patches is about 140 μm and each patch is about 100 μm wide. In Fig.42 the presubiculum (PR) is seen as a narrow band of tissue between the postsubiculum and subiculum. There are no comparable patches in its outer layers. The staining in the outer layers of the presubiculum can be rather irregular as shown in Fig.42 but in the sections cut so far there has been no clear-cut evidence for a regular set of patches in its staining. Fig.42 also provides evidence of a patchy staining pattern in the stratum griseum intermediale (SGI) of the superior colliculus. The patches which are between 100 and 200 μm wide will be described in more detail later.

In horizontal sections the postsubiculum is only present at relatively dorsal levels, i.e., above the level where most of the sections were cut in accordance with the practice of Mellgren and Blackstad.[153] In the sections passing through the postsubiculum there was evidence of a patchy staining in the outer layers as demonstrated in Fig.44 This is a horizontal section from an "unanaesthetised" mouse. In layer I of the postsubiculum there are three purple patches separated by three

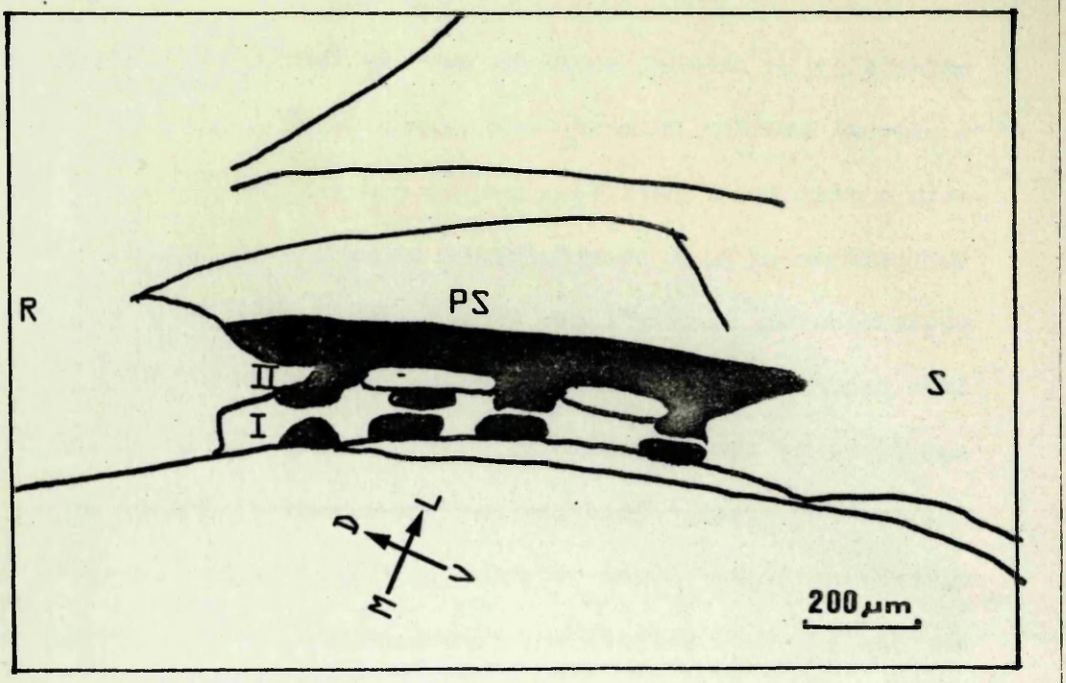


Fig.43 Coronal section through postsubiculum showing laminar arrangement of patches. (Perfusion fixed unanaesthetised mouse)

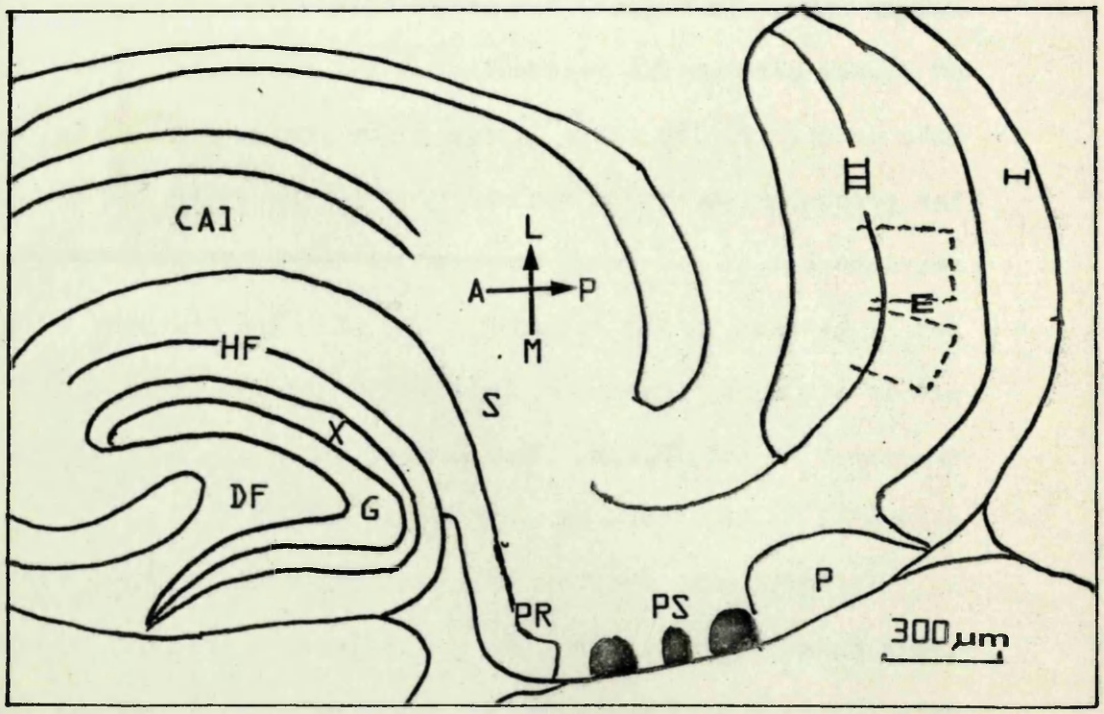
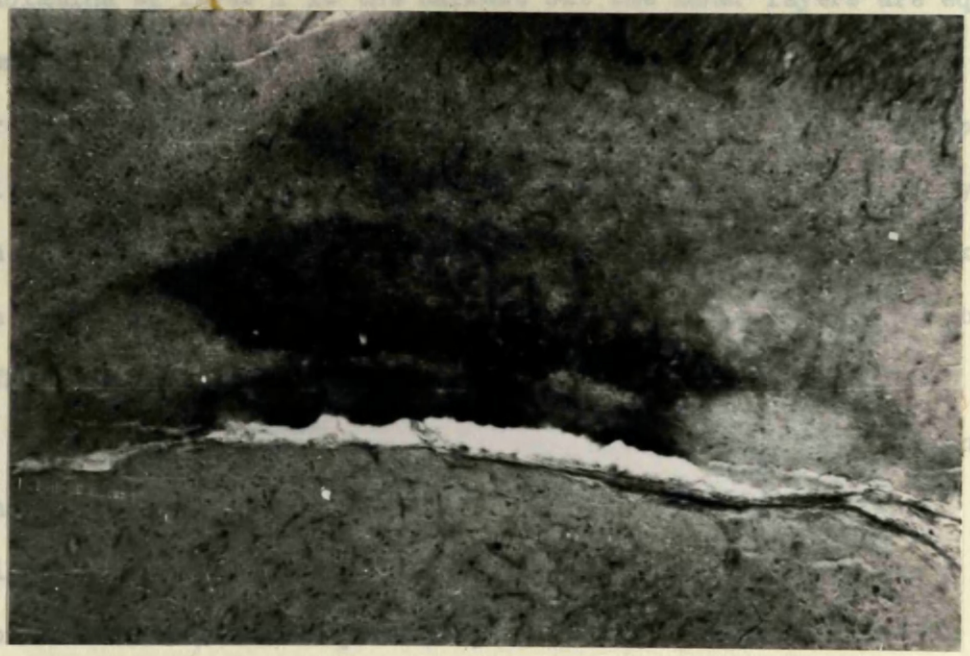


Fig.44 Horizontal section through dorsal hippocampal formation showing patches in layer I and II of the postsubiculum. (Unanaesthetised mouse)





orange-brown patches. The purple patches have a diameter of about 60 μm while the orange-brown patches have a width of 80 to 140 μm. The outer sublamina of layer I is the darkest but the inner layers are equally densely stained. The resolution in this Fig. is lower than in the previous two because the brain was frozen without having been perfused with a fixative. The staining in the entorhinal cortex is very irregular in the outer part of layer III, but there are two areas of darker staining in the middle of the entorhinal cortex, each with a width of about 200 μm. (These are outlined by dotted lines).

Finding the patches in both horizontal and coronal sections implies that in the postsubiculum as well as in the dentate fascia the patches of high phosphorylase a activity are in the form of ovoid discs. This was confirmed when surface parallel sections were cut. Due to the convoluted nature of the hippocampal region it was difficult to flatten the post-subiculum properly, especially in the small mouse brains, which were very easily damaged. Therefore in Fig. 45 where the brain has not been perfectly flattened the section passes through the dark layer I and the paler layer II. The presubiculum is hidden within the hippocampal fissure. The dark patches in the postsubiculum are discrete entities each with a diameter of 80 - 150 μm. They are arranged in an irregular manner and are surrounded by paler cortex. The distance between patches varies from 0 to 200 μm. Some, but not all, of the patches have a blood vessel within them. There is also evidence of some patches of similar size in the subiculum.

By contrast the pre- and parasubicular areas have not provided any consistent evidence of a modular arrangement. The different layers nearly always appeared relatively uniformly stained at least in sections cut roughly at right angles to the long axis of the hippocampus. In surface parallel sections there was some evidence of the parasubiculum being subdivided into modules with a width of about 200 μm, but the

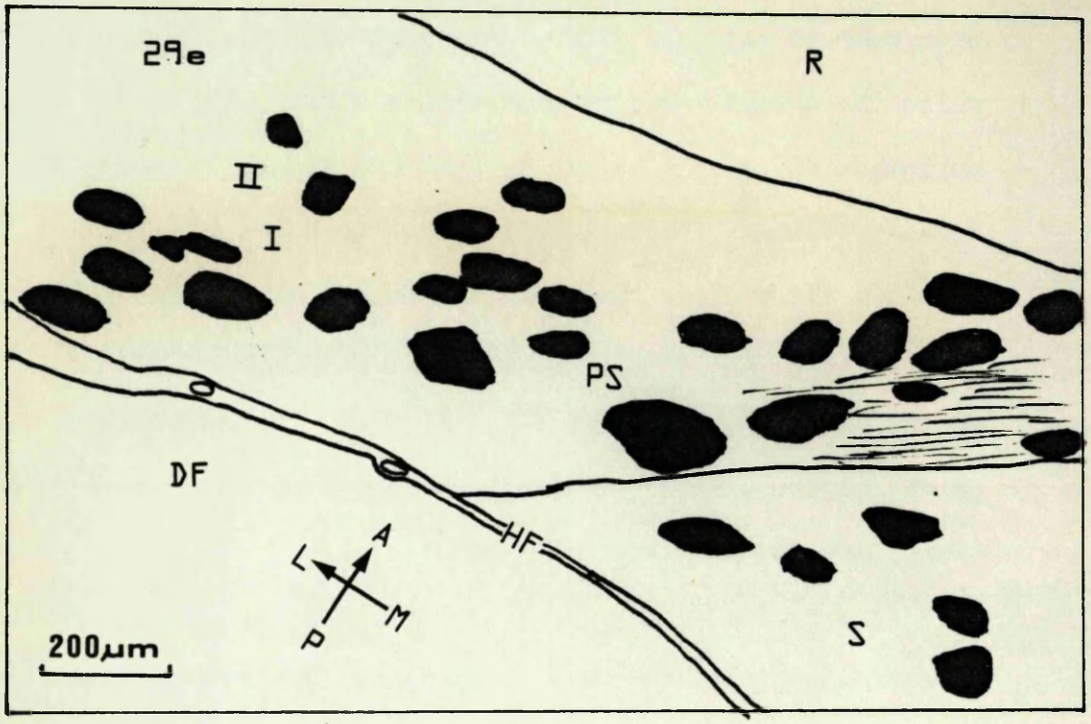


Fig.45 Surface parallel section mainly passing through layer I of post-subiculum showing the ovoid patches of higher phosphorylase a activity. (Unanaesthetised mouse)

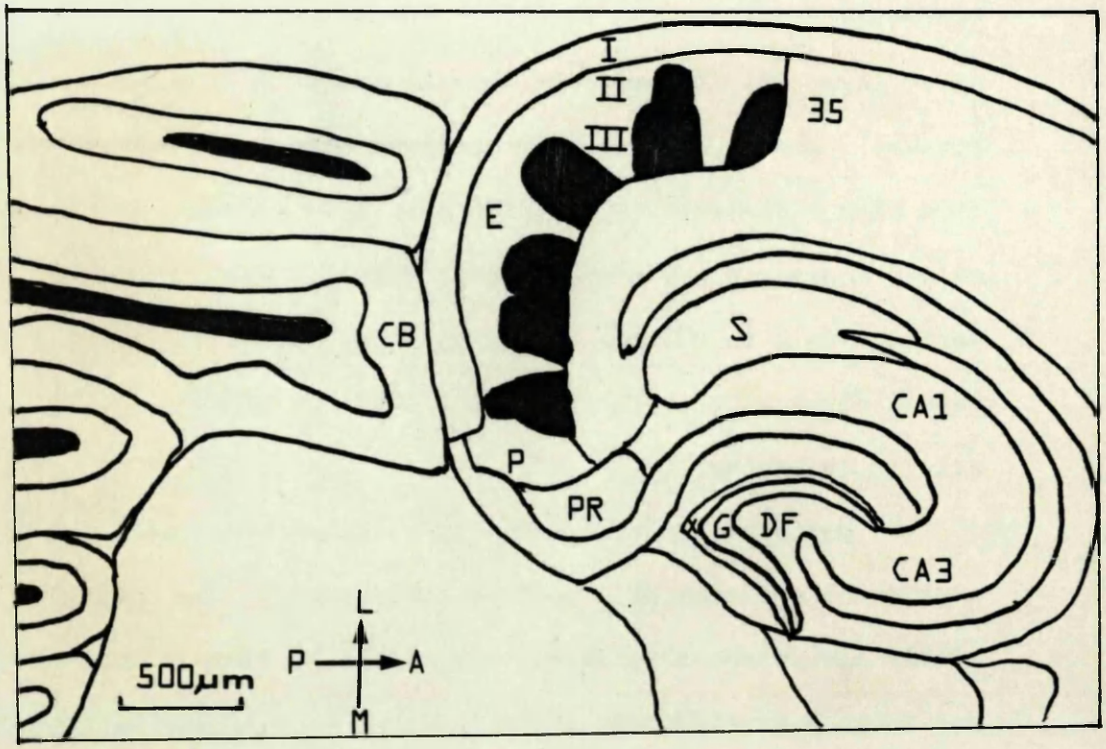


Fig.46 Horizontal section through the posterior hippocampus showing evidence of modular organisation in layer III. Thin vertical strips are observed in the stratum moleculare of the cerebellum (CB). (Unanaesthetised mouse)





patches were only observed in the top of layer II in one brain and they may have been artefacts.

Entorhinal cortex

This area also has two sets of patches which are apparently independent of each other as they have not been seen together in sections from any one brain. One set is restricted to layer II and the other mainly to layer III. The layer III patches are illustrated in Fig.46 a horizontal section through the posterior hippocampus taken from an "unanaesthetised" mouse. The patches have an irregular spotty appearance and although mainly restricted to layer III they sometimes extend into layer II. The dark patches have a width of 200 - 250 μm and are usually separated by pale areas of varying width ranging from 50 to 200 μm. Occasionally however two of the patches seem to be fused together and have no pale area between. So far these layer III patches have only been observed in horizontal sections from the brain of one "unanaesthetised" mouse. Layer III patches have also been observed in the entorhinal cortex in sections from the brains of two "unanaesthetised" rats. A representative section is shown in Fig.47. Dark patches with diameters ranging from 100 to 300 μm are found in both the medial and lateral parts of the entorhinal cortex.

The patches are irregular in shape but have a roughly oval or circular outline. They are not arranged in any geometrical pattern. As Fig.46 was taken at low magnification it includes part of the cerebellum. No patches have been observed in the cerebellum so far but thin vertical bands of high phosphorylase activity have been observed in the stratum moleculare of the cerebellum as shown in Fig.46. These bands will be described in more detail later.

The layer II patches found in the entorhinal cortex are illustrated in Fig.48 which is a horizontal section from the brain of a

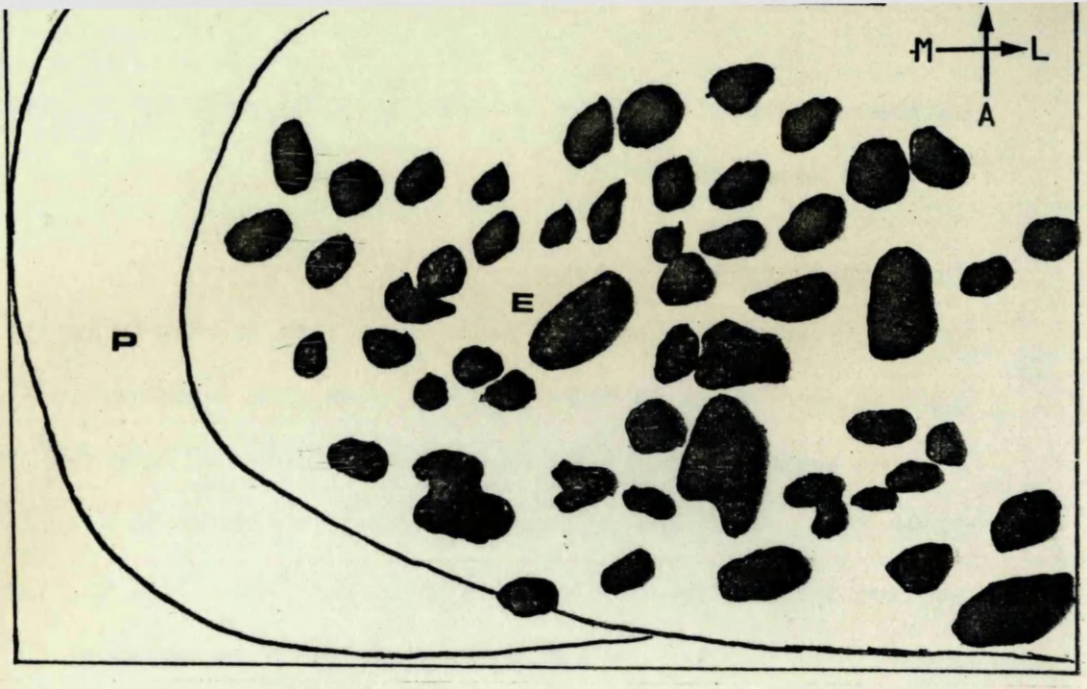


Fig.47 Surface parallel section through layer III of the entorhinal cortex showing large ovoid patches. (Unanaesthetised rat)

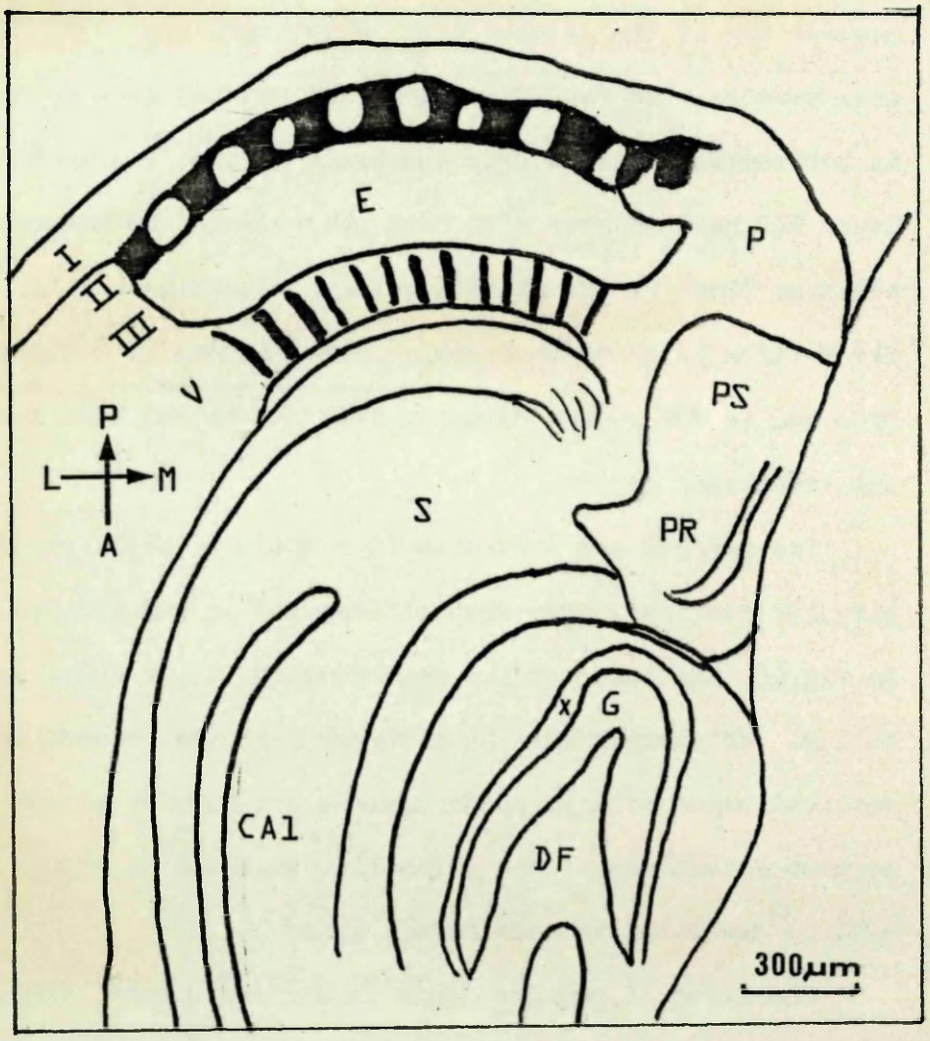
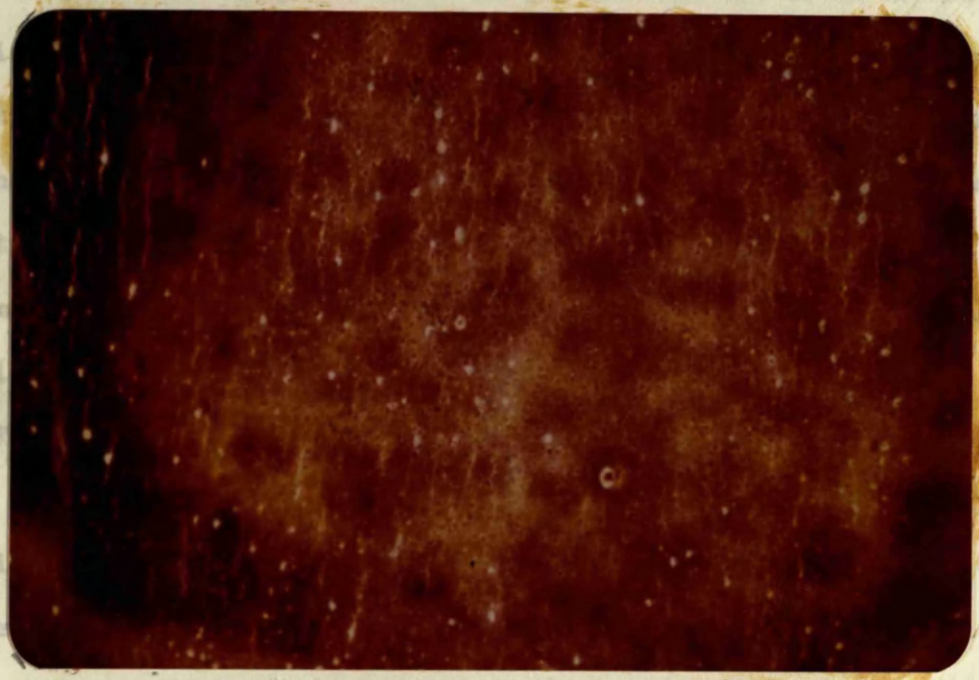


Fig.48 Horizontal section through the hippocampal formation showing ten indistinct patches of higher phosphorylase a activity in layer II. (Pentobarbitone anaesthetised mouse)





pentobarbitone anaesthetised mouse. Ten rather indistinct dark patches occur in layer II. They are about 100 μm wide and are often separated by pale areas of a similar width. However the pale areas are sometimes indistinct or very narrow and as a result the patches often do not have distinct borders. In surface parallel sections the patches are equally indistinct but usually take the form of ovoid areas 100 μm wide and 200 μm long. They are often fused together and difficult to distinguish especially in the medial part of the entorhinal cortex.

Fig.48 also illustrates the finding that there are pale cords of cells separated by areas of higher phosphorylase activity apparently containing radially running fibres. There does not appear to be a one to one correlation between the dark bands of layer V and those of layer II as there are 13 bands in layer V of the section shown in Fig.48 and only 10 in layer II.

Cingulate cortex

Although not part of the hippocampal formation, the cingulate cortex is introduced at this point because it is a part of the limbic cortex that acts as an important relay station for information passing between the hippocampal region and neocortical areas.[56] The posterior part of the cingulate cortex is formed by the retrosplenial areas 29 a-d and the anterior forms part of the prefrontal or medio-dorsal projection cortex.[235] In coronal sections through the posterior cingulate cortex of rats which had been anaesthetised with urethane prior to decapitation, bands of high phosphorylase a activity are observed in area 29C (Fig.49). These bands are found in layers II, III, IV and upper V. Layer I is uniformly stained while in layer IV the bands are less distinct because of a comparatively high background activity. The bands are 60 - 70 μm wide and are separated by pale areas with widths varying between about 40 and 80 μm.

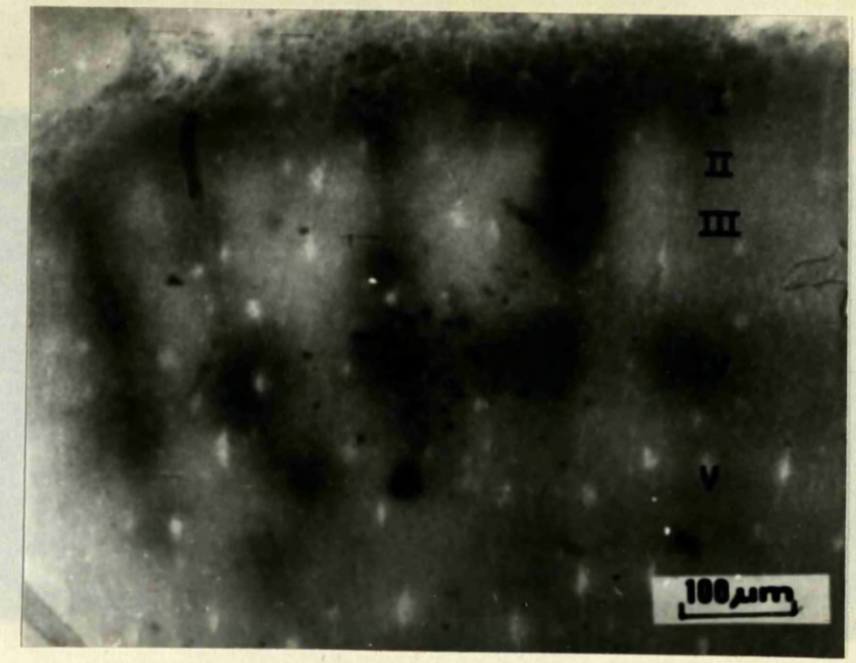
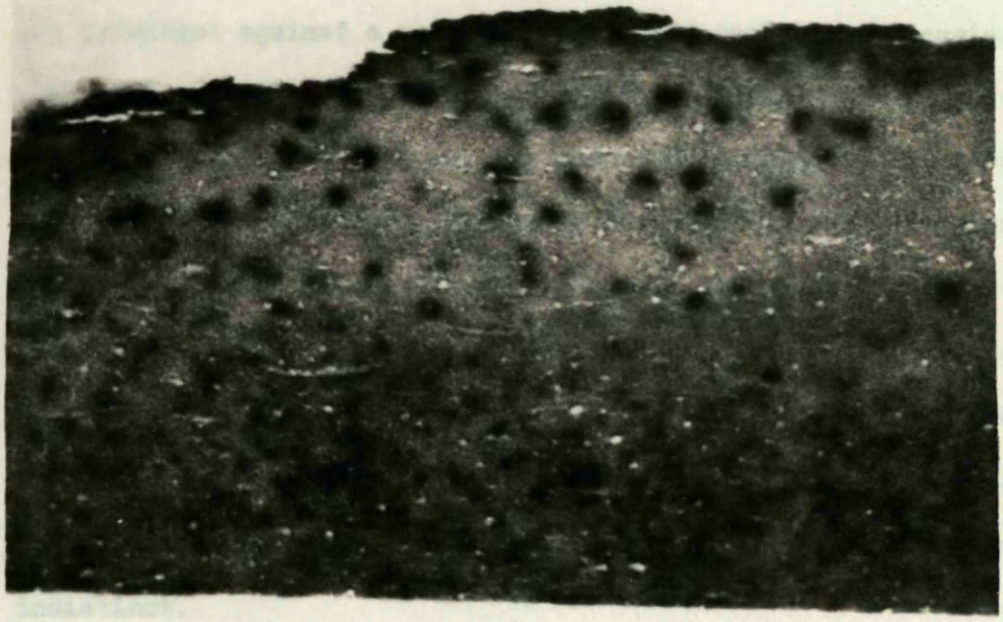
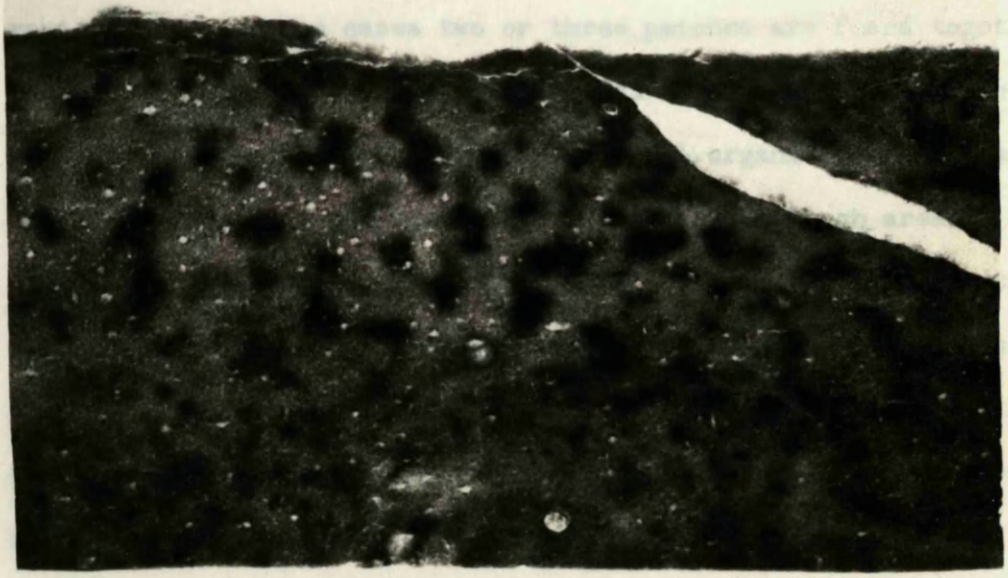


Fig.49 Coronal section through area.29C showing four vertical bands of high phosphorylase a activity which occur in layers II, III, IV and upper V. (Urethane anaesthetised rat)

Fig.50 Surface parallel section passing through layer II of area 29C showing patches of high phosphorylase a activity. (Urethane anaesthetised rat) X52

Fig.51 Surface parallel section passing through layer II of area 24 showing patches of high phosphorylase a activity. (Urethane anaesthetised rat) X52





In surface parallel sections through layer II circular or ovoid patches of high activity are observed in the cingulate cortex in the area apparently corresponding to area 29C (Fig. 50). The dark patches are prominent against a pale background and each has a diameter of between 60 and 70 μm. The distance between patches is variable and ranges between 40 and 120 μm. As the patches have only been observed in urethane anaesthetised animals it seems likely that they correspond to the bands seen in coronal sections. If this is the case then it indicates that in 29C there are cylinders of high phosphorylase a activity each with a diameter of about 70 μm. There has also been an indication of similar cylinders of high activity in area 29C in sections from urethane anaesthetised mice but so far those observed have been indistinct.

In surface parallel sections from urethane anaesthetised rats dark patches are also observed in the anterior cingulate cortex (area 24), Fig. 51. These patches are bigger than those found in area 29C having diameters of between 60 and 140 μm. Again the distance between them is variable and in some cases two or three patches are fused together. As in 29C they are clearly outlined against a pale background. The patches may indicate that area 24 has a cylindrical organisation similar to area 29C. So far no coronal sections have been cut through area 24 of the urethane anaesthetised rat to test this possibility. There are also patches in the neocortical areas lateral to the cingulate. These patches have a diameter of between 40 and 80 μm and have a similar appearance in coronal sections so that they resemble spheres more than cylinders.

General architectonics

The various architectonic areas of the hippocampal region have consistent differences in their colour when iodine is used to stain the

glycogen produced in the phosphorylase a incubation medium. There are some variations in the intensity and shade of staining in sections taken from different brains, but the differences between various areas are sufficiently consistent to be of value in identifying the areas especially when they have been sectioned in unusual orientations or where the section has only passed through a small part of the area. The differences are shown in Fig.52 which is a representative section cut in the horizontal plane from a block taken from an "unanaesthetised" rat.

The granule cell layer (G) and the pyramidal cell layers (SP) of the hippocampus are pale purple and dotted with dark cell nuclei. Immediately above and below them lie thin purple layers with comparatively few cell bodies and which contain afferent fibres running in the plane of the layer of cell bodies. These layers are thickest in the CA3 area where the mossy fibre afferents from the dentate fascia terminate in the stratum lucidum (SLu) and among the basal dendrites of the pyramidal and basket cells. They are thinnest in the CAI area which has no mossy fibre input. The layer immediately beneath the granule cell layer has a particularly deep purple colour. In the hippocampus the stratum radiatum and oriens also have a fairly deep purple colour. In the dentate fascia the outer layers of the stratum moleculare are a brownish colour that has more of a purple tinge than the stratum moleculare of the hippocampus. In the hippocampus this layer has a more orange colour similar to the outer layer of the three subicular areas. The middle layers of the presubiculum are also an orange brown colour in contrast to the purple parasubiculum and subiculum. At this level of the brain the postsubiculum and area 29e are not sectioned. Layer III of the entorhinal cortex is a pale orange brown while the outer two layers are a darker brown and the inner three layers are a more purple colour. There is an abrupt change of colour at the edge of the entorhinal cortex in its outer three layers where it meets the neocortical area 35.

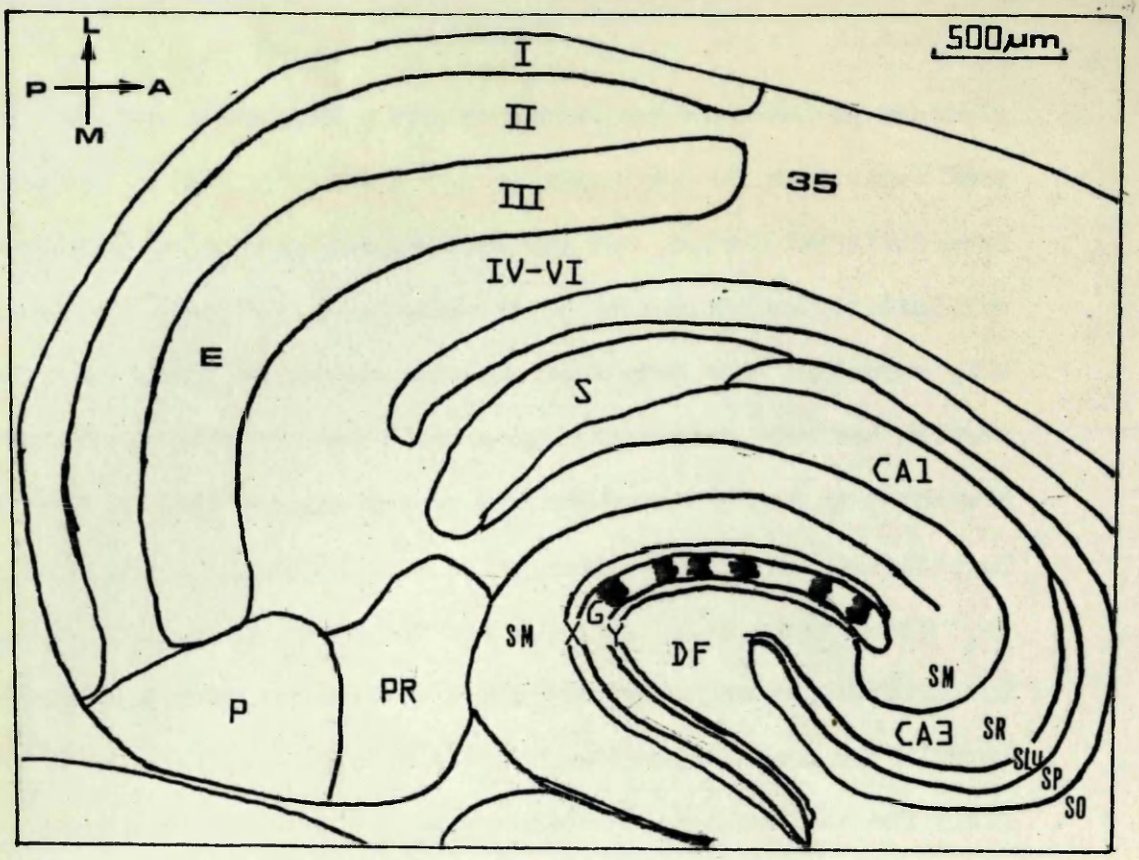


Fig.52 Representative horizontal section through the posterior hippocampal formation. (Unanaesthetised rat)

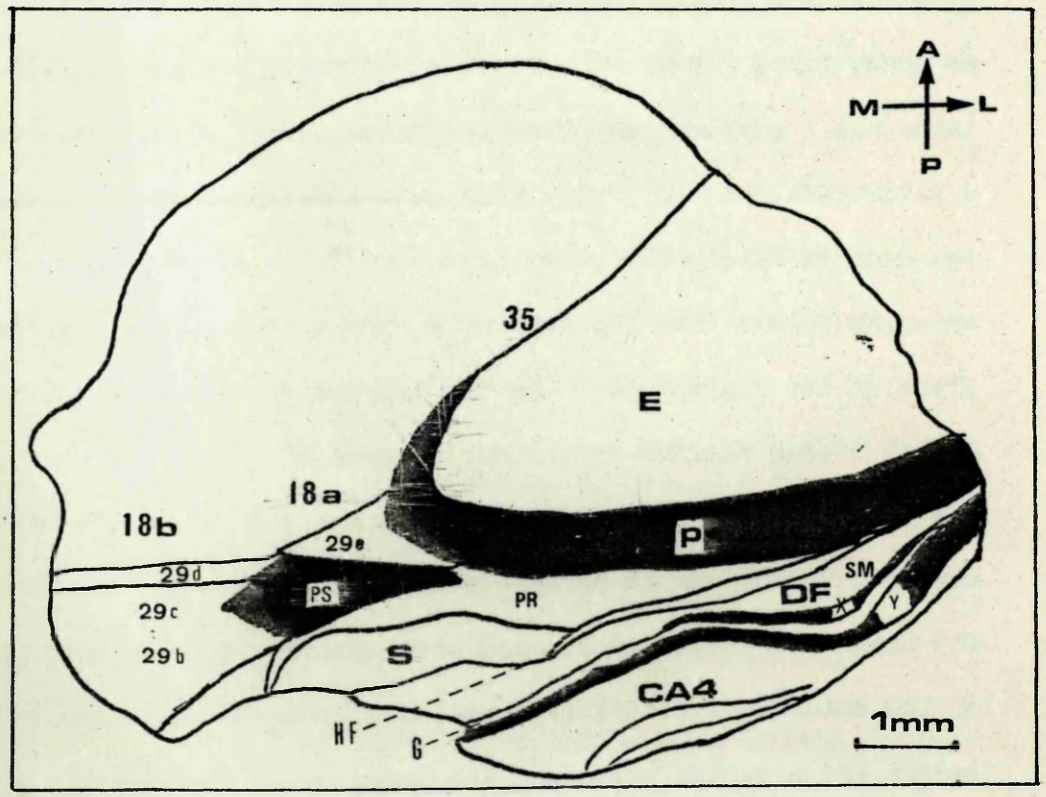
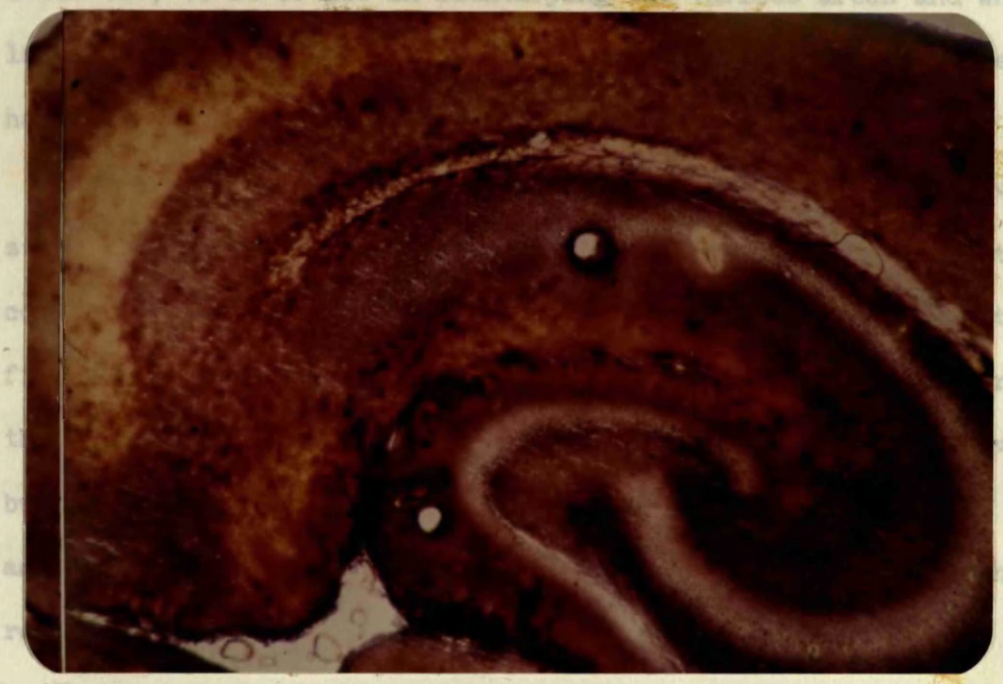


Fig.53 Representative surface parallel section through the hippocampal region cut at a depth of 350 µm. (Unanaesthetised rat)





The differences in the staining pattern are sufficiently consistent and clear-cut, to be of use in identifying the various areas and which layers the section has passed through, when surface parallel sections have been cut.

As described previously surface parallel sections are of value in studying both cortical architectonics and the modular organisation of cortical areas. Because most histological techniques involve tissue fixation previous workers have not been able to flatten the surface of the hippocampus to cut tangential sections and instead they have had to build up "unfolded" maps by reconstructions based on series of coronal and horizontal sections.[220] Therefore in the present study the spatial relationship of the areas making up the hippocampal region was studied by flattening the posterior pole of the brain including the partially unfolded hippocampus and cutting surface parallel sections. A representative section is shown in Fig.53 which is also a section taken from a brain of an "unanaesthetised" rat. The section was cut at a depth of 350 μm and so passes through layer III of the entorhinal cortex (E). The various areas could be recognised because of their characteristic colour and size and because of the close resemblance of the observed pattern to the "unfolded" maps constructed by a number of workers.[220,253,20]

Along the posterior edge of the patchy, dark orange entorhinal cortex lies the purple, more homogeneously staining parasubiculum (P). Next to the parasubiculum lies the thin strip of the presubiculum (PR) which extends out medially to separate the mauve coloured subiculum (S) and the brown area corresponding to the postsubiculum (PS). Wedged between the postsubiculum and the parasubiculum lies a small, pale, yellow/orange area apparently the same as Blackstad's area 29e.[20] On their anterior edge, area 29e and the entorhinal cortex (E), are bounded by the neocortical areas 35, 17, 18 and 18a, while the subiculum (S) is bounded by the areas of cingulate cortex 29 a-d.

The borders of the various architectonic areas in the neocortex cannot be distinguished in sections taken from layer III of "unanaesthetised" rats. At the posterior aspect the presubiculum (PR) and parasubiculum (P) are separated from the dentate fascia (DF) by the hippocampal fissure (HF). The stratum moleculare only has a distinctive purple inner layer (X) in its lateral half. By contrast, the equally dark layer (Y), where septal and catecholaminergic fibres terminate among the cell bodies of the inhibitory basket cells, lies directly underneath the pale granule cell layer (G) for the whole length of the dentate fascia. Next to this lies the hilus of the dentate fascia which Lorente de No[143] termed CA4 as it contained the terminal cells of the stratum pyramidale of the hippocampus.

Other Brain Structures with a Modular Organisation

Evidence of a modular organisation has been found in a number of structures apart from the cerebral cortex with both histochemical[84] and anatomical methods.[259] Of these structures the ones that have been most deeply studied are the superior colliculus,[110] caudoputamen[95] and cerebellum.[150] These structures were also studied in the present work and evidence was found of a modular organisation in each.

Superior colliculus

The superior colliculi from the brains of 6 "unanaesthetised" and 6 "pentobarbitone anaesthetised" mice were examined by cutting sections in the coronal, sagittal and horizontal planes and by partially flattening them to allow surface parallel sections to be cut. The sections were incubated in phosphorylase a incubation medium and stained by iodine in the normal way.

Dense patches of high phosphorylase a activity were observed in the intermediate grey layer (SGI) of the superior colliculus in many coronal sections from both "anaesthetised" and "unanaesthetised" animals.

However in coronal sections from one "anaesthetised" mouse no patches were observed. As seen in Fig.54 the patches were 200 to 400 μm wide and were separated by pale narrow zones. The patches are spaced at fairly regular intervals and sometimes showed a medial to lateral increase in size. Thus of the seven patches in Fig.54 the ones on the medial edge are approximately 200 μm wide while those on the lateral edge are about 400 μm wide. The patches often give the appearance of being two smaller patches that have fused together. In sagittal sections (Fig.55) the patches appear very similar being again 200 - 400 μm in size. However the pale zones between are often indistinct or not present at all where two patches are fused together. Again, the patches were seen in sections from both "anaesthetised" and "unanaesthetised" animals.

The reason for the patches being fused together or unclear in some brains was found when surface parallel sections were cut through the partially flattened superior colliculus. Two similar types of pattern were observed. One consisted of a continuous network of dense bands that spread over the whole area of the superior colliculus. This is illustrated in Fig.56 . Because the superior colliculus was not completely flattened the network pattern is not present at the medial edge of this section. The dark network encloses patches of lower activity. This network appearance of the high phosphorylase activity will mean that sections in both the coronal and sagittal planes would show discrete patches of activity but at times the patches would appear to be fused together when the section passed through a junction in the network.

The other type of pattern observed is shown in Figs.57 and 58 A. In these sections the phosphorylase a activity takes the form of curving bands or slabs that are similar in form to the ocular dominance columns or slabs found in layer IV of the primate visual cortex. Again the superior colliculus was not perfectly flattened and so in any one

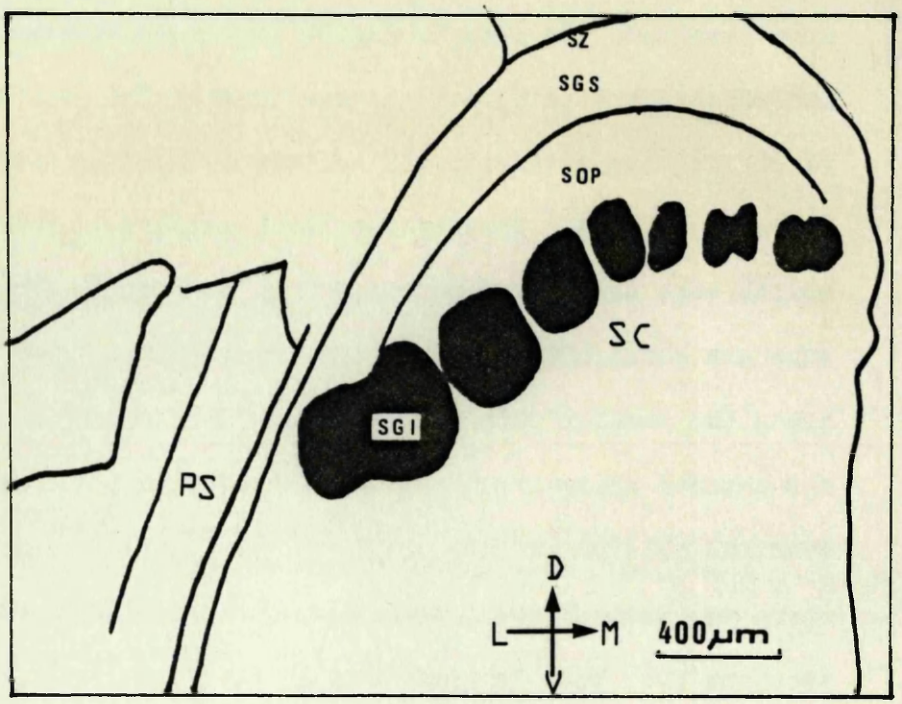


Fig.54 Coronal section through superior colliculus showing patches of high activity in the intermediate grey layer (SGl). (Unanaesthetised mouse)

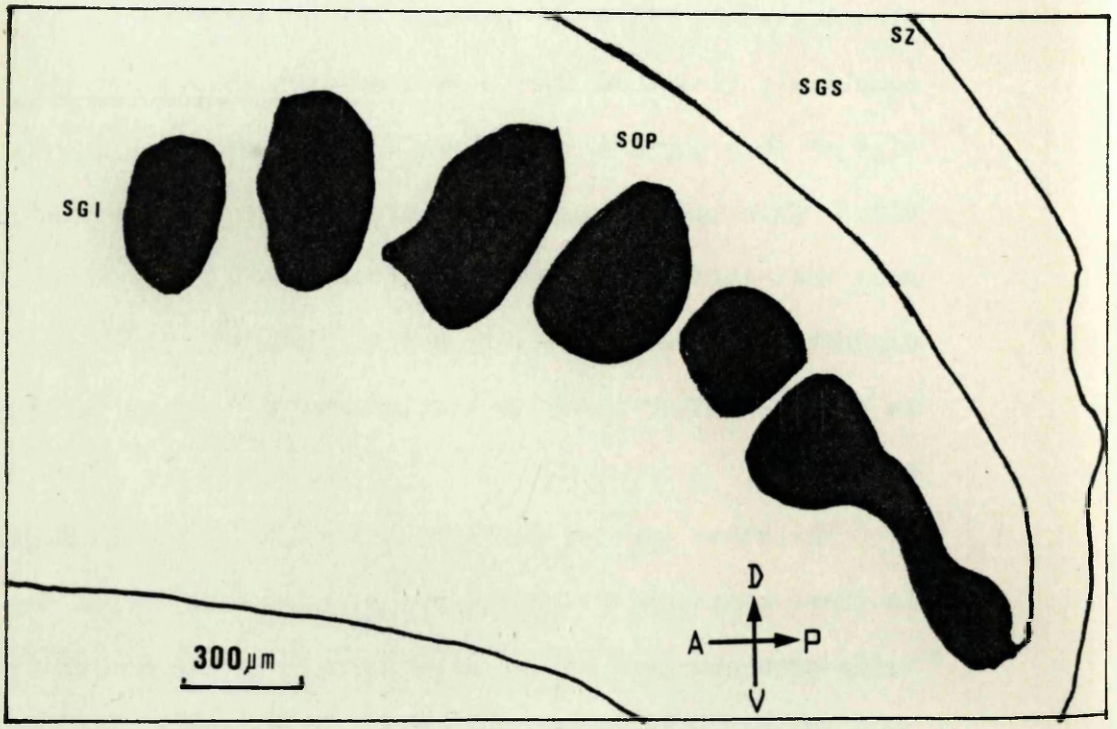


Fig.55 Sagittal section through superior colliculus again showing patches of high phosphorylase a activity in the SGl. (Unanaesthetised mouse)



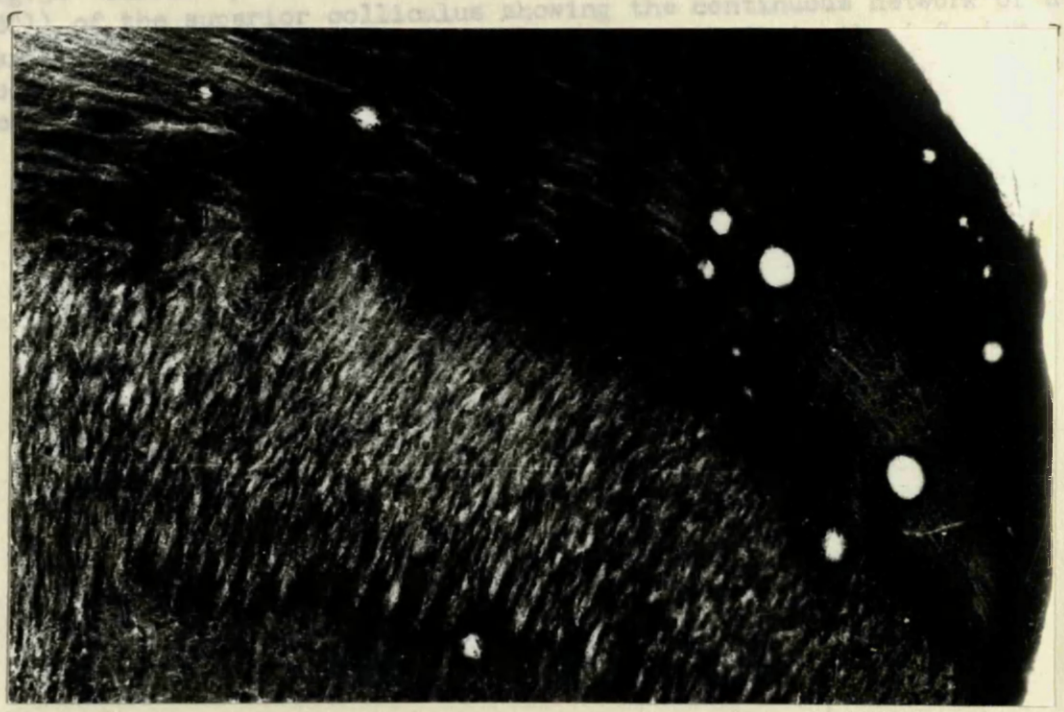




Fig.56 Surface parallel section through the intermediate grey layer (SG1) of the superior colliculus showing the continuous network of dense bands of high staining. The pale area to the left is the inferior colliculus and the midline is at the top of the section. (Unanaesthetised mouse)

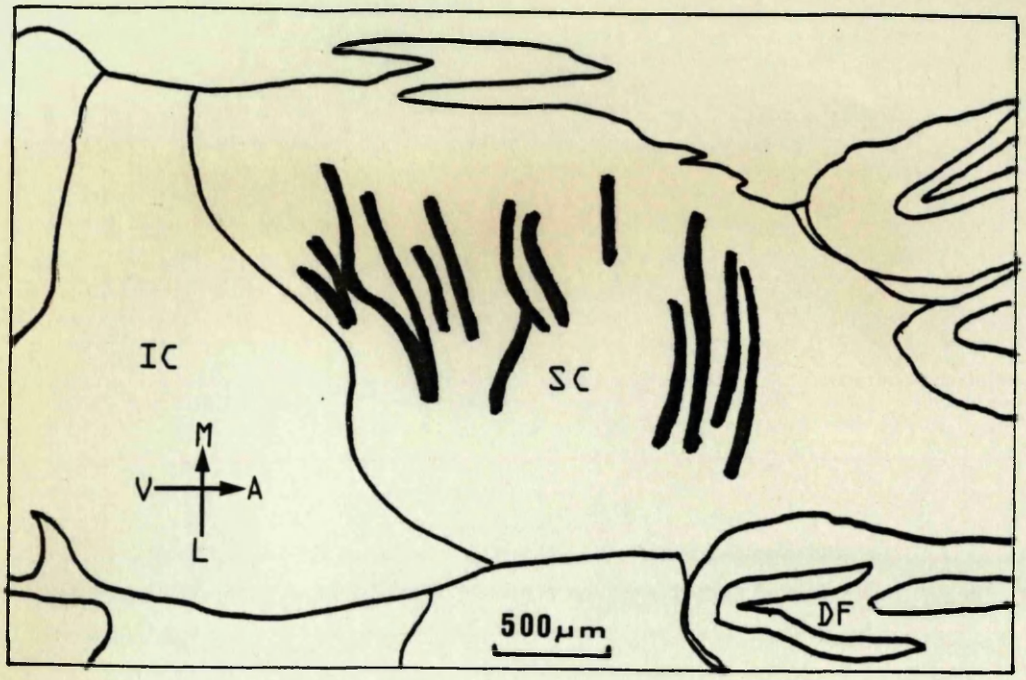


Fig.57 Surface parallel section through SG1 of the superior colliculus showing curving bands of high staining. This section is shown diagrammatically above. (Pentobarbitone anaesthetised mouse perfused with 1% paraformaldehyde)

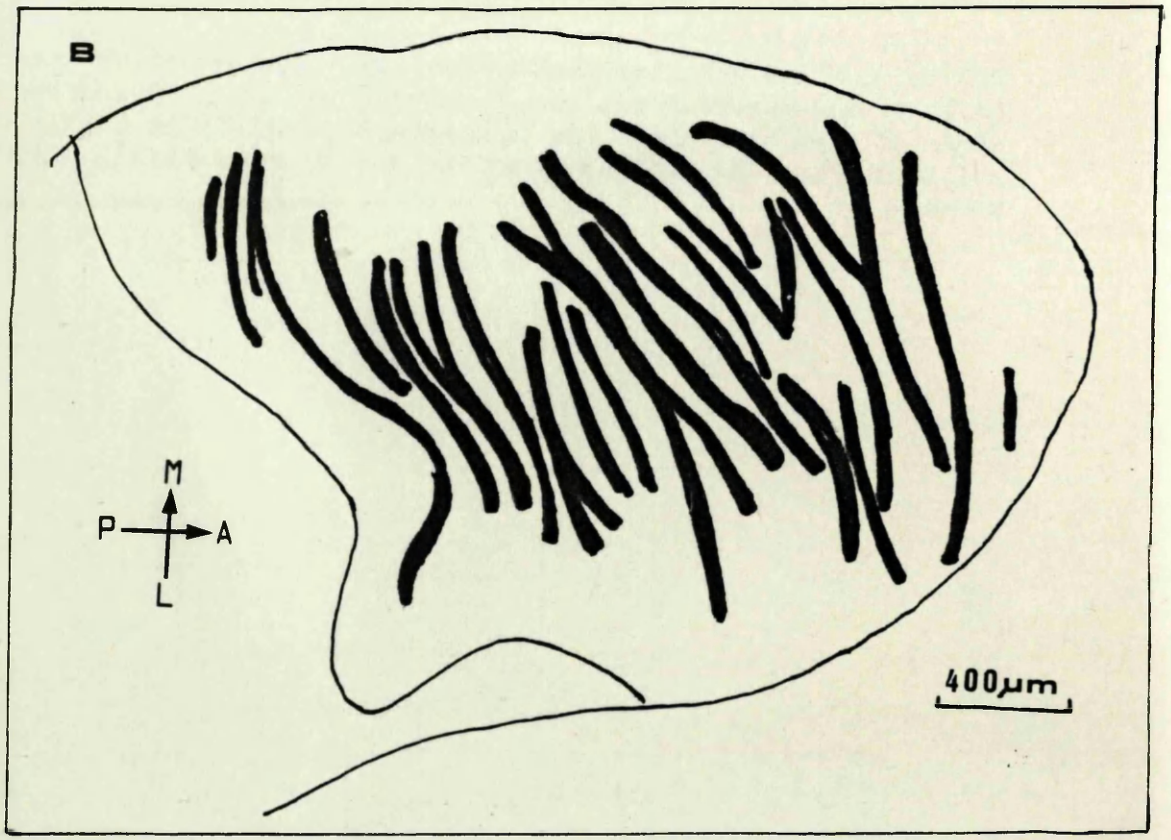
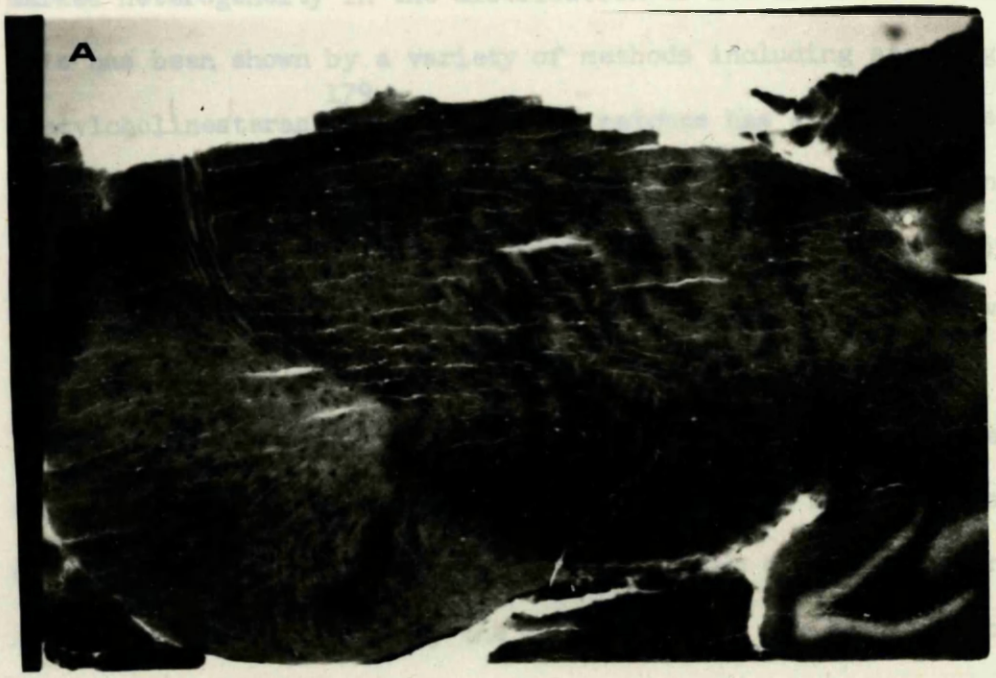


Fig.58 A Adjacent section to that shown in Fig.57
Fig.58 B Reconstruction of banding observed in SG1 obtained by tracing the bands in 7 serial sections two of which are shown opposite.




A

section the bands of high activity are clear in some parts of the section but not in others. Along the medial and lateral surfaces where the curvature of the collicular layers is most pronounced, the bands are least distinct in the sections illustrated. However by tracing a series of sections onto acetate sheets an overall picture of the pattern was obtained and this is shown diagramatically in Fig.58B. The bands occur in all parts of the superior colliculus and are orientated predominantly in a transverse direction. The bands are about 200 μm wide and are sometimes branched. The bands are not very distinctly outlined but they usually appear to run independently of each other and are not fused together in a network of the type described above. Sagittal sections through this pattern would have a patchy appearance but coronal ones would not. This is presumably the reason why coronal sections through one brain did not give any evidence of a patchy staining pattern.

Caudoputamen

The caudoputamen (striatum) in the rat and other mammals has a marked heterogeneity in the distribution of its afferent terminals.[95] This has been shown by a variety of methods including staining for acetylcholinesterase.[179] A mosaic of patches has also been observed in the present study when sections have been incubated for phosphorylase a activity. The patches are irregular in shape and size (Fig. 59) but can be followed through a number of serial sections. They have been observed in both anaesthetised and unanaesthetised brains but no detailed examination of the patches was made. Following Herkenham's[95] description of a method whereby sections from the striatum of one animal can be used to look at the distribution of afferent terminals and a variety of receptors, the description of any one histological marker alone is of relatively little value. The phosphorylase a patches were observed in both coronal and horizontal sections and so form a three dimensional mosaic.

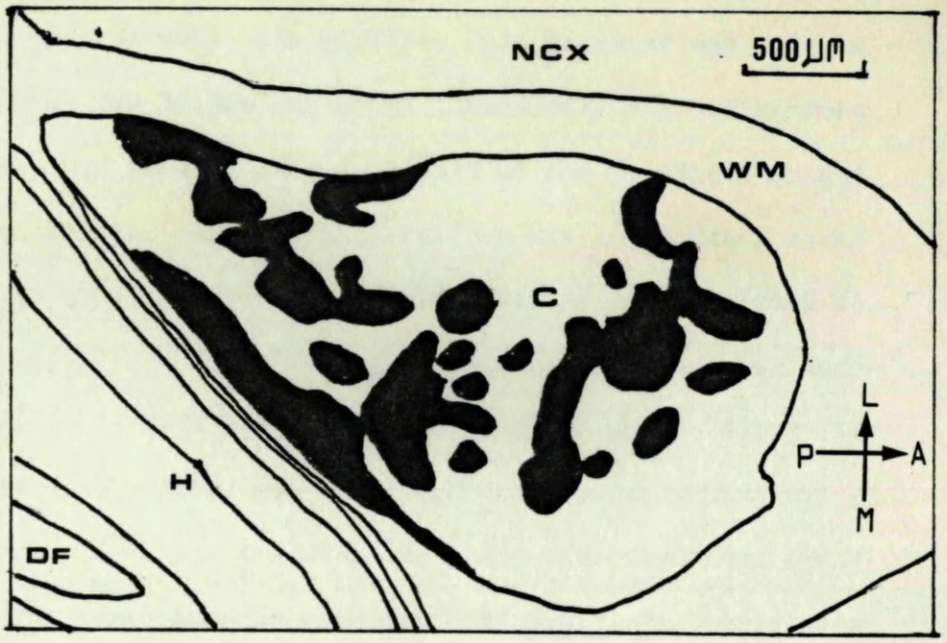


Fig.59 Horizontal section through the caudoputamen showing mosaic of dense patches. (Pentobarbitone anaesthetised mouse)

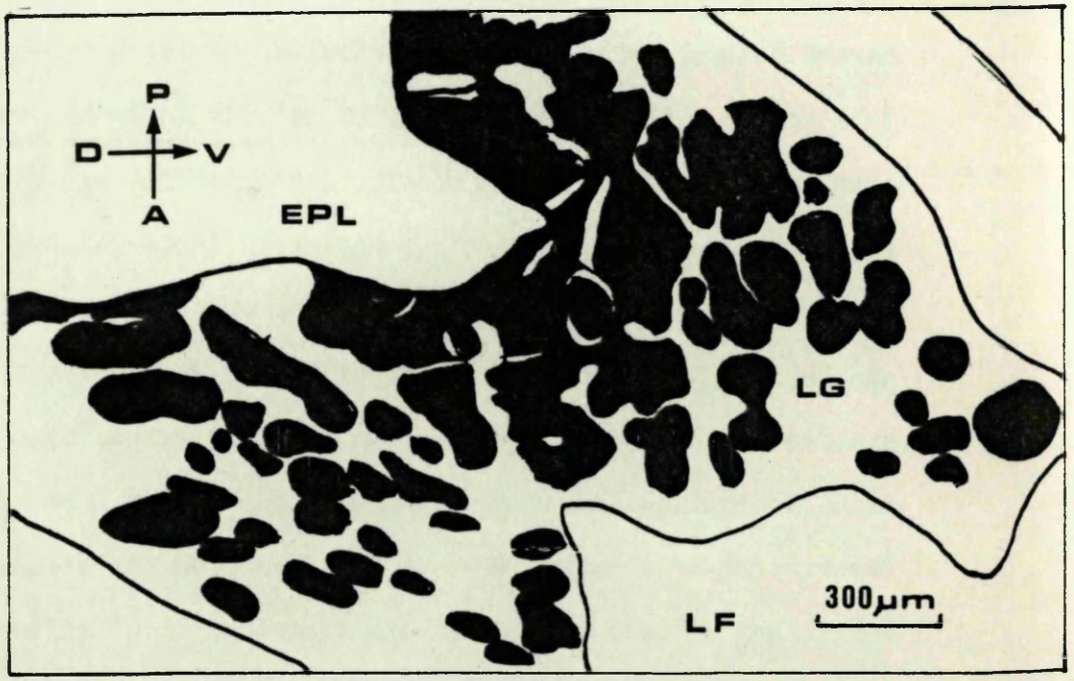
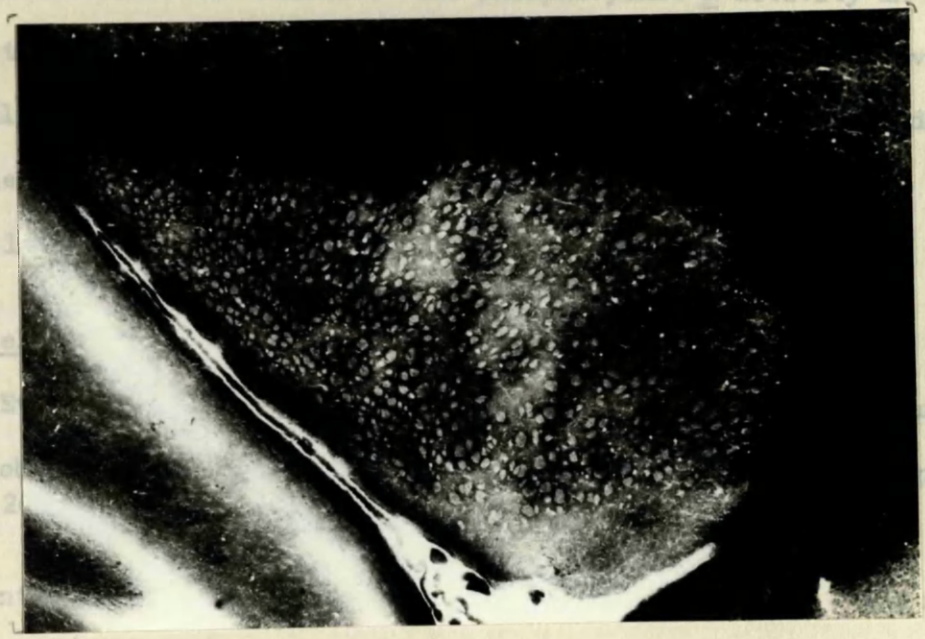
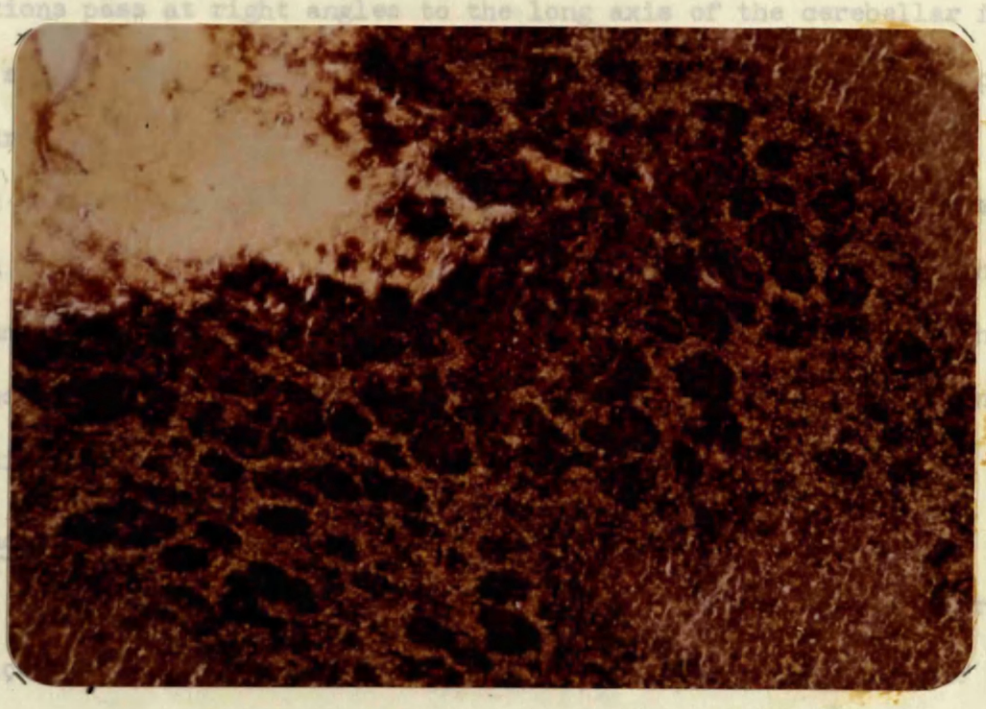


Fig.60 Partially flattened section through the olfactory bulb showing the high phosphorylase a levels in the hollows of the glomeruli. (Urethane anaesthetised rat)





As mentioned earlier the caudoputamen has a different appearance in sections from the brains of "pentobarbitone anaesthetised" as opposed to "unanaesthetised" animals. The phosphorylase a activity in sections from the former group is higher and the patches of higher activity usually take up a greater proportion of the caudoputamen than do the patches observed in sections from the "unanaesthetised" group. (Compare Figs. 15 and 16).

Cerebellum

Evidence of banding associated with input to the cerebellum has been observed both in studies of the mossy fibre input to the granule layer [269] and the climbing fibre input to the molecular layer. [86] In the present study the cerebellum was only sectioned in a few animals and so the results are very preliminary. No evidence of banding was observed in the granule layer but vertically oriented bands of high activity were observed in the molecular layer as shown in Figs. 46 and 61 . Both Figs. are of horizontal sections through the cerebellum. At the level shown the sections pass at right angles to the long axis of the cerebellar folia and so are in approximately the same plane as the Purkinje cell apical dendrites. The vertical bands are very narrow and are so closely packed that it is often difficult to distinguish them as separate entities. When they can be observed separately their width is found to be only 30 - 40 μm. These bands have been observed in sections taken from both anaesthetised and unanaesthetised animals but they were not present in sections from all brains of either group.

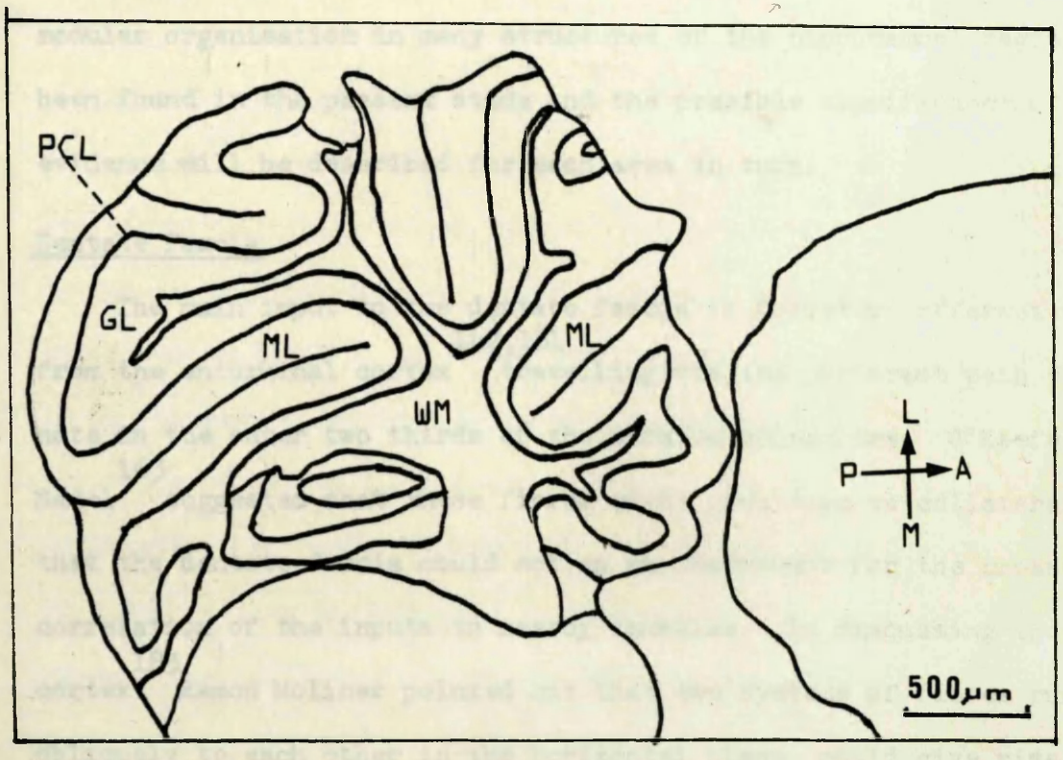
Olfactory bulb

The structure of the brain which has the most obvious modular organisation is the olfactory bulb. Nerves arriving from the olfactory mucosa terminate in discrete clusters or glomeruli in the second layer of the olfactory bulb. [181] Each glomerulus of nerve endings is surrounded by a

bowl-like collection of interneurones. Sections passing through the olfactory bulb were incubated for phosphorylase a to demonstrate this glomerular arrangement and compare it with the staining pattern of the barrels or macroglomeruli of the somatosensory cortex. The glomeruli are shown in Fig.60 which is a partially flattened section through the olfactory bulb. Each glomerulus of nerve endings appears as a patch of relatively high staining intensity surrounded by a pale zone that corresponds to the bowl of interneurones.



Fig.61 Horizontal section through the cerebellum showing vertical bands of high phosphorylase activity in the molecular layer (ML). (Unanaesthetised mouse)



DISCUSSION

Anatomical studies have shown that phylogenetically ancient laminar structures such as the cerebellum[36] and superior colliculus[110] (optic tectum) and more recent structures such as the neocortex[116] have a distinct modular organisation produced by discrete bundles of afferent fibres. The segregation of afferent fibres seems to be a general organisational principle[158] whenever two separate afferent inputs are mapped onto a laminar neural structure by fibres terminating in the same layer. Thus when an extra eye is grafted onto a tadpole[42] the afferent fibres forming the input from the two ipsilateral eyes are segregated to terminate in discrete slabs, analogous to the ocular dominance slabs of the cat visual cortex,[136a] so that the input from each eye remains separate but is topographically represented over the surface of the optic tectum. The hippocampus is organised into analogous slabs or lamellae[6] but the width of these lamellae is not known.[56] Neither is it known if the lamellae are subdivided into modules analogous to the cylindrical columns composing the cat visual cortex. Histochemical evidence of a modular organisation in many structures of the hippocampal region has been found in the present study and the possible significance of this evidence will be described for each area in turn.

Dentate fascia

The main input to the dentate fascia is formed by afferent fibres from the entorhinal cortex[147,181] travelling via the perforant path to terminate in the outer two thirds of the stratum moleculare. O'Keefe and Nadel[163] suggested that these fibres might give rise to collaterals so that the dentate fascia could act as an instrument for the cross-correlation of the inputs to nearby lamellae. In discussing the neocortex[183] Ramon Moliner pointed out that two systems of fibres running obliquely to each other in the horizontal plane, could give rise to a

moiré pattern with nodes of high fibre density and hence give the appearance of regular repeating units arranged in a vertical plane. If O'Keefe's hypothesis[163] was correct then this might also apply to the dentate fascia.

However in the neocortex all the evidence for modules or columns points to their being formed by afferent fibres running predominantly in a direction orthogonal to the surface.[223] These fibres are of both intrinsic and extrinsic origin. The results described in this section suggest that there may also be fibres running at right angles to the surface of the dentate fascia which give rise to modules. A regular organisation of patches was observed in the granule cell layer and the stratum moleculare. As these were observed in series of both coronal and surface parallel sections they are thought to have physiological significance. The arrangement of the patches in the stratum moleculare was too irregular to have been produced by the interaction of horizontally running fibres while in the granule cell layer the high density of the cell bodies prohibits fibres from running in a horizontal direction. As there is no record of vertically ascending extrinsic afferent fibres, the only morphological basis of modules in the dentate fascia is the presence of interneurones.

Cajal[182] described four types of interneurones, in the dentate fascia and underlying hilus, which had vertically ascending axons terminating in the stratum moleculare. These cells are similar to the ones found in the hippocampus (Fig. 13). Two of them have their cell bodies located in the stratum moleculare while one is located in the hilus. The fourth type is a basket cell, the soma of which occurs in or below the granule cell layer. Its axon ascends into the stratum moleculare but branches to give clusters of terminals at regular intervals in the granule cell layer. The terminal field of the axon in the stratum moleculare is apparently confined to a width of about 100 μm. Thus this cell may form the basis of a modular organisation in both the granule cell layer and

the molecular layer. This hypothesis is consistent with the electrophysiological experiments of Lomo[139] which showed that the lamellar organisation in the dentate fascia was produced by lateral inhibition thought to be mediated by the basket cells which have been shown to contain the inhibitory neurotransmitter GABA.[188]

CA1 and CA3 areas

In the mouse CA1 area, patches of relatively high phosphorylase activity were observed in the suprapyramidal layers. Although the patches were not clearly outlined they were regularly arranged and those centred on the stratum lacunosum (str. lac.) were observed in both coronal and sagittal sections. Thus the patches found in and below the str. lac. are thought to be a reflection of physiological activity and not artefacts. The patches in the stratum radiatum (str. rad.) have the same repeat interval as those centred on the str. lac. and they may have the same morphological basis. As the str. lac. patches have a similar shape in coronal and sagittal sections they apparently represent cylinders of high phosphorylase a activity similar to those observed in the dentate fascia.

Physiological studies have shown[5,6] that all the major pathways in the hippocampal formation are arranged in a plane parallel to the lamellae. Lorente de No[143] described a longitudinal association pathway but this was mainly formed by pyramidal cells in the CA2 subfield and so could not give rise to a periodic subdivision of the CA1 subfield. Thus in the CA1 field as in the dentate fascia the most likely basis of a cylindrical organisation is the presence of interneurones with ascending axons that terminate in a relatively discrete area.

In the CA1 area of the mouse Lorente de No[143] described the presence of 12 different types of interneurones. Of these six had ascending axons which terminated in the suprapyramidal layers. These cells are reproduced in Fig. 13. Cells 5 and 6 are confined to the suprapyramidal

layers and have short axons that terminate near the cell bodies. Of the other four cells, 1 and 3 are basket cells and presumably contain the inhibitory neurotransmitter GABA.[189] Cell 2 has dendrites passing through all the layers of the hippocampus and its axon terminates in the outer part of str. rad. and in the two outermost sublayers. Cell 4 is more specific having its dendrites confined to the str. oriens (SO) and its axon confined to the str. rad. With regard to the patches centred on str. lac., cell 1 could not produce modules centred on str. lac. as its axon terminates mainly in the pyramidal cell layer and in the molecular layer, neither could cell 4 as its axon is confined to the str. rad. The other four cells could all in theory contribute to a modular organisation in the upper three layers of the CA1 area. Cell 3 is especially interesting in this respect, because not only is it similar to the basket cell, which it was suggested might give rise to a modular organisation in the dentate fascia, but its dendrites are restricted to str. oriens and so it receives a specific input. Apart from axon collaterals and the axons of local interneurones the main input to the str. oriens is a subicular input which arrives via the alvear path.[143,19]

Both Cajal[181] and Lorente de No[143] described an alvear path which contained axons of both subicular and CA1 pyramidal cells travelling towards the fimbria. However subsequent anatomical studies using axon degeneration techniques did not support their findings[36] and it is only within the last few years that its existence has been confirmed by both neuroanatomical[19] and electrophysiological techniques.[2] The fibres in this pathway synapse with the basal dendrites of cells in the pyramidal cell layer but they also give rise to axon collaterals which ascend vertically between the pyramidal cell bodies to terminate among the apical dendrites.[143] The vertical axons observed in the present study probably correspond to the collaterals. Therefore the input from the subiculum may give rise to a vertical modular organisation in the CA1

field, both directly through the action of axon collaterals, but mainly through activating interneurones such as cells 2 and 3 which have dendrites in the str. oriens.

The patches observed in str. rad of a few sagittal sections may be related to cell 4, the axon of which is restricted to the str. rad. but further experiments are required to confirm the occurrence of patches confined to the str. rad. Similarly the patches observed in str. mol. were only observed in coronal sections through the posterior hippocampus and further experiments are required to define their shape and confirm that they are not artefacts. The fact that they were only seen in sections which were cut in a plane roughly parallel to the long axis of the hippocampus suggests that they may not represent modules but instead slabs or lamellae. Both types of basket cell observed in the CA1 field have axons ascending to the str. mol.[143] Therefore in theory they could provide the morphological basis for a physiological focusing of the afferent input to str. mol. to produce narrow slabs with a thickness of about 150 μm.

The lack of any regular patches of phosphorylase a activity in the CA3 field does not mean that this field lacks a modular organisation. According to Lorente de No[143] similar types of interneurone are found in the CA3 field as in the CA1 field. Therefore it is possible that modules exist there also. However according to Vinogradova[234] the limbic system may be regarded as consisting of two big interconnected circuits. The main one is an information processing circuit involving areas of the periallocortex and the CA1 field. The other is a regulatory circuit which involves the hypothalamus and reticular formation and includes the CA3 field. As Mountcastle's[158] hypothesis of a modular organisation only covers those brain structures involved in information processing, it may well be that any modular organisation in the CA3 field would not be as important or as obvious as in other hippocampal areas.

Subiculum

According to Cajal,[181] one of the most typical features of the subiculum in humans and the higher mammals is the presence of cellular clusters separated by ascending bundles of white matter in the layer of small pyramids. One of his Figs illustrating this is reproduced in Fig. 12. He also described islands of small closely packed interneurones in the superficial layers. He did not find any evidence of these cellular islands in the mouse, but in the rat Mellgren and Blackstad[153] described discrete groups of layer III pyramidal cells associated with higher levels of a dehydrogenase enzyme (NADPH-DH) and separated by paler zones apparently containing bundles of radially running fibres. In the present study phosphorylase a levels sometimes showed a banded arrangement in layer III suggesting a similar functional arrangement to that found in the rat by Mellgren and Blackstad.[153] The patches of phosphorylase a observed in layer II in a few sagittal sections may also be indicative of vertically ascending bundles of fibres dividing layer II into semi-discrete islands. Thus the evidence from this and other studies indicates that the subiculum has a modular organisation determined by vertically running fibres.

One of the major inputs to the subiculum is afferent fibres arriving from the CA1 area as the majority of CA1 pyramidal cells give rise to axons that terminate in the subiculum.[63,220] These axons travel in the alveus and then ascend vertically to terminate[143] in layers II - IV of the mouse subiculum particularly in the part nearest the CA1 field (prosubiculum). Lorente de No[143] described interneurones in the subiculum that he considered were analogous to the basket cells of the CA1 field and these may also be involved in producing a modular organisation. One of the cells he illustrated had its axonal plexus located within a relatively narrow horizontal zone on one side of the cell body in a position that would be ideal for providing lateral inhibition.

Post-, para- and presubiculum

In describing the human presubiculum Cajal[181] stated that the small pyramidal and fusiform cells were arranged in undulations. The areas of lower density were associated with bundles of ascending fibres. Bundles of ascending fibres also occurred in the layer of medium and large pyramids (layer III) which in places led to the cells being arranged in vertical rows. In the cat Rose and Woolsey[194] subdivided the presubiculum into two fields one of which (the postsubiculum) was characterised by having nests or clusters of rounded cells in its outer layers. However most workers do not recognise two separate subfields and when Blackstad[20] described clusters of cells in the rat presubiculum he did not label it the postsubiculum. More recently a study[191] of the thalamic afferent fibres from the lateral dorsal nucleus indicated that they terminate in vertical bands extending through layers I - IV of the part of the presubiculum apparently corresponding to the postsubiculum. Furthermore a recent immunohistochemical study[271] of neurotensin containing afferent fibres indicated that these fibres terminate in discrete patches in layer I of the rat postsubiculum.

Finding cylinders of high phosphorylase a activity in the outer two layers of the postsubiculum implies that at least some of the fibres terminating in layer I such as the neurotensin containing fibres, terminate in discrete modules apparently with a diameter of between 80 - 150 μm. Furthermore the phosphorylase a activity indicates that these fibres terminate in three discrete sublayers within layer I each of which is associated with altered phosphorylase a activity. This implies that there is a very high degree of specificity in the termination of afferent fibres. A similar specificity is also observed in the stratum moleculare of the dentate fascia which can also be divided into three sublayers each of which has a distinct grouping of afferent fibre terminals.[36] The banding observed when the terminal field of afferents

from the lateral dorsal nucleus were studied[191] extended throughout the outer four layers of the presubicular area. Therefore it is likely that there is a modular organisation stretching through these four layers and that other afferents involved in information processing such as those from the subiculum[206] also have a modular organisation.

In the present study there was no evidence of a modular organisation in the part of the presubiculum outwith the postsubiculum. Other studies have not differentiated between the two sub-areas and so it is uncertain at present whether the presubiculum does have a modular organisation other than in the postsubiculum. Similarly at present there is no evidence of a modular organisation in the parasubiculum. As the parasubiculum is though to have developed in conjunction[36] with the prefrontal cortex it is apparently involved in information processing and so a modular organisation in this structure would be expected.

Entorhinal cortex

Clusters of cells also occur in layer II of the lateral entorhinal cortex of the rat[20] and in a histochemical study using NADPH-DH Mellgren and Blackstad[153] provided evidence of a modular organisation in layer II of the medial entorhinal cortex. In layer II the NADPH-DH activity was arranged in narrow patches of high activity separated by intervening areas of lower activity. Although the size of these patches was not given they appear to be comparable to the layer II patches of high phosphorylase a activity observed in mice and rat brains in the present study. Phosphorylase a patches were also observed in layer III. These were larger than the layer II patches as they had a width of about 250 µm. So far they have only been found in a few brains and so their significance is uncertain. However some of them may correspond to the islands of dense dopaminergic innervation which have been described in layers II and III of the rat lateral entorhinal cortex.[39,60]

Blackstad[20] showed that contralateral afferent terminals were

concentrated in the layer II cell islands and so exhibited a modular organisation. Lorente de No[142] described three types of vertically ascending afferent fibre terminating within the entorhinal cortex. One type terminated solely in layer II while the other two types terminated in the outer three or four layers. In each case the terminal plexus was restricted to a band a few hundred μm wide and so was suitable for producing a modular organisation. Therefore the evidence discussed above suggests that the afferents to the entorhinal cortex are associated with at least two sets of modules one of which is restricted to layer II while the other involves the upper three or four layers.

The bundles of afferent fibres separating the cords of cells in layer V give rise to the perforant path[181] which is thought to be specifically organised so as to produce the hippocampal lamellae.[139] However at present the relationship between the entorhinal modules and the bundles of efferent fibres is unknown, although the perforant path is largely derived from the axons of layer II stellate cells.[181]

Cingulate cortex

The cingulate cortex in the rat as defined by Vogt and Peters[235] includes both the retrosplenial and prefrontal cortex. In the monkey retrosplenial limbic cortex Goldman and Nauta[79] described a most striking regular periodicity in the terminal fields of cortico-cortical fibres. Their autoradiographs, showing the transport of tritiated amino acids, indicated that the fibres terminated in bands with a width of 200 - 400 μm that alternated with 400 - 500 μm wide unlabelled spaces. In the rat also, cortico-cortical fibres terminate in a columnar fashion[18] in the outer layers of at least one part of the retrosplenial cortex (area 29 C). This is the area where cylinders of high phosphorylase a activity were observed in the present study. Both the afferents from the caudal part of the prefrontal cortex (area 24)[18] and those arriving directly from the dorsal part of the subiculum terminate in a columnar fashion.[215]

Sorensen[215] suggested that the vertical bands of subicular afferents might be in register with the dendritic bundles which have been described in this area of the cortex. Thus there is strong evidence of a columnar organisation dependent on vertically ascending afferent fibres and this is apparently reflected by the phosphorylase a levels. The phosphorylase a staining also provided evidence for columns in area 24. Area 24 probably does have a columnar organisation as its efferent fibres terminate in a columnar fashion in 29 C.[18] However more experiments are required before it can be asserted that phosphorylase a activity does indeed reflect a columnar organisation there.

Cortical architectonics

In a histochemical study of the architectonic organisation of the hippocampal formation Mellgren and Blackstad[153] showed that all the dehydrogenase enzymes they studied had patterns of activity that conformed to and helped confirm the known architectonic organisation. They did not study phosphorylase activity but Shimizu,[204] in his study of total phosphorylase activity in the rat brain, stated that the colour and intensity of the iodine stained reaction product was related to the architectonic organisation and that the colour of staining was fairly constant in any one area in sections from different brains. In studying phosphorylase a activity in relation to chronic hypoxia Ibrahim[112] showed that phosphorylase a activity was consistently related to the architectonic organisation of the hippocampus as viewed in coronal sections. Therefore these studies support the conclusion reached in the present study that, after incubation for phosphorylase a, both the intensity and in a general way the colour, of the I_2 stained reaction product are related to the architectonic organisation.

Modular organisation of non-cortical structures

In the intermediate grey layer of the superior colliculi most of the anatomical inputs studied terminate in a modular fashion. Thus the

input from the prefrontal cortex,[78,18] frontal eye fields,[89] somatosensory cortex,[259] substantia nigra,[83] spinal cord and the trigeminal input,[110] all have a modular pattern of termination. There must be some overlap of these inputs and it is thought that the cortical and trigeminal somatosensory inputs at least are in register.[110] The trigeminal input only terminates in the rostral 60 - 70% of the colliculus whereas the nigrocollicular input reaches virtually the entire structure.[83] With all these modular inputs there is little doubt that the bands of phosphorylase a activity observed in the present study do represent the terminal field of at least one group of afferent fibres. One possibility would be that they represent input from the substantia nigra but to be certain of this it would be necessary to carry out a neuroanatomical study with the transport of tritiated amino acids and compare the silver grain density with the phosphorylase a staining in the same section. None of the previous anatomical studies have looked at the modular organisation of the anatomical inputs in surface parallel sections, but the surface parallel organisation has been reconstructed[83] for the input from the substantia nigra. It was found that the input terminated in discrete wavy bands reminiscent of the ocular dominance slabs observed in layer IV of the visual cortex.[136] Acetylcholinesterase activity also exhibits a patchy staining pattern when demonstrated histochemically in coronal sections.[84] It appears to have a complementary organisation to the phosphorylase a patches described above which would fit neatly between the phosphorylase a patches. Graybiel[84] reconstructed the staining pattern to form the pattern that would occur in surface parallel sections and showed that the cholinesterase activity had a banded appearance similar to that observed with phosphorylase a activity in the present study. Thus it is possible that the phosphorylase a and cholinesterase staining patterns may be arranged in an interdigitating fashion.

The caudoputamen (striatum) is also thought to have an irregular

modular organisation as the input from different cortical areas and other structures terminates in small discrete areas.[95] Thus the input from the sensori-motor cortex[117] terminates in the form of interrupted clusters, strips and bands as does the input from the frontal cortex. Jones[117] interpreted this to mean that individual corticostriatal fibres terminated in a very restricted manner and that the terminal ramifications of fibres from one cortical area may alternate in the caudoputamen with those arising in other areas. The terminal fields of the input from the frontal cortex has been shown[179] to correspond to patches of high acetylthiocholinesterase staining which apparently fill in the spaces left by the terminals from the parafascicular input and areas containing a high density of opiate receptors.[95] In the present study a mosaic of patches and strips of high phosphorylase _a_ activity was observed in sections through the caudoputamen. There can be little doubt that these have some physiological significance but it is not known which fibre system they may correspond to. Preliminary observations suggest that the pattern of staining in sections from the "anaesthetised" and "unanaesthetised" groups of mice may be different and so it is possible that anaesthesia affects the input to the caudoputamen in a way that has an affect on the postmortem levels of phosphorylase _a_. Thus an analogous situation may occur to that observed in the neocortex where different systems of modules were observed in sections from the two groups of mice.

There is also evidence of a modular organisation in the cerebellum. Thus the trigeminocerebellar mossy fibre input terminates in a spatially precise mosaic in the granule cell layer.[269] Similarly the olivocerebellar projection mediated by climbing fibres which terminate in the molecular layer has a parasagittal zonation. Staining for acetylthiocholinesterase also gives evidence for a patchy distribution of afferent fibres.[150] The narrow bands of high phosphorylase _a_ activity observed

in the present study are not directly related to any of the above patterns of organisation as the phosphorylase a bands are much narrower. However the phosphorylase a bands may be related to the climbing fibre input which terminates in narrow vertical bands to form rows of synapses along the apical dendrites of the Purkinje cells.

SECTION III PHOSPHORYLASE A AS A MARKER OF FUNCTIONAL ACTIVITY

Introduction

Results from the previous two sections have shown that the postmortem phosphorylase a levels do reflect changes produced in the neocortex by anaesthesia and that the histochemical method is of value in studying the modular organisation of the brain. Both these findings indicate that postmortem phosphorylase a levels reflect some aspect of functional activity but the above results do not indicate how closely phosphorylase a activity is linked to electrical activity or how easily the postmortem levels can be manipulated by physiological stimuli. Therefore in this section an attempt was made to alter phosphorylase a activity directly by applying penicillin to the cortex in order to induce an epileptiform focus. As well as providing a method whereby cortical activity can be altered under experimentally controlled conditions, this procedure allowed a direct comparison to be made with results obtained by ^{14}C-2-deoxyglucose autoradiography. In the first part of this section the results from control anaesthetised and unanaesthetised rats will be described.

RESULTS

Unanaesthetised rats

Sections were taken from the brains of 8 rats killed by a blow on the back of the spine and 4 rats decapitated immediately after the inhalation of ethyl chloride vapour. The pattern of staining observed after incubation for phosphorylase a activity was basically the same and so both groups of sections will be referred to as sections from "unanaesthetised" rats.

When surface parallel sections were cut through layer IV the primary sensory areas had a significantly higher level of activity than the

surrounding cortex. This is shown in Fig.62. The primary somatosensory cortex does not have a high level of staining throughout its entire extent. Only the sub-areas receiving a direct thalamic input from the ventrobasal complex are strongly stained. Thus the different parts of the ratunculus described by Welker[244] are distinctly outlined. Going in a medial to lateral direction the areas corresponding to the input from the hindpaw (HP), forepaw (FP), lower lip (LL) and head (HD) are indicated on the adjacent diagram as is the whisker barrel region (WB). The small area lateral to the representation of the head is in the position of area 40, the secondary somatosensory cortex. Beyond this the dark area marked by (K) is an artefact caused by the section folding over on itself.

In coronal sections through the somatosensory cortex, layer IV and the base of layer III have the highest staining intensity. The staining in layer IV is not homogeneous, because as well as the discontinuities caused by the barrels, the different parts of the ratunculus are separated by thin pale bands. This produces islands of high activity with a width of 1 mm or more. Layer Vb is also distinct because of its high staining intensity relative to the two adjacent layers. Like them it is homogeneously stained. There is no indication of the modular pattern, caused by the specific thalamic afferents, in layer Va although the pattern can be discerned in the base of layer III.

Urethane and pentobarbitone anaesthetised rats

Sections from the 4 rats anaesthetised with urethane are similar to those from the "unanaesthetised" rats except that those from the urethane group have a relatively higher staining intensity in all layers. Layers IV and Vb are still distinguishable by having a higher staining intensity than the adjacent layers. In surface parallel sections the primary sensory areas still have a higher activity than the surrounding cortex although there is not such a pronounced difference in intensity

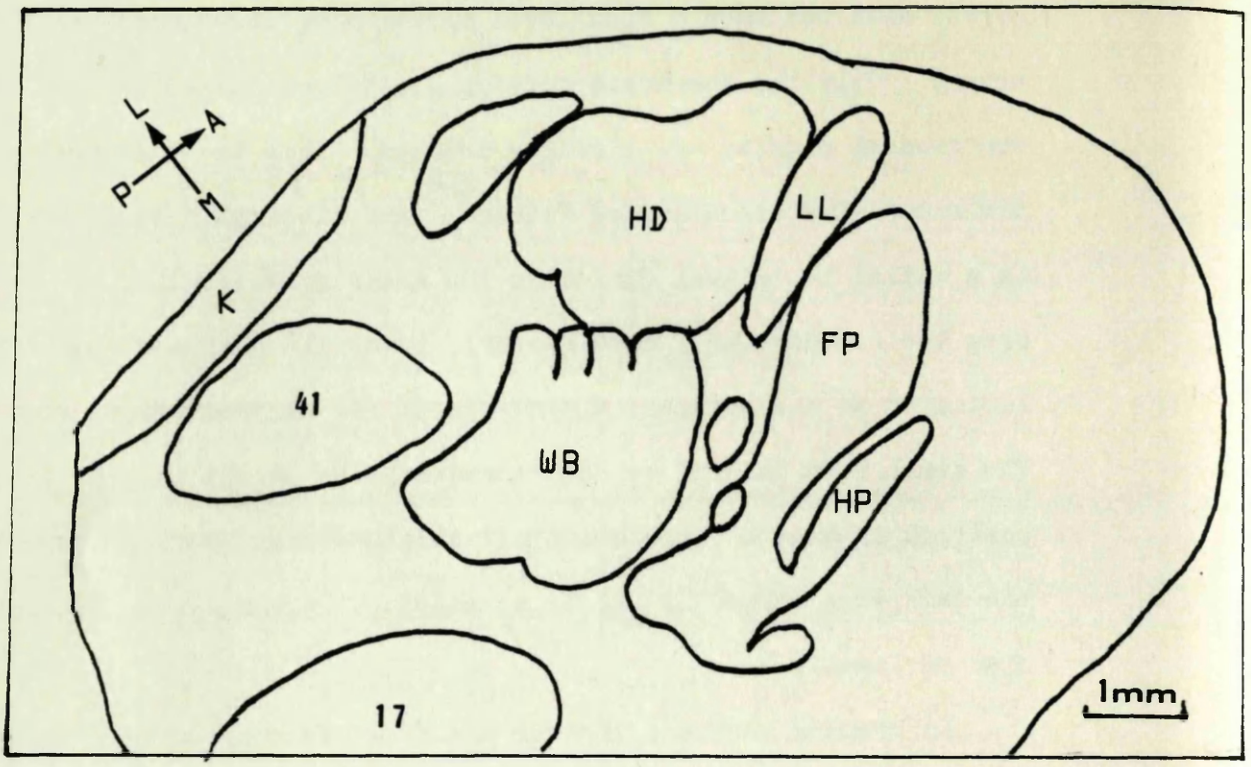
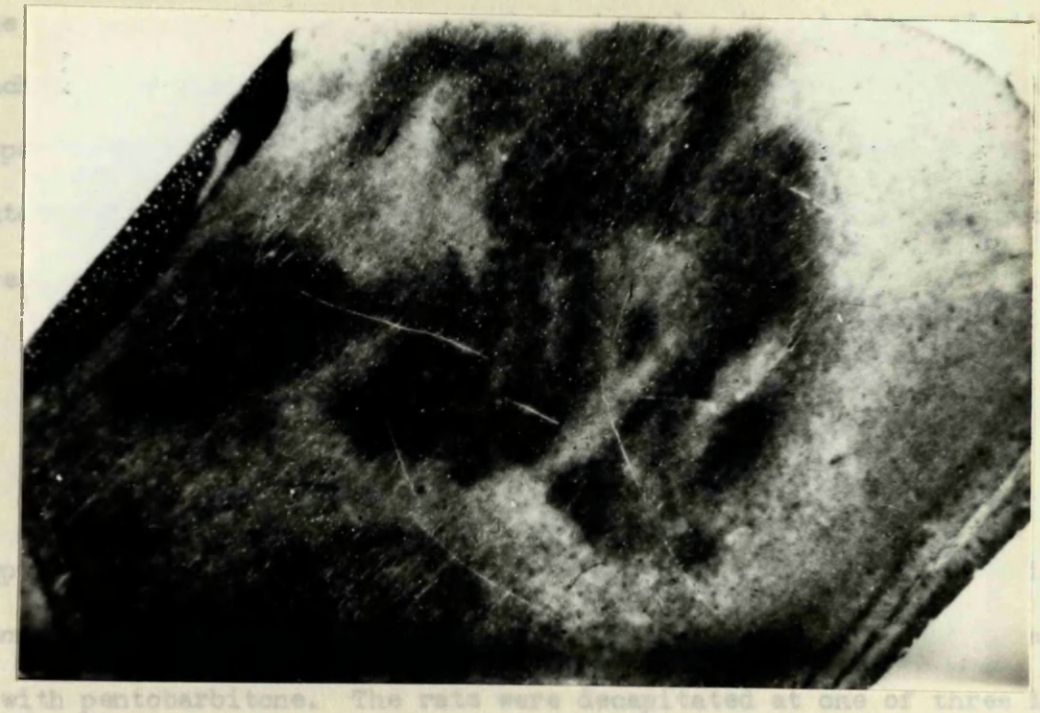
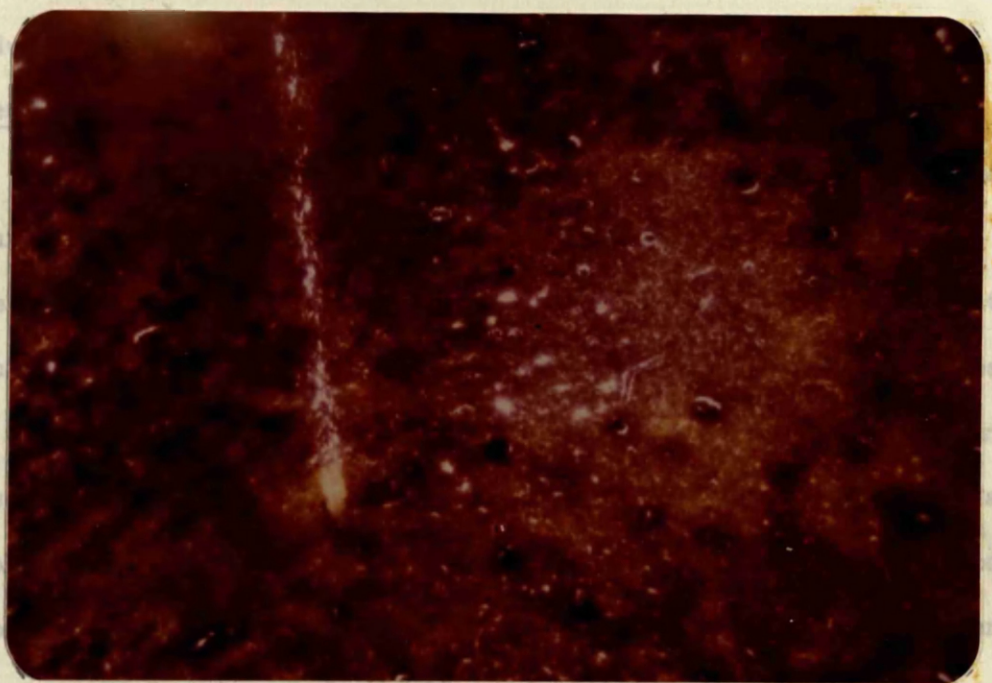


Fig.62 Surface parallel section through layer IV of the rat neocortex showing the relatively high phosphorylase a activity observed in the primary sensory areas. (Unanaesthetised rat)

Fig.63 Surface parallel section through the base of layer I showing area of purple staining in area of cortex immediately under where a pledget of penicillin soaked paper had been applied. (Urethane anaesthetised rat) X82





as there was in sections from the unanaesthetised group. In surface parallel sections from the two pentobarbitone anaesthetised rats the primary sensory areas are also outlined by having a higher activity than the surrounding cortex. However the ratunculus is not observed with such clarity because layers IV - VI are not as intensely stained as the upper three layers. In both groups of "anaesthetised" rats small patches with a diameter of 60 - 120 μm are seen especially in the motor areas of the cortex.

Changes in Phosphorylase a Activity Produced by an Epileptiform Focus

Sections were cut from the brains of 13 rats which had had an epileptiform focus induced by the application of penicillin to the primary somatosensory area. Of these rats 9 were anaesthetised with urethane and 4 with pentobarbitone. The rats were decapitated at one of three intervals after the application of penicillin. These intervals were 5 - 10 min., 30 min. - 1 hour and 4 - 5 hours. In all cases where sections were stained for phosphorylase a electrical monitoring of the cortical activity had been used to ensure that epileptiform activity had been produced.

Interval of 5 - 10 min.

This activity was characterised by interictal spikes which had a relatively high voltage and a duration of less than 100 msec.[100] There were 5 rats in this group one of which was anaesthetised with pentobarbitone. In each case penicillin was applied by a pledget placed on the surface. Of these rats, three including the pentobarbitone anaesthetised animal gave no evidence of an epileptiform focus when surface parallel or coronal sections were cut through the somatosensory cortex. In one rat a small purple spot with a diameter of 200 μm was observed in serial sections through layer II in a position directly above the projection area of the primary input from the forepaw. This spot was

not observed in sections below the upper part of layer III or in layer I. In the fifth rat a larger purple spot with a diameter of 500 μm was observed in layer II in serial surface parallel sections. (Fig.63) This spot was directly beneath the position where the tiny pledget of penicillin-soaked filter paper had been placed as it was in the part of the primary somatosensory cortex corresponding to the forepaw area. (Fig.63) The spot was largest in layer I where it was about 800 μm in diameter and gradually decreased in size until by the base of layer III it had disappeared altogether. The phosphorylase activity within the purple spot actually seemed to be lower than the surrounding cortex. The activity was also comparatively homogeneous while the surrounding cortex had a brown colour and exhibited patches of higher activity with a diameter of 60 - 120 μm.

Interval of 30 min. - 1 hr

Of the 5 rats in this group 3 were anaesthetised with pentobarbitone and 2 with urethane. In each case a focus of epileptiform activity was induced by iontophoresing penicillin from a pipette, the tip of which was in or near layer IV. In none of the rats was there any evidence of an epileptiform focus in sections incubated for phosphorylase a activity. In 3 of the rats the tip of the penicillin pipette was marked by a small spot of pontamine sky blue. This was surrounded by a pale area and sometimes by a thin outer ring of damaged cells with high phosphorylase a levels. The outer ring had a diameter of between 200 and 400 μm. The pale area surrounded by a dark ring of damaged cells could be followed down through serial sections from layer II. Similar cylinders of abnormal activity were also associated with the various glass electrodes used for recording and so these narrow cylinders of altered enzyme activity indicated the line of the electrode track.

Interval of 4 hr.

The 3 rats in this group were anaesthetised with urethane.

Penicillin was applied by a pipette in one rat and by a paper pledget in the other 2 animals. In one of the latter a dark focus of high activity was observed in layers I to III. The focus was largest in layer I being about 1 mm in diameter, was smaller in layer II where it was between 600 and 700 μm and had disappeared completely by the base of layer III. Within the dark purple focus both the neuropil and cell bodies were strongly stained. This is shown in Fig.64 which is a surface parallel section through layer II. However in the other rat where a pledget of penicillin had been applied there was no evidence of any abnormal activity in the forepaw area of the somatosensory cortex. In the third rat where penicillin had been applied by pipette there was a cylinder of altered phosphorylase a activity in the upper three layers of the forepaw area. This cylinder had a diameter of between 800 μm and 1 mm being slightly wider in layers I and II. At a depth of 500 μm the centre of the cylinder was formed by a spot of pontamine sky blue marking the pipette tip (see Fig.65). The cylinder of abnormal activity ended within a few hundred microns of this depth at the top of layer IV. (In the somatosensory cortex layer IV is located between the depths of 650 and 800 μm). Smaller patches of altered phosphorylase a activity were observed in areas of the cortex both near and distant from the forepaw area (see Fig.65). These were about 200 μm in diameter and could be followed through serial surface parallel sections. However similar patches were also observed in control brains and so they are apparently not caused by the initiation of epileptiform activity.

Locating the micropipette tip in relation to the anatomical organisation

Three methods were used to obtain the location of the electrode tip. The most accurate method employed was the iontophoresis of pontamine sky blue from the pipette tip. Initially this was carried out by passing current through the electrode at the beginning of the experiment. However it was found that during long experiments the dye spot disappeared

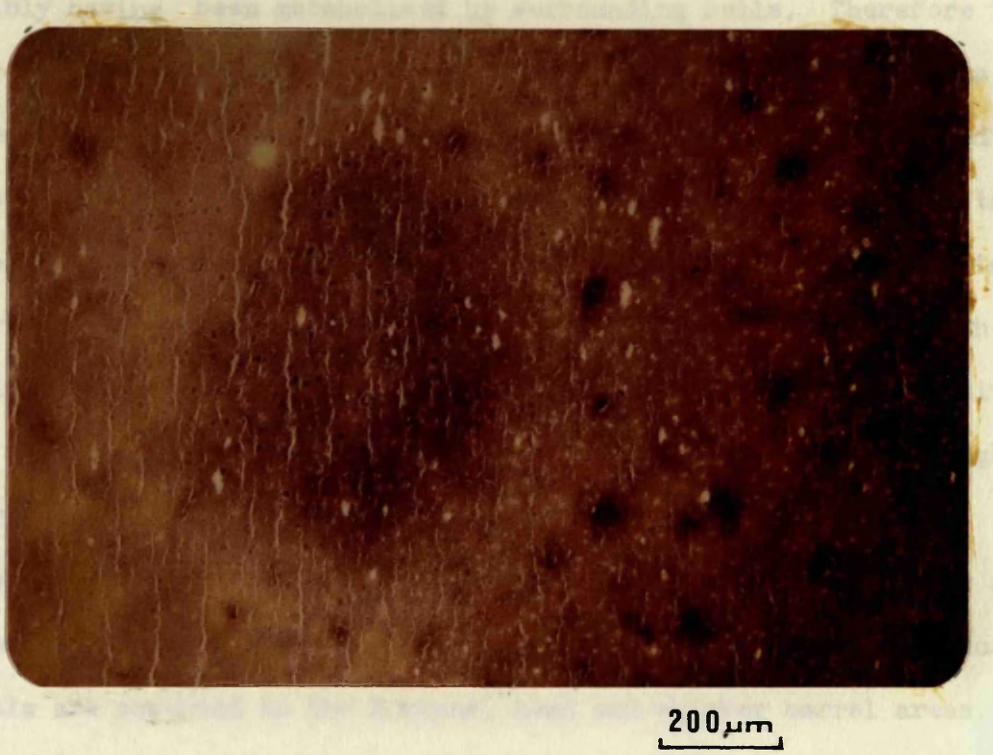


Fig.64 Surface parallel section through rat somatosensory cortex cut at a depth of 280 µm. Area of dense purple staining is observed in the region of the epileptiform focus. (Urethane anaesthetised rat, perfusion fixed)

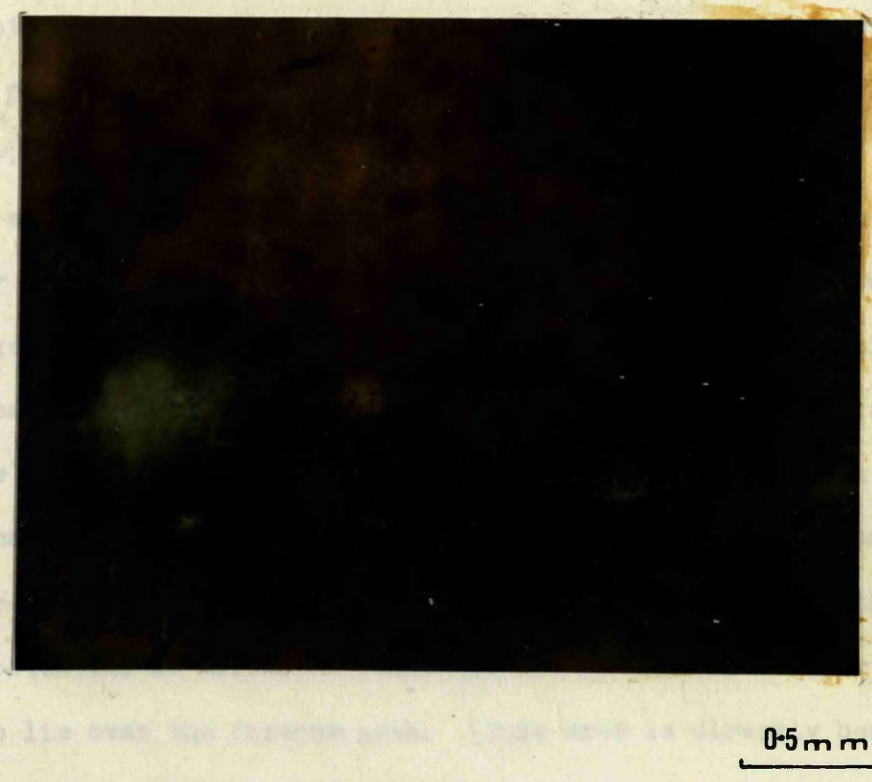


Fig.65 Surface parallel section through the rat somatosensory cortex cut at a depth of 500 µm. The position of the electrode tip is marked by a spot of pontamine sky blue, surrounding which is an area of damaged tissue with altered phosphorylase a activity. (Urethane anaesthetised rat)

possibly having been metabolised by surrounding cells. Therefore in later experiments the dye was ejected shortly before the animal was to be decapitated. When this procedure was used the dye spot remained in position even after the section had been incubated for 90 min. in the phosphorylase a incubation medium. When the pipette tip had been in layer IV, the dye spot could be observed directly in relation to the architectonic organisation which is indicated by phosphorylase activity. This is shown in Fig.66 which is a surface parallel section through layer IV that has been incubated for 30 min. to demonstrate total phosphorylase activity. In this section the primary somatosensory areas are evident because of their higher levels of activity and individual barrels are observed in the forepaw, head and whisker barrel areas. These landmarks allow the blue spot to be accurately located in terms of the sensory ratunculus described by Welker [244] and confirm that the penicillin pipette was indeed in the forepaw area. Sections stained for phosphorylase a activity also allow the sensory areas to be observed, but in pentobarbitone anaesthetised rats especially, the ratunculus is often not clearly demarcated and it is useful to have at least one section passing through layer IV incubated for total phosphorylase activity.

In experiments where the pipette or electrode had been in position for 1 hr or more, the electrode track was indicated by a narrow cylinder of damaged cells with high phosphorylase a activity. This cylinder ended near where the micropipette tip had been and so allowed a fairly accurate estimate to be made of where the tip had been both in terms of anatomical areas and cortical depth. The third method used was to place a silver-ball electrode onto the cortical surface and record the forepaw evoked potential at different positions within the area of cortex estimated to lie over the forepaw area. (This area is directly beneath the junction of the sagittal ridge and the coronal suture.) The point within this area where the evoked potential has the shortest latency

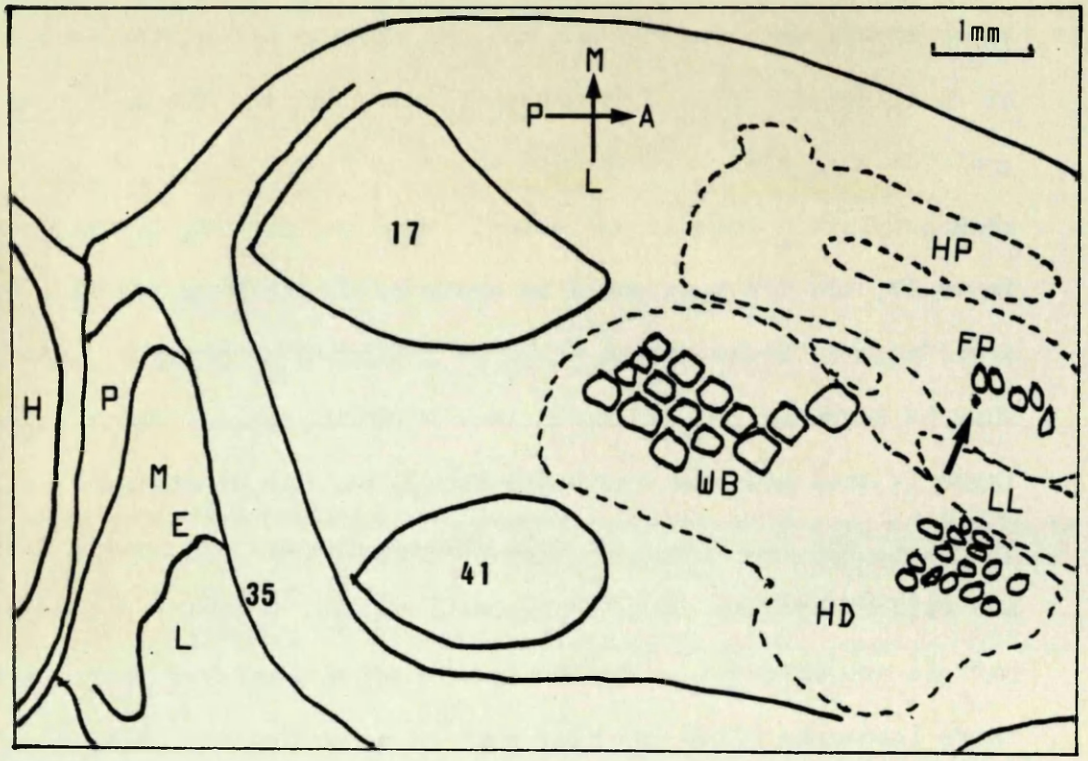


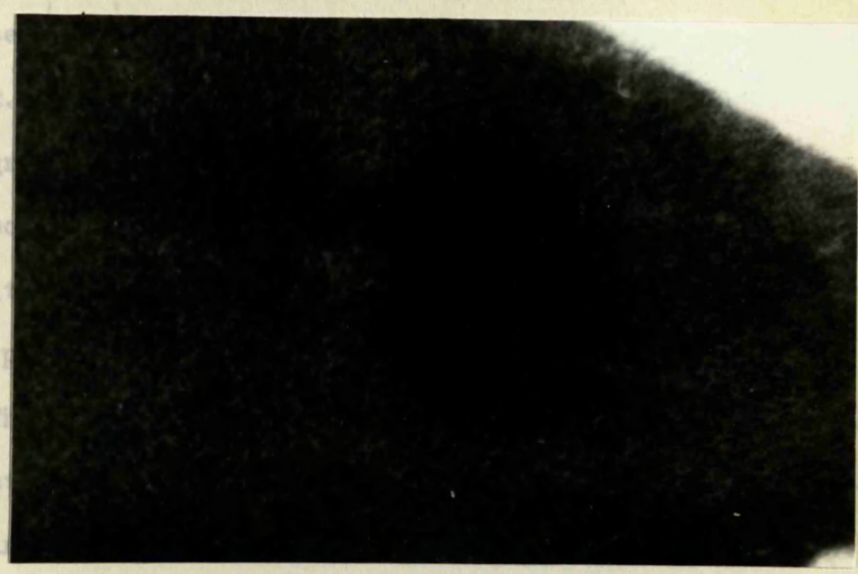
Fig.66 Surface parallel section through layer IV of the rat neocortex incubated to demonstrate total phosphorylase. The pontamine sky blue spot marking the electrode tip is marked by an arrow.

Fig.67 Autoradiograph taken from a tangential section through layer II/III of the rat neocortex. The central area of high grain density indicates an increased ^{14}C2DG uptake associated with the epileptiform focus. X 18

is taken to be the centre of the forepaw area. The advantage of this method is that it allows the position of the pipette tip to be localised before the experiment begins. The three different methods of locati[illegible] all be use[illegible]



[illegible] methods [illegible] used to stu[illegible] of the rat[illegible] be made bet[illegible] 2-d[illegible] te after pla[illegible] surface of [illegible] for the [illegible] In the autoradiographs prepared from tangential sections the primary focus appeared as a discrete area of increased glucose uptake with a diameter of about 2 mm. The increased glucose uptake was restricted to the upper 4 layers of the cortex. Fig.67 shows the focus of increased activity in a tangential se[illegible] ing the cortex. [illegible] has a very gr[illegible] individual cell bo[illegible] horylase a activi[illegible] ly seen as dense p[illegible]



Th[illegible] ing the epilep[illegible] ncreased by usi[illegible] by Goochee.[80] This is a computer system which allows the density of silver grains to be measured automatically over tiny areas of the autoradiograph, the

is taken to be the centre of the forepaw area. The advantage of this method is that it allows the position of the pipette tip to be localised before the experiment begins. The three different methods of locating the micropipette tip were complementary and could all be used in the one experiment.

Comparison between phosphorylase a and ^{14}C-2-deoxyglucose methods

One experiment was performed where the ^{14}C-2-DG method was used to study a penicillin induced epileptiform focus in the forepaw area of the rat somatosensory cortex. This allowed a direct comparison to be made between the ^{14}C-2-DG and phosphorylase a methods. The radioactive 2-deoxyglucose was injected into the rat's femoral vein one minute after placing a tiny pledget of penicillin-soaked filter paper on the surface of the cortex. The rat was maintained under halothane anaesthesia for the following 30 min. before being decapitated. In the autoradiographs prepared from tangential sections the primary focus appeared as a discrete area of increased glucose uptake with a diameter of about 2 mm. The increased glucose uptake was restricted to the upper 4 layers of the cortex. Fig.67 shows the focus of increased activity in a tangential section cut through layer II/III after partially flattening the cortex. The focus is clearly outlined but the autoradiograph has a very grainy appearance and it is not possible to distinguish individual cell bodies. By contrast in the section incubated for phosphorylase a activity and shown above Fig.64 the cell bodies can be clearly seen as dense purple spots.

The contrast between the density of silver grains overlying the epileptiform focus and the density in normal cortex can be increased by using an Image Enhancement system such as that described by Goochee. This is a computer system which allows the density of silver grains to be measured automatically over tiny areas of the autoradiograph, the

value recorded and a colour image of the autoradiograph composed where the density of silver grains is indicated by a predetermined colour code. Fig. 68 shows the image produced by this system of the autoradiograph shown at higher power in Fig. 67 . At the top of Fig. 68 the colour code is shown calibrated in arbitrary units. As well as the orange-yellow area indicating the site of the primary focus there is also an indication of increased activity in the cortex immediately in front of the focus. This is also a discrete area of higher activity and so was not caused by the spread of the penicillin. When the diagram of the primary sensory areas is superimposed over the image (see adjacent diagram) and the primary focus is centred over the forepaw area the adjacent area of high activity is found to be in the part of the motor cortex corresponding to the forepaw area as described by Hall and Lindholm.[87] The strip of increased activity in Fig. 68 may indicate an increased level of activity in the somatosensory hindpaw and motor hindpaw and vibrissae regions. Evidence of the primary and secondary foci has almost completely disappeared in autoradiographs of sections passing through layer V as shown in Fig. 69 . The grain density in the somatosensory and motor areas is barely above background levels. The rim of high grain density which passes right round the peripheral part of the autoradiograph is caused by the imperfectly flattened section passing through layer IV. Layer IV has a comparatively high resting uptake of glucose[40] and so it also has a higher grain density in autoradiographs taken from control animals.

Enzyme Localisation

Tissue fixation

Using histochemical methods the localisation of the enzyme under study can be preserved by fixing the tissue, usually by perfusion, prior to carrying out the incubation procedure. Phosphorylase *a* is a very sensitive enzyme and even mild organic fixatives such as absolute

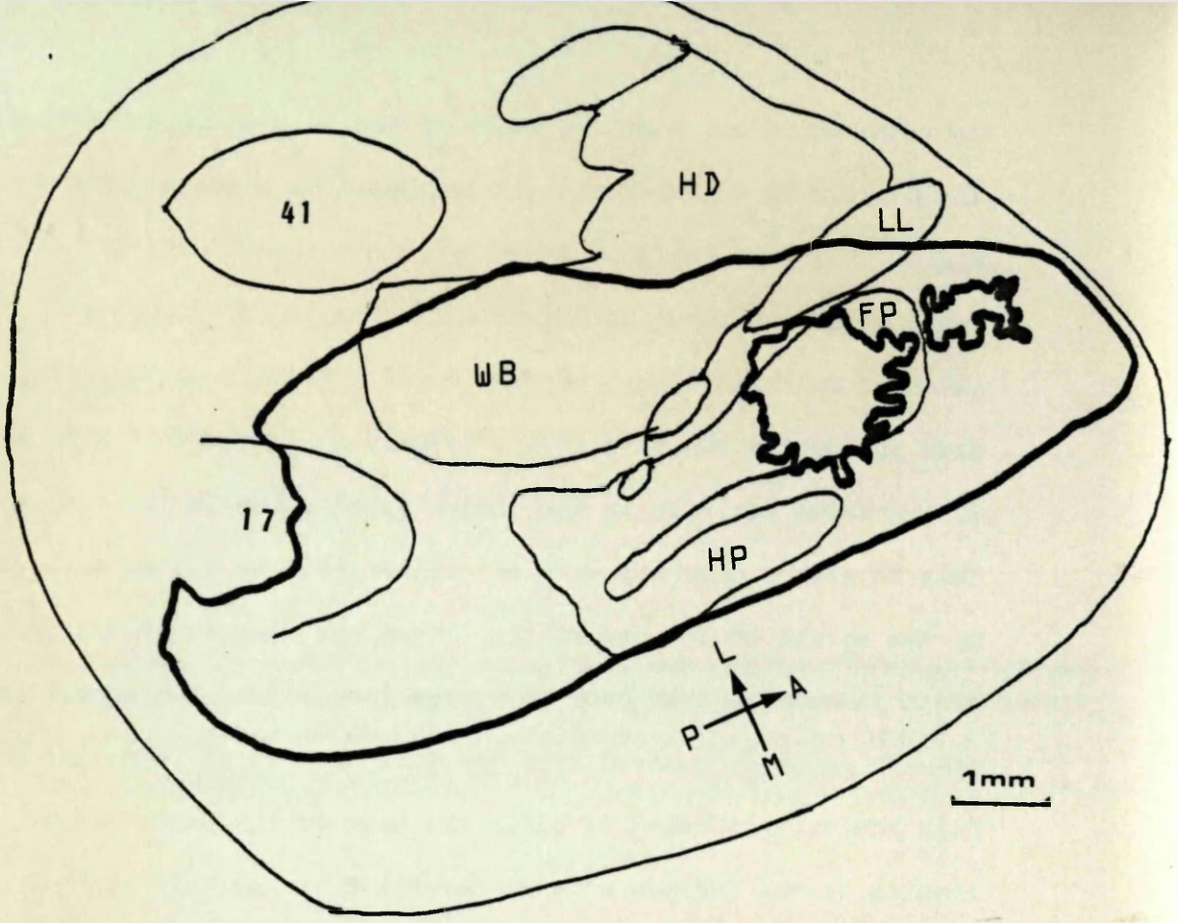


Fig.68 Television image produced by the Image Enhancement system of the autoradiograph shown at higher power in the previous figure. The diagram above shows an inverted outline of the image in relation to the architectonic organisation of the rat neocortex.

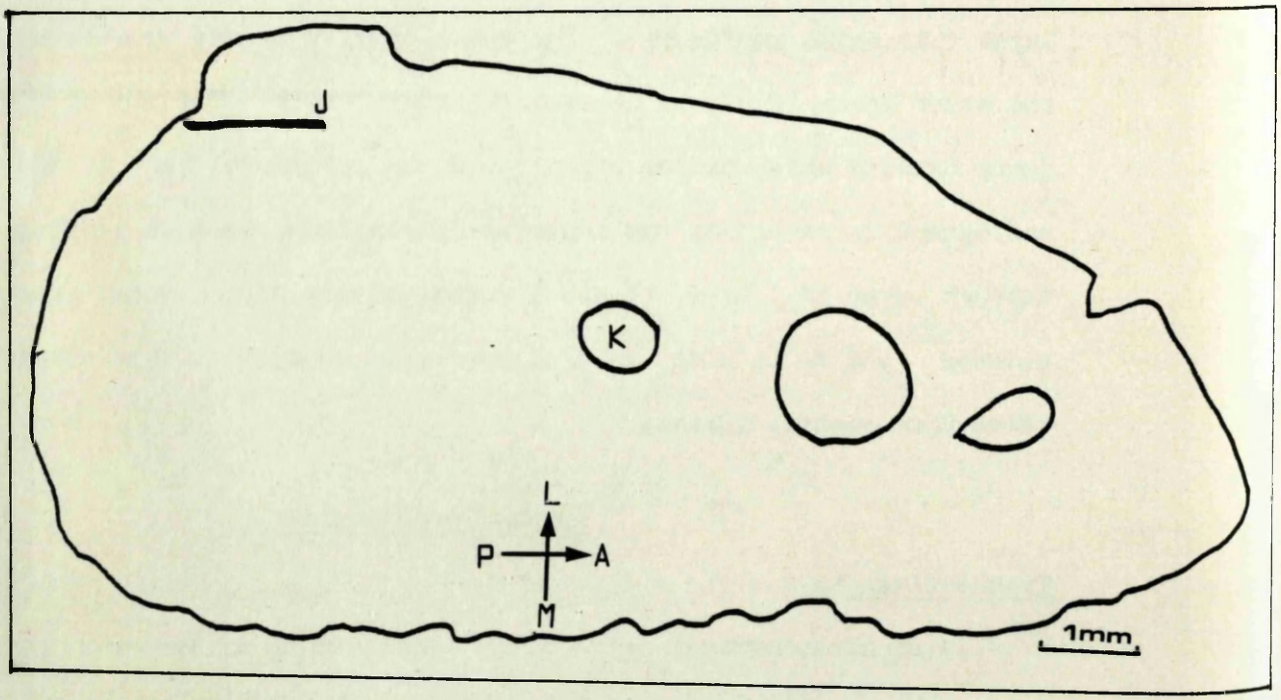
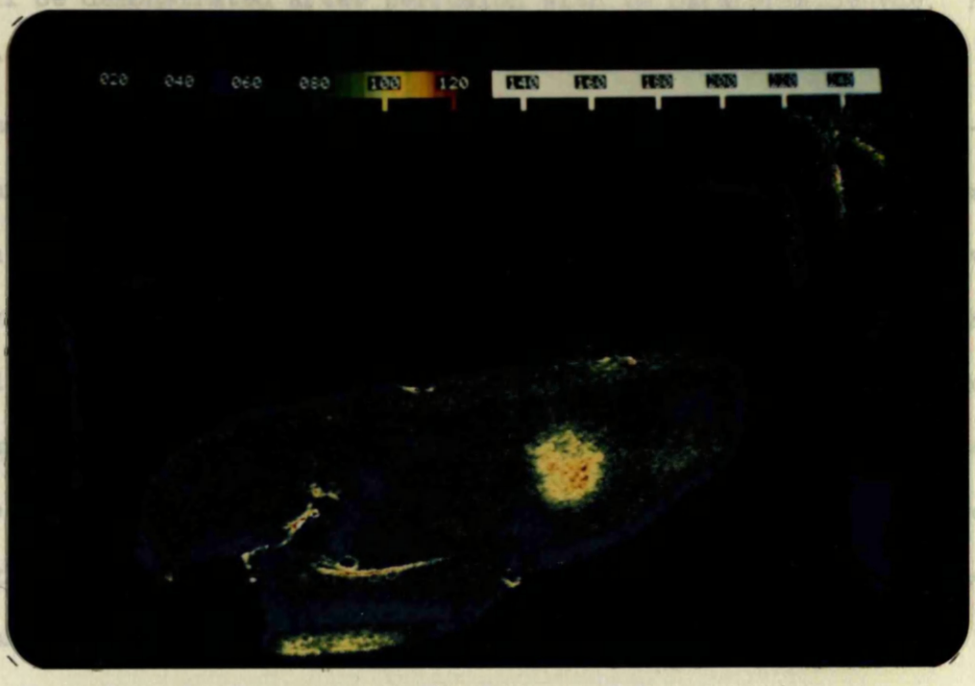


Fig.69 Television image of the autoradiograph formed by a tangential section cut at the top of layer V from the same cerebral hemisphere as in the previous two Figs. K and J mark artefacts caused by a bubble and a fold in the section respectively.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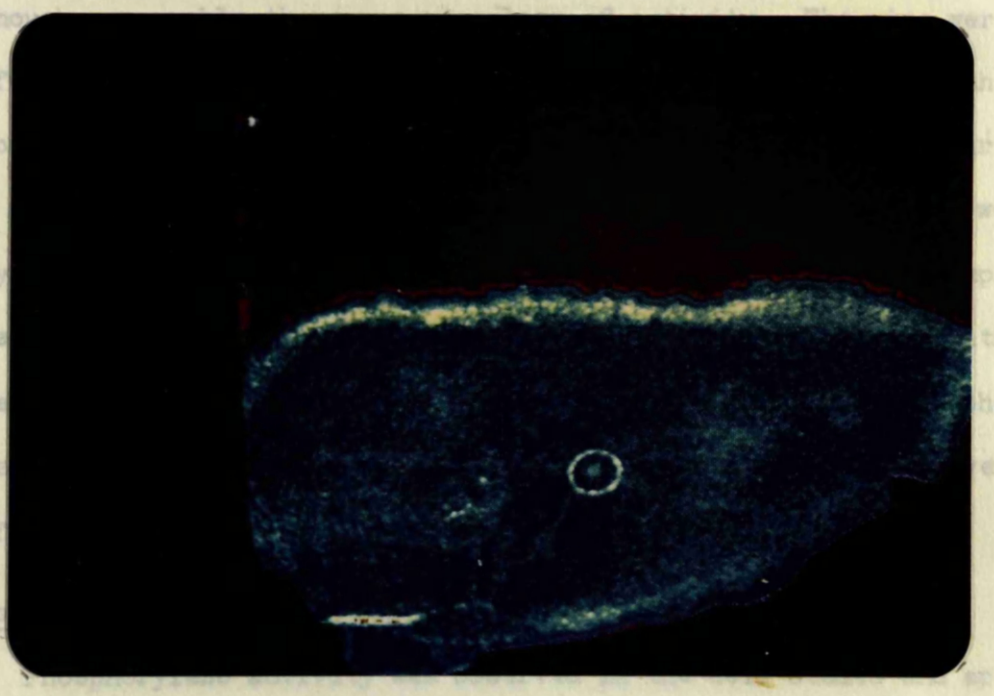


alcohol or acetone cause some loss of enzyme activity.[226] Lindberg[138] has shown that high levels of phosphorylase activity in the liver can still be demonstrated after perfusion with 1% paraformaldehyde. However use of this fixative alone is not optimal, largely because formaldehyde does not react with carbohydrates. With immunohistochemical studies many antigenic sites are denatured by strong fixatives such as glutaraldehyde and a new fixative has been developed[149] which provides good fixation of both proteins and carbohydrate but which is mild enough to preserve enzyme activity. With this fixative hydroxyl groups of sugar residues are oxidised to aldehyde groups by the periodate ion which acts as a weak oxidising agent. These aldehyde groups on glycoproteins and carbohydrates are then cross linked by lysine. (Each molecule of lysine has two reactive sites.)

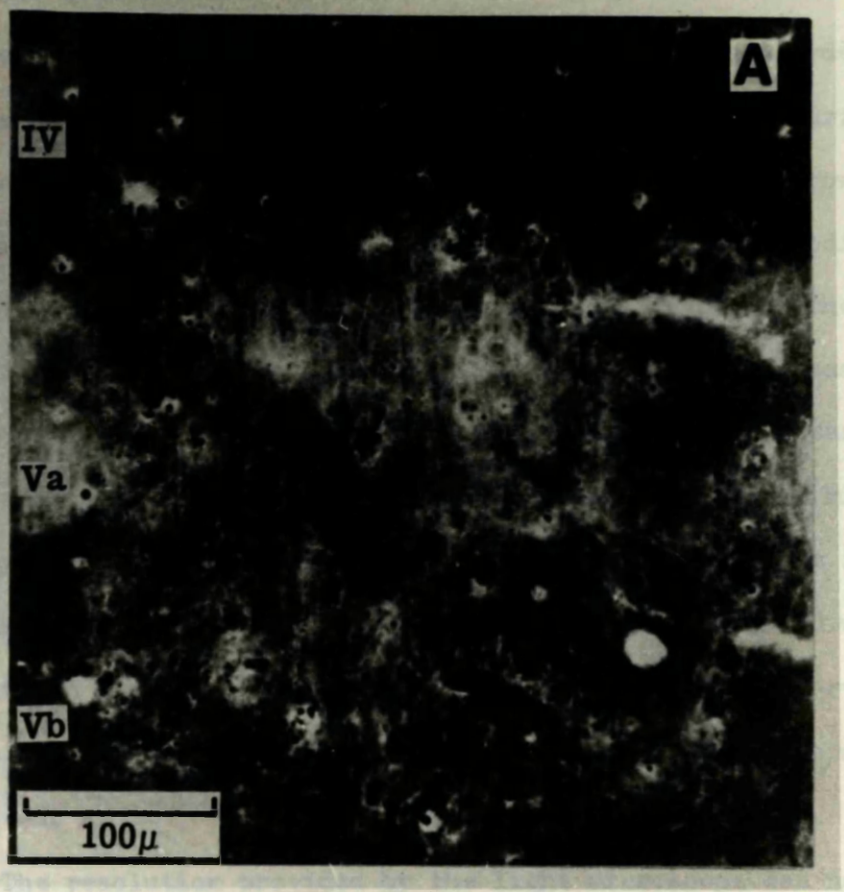
Therefore 6 mice were perfused with different concentrations of the different reagents ranging from 0.005 M sodium periodate and 1% paraformaldehyde to 0.01 M sodium periodate and 2% paraformaldehyde. None of the mixtures caused any marked inactivation of the enzyme although presumably there was some loss of activity. The mice were perfused transcardially after being transiently anaesthetised with ethyl chloride or ether vapour from a pad of cotton wool placed on their nose and mouth. As expected, the pattern of phosphorylase a staining was very similar to that found in sections from unfixed brains. (Compare Figs. 18 and 20). In sections from the fixed brain the barrels within layer IV of the somatosensory cortex had comparatively high phosphorylase a levels but this increased activity did not spread into layer V or for more than a short distance into layer III.

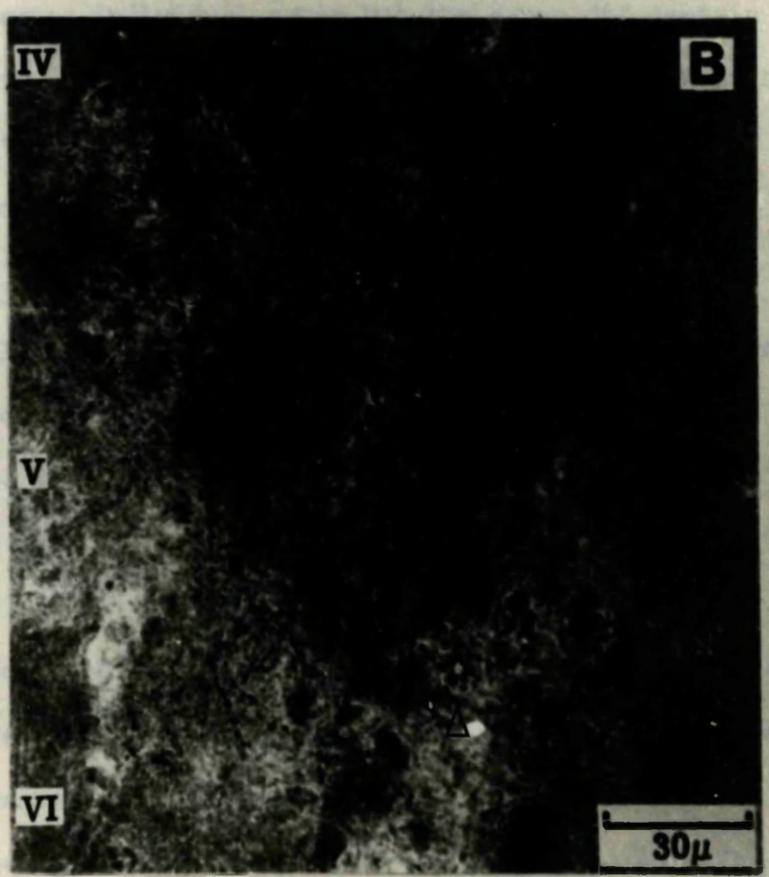
Cytological location

Phosphorylase activity was observed in the cell bodies and apical dendrites of pyramidal cells in the rat sensori-motor cortex even in sections from unfixed brains. (Fig. 70). However the apical dendrites

Fig.70 Coronal section through sensori-motor cortex of a rat that had been anaesthetised with urethane and then decapitated. Moderately high levels of phosphorylase a activity are seen in the layer V pyramidal cell bodies and their apical dendrites which are ascending to layer I.

Fig.71 Coronal section through sensori-motor cortex of a mouse which had been anaesthetised with sodium pentobarbitone and then perfused transcardially with a solution containing 1% paraformaldehyde. Again, cell bodies and dendritic processes of neurones in layer V have a moderately high level of phosphorylase a activity.


A
IV
Va
Vb
100μ


IV
B
V
VI
30μ

of cells were not seen in sections from the brains of mice unless they had been fixed with the mixture containing paraformaldehyde and periodate-lysine described above. In fixed sections both long dendrites (marked by small arrowheads) and cell bodies (marked by large arrowheads) were observed. (Fig.71) In both fixed and unfixed sections the cell bodies had a grainy appearance caused by large granules of glycogen, whereas the dendrites were much more uniformly stained. In the hippocampus pyramidal cells with their branched apical dendrites were clearly seen in the various sub-areas of the ammon's horn and subiculum (Fig.72). All the pyramidal cell bodies were strongly stained. However in the thalamic nuclei of mice brains, cell bodies were only observed with any clarity in sections from fixed brains (Fig.73). Therefore in the mouse brains the localisation of the reaction product was markedly improved.

The resolution provided by the light microscope was not adequate for viewing individual axons but the axons did contain phosphorylase activity, as the white matter, particularly in baboon brains, contained relatively high levels of phosphorylase activity, only some of which was located in glial cells.

Enzyme mobility in unfixed sections

Although individual cells could be observed in sections from unfixed rat brains the enzyme molecules did seem to be quite mobile and able to diffuse a considerable distance within the section. Evidence of its mobility was observed in surface parallel sections incubated for either phosphorylase a or total phosphorylase activity. In sections from some rats artefactual patterns of high phosphorylase a activity occurred. These often had very distinct outlines and a pale spotty appearance (Figs.74 and 75). The pale spots indicated where the enzyme had been leached away from its original location. The patterns caused by these incubation artefacts were entirely unrelated to the anatomical

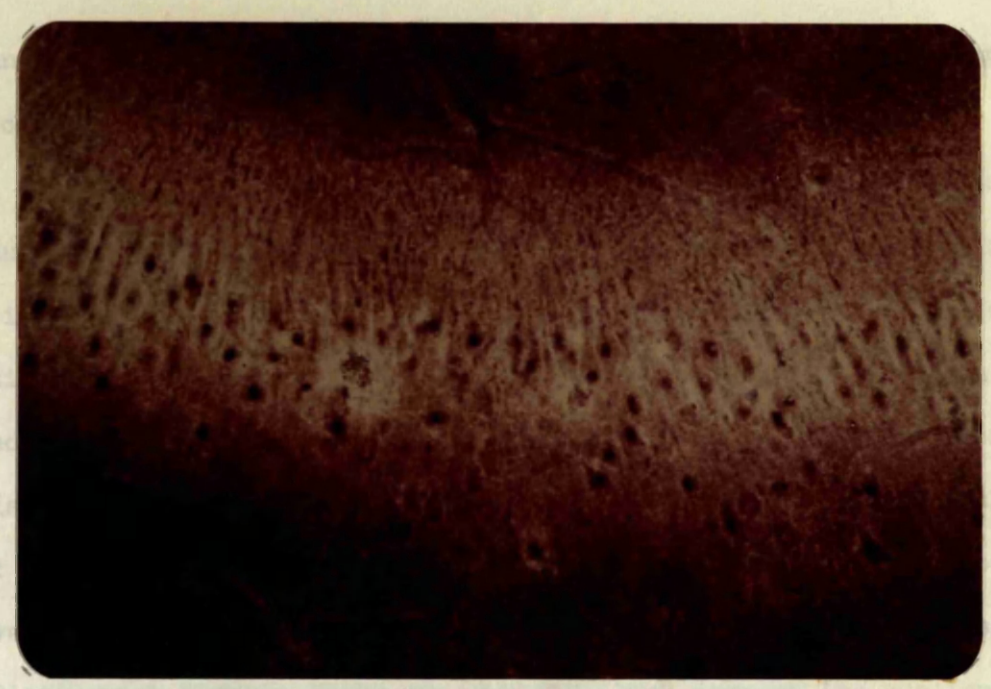


Fig.72 Horizontal section through hippocampal CA3 field showing that phosphorylase a activity is observed in all cells, both in the somae and in the apical dendrites. (Unanaesthetised rat)



Fig.73 Coronal section through the lateral thalamic nucleus showing moderately high levels of phosphorylase a in the cell bodies. (Lightly anaesthetised mouse perfused with paraformaldehyde-periodate-lysine mixture)

organisation of the brain and often one pattern would cross many architectonic boundaries.

It was not realised immediately that the patterns were artefacts because a series of sections often had very similar patterns and in a few instances it appeared to be possible to follow a pattern from one section through to the next. The patterns were usually aligned in the direction of a major axis. This could have been due either to a preferential organisation of certain components within the brain or to the fact that the sections were incubated while in a vertical direction and so were subjected to a linear force parallel to the plane of the section. To distinguish between these two possibilities some of the sections were rotated through 90° in the vertical plane. It was found that the patterns of high activity retained their major axis in the direction of the gravitational field and so were rotated through 90° with respect to the long axis of the brain. This proved that the patterns were caused by an artefactual process. As the patterns were also observed in sections from rat brain incubated for total phosphorylase activity, it seems likely that they are due to diffusion of the enzyme within the section during the incubation period.

These patterns were virtually never seen in sections from mice brains and were not observed in sections from rats which had been perfused with the mixture of paraformaldehyde-periodate-lysine. Therefore in the second series of rats studied in relation to the induction of an epileptiform focus all the brains were perfused with fixative prior to freezing.

Use of undamaged tissue

An alternative approach to improving the localisation of the reaction product is to use tissue that has not been damaged by the processes of freezing and sectioning. However Ibrahim[112] showed that, although incubating blocks of tissue improved the localisation, the



Figs.74 and 75 Surface parallel sections through unfixed rat neocortex showing artefactual staining patterns caused by the enzyme mobility.



incubation medium had a very limited permeability and phosphorylase activity could only be demonstrated at the surface of the block.

To obtain an estimate of how far the incubating medium could effectively penetrate, very thick (300 μm) sections were cut through the rat hippocampal formation using a vibratome. The sections were mounted on coverslips and incubated as usual for 10 min. The levels of phosphorylase a activity demonstrated were only slightly higher than those in 24 μm sections. When the sections were viewed through the coverslip glass no evidence of any phosphorylase activity was observed in the part of the section within about 100 μm of the coverslip glass. Therefore the effective penetration of the incubating medium does not seem to be much greater than 24 μm.

In an attempt to obtain better penetration of the incubation medium into undamaged brain tissue, two rats were perfused transcardially for an hour with Meijer's[152] incubation medium. The medium was heated to 39°C before entering the heart by using a water jacket designed for the Langendorf rabbit heart preparation. The rats were kept in a bath of heated saline throughout the period of perfusion. However, despite a good perfusion initially with complete clear-out of the blood, no phosphorylase a activity could be demonstrated in the brain after sections had been cut and stained for iodine. Therefore this procedure was not continued.

Change in Phosphorylase a Levels with Time Interval before Freezing

The alterations in phosphorylase a levels in the rodent brain caused by varying the time between death and freezing have been examined quantitatively in a number of biochemical studies.[26,68] Therefore in the present work only a few qualitative experiments were carried out in order to confirm that the phosphorylase a levels demonstrated histochemically showed the same changes as have been described biochemically. Two urethane anaesthetised rats were used along with 3 mice, 2 of which

were unanaesthetised and the third was anaesthetised with pentobarbitone.

To achieve as rapid removal of the brain as possible one rat was anaesthetised with urethane and the skin was cut away from the top of the skull. Then the head was sliced in half using a sharp steel blade as a guillotine. The two hemispheres were scooped out of the skull with a spatula and dropped into liquid nitrogen; the first in 10 sec. and the second in 15 sec. In sections from both hemispheres that had been incubated in the normal way there were very high levels of phosphorylase _a_ in all parts of the cortex. An example of this is shown in Fig.76 which is a coronal section through the sensori-motor cortex. The levels of phosphorylase _a_ are nearly as high as in sections incubated for total phosphorylase activity.

Similar results were obtained with two unanaesthetised mice which were killed by a blow on the spine. The brain was partially exposed by cutting away some of the skin and then cutting into the back of the skull with sharp pointed scissors. The head and shoulders of the first mouse were immersed into liquid N_2 about 15 sec. after being killed and the head of the second mouse was immersed in isopentane at -130°C 10 sec. after its death. Again, unusually high levels of phosphorylase _a_ activity were observed in all parts of the brain that were sectioned.

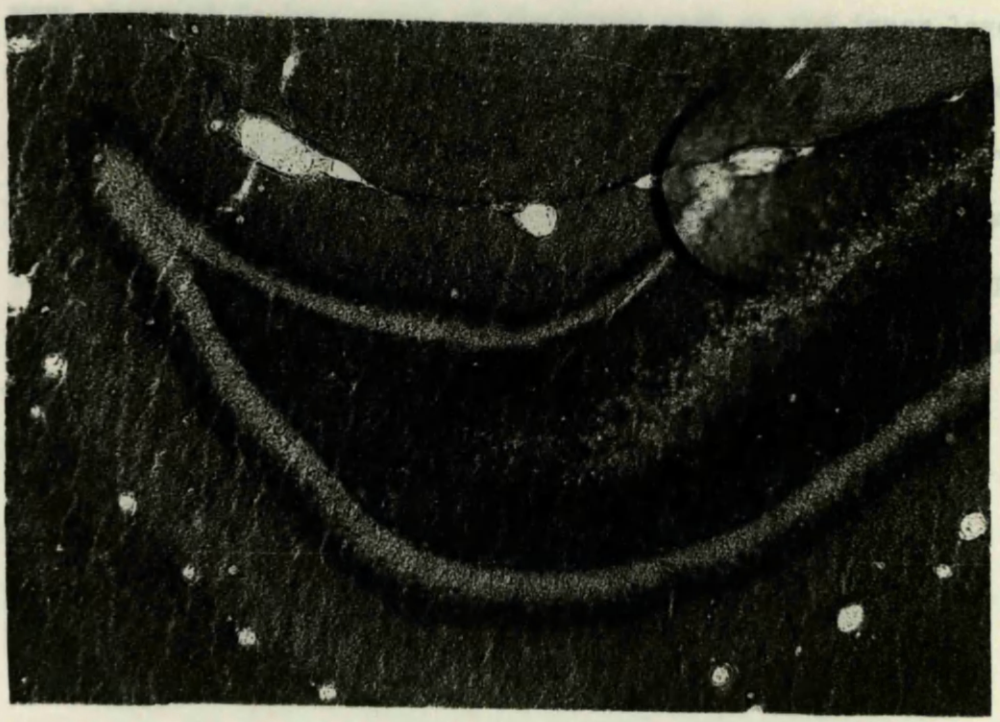
The brain of small rodents can be frozen with little or no change in the metabolites if liquid N_2 is poured directly onto the exposed skull of anaesthetised animals.[176] This procedure was used with one rat and one mouse. Sections from the rat brain were dropped directly into cold absolute alcohol, while those from the mouse were dropped directly while still frozen into acetone that had been cooled to below 0°C by pouring in liquid N_2. In all the sections of the rat and mouse brains studied there were low levels of phosphorylase _a_ in most areas of the brain. This was partly due to inactivation of the enzyme by the alcohol or acetone.[226] However the inactivation due to the fixative was only part

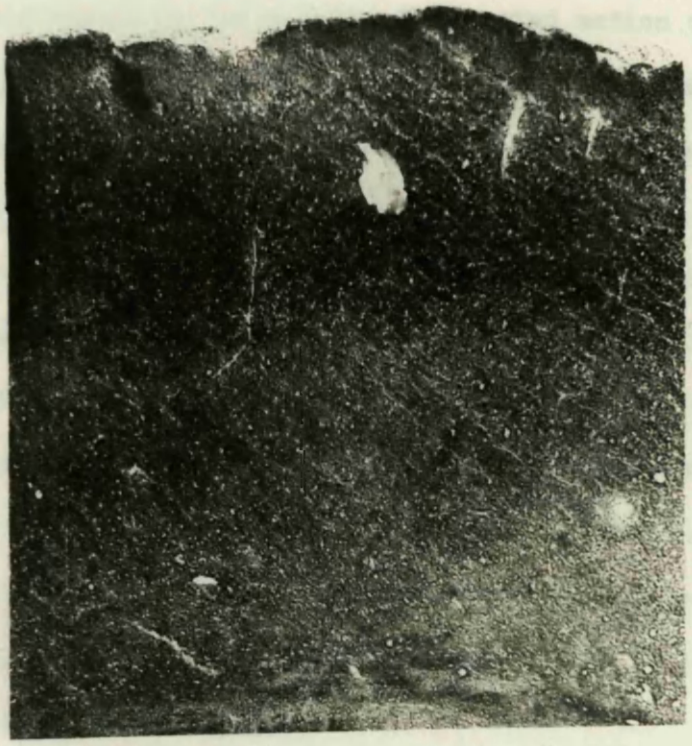


Fig.76 Coronal section through the sensori-motor cortex of a brain that had been frozen 15 sec. after decapitation. (Urethane anaesthetised rat)

Fig.77 Inverted coronal section through the dentate fascia of a brain that had been frozen by pouring liquid N_2 directly onto the exposed skull. (Pentobarbitone anaesthetised mouse) X 80

Fig.78 Coronal section through the sensori-motor cortex of the same brain as described in the previous Fig. X 52





of the reason for the low activity because high phosphorylase a levels were observed in some parts of the brain such as the inner layer of the stratum moleculare of the dentate fascia (Fig. 77). In the neocortex there were low levels of phosphorylase a but there was still evidence of stratification in the cortex. For example in the sensori-motor cortex shown in Fig. 78 there were higher levels in the outer part of layer I, layer IV and the base of layer III and in layer Vb than there were in the intervening layers.

DISCUSSION

a. Biochemical background

The brain functions through the integrated action of its billions of neurones communicating with each other via the release of neurotransmitters and other chemical messengers. Neurotransmitters have two main classes of effect.[154] The first is where the chemical messenger produces a very rapid and transient conformational change at a receptor linked to the properties of a membrane ionic channel. The classic example of this is the action of acetyl choline on the nicotinic receptor. The other class of effect is of longer duration than the first and operates through regulating certain aspects of the cell's metabolism. For example, the β adrenergic receptor is thought to be linked to an adenylate cyclase, a GTPase and certain methylating enzymes which alter the microviscosity of the membrane.[154]

In general neurotransmitters or neuromodulators exerting a comparatively long-lasting effect act mainly through the second messengers Ca^{2+},

CAMP or CGMP and the protein kinases and phosphatases whose activity they control.[85] Protein kinases catalyse the addition of phosphate groups to serine and threonine residues while phosphatases catalyse their removal.[38] Following the discovery in 1968[241] of CAMP-dependent protein kinase, there has been a rapid acceleration in the number of proteins which have been found to undergo reversible phosphorylation. About 35 enzymes and a large number of other proteins[38] are known to be regulated in this manner.

Indeed protein phosphorylation is the major general mechanism by which intracellular events in mammalian tissues are controlled by external physiological stimuli.[38] Different cellular functions are controlled by common protein kinases and phosphatases. These form an integrated network of regulatory pathways mediated by phosphorylation-dephosphorylation that allows diverse cellular events to be coordinated by neurotransmitter and neurohormonal action.[85]

As a result energy metabolism and neuronal function are inextricably linked and it is not possible to draw a dividing line between them. Most neurotransmitters are synthesised by making relatively simple alterations to basic metabolites such as those in the tricarboxylic acid cycle. Thus the non-essential amino acids glutamate and aspartate are synthesised from glucose, and the presence of a single enzyme, glutamic acid decarboxylase (GAD), converts glutamate into the inhibitory transmitter γ amino butyric acid (GABA).[208] This direct linkage of neurotransmitter and energy metabolism through the use of common metabolic pathways is efficient because not only does it save on the duplication of metabolic machinery but because most of the energy produced by oxidative phosphorylation is used in signal transmission between neurones. Up to 40% of the ATP generated is used to support the active transport of Na^+ and K^+ ions alone.[254]

Enzyme phosphorylation was first discovered to have a role in

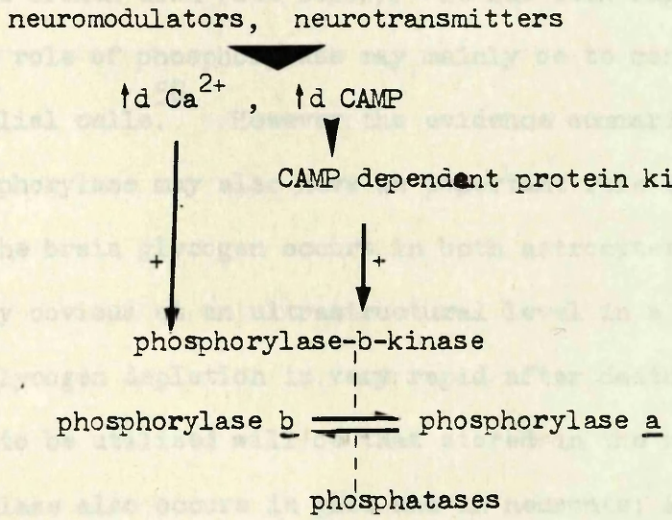
controlling a metabolic system during studies of glycogen breakdown in skeletal muscle.[131] Krebs found that the enzyme responsible for breaking down glycogen - glycogen phosphorylase - was activated by being phosphorylated by phosphorylase-b-kinase. Glycogen phosphorylase exists in two forms -

i. as the phosphorylated form phosphorylase a which is active in the absence of AMP

ii. as the nonphosphorylated form phosphorylase b which requires quite high levels of AMP before becoming active.

The relative amount of each is controlled by phosphorylase-b-kinase whose activity in turn is controlled by Ca^{2+} levels and CAMP dependent kinase.

Fig. 80



When muscle is stimulated electically, calcium ions released from the sarcoplasmic reticulum[219] activate phosphorylase-b-kinase. As a result the phosphorylation state of glycogen phosphorylase is increased to provide the ATP required to sustain muscle contraction. Thus in muscle the level of phosphorylase a accurately reflects the state of metabolic activity as it is of central importance in linking glycolysis to the energy requirements of the cell.[219]

The brain largely relies directly on glucose transported across the

blood-brain barrier for its energy supplies.[208] However as in muscle, glycogen is the single largest energy reserve[178] and phosphorylase may be important for the short-term control of glucose levels. Thus people suffering from defects in glycogen metabolism show mental retardation.[13] Increased neuronal activity requires increased ATP production[213] just as in muscle and in the brain this increased activity is also associated with increased protein phosphorylation.[92a, 155] Thus electrical stimulation is associated with alterations in the phosphorylation state of synaptic membrane proteins such as protein I[161] as well as metabolic enzymes such as pyruvate dehydrogenase[31] and glycogen phosphorylase.[155] There is evidence that the phosphorylation of phosphorylase is brought about in neurones in the same fashion as it is in muscle so that intrinsic glycogen can be broken down (see below). It has been suggested that the metabolic role of phosphorylase may mainly be to control glycogen breakdown in glial cells.[58] However the evidence summarised below indicates that phosphorylase may also have an important role in neuronal metabolism.

In the brain glycogen occurs in both astrocytes and in neurones.[216] It is only obvious at an ultrastructural level in a few neurones[173] because glycogen depletion is very rapid after death[145] and the first glycogen to be utilised will be that stored in the neurones themselves. Phosphorylase also occurs in glia and in neurones; in their apical dendrites, somae and in their axons.[112] In neurones, structures analogous to the sarcoplasmic reticulum, are thought to be stores for Ca^{2+} ions. Thus the smooth endoplasmic reticulum and mitochondria are significant Ca^{2+} buffers[21] as are the subsurface cisterns found in postganglionic neurones.[73] Addition of caffeine[73] to the preparation depletes the latter structures of their Ca^{2+} and is associated with a prolonged afterhyperpolarisation of the action potential suggesting that in neurones these stores may be used in a similar manner to muscle sarcoplasmic reticulum. Ozawa[164] showed that brain phosphorylase kinase is activated by Ca^{2+} and

other biochemical studies[207] have shown a predominantly neuronal localisation for the two major Ca^{2+} dependent protein phosphorylation systems in the brain.

Experiments using the rabbit vagus nerve[134a] provided evidence that Ca^{2+} influx associated with the action potential causes increased glycogenolysis due to increased activity of phosphorylase-b-kinase. Calcium entry was also found to increase the rate of mitochondrial oxidative phosphorylation. Similar results were obtained by different groups of workers using the hippocampal slice preparation. For a period of up to 30 min. after long term potentiation had been elicited there were increased Ca^{2+} levels.[14] It was suggested that this would activate phosphorylase-b-kinase as this enzyme had been found to phosphorylate a small synaptic protein[30] which turned out to be the alpha subunit of pyruvate dehydrogenase.[31] This enzyme plays a very important role in mitochondrial metabolism and influencing it could have an affect on Ca^{2+} sequestration and hence of synaptic potency. At least one other membrane protein is also altered by long term potentiation[15] and in another preparation Gordon[81] has provided evidence that the characteristics of the acetyl choline receptor are altered by phosphorylation mediated by a membrane-bound kinase. A large increase in the level of phosphorylase a occurs within a few hundred msec of the Ca^{2+} influx associated with the cardiac action potential.[227] Thus phosphorylase apparently participates in a rapidly acting system whereby electrical activity is transduced into a variety of metabolic changes some of which may be involved in altering synaptic efficacy.

As mentioned above phosphorylase activity is also controlled by CAMP levels.[38] Biochemical studies have shown that cytosol and membrane fractions from various regions of the brain contained only 2 specific CAMP receptor proteins.[242] These were the regulatory units of CAMP-dependent kinases. One of these kinases can phosphorylate at least ten proteins from the synaptosomal plasma membrane fraction alone.[185] Some

of these proteins have been identified[85] and one of the proteins that undergo phosphorylation may correspond to phosphorylase.[178] A number of neuromodulators and putative neurotransmitter systems mediated by non-specific fibres are known to have receptors linked to adenyl cyclase and so affect CAMP levels.[85] These include histamine (H_2), serotonin, octopamine, prostaglandin E_1, adrenalin (β), noradrenalin (β_2) and dopamine. There is also evidence that some if not all affect phosphorylase activity in the brain.[4,178] There is evidence that putative neurotransmitters of specific interneurones such as vasoactive intestinal peptide (VIP) also stimulate adenylate cyclase[22] and as a result may also activate phosphorylase. Thus phosphorylase levels may be linked to information processing activity in the brain as well as general regulatory systems through the CAMP and Ca^{2+} second messenger systems.

Protein phosphorylation is also involved in long term aspects of neuronal regulation, the effects of which may be measureable hours after they were induced.[148,85,30] There is evidence[38,148] to suggest that the protein phosphatases are involved in the long term aspects. Comparatively little work has been carried out on the function of the phosphatases[256] but there is a suggestion of their being involved in the long term regulation of phosphorylase activity[66] and at least one hormone affecting glycogen metabolism is thought to act through regulating phosphatase activity by a cellular messenger other than Ca^{2+} or CAMP.[38]

Consequently, phosphorylase levels may show rapid fluctuations with the time-scale of an inhibitory postsynaptic potential[227] superimposed on more long-term fluctuations.[67,66] The rapid fluctuations can only be shown by rapid in situ freezing of the tissue[227] and although, it should be possible to achieve this in small rodent brains by freezing across the skull,[176] this method prohibits any physical manipulation of the brain prior to freezing. As the primary object of this research was to look for evidence of modular organisation within the various architectonic

regions of the rodent cerebral cortex, it was felt more important that the cortex should be flattened to allow surface parallel sections to be cut than that in vivo levels of phosphorylase should be studied. It was expected that the postmortem levels of phosphorylase a would reflect its long-term fluctuations.

On decapitation the CAMP and Ca^{2+} levels in the brain both increase immediately and phosphorylase activity reaches a maximum less than 10 sec. after death.[58,26] Thereafter the ATP levels fall to low levels[146] and, as ATP is required for protein kinases to add phosphate groups to their substrates, after a few minutes the phosphatases alone will control the levels of protein phosphorylation. Thus the postmortem levels of phosphorylase a in the brain may reflect an aspect of the long term regulation of metabolism by the protein phosphorylation system, as mediated by the phosphatases, whereas in vivo levels of phosphorylase a will reflect the state of the short term regulatory system mediated mainly by Ca^{2+} and cyclic adenosine 3'5'-monophosphate dependent protein kinases.

Anaesthesia

The author originally had reservations about whether the variations in the phosphorylase activity observed histochemically did represent changes in physiological activity or were merely postmortem artefacts. Some of the patterns observed in surface parallel sections did turn out to be artefacts and so it was important to have criteria with which to judge if a specific staining pattern was an artefact or not. A pattern was only judged to be meaningful i) if it could be followed through at least three serial sections, ii) if a three dimensional representation of the pattern could be formed by observing related staining patterns in sections from at least two and preferably three different orientations, iii) if there was a known anatomical basis for the three dimensional structure obtained.

Using these criteria to define physiologically meaningful results

evidence was obtained that the postmortem levels of phosphorylase a were related to functional activity. Thus in coronal and surface parallel sections from the cortex of "unanaesthetised" mice and rats layer IV of the primary sensory areas, where the specific thalamic afferents terminate, had a characteristically high level of phosphorylase activity. This pattern of staining was not observed in sections from pentobarbitone anaesthetised mice and rats and so even short periods of anaesthesia (10 min.) have an affect on the postmortem levels of phosphorylase a. In sections from some of these brains the staining pattern as observed in surface parallel and coronal sections represents cylinders of high phosphorylase activity. These indicate that there are cylinders of tissue with a comparatively low level of active phosphatases. Thus after decapitation the active phosphorylase is inactivated more slowly in these cylinders of tissue than in the surrounding areas. By contrast in sections from urethane anaesthetised rats and mice, high levels of phosphorylase a are observed in all layers and areas of the cortex, implying that there is a low level of phosphatases in all parts of the cortex as compared to the levels in "unanaesthetised" brains.

Epileptiform activity

The results obtained in studying an epileptiform focus were at first sight rather inconsistent. The only consistent finding was that areas of cortex where cells had been damaged an hour or more prior to death showed distinct changes in their level of phosphorylase a activity. As some damage was always associated with the insertion of the glass microelectrode, incubating the sections for phosphorylase a activity provides a method for the accurate tracing of microelectrode tracks in relation to the known architectonic organisation. This finding is consistent with earlier reports of altered phosphorylase activity being associated with damaged cortical tissue containing small shrunken cells.

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Folbergrova has made biochemical measurements of phosphorylase a

levels associated with the induction of convulsive activity by a number of irritant chemicals other than penicillin.[66,67] In general her results showed that there was a transient increase in phosphorylase a levels associated with the induction of an epileptogenic focus followed by a decrease in phosphorylase a levels to background levels or below. Phosphorylase phosphatase levels were not measured directly but it was suggested[66] that increased phosphatase activity may have been one of the reasons for the secondary fall in phosphorylase a levels. Consequently it was expected that the postmortem levels of phosphorylase a would be altered by a penicillin induced epileptiform focus and that this could be demonstrated histochemically.

Nevertheless no consistent change in postmortem phosphorylase a activity was demonstrated histochemically. In two out of the five rats decapitated after 5 - 10 min., areas of purple staining were observed in a position corresponding to the location of the centre of the epileptiform focus. The areas were distinctive because of the purple colour which was in contrast to the brown surrounding areas and also because of the homogeneity of the staining compared to the surrounding cortex. The actual level of phosphorylase a within the area was not markedly different from the surrounding cortex. The change in colour of the iodine-stained polysaccharide occurring in the section implies that there had been an alteration in the relative activity of branching enzyme[224] and was probably unrelated to any change in phosphorylase a. In only one brain out of the 13 studied was there a marked change in the levels of phosphorylase a that could not be explained in terms of damage caused by the insertion of a micropipette. In sections from the brain of a rat where epileptiform activity had been induced 4 hrs before decapitation, by application of a penicillin soaked pledget, an area of dark purple staining was observed in layers I and II. However this may have been caused by mechanical damage to the layers immediately beneath

the pia occurring when the dura was being removed prior to the application of the penicillin. Electrophysiological studies[100] have indicated that the primary site of action of topically applied penicillin is probably layer IV and so if the area of altered phosphorylase activity was caused by the epileptiform focus it should have also been observed in layers III and IV in an analogous way to the region of altered metabolic activity observed in the ^{14}C2DG experiment.

The pyramidal cells associated with an epileptiform focus exhibit hyperactivity which can lead to the production of a secondary epileptogenic focus in areas receiving a strong excitatory input from pyramidal cells in the primary focus.[133] Thus in the ^{14}C2DG experiment there was evidence of increased glucose uptake in areas of the neocortex which would be expected to have an important afferent input from neurones in the forepaw area primary focus. This confirmed previous reports[40] of increased deoxyglucose uptake being associated with the spread of epileptic hyperexcitability from a primary penicillin induced focus. However no evidence of altered postmortem phosphorylase _a_ levels was found in the areas where secondary foci would have been produced in the rat brains. Therefore it was concluded that the postmortem levels of phosphorylase _a_ were not appreciably altered by the induction of a primary or secondary epileptiform focus associated with the application of penicillin. As a result the postmortem levels of phosphorylase _a_ cannot be used as a histochemical marker to trace anatomical pathways associated with the spread of epileptic hyperexcitability.

Postmortem phosphorylase _a_ activity is determined by the level of active phosphatases and at present it is not possible to predict which in vivo alterations of brain activity will be associated with a change in postmortem phosphatase activity. From a consideration of the biochemical background it would be expected that phosphorylase _a_ would be a marker of alterations in neural activity and would return to background levels when-

ever a new steady state had been reached. Thus steady-state electrical stimulation of the rabbit optic nerve at frequency of 12 min^{-1} for 20 min was not associated with any alteration in phosphorylase a activity measured biochemically[27] whereas the initial induction of an epileptiform focus was associated with altered activity. However it appears that although the protein kinases participate in a regulatory system mediated largely by Ca^{2+} and CAMP that responds to dynamic changes in neural activity, the phosphatases participate in a longer-term regulatory system that is not properly understood.[256] Studies on oocyte maturation indicate that phosphatase activity can have a very important role in cellular function.[109] Preliminary experiments on stimulation of the mouse visual cortex described in Section I may indicate that the phosphatases are linked to some types of physiological stimuli and this line of investigation should be worth further study.

Long-term changes in phosphorylase a levels

Although the postmortem levels of active phosphorylase were not altered by epileptiform activity studying the phosphorylase a levels histochemically is still a useful technique to use in conjunction with electrophysiological recording. Thus in experiments lasting for more than an hour the micropipette produced sufficient tissue damage for the electrode track to be observed histochemically, while the position of the tip could be located by a dye such as pontamine sky blue. When surface parallel sections through layer IV were cut and some incubated for total phosphorylase and some for phosphorylase a activity, then a detailed outline of the primary sensory areas was obtained, down to the level of individual barrels. Therefore the electrode tip could be accurately located in relation to the known anatomical organisation. Phosphorylase a histochemistry should also be of use in studying chronic epileptiform foci which are associated with cell death and gliosis as the postmortem levels of phosphorylase a would be relatively high. Previous studies by

Ibrahim[112] have shown that phosphorylase a levels are markedly altered by various forms of chronic hypoxia. Experiments involving the biochemical assay of total phosphorylase in micro-samples obtained from tangential sections of the mouse barrel cortex[47] showed that total phosphorylase levels in the barrel hollows were altered several days after vibrissal removal even in mature animals and this presumably would be true of phosphorylase a levels as well.

Reaction product localisation

Biochemical studies[202] have shown that phosphorylase normally exists in cells as part of an insoluble complex where it is bound to glycogen. However in the presence of either abnormally high or abnormally low concentrations of glycogen the phosphorylase dissociates and becomes water soluble In sections of brain placed in the incubation medium the glycogen levels will initially be almost negligible and by the end of the incubation they will be very high. Therefore for at least part of the time the phosphorylase will be in a soluble form. This was found to be the case and in sections from the rat brain especially, the phosphorylase was able to diffuse for considerable distances within the section. Some diffusion of phosphorylase or the reaction product occurred in sections from mouse brain as well and a level of localisation necessary to observe cell bodies and their processes was only achieved in sections from brains that had been rapidly perfused with a solution of paraformaldehyde and periodate-lysine. Rapid perfusion with the fixative will help to preserve intrinsic glycogen and this will be held in its proper position along with the phosphorylase by the periodate-lysine mixture which is a carbohydrate fixative.[149]

Perfusion with this mixture for 5 - 10 min. should provide adequate fixation for examination under the electron microscope and so it should be possible in future to study brain phosphorylase activity in relation to neuronal ultrastructure. Ultrastructural studies[226] have already been

carried out on skeletal muscle where it was found that tissue preservation was reasonable even after the tissue had been in the incubation medium for 30 min. Studies at the ultrastructural level will provide an answer to the question of whether phosphorylase occurs in synaptic terminals or not. At present the high levels of phosphorylase observed in parts of the brain with few cell bodies such as the hollows of the olfactory glomeruli and whisker barrels or the layer of apical dendrites in the hippocampus as well as the presence of phosphorylase in axons suggests that there may be a high concentration of phosphorylase in both pre- and postsynaptic elements.

Measurement of in vivo levels of phosphorylase a

The few experiments carried out to study the speed with which phosphorylase a levels altered after decapitation were consistent with the biochemical observations that there is a large increase in phosphorylase a levels within 10 sec. of death.[26,68] Therefore the only way to measure in vivo levels of phosphorylase a is to freeze the brain in situ. This can be done either by pouring liquid N_2 onto the bone,[176] or for even more rapid freezing, isopentane at -150°C can be poured directly onto the dura.[68] Both these methods allow the in vivo levels of metabolites such as ATP or regulatory enzymes such as phosphorylase a to be studied biochemically. It should also be possible to measure in vivo levels of phosphorylase a histochemically provided the sections are not allowed to thaw out before the kinases and phosphatases have been inactivated by acetone dehydration. Preliminary experiments suggest that in vivo phosphorylase levels can be measured histochemically and it should be possible by using this method to study the formation of both primary and secondary epileptiform foci induced in the rat cortex.

It may also be possible to study in vivo phosphorylase levels at an ultrastructural level provided thin slices of tissue are used in conjunction with a cryoprotectant such as dextran. Thus in theory it

should be possible to study the changes in phosphorylase a associated with long term potentiation of hippocampal synapses[31] by using the hippocampal slice preparation. This preparation would allow the high frequency stimulation of afferent pathways necessary to produce long term potentiation and this could be followed by virtually instantaneous freezing in a quenchant such as liquid propane. Subsequently the slice could be dehydrated in acetone at -80°C before being brought to room temperature for the introduction of metabolic inhibitors and immersion in the incubation medium for phosphorylase a.

GENERAL DISCUSSION

The group-selective theory of higher brain function

In the history of biology whenever a phenomenon involving some sort of learning process has been studied it appears that an instructive theory was first proposed to account for the underlying mechanisms.[114] In each case this has later been replaced by a selective theory. The best example of this is Lamarck's theory of individuals adapting to their environment, being replaced by Darwin's theory of natural selection. This theory is the unifying theory of biology and along with the discovery of the chemical structure of DNA has provided a basis for studying biological phenomena at every organisational level. Thus for populations of bacteria the belief that their resistance to antibacterial agents was acquired by adaptation has been replaced by a model involving the selection of naturally occurring mutants. Similarly in individual vertebrates the formation of antibodies was originally thought to be due to antigen molecules forming a template that "instructed" immunoglobulin molecules to fold into a complementary conformation. However this hypothesis has been replaced by a selective mechanism whereby antigens are "recognised" by a small proportion of the large pool of antibodies already existing in the mature vertebrate.[114,57]

The first suggestion that the learning processes of the brain might also involve a selectionistic mechanism was made by Jerne in 1967.[114] Since then this idea has been developed and has recently been explicitly formulated by Edelman.[57] Already this theory is beginning to supplant the earlier instructive theories of learning such as that proposed by Brodal.[29]

An essential aspect of the theory is that the parts of the brain involved in information processing, should be composed of vast numbers of replicated neuronal groups which would form a primary repertoire analogous to the nucleotide codons of the genetic code. Mountcastle[158]

has proposed that in the cerebral cortex these neuronal groups are the minicolumns and macrocolumns which have been described by a number of workers.[158,107] The minicolumns each contain a few hundred cells and are thought[158] to be formed by the migration of neurones from the germinal epithelium of the neural tube along the radial glial cells to form a vertically oriented cord stretching throughout the depth of the cortex. The smallest number of neurones that this cylinder or minicolumn could contain is 110. This is the number of neurones counted in vertical cylinders with a diameter of 30 μm by Powell and his colleagues.[192] They found that this figure is almost invariant between different neocortical areas and different species of animals except for the striate cortex of primates where it is 260. Apart from this exception, a given area of cortex in any mammalian species will contain the same number of neurones. In the human, where there is also evidence of a columnar organisation,[99] Mountcastle[158] estimated that there would be about 600 million minicolumns and about 600,000 macrocolumns each composed of hundreds of minicolumns. The number of macrocolumns is comparable to the 10^6 variations possible in the gamma globulin macromolecule that subserves immune phenomena.[57] Therefore the large number of columns in the cortex is consistent with the hypothesis that the substrates of higher brain function, like immunological processes, interact selectionistically. If as Lorente de No suggested[141] it is possible to consider the mouse cortex as a model for the human cortex then Edelman's ideas will also apply to the mouse brain.

Number of columns in the mouse neocortex.

The total area[34] of the mouse neocortex from both hemispheres is about 70 mm^2. According to Szentagothai[223] the dominant feature of cortical organisation is its division into corticocortical columns each with a diameter of 250 μm. An anatomical study has confirmed the existence and size of these columns in the mouse neocortex.[252] Assuming

that these columns are packed closely together in a hexagonal lattice as suggested by Braitenberg[24] and that each has an area of 0.05 mm then the total number of corticocortical columns that could exist in the mouse neocortex is 1400. Edelman[57] has stated that the group-selective theory would not be tenable unless there are more than a few thousand neuronal groups apart from those forming the primary sensory repertoire. Therefore it is important to try and obtain an exact figure for the number of macrocolumns and minicolumns that actually exist in the mouse neocortex.

In the results described in Section I the number of cylinders of high phosphorylase a activity in the somatosensory and auditory areas was 100 and 40 respectively. It was suggested that these represented corticocortical columns. If this is true then the areas of low activity in between would also represent corticocortical columns because there has been no evidence to suggest that there is not an equal density of corticocortical afferents in all parts of the cortex. Similarly in the three visual areas where there were about 45 cylinders of high phosphorylase a activity, this would only represent half the number of columns produced by the afferent input, which in this case may have been specific thalamic. In the primary somatosensory cortex the specific thalamic input gives rise to 220 barrels each of which is thought to be associated with a thalamic column.[266] This figure is about twice the number of cylinders of high and low phosphorylase activity because many of the barrels only have a diameter of about 100 μm. As the barrel cortex is a highly specialised area the thalamic columns in this area are probably not representative and it is likely that in most cortical areas the diameter of columns is nearer 250 μm. Therefore it is estimated that the total number of corticocortical columns in the three sensory areas on one side of the neocortex is 90 visual plus 200 somatosensory plus 80 auditory to give a total of 370. As the sensory areas only make up

about half the neocortical surface the total number of corticocortical columns on both sides of the neocortex is estimated to be 1480. If, as suggested in SECTION I, the thalamic afferents form a distinct but overlapping system of columns then the number of columns in the mouse neocortex could be nearly 3000.

Compared with the human neocortex this is a very small number and for the group-selective theory to be correct the basic unit of selection must be smaller than a column with a diameter of 250 μm. According to Mountcastle[158] the smallest selection unit would be a narrow vertical cylinder containing about 120 cells. As each column with a diameter of 250 μm contains about 10,000 cells there could be 80 minicolumns within any one column. In the LITERATURE REVIEW it was stated that based on the cell counts of Pasternak and Woolsey[166] it would only be possible to form the neurones in layer IV into 20 identical subgroups. However if, as suggested in the present study, there are two sets of overlapping columns in the mouse barrel cortex, then the type Y and D cells might be primarily concerned with integrating corticocortical input. If this is true then there may be at least two different types of minicolumns in the barrel cortex each of which contains roughly 120 cells.

Theoretical grounds for assuming that the basic unit of the mouse neocortex is indeed as small as 120 cells is provided by comparing the number of retinal ganglion cells and number of neurones in area 17 as shown below.

Table 3

	Mouse	Rat	Cat
number of retinal ganglion cells[200]	6×10^4	11×10^4	2×10^5
surface area of primary visual cortex (mm^2)[34,132,229]	2	6	380
approximate number of cortical neurones[192]	2.5×10^5	7.5×10^5	475×10^5
cortical magnification	4	7	247

Thus in the mouse visual system there are only four times as many cortical neurones in area 17 as there are retinal ganglion cells and even with a cortical unit as small as 120 cells the input will be processed in terms of the input from a pool of 30 ganglion cells. In the cat a pool of 40 retinal ganglion cells will be processed by a column of cells with a diameter of 250 μm. It is unlikely that the basic unit of retinal ganglion cells even in the mouse retina is any bigger than 30 cells.

Number of modules in the hippocampal formation.

The most typical function of the forebrain appears to be in the perception of goals and goal priorities, as well as in the patterning of behavioural strategies serving the pursuit of these goals.[160] To do this the brain must be able to construct models of the external environment.[162] According to O'Keefe[163] all model building proceeds in two stages. In the first the brain must select and interpret sensory data to generate a set of "facts", and only in the second stage can the model actually be built out of these facts. The fundamental role of the hippocampal region in the selective registration of information[169,43] implies that it is largely involved in the first stage while the actual model building will occur in the neocortical areas. The capacity of the hippocampal formation for the selective processing of afferent information can be judged from a consideration of the number of cells present, the diversity of cell types and the size and number of the basic organisational units.

Swanson and Cowan[220] published a surface reconstruction of the rat hippocampal formation and gave measurements of the surface area of all the areas contained within it. A similar study has not been made of the mouse but West and Andersen[248] made an allometric study of the rat and mouse area dentata and concluded that the mouse area dentata was just a smaller version of the rat's. As they measured the area of this structure in the rat and mouse it is possible to estimate the surface area of the other structures in the mouse hippocampal formation. This

is summarised in Table 4. The surface reconstruction of the rat hippocampal formation is illustrated in Fig. 1 A in relation to the neocortex. This shows that the surface area of the hippocampal formation is nearly as great as that of the neocortex. The density of cells in the area dentata at least,[248] is nearly as great as in the neocortex (1.3×10^5 mm^{-2} as opposed to 1.55×10^5 mm^{-2}).

Although there is a greater morphological diversity of cell types in the neocortex than in the hippocampus, Lorente de No[143] described 12 types of neurones in the CA1 area and studies by Cajal[182] and more recently Amaral[3] suggest that there are a similar number of interneurones in the area dentata. Immunohistochemical studies[59] have indicated that the same types of putative neurotransmitter are present in the hippocampus as in the neocortex. Thus the hippocampus cannot be considered a "simple" cortex and in terms of putative neurotransmitters it may be just as complicated as the neocortex.

Table 4

Approximate surface area (mm^2) of the fields within one side of the hippocampal formation of the rat and mouse.[220,248]

	rat	mouse
hippocampal formation	60	17
area dentata	13.5	3.7
CA3 field	15	4.3
CA1 field	11	3.1
subiculum	5.5	1.6
presubiculum	4.5	1.3
postsubiculum	1.5	0.4
parasubiculum	1	0.3
entorhinal cortex	9	2.6

Using the estimates of the surface areas of the various fields and the estimates of the module size described in SECTION II it is possible to obtain an approximate idea of the number of modules in each area of the mouse hippocampal formation.[220] As the rat hippocampus is 7 mm long the mouse hippocampus will be about 2 mm long and an estimate of the number of lamellae can be made using this figure. This is summarised in Table 5 below.

Table 5

Approximate number of modules in each field of the mouse hippocampal formation

name of field	size of module	no. of modules
entorhinal cortex	250 μm diameter cylinder	50
postsubiculum	70 μm diameter cylinder	50
subiculum	140 μm diameter cylinder	100
CA1 field	160 μm diameter cylinder	155
dentate fascia	40 μm wide lamellae	50

The modular organisation observed in sections of the rodent hippocampal formation is summarised in Fig. 79.

There are two main efferent paths from the hippocampus.[56] One involves the output of CA3 cells the axons of which leave via the fornix to terminate in the septum. The other output path is via the subicular areas. Kornhuber[129] suggested that both of these pathways might be involved in memory formation but a more recent study has shown that extensive bilateral destruction of the fornix in man may occur without any gross impairment of memory.[264] This implies that the subicular pathway may have a more important role in memory formation. This suggestion has been backed up by a recent electrophysiological study of the hippocampal efferent systems in the rat.[63]

The subiculum is connected to the mamillary bodies[56] and to other

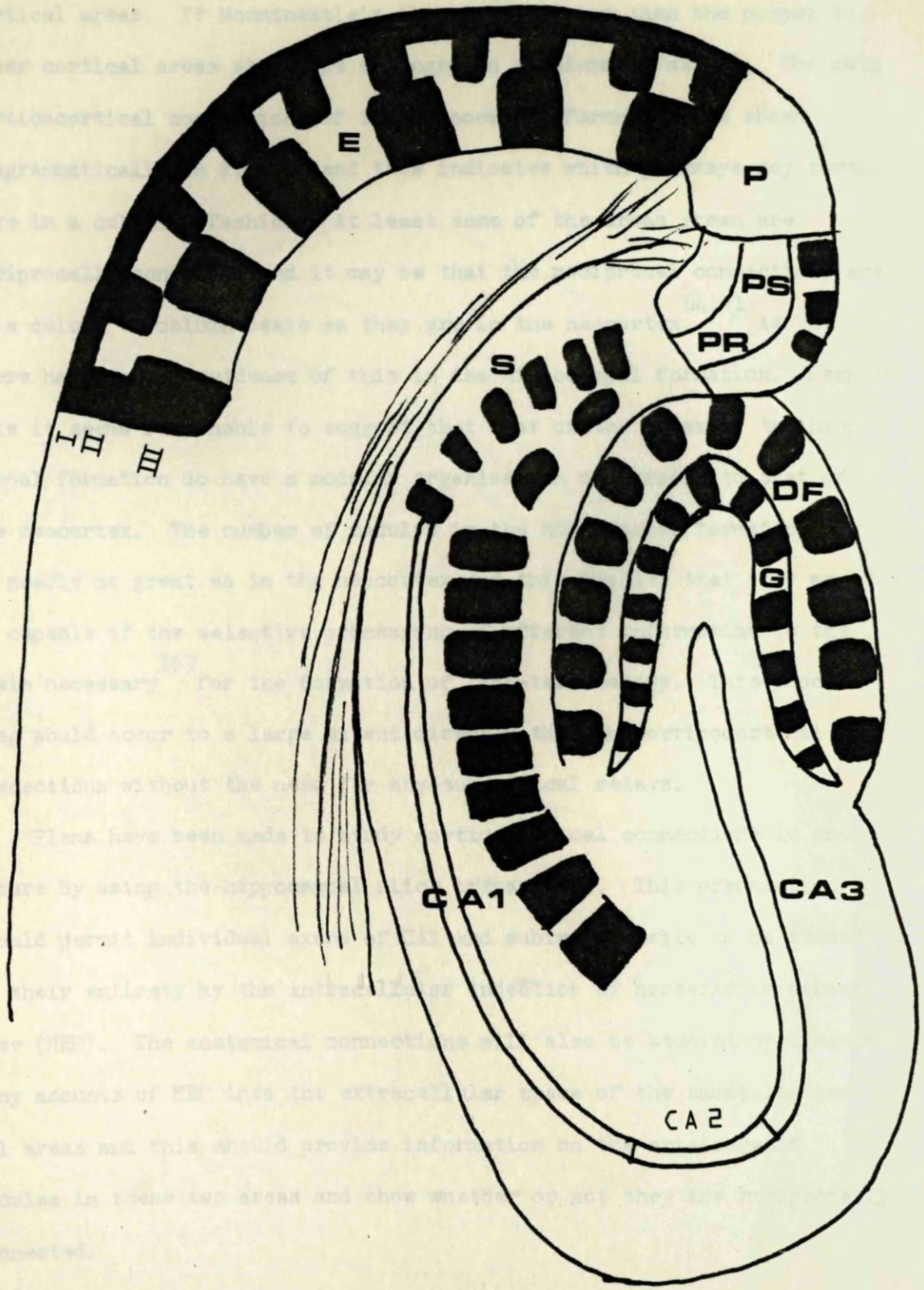

Fig.79 Summary diagram of modular organisation in rodent hippocampal formation as observed in sections incubated to demonstrate phosphorylase a activity.

cortical areas. If Mountcastle's theory is correct then the output to other cortical areas should be arranged in a columnar fashion. The main corticocortical connections of the hippocampal formation are shown diagrammatically in Fig. 81 and this indicates which pathways may terminate in a columnar fashion. At least some of the areas shown are reciprocally connected and it may be that the reciprocal connections are on a column to column basis as they are in the neocortex.[64,71] As yet there has been no evidence of this in the hippocampal formation. Despite this it seems reasonable to suggest that most of the areas of the hippocampal formation do have a modular organisation comparable to that of the neocortex. The number of modules in the hippocampal formation may be nearly as great as in the neocortex and this implies that they would be capable of the selective processing of afferent information to the brain necessary[169] for the formation of long-term memory. This processing would occur to a large extent directly through corticocortical connections without the need for any subcortical relays.

Plans have been made to study corticocortical connections in the future by using the hippocampal slice preparation. This preparation should permit individual axons of CA1 and subicular cells to be traced in their entirety by the intracellular injection of horseradish peroxidase (HRP). The anatomical connections will also be studied by ejecting tiny amounts of HRP into the extracellular space of the subicular and CA1 areas and this should provide information on the existence of modules in these two areas and show whether or not they are reciprocally connected.

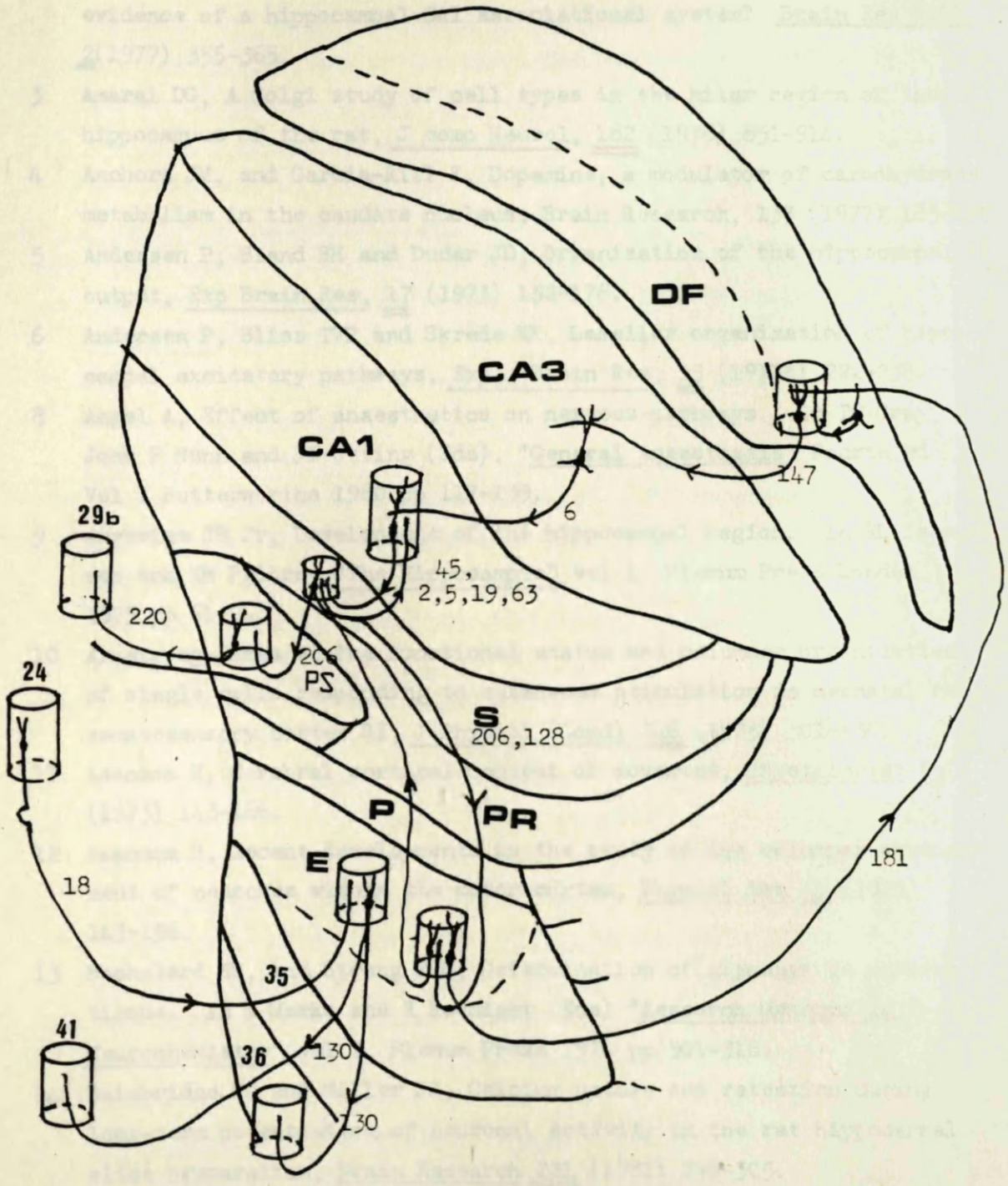

Fig.81 Summary diagram showing some of the corticocortical connections, associated with the hippocampal formation, which may terminate in a columnar fashion. The connections are drawn on a column to column basis, but although this arrangement has been shown in the neocortex, as yet there is no evidence of it in the hippocampal formation. The numbers beside the pathways cite the articles demonstrating their existence.

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Early histochemical changes in epileptogenesis.

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M.N. Wallace & O. Holmes.

A histochemical method is being developed for studying intracellular biochemical changes in the evolution of an epileptic focus; the method is also useful for elucidating the structural organisation of the cerebral cortex. The activity of glycogen phosphorylase, an enzyme, whose activation is apparently related to synaptic potentiation[1] is examined with Meijer's technique.[2] Penicillin is applied via a micropipette to layers IV or V of the somatosensory area of the cortex in the anaesthetised rat in order to produce epilepsy. Subsequently the cortex is dissected away from underlying structures, flattened, frozen and sectioned horizontally. Fifteen minutes after the application of penicillin the method records areas of high enzymic activity. Some of these areas correspo to the area of the primary sensory ratunculus within layer IV.[3] After an hour of epileptic activity horizontal laminae that are about 20μ thick can be seen. These often have sharply circumscribed boundaries but sometimes have ragged edge with horizontally running fingerlike projections suggestive of dendritic bundles

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