INVESTIGATIONS INTO THE POSSIBLE ROLE OF N-ACETYLSEROTONIN AS A NEUROTRANSMITTER

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ABSTRACT

A possible role for N-acetylserotonin (NAS) as a central nervous system neurotransmitter was investigated. Of the generally accepted criteria which are used to define transmitter function, those of localization and physiologic effect were pursued. Various immunohistochemical techniques were used to show the hippocampal distribution of NAS. This distribution was found to be distinctly different from that of melatonin and that reported for serotonin. NAS was located in sub-micron sized structures immediately adjacent to the hippocampal pyramidal cells in areas CA1 and CA3. There was also evidence for cells containing substantial quantities of NAS in the CA4/dentate region. Some fibres could also be identified here. The presence of NAS in the hippocampus of the rat was confirmed by gas chromatography-mass spectrometry (GCMS). Attempts to identify an extra-hippocampal source for the NAS found in hippocampus by use of lesions did not provide evidence that hippocampal NAS is dependent on an intact projection from brainstem via the fimbria-fornix pathway. A study using the 5HT synthesis blocker, p-chlorophenylalanine, showed that hippocampal NAS content can be reduced by this drug, as measured by GCMS. Immunohistology was further used to

identify a population of cells which might be expected to respond to NAS, those being the pyramidal cells of the hippocampus. In the hippocampal slice preparation, NAS and 5HT were equally potent in inhibiting glutamate-induced cellular activity, as measured extracellularily. Melatonin could not affect the firing rate of the pyramidal cells. It is concluded that this line of research may be useful in further defining the functional role of NAS in the CNS of rat.

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INTRODUCTION

It is generally accepted that many neurons of the brain and peripheral nervous system communicate with each other through the release of some transmitter substance from their terminals. A number of chemical substances, present in brain, have been shown to have excitatory or inhibitory effects on some neurons. The transmitters associated with most brain neurons have yet to be identified (Storm-Mathisen, 1977). There is considerable evidence that the low molecular weight monoamines, GABA, glycine, acetylcholine, serotonin, norepinephrine, dopamine, histamine and glutamine may act as neurotransmitters (NT's) at certain synapses in the brain. In recent years, many additional neurotransmitter/neuromodulator (NT/NM) candidates have been proposed, among them the subject of this present thesis, N-acetylserotonin (NAS).

Identifying a substance as being a neurotransmitter at a particular group of synapses depends on satisfying several criteria (Curtis, 1979), including: 1) application of the substance to the synapse should duplicate the actions of the natural transmitter. 2) the substance should be demonstrated to be present at the site of proposed action, in sufficient quantity for sustained activity. 3) the

mechanisms for synthesis and degradation of the substance should be demonstrable in appropriate locations. 4) the release of the substance from the suspected pre-synapse should be demonstrable in response to appropriate stimuli. 5) a specific, high affinity receptor should be present , and demonstrable, on the target cells. Of the criteria listed, the first is generally accepted as being the most significant (Storm-Mathisen, 1977) since it involves demonstration of an effect, where the others only indicate a potential.

It is intuitively obvious that any NT candidate should have appropriate effects on a target cell, and that these effects should be identical, in every respect, to the endogenous substance. Not only must the same effects be observed in the gross sense, but there should be correspondence with respect to such things as time course, permeability and conductance changes, and even with interactions of agonist and antagonist drugs. This criterion is, with certain limitations, readily amenable to investigation. Technically, it is not difficult to monitor the activity of a single neuron or, if desired, a small group of neurons. Responses of a cell can be accurately observed with respect to time course, permeability changes, and conductance changes for specific ions. As well, responses to drugs and NT candidates can be monitored.

The following factors should be considered in designing a series of experiments to test a NT/NM candidate: 1) there must be a way to identify the population of neurons of interest; 2) it should be possible to monitor ongoing activity of these neurons in the intact animal to determine the response of the cell to its natural synaptic inputs vis a vis activity, conductance, etc., as well as to determine the effects of applied drugs, including the NT candidate. This presupposes some understanding of the electrophysiologic architecture associated with the neuron of interest. 3) some model must be used to apply the NT candidate to the cell, in a fashion approximating nature, to determine whether or not a physiological effect exists.

Demonstration of Neurotransmitters at the Synapse:

Before the development of the formaldehyde condensation technique for the demonstration of intra-tissue distribution of monoamines by fluorescence microscopy (Carlsson et al, 1961, Falck, 1962), it was not possible to show the association of a putative NT with a possible target cell. In this technique, catecholamines and serotonin react with formaldehyde vapour to form ultraviolet fluorescing molecules belonging to the isoquinoline and tetrahydro-beta-carboline groups, respectively. Some differentiation was possible within this group of compounds

so that serotonin could be identified as separate from the catecholamines by the colour at which its derivative fluoresced. Extensive studies were undertaken over the next decade to describe the contribution that cells which contained these substances made to the architecture of the brain (Ungerstedt, 1971, review). Work on improving the sensitivity led to the development of the glyoxylic acid technique (Lindvall and Bjorklund, 1974). With this new technique, and its subsequent modifications (Loren et al, 1976, Watson and Barchas, 1977), more pathways could be resolved, and known pathways described more completely and in greater detail.

During this same time, others were using alternate approaches in answering the same questions of localization. Labelled precursors to suspected NT's were injected, with tissues dissected out and assayed for radiolabelled products (McGeer and McGeer, 1964). Assays for the endogenous substances improved over time, allowing microasssays to be performed on ever-decreasing quantities of tissue (Palkovits, 1976). These latter techniques, however sensitive, lacked the precise localization afforded by histofluorescence. The situation improved only marginally until the application of immunohistologic techniques to amine localization (Hartman and Udenfriend, 1969, 1972, Gershon et al, 1977). Of neccessity, the early work in the

field inferred the presence of the amine by localizing a component or components of its synthetic pathway (Joh et al, 1975, Hartman, 1973). Although correlations with known amine distributions were good, the nature of the procedure made data, when uncorroborated by other techniques, of limited value in describing new amine distributions. Most recently, techniques have been developed for the production of antisera directed against some of the monoamines (Grota and Brown, 1974). With appropriate characterization and suitable controls these sera have been used to directly identify the locations within CNS of melatonin (Bubenik et al, 1976), NAS (Porietis et al, 1978), serotonin (5HT) (Lidov et al, 1980, Steinbusch et al, 1978) and dopamine (DA) (Porietis et al, 1977).

Electrophysiology:

Since the criterion of physiologic activity is critical, it is fortunate that a system exists in which the NT candidate of present interest, namely NAS, can be studied. This system is the in vitro hippocampal slice (Yamamoto and McIlwain, 1966).

The hippocampus is a convenient structure because it contains NAS, has an unusually well defined cytoarchitecture, and many of the electrophysiologic

interactions between cells are known (MacVicar and Dudek, 1980, Andersen ,1975, Andersen et al, 1973, 1971, 1964a,b, Bliss and Lomo, 1973). In addition, a secondary lamellar structure is present so that many of the interconnections between cells may be maintained in preparations such as the hippocampal slice.

Hippocampal Cytoarchitecture:

The hippocampus is part of the archicortex, anatomically associated with the medial wall of the lateral ventricles. The structure is usually subdivided into the hippocampus proper (Ammons Horn), the dentate gyrus, and the subicular complex (continuous with entorhinal cortex). The hippocampus proper has been classically described in four divisions, CA1 through CA4 (Lorente de No, 1934). More recent anatomical and physiologic data favour the division of the hippocampus into superior, (CA1), and inferior, (CA2,3) regions, with the dentate (CA4) being considered separately (Blackstad, 1956). The most common notation uses only CA1,CA3, and dentate.

The layering of the hippocampus is different from that of cerebral cortex. In CA1 and CA3, progressively deeper layers are alveus, stratum oriens (SO), stratum pyramidale (SP) (pyramidal cell layer), stratum radiatum

(SR), stratum lacunosum moleculare (SM), ending with the hippocampal fissure. The dentate area is somewhat simpler, consisting of a mossy fibre layer, a granule cell layer (GCL), and a polymorph cell layer (PCL) in the hilus of the dentate.

Other work in the hippocampus has defined another compartmentalization of function within the structure. Both physiological (Andersen et al, 1971) and anatomical (Blackstad et al, 1970) studies have suggested a segmented or lamellar structure analogous to the columnar organization of the motor and visual cortices. A further analogy to those particular areas of brain is the phenomenon of inhibition occuring on either side of an excited column/lamella.

Input:

The main input to the hippocampus is from the entorhinal cortex via the perforant path. This route passes through the subicular complex and terminates mainly on granule cells in the dentate gyrus. Electrophysiologic investigations show a significant proportion originate contralaterally (White et al, 1976). This contralateral input becomes more significant when ipsilateral inputs are damaged (Steward, 1976). The granule cells, in turn,

provide input to the pyramidal cells of the CA3 region, but only minimally innervate CA1. There is some direct input from entorhinal cortex via the perforant path to the CA3 region (Nafstad, 1967). There is no input to the CA3 region from CA1 (Andersen et al, 1973). The main input to CA1 pyramidal cells is excitatory from axon collaterals from CA3 pyramidal neurons. There have been reports of perforant path inputs to CA1 (Angevine, 1975), but these inputs, if they exist, are few (Steward, 1976). CA1 pyramidal cells are thought to excite nearby CA1 neurons in a positive feedback arrangement (Andersen, 1975). Input to CA1 from the cingulum has been described anatomically (Segal and Landis, 1974) but electrophysiologic data are not available.

Both rat and cat have been shown to have septal afferents to the hippocampus (Segal and Landis, 1974, Siegal and Tassoni, 1971). There is however, no widely accepted view regarding the areas of hippocampus receiving this input. Most recent findings in the rat (Rose 1976) suggest a projection from the medial septal nucleus primarily to the molecular layer of the dentate and secondarily to the stratum radiatum of CA3.

Several brainstem nuclei, including the raphe and locus coeruleus (LC) have been shown to project to the

hippocampus (Pasquier, and Reinoso-Suarez, 1977). LC projects primarily through the cingulum and fornix to the dorsal hippocampus, with terminal fields in stratum lacuñosum moleculare of CA1 (Pasquier and Reinoso-Suarez, 1978). No catecholamine (CA) projection to dentate granule or polmorph cells was demonstrated. Raphe inputs project via the same pathway with terminations in stratum lacunosum moleculare and stratum radiatum of CA1 and 3, as well as in the polymorph cell layer of the dentate. No projection to the stratum pyramidale was found (Pasquier and Reinoso-Suarez, 1978).

Outputs:

The main efferents from the hippocampus originate in the CA1 and CA3 regions (Swanson and Cowan, 1977). In the rat, these efferents have two main projections, one to the lateral septum, the other to the subicular complex. There is evidence for other projections, but their terminations are not well defined. Hippocampal efferents seem to differ widely from species to species, so that data from one cannot be used in another (Swanson and Cowan, 1977).

The literature in hippocampal electrophysiology is extensive, due largely to the attractiveness of its highly repetitive organization. Several putative NT's have been

tested in hippocampus, (Storm-Mathisen, 1977), and many of the effects of the endogenous transmitters have been extensively reported (Segal, 1980, White et al, 1979, Spencer et al, 1976, Curtis et al, 1970, Bland et al, 1974, Stefanis, 1964, Wang et al, 1979, Schwartzkroin and Andersen, 1975). In addition, the slice preparation is uniquely suited for this type of study because of the easy access afforded to the CA1 and CA3 pyramidal cells for both recording and application of NT candidates, their analogs, and other pharmacologic agents.

There are several techniques in common use for applying substances of interest to neurons. These include perfusion, microdroplet application and microiontophoresis. Perfusion lacks precise spatial and temporal control although with appropriate interpretations, useful data can be obtained. The substance is applied or withdrawn over relatively long periods of time, with the measured response usually one of very short duration i.e. depolarizations or spike activity. A second undesirable consequence of the technique is that all of the neurons within the preparation are exposed, and combined with the slow time-course of application, this precludes differentiation not only between pre- and post-synaptic effects, but also between mono- and poly-synaptic phenomena.

The microdroplet technique differs from perfusion in these respects: it is much shorter in time of onset and offset, and the effective area exposed to drug is much smaller, although it may still include a polysynaptic chain leading to the observed results. The shorter times involved in the microdroplet application technique allow some differentiation of mono- vs. polysynaptic events, but it is still far from satisfactory in this respect. The most sophisticated approximation to the synapse is iontophoresis. With this technique, both parameters of time and space can be very rigidly controlled. It is still only an approximation and falls far short of mimicking the synaptic cleft. In addition to the relatively great distances still involved (microns vs. nanometers) the final concentration of NT or drug at the receptor is still unknown, and more importantly, the special relationship between pre- and post-synapse does not exist. Neither microiontophoresis nor microdroplet application, in themselves, can be used to differentiate between pre- and post-synaptic events, although ion manipulations can give some information in this regard.

Glutamate:

Glutamate (l-glutamic acid) meets several of the neccessary criteria for being an excitatory neurotransmitter

in mammalian CNS (Curtis, 1979). Glutamate has been shown to be released during electrical stimulation of the excitatory mossy fibre pathway which connects granule cells in the dentate gyrus of the hippocampus to the pyramidal cell dendrites in CA3 and, to a lesser extent, in CA1 (Crawford and Connor, 1973). It has also been shown that application of glutamate to pyramidal cells and granule cells causes an increase in the frequency of firing in these cells (Biscoe and Straughan, 1966, Dudar, 1972, Spencer et al, 1976). Glutamate excitation of CA1 pyramidal cells in the slice preparation has been studied in detail (Schwartzkroin and Andersen, 1975). These investigators found that the entire dendritic tree as well as the cell body were glutamate sensitive, with no differences in sensitivity between the various areas of the cell. These findings are reflected by the work of Nitsch and Okada(1979), who demonstrated the different concentrations of glutamate in various layers of the hippocampus. Although significant differences were found between some layers, the values for individual layers rarely differed from the mean value by more than 20%.

Metabolism:

The demonstration of the synthetic and degradation pathways of a NT candidate require the following: (i)the synthetic mechanism should be demonstrable in its entirety

within the structures, presumably neurons, or parts of neurons where the substance is to be stored prior to its release (ii)the synthetic machinery should be demonstrated to be adequate to the function proposed (iii)the control of the synthetic pathway, especially if it is under product inhibition, should be determined (iv)mechanisms must exist for terminating the action of a NT. These may include reuptake, conjugation-excretion, breakdown, or any other effective inactivation step (v)all products of metabolism should be identified. The difficulties in defining metabolism to this extent are discouraging. Most putative NT's have at least two possible synthetic routes, and many act as intermediates in the synthesis of other active substances. The necessary complexity of degradation pathways makes it impractical to monitor all inactivation products. In most instances localization is limited to the main synthetic pathway, at least the rate-limiting enzyme or the enzyme which is last in the chain for the particular substance, and the major degradation product. This partial information is far from conclusive, but is usually accepted with strong arguments regarding the criteria of activity, release, and specific receptors.

There is increasing evidence that specific local reuptake and recycling is a major contributor to the termination of many NT responses. If the specificity and

import of this function is confirmed, specific uptake of labelled NT candidates may provide valuable evidence for NT function.

Release:

A demonstrable release of a substance in response to appropriate stimulation is strong evidence in favour of a NT/NM role for that substance. This criterion, along with an observable response, may be considered the two most important of the list (Storm-Mathisen, 1977). Release, of course, implies that presence has already been shown, and evidence that a release mechanism exists is neccessary to support the hypothesis that the substance is involved in communication between cells. The major limitation in regard to demonstrating release is again one of resolution. The most sophisticated techniques (Wieraszko and Lynch, 1979) still must rely on the NT output of a very large number of cells. Even if this were to be discounted, the possible artifacts due to incorporation of label, glial uptake, and nonspecific uptake/release remain worrisome. The authors of the cited paper have included controls and have cited other literature, which addresses these concerns in other systems (Kuhar and Snyder, 1970, Iversen and Storm-Mathisen, 1976, Taxt et al, 1977).

The Receptor:

A receptor in this sense is more accurately described as specific, high affinity binding to some cell component by the ligand in question. Probably the most extensively studied binding site in the nervous system is that for acetylcholine(ACh), followed closely by one for dopamine. Both of these binding sites are usually identified by their binding of a snake venom toxin and synthetic analogs, respectively, rather than by binding the native molecules. The same is true of recent work using labelled LSD to localize and quantify a binding site for 5HT (Hamon et al, 1980, Peroutka and Snyder, 1979).

Studies of binding sites have some inherent difficulties associated with them. Many cells can take up a variety of substances indiscriminately. Even in cell fractions, this capability for non-specific binding exists and must be controlled for. It is also necessary to demonstrate displacement in a dose-related manner. Since the ligands are chosen for their high affinity relative to the native substance, in order to make the assay more sensitive, the displacement by the native substance can be difficult to show directly.

Neuropharmacology of Serotonin (5-HT):

Histofluorescence and other techniques have been used to map the distribution of presumed 5HT neurons in rat brain. Functions have been suggested for 5HT based on this evidence in conjuction with pharmacologic data. The model of the 5HT synapse used in these investigations of drug actions, is fairly conventional. Drugs can be used to assess 5-HT function in the following ways: 1)direct agonist action at a post-synaptic receptor; 2)direct antagonist effect at a post-synaptic receptor; 3)agonist or antagonist effects at the pre-synaptic receptor, i.e. feedback or axo-axonal interaction; 4)5HT concentration increases brought about by precursor loading or MAO inhibitors; 5)5HT content decrease by 5HT releasing drugs, reuptake inhibitors, or synthesis inhibitors; 6)5HT neurotoxins.

There are two main classes of drugs which may act as 5HT mimetics directly at the receptor: indoles, including bufotenine., N,N dimethyl 5methoxytryptamine as examples (Green and Grahame-Smith, 1978, Haigler and Aghajanian, 1977, Bennett and Snyder, 1976) and certain substituted piperazines, including quipazine (Green et al, 1976, Hong et al, 1976), MK 212(Clineschmidt and McGuffin, 1978). These last two drugs are postulated to have pre-synaptic effects

also (Clineschmidt et al, 1978), although there are drugs from this group with nearly pure post-synaptic effects (Fuller et al, 1979).

5HT antagonists include metergoline (Fuxe et al, 1978), methysergide (Gyermek, 1961), cyproheptadine(Stone et al, 1961), and LSD (Boakes et al, 1969), among others. These compounds often have mixed agonist/antagonist activity (Haigler and Aghajanian, 1977), making their antagonist role unclear. This issue has received considerable attention (Monachon et al, 1972, Fuller and Steinberg, 1976, Jacoby et al, 1975, 1976, D'Amico et al, 1976) but remains unresolved. The pre/post synaptic relationships of these drugs have not been demonstrated.

Brain 5HT concentrations can be manipulated by loading with the precursors l-tryptophan or 5-hydroxytryptophan, although this process has undesirable metabolic side-effects (Gal et al, 1978, Yunger and Harvey, 1976). Monoamine oxidase inhibitors are useful in increasing 5HT stores, but none are known that are specific to 5HT.

Reserpine and tetrabenazine, as well as other agents, are capable of releasing stores of 5HT, but are so non-specific as to be of limited utility in studying 5HT

function. On the other hand, several specific inhibitors of 5HT reuptake are available, including fluoxetine (Wong et al, 1974, Fuller et al, 1974a,b), zimelidine (Ross et al, 1976), pirandamine (Pugsley and Lippmann, 1976), and fluvoxamine (Claassen et al, 1977).

Synthesis inhibitors act most effectively on tryptophan hydroxylase. PCPA and related compounds are the most commonly used for this purpose (Trulson and Jacobs, 1976).

5,6Dihydroxytryptamine (5,6DHT) and 5,7dihydroxytryptamine (5,7DHT) are transported specifically into 5HT neurons, and destroy these cells. These compounds are used extensively in chemical lesioning of 5HT sytems. An excellent review is available in the Annals of the New York Academy of Science, Vol 305, 1978, by several authors.

N-acetylserotonin:

NAS was first identified in its role as an intermediate product in the synthesis of melatonin (5 methoxy n-acetyl tryptamine) from tryptophan (Lerner et al, 1958, Weissbach et al, 1961). In the pineal gland tryptophan is hydroxylated by tryptophan hydroxylase to 5-hydroxy tryptophan, then decarboxylated by l-amino acid

decarboxylase to 5HT. 5HT is acetylated by the enzyme rate-limiting to this pathway, N-acetyltransferase (NAT) (E.C. 2.3.1.5). Tryptophan hydroxylase and l-amino acid decarboxylase are widely distributed in brain (Lovenburg et al, 1973, Revson, 1973). NAT has also been demonstrated in several brain areas, with notably high concentration in cerebellum (Paul et al 74). Pineal NAT has been shown to be under noradrenergic control and to vary in activity with a circadian rhythm (Brownstein et al, 1973). No circadian variation has been demonstrated in extrapineal brain NAT (Moore, 1975). Brain NAT is similar to pineal NAT as opposed to liver NAT in that the former can both be induced by adrenergic agonists (Friedhoff and Miller, 1977). It is attractive to think that NAS in brain and pineal share a common synthetic pathway. much as in the case of the catecholamines. The fact that the general distribution of 5HT and NAS in brain differ discourage the conclusion that NAS functions as some inactivation product of 5HT (Lidov et al, 1980, Bubenik et al, 1976). More important for the present thesis, the distribution of 5HT and NAS in the hippocampus of the rat are distinctly different (Lidov et al, 1980, Porietis et al ,1978). Another possibility that brain NAS functions as a melatonin precursor, is challenged by the lack of data supporting the localization of the enzyme which converts NAS to melatonin, namely HIOMT, in brain areas outside of the pineal and retina (Cardinali and

Wurtman, 1972). There is, in addition, a marked absence of melatonin in several brain areas where high concentrations of NAS have been found (Bubenik et al, 1976, Porietis et al, 1978).

Brain content of 5HT seems to be limited by the availability of substrate, tryptophan (Fernstrom and Wurtman, 1973). Given the direct relationship between 5-HT , the immediate precursor of NAS, with intake of tryptophan, it seems likely that maximum brain content of NAS would be similarily regulated, with the function of NAT being to down-regulate NAS content. An alternate synthetic pathway for NAS, which bypassed 5HT, with some mechanism for a relatively greater uptake of tryptophan would be the only mechanism for dissociating NAS from 5HT synthesis. An active, aggressive incorporation of 5HT into NAS would appear as a fall in 5HT concentration, much as is does in pineal. I am unaware of a situation where two major synthetic pathways converge on a pair of compounds so closely related as these two. In hippocampus, the 5HT content exceeds that of NAS by an order of magnitude (Narasimhachari, personal comm). This abundance of precursor tends to contraindicate an alternate, unnecessarily complex, pathway for NAS synthesis.

There is an enzyme (aryl acylamidase) in brain which

is capable of de-acetylating NAS to 5HT (Paul et al, 1976). This enzyme is regulated by 5HT and its analogs, suggesting that it has some function in the indoleamine system, even if not as a specific inactivator of NAS (Oommen and Balasubramanian, 1979).

As noted previously, uptake by neurons is rapidly becoming a legitimate mechanism for terminating the action of a NT. There are reuptake blockers which differentiate 5HT from other amines, i.e. fluoxetine (Fuller and Wong, 1977). It will be interesting to compare NAS to 5HT with respect to their uptake, and to their interaction with 5HT uptake blockers. 5,6DHT and 5,7DHT are already known to share the uptake mechansim for 5-HT (Baumgarten and Schlossberger, 1973) and once taken up are known to cause destruction of 5HT neurons. The effects of these on brain NAS content are the subject of ongoing investigation (Pang, personal comm). It seems likely that 5,6 DHT and 5,7 DHT will reduce brain content of NAS as a consequence of 5-HT reductions.

Presence of NAS in Brain:

Early studies utilizing radioenzyme assays concluded that brain content of NAS outside the pineal was insignificant (Saavedra et al, 1973). NAS has been

demonstrated by immunohistology to be present in several brain areas including cerebellum, the root of the trigeminal nerve, pontine reticular formation, and hippocampus (Bubenik et al, 1976, Porietis et al, 1978). Ongoing work has identified and quantified NAS within neurons in many brainstem nuclei (Pulido, personal comm, manuscript in preparation). The hippocampal localization forms a part of the present thesis and will be discussed in detail in a later section.

Release of NAS from neurons:

There are no data available on this subject.

NAS receptors:

Highly specific, high affinity binding of radiolabelled NAS has been demonstrated in several brain areas, including hippocampus and cerebellum (Niles, personal comm). Available data show binding affinities for NAS to be comparable to those for 5HT. The number of NAS binding sites in cerebellum exceeds the number there for 5HT. Similar comparisons have yet to be made in hippocampus. Hippocampal NAS binding has been shown to be greater than that for melatonin in the same tissue. This is an active area of study and data will be forthcoming.

A strategy for the investigation of the function of NAS in the nervous system of the rat:

1) Does NAS in brain have a distribution different than that of 5-HT, its presumed precursor, and of melatonin, to which it is converted in the pineal? Preliminary evidence indicates that NAI's may be present in the hippocampus of the rat. The aim of the histologic work in the present thesis is to investigate the comparative distribution of NAS and melatonin within the hippocampus, and to compare these data with the published reports of hippocampal 5-HT distribution.

2) In those areas where NAS can be demonstrated exclusive of 5-HT or melatonin, can adequate resolution of the NAS-containing structures be obtained to suggest a function for NAS in this brain area? A high resolution immunohistochemical technique for localizing NAS was developed using Protein-A peroxidase as a secondary reagent. This was applied to histologic slices of rat brain hippocampus in order to observe the relationship of NAS-like immunoreactivity to the identifiable cells and structures in hippocampus.

3) Can an appropriate cell population be identified for purposes of electrophysiologic investigation of the effects of NAS? The criteria used in the histological investigation to identify cells of possible interest were: i)staining pattern to be reasonably consistent with NAS acting as an input to the cells of interest. This includes the exclusion of a melatonin precursor function(i.e. no melatonin detectable at the site), and a geometry of staining relative to the cell and its dendrites suggestive of a possible link. ii)the cells should be identifiable when live, so that they are easily located for electrophysiologic study, iii)an extensive electrophysiologic and pharmacologic literature should exist concerning these cells, so that appropriate comparisons could be made in an effort to avoid artefact, non-physiologic situations etc., iv)if possible the area of interest should be adaptable to an in vitro paradigm i.e. tissue culture or thin slice, to facilitate access.

4) What, if any, response can be demonstrated in cells which, from histologic data, may be associated with NAS-containing structures? Extracellular recordings were made while applying NAS to the cell at an appropriate site as determined from the histology. The effects of NAS and its structural analogs on cellular activity were determined. Most neurons do not exhibit spontaneous activity. If the

population of interest is among these, and NAS does not prove to be excitatory, some procedure to induce activity would have to be applied. Appropriate procedures could be found in the literature and should be defensible as an approximation of a physiologic action.

5) What can be determined about the cells of origin of the NAS in hippocampus? The fimbria/fornix, a major input to the hippocampus was lesioned, bilaterally and unilaterally, to see if any changes in NAS content within the hippocampus could be observed by immunohistology.

6) Can the presence of NAS in hippocampus be confirmed through other procedures? A collaboration was established with Dr. Narasimhachari, U. North Carolina, to assay NAS content in hippocampus in animals treated with the 5HT synthesis inhibitor, p-chlorophenylalanine (PCPA), and in untreated, control, animals, using the method of gas chromatography- mass spectrometry.

MATERIALS AND METHODS

I Immunohistology

Primary antisera:

Four separate antisera were used. The processes involved in the production and characterization of two of these, anti-N-acetylindole (a-NAI) and anti-melatonin (a-Mel) have been published (Grota and Brown, 1974, Pang et al, 1976). The remaining antisera, both directed against NAS specifically, were produced by immunization of rabbits with an NAS-bovine serum albumin(BSA) conjugate. The conjugate was prepared by coupling NAS to BSA via a para-carboxy benzyl bridge (DeSilva and Snieckus, 1978). Injection and harvesting were as in the papers cited above. Characterization data regarding these antisera , as determined by radioimmunoassay, are shown in Appendix I (work of Brown and Grota, unpubl).

Tissue preparation:

Early studies used male Wistar rats, approximately 200g. Since the rats proposed for use in electrophysiology were a different strain (Royal Victoria Hospital), the work

with the specific a-NAS sera was repeated in this strain. Again, male rats weighing about 200g were used.

The animals were decapitated, the brain removed quickly and placed on dry ice. After thorough freezing, the tissue was mounted on a block and placed in a Cryostat (American Optical) to equilibrate to -13 degrees C. Sections were cut at 10-12 microns, with orientation in the coronal plane. Acid-alcohol cleaned slides were used throughout. Touching the room temperature slide to the frozen section caused the section to adhere to the slide. The sections were then dried under a stream of air at room temperature for 30 minutes. The sections were rehydrated briefly in isotonic Tris-buffered saline(TBS), pH 7.2, then processed immediately.

Staining techniques:

Two indirect staining techniques were employed. In both cases, the primary antiserum was applied, in 1:5 dilution in TBS, to the prepared sections. The sections incubated at room temperature, in a water vapour saturated atmosphere, for 40 minutes. The slides were rinsed in buffer, 3x5min., then subjected to the secondary reagent.

For fluorescence microscopy, the secondary reagent

was a commercial preparation of goat antiserum to rabbit 7s gamma globulin, coupled to fluorescein isothiocyanate (FITC), (Hyland). This reagent was diluted 1:5 in TBS, and was applied to the sections. The sections were incubated for 30 minutes under the same conditions as the primary serum. The sections were washed with buffer, 3x5min, coverslips were mounted using 50% glycerol in phosphate buffered saline(PBS), pH 7.8. The sections were observed through a Leitz Ortholux microscope equipped with epi-illumination fluorescence optics. Dichroic excitation filters were combined to produce narrow-band excitation at 495nm. Emitted light was passed through a 515nm barrier filter and then into the ocular/camera head. Photographs were taken of areas showing staining. Exposure was automated, and exposure times recorded. Films used were Tri-X (Kodak), developed for ASA 1600, and High Speed Ektachrome (Kodak), developed for ASA 800. Identical areas of adjacent control sections were identified and photographed with manual exposure, using times corresponding to the automated exposure for each area of interest.

In some of the studies using specific a-NAS sera, an alternate secondary reagent was used, protein-A peroxidase (PrAP). This reagent was used diluted 1:10 in the second incubation, replacing the FITC conjugate. Incubation times were the same as for FITC. After the incubation, slides were

washed 3x5min in TBS, then 1x5min in PBS. The sections with attached immune complexes were then fixed in 2% glutaraldehyde in PBS, pH 7.4, for 30 minutes. Sections were washed for 30 minutes in several changes of PBS. They were then incubated with a 0.125mg/ml solution of diamino-benzidine(DAB) in PBS, freshly prepared. This solution was replaced after 20 minutes with a second, freshly prepared, solution of DAB, with 0.115% hydrogen peroxide. Slides were incubated for an additional 30 minutes, briefly rinsed in PBS, then immersed in 1% osmium tetroxide for 10-20 minutes. The slides were then thoroughly washed, mounted with Permount(Fisher) and examined. The same microscope was used, but with transmitted light optics. All exposures were timed automatically, since the background density was the same in immune and contol slides. Photographs were compared as before to define the extent of specific staining.

Staining Procedures:

During the early part of the study, a specific a-NAS serum was not available. To localize NAS immunoreactivity, it was necessary to use two antisera, one specific for melatonin, the other binding NAS and melatonin equally well (Bubenik et al, 1976). Each observation then consisted of three parts: 1)a-NAI serum staining 2)a-melatonin serum

staining and 3)staining with absorbed sera(see Controls section). Interpretation was as follows: melatonin content was given by the difference between staining with a-mel control and a-mel serum. NAS, without melatonin present, was defined as the difference between staining with the control and a-NAI sera, less any specific a-mel reaction. If a-NAI and a-mel stained equally well, the content was described as melatonin. If specific a-NAI staining was greater than that of a-mel, but there was specific staining with a-mel, a mixture of NAS and melatonin was inferred.

When the specific a-NAS became available, a direct comparison with a-NAI was made to confirm the earlier results. The a-NAS serum, with controls, was subsequently used alone.

Controls:

With each new procedure, and with each new batch of immune reagents, the following controls were performed: 1) primary antiserum was omitted from the procedure. 2)pre-immunization rabbit serum was used as the primary reagent 3)the specific antisera were pre-incubated with an appropriate ligand at high concentration, before applying to the sections. If the first two controls were negative, only the third was continued in subsequent work.

Preparation of Protein-A Peroxidase:

The PrAP reagent was prepared while the author was visiting the laboratory of Dr. A.O. Jorgenson, Dept. of Anatomy, U of Toronto. The procedure used was modified from Dubois-Dalcq et al, 1977, and is as follows:

Day 1

-dissolve 10 mg. peroxidase (HRP type VI, Sigma) in 1.0 ml freshly prepared 0.3M Na-Bicarbonate, pH 8.1

-add 0.2 ml of absolute ethanol with 1% 1-fluoro, 2,4 dinitro benzene (Eastman)

-stir gently in the dark, 1h at room temperature(RT)

-add 1.0 ml 0.08M Na-meta periodate in distilled water

-mix for 30 min as before

-add 1.0 ml 0.16M ethylene glycol

-mix for 1h as before

-dialyze in darkness at 4C against 0.3M Na-Bicarbonate, pH 8.1, overnight

with several changes of large volumes

Day 2

-change dialyzing buffer to 0.3M Na-Bicarbonate, pH 9.5, 2x1h, in darkness at 4 C

-warm to RT

-adjust pH to 9.5 with 0.1N NaOH

-centrifuge for 20 min at 12,000xg to remove any precipitate

-add 5mg Protein-A (Pharmacia) to supernatant

-check pH, adjust to 9.5 if neccessary

-stir gently in darkness, 3h at RT

-cool to 0 C on ice

-add 5mg $NaBH_4$

-stir gently in darkness, 3h at 4 C

-dialyse against TBS(.15M NaCl, .01M Tris,pH 7.4) in darkness overnight at 4 C, with several changes

Day 3

-change buffer, leave for 2h

-concentrate contents of dialysis bag to a volume of 1.0 to 1.5ml, using Sephadex G-200

-centrifuge at 20,000xg for 20 min

-apply supernatant to Sephadex G-100 column(100x1.5cm)

-set flow to 7.2ml/hr, with a pressure head of 40cm

-collect fractions 1-45 at 1.2 ml each, excess at 5m..

Day 4

-measure optical density (O.D.) at 280/403nm.

-collect and pool fractions with OD280 greater than .25

The nature of the product was investigated by SDS gel electrophoresis, as a secondary quality control.

On the discrimination of specific vs. non-specific staining:

Much of the immunohistologic data to follow rely on the differentiation of a specific antibody-to-antigen reaction from a non-specific binding of the primary reagent, secondary reagent, or marker, to some component of the tissue slice. Due to a certain variability in the results given by the immunostaining technique, including variability in controls, I would like to outline the criteria of specificity used in reporting these present data:

1)Reproducibility: Although not sufficient as a definition of specificity, a high degree of reproducibility was required before any staining pattern was reported here as being specific. All of the figures presented are representative of at least five samples, each from a different animal and processed separately from the others, but simultaneously with the appropriate controls. 2)Pattern: Within undamaged tissue areas, a concentration of stain relative to overall background, was always investigated further as a possible specific reaction. Those areas which satisfied the criterion of reproducibility were then considered in comparison to absorbed controls. 3)Absorbtion: In theory, the reaction which identifies the presence of the antigen of interest occurs between a binding site on the immunoglobulin, which is capable of recognizing some part of

the antigen, and the antigen in the tissue, or an immunologically identical (by definition), substance. If a large excess of the antigen, in soluble form, is present at or before the time of the tissue reaction, this excess antigen should successfully compete for the immunoglobulin binding sites, and at least reduce, if not eliminate, the antibody/tissue-antigen reaction. Any surviving staining, although it may be reproducible, represents the localization of a substance not immunologically related to the antigen of interest. With each new primary antiserum, an additional control was used to assess the effect of adding a substance to the buffered antiserum. A compound of similar molecular weight and acid/base properties, dopamine, was added to a aliquot of buffered antiserum. An assumption was made that the pH, molarity, and any other non-immunological effects would be similar for the two compounds. The antigen-absorbed control was only considered valid if the unrelated compound did not effect staining. This proved to be the case in all trials.

Through the observation of several hundreds of sections, a subjective, but highly practiced, image of residual staining was formed, and used subsequently in asessing specific staining. Any of the residual staining, be it directed against antigens other than that of particular interest, or be it random precipitation of a

reagent or marker, will be referred to an non-specific. Any staining in an area of obvious tissue damage was ignored since damage has been seen to regularily, and without discrimination, stain with primary and secondary reagents, as well as with marker, and sometimes even in an absorbable fashion.

Assay of NAS by GCMS:

Male Wistar rats, about 200g each, were treated with the 5HT synthesis inhibitor, PCPA (Sigma), 350mg/Kg/day, divided into two doses daily, for a period of three days. Control animals were injected with like volumes of saline on the same schedule. Animals were killed by decapitation, twelve hours after the last drug injection. The brain was removed quickly and both hippocampi were dissected out. This tissue was sealed in coded vials, then placed on dry ice. The samples were transported frozen to the lab of Dr. Narasimhachari, where they were processed for analysis by GCMS. NAS was measured using single ion monitoring, with a deuterated internal standard. The drug-treated group was compared to controls by t-test analysis.

II Electrophysiology

The hippocampal slice was prepared after the method

of Yamamoto and McIlwain, 1972. Male, Royal Victoria Hospital rats, approx 200g, were decapitated after light ether anaesthesia. The brain was quickly removed and placed in ice-cold medium. The hippocampus from one hemisphere was dissected out while under medium and placed on a tissue chopper. Slices were made at right angles to the long axis of the hippocampus, at a thickness of 350microns. When 6 or 7 slices had been collected, they were transferred to the recording chamber, which had been prepared earlier. The temperature in the chamber was maintained at 34 degrees C, and monitored by a local thermocouple. The chamber was filled with medium, and water-vapour saturated 95% oxygen was continually passed over the surface of the medium. The slices were supported at the air-medium interface by a nylon mesh. The recording chamber had a sintered Ag/AgCl bath ground built in. All manipulations of the slice and electrodes in the chamber were observed through a variable-magnification microscope mounted above the chamber. The slices were allowed to recover for at least 30 minutes. The medium in the chamber was then replaced with fresh medium, the slices allowed a further 10 min equilibration, then the experiments were begun.

Recordings were always made from the pyramidal cell layers of CA1, about 100microns deep to the surface of the slice. The recording electrode was positioned first,

followed by the iontophoretic electrode. Placement was considered successful when a high firing frequency was detected in response to a sustained 100nA glutamate ejection.

The drug-testing paradigm was as follows: a glutamate current was selected that caused a plateau of firing to be reached at high frequency with short (5 second) pulses. A minimum of two, 5 sec, glutamate pulses were applied at short intervals. Immediately following these baseline pulses, glutamate was applied simultaneously with the test drug. The length of the application was approximately 5 sec. This sequence was repeated for each current value of test of drug, usually in duplicate. Test drugs were applied from 0nA, in increments of 10nA, to blocking values, or until firing was effectively shut down. At each electrode placement, NAS was tested first, followed by at least one other drug. Recordings were made of the observed extracellular activity with a synchronized voice cue. The tape was played back and data collected on firing frequency.

Analysis of Frequency Data:

The electrophysiology literature cited in this thesis is representative of that area of research in that

statistics are infrequently used in data analysis. Data are usually presented as tracings from pen-recorders or photographs of oscilloscope traces. Obvious differences in frequency of cellular responses can be easily interpreted in this fashion. The recording and presentation of numerical frequencies, or of inter-spike interval data is seen in some literature (MacDonald and Nistri, 1978, deMontigny and Aghajanian, 1978). I could not find any discussion in the literature concerning the validity of applying any statistical analysis to these numerical data. Some discussion of this question does appear in the PhD. theses of J. Kalaska (U of Toronto) and J.F. MacDonald (U British Columbia). In both cases the point is made that there is no evidence supporting the hypothesis that numerical data derived from electrophysiologic experiments are distributed normally, or in any other definable distribution.

Each plotted point represents the arithmetic mean of several observations, taken over a short time period. There is evidence that, at least in the case of glutamate and some of its analogs, the cellular response may be directly related to the concentration of the drug at the surface of the cell (MacDonald and Nistri, 1978). Given a constant spatial relationship between cell and electrodes and a fixed error associated with the current generating device, a normally distributed drug concentration can be assumed at

the cell surface, over increments of time. On the basis of this argument, a standard error of the mean was calculated for each data point, and subsequently indicated on the graphs. Even if the arguments given above are incorrect, this calculated value of standard error gives some indication of the variability at each data point.

Within each placement, all parameters save ejection current were assumed to be constant. The relative response of each cell group was plotted on semi-log co-ordinates against test drug ejection current. This relationship of log(dose) to response is well established for many receptor-effector systems (Endrenyi, 1976, Perkins, 1973) and was used here by convention. Within each group of data (one placement), all points which appear on the positive slope of the curve were used to calculate a log-transformed linear regression. This calculation does not require any assumptions regarding the distribution of the individual data points, only the assumption that they lie on a straight line. That this assumption was likely correct was reinforced by the high values of the coefficients of regression, the values of which are related to the statistical confidence in the parameters of slope and intercept which define the line.

At this point, the problem of comparing lines is

addressed. If two sets of lines are each taken from normally distributed populations of lines, each population having the same variance, the slope and intercept values for those lines can be compared by conventional parametric statistics such as the t-test (Siegel, 1956). The populations of the linear data in the present report are inadequately defined with regard to distribution and variance to make this type of comparison valid. The most appropriate test for these data must be free of these assumptions, yet take advantage of the fact that the data comparing 5HT and NAS are paired. The Wilcoxon matched-pairs, signed rank test fulfills these criteria (Siegel, 1956). With small sample sizes, as in the present case, this test has a power-efficiency value of 95% relative to the t-test (Mood, 1954). The calculation procedure for this test is explained in Siegel(1956).

Preparation of medium:

The chemicals used in the preparation of the medium are listed in appendix II. Stock solutions of Na-K-PO4, Mg, and Ca, were prepared. These were diluted and mixed in appropriate volumes of deionized water. To this mixture was added dry sodium-bicarbonate and glucose. The final concentrations, in millimoles, of the constituents was Na-151.25, K-4.5, Cl-132.5, Ca-2.0; Mg-2.0, phosphate-1.25,

sulphate-2.0, Na-bicarbonate-2.0, and glucose-10.0.

Preparation of Electrodes:

Recording electrodes were prepared from 1.2 mmO.D. borosilicate glass capillaries. They were pulled to fine tips on a DKI pipette puller, then broken back to approximately 1 micron diameter, under direct microscopic observation. These electrodes were pressure filled with 4M NaCl.

Iontophoretic electrodes were prepared from commercially prepared 7-barrel blanks (F. Haer). These electrodes were pulled on the same apparatus, and had their tips broken back to approximately 3 microns diameter. The tips of all barrels were filled with distilled water by capillary action. The drug solutions were then used to backfill the various barrels. The centre barrel was filled with medium to serve as the current balance channel. The filled electrode was mounted on the holder, tested for short circuits, then time was allowed for the drug to diffuse into the tips.

Drugs were prepared as follows: Glutamate(Sigma), free base in distilled water, saturated; NAS(Sigma) in distilled water, saturated; melatonin in

10%methanol/distilled water, 100mM; 5HTcreatinine sulphate in distilled water, 100mM.

Wiring:

Iontophoresis was controlled by a 6-channel programmable microiontophoresis unit (Dagan); six independent channels, anion/cation with separate retaining current controls, and an automatic current balance channel. The recording electrode was held in an Ag/AgCl electrode holder(WPI). Signals were amplified by a high gain AC preamplifier (Grass), and displayed on an oscilloscope. A direct recording was made from the preamplifier output. At playback, the record was filtered, if necessary, then passed through an amplitude discriminator. The output of this device was used to drive a decade counter. The counter was operated by a timer in a bistable mode, counting for 2 seconds, displaying counts for 1 second, then repeating.

RESULTS

Histology of NAS-like immunoreactivity:

Fluorescence with a-NAI and a-Mel:

Fig. 1 shows adjacent coronal sections of rat hippocampus. The magnification factor is low (30x to print) so as to show almost the entire hippocampus. See the key diagram for orientation. Fig. 1a shows a section which was incubated with buffered anti-N-acetylindole(a-NAI) serum. In this figure, there is a very strong association of specific (as defined in Methods section) fluorescence with the area identified as the stratum pyramidale of CA1(SP-CA1). There is also specific fluorescence associated with the SP-CA3, but the intensity is lower than that found in the CA1 area. The intensity of fluorescent stain shown in association with the polymorph cell layer of CA4/dentate is similar to that seen in SP-CA3. The diffuse staining appearing elsewhere on the section was defined as non-specific, using the criteria of pattern, reproducibility, and immune absorbtion, which were elaborated in the Methods section. No detail regarding the cellular or sub-cellular localization of the fluorescence

Key diagram for Fig. 1a,b,c

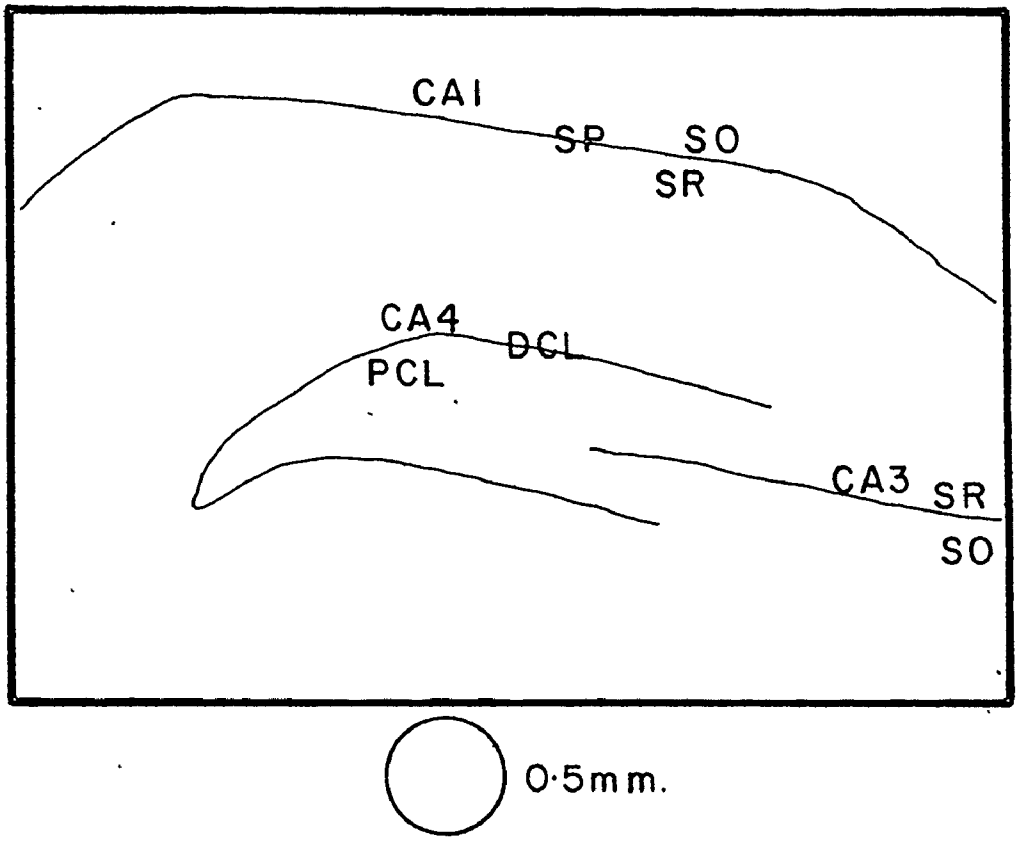
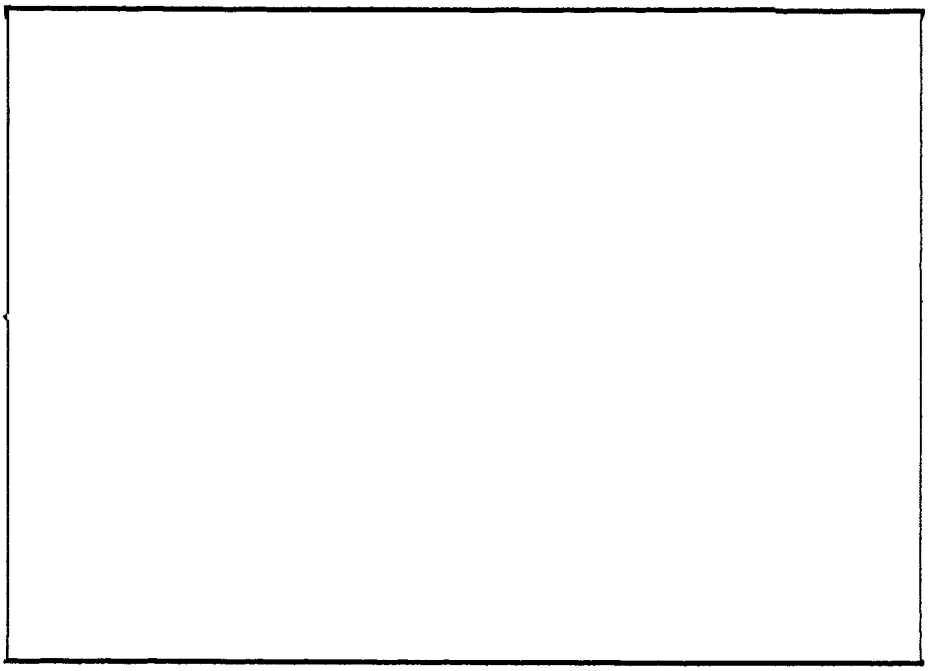
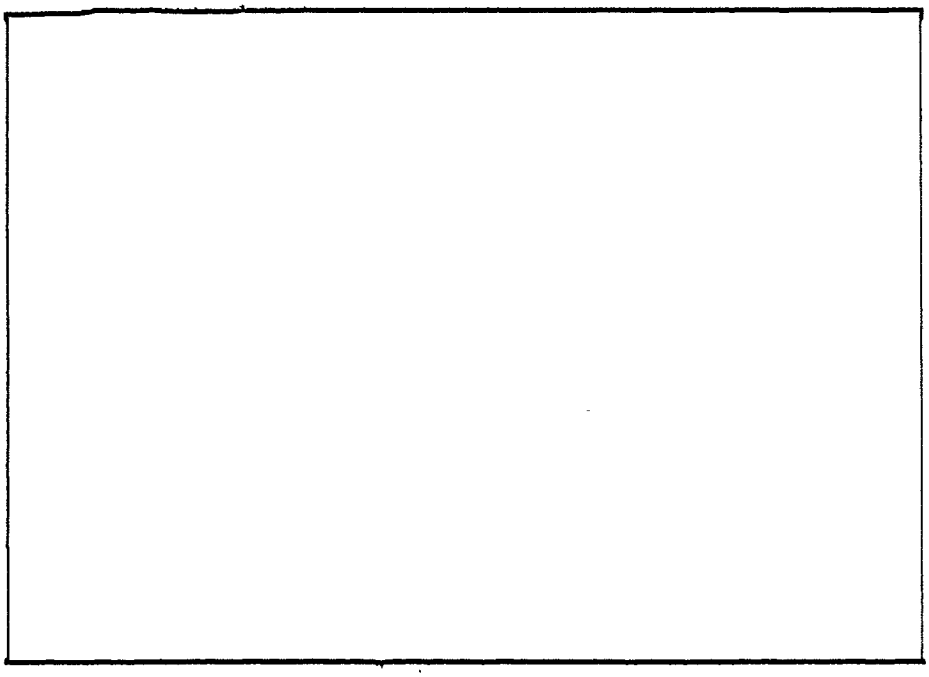


FIGURE 1

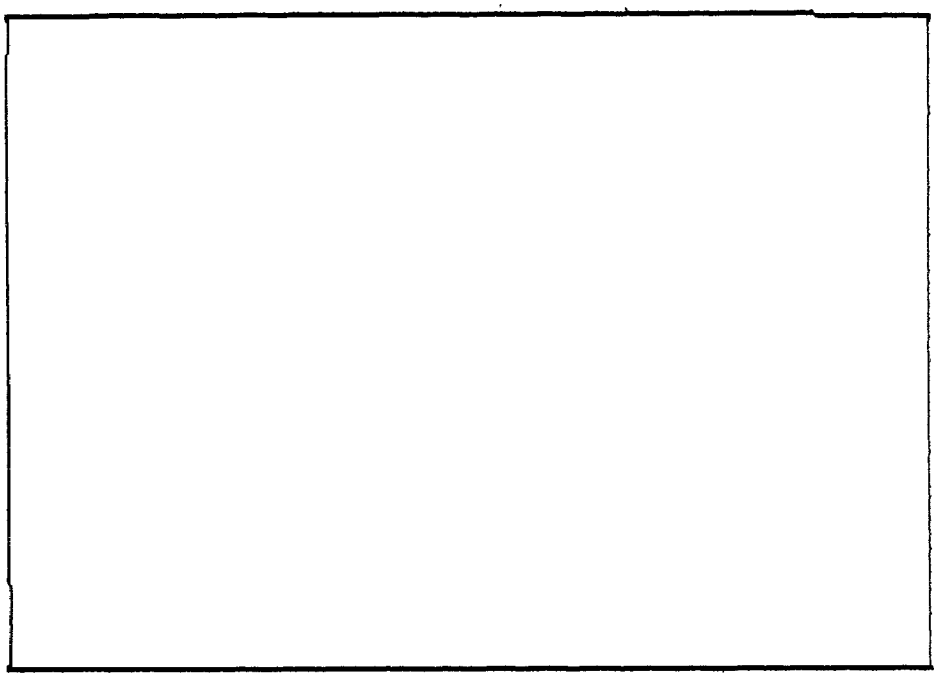


1a. Immunofluorescence (IF) of N-acetylindoles



1b. IF of melatonin

FIGURE 1 (cont)



1c. IF of N-acetylindoles (NAI's)-absorbed

can be seen at this level of magnification. Fig. 1b shows an adjacent section to that in 1a, here stained with anti-Melatonin (a-Mel) serum as the primary reagent. With the exception of the primary reagent used, tissue processing of the two sections was identical, as was the photographic recording. Note that the areas defined as staining specifically with the a-NAI serum shown in Fig.1a, do not show specific staining when the primary reagent is directed against melatonin. The dispersed staining in other areas of the section is non-specific, by the stated criteria. It is interesting to note that the non-specific background staining with the a-Mel processing, seen in 1b, is less than with the a-NAI processing, seen in 1a. This is not a consistent finding, as will be shown, and serves to illustrate the variability of the technique. Fig. 1c shows the result of treatment of an adjacent section with a-NAI serum which had been pre-incubated (absorbed) with 50mM NAS in the dilution buffer, for approximately ten minutes before application to the section. No specific staining is evident anywhere on the section. Only a uniform, faint glow, also present in tissues that have no treatment whatever, permits visualization of tissue landmarks. Of interest is the observation that the limited non-specific background seen in 1a is absent in this control. This interpretation of specificity vs. non-specificity will be addressed again in the Discussion.

The data presented in Fig. 2 are analogous to those in Fig. 1. Shown are three adjacent control sections of rat hippocampus, illustrating at a medium power of magnification (120x), the localization of NAI-like immunoreactivity in the CA1 region. Again the first figure, 2a, shows the pattern of staining obtained with the a-NAI serum. The key diagram shows SP-CA1 to be about 50 microns wide in this orientation. The most intense staining is contained within this zone, appearing to be in areas 5 microns or less in diameter. Further detail of this localization cannot be seen at this magnification. There is some staining which appears outside the 50 micron band and seems similar to that within SP-CA1. The variability of the presence and distribution of this stain, as well as data from high power examination, identifies this stain as largely, if not exclusively, artifact. The area of unusual appearance in the lower right of the figure is an area of tissue damage, and should be disregarded.

Fig. 2b shows the adjacent section, treated with the melatonin-specific antiserum (a-Mel). SP-CA1 is devoid of any staining. The diffuse glow in stratum radiatum (SR) is not specific, but appears largely due to tissue damage attributable to the acetone dehydration process used in some of this earlier work. The large, orange coloured blot is due to some impurity on the slide or cover slip, and is

Key diagram for Fig. 2a,b,c

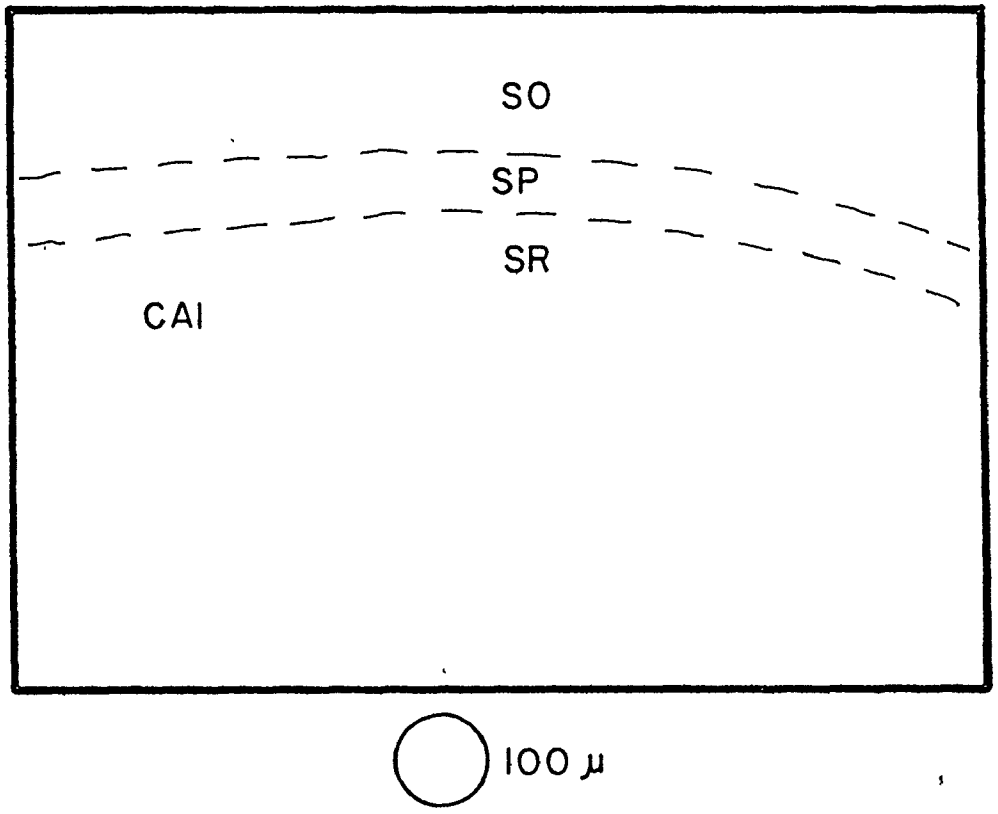
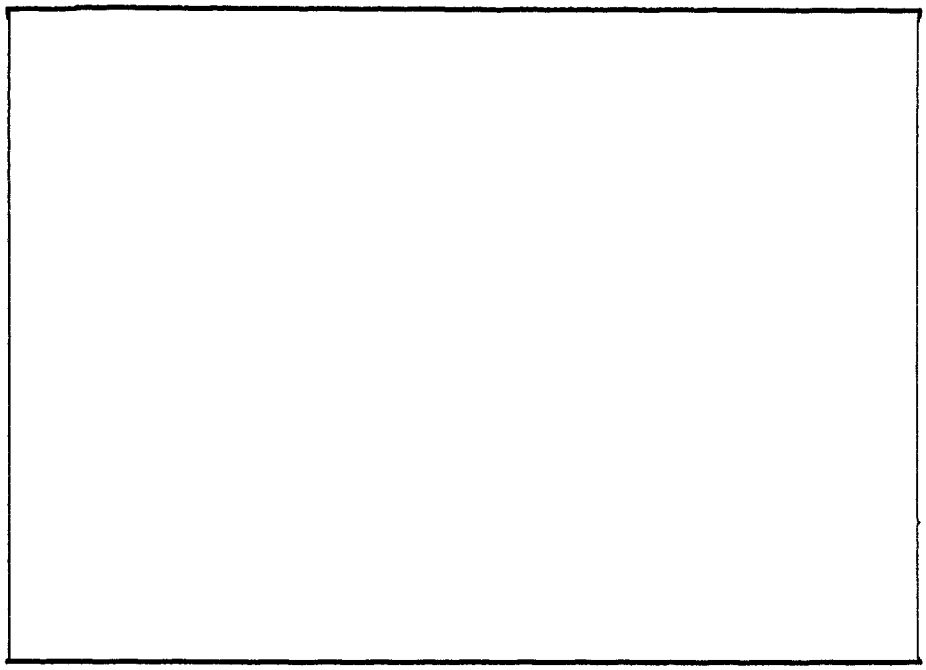
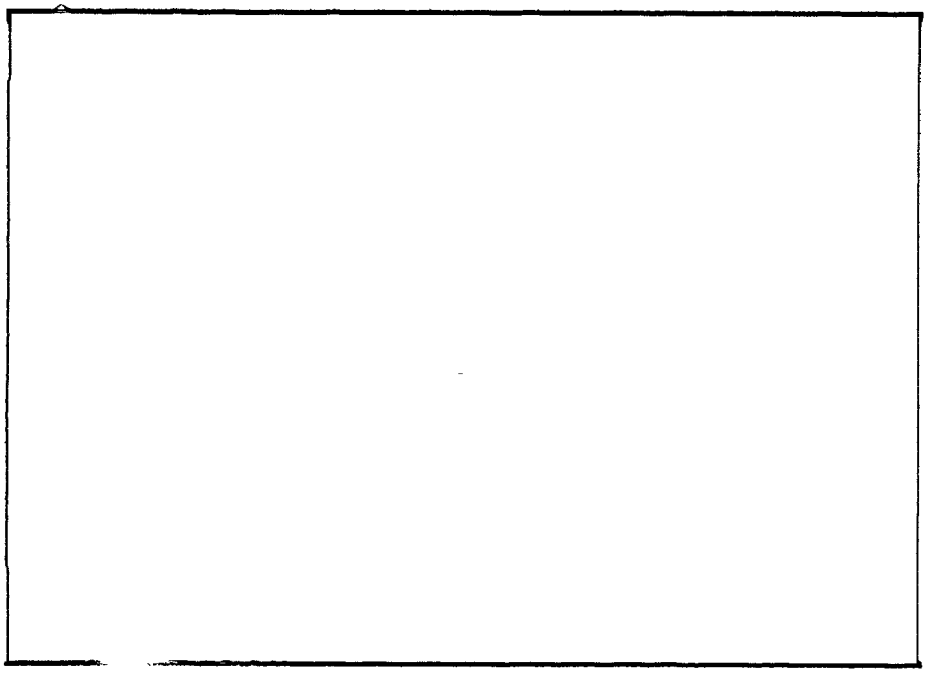


FIGURE 2



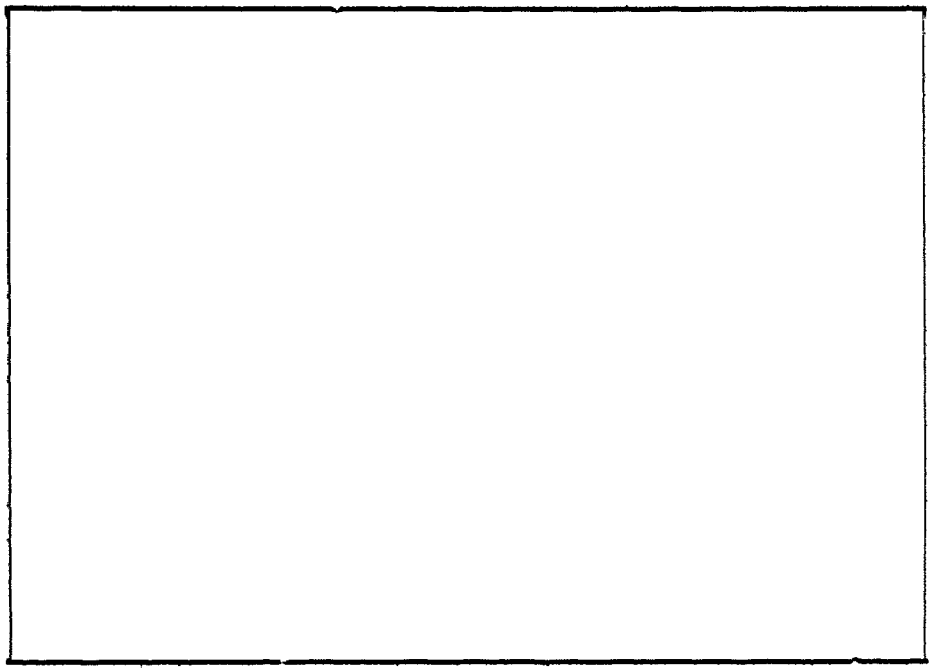
2a. IF of NAI in CAI pyramidal cells



2b. IF of melatonin – CAI

COLOURED PICTURE

FIGURE 2 (cont)



2c. Absorbed anti-NAI, CAI region

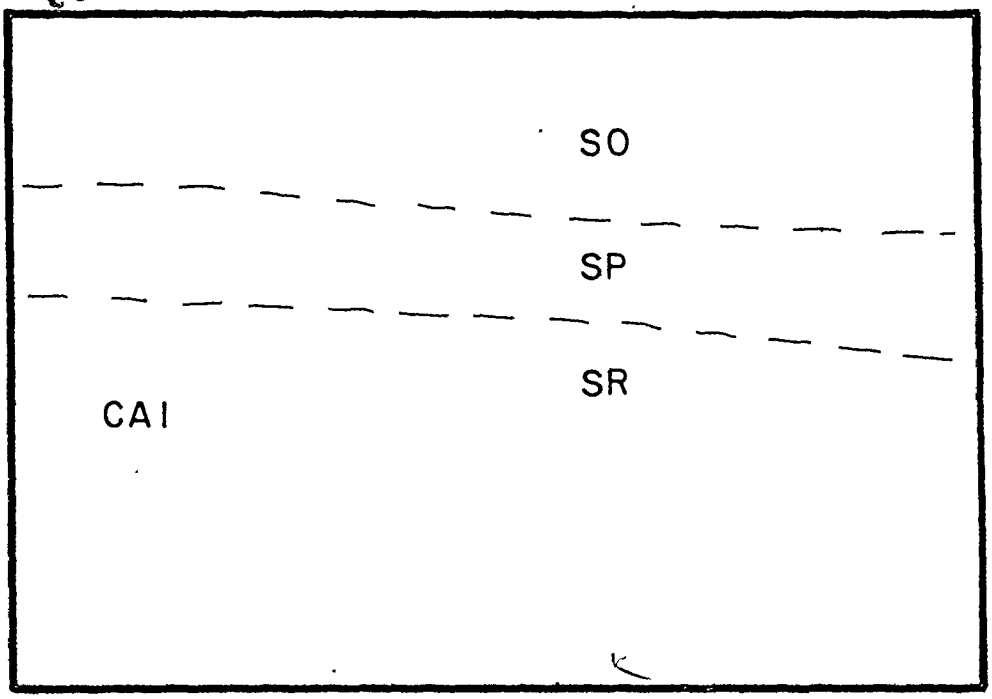
COLOURED PICTURE

entirely out of the plane of focus of either surface of the tissue section.

Fig. 2c again shows an a-NAI stained section, absorbed with NAS. Note the random deposition of fluorescent particles, about 1-2 microns in diameter, throughout the field. These particles have no consistent association with any structures or tissue areas, and vary from section to section. They are often invisible at lower magnifications (see Fig. 1c).

The pair of micrographs in Fig. 3 shows some of the details of localization of the specific staining in SP-CA1. The 50 micron wide SP-CA1 passes through Fig. 3a horizontally, just above the midline. The clearest distribution is associated with two CA1 pyramidal cells approximately a third over from the left border of the field. These two cells (ca.12 microns ea.) are surrounded by sub-micron size dots of fluorescence. The individual points can only be resolved when in sharp focus. The large fluorescent patches at the right of the field are likewise made up of individual dots, but appear as larger structures due to the fact that they are not in the same plane of focus as the left hand side of the field. These individual dots of fluorescence occupy structures that are below the resolution of the present technique. There are no

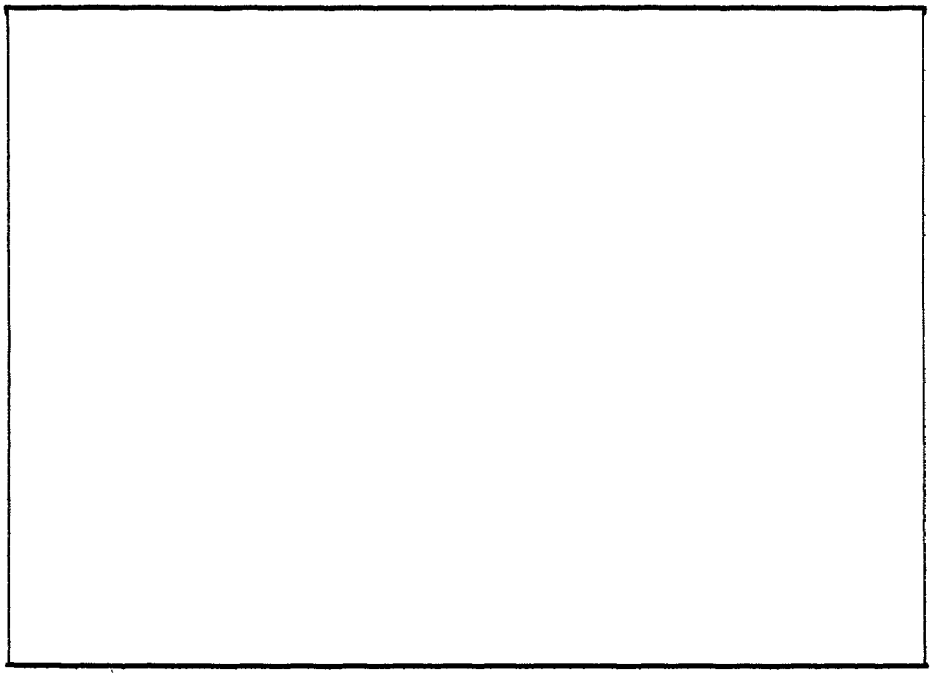
Key diagram for Fig. 3a,b



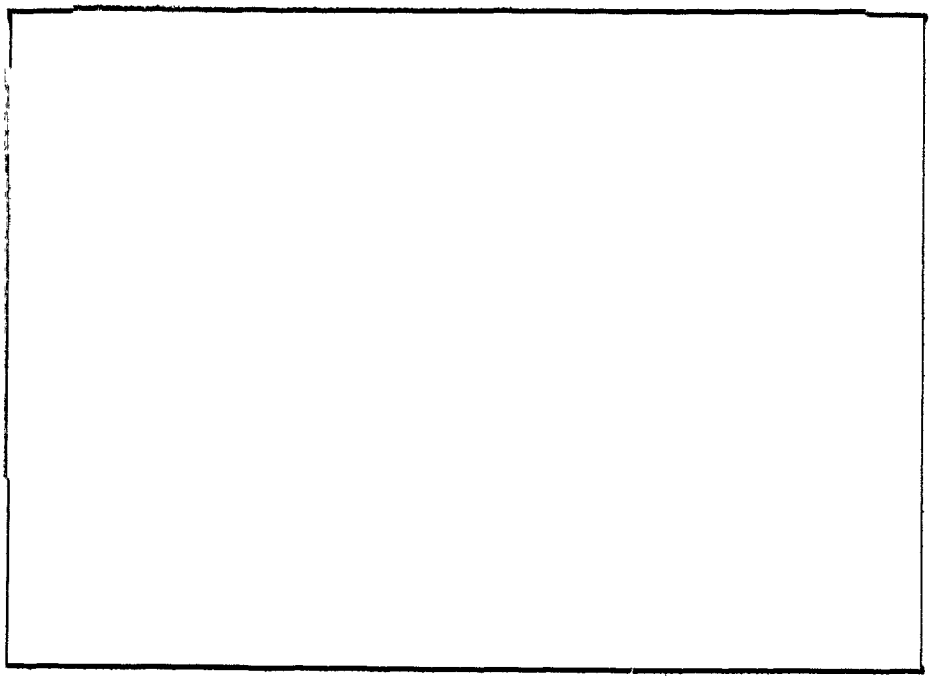
○ 20μ

?

FIGURE 3



3a. Anti-NAI IF , CAI pyramidal neurons



3b. Absorbed control for above

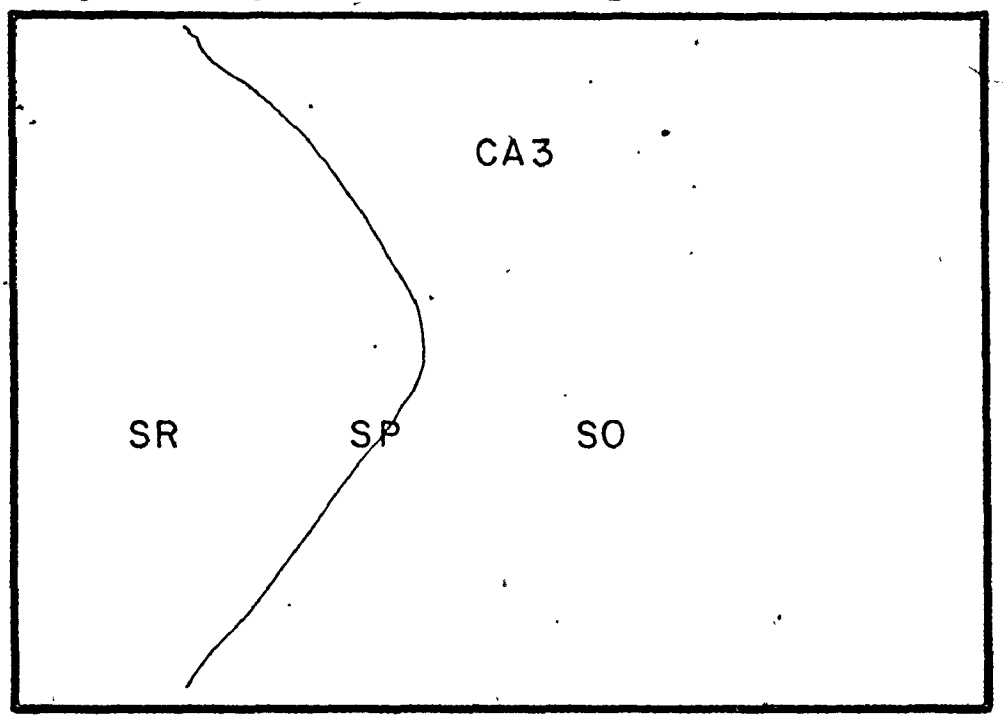
COLOURED PICTURE

observations indicating the presence of NAI immunoreactivity within pyramidal cells. In those instances where structures are sufficiently well preserved, the fluorescence appears to surround the pyramidal cells. No filled cells or fluorescing fibres were found with this technique. The fluorescence in SR is without consistent pattern or substructure, leading to its being defined as non-specific. This fluorescence was also less responsive to absorbtion (see Fig. 3b), thus reinforcing the conclusion about its non-specificity.

Fig. 3b shows an analogous section, treated with absorbed a-NAI as the primary reagent. Arranged along the horizontal midline are several identifiable cell bodies of pyramidal neurons of CA1, none of which have any associated fluorescence. Note the presence of non-absorbable fluorescence in SR, below.

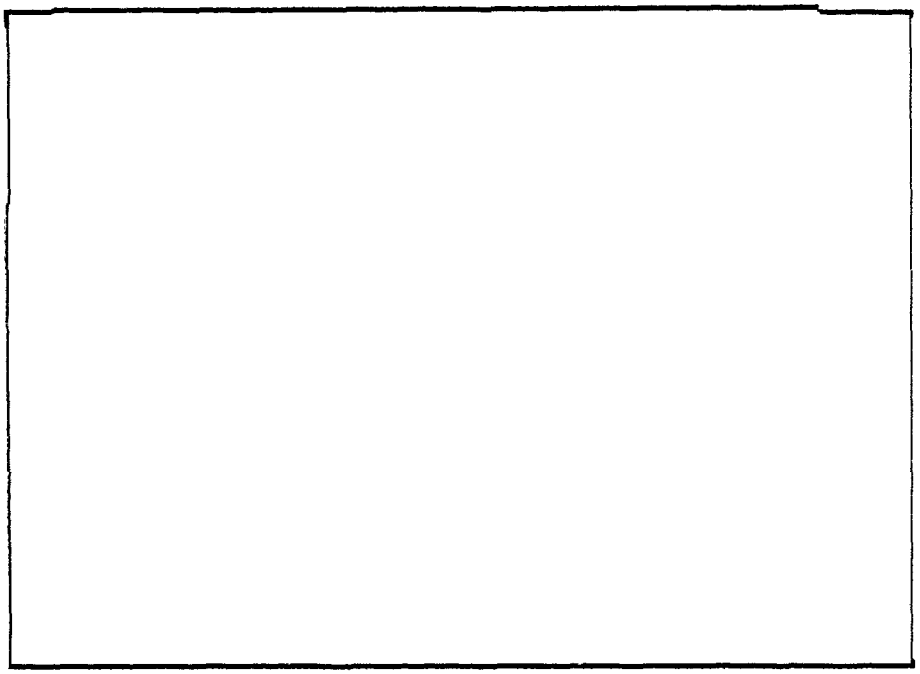
Fig. 4 shows the CA3 area at intermediate magnification. Refer to the key diagram for orientation. Fig. 4a shows the result of staining with a-NAI serum. The CA3 pyramidal cells have associated with them, fluorescent dots about 2 microns in diameter. These dots appear around the cell bodies, and in this figure, on the border between SP and stratum oriens(SO). With the exception of this staining of the SP/SO border, the staining patterns in CA1

Key diagram for Fig. 4a,b

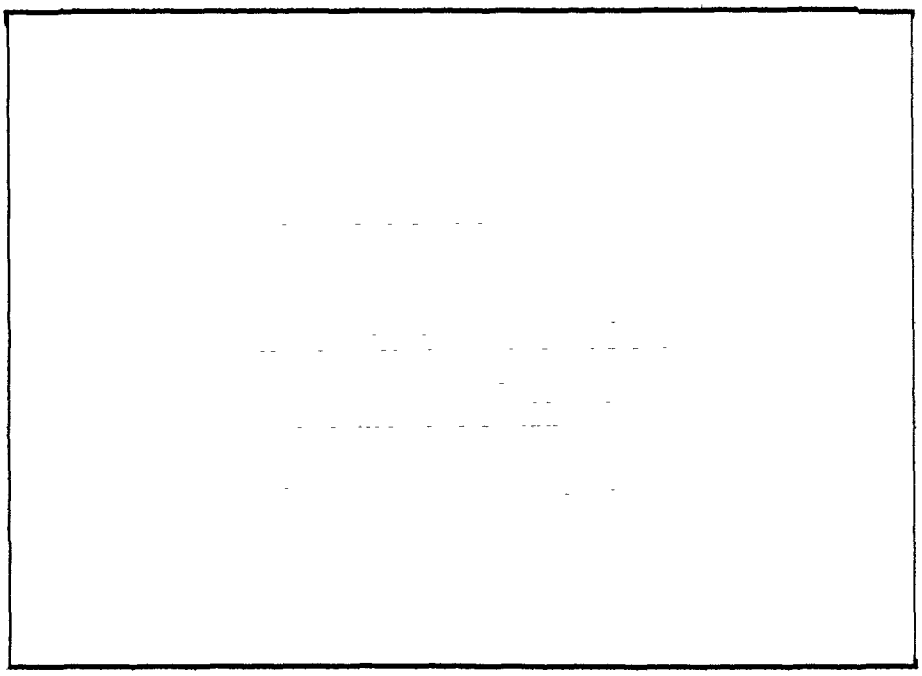


○20 μ

FIGURE 4



4a. CA3 pyramidal layer , anti-NAI IF



4b. Absorbed control

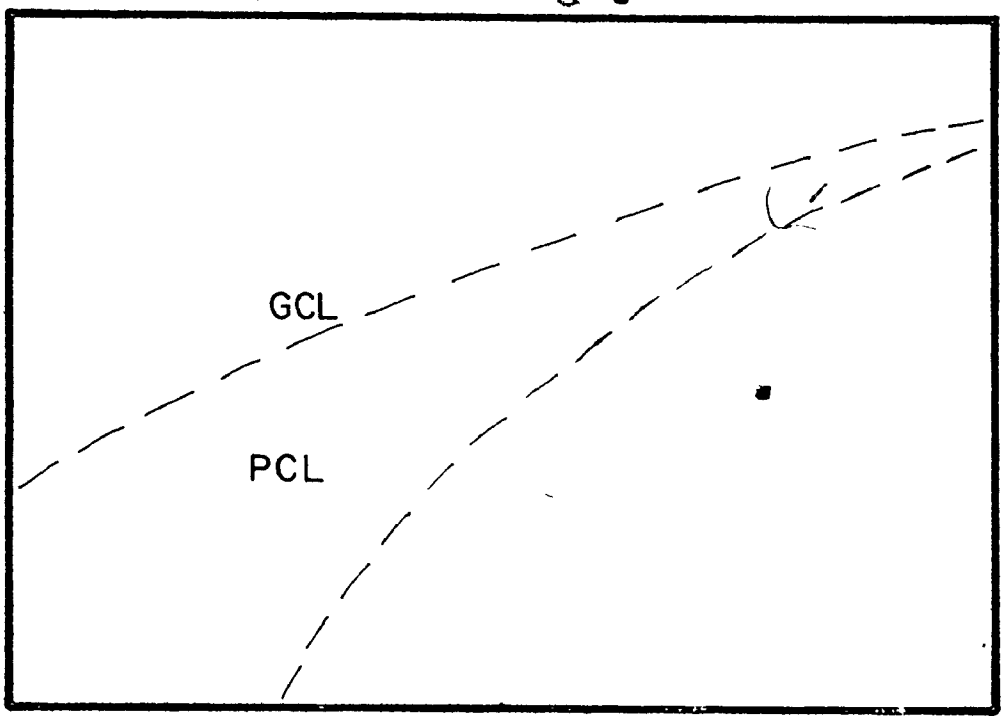
COLOURED PICTURE

and CA3 are identical. A non-specific staining appears in SO and SR as variably sized dots of fluorescence, with no constant arrangement and with inconsistent presence. Fig. 3b, stained with pre-absorbed a-NAI serum, shows some remaining glow in SP-CA3 and non-specific deposits remaining in all layers, seen here with variable size and random distribution.

Fig. 5 shows intermediate magnification views of the CA4/dentate area. Fig. 5a shows the result of staining in this area using a-NAI serum. The staining is confined to the polymorph cell layer in the hilus of the dentate, extending slightly into the granule cell layer. The granule cells of CA4 are structurally, though not functionally, analogous to the pyramidal cells in CA1 and CA3. In this figure, the stain appears as small dots of fluorescence, 1-2 microns in diameter. No fluorescence-filled cells were seen. This figure is relativly free of non-specific stain deposits, illustrating the variability used to define staining non-specificity.

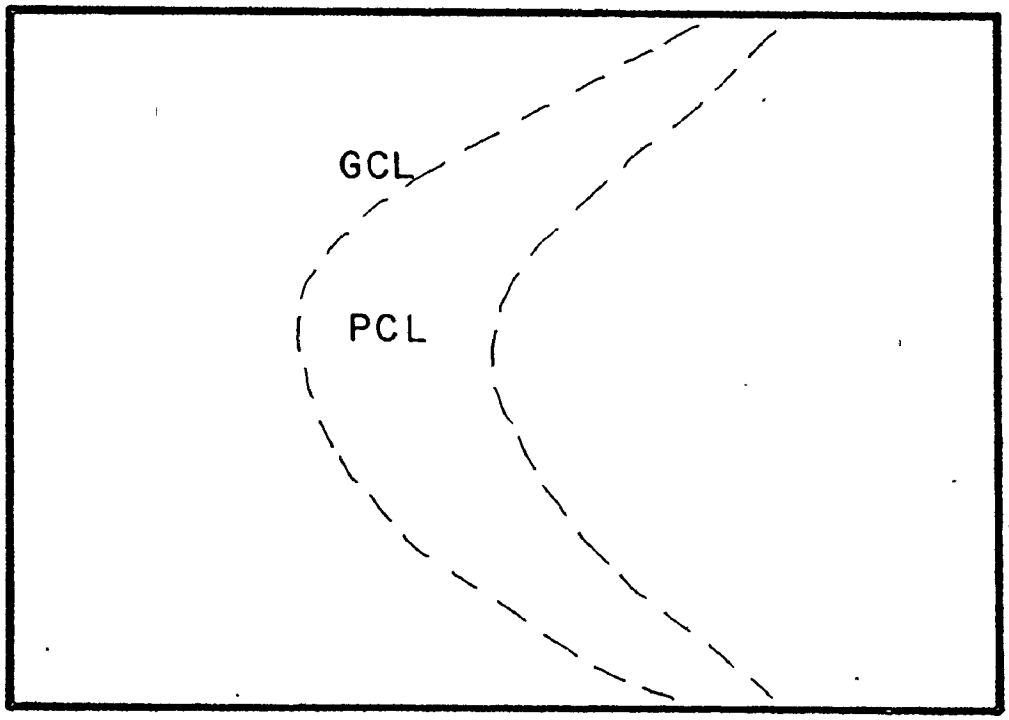
Fig 5b shows the absorbed control to 5a. Although a faint glow remains in the area of specific staining of 5a, its intensity is markedly diminished. Very few distinct points of fluorescence are seen. A random, non-specific deposition of FITC is seen throughout the section, showing

Key diagram for Fig. 5



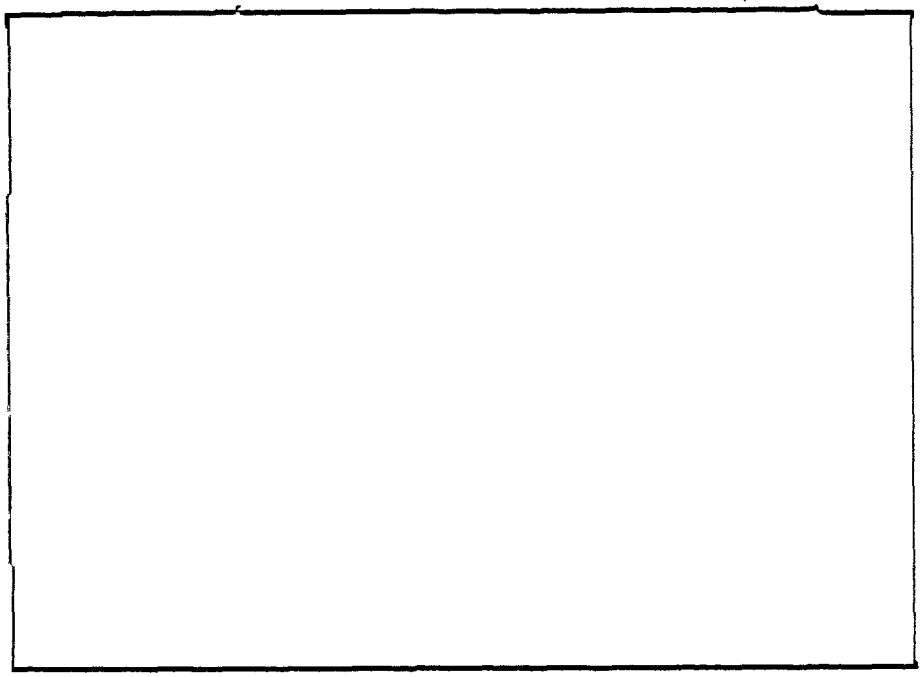
○ 20 μ

a

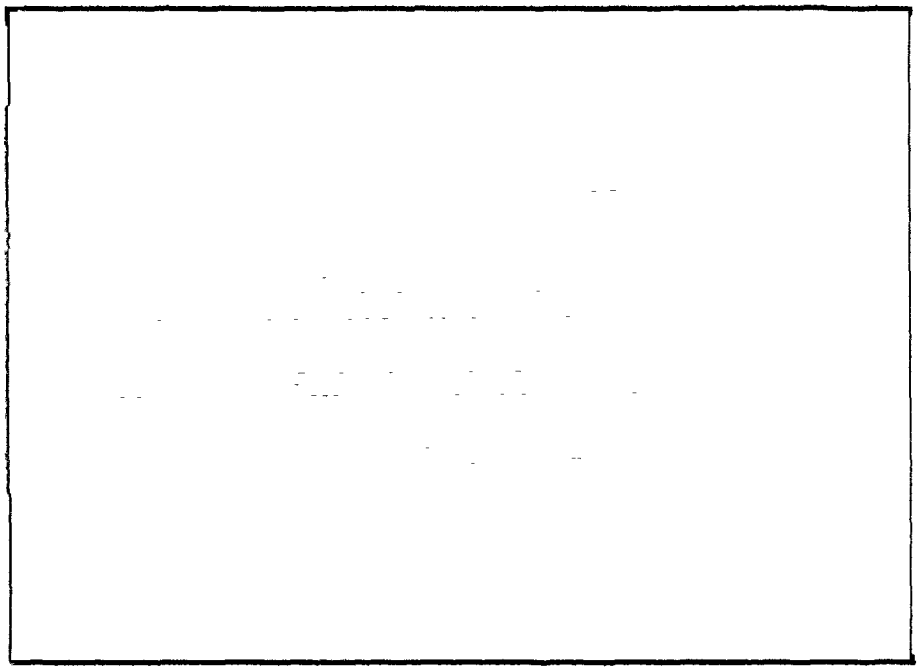


b

FIGURE 5



5a. Dentate gyrus, anti-NAI IF



5b. Control section, NAS-absorbed

COLOURED PICTURE

the variability of this phenomenon.

Fig. 6 is a high magnification micrograph of the CA4/dentate area, showing mainly the polymorph cell layer. The cells of this layer can been seen as dark circular shapes, varying in size from approximately 2-10 microns. The specific fluorescence in this area consists of single dots, 1-2 microns in diameter, sometimes coalescing to larger areas of fluorescence. These larger areas remain resolveable as several separate dots with through-focussing. There are some observations in the polymorph cell layer that cell bodies may be filled with specific stain. This layer consists of many cell types in irregular arrangements, so no ordered gross pattern of staining is expected. The stain-filled cells are not found with high frequency, representing perhaps 1%(subjective) of the cells in this layer. No fine structural observations are available which might identify these cell-like structures further.

Specific a-NAS antisera: Fluorescence:

Fig. 7 shows a pair of micrographs depicting SP-CA1, stained using the specific a-NAS serum as a primary reagent. This is in many ways a duplication of the previous work with the bispecific a-NAI, and was done largely as a test measure for the newly available specific antisera. The overall

Key diagram for Fig. 6

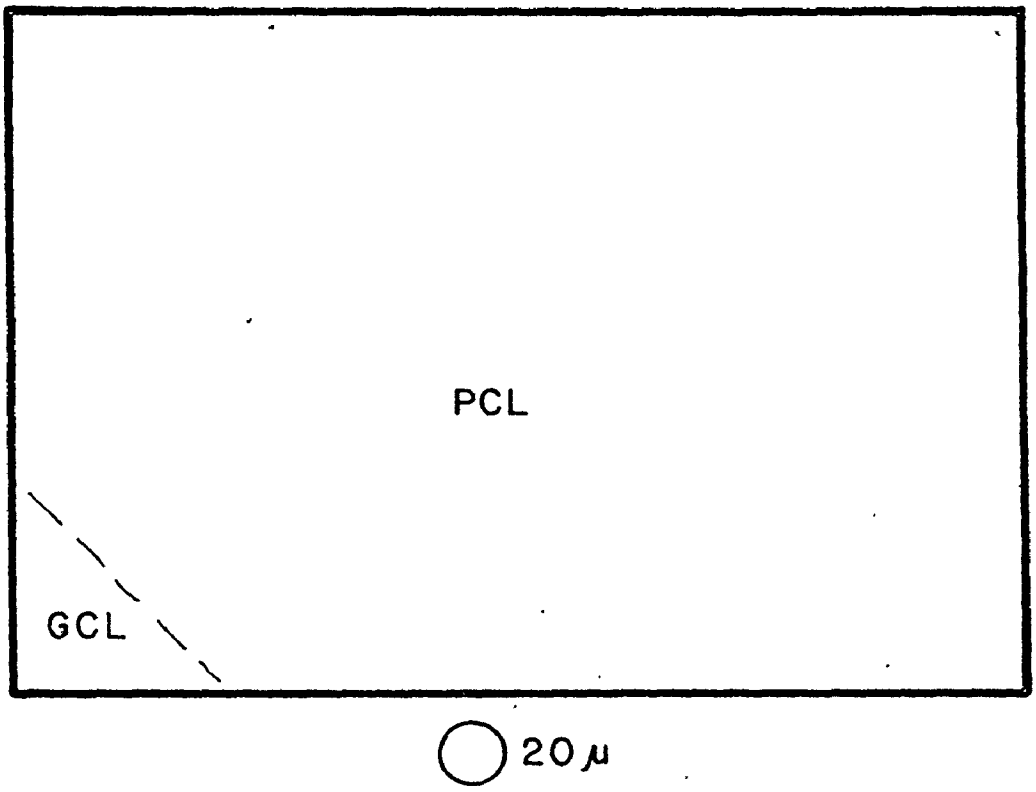
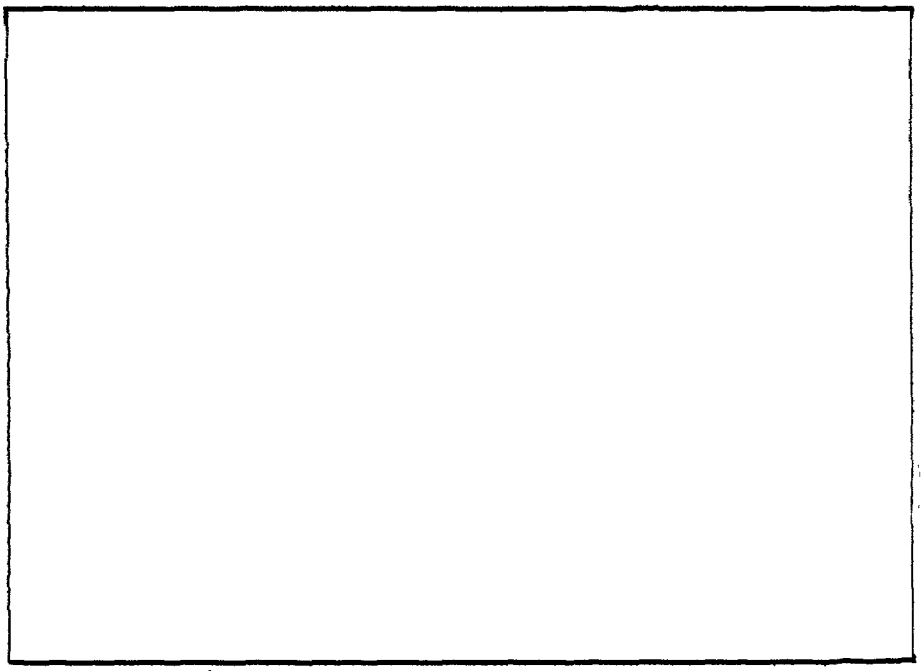


FIGURE 6



6. Dentate , anti-NAI , detail

COLOURED PICTURE

Key diagram for Fig. 7

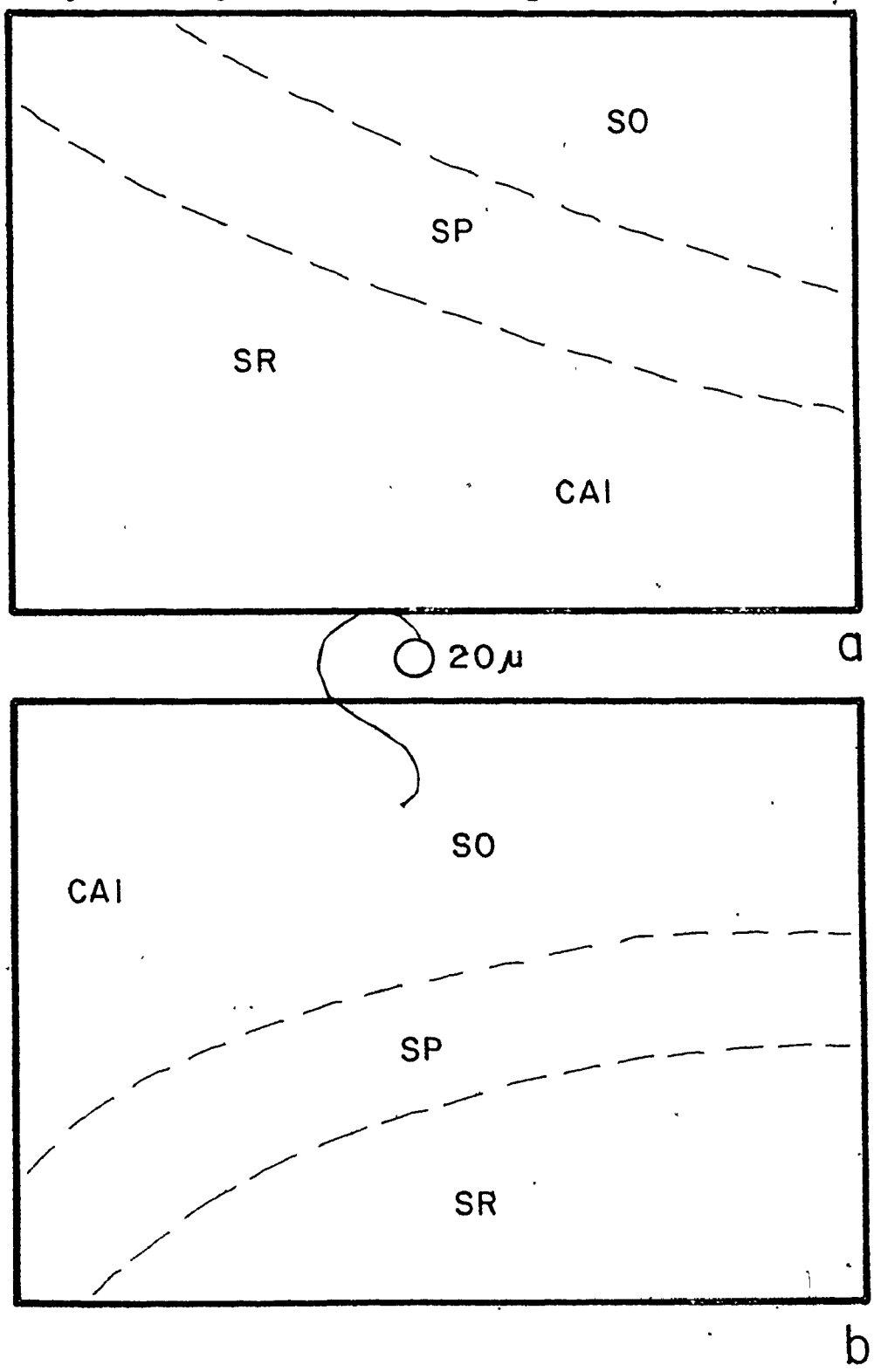
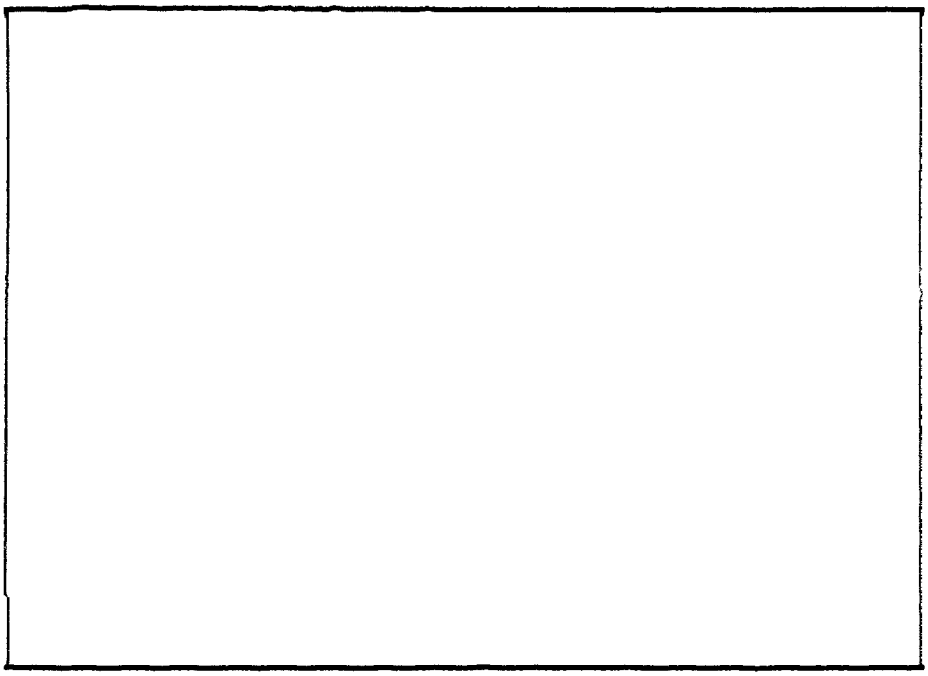
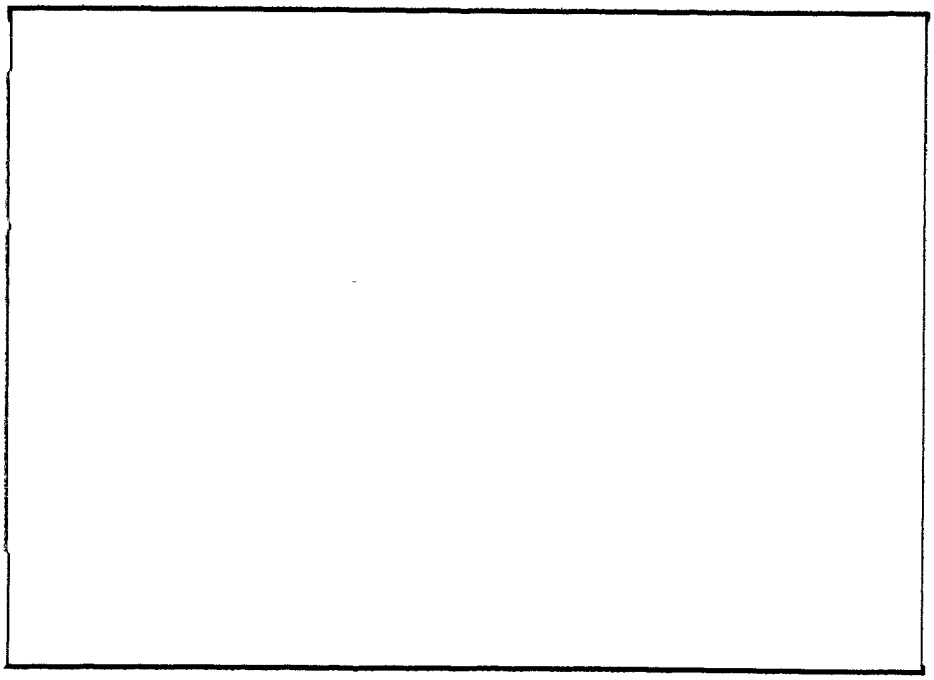


FIGURE 7



7a. Specific anti-NAS IF, CAI



7b. Absorbed control

COLOURED PICTURE

background tissue glow is higher in this pair of micrographs, due to a slightly thicker than usual section. The pyramidal cells appear as dark profiles, 8-10 microns in diameter. The specific staining can be seen as 1-2 micron diameter fluorescent dots, located within SP, but outside, and apparently surrounding, the pyramidal cells themselves. Occasionally, a group of these dots would appear as a single, larger structure, but accurate focussing could always resolve the separate entities making up the staining. No stain-filled cell like structures were seen in this area. There is no specific staining in SR below, or SO above. Fig. 7b shows the NAS-absorbed control in a slightly different orientation from 7a(see key). There is no punctiform staining in SP. The orange-coloured dots appearing in this micrograph are endogenous neural pigments and have no association with immunohistologic processing (U. DeBoni, personal comm).

Fig. 8 shows a medium power magnification view of specific NAS-immunoreactive(NASI) staining in SP-CA1 and a corresponding absorbed control. In 8a, many of the pyramidal cells of SP-CA1 are in the plane of focus. While none of these cell bodies stain, the spaces between them are well filled with specific staining. This staining appears again as small (1-2micron) points of fluorescence, here often coalescing into larger fluorescent structures that

Key diagram for Fig. 8

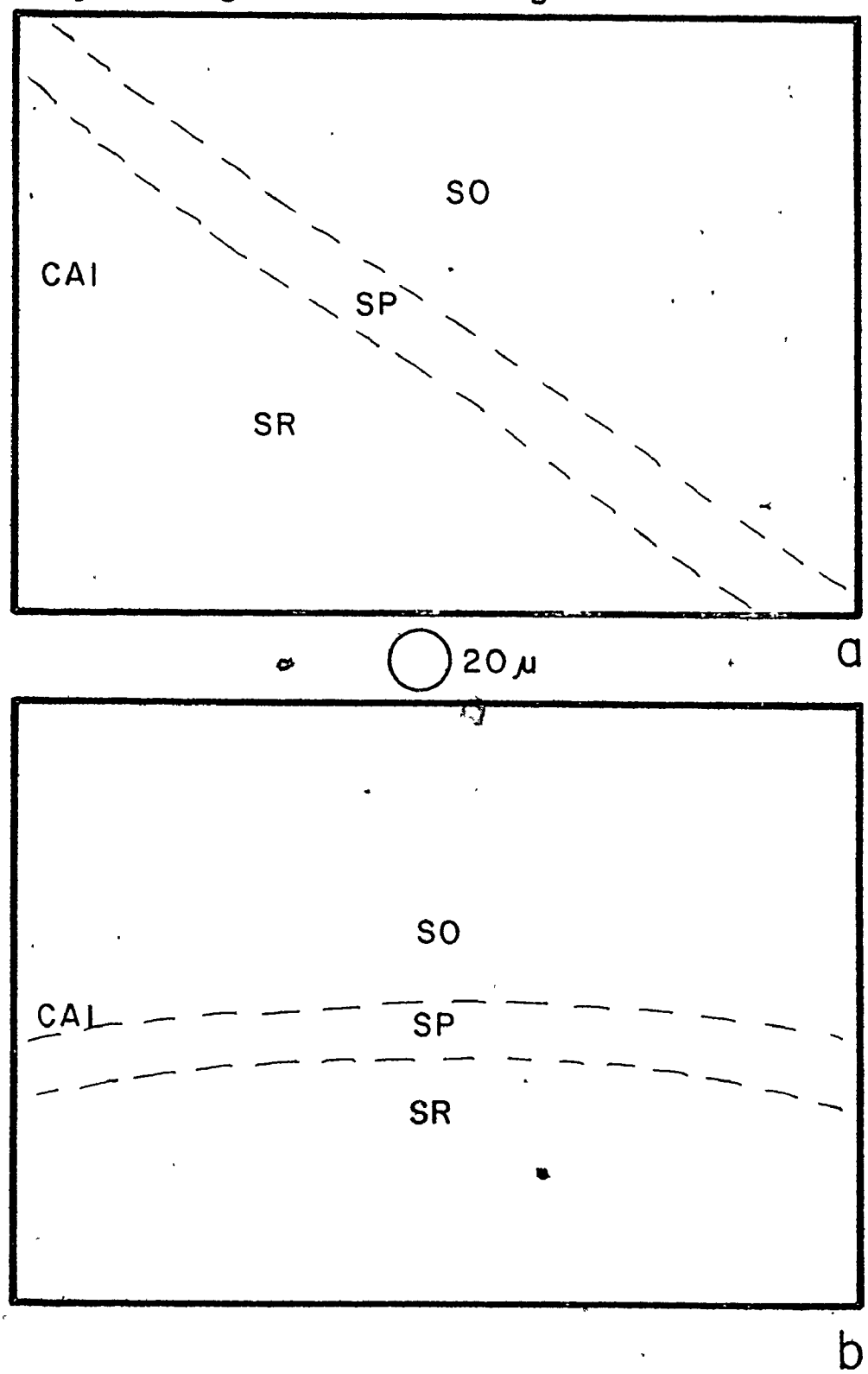
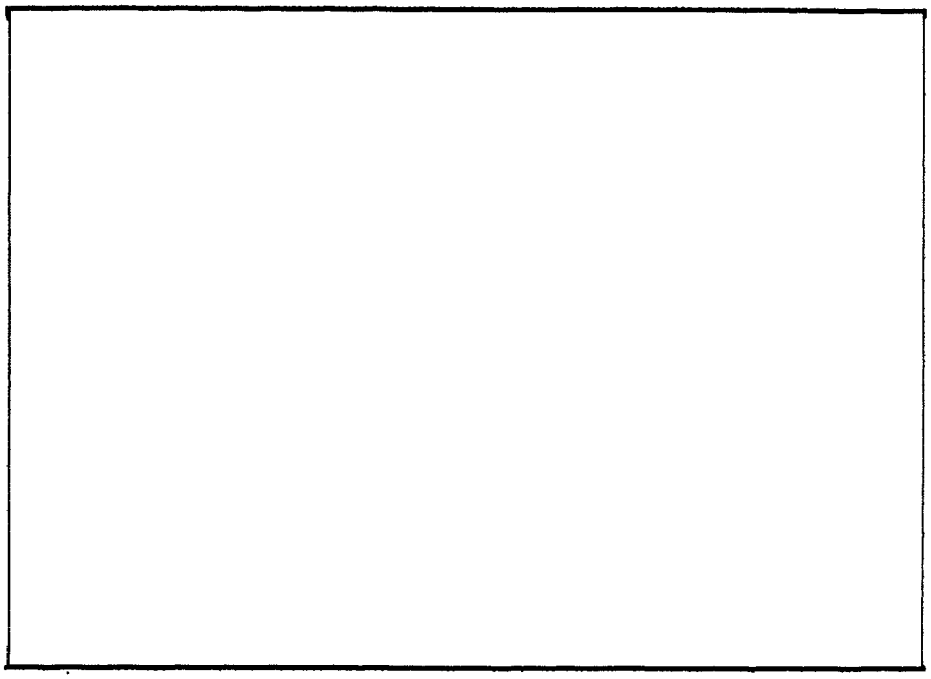
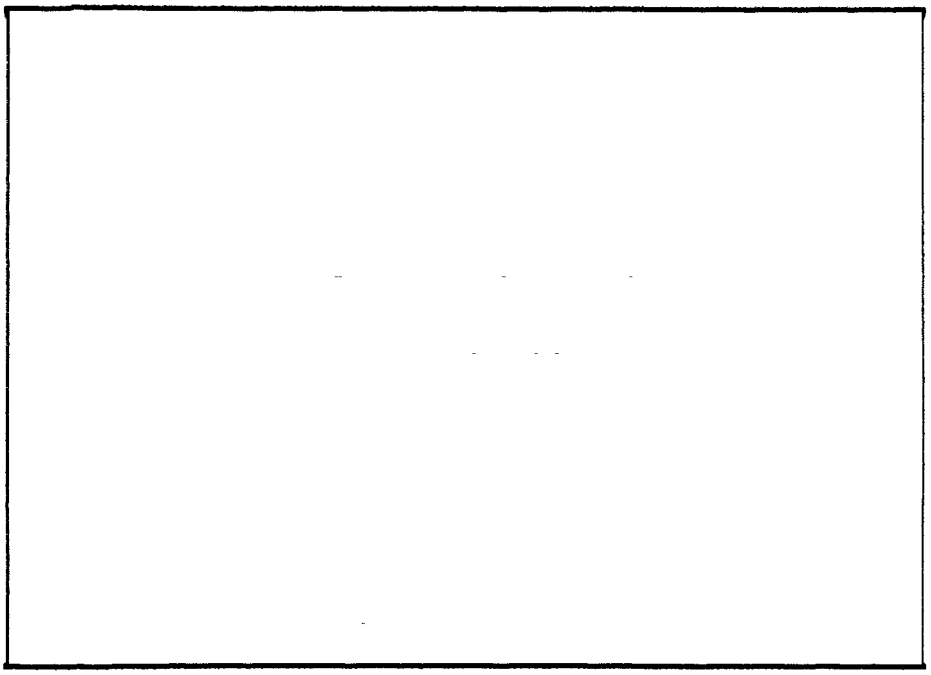


FIGURE 8



8a. CAI pyramidal layer, anti-NAS



8b. NAS-absorbed control

COLOURED PICTURE

remain resolveable into their components by through-focussing. SO, above, and SR, below, have limited non-specific staining. Fig. 8b shows the NAS-absorbed control section. Some staining in SP resembling that in the immune section remains, but its intensity is far below that in the immune section. This seems to be the result of incomplete absorbtion, a regularily observed occurence in areas of high tissue concentrations of antigen. At the low intensity of staining seen in this incompletely absorbed section, a discrete, fine punctiform staining around the pyramidal cell bodies is apparent. This remaining, presumably specific, staining pattern reinforces the conclusions about the distribution that are obtained from the heavily stained immune slides, in which fine details are difficult to photograph.

Fig. 9a shows the staining in SP-CA3 using the specific a-NAS serum. The overall background glow is high due to the thicker than usual section (15 micron). The cell bodies of SP are readily discernable, and do not appear to be stain-filled. The specific staining is manifest as submicron fluorescent points which surround pyramidal cell bodies in significant numbers. There is no specific staining in SO or SR, and non-specific staining , excluding background glow, is negligible over the entire section. Fig. 9b shows the NAS-absorbed control. There is no stain

Key diagram for Fig. 9a,b

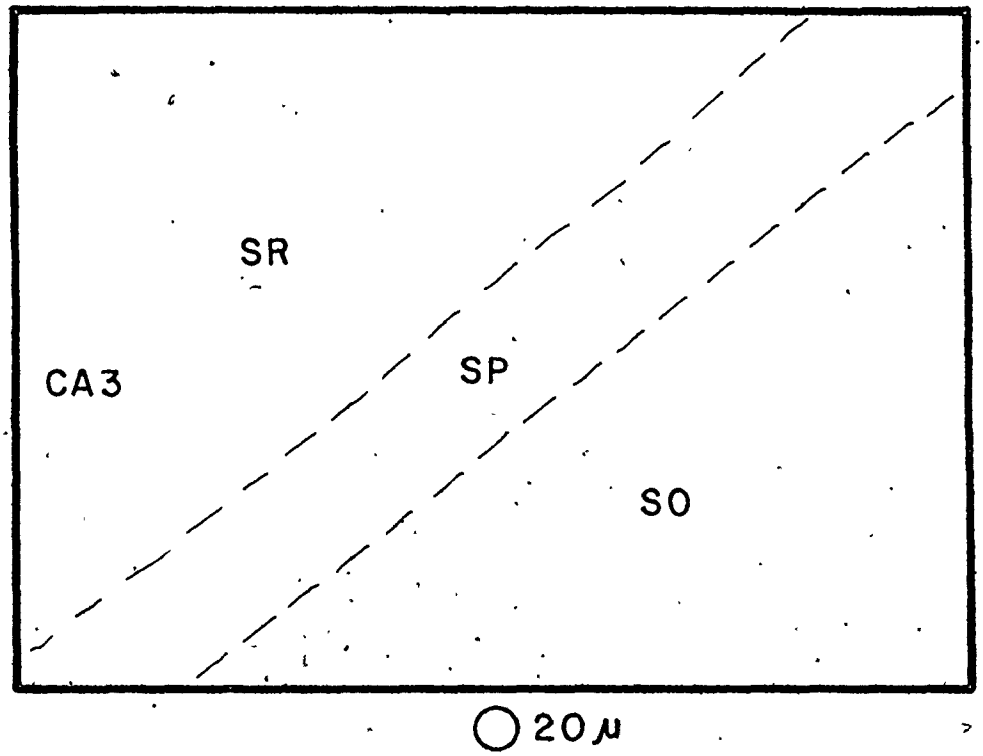
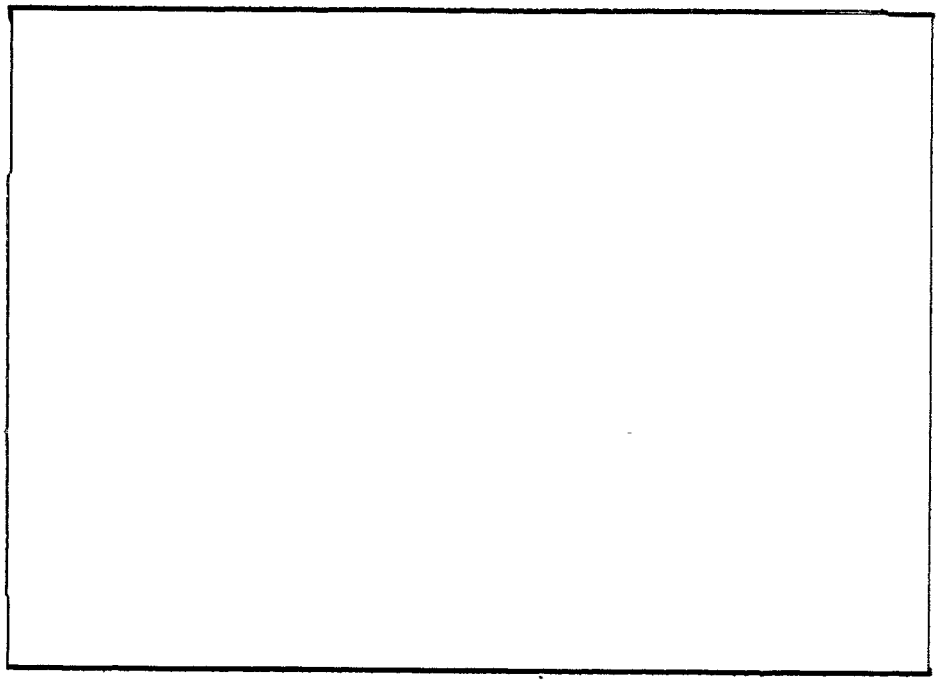
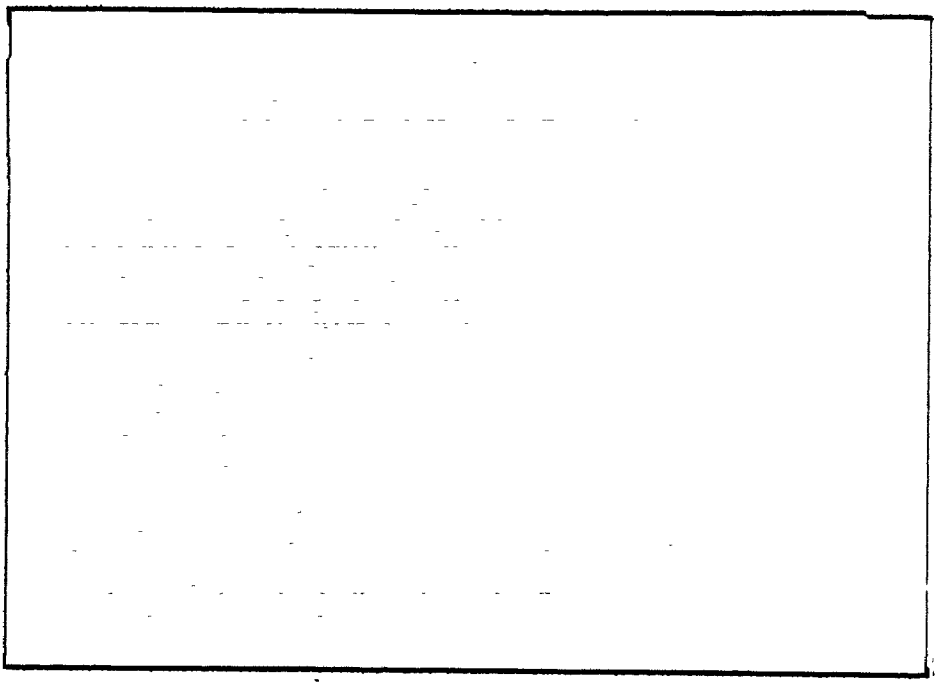


FIGURE 9



9a. CA3, anti-NAS



9b. NAS-absorbed control

COLOURED PICTURE

surrounding the pyramidal cells of CA3. Other than background glow, there are only very few deposits of stain material, readily identified as non-specific by the stated criteria.

Fig. 10 illustrates, at medium magnification, the staining by the specific a-NAS serum in the CA4/dentate region. A large portion of the polymorph cell layer is shown in the immune serum treated section depicted in 10a. The staining is mostly in the form of very small, discrete packets of fluorescence which appear in the spaces among the readily seen larger cells of the polymorph cell layer. None of these larger cells appear stain-filled. A small amount of stain appears in the granule cell layer in the upper left of the figure. Of special interest are the several groups of fluorescence appearing in the lower half of the figure. There are several fluorescing structures which may be small, stain-filled cell bodies. The two large fluorescent collections, just below the horizontal midline are artifacts. Overall non-specific staining is very light. There is also evidence of fibres in this area, as several fluorescent dots appear in a line, without any visible structure constraining them to this configuration. Resolution of these structures, putative cells and fibres, is not possible using the fluorescence technique.

Key diagram for Fig. 10a,b

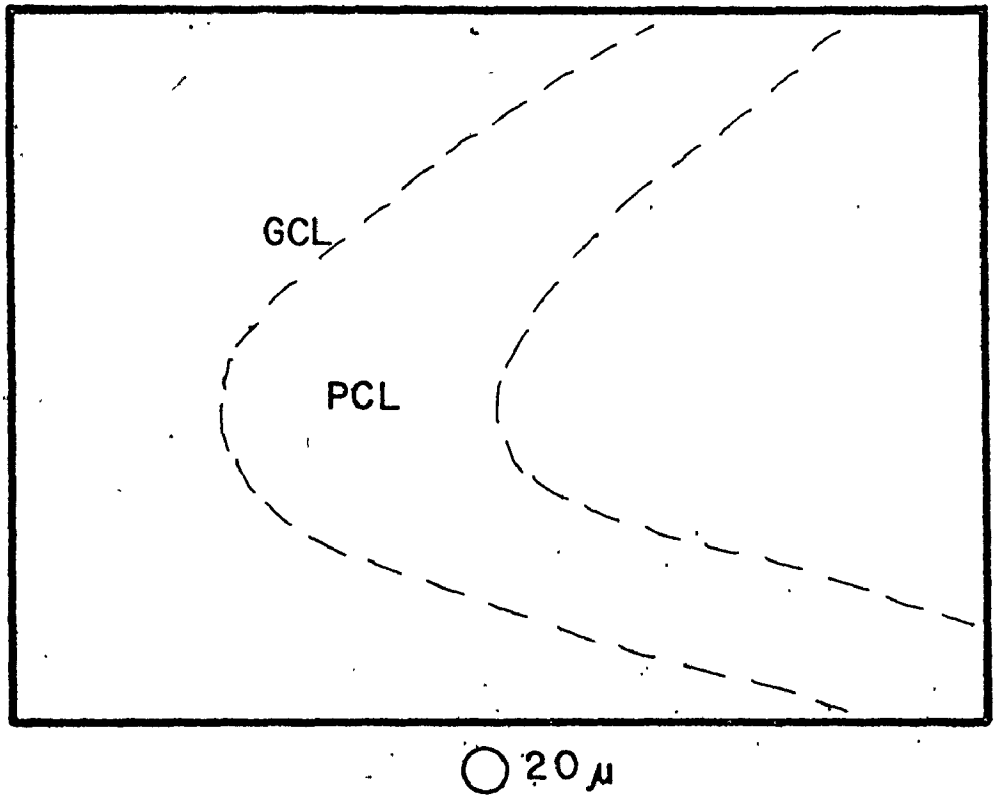
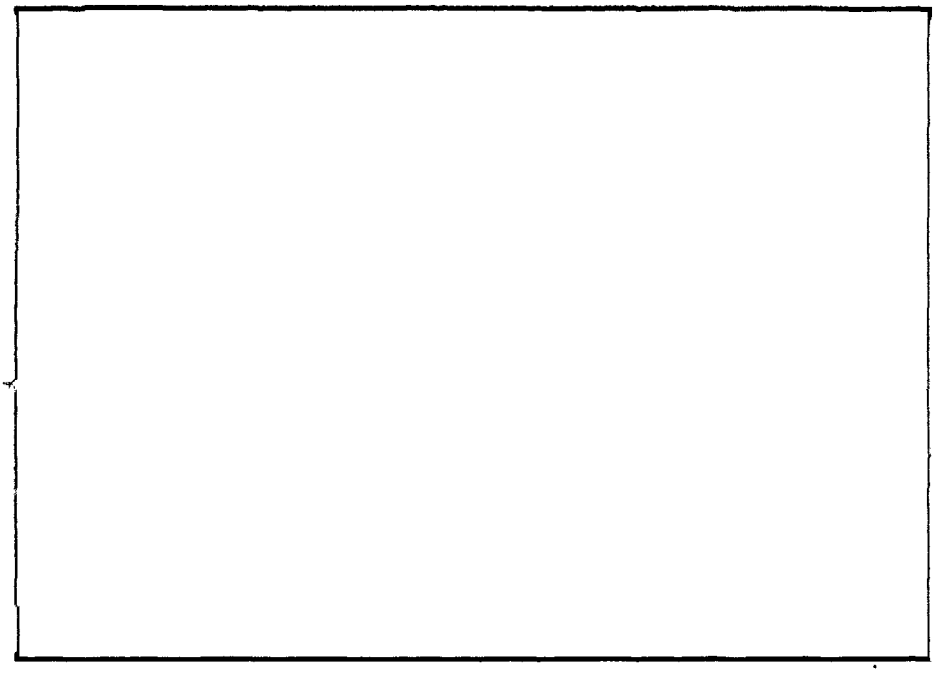
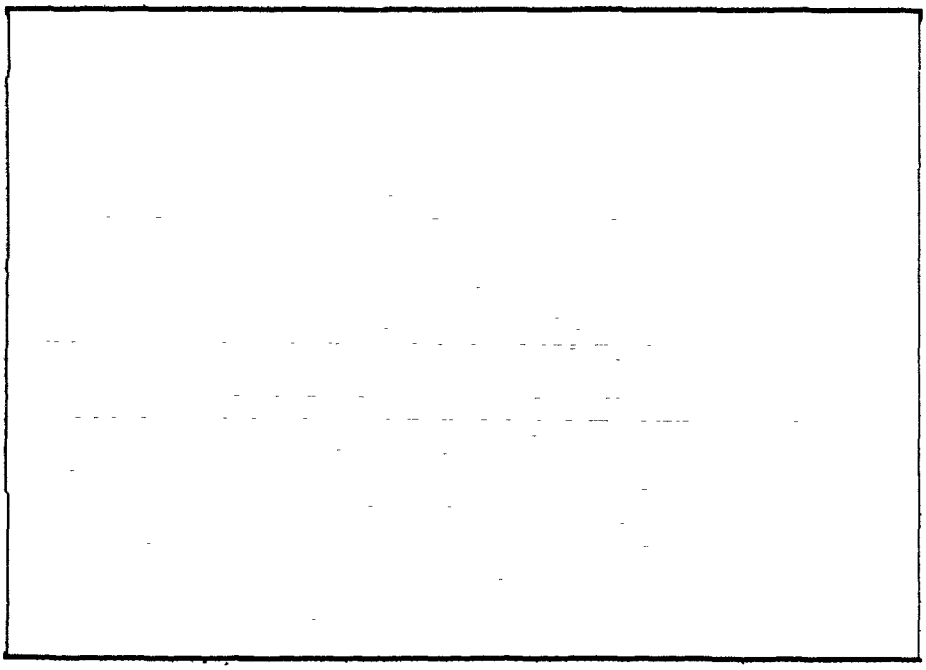


FIGURE 10



10a. Dentate gyrus, anti-NAS IF



10b. NAS-absorbed control

COLOURED PICTURE

Fig. 10b shows the NAS-absorbed control for 10a. The right hand portion of the figure shows tissue damage. Note a marked diminution of staining in all areas described as having specific staining in 10a. No cell-body sized structures appear fluorescent. Some of the inter-cell punctiform staining persists, although it is far less intense in the control figure.

Protein-A Peroxidase as a secondary reagent:

Protein-A/Peroxidase(PrAP) was prepared as described in the Methods section. Both major components in this coupling reaction have molecular weights approximating 40K. The coupling procedure involves blocking the amino groups on peroxidase (Nakane and Kawaoi, 1975). The sugars on the enzyme molecule are then oxidized to aldehydes, which will react with amino groups. Since no amino groups remain on the peroxidase, the only condensation reaction which occurs is between the aldehyde on the peroxidase and the amino group on the Protein A. The reaction mixture was optimized to encourage a 1:1 reaction, and SDS polyacrylamide gels were used to test the molecular weight of the products. The major product has a molecular weight of approximately 80K. This confirms that the major product is a 1:1 conjugate of protein-A and peroxidase. This conjugate was used as a secondary reagent in the following immunohistologic studies.

Staining with PrAP and specific a-NAS:

Fig. 11 shows, at low magnification, the result of staining using PrAP and the specific a-NAS serum. In Fig. 11a, the stain can be seen to be associated with SP-CA1, although details of the staining are not apparent. The stain in the corpus callosum is non-specific as are the large patches of low density staining in SR. Fig. 11b shows the adjacent control section, using a primary reagent pre-absorbed with NAS. Although SP-CA1 can be located, there is no stain associated with it . Note the staining in the corpus callosum, which is largely unaffected. This artifact persists even if no primary reagent is used, so is readily identifiable as non-specific. The large dark area at the right of the figure is an area of damaged tissue.

A higher magnification view, as seen in Fig. 12a, stained here with a-NAS serum, shows the stain to be localized in small (1-2micron) structures, often in groups, but never filling an identifiable pyramidal cell. Pyramidal cells appear as pale structures, 8-12 microns in diameter. There is some deposit of stain in SO and SR, but the variability of this observation makes a definitive statement regarding its specificity difficult. Fig. 12b shows the NAS-absorbed control for 12a. The staining in SP-CA1 is nearly eliminated, as is that over SO.

Key diagram for Fig. IIa,b

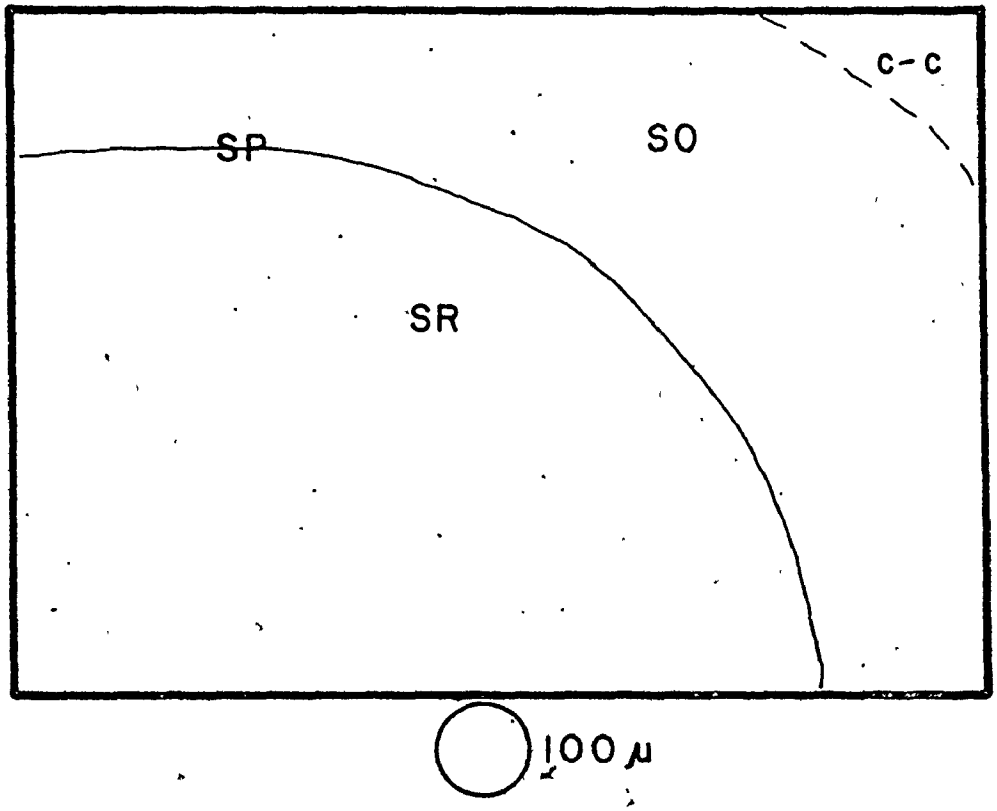
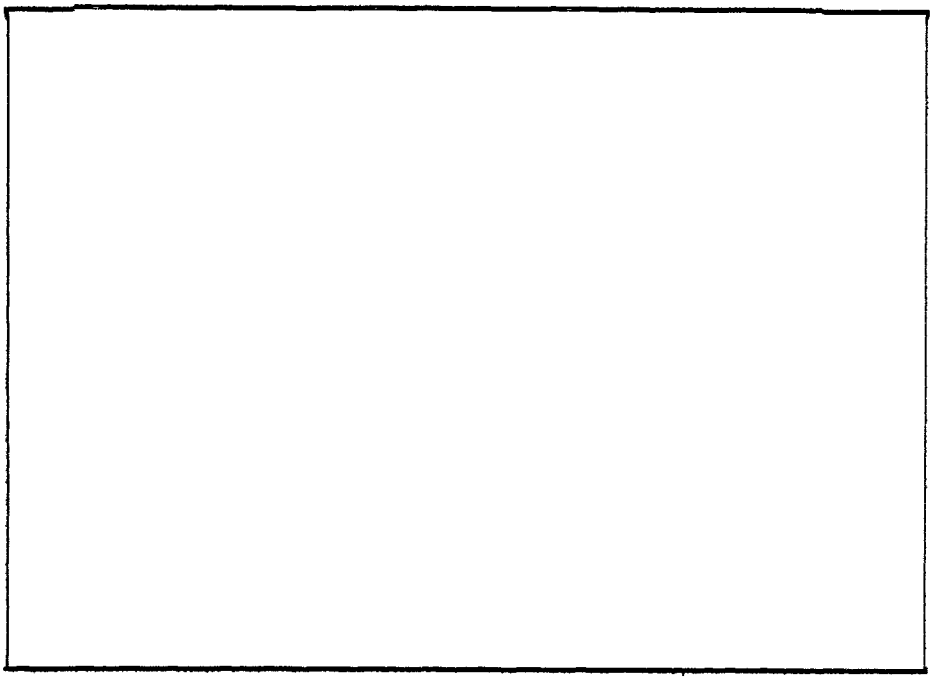
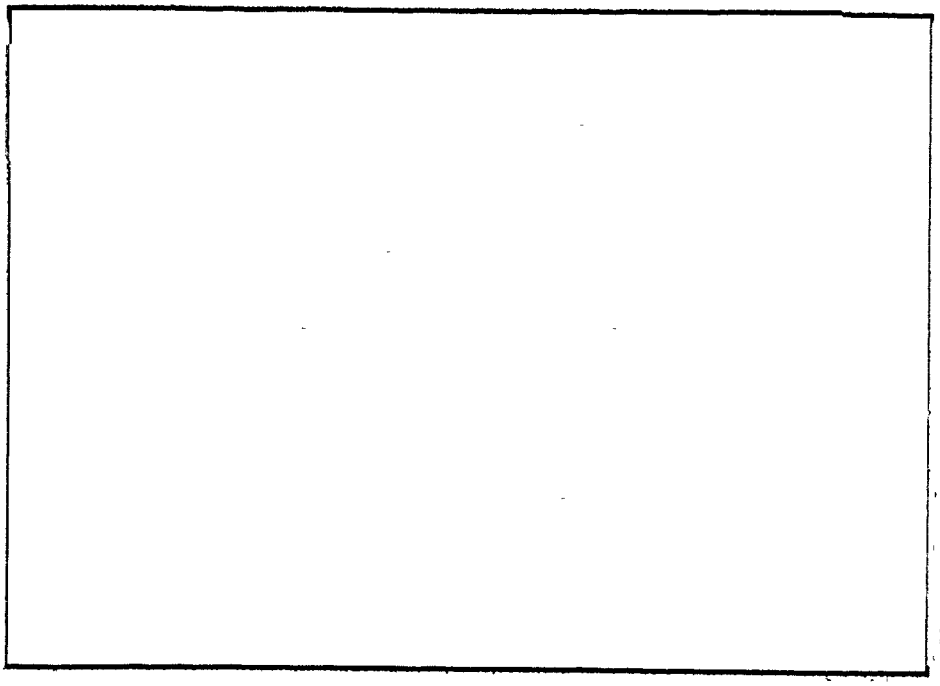


FIGURE II



IIa. Peroxidase-labelled anti-NAS , CAI



IIb. Control , NAS - absorbed

COLOURED PICTURE

Key diagram for Fig. 12a,b

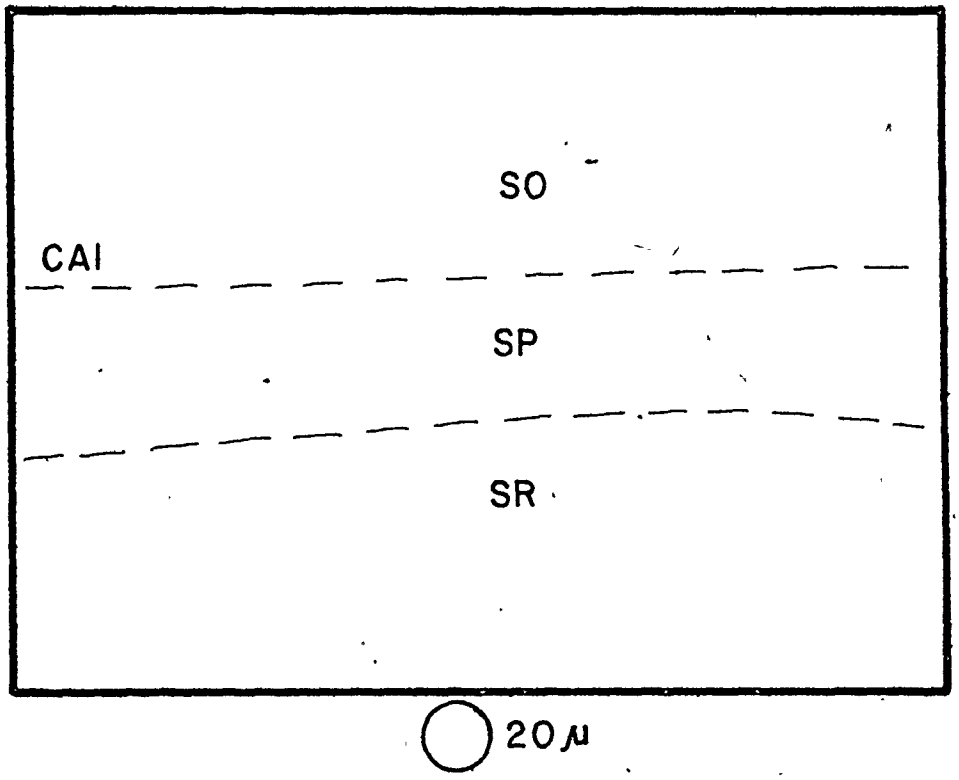
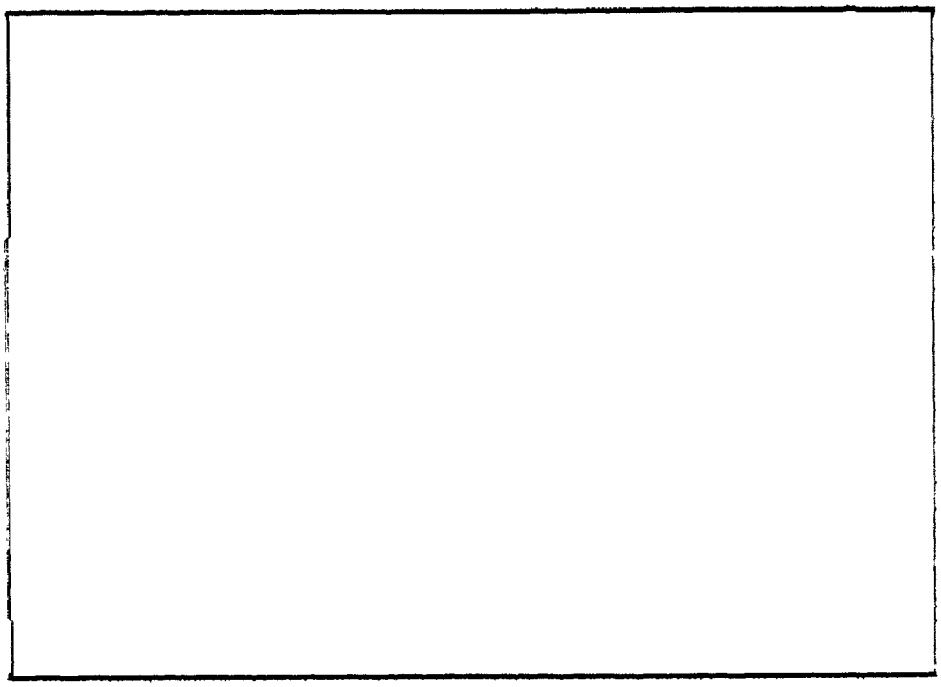
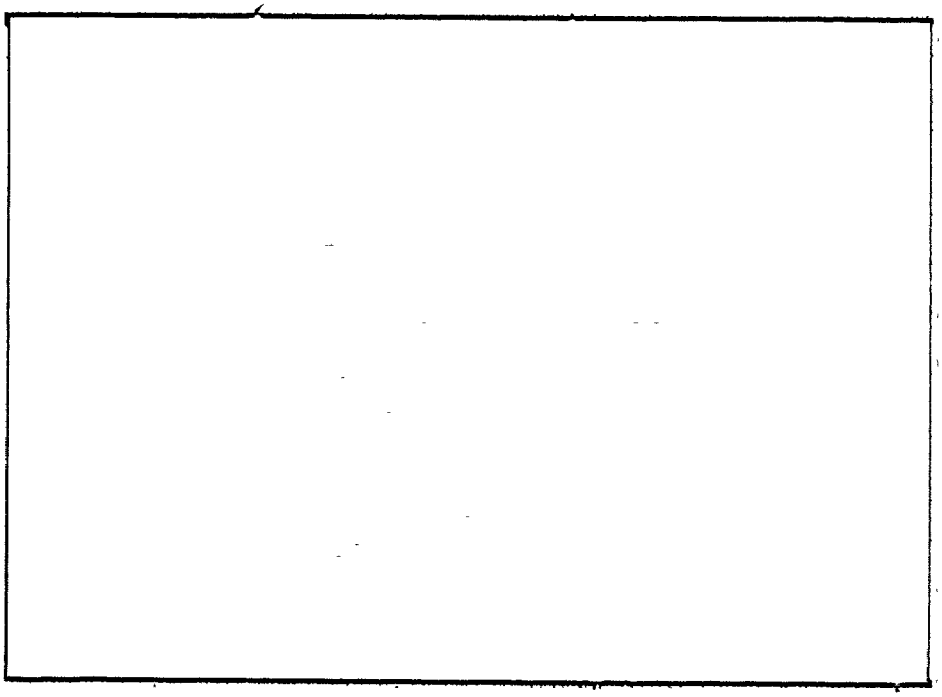


FIGURE 12



12a. CAI, anti-NAS / peroxidase



12b. NAS-absorbed control

COLOURED PICTURE

Anti-NAS and PrAP: evidence of fibre and cell-body staining:

Fig. 13 demonstrates the staining in the CA4/dentate region. Much of the specific staining in this region consists of discrete deposits of stain, ca. 1-2microns in diameter. These tend to surround the larger (ca. 10micron) cells of the polymorph cell layer. As in the fluorescence micrographs, there are some collections of stain in the polymorph cell layer that are very suggestive of cell bodies. One in particular, near the centre of this figure, is also associated with a densly filled fibre, enhancing the image of a cell-like structure. There is no evidence of cell-like structures in the NAS-absorbed control, Fig. 13b, but the fibre staining persists, although at diminished density. This particular figure shows the absorbed stained fibres at their maximum density, that is to say when the absorbtion has been least effective. The overall conclusion is that much, if not all, of the staining of fibres here is specific.

High magnification study of cell bodies and fibres in CA4:

Fig. 14 is a very high magnification micrograph of a collection of fibres in the polymorph cell layer of dentate. Several large, unstained, cells can be seen as light circles, approximately 8-10 microns in diameter. There are

Key diagram for Fig. 13

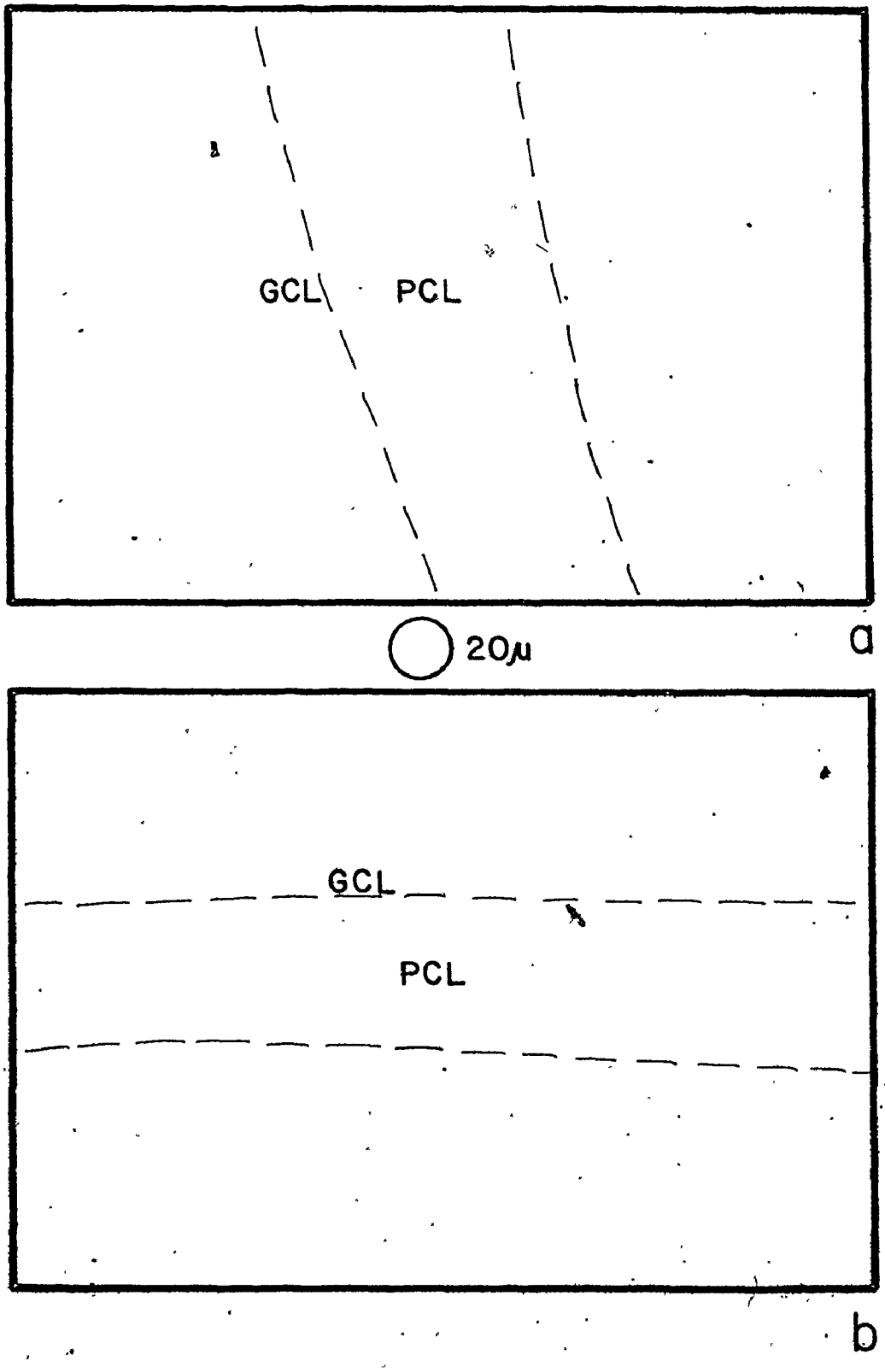
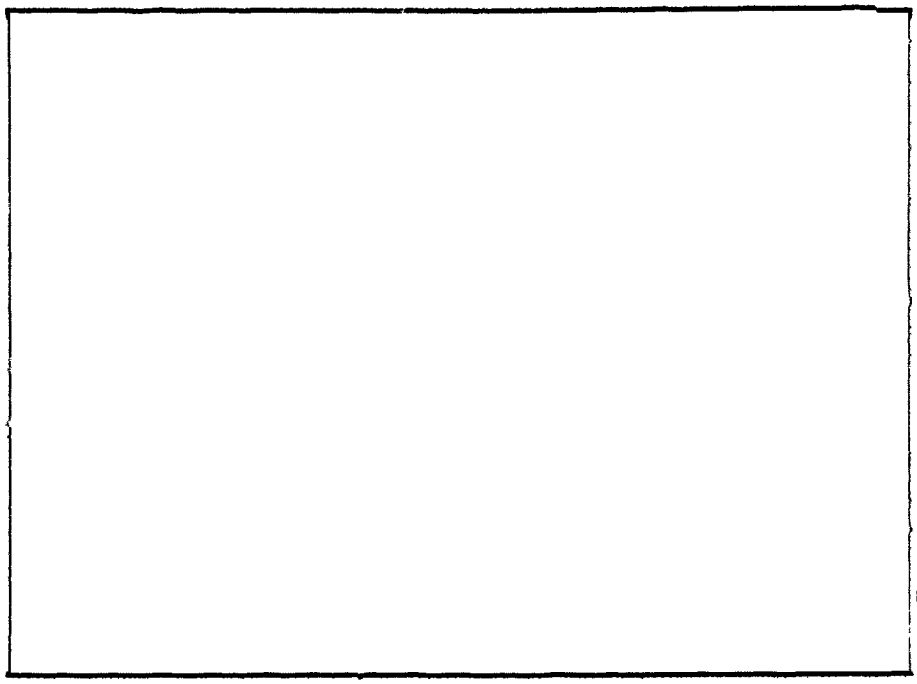
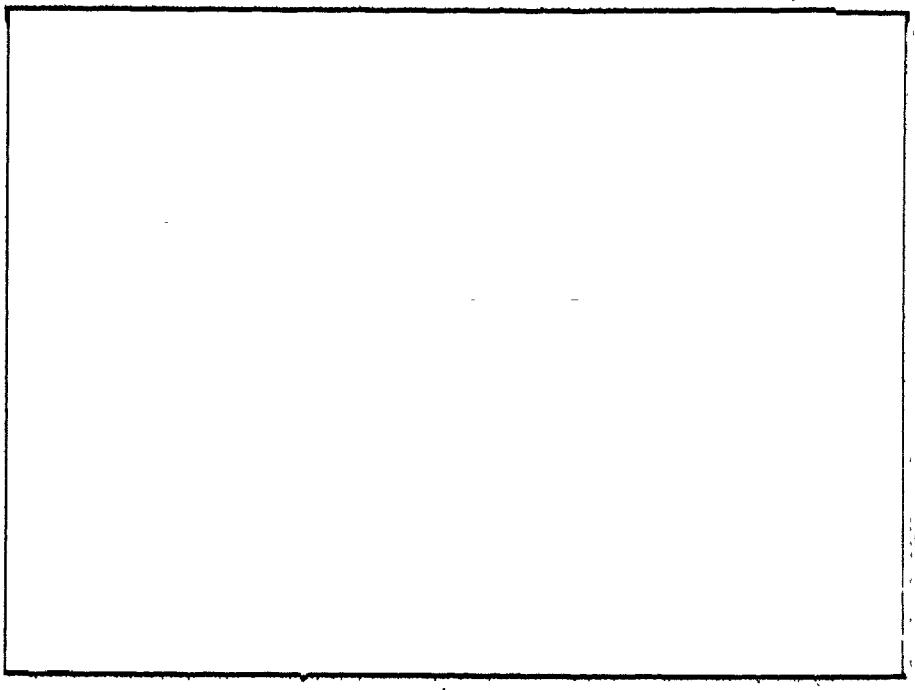


FIGURE 13



13a. Dentate , anti-NAS / peroxidase



13b. NAS-absorbed control

Key diagram for Fig. 14/15 scale

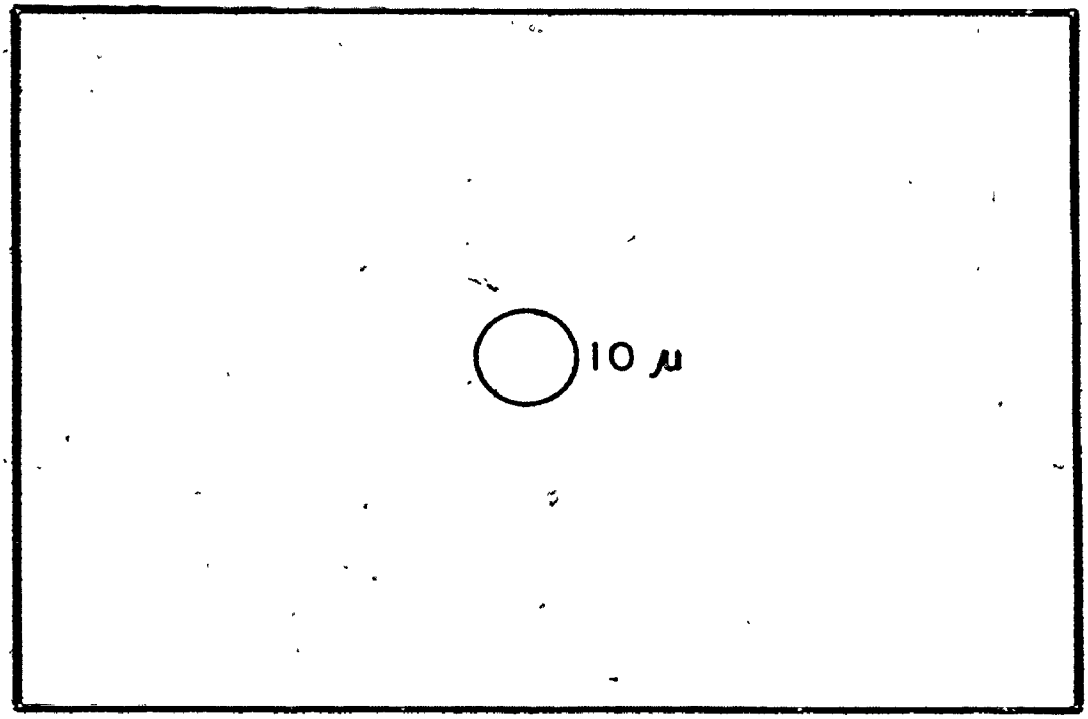
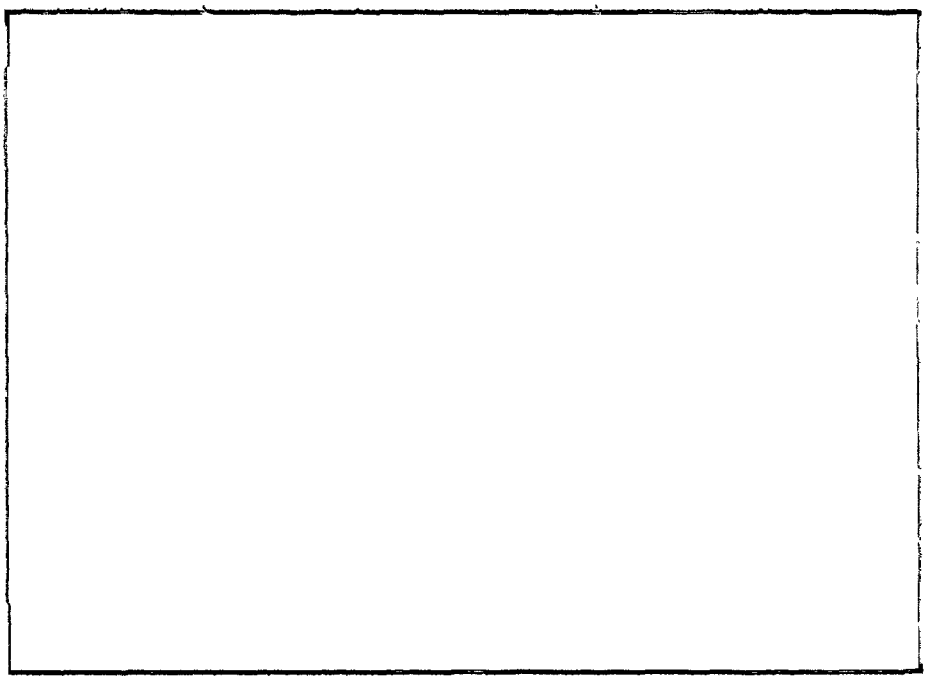


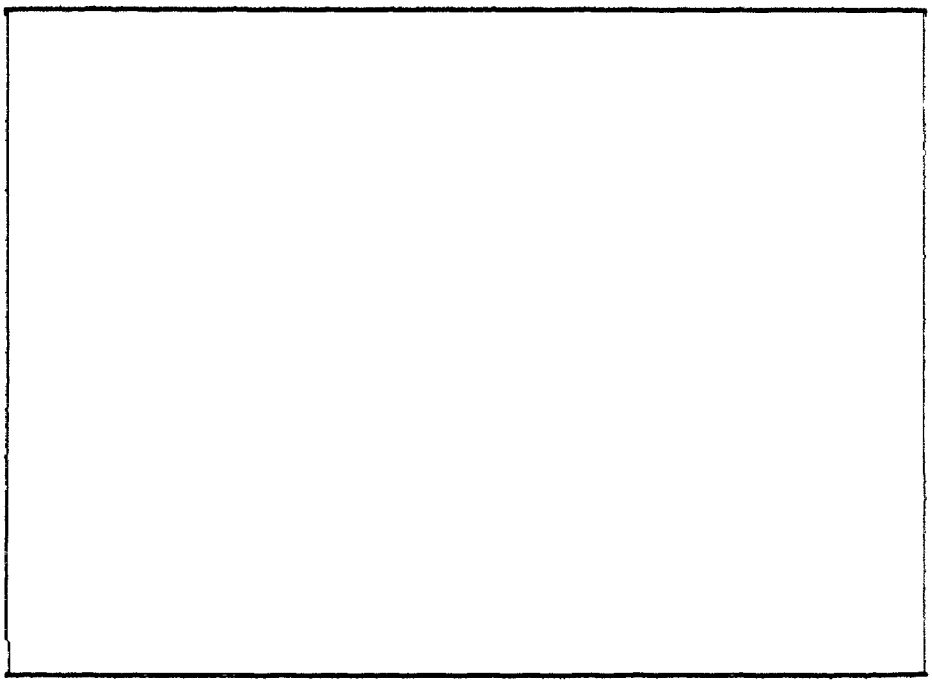
FIGURE 14



14. Dentate, detail, anti-NAS

OURED PICTURE

FIGURE 15



15. Dentate , detail , anti-NAS

COLOURED PICTURE

small, 1-2 micron, deposits of stain material associated with some of these cells. The centre of the figure contains a system of converging fibres,each about 1 micron across, some up to 40 microns long, which seem to have a common terminal/origin. On close examination, an apparent cell body is visible at the junction of these fibres and extending below. The left border of this shadow is defined by a row of five small stain deposits. Although single fibres were found in absorbed control sections, no structures resembling the present one were seen.

Another interesting finding is illustrated in Fig. 15, also from the polymorph cell layer of the dentate. The feature of this micrograph is the eccentrically located structure, ca. 8 microns across, resembling a neuronal cell body with a single process. The deposit of stain over the putative cell body is very heavy, and is made up of a large number of submicron-sized deposits. Like structures were not observed in any of the NAS-absorbed control sections.

Confirmation of NAS in hippocampus by GCMS:

Significant quantities of NAS were detected in rat hippocampi, using the method of GCMS. Control animals had a mean hippocampal NAS concentration of 496ng/g with a standard error of 168ng/g. Animals treated with PCPA as

described in Methods had hippocampal NAS concentrations of 79ng/g with a standard error of 65ng/g. These mean values were significantly different by t-test at p=0.05.

Lesion study:

As a first attempt to address the question of the source of the NAS in hippocampus, a collaboration was established with S.Suzuki, Dept of Psychology, McMaster U. The hippocampi of 7 bilaterally fornix lesioned rats were examined by immunohistology, and compared to similarily processed, sham lesioned hippocampi. Lesions were verified by behaviour (part of S.Suzuki's thesis) and by conventional histology. No differences in NAS immunoreactivity could be found between lesioned and sham-lesioned animals, given the variability of the immunostaining technique. To aid in eliminating that variability, 6 animals were prepared having unilateral fornix lesions. Fig. 16 (from Konig and Klippel, 1963) shows the level at which the lesion was placed. This area of fornix is well anterior to the hippocampus, and was chosen for the compactness of the projection in this region, and for its distance from the hippocampus proper, avoiding secondary damage via interruption of blood supply or local gliosis. The rectangle superimposed on this figure corresponds to the area shown in Fig. 17.

Key diagram for Fig. 16

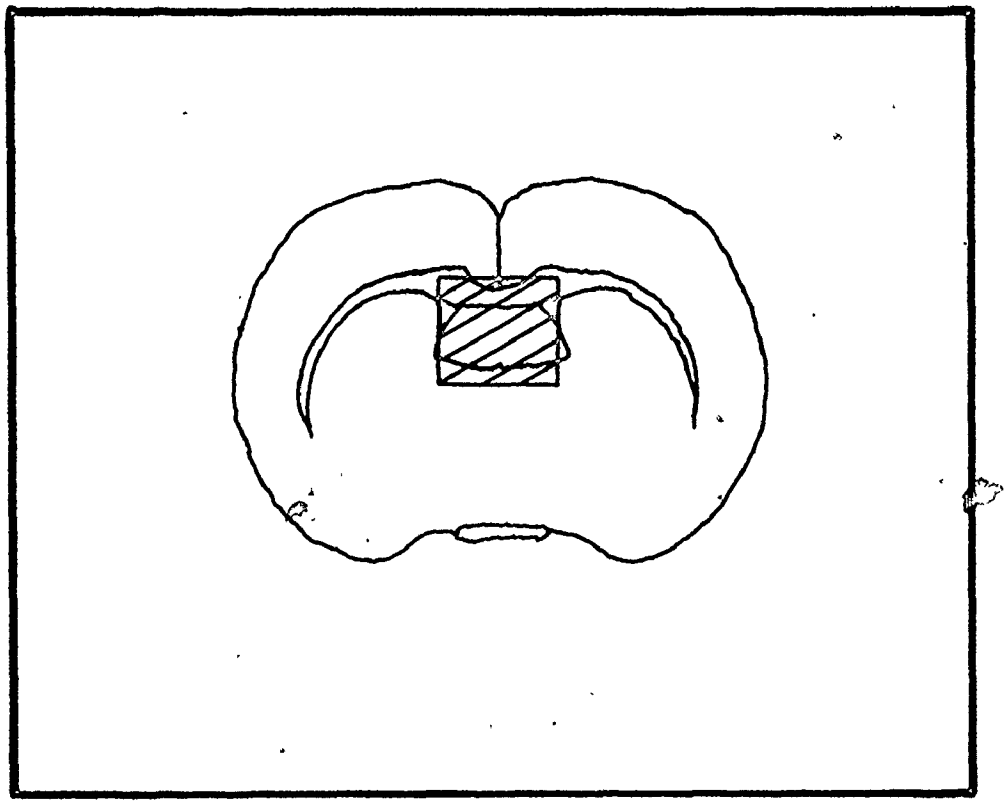
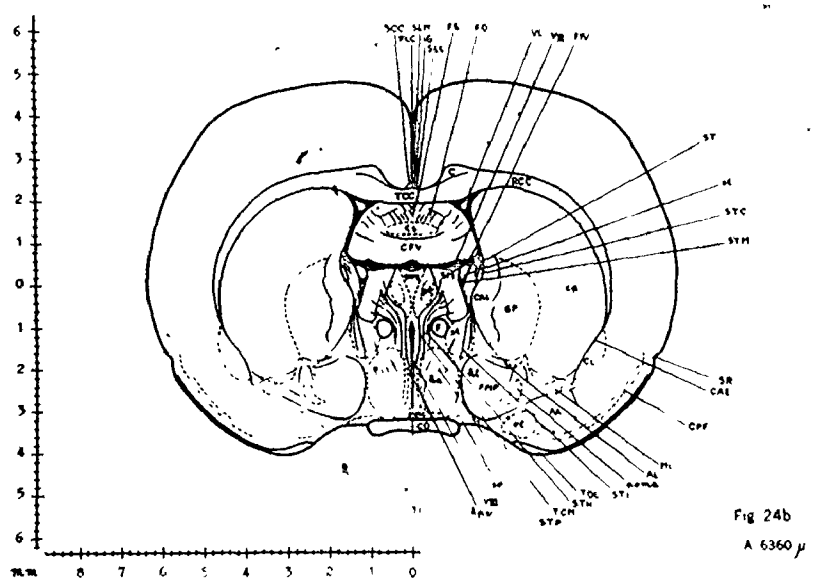


FIGURE 16

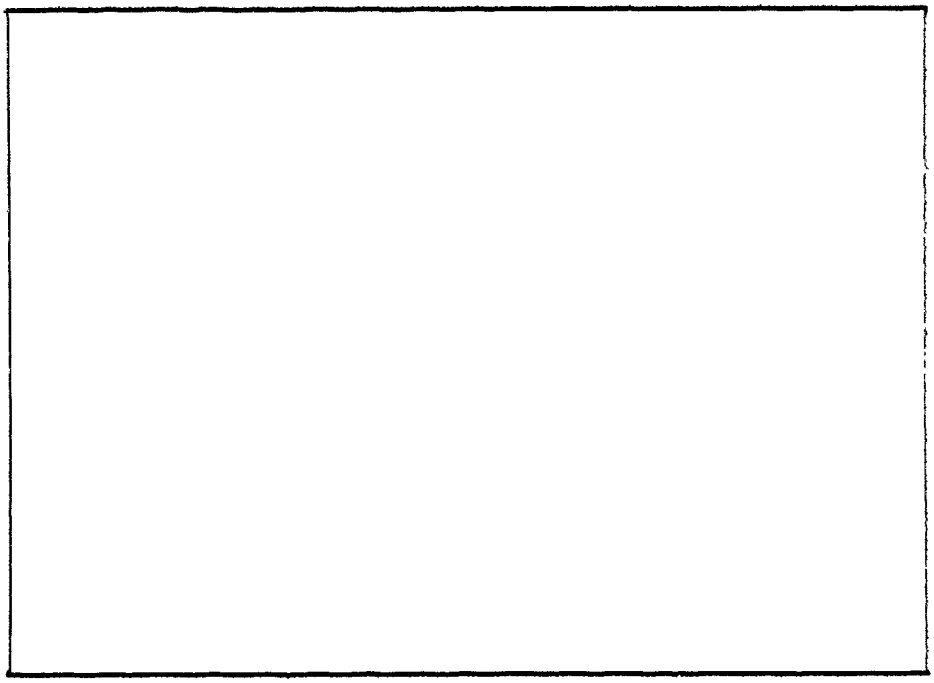


16. from Konig & Klippel -

Fig. 17 shows a representative unilateral lesion. All lesions were placed on the right side of the animal. The rationale was to cause maximum destruction on one side with minimal disturbance of the other side. In this figure, it is apparent that the left side is spared. No gross disturbance, cautery, or gliosis is detectable. The right half of the fornix pathway is almost completely destroyed. Approximately 70% of the tissue mass is missing and the remainder is heavily infiltrated with glial scars, indicating substantial damage.

Comparison of lesioned to unlesioned sides of hippocampus vis a vis NAS immunoreactivity showed no consistent differences in any of the areas CA1, CA3, or dentate. A pair of representative micrographs is seen in Fig. 18, showing a comparison in SP-CA1. Fig.18a shows the unlesioned side, 18b the lesioned side. In both, the distribution of stain is in small, discrete structures among the pyramidal cells. The total density of staining is approximately equal, and any difference is well within the variability of the technique on a single section. In each of 6 animals, 3 standard sites were selected on each side of the hippocampus, and compared to the contralateral side. At least 4 sections were analyzed per animal. No significant differences were found between any pairs of examinations by subjective criteria.

FIGURE 17



17. Site of unilateral lesion

Key diagram for Fig. 18a,b

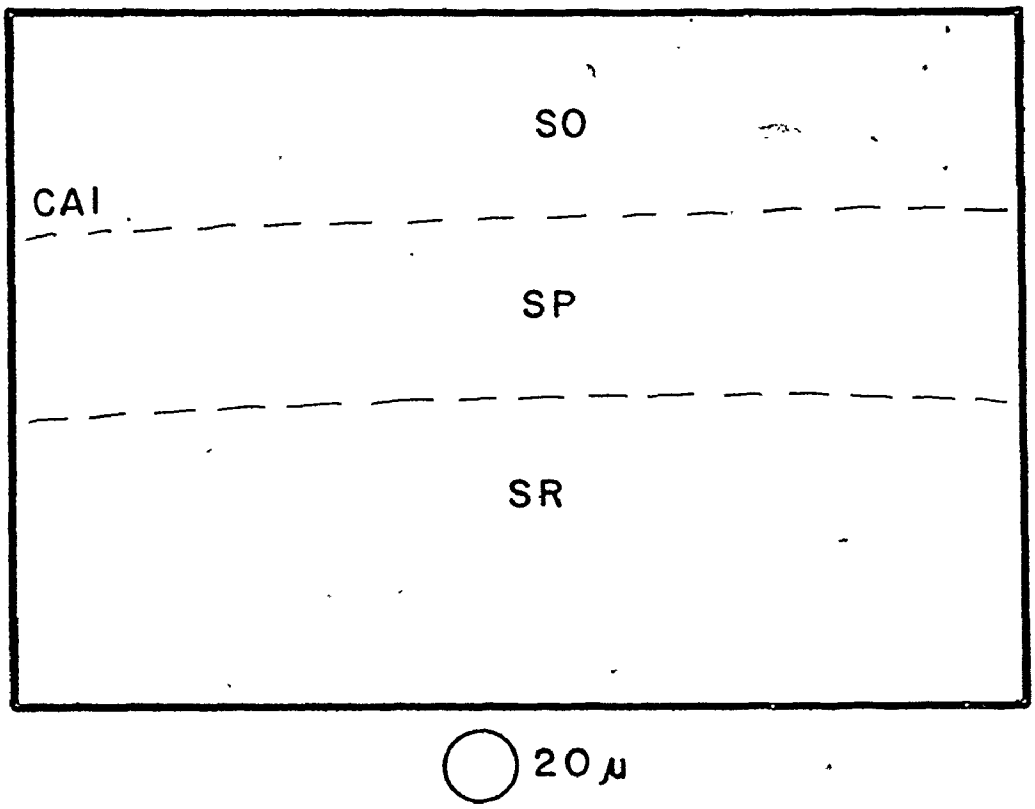
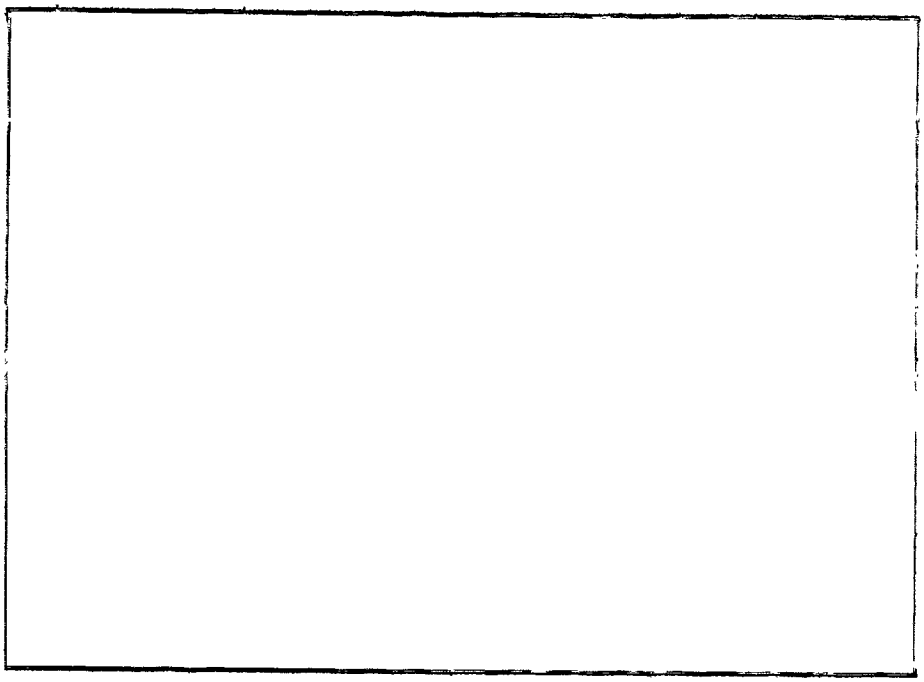
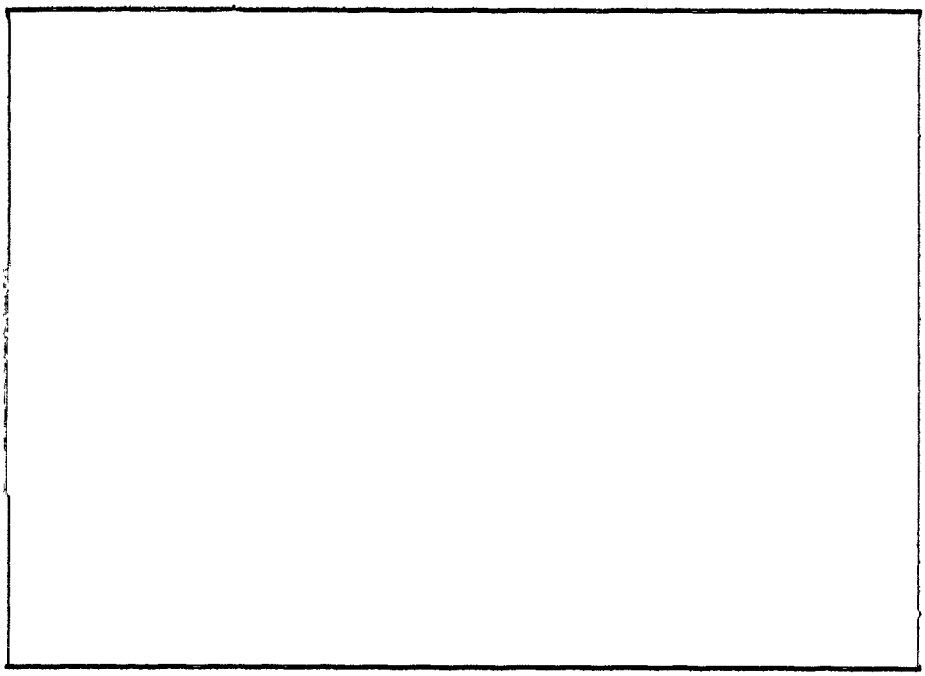


FIGURE 18



18a. Unlesioned side, CA1



18b. Lesioned side, CA1

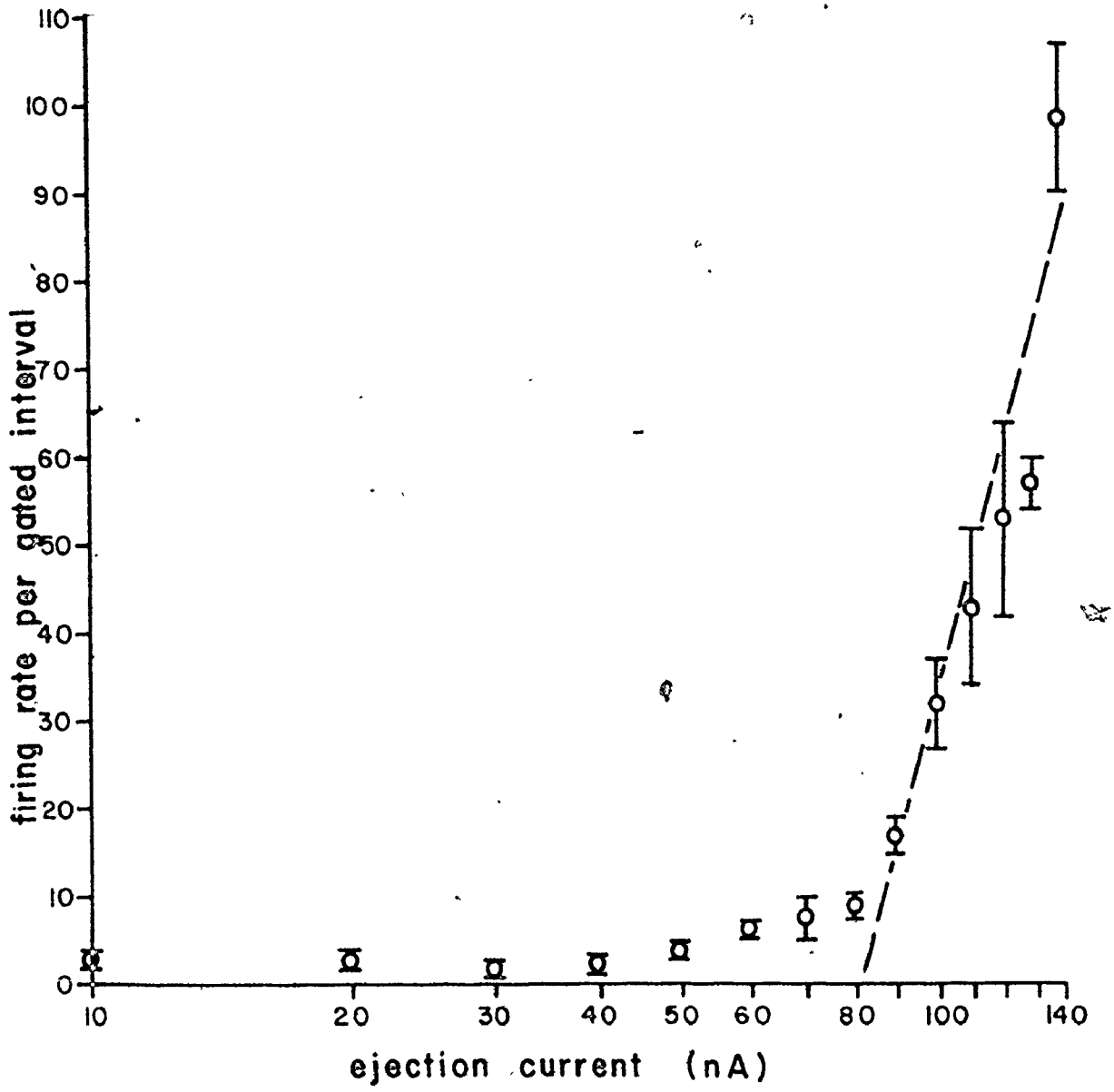
COLOURED PICTURE

Electrophysiology:

Glutamate response:

Recording and drug delivery electrodes were placed under visual observation. No attempt was made to discriminate glutamate-responsive cells (the large majority) from those cells which did not respond. A successful placement was arbitrarily defined as one in which an increase in firing rate could be elicited using a 100nA glutamate ejection current. Fig. 19 shows the firing rate of a cell in response to glutamate over the range 10-140nA. The abcissa is calibrated in units of log10(ejection current), approximating the concept of the log(dose) scale used in standard pharmacokinetics. The ordinate is a measure of the response of the cells, expressed as the number of extracellular spikes detected over the sampling time of the counting device. Each point on the graph shows the mean and standard error of several measurements at a single placement. A linear regression was calculated from the last six points, those which were judged to be on the linear portion of the rise of the curve. The line defined by that calculation appears on the graph, with a coefficient of regression(.887) a slope (freq/log10(ejection current)=358), and a y-intercept (-68).

FIGURE 19, GLUTAMATE RESPONSE



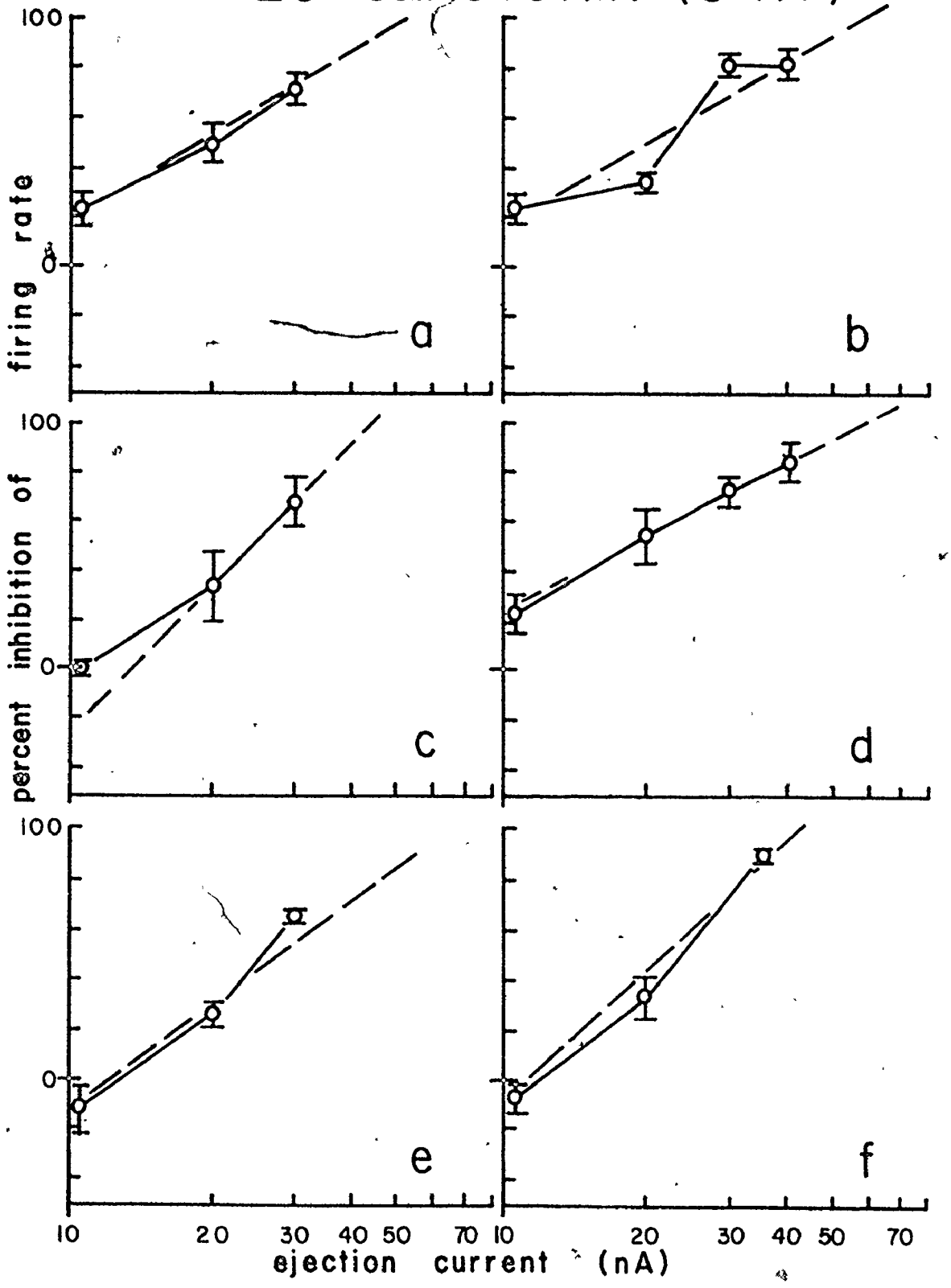
The shape of this curve resembles closely a typical log(dose)-response curve. There is little or no significant effect until the threshold. Further, the rise is exponential (seen as linear on this plot). In the present studies the glutamate ejection currents used ranged between 100nA and 140nA, this range of current resulting in initial firing rates of approximately 60 spikes per interval at the start of the experiment.

5HT Response:

Serotonin (5HT) is widely accepted as having a neurotransmitter or neuromodulator effect on the hippocampal pyramidal cells. This was supported by the present study. Fig. 20 shows plotted data from six placements where different 5HT currents were iontophoresed simultaneously with a constant current of glutamate. The abcissa represents the 5HT ejection current, on a logarithmic scale. The ordinate is a calculated value of mean percent inhibition of glutamate-induced cell firing for the corresponding 5HT ejection current. Each point represents the mean of multiple readings taken at one placement, and within a short time period. A calculated standard error is indicated.

The cells at all six placement sites showed a potent

FIGURE 20 SEROTONIN (5-HT)



inhibition of glutamate-induced firing in response to iontophoresed 5HT. Of the six responses, three (a,b,d) were apparent at the lowest 5HT ejection current(10nA); the other three cell groups did not respond at this current. All cell groups showed a marked response at a 20nA ejection current, although the response was variable both within cells (the standard error), and between cells (range of 23-55% inhibition). Of the six cell groups tested, five showed unremitting increases in inhibition with increasing eject currents. The one cell group (b) showed no further inhibition above the 30nA dose.

Fig. 20 also shows the calculated regression lines for the six curves in 20 (broken lines). The regression line parameters for 5HT are summarized in Table 1. The letters a-f are used to identify the original curves in Fig. 20, the corresponding regression lines, and the tabulated data in Table 1. The effective dose-50% (ED50) value appearing in Table 1 is equivalent to the 50%-inhibition value given by the regression equation and is included to represent a standard useful dose for the reagent, for purposes of comparison to other substances.

NAS Response:

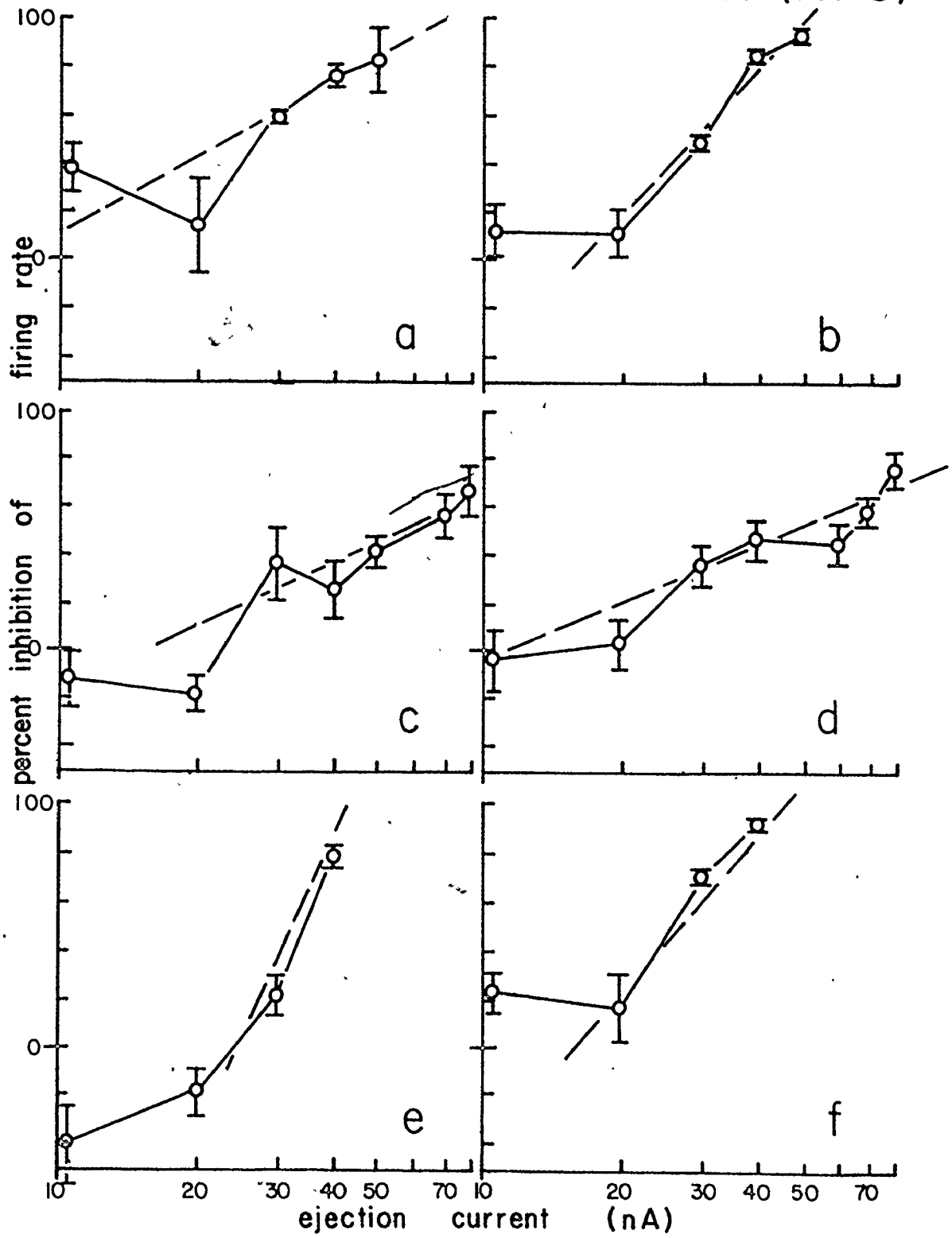
Fig. 21 shows analogous data for the substance of

TABLE I

PARAMETERS OF REGRESSION ANALYSIS OF NAS AND 5HT

curve	5HT			NAS		
	slope	intcpt	ED50	slope	intcpt	ED50
a	116	-97	18	105	-95	23
b	110	-93	20	203	-248	29
c	188	-211	24	126	-177	63
d	103	-80	18	76	-74	44
e	141	-154	27	483	-693	34
f	181	-192	22	111	-88	17

FIGURE 21 N-ACETYLSEROTONIN (NAS)



primary interest, NAS. The axes are the same as in the previous figure and individual data points are calculated as before. The curves are again labelled a-f, according to the appropriate grouping of data; i.e. 5HT(a) and NAS(a) represents data from the same group of cells. Comparisons between the effects of these two drugs were made only using paired data, where information on both effects was gathered at one placement. The low dose response to NAS varies over a wide range from moderate excitation (e) through no effect (b,c,d) to moderate inhibition (a,f). The high variability of each of these data points is relatively greater than was found in the 5HT case. The response of the individual groups appear to converge at the 20nA dose, the curves showing initial inhibition showing less inhibition, and the one curve showing significant excitation projecting to a non-significant level of excitation. The individual variability remains high. Over the next dose interval, all six curves are remarkably parallel. Above 30nA, two distinct groups form, the larger group having an unremitting increase in inhibition with increased ejection currents, with high slope (a,b,e,f). Two of the curves (c,d), however, follow a different course, having an initially lower slope than the others, but establishing an equally steep slope in higher dose ranges. The variability of the data, as well as the small sample size, do not allow valid inferences to be made regarding the distribution of the

types of responses.

Fig. 21 also shows the regression lines calculated for the NAS data. Again, only the data points after the initiation of the upward slope were used in the calculations. The various parameters defining the regression lines, as well as a calculated ED50, assuming again an absolute efficacy, for each line appears in Table 1.

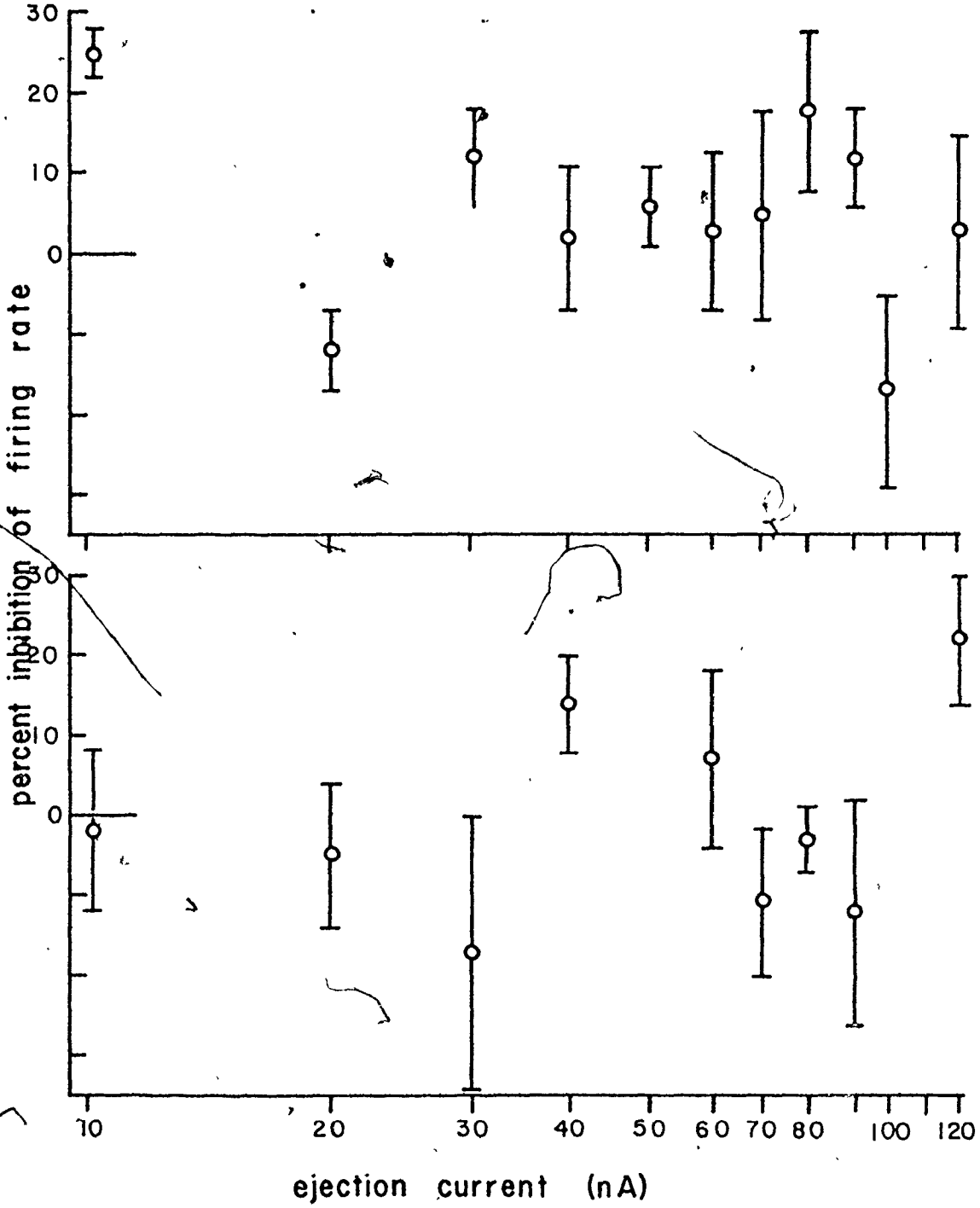
Melatonin response:

The effects of iontophoretically ejected melatonin on CA1 pyramidal cells in the hippocampus are shown in Fig. 22. The axes are labelled as in Fig. 20. Two cell groups were tested, and data points calculated as for 5HT and NAS. Although a great variation in frequency of firing can be seen, this variation seems independent of the dose of melatonin, and in any case does not differ from baseline significantly at ejection currents up to 120nA.

Comparison of 5HT and NAS responses:

Only two sets of data were compared statistically, those showing the 5HT effects and those showing NAS effects. The actual comparisons were made between the calculated

FIGURE 22 MELATONIN



regression lines, and the calculated ED50 values from those regression lines. The melatonin data were too few in number to allow comparison, and the difference in the slopes of those two lines from the slopes of the 5HT or NAS curves is so obvious over the dose range tested, that statistical analysis appears superfluous.

All of the matching parameters in Table 1 were compared by the Wilcoxan Matched-Pairs Signed Ranks Test. There were no differences found between the two blocks of data, representing the 5HT and NAS effects respectively. The differences between the ED50 values for the two blocks approached significance at the p=0.10 level, but this is not sufficient to establish a difference between these two groups of data.

DISCUSSION

Immunohistology of NAI's:

The use of the bispecific antisera in localizing NAI's by immunofluorescence (IF) is well established in this laboratory (Bubenik et al, 1974). Early mapping work suggested the presence of NAI-like immunoreactivity in the hippocampal formation of the rat (G.A. Bubenik, personal comm). When an antiserum specific to melatonin (Mel) became available (Grota and Brown, 1974, Pang et al, 1976) a technique was developed to differentiate immunoreactive Mel from immunoreactive NAS using the two antisera (Bubenik et al, 1976, see also Methods). The results, as given in the present thesis, indicate that NAS is present in the hippocampus in a regular pattern, and that if Mel is present, it is present only in very low concentrations relative to content in other areas where it has been identified by the IF technique (Bubenik et al, 1976). The synthesis of new conjugates such as NAS-PCB (DeSilva and Sniekus, 1978) led to the development of antisera which bind NAS specifically relative to other indoles (see Appendix I). The subsequent re-investigation of the NAS distribution in hippocampus gave results identical to those found with the

two antisera technique. A further refinement of the staining procedure, using PrAP as a secondary reagent, permitted better preserved tissue sections in which the localization of immunoreactive NAS could be determined with higher resolution. The staining for NAS in the pyramidal cell layers of CA1 and CA3 was associated with, but apparently not within, the pyramidal cell bodies. The stain was in structures on the order of a micron or less in diameter. These structures often would define a pyramidal cell body by surrounding it. No fibres were evident in these regions. There was no specific staining in CA1 or CA3 other than that associated with the cell bodies in the pyramidal layer. In CA4/dentate specific staining was found in the polymorph cell layer, with occasional observations of stain in the granule cell layer. The large proportion of staining was morphologically similar to that in CA1/CA3, that is the stain was in micron and submicron sized units, not appearing to be within cell bodies. An interesting finding in the later studies utilizing PrAP with the related preservation of tissue, was staining of cell bodies and fibres in the dentate. This staining was clearly different from the punctiform staining described earlier. Entire cell bodies would appear to be well filled with stain material confined to the exact contours of the cell, and sometimes extending into an associated process. Interestingly, none of the cell bodies which stained for NAS content showed a close

association with the submicron-type NAS-staining structures. Fibres appearing to be filled with immunoreactive NAS could also be visualized in the CA4/dentate region. A few fibres were associated with cell bodies, but most were not. These fibres would occasionally run into the granule cell layer. It seems probable that the reason that these fibres could not be visualized by IF is the improvement in the quality of tissue preservation afforded in the PrAP technique. This may also explain the identification of cell-body structures using PrAP as opposed to inconclusive findings for cell bodies using IF.

The finding that the hippocampus of the rat contains significant quantities of NAS has been corroborated with the technique of Gas Chromatography-Mass Spectrometry(GCMS). Total content of NAS in hippocampus was approximately 1/10th that of 5HT(Dr. Narasimhachari, personal comm.). The question of a corroboration of the distribution remains. The in vitro binding characteristics of the antiserum are given in Appendix I. From this information it follows that this serum is capable of binding NAS, apparently in preference to other indoles. Pre-incubation of the diluted serum in the presence of dissolved NAS caused a marked reduction in the ability of the mixture to stain for immunoreactive NAS. Pre-incubation with another substance, dopamine, replacing NAS in the mixture, did not affect the

staining. This supports the hypothesis that a NAS-specific site located on the IgG molecules in the serum is responsible for the localization of the marker material during immunohistochemical processing. This, combined with the *in vitro* binding data, strongly suggest that the substance localized was indeed immunoreactive NAS. This control, however, is not definitive for establishing specificity (Swaab et al, 1977). There remains a discrepency in the dilution factors between the *in vitro* crossreactivity testing and the immunohistologic technique which affect the definition of crossreactivity. A substance with negligible crossreactivity at high concentration may have a very different activity at the low concentrations used in immunohistology. A way of verifying the *in vitro* crossreactivity for immunohistology is to do similar studies using immune staining as the measured variable. Various structural analogs, as well as structurally unrelated compounds known to be present in the test tissue, are tested quantitatively for their ability to reduce the intensity of the staining. In this way a staining-suppression curve can be constructed which is analogous to the antigen-displacement curve used in the radioimmune assay. The work done by others in our laboratory with this procedure has not shown significant crossreactivity of the a-NAS sera on tissue slices, (O. Pulido, personal comm).

The finding that NAS could be localized in the hippocampus in a distribution distinct from that of Mel suggests that its presence there is for some function other than to act as a Mel precursor. Of equal significance is the fact that the NAS distribution is very distinct from the 5HT distribution, the latter being a diffuse distribution associated only with the dendritic arborization of the pyramidal cell (Lidov et al, 1980, Pasquier and Reinoso-Suarez, 1978) while the former is definitely associated with the cell bodies, and does not appear to be present in the dendritic areas.

There is a substantial literature supporting a NT function for 5HT on the pyramidal cells of the hippocampus (Hamon et al, 1980, Segal, 1976, 1980, Samanin et al, 1978, Bennett and Snyder, 1976, Jahnsen, 1980). Of special interest is the fact that the pyramidal cell bodies are very sensitive to the application of 5HT in spite of the fact that 5HT has not been found near the cell body layers in CA1/CA3 (Segal, 1980, Lidov et al, 1980). It is possible that the methods for detecting 5HT are not sufficiently sensitive to demonstrate 5HT proximal to the cell body. With the total hippocampal content of 5HT being approximately ten times that of NAS, the recently available immunohistologic techniques for 5HT (Steinbusch et al, 1978), and their application in the hippocampus (Lidov et

al, 1980), this explanation seems unlikely. The most likely explanation for this combination of observations is that NAS has some function in rat hippocampus, independent of 5HT or Mel. Because 5HT is well established as a NT/NM in hippocampus, a working hypothesis considering NAS as a NT/NM is not unreasonable.

The definition of a substance as a NT requires several criteria to be fulfilled. It has been demonstrated that NAS has a localization appropriate for a NT. It is found in high local concentrations in a regular arrangement around cells which are known to respond to electrical (MacVicar and Dudek, 1980b) and chemical (Segal, 1980, Schwartzkroin and Andersen, 1975) stimuli. Its localization cannot be justified in terms of its acting only as a precursor or breakdown product, at least in its most likely synthetic/degradation pathway. These conclusions led directly to the studies of the effects of NAS on the electrical activity of one of the cell types with which it is associated in hippocampus, the CA1 pyramidal neurons.

Electrophysiology:

The CA1 pyramidal neuron is known to respond to glutamate, predominantly by excitation (Schwartzkroin and Andersen, 1975), and to 5HT with inhibition (Segal, 1980).

Although some analogs of both of these drugs have been tested in the CA1 pyramidal cell (Spencer et al, 1976, Gusel and Mikhailov, 1980) NAS has not been included in these reported studies. Since NAS is structurally similar to 5HT, and the latter is generally accepted as having inhibitory effects on responsive neurons, the assumption was made that if NAS had an effect, it might likely be inhibitory. This was confirmed experimentally. Since the hippocampal CA1 cells in the slice rarely exhibit spontaneous activity, a paradigm for inducing cellular electrical activity was devised. Glutamate-induced CA1 excitation was readily elicited by microiontophoretic application using ejection currents commensurate with those reported in the literature (Schwartzkroin and Andersen, 1975, MacDonald and Nistri, 1978)

In order to establish a relationship to the literature, the effects of 5HT in the present test system were observed. 5HT was shown to be highly effective in inhibiting the glutamate response in all cells tested. These same cells were immediately tested for responsiveness to the application of NAS. In general, the response to NAS and that to 5HT are very similar. By subjective observation, both had a short time of onset, with no prolonged after-effects. Both inhibit glutamate induced firing of CA1 pyramidal neurons in a dose-responsive

fashion, and over the same range of ejection currents. Statistically, the responses to the two compounds are not distinguishable. The individual variation in the responses could be due to differences in target cells, differences in electrode placement, differences in electrode characteristics, or some unidentified variable.

As an adjunct to the search for Mel in the hippocampus, the effects of microiontophoretically applied Mel on hippocampal CA1 pyramidal neurons was investigated. Over the range of ejection currents in the present study, no effect was seen. This reinforces the conclusion of the histologic finding where Mel content in the hippocampus could not be demonstrated.

Although the results from iontophoretic studies are internally consistent, and show interpretable results, some technical problems which bear on the results should be discussed. 1) Extracellular recording. Extracellular recording usually detects the responses of a number of cells. Natural heterogeneity among these cells may tend to increase the variation of the measured response, perhaps sufficiently to obscure a significant, but small difference. For example, a ceiling response is difficult to interpret. It may be a result of a true maximal response achieved by all cells or it may be due to some cells in the population

reaching a ceiling effect, and others never having been affected at all, or innumerable variations on this idea. This is compounded by the fact that these different cells will be at different distances from the drug delivery electrode, and if response is concentration-dependent, as seems to be the case, the responses will vary according to the different concentrations at the various cells. 2) The Drug Delivery Electrode: Since two separate electrodes are used, and the electrode tips are not visible within the tissue, their relative positions cannot be observed, or consequently, controlled. A subjective assessment of proximity by ejection current artifact and sensitivity to glutamate ejection, as well as a lack of latency to the glutamate response were the only observations made for placement. Response is related to concentration at the receptor, and concentration is related to distance of the delivery electrode from the cell (MacDonald and Nistri, 1978). This situation introduces a variable which makes comparisons between placements questionable, if not totally unjustifiable. 3)Quantification of Data: In addition to the fact that several cells were being measured at the same time, the quantification procedure did not attempt to measure such things as changes in the height of the spike responses. The amplitude discriminator was set with its lower limit just above background noise, so that amplitude change responses high above background would not be

detected. The counting device was gated to count for a time interval approximately two seconds long, so it is unlikely that low frequency firing changes remained undetected, unless they are masked by a substantial high frequency signal.

Keeping in mind these drawbacks and assessing only paired data, it can be concluded with reasonable certainty that NAS and 5HT are equally potent in reducing the frequency of glutamate- induced firing in CA1 pyramidal neurons in the hippocampal slice. A more precise comparison of potency and an investigation of efficacy would be better investigated in a similar drug application paradigm but substituting intracellular recording from a single neuron. This then is the obvious direction for future studies to take.

Lesions:

One of the classical methods for determining the source of a substance in the brain is to lesion either cell body groups or fibre tracts while monitoring the disappearance of the substance of interest (Jones and Hartman, 1978). The hippocampus has two major input pathways. One is the perforant path, originating from and passing through the entorhinal cortex adjacent to the

hippocampus. The second is the fimbria-fornix pathway. This pathway is associated with most of the brainstem projections to the hippocampus, and in particular with the Raphe-hippocampal projection of 5HT (Pasquier and Reinos-Suarez, 1978). It is of particular interest therefore, that lesions in the fimbria-fornix, as reported here, do not seem to affect either the content or distribution of NAS within the hippocampus. More extensive and more precise lesion studies are necessary for definitive conclusions regarding any external sources for NAS. The finding that NAS-containing cell bodies with associated fibres can be visualized within the hippocampus indicates that at least some of the CA1/CA3 associated NAS may be of hippocampal origin.

CONCLUSIONS

Two of the criteria required to establish a putative neurotransmitter function for NAS have been investigated. Hippocampal NAS is distributed in a discrete pattern of high local concentrations in association with pyramidal neurons of CA1/CA3, as well as in and about polymorph cells in the CA4/dentate region. This distribution of NAS does not resemble that of 5HT as reported in the literature and no Melatonin could be detected in any part of the hippocampus. NAS presence was independently confirmed by GCMS. The pyramidal cells in CA1 have been shown to respond to NAS in a similar fashion as they do to 5HT, both from the present 5HT studies and from the literature on 5HT physiology and pharmacology. These data support the hypothesis that NAS may be a neurotransmitter or neuromodulator in the hippocampus of the rat, although extensive investigations in electrophysiology, receptor binding, metabolism, and to a lesser extent, histology, must still be performed.

APPENDIX I

CROSSREACTIVITY OF ANTI-NAS ANTISERUM BY IMMUNOASSAY

Ligand	crossreactivity relative to NAS
N-acetylserotonin (NAS)	100
NAS-PCB	555
Melatonin-PCB	5
N-acetyltryptamine	0.6
5-methoxytryptamine	0.02
Serotonin (5HT)	0
N-acetyltryptophan	0
5-methoxytryptophan	0

These data are provided through the courtesy of Drs. G.M. Brown, and L.J. Grota.

APPENDIX II

PREPARATION OF PHYSIOLOGIC MEDIUM

Stock Solutions:

A:	NaCl	36.239g
	$NaH_2PO_4.H_2O$	0.86g
	KCl	1.677g
B:	$MgSO_4$	12.0g
C:	$CaCl_2.2H_2O$	14.7g

-all of the above are dissolved in 500 ml deionized water

Preparation:

To approximately 400 ml deionized water in a 500 ml volumetric flask, add 1.0921g NaBicarbonate. Mix until dissolved. Add 0.9008g d-glucose and mix. Add 50 ml stock A, and 5 ml each of stocks B and C. When the solution is clear, make volume up to 500 ml with deionized water. Oxygenate for at least 30 minutes with 95% oxygen/5% carbon dioxide, verify pH 7.35, and use immediately.

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