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NEUROCHEMICAL CHARACTERIZATION OF 5-HYDROXYTRYPTAMINERGIC NEURONS TERMINATING IN THE NEURAL AND INTERMEDIATE LOBES OF THE PITUITARY GLAND

By

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ABSTRACT

Neurochemical Characterization of 5-Hydroxytryptamine Neurons Terminating in the Neural and Intermediate Lobes of the Pituitary Gland

by

Nancy J. Shannon

Immunocytochemistry has revealed that nerve fibers within the neural and intermediate lobes of the pitiutary gland contain 5-hydroxyryptamine (5-HT). The purpose of this study was to begin to characterize these neurons by determining if 5-HT is synthesized within them and by establishing the location of their cell bodies of origin. Since neurochemical techniques were to be used, biochemical indices of 5-HT neuronal activity were first evaluated.

A comparison of these indices in rat brain regions containing 5-HT nerve terminals following electrical stimulation of their cell bodies revealed that the concentration of the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA), the ratio of the concentrations of 5-HIAA to 5-HT and the rate of accumulation of the 5-HT precursor 5-hydroxytryptophan (5-HTP) following inhibition of aromatic L-amino acid decarboxylase provide valid indices of 5-HT neuronal activity while the pargyline-induced accumulation of 5-HT and decline in 5-HIAA concentrations do not.

Because administration of the 5-HT precursor tryptophan increased 5-HTP accumulation and 5-HT and 5-HIAA concentrations while leaving catecholamine synthesis unaltered, it was felt that this manipulation might be useful in some

investigations of regions with a sparse 5-HT innervation. Furthermore, experiments utiizing the 5-HT uptake inhibitor fluoxetine and electrical stimulation suggested that tryptophan administration does not increase 5-HT release and, parenthetically, that 5-HIAA is primarily formed from intraneuronal 5-HT prior to release and reuptake. Exposure of brain tissue extracts to sulfatase hydrolysis to determine if sulfoconjugation of 5-HT and 5-HIAA obscured their electrochemical detection by high performance liquid chromatography revealed no increase in "free" indoleamines.

Following the examination of biochemical estimates of 5-HT neuronal activity some of these techniques were used to investigate 5-HT-containing fibers in the pituitary gland. Detection of 5-HTP accumulation in the neurointermediate lobe indicated 5-HT synthesis in this region and, as in other 5-HT neurons, the rate of synthesis was increased by tryptophan administration and electrical stimulation. This conclusion was extended to both divisions of this lobe with an experiment involving repeated fluoxetine administration. The cell bodies of origin of these neurons were localized to the brainstem through a series of lesioning and electrical stimulation studies.

to my husband, Greg, for supporting me through this endeavor and for encouraging me to embark on others

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
AL	anterior lobe of the pituitary gland
ALAAD	aromatic L-amino acid decarboxylase
ARC	arcuate nucleus
DA	dopamine
5,7-DHT	5,7-dihydroxytryptamine
DMN	dorsomedial nucleus of the hypothalamus
DOPA	3,4-dihydroxyphenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid
DR	dorsal raphe nucleus
5-HIAA	5-hydroxyindoleacetic acid
HPLC	high performance liquid chromatography
HPLC-EC	high performance liquid chromatography with electrochemical detection
HRP	horseradish peroxidase
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
HVA	homovanillic acid
IL	intermediate lobe of the pituitary gland
ME	median eminence
MHPG	3-methoxy-4-hydroxyphenylglycol
MOPS	3[N-morpholino]propanesulfonic acid
MPN	medial preoptic nucleus
MR	median raphe nucleus
αMSH	α-melanocyte stimulating hormone
NE	norepinephrine
NIL	neurointermediate lobe of the pituitary gland

NL	neural lobe of the pituitary gland
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PNC	p-nitrocatechol
PSCN	preoptic suprachiasmatic nucleus
RL	nucleus raphe linearis
RM	nucleus raphe magnus
RO	nucleus raphe obscurus
RPa	nucleus raphe pallidus
RPo	nucleus raphe pontis
SCN	suprachiasmatic nucleus

INTRODUCTION

5-Hydroxytryptamine (5-HT, serotonin) was first found in the brain in 1953 by Twarog and Page, and it was noted that concentrations of the amine varied among brain regions (Amin et al., 1954; Bogdanski et al., 1957). A decade later, using the technique of formaldehyde-induced fluorescence, Dahlström and Fuxe (1964) demonstrated that this amine is located in discrete groups of cell bodies and hypothesized that these perikarya were the origin of 5-HT axons projecting throughout the brain and spinal cord. These observations have spurred a number of investigations in the anatomy and biochemistry of 5-HT in the central nervous system. This introduction will summarize the results of these studies regarding the location of both "classical" 5-HT cell bodies in the brainstem and recently discovered 5-HT cell bodies in the hypothalamus, will describe their projections in the brain and spinal cord and will focus on the 5-HT innervation of the neural and intermediate lobes of the pituitary gland. Finally, the neurochemistry of 5-HT will be discussed.

I. 5-HT-Containing Cell Bodies

As mentioned above, the first evidence that 5-HT is contained within specific groups of neuronal perikarya was derived from experiments employing formaldehyde-induced fluorescence. While this technique has proven to be extremely valuable in 5-HT research, it has certain disadvantages. Since this method necessitates freeze-drying tissue, the histological quality of the tissue section is compromised. Furthermore, the fluorescent reaction product, a

β-carboline, degrades very rapidly upon exposure to light, thereby causing the fluorescent label to diminish during microscopic examination. Consequently, several other anatomical techniques have also been employed in order to confirm the earlier findings. While studies based on [^{3}H]5-HT uptake and on immunocytochemistry have their own drawbacks, such as the possibility of non-specific labeling, the results obtained with all of these experimental techniques are in good agreement. The description of 5-HT-containing cell bodies in the brainstem is covered by several excellent reviews (Azmitia, 1978; Steinbusch and Nieuwenhuys, 1983; Willis, 1984) and will be briefly summarized here, while the evidence for 5-HT-containing perikarya in the hypothalamus will be discussed in greater detail.

A. Brainstem

Cell bodies of neurons containing 5-HT reside predominantly, but not exclusively, in the raphe nuclei of the medulla, pons and mesencephalon. As the name "raphe" (from the Greek, rhaphe, meaning "seam") implies, the structures are located along the midline of the brain and are depicted in a midsagittal view in Figure 1. The caudal most raphe nuclei, nucleus raphe pallidus and nucleus raphe obscurus, roughly correspond to the B_1 and B_2 cell groups, respectively, of Dahlström and Fuxe (1964) and are situated entirely within the medulla oblongata. The nucleus raphe magnus is more rostrally located in the medulla and extends into the pons and contains many of the cells of group B_3. Lying entirely within the pons and synonymous with the cell group B_5 is the nucleus raphe pontis. The most rostral raphe nuclei, nucleus raphe dorsalis (dorsal raphe nucleus), nucleus raphe medianus (median raphe nucleus, nucleus centralis superior) and nucleus raphe linearis, reside within the mesencephalon and approximately correspond to cell groups B_7 (nucleus raphe dorsalis) and B_8 (nucleus raphe medianus and nucleus raphe linearis).

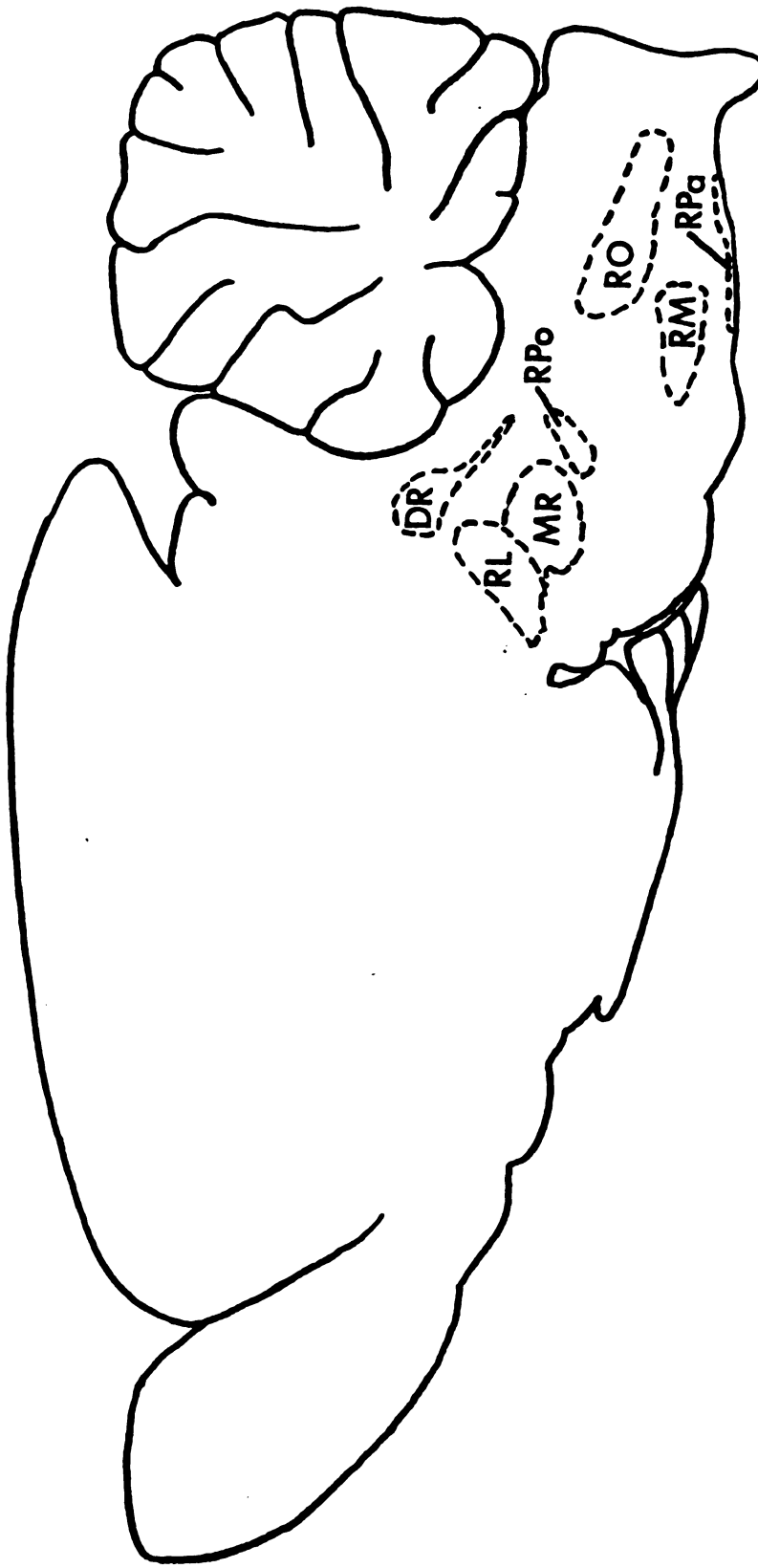

Figure 1. Midsagittal view of the rat brain indicating the brainstem raphe nuclei. DR, dorsal raphe nucleus; MR, median raphe nucleus; RL, nucleus raphe linearis; RM, nucleus raphe magnus; RO, nucleus raphe obscurus; RPa, nucleus raphe pallidus; RPo, nucleus raphe pontis. (Modified from Paxinos and Watson, 1982).

Additional regions containing 5-HT cell bodies outside of the raphe nuclei are described by the Swedish investigators and other neuroanatomists. The area postrema contains some glia cells with 5-HT, the medullary group B_4 is made up of a few cells under the fourth ventricle, and group B_6 lies in the pons under the rostral portion of this ventricle. Group B_9 is situated in the mesencephalon lateral to the interpeduncular nucleus and within and around the medial lemniscus. In addition to these relatively discrete groups, 5-HT perikarya are found scattered lateral to most of the raphe nuclei.

Wiklund et al. (1981) provide more quantitative information on the distribution of 5-HT cell bodies. In a formaldehyde-induced fluorescence study of the cat brainstem, they estimate that 77.5% of 5-HT perikarya are contained within the raphe nuclei while the remainder of 5-HT cell bodies reside in other regions of the brainstem. They also find an uneven distribution of 5-HT cells within the raphe nuclei such that the dorsal raphe nucleus alone contains approximately 40% of all 5-HT perikarya, which is more than the combined total of the other raphe nuclei. Conversely, not all cells of the raphe nuclei contain 5-HT. Of the total number of neuronal cell bodies within the dorsal raphe nucleus, Wiklund and coworkers estimate that 70% label for this amine. The proportions of cell bodies containing 5-HT is less in the other raphe nuclei and are listed in Table 1.

B. Hypothalamus

While there is an occasional report of 5-HT-containing cell bodies within various hypothalamic nuclei of the rat (e.g., arcuate nucleus, Kent and Sladek, 1978), similar results have been obtained in only the dorsomedial nucleus of the hypothalamus by several investigators using a variety of techniques. Fuxe and Ungerstedt (1968), using the formaldehyde-induced fluorescence method,

TABLE 1

The Proportion of Cell Bodies Within Raphe Nuclei that Contain 5-HT

Nucleus	Cells Containing 5-HT
N. raphe pallidus	50%
N. raphe obscurus	35%
N. raphe magnus	15%
N. raphe pontis	10%
N. raphe dorsalis	70%
N. raphe medianus	35%
N. raphe linearis	25%

From Wiklund et al. (1981).

demonstrated 5-HT cell bodies in the dorsomedial nucleus following intracerebro ventricular infusion of exogenous 5-HT and administration of a monoamine oxidase inhibitor. These results were confirmed in studies showing that pretreatment with a monoamine oxidase inhibitor followed by extensive intracerebroventricular perfusion with [^{3}H]5-HT results in uptake of the label by cell bodies situated in the ventral portion of the dorsomedial nucleus (see Figure 2; Chan-Palay, 1977; Beaudet and Descarries, 1979). The radiolabeled cells, representing about 7% of the total number of cells in the dorsomedial nucleus, were still observed following the addition of nonradioactive norepinephrine to the [^{3}H]5-HT perfusion solution, but were not labeled by perfusion with [^{3}H]norepinephrine. This indicates that the labeled perikarya are probably not catecholaminergic cells that simply incorporated exogenous 5-HT.

Immunocytochemical studies performed without the addition of exogenous 5-HT provide further evidence for 5-HT in cell bodies of the dorsomedial nucleus. Using independently derived antibodies to 5-HT, several groups visualized 5-HT in cell bodies of the dorsomedial nucleus of both rats and cats following pretreatment with a monoamine oxidase inhibitor and the 5-HT precursors, tryptophan (Frankfurt et al., 1981; Steinbusch et al., 1982; Sakumoto et al., 1984) or 5-hydroxytryptophan (5-HTP; Sakumoto et al., 1984). Furthermore, a unilateral injection of a 5-HT neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), into the dorsolateral hypothalamus of the rat markedly decreased the number of 5-HT-immunoreactive cell bodies in the ipsilateral dorsomedial nucleus (Frankfurt and Azmitia, 1983). A similar injection of a catecholamine neurotoxin, 6-hydroxydopamine, was without effect, confirming that these hypothalamic cells are not catecholaminergic. Likewise, the probability that other, non-catecholaminergic neurons of the dorsomedial nucleus simply take up 5-HT released from neurons projecting to the hypothalamus was shown to be low in an experiment in which

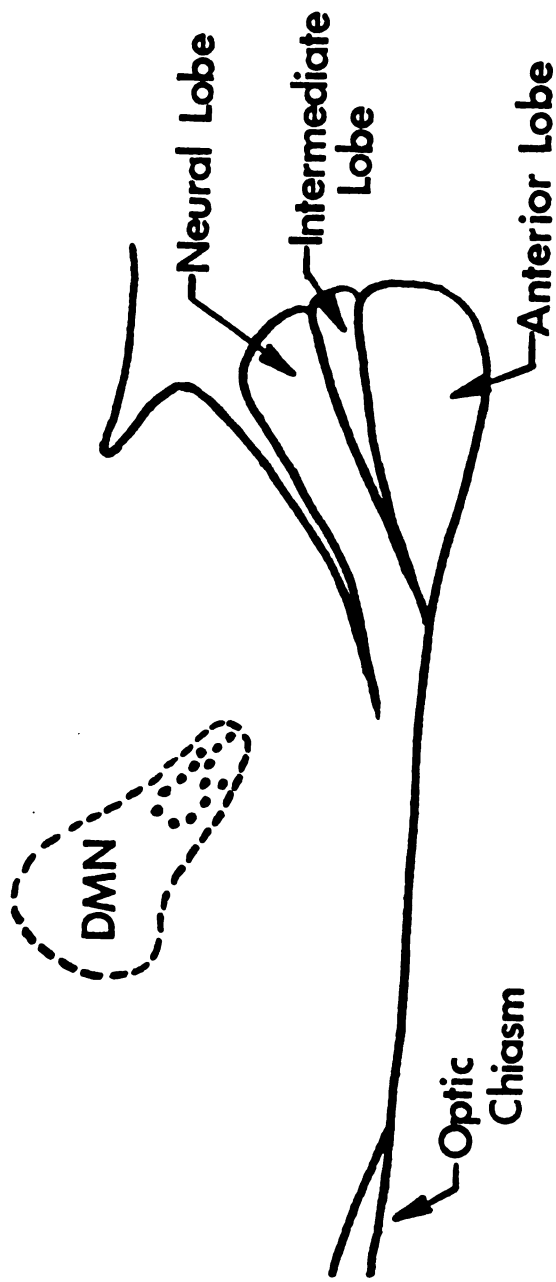

Figure 2. Sagittal view of the hypothalamus indicating the location of 5-HT-containing cells in the dorsomedial nucleus (DMN).

5,7-DHT was injected into the rostral mesencephalon (Frankfurt and Azmitia, 1983). In this study, the neurotoxin depleted the hypothalamus of large, 5-HT-immunoreactive varicosities but did not affect the 5-HT-containing cell bodies of the dorsomedial nucleus.

Taken together, these results provide substantial evidence for the presence of 5-HT in cell bodies of the hypothalamus. It is rather disconcerting, however, that 5-HT in these cells could be visualized only after the administration of exogenous 5-HT and/or pharmacological manipulations designed to enhance the synthesis of 5-HT or to prevent the degradation of this amine. This contrasts markedly with studies of the brainstem 5-HT-containing cell groups in which 5-HT could be easily visualized without these manipulations. Size also differentiates the 5-HT-containing perikarya of the dorsomedial nucleus from those of the brainstem. While the former are only about 10 µm in diameter (Beaudet and Descarries, 1979; Frankfurt and Azmitia, 1983), the latter range from 20 to 40 µm in diameter (Steinbusch and Niewenhuys, 1983). Therefore, while cells of the hypothalamic dorsomedial nucleus appear to contain 5-HT, they differ from the 5-HT-containing cells of the brainstem.

II. 5-HT Neuronal Projections

5-HT neurons are believed to subserve a variety of behavioral, endocrinological and autonomic functions, and the range of 5-HT neuronal projections is consistent with this functional diversity. Save for a very few areas (e.g., optic chiasm and corpus callosum; Steinbusch and Nieuwenhuys, 1983), each region of the brain and spinal cord is innervated by 5-HT cells. While this breadth of 5-HT neuronal projections was noted with the first formaldehyde-induced fluorescence studies of the central nervous system, it was only later with the use of neuroanatomical tracing techniques that attempts were made to link particular

5-HT terminal regions with their cell bodies of origin. Investigations of neuronalprojections initially employed the Nauta degeneration technique, anterograde tracing techniques using tritiated amino acids or retrograde tracing techniques using horseradish peroxidase (HRP) or fluorescent tracers. While these studies adequately determine if the raphe nuclei project to a particular region of the central nervous system or if a certain area receives projections from the raphe nuclei, they say nothing of the neurotransmitter content of the projection cells. Given the data presented earlier which show that only a small percentage of raphe cells contain 5-HT and that about a quarter of 5-HT cells lie outside of the raphe nuclei, this is an important limitation. Consequently, some researchers have employed other strategies to look specifically for 5-HT-containing projections. One method combines the use of the above tracing techniques with the administration of neurotoxins which selectively destroy 5-HT neurons (e.g., 5,7-DHT; Björklund et al., 1975). "Double-labeling" is another approach in which retrogradely transported label (e.g., wheat germ aglutinin) is injected and the labeled cell bodies are checked for 5-HT immunoreactivity. The following sections briefly summarize the results of these studies in the spinal cord and in the brain, and then provide a detailed account of 5-HT innervation of the pituitary gland.

A. Spinal Cord

Each level of the spinal cord receives 5-HT innervation (Willis, 1984). 5-HT neurons in the nuclei raphe magnus, pallidus and obscurus project extensively throughout the cord and terminate in the dorsal and ventral horns and in the intermediolateral cell column. The dorsal raphe nucleus and the B_9 cell group (around the mesencephalic medial lemniscus) send 5-HT projections to the cervical spinal cord, and the nucleus raphe pontis also contains a few 5-HT cells with descending processes.

B. Brain

As in the spinal cord, 5-HT-containing fibers have been visualized in practically every region of the brain (Azmitia, 1978; Parent, 1981; Steinbusch, 1981; Steinbusch and Nieuwenhuys, 1983). The specific cell groups of origin of these processes have been determined in some cases, and, while there are some exceptions, the cell bodies generally reside in the mesencephalon.

5-HT cells in the dorsal raphe nucleus project to the striatum, globus pallidus, septum, nucleus accumbens, olfactory tubercules, hippocampus, thalamus, habenular nuclei, hypothalamic nuclei, subcommissural organ, mammilary bodies, substantia nigra, ventrolateral geniculate nucleus, amygdala, interpeduncular nucleus, piriform cortex and temporoparietal cortex. The median raphe nucleus contains 5-HT neurons which project to the olfactory bulb, anterior olfactory nuclei, septum, thalamus, hippocampus, hypothalamic nuclei, mammilary bodies, habenular nuclei, interpeduncular nucleus, frontal cortex, cingulate cortex and entorhinal cortex. While the ascending 5-HT projections from the nuclei raphe pontis and raphe linearis are not well-defined, they are believed to communicate with other raphe nuclei. A description of specific ascending projections from the B_9 cell group is also lacking.

In addition to these projections to distant rostral sites, the raphe nuclei are also linked to each other. The nuclei raphe pallidus, raphe obscurus and raphe magnus are innervated by the dorsal and median raphe nuclei, while only the dorsal raphe nucleus projects to the nucleus raphe pontis. The dorsal raphe nucleus, in turn, receives projections from the nuclei raphe magnus, raphe pontis and raphe linearis, and the median raphe nucleus appears to contain processes from the dorsal raphe nucleus and the nucleus raphe linearis. The latter nucleus receives reciprocal connections from the dorsal and median raphe nuclei. While

each of the raphe nuclei contain 5-HT fibers and terminals, it is not known whether the inter-raphe connections are made, in fact, by 5-HT neurons.

In contrast to those of the brainstem cells, the projections of the putative 5-HT-containing perikarya of the dorsomedial nucleus of the hypothalamus have not yet been traced beyond the boundaries of that nucleus. The axons of these cells have a very small diameter when compared with other 5-HT containing fibers and appear to project ventrolaterally (Beaudet and Descarries, 1979; Frankfurt and Azmitia, 1983).

It should be noted that 5-HT neurons also project to non-neuronal tissue in the brain. In particular, the ependyma of each cerebral ventricle contains 5-HT processes (Steinbusch, 1981), and pial vessels are innervated by 5-HT fibers originating in the midbrain raphe nuclei (Scatton et al., 1985).

C. Pituitary Gland

The mammalian pituitary gland is composed of three divisions (shown in Figure 2) which vary both in secretory products and in innervation (Goodman, 1980). At one end of the spectrum is the anterior lobe. This division contains cells which synthesize and secrete follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, adrenocorticotropic hormone, prolactin and growth hormone. While the anterior lobe does receive some peripheral innervation from the autonomic nervous system, it receives no direct neuronal input from the brain. At the other end of the spectrum lies the neural lobe. Its secretory products, vasopressin (antidiuretic hormone) and oxytocin, are not manufactured by cells intrinsic to this lobe, but rather these hormones are synthesized mainly in cell bodies of the hypothalamic paraventricular and supraoptic nuclei and are transported down axons of these cells to the neural lobe. In addition, the neural lobe receives neuronal input from the brain and the autonomic nervous system. Intermediate, both in location and in attributes, to

the anterior and neural lobes is the intermediate lobe. While this division is only a very thin zone in humans (Wheater et al., 1979), it is well developed in some species such as the rat (Howe, 1973). Like the anterior lobe, the intermediate lobe contains cells, proopiomelanocorticotrophs, which synthesize and release the peptides α-melanocyte-stimulating hormone and β-endorphin, among others. Like the neural lobe, the intermediate lobe receives both central and peripheral innervation.

5-HT innervation of the pituitary gland was first intimated by studies which biochemically measured this amine in pituitary glands of the pig (Björklund et al., 1967), cat (Björklund and Falck, 1969), calf (Piezzi et al., 1970) and rat (Piezzi and Wurtman, 1970; Saavedra et al., 1975). Since the amine concentrations determined in the pituitary gland may also be attributed to 5-HT in the blood (Erspamer, 1966), in rodent mast cells (Erspamer, 1966) or in glial cells (Steinbusch and Nieuwenhuys, 1983), however, more direct evidence for neuronal 5-HT was necessary.

Recently, using immunocytochemistry, several groups have localized 5-HT to nerve fibers within the pituitary gland. Steinbusch and Nieuwenhuys (1981) found 5-HT immunofluorescence in fibers of the neural lobe of the rat pituitary gland, but failed to detect 5-HT in the intermediate or anterior lobes. The 5-HT fibers in the neural lobe were most highly concentrated in the region adjacent to the intermediate lobe. Slightly different results were obtained by Sano et al. (1982), who obtained immunohistochemical staining evidence for 5-HT innervation of both the neural and intermediate lobes of the cat pituitary gland following pretreatment with a monoamine oxidase inhibitor. The pattern of neural lobe innervation was similar to that obtained by Steinbusch and Nieuwenhuys and 5-HT processes were found throughout the intermediate lobe. The demonstration of 5-HT immunoreactive fibers in the cat intermediate lobe but not

in that of the rat may have been due to species differences, to the use of a monoamine oxidase inhibitor in the cat study, or to different sensitivities of immunofluorescence versus immunohistochemical staining. The last possibility is the most likely because Westlund and Childs (1982), with a more sensitive immunohistochemical staining method, demonstrated 5-HT-containing fibers in the neural and intermediate lobes of the rat pituitary gland. These observations have been confirmed in rat (Friedman et al., 1983; Léránth et al., 1983; Mezey et al., 1984; Saland et al., 1986) and in mouse, guinea pig and bat (Payette et al., 1985), and a radioautographic study has demonstrated [^{3}H]5-HT uptake by neuronal processes of the rat neural and intermediate lobes (Calas, 1985). Furthermore, a study with electron microscopy suggests that 5-HT-immunoreactive fibers form synaptic connections with β-endorphin-immunoreactive cells, the proopiomelanocorticotrophs of the intermediate lobe (Léránth et al., 1983).

Whereas the issue of 5-HT innervation of the neural and intermediate lobes of the pituitary gland appears to be settled, it is still unclear whether 5-HT processes reside within the anterior lobe. Some investigators report 5-HT immunoreactive processes within this region (Westlund and Childs, 1982; Léránth et al., 1983) while other groups find no evidence for this (Sano et al., 1982; Payette et al., 1985; Saland et al., 1986). There are, however, data demonstrating uptake of 5-HT into non-neuronal cells of the anterior lobe, at least some of which have been identified as gonadotrophs (Nunez et al., 1981; Johns et al., 1982; Payette et al., 1985).

Uptake of blood-borne 5-HT has also been suggested as the source of this amine in neuronal processes of the intermediate lobe (Saland et al., 1985, 1986). The findings that repeated injections of fluoxetine, a 5-HT uptake inhibitor, or of 6-hydroxydopamine, a catecholaminergic neurotoxin, decrease 5-HT-immunoreactivity in the intermediate lobe but not in the neural lobe of the

rat pituitary gland have been interpreted as evidence for 5-HT synthesis in the neural lobe and for uptake of preformed 5-HT into catecholaminergic neurons of the intermediate lobe (Saland et al., 1985, 1986). These results are inconsistent with the observation by Payette et al. (1985) that 6-hydroxydopamine administration does not alter 5-HT-immunoreactivity in the mouse intermediate lobe. Furthermore, the conclusions of Saland and coworkers are not entirely supported by the fact that p-chorophenylalanine, a 5-HT synthesis inhibitor, decreases 5-HT-immunoreactivity in both the neural and intermediate lobes (Saland et al., 1986). The authors suggest that this discrepancy may result from a p-chlorophenylalanine blockade of 5-HT synthesis extrinsic to the pituitary and thus removal of a source of 5-HT for uptake into the intermediate lobe, but it appears that further studies are necessary in order to resolve this issue.

Another incomplete area of research in the investigation of 5-HT innervation of the pituitary gland is the determination of the source of this innervation. While no 5-HT-specific neuroanatomical tracing studies have been conducted, some biochemical investigations have been undertaken in an attempt to determine the cell bodies of origin of these 5-HT-containing fibers. So far, this work has emphasized the intermediate lobe.

Since bilateral superior cervical ganglionectomy does not alter 5-HT-immunoreactivity in the intermediate lobe of the pituitary gland (Friedman et al., 1983), the 5-HT innervation of this lobe most likely arises in the brain. This hypothesis is borne out by studies involving pituitary stalk transection which show that, one week following surgery, 5-HT concentrations in the intermediate lobe decline to about 50% of control values (Friedman et al., 1983; Palkovits et al., 1986). This manipulation had no significant effect on 5-HT levels in the neural lobe, perhaps because of inter-animal variation (SEM of 50%). Alternatively, 5-HT in the neural lobe may, and the residual 5-HT in the intermediate lobe has

been reported to (Palkovits et al., 1986), reside in blood-borne elements and in mast cells. It is doubtful, however, that the blood is an important source of 5-HT in the intermediate lobe since this division of the pituitary is practically devoid of blood vessels (Howe, 1973). Perhaps the 5-HT-containing glial cells described by Steinbusch and Nieuwenhuys (1983) also contribute to the 5-HT concentrations measured in these lobes.

One study has been performed in an attempt to determine which brain region(s) contribute to the 5-HT innervation of the intermediate lobe. Mezey and colleagues (1984) found that hypothalamic deafferentation according to the technique of Halasz and Pupp (1962), knife cuts separating the mesencephalon from the diencephalon and lesions of the dorsomedial nucleus of the hypothalamus each reduced 5-HT-immunoreactivity in the intermediate lobe. In addition, knife cuts alone lowered the concentration of 5-HT in the intermediate lobe to 75% of control values, and knife cuts plus lesions of the dorsomedial nucleus reduced concentrations of this amine to 50% of control values. It should be noted that none of these manipulations altered 5-HT in the neural lobe, again a result which may be explained by non-neuronal sources of 5-HT confounding the data. The authors conclude from these lesion studies that both the midbrain raphe nuclei and the dorsomedial nucleus of the hypothalamus contribute to the 5-HT innervation of the intermediate lobe. This conclusion is inconsistent with tracing studies which found no labeling of cells in the dorsomedial nucleus following introduction of the retrograde tracer HRP into the pituitary gland (Sherlock et al., 1975; Broadwell and Brightman, 1976). Further experimentation is needed to resolve this conflict concerning 5-HT innervation of the pituitary gland.

III. 5-HT Neurochemistry

As depicted in Figure 3, 5-HT is synthesized from the essential L-amino acid, tryptophan, through a stepwise process utilizing the enzymes tryptophan hydroxylase (EC 1.14.16.4) and aromatic L-amino acid decarboxylase (ALAAD; EC 4.1.1.28). Tryptophan is obtained from the diet and transported by the blood largely bound to plasma proteins (Chaouloff et al., 1985, 1986) to the brain where free tryptophan competes with other large neutral amino acids (e.g., tyrosine, phenylalanine, leucine, isoleucine, valine) for carrier-mediated transport across the blood-brain barrier (Fernstrom, 1983). Once inside 5-HT neurons, tryptophan is hydroxlyated at the 5 position by the rate-limiting enzyme tryptophan hydroxylase to form 5-hydroxytryptophan (5-HTP). This step requires molecular oxygen and utilizes reduced tetrahydrobiopterin as an electron donor (Lovenberg and Kuhn, 1982). 5-HTP is then rapidly decarboxylated to form 5-HT by the ubiquitous enzyme ALAAD which uses pyridoxal-5-phosphate as a cofactor (Lovenberg et al., 1962). Following synthesis 5-HT may be stored in vesicles, released to act on receptors or degraded to one of several metabolites (Green and Grahame-Smith, 1975; Fernstrom, 1983). The major metabolite of 5-HT is 5-hydroxyindoleacetic acid (5-HIAA) which is formed in a two-step reaction sequence by monoamine oxidase (EC 1.4.3.4) and aldehyde dehydrogenase (EC 1.2.1.3). Minor metabolites of 5-HT include 5-hydroxytryptophol which is produced by the sequential actions of monoamine oxidase and alcohol reductase (EC 1.1.1.2), and 5-hydroxykynuramine which is synthesized from 5-HT via the enzyme indoleamine 2,3-dioxygenase.

Factors influencing the rate of synthesis of 5-HT have been extensively reviewed (Hamon and Glowinski, 1974; Green and Grahame-Smith, 1975; Lovenberg and Kuhn, 1982; Fernstrom, 1983) and have been shown to produce their effects by altering tryptophan hydroxlyase activity. Unlike tyrosine hydroxlyase,

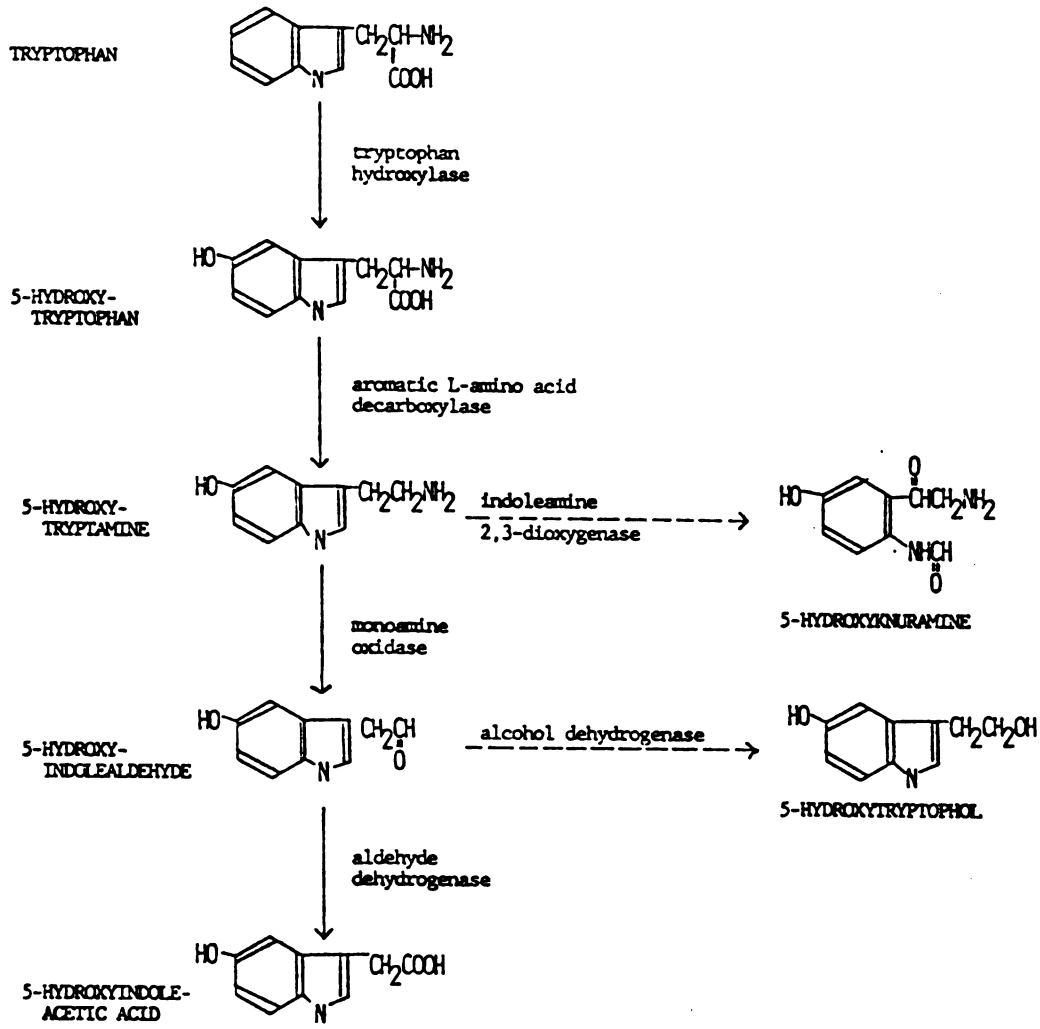

Figure 3. The synthetic and degradative pathways of 5-HT.

the rate-limiting enzyme in catecholamine biosynthesis, tryptophan hydroxlyase does not appear to be affected to an appreciable extent by end-product inhibition. Rather, the activity of this enzyme appears to be influenced by substrate availability. While insufficient concentrations of the reduced pterin cofactor could conceivably affect the rate of 5-HT synthesis, and decreases in arterial oxygen have been shown to reduce tryptophan hydroxlyase activity (Davis and Carlsson, 1973), tryptophan is clearly the substrate most important in determining the rate of 5-HT synthesis. Since the K_m of tryptophan hydroxlyase for tryptophan is approximately the same as the concentration of brain tryptophan (approximately $1\text{-}5 \times 10^{-5}$M; Fernstrom and Wurtman, 1971), one might predict that alterations in the availability of this substrate would markedly alter the activity of the enzyme. Indeed, this appears to be the case. A number of studies have shown that diets deficient in tryptophan decrease brain levels of 5-HT (e.g., Culley et al., 1963) and conversely, other investigations have demonstrated that diets with enhanced tryptophan contents or parenteral administration of tryptophan elevate the concentration of 5-HT in the brain (e.g., Green et al., 1962; Fernstrom and Wurtman, 1971).

In addition, the activity of 5-HT neurons also appears to play a role in the rate of 5-HT synthesis. Disruption of impulse flow by transection of these neurons has been shown to decrease the activity of tryptophan hydroxlyase (Carlsson et al., 1973), and increases in 5-HT neuronal activity produced by electrical stimulation of these neurons in animals pretreated with an ALAAD inhibitor increases the rate of 5-HTP formation during the applied stimulation and for at least 30 minutes thereafter (Herr et al., 1975; Bourgoin et al., 1980; Boadle-Biber et al., 1983, 1986; Duda and Moore, 1985). It has been hypothesized that the increased activity of tryptophan hydroxlyase observed following electrical stimulation is the result of a stimulation-induced influx of calcium which

increases the affinity of this enzyme for tryptophan and reduced pterin cofactor (Knapp et al., 1975).

STATEMENT OF PURPOSE

Immunocytochemical studies have revealed the presence of 5-hydroxytryptamine-containing nerve axons and terminals in the neural and intermediate lobes of the pituitary gland. Little is known of these neurons, however. The purpose of the present studies is to begin to characterize these 5-hydroxytryptamine-containing neurons by determining if 5-hydroxytryptamine is synthesized within them and by establishing the location of their cell bodies of origin. Since neurochemical techniques will be used to address these issues, biochemical indices of 5-hydroxytryptaminergic neuronal activity will first be rigorously evaluated.

MATERIALS AND METHODS

I. Materials

A. Animals

Male Long-Evans rats (200-225 g initial weight; Charles River Breeding Labortories, Wilmington, MA, and Blue Spruce Farms, Inc., Altamont, NY) were housed 4 to a cage in a temperature- ($22^{o}C$) and light-controlled (lights on between 7:00 a.m. and 7:00 p.m.) room with food (Wayne Lablox) and tap water available ad libitum. Rats receiving brain lesions were also provided with fresh fruit and a mash of ground rat chow mixed with table sugar and water.

B. Drugs

The following drugs were dissolved in 0.9% saline: fluoxetine hydrochloride (kindly supplied by M. Forman of Eli Lilly Co., Indianapolis, IN), NSD 1015 (m-hydroxybenzylhydrazine hydrochloride, Sigma Chemical Co., St. Louis, MO), pargyline hydrochloride (Regis Chemical Co., Morton Grove, IL). Chloral hydrate (Sigma Chemical Co.) and L-tryptophan methyl ester hydrochloride (Sigma Chemical Co.) were dissolved in water. A 10 µg/µl solution of 5,7-dihydroxytryptamine (5,7-DHT, Calbiochem-Behring Corp., La Jolla, CA) was prepared in 0.3% saline with 0.1% ascorbic acid for intracranial injection of 1 µl per injection site. Probenecid (Sigma Chemical Co.) was dissolved in 1 N sodium hydroxide, and the pH of the final solution adjusted to 10 with hydrochloric acid. The anesthetic Equithesin was prepared by stirring 42.51 g chloral hydrate, 9.72 g pentobarbital sodium (Sigma Chemical Co.) and 21.26 g magnesium sulfate in 443 ml of warm propylene glycol. After these compounds were completely dissolved,

120 ml of 95% ethanol was added and the total volume was brought up to 1000 ml with water. Chloral hydrate, NSD 1015 and pargyline were administered in volumes of 1 ml/kg, fluoxetine and tryptophan in volumes of 2 ml/kg, and Equithesin and probenecid in volumes of 3 ml/kg.

II. Methods

A. Electrical Stimulation

At 25, 40 or 70 minutes before sacrifice the animals were anesthetized with chloral hydrate (400 mg/kg, i.p.). Chloral hydrate anesthesia was chosen because it does not alter the concentration of the 5-HT metabolite 5-HIAA in cerebroventricular perfusates of rats (Nielsen and Moore, 1982) and it does not affect single unit activity of dorsal raphe neurons in cats (Trulson and Trulson, 1983). In some cases, when drugs were to be administered intravenously to ensure rapid onset of their actions, a femoral vein was catheterized with polyethylene tubing (PE-10 Intramedic^{R} polyethylene tubing, Clay Adams, Parsippany, NJ).

The animals were then placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) with the incisor bar set 3 mm below the intraaural line and prepared for electrode implantation by exposing and drilling through the skull. For stimulation of the dorsal raphe nucleus a coaxial bipolar stainless steel electrode (NE-100, Rhodes Medical Intruments, Inc., Woodland Hills, CA) was lowered at a 25^{o} angle to avoid the sagittal sinus so that the tip was located in this nucleus: A 0 mm, L 0 mm, H -1.0 mm (Kŏnig and Klippel, 1967). The same electrode was used to stimulate the pituitary stalk, but in this case it was positioned midsagittally 2.5 mm posterior to bregma and lowered to the base of the brain so that the tip was located in the pituitary stalk. Concurrent stimulation of the dorsal and median raphe nuclei was accomplished by gluing together two NE-100 electrodes such that the tip of one was positioned in the

dorsal raphe nucleus and the tip of the other was located in the median raphe nucleus (A 0 mm, L 0 mm, H -2.5 mm) when the electrodes were lowered at a 25° angle. Two NE-100 electrodes were also glued together to bilaterally stimulate the dorsomedial nucleus of the hypothalamus (A +4.0 mm, L ±0.5 mm, H -2.5 mm). The location of the stimulating electrode(s) was confirmed either by examination of fresh tissue (pituitary stalk, dorsomedial nucleus) or of histological sections (dorsal raphe nucleus, median raphe nucleus).

The raphe and hypothalamic nuclei and the pituitary stalk were stimulated for 15, 30 or 60 minutes with cathodal monophasic pulses of 1 msec duration and 0.3 mA current at 5 or 10 Hz, parameters previously shown to significantly increase concentrations of 5-HIAA (Sheard and Aghajanian, 1968) and 5-HTP (Duda and Moore, 1985) in the rat forebrain. Current was generated by a Grass stimulator (Model SD9) and delivered via a Grass constant current unit (Model CCU 1A).

B. Chemical and Electrolytic Lesions

Surgery was performed on Equithesin-anesthetized animals with the aid of a stereotaxic apparatus 7 to 10 days before sacrifice. Sham surgery consisted of exposing and drilling through the skull. 5-HT perikarya of the raphe nuclei were lesioned with the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT). In the case of the dorsal and median raphe nuclei a 22 gauge stainless steel cannula was lowered to A +0.5 mm L 0 mm H -3.0 mm, and a 27 gauge stainless steel injection cannula attached to a 5 µl Hamilton syringe was inserted into the guide cannula. One microliter of a 5,7-DHT solution was rapidly injected and the guide cannula-injector assembly was held stationary for an additional 5 minutes. This process was repeated at coordinates 1 and 2 mm dorsal to the first injection site. The guide cannula was then raised out of the brain, moved 1 mm caudal, lowered and the series of 5,7-DHT injections was duplicated. The nuclei raphe pontis and

raphe magnus were lesioned in a similar fashion, except that 5,7-DHT was initially injected at A +0.5 mm L 0 mm H -4.0 mm (Paxinos and Watson, 1982). Injections were then made 1 and 2 mm dorsal to this site, and this procedure was repeated 1, 2 and 3 mm caudal to the placement of the initial injection.

Because of the possible spread of 5,7-DHT following injection, lesions of the dorsomedial nucleus of the hypothalamus were performed electrolytically rather than chemically to ensure that the lesion was confined to this nucleus. Lesioning electrodes were constructed of size 0 insect pins insulated to 1 mm of the tip with a quick-drying enamel. Two electrodes were secured together in a parallel arrangement with dental acrylic so that their tips were 1 mm apart. The electrode assembly was lowered into the bilateral dorsomedial nuclei (A +4.0 mm, L $\pm$0.5 mm, H -2.5 mm), and 0.5 mA of anodal current was applied for 15 seconds.

Following all surgery, Sulf-U-Dex^{R} (McCullough Cartwright Pharmaceutical Corp., Highland Park, IL) was sprinkled over the wound, the scalp was closed with stainless steel wound clips and 0.1 ml of Combiotic^{R} (Pfizer, Inc., New York, NY) was injected intramuscularly.

Correct placement of lesions was ascertained at the time of sacrifice by examination of fresh tissue (dorsomedial nucleus) or of histological sections (raphe nuclei).

C. Platelet Isolation

Immediately before sacrifice animals were anesthetized with chloral hydrate (400 mg/kg, i.p.), and 1 ml of blood was drawn from the heart into a plastic syringe using an 18 gauge needle. Blood samples were placed in plastic 12x75 mm tubes containing 110 µl of 3.8% sodium citrate (blood:sodium citrate:: 9:1) and kept in a 37^{o}C water bath until all samples were collected (up to 180 minutes). The samples were then centrifuged at 4950 x g for 1.5 minutes, and 100

µl of the supernatant (platelet enriched plasma) were placed in 1.5 ml polyethylene microtubes and centrifuged in a Beckman Microfuge™ (Beckman Instruments, Inc., Palo Alto, CA) at 8730 x g for 5 minutes. The resultant supernatant was discarded and the platelets were resuspended and washed by vortexing in 1000 µl of 0.9% saline containing 0.01% EDTA. The samples were again centrifuged at 8730 x g for 5 minutes, the supernatant poured off and the platlets resuspended by vortexing in 1000 µl of HPLC mobile phase (described below). Following sonication for 3 seconds (Sonicator™ Cell Disruptor, Heat Systems-Ultrasonics, Inc., Plainview, NY), the samples were centrifuged for 5 minutes at 8730 x g and a 60 µl sample of the supernatant was removed and stored at -20°C until analyzed for 5-HT by high performance liquid chromatography with electrochemical detection (HPLC-EC). The protein content of the pellet was determined according to the method of Lowry et al. (1951).

D. Tissue Dissection

Following treatment the rats were decapitated, their brains and pituitary glands were removed rapidly and placed on a cold plate, and a 5 mm section of thoracic spinal cord was dissected. A frontal cut through the brain was made with a razor blade just posterior to the mammilary bodies and the portion of the brain anterior to the cut frozen on dry ice. Frontal brain sections (600 µm) were prepared in a cryostat and punches of discrete regions (nucleus accumbens, central nucleus of the amygdala, dorsomedial nucleus of the hypothalamus, suprachiasmatic nucleus, preoptic suprachiasmatic nucleus, medial preoptic nucleus, arcuate nucleus, median eminence) were made with stainless steel cannulae of 500 or 1000 µm inner diameter according to a modification (Lookingland and Moore, 1984) of the micropunch technique of Palkovits (1973). These regions were selected for analysis because they are known to receive 5-HT projections, in some cases from the dorsal raphe nucleus (Azmitia, 1978), and

because they are representative of limbic, basal ganglia and hypothalamic structures. With the aid of a dissecting microscope, the neurointermediate lobe of the pituitary gland was separated from the anterior lobe with fine forceps. When the neural and intermediate lobes were to be analyzed separately, the neurointermediate lobe was placed on a glass slide, frozen on dry ice and dissected according to a modification of the technique of Lookingland et al. (1985).

Once dissected, the tissue was placed in a polyethylene microsample tube containing 60 µl of HPLC mobile phase, sonicated for 3 seconds, centrifuged and stored at -20°C. The supernatant was analyzed by HPLC-EC, and the protein content of the pellet was determined by the method of Lowry et al. (1951).

E. Histology

In studies involving electrical stimulation of the raphe nuclei, brain tissue posterior to the mammilary bodies was preserved in a 10% buffered formalin solution until it was sectioned (100 µm) in a cryostat. The sections were stained with cresyl violet (cresyl echt violett, Chroma-Gesellschaagt, Schmid and Co.) and examined under a microscope to determine the location of the stimulation site. An example of an electrode track representing the site of stimulation is shown in Figure 4.

F. Measurement of Monoamines

5-HT, 5-HTP, 5-HIAA, dopamine (DA) and 3,4-dihydroxyphenylalanine (DOPA) were measured with an HPLC-EC system. Fifty microliters of the sample supernatant were injected onto a C_{18} reverse phase column (4.6 mm i.d. x 25 cm; 5 µm spheres, Biophase ODS, Bioanalytical Systems, Inc., West Lafayette, IN) which was protected by a precolumn cartridge filter (10 µm spheres, 4.6 mm i.d. x 3 cm, Bioanalytical Systems, Inc.). The HPLC column was coupled to an electrochemical detector (LC4A, Bioanalytical Systems, Inc.) equipped with a TL-5 glassy carbon electrode set at a potential of +0.75 V relative to a Ag/AgCl

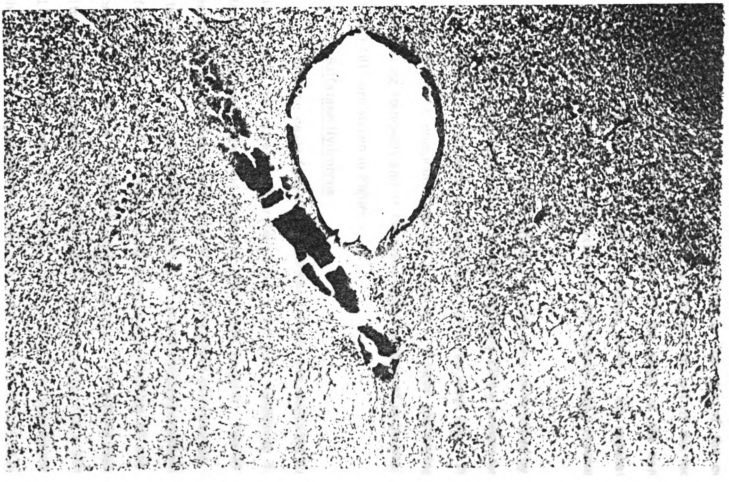

Figure 4. A frontal brain section showing an electrode track terminating in the dorsal raphe nucleus (x27).

reference electrode. The HPLC mobile phase consisted of 0.05 M phosphate/0.03 M citrate buffer adjusted to a pH of 2.7, 0.1 mM EDTA, 1.5 mM sodium octyl sulfate and 25% methanol. Depending on the age of the column, the concentrations of the mobile phase components and the pH of the mobile phase were altered slightly to attain separation of the peaks of the compounds of interest while minimizing the total retention time. All separations were performed with a flow rate of approximately 0.8 ml/min and a pressure of about 2500 psi.

The amounts of compounds in a sample were determined by comparing sample peak heights measured by a Hewlett-Packard 3390A Integrator (Hewlett-Packard, Avondale, PA) with standards that were run each day. The limit of sensitivity for each compound was 20 to 30 pg. Sample chromatograms of a mixture of 1 ng standards and of the dorsomedial nucleus of an animal pretreated with NSD 1015 are shown in Figure 5.

G. Sulfatase Hydrolysis

In some cases following dissection, tissue was sonicated for 3 seconds in 45 µl of 1 mM citrate buffer (adjusted to pH 2.7) containing 0.2% sodium metabisulfite, centrifuged and stored at -20°C until analysis. Two 20 µl aliquots of each sample supernatant were placed in polyethylene microsample tubes which contained 35 µl of MOPS buffer (0.2 M 3[N-morpholino]propanesulfonic acid buffer adjusted to pH 7.1 at room temperature and containing 30 mM magnesium chloride, 10 mM EGTA and 20 mM dithiothreitol) and 3 µl spiking solution (300 pg p-nitrocatecholsulfate in 3 µl MOPS buffer). Two microliters of a 50% glycerol - 0.01 M Tris solution, pH 7.5, was also added to the "non-hydrolyzed" aliquot of each sample. The "hydrolyzed" aliquots of each sample received an addition of 2 µl of the glycerol-Tris solution which contained 9.9 milliunits of sulfatase. The sulfatase (EC 3.1.6.1) was purchased from Sigma Chemical Co. in a partially

Figure 5. Sample HPLC-EC chromatograms obtained following the injection of standards and of brain tissue. A mixture of 1 ng standards (left) or a sample of the dorsomedial nucleus of the hypothalamus of a rat pretreated with NSD 1015 (100 mg/kg, i.p., 30 minutes prior to sacrifice) (right) were injected onto a HPLC-EC column. Details of the HPLC-EC systems are described in the "Materials and Methods" section. DA, dopamine; DOPA, 3,4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; HVA, homovanillic acid; NE, norepinephrine.

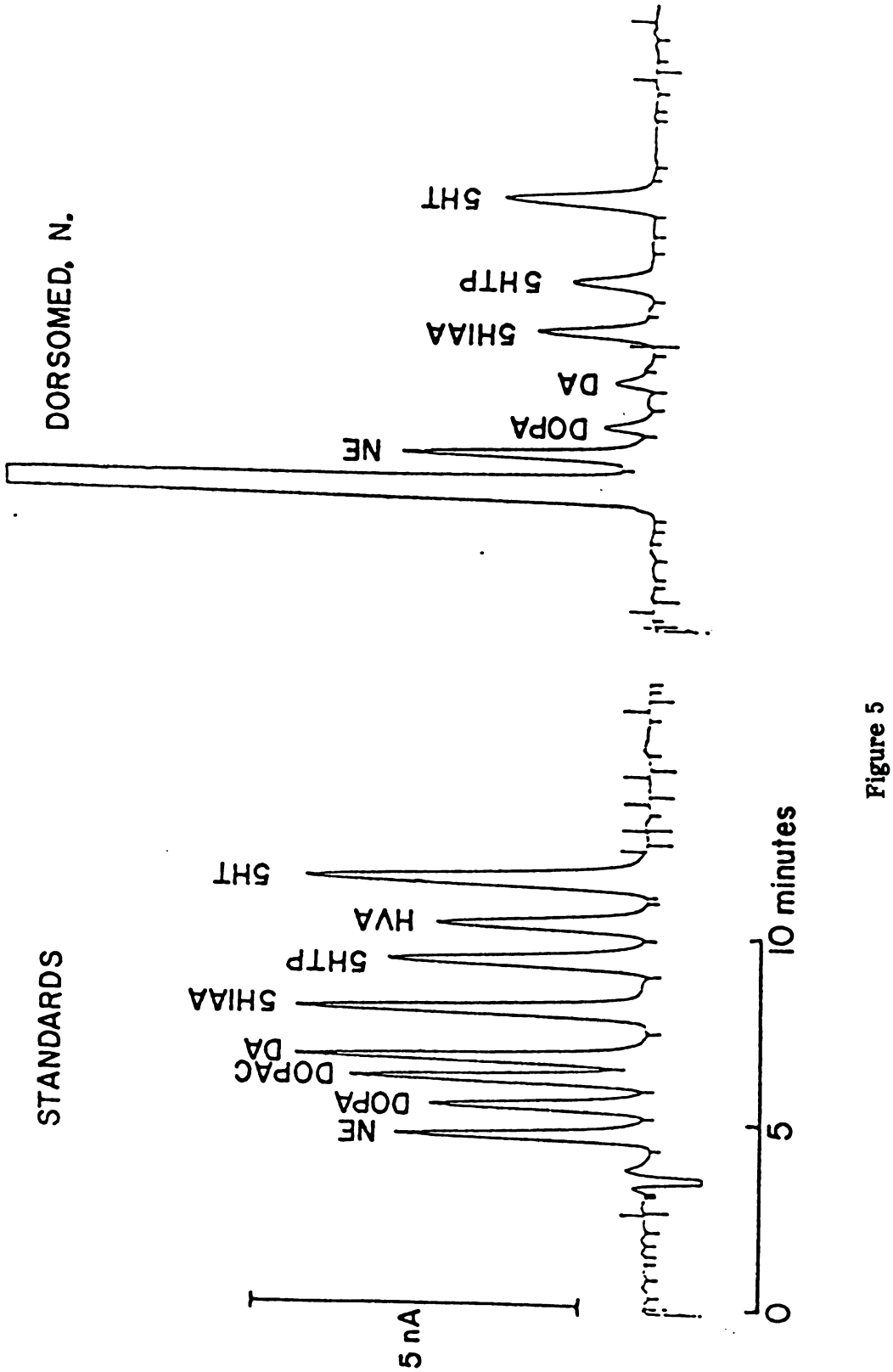
DORSOMED. N.
5HT
5HTP
5HIAA
DA
DOPA
NE
STANDARDS
5HT
HVA
5HTP
5HIAA
DA
DOPAC
DOPA
NE
5 nA
0
5
10 minutes

Figure 5

purified form obtained from Aerobacter aerogenes, and the enzyme was dialyzed against the glycerol-Tris solution before use. The samples were incubated for 3 hours at room temperature before the reaction was stopped by the addition of 20 µl of 1 M citrate in 100% methanol.

H. Statistical Analysis

The absolute values from each region were analyzed with Student's t-test when comparing two groups and with one-way analysis of variance followed by the Student-Newman-Keuls test (Steel and Torrie, 1980) when comparing three or more groups. Where appropriate, data were examined for interactional effects with a 2x2 factorial analysis. Differences with a probability of error of less than 5% were considered significant. For comparative purposes some data are presented as a percentage of control values.

RESULTS

I. BIOCHEMICAL INDICES OF 5-HT NEURONAL ACTIVITY

A. Comparison of Biochemical Indices of 5-HT Neuronal Activity Following Electrical Stimulation of the Dorsal Raphe Nucleus

Several biochemical indices of 5-HT neuronal activity are currently in use. Following the observation that the concentration of the 5-HT metabolite 5-HIAA increases in the forebrain following electrical stimulation of midbrain 5-HT cell bodies (Aghajanian et al., 1967; Sheard and Aghajanian, 1968; Kostowski et al., 1969; Sheard and Zolovick, 1971; Curzon et al., 1978), concentrations of 5-HIAA and the ratio of the concentrations of 5-HIAA to 5-HT have been employed to estimate the activity of 5-HT neurons. Likewise, it has been demonstrated that following administration of an ALAAD inhibitor such as NSD 1015, the 5-HT precursor 5-HTP accumulates with time at a rate that is proportional to 5-HT neuronal activity (Herr et al., 1975; Bourgoin et al., 1980; Boadle-Biber et al., 1983, 1986; Duda and Moore, 1985). Therefore, the concentration of 5-HTP following NSD 1015 administration has served as an index of the activity of these neurons. Finally, the observation that the concentrations of 5-HT and 5-HIAA increase and decrease, respectively, following the inhibition of 5-HT degradation with a monoamine oxidase inhibitor such as pargyline (Tozer et al., 1966; Neckers and Meek, 1976) has prompted some investigators to interpret any alterations in the pargyline-induced increase in 5-HT or decline in 5-HIAA concentrations produced by an experimental manipulation as changes in 5-HT neuronal activity.

While the results of some of these methods have been compared (Morot-Gaudry et al., 1974; Van Loon et al., 1981; Johnson and Crowley, 1982), these

evaluations were performed under only "basal" activities of 5-HT neurons (i.e., without defined alterations in impulse traffic in these neurons). The purpose of the present study was to compare different biochemical indices of 5-HT neuronal activity under conditions of changing neuronal impulse traffic produced by electrical stimulation of these neurons, and to determine which of these methods is the most appropriate for use in selected brain regions.

In each of these experiments animals received an intravenous drug injection concurrent with the onset of electrical stimulation of the dorsal raphe nucleus for 0, 15 or 30 min. Stimulating current was delivered at a rate of 0 Hz (sham controls), 5 Hz or 10 Hz. Animals were decapitated immediately upon the cessation of stimulation, and the concentrations of 5-HT, its precursor, 5-HTP, or its metabolite, 5-HIAA, were determined in various brain regions. To facilitate comparisons among studies, a composite of the results obtained in the experiments from a selected brain region, the nucleus accumbens, is represented in Figure 6. Each study is described individually in greater detail below.

1. Measurements of the concentrations of 5-HIAA and 5-HT

Electrical stimulation of the dorsal raphe nucleus of saline-treated animals increased the concentrations of 5-HIAA in brain regions containing 5-HT nerve terminals in a frequency-dependent manner. In the nucleus accumbens shown in Figure 6A, 15 min of stimulation at a frequency of 5 Hz significantly increased 5-HIAA concentrations above control (0 Hz) values, and stimulation at 10 Hz produced a more marked increase. Similar results were obtained in other regions and are shown with those of the nucleus accumbens in Table 2. When electrical stimulation at either 5 or 10 Hz was carried out for 30 min a slight increase in 5-HIAA concentrations as compared to the 15 min values was observed in most brain regions.

Figure 6. The effect of electrical stimulation of the dorsal raphe nucleus on various biochemical indices of 5-HT neuronal activity in the nucleus accumbens. A) Effect on the concentration of 5-HIAA. Saline (1 ml/kg) was administered through a femoral vein catheter concurrent with the onset of electrical stimulation (monophasic pulses of 1 msec duration and 0.3 mA current) of the dorsal raphe nucleus at "zero time", and animals were sacrificed at 15 or 30 min thereafter. B) Effect on the concentration of 5-HT. The data were obtained from the same animals as in panel A. C) Effect on the ratio of the concentrations of 5-HIAA to 5-HT. The data were obtained from the same animals as in panels A and B. D) Effect on the accumulation of 5-HTP. The experimental protocol was the same as in panel A, except that NSD 1015 (25 mg/kg) rather than saline was administered at zero time. E) Effect on the pargyline-induced increase in the concentration of 5-HT. The experimental protocol was the same as in panel A, except that pargyline (30 mg/kg) rather than saline was administered at zero time. F) Effect on the pargyline-induced decline in the concentration of 5-HIAA. The data were obtained from the same animals as in panel E. Symbols (□, 0 Hz; △, 5 Hz; ○, 10 Hz stimulation frequencies) represent the mean and vertical lines $\pm$ 1 SEM of 7-12 determinations, expressed as a percentage of control values at the corresponding times. Where no vertical line is depicted the SEM is less than the radius of the symbol. Solid symbols represent those values that are significantly different from 0 Hz (control) at the same time point ($p < 0.05$). *, significantly different from 5 Hz at the same time point ($p < 0.05$).

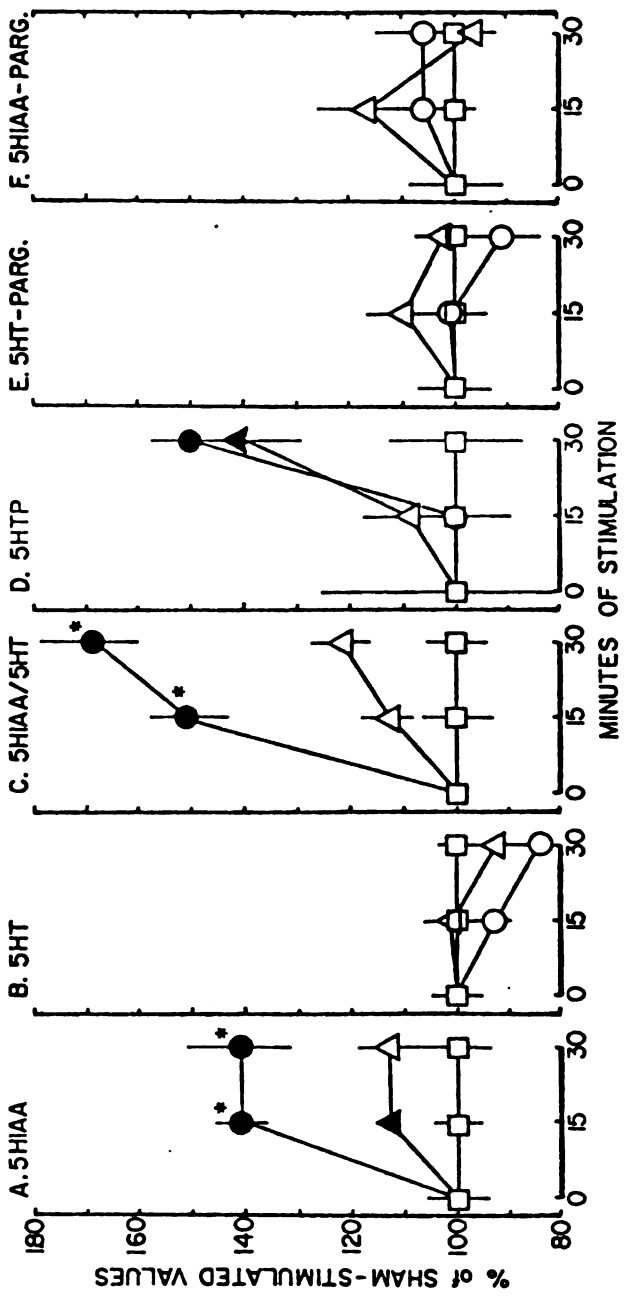
A. 5HIAA
B. 5HT
C. 5HIAA/5HT
D. 5HTP
E. 5HT-PARG.
F. 5HIAA-PARG.
% of SHAM-STIMULATED VALUES
180
160
140
120
100
80
0
15
30
MINUTES OF STIMULATION

Figure 6

TABLE 2

Concentrations of 5-HIAA in Selected Brain Regions Following Electrical Stimulation of the Dorsal Raphe Nucleus

Region	Min of Stimulation	Rate of Stimulation		
		0 Hz	5 Hz	10 Hz
N. Accumbens	0	7.77±0.45	---	---
	15	8.09±0.42	9.14±0.19[a]	11.42±0.40[a,b]
	30	8.83±0.61	9.98±0.58	12.48±0.87[a,b]
Amygdala	0	4.90±0.26	---	---
	15	5.11±0.34	5.60±0.23	6.38±0.38[a]
	30	5.19±0.35	6.15±0.27	7.70±0.45[a,b]
Suprachiasmatic N.	0	4.48±0.34	---	---
	15	4.26±0.20	4.83±0.21	5.44±0.29[a]
	30	4.58±0.39	5.67±0.29	5.51±0.32
Dorsomedial N.	0	7.43±0.36	---	---
	15	7.90±0.45	8.91±0.34	9.70±0.32[a]
	30	8.18±0.51	9.58±0.25	10.89±0.41[a]

Experimental procedures are as described in the legend to Figure 6A. Values represent the mean ± 1 SEM of 7-12 determinations, expressed as ng 5-HIAA/mg protein. [a], significantly different from 0 Hz (control) at the same time point ($p < 0.05$). [b], significantly different from 5 Hz at the same time point ($p < 0.05$).

In the same group of animals, electrical stimulation of the dorsal raphe nucleus slightly reduced the regional concentrations of 5-HT (Figure 6B and Table 3), but this effect was statistically significant only in the amygdala after 15 min of 10 Hz stimulation. Electrical stimulation of the ascending 5HT neurons did, however, increase the ratio of the concentrations of 5-HIAA to 5-HT (Figure 6C and Table 4). While in the case of the nucleus accumbens (Figure 6C) the values obtained with stimulation at a frequency of 5 Hz were not significantly different from control values, stimulation at 10 Hz significantly increased the ratio of the concentrations of 5-HIAA to 5-HT after both 15 and 30 min, with a tendency for the 30 min values to be greater than the 15 min values. Table 4 shows that the results obtained in other regions were consistent with those of the nucleus accumbens.

2. Measurement of the rate of 5-HTP accumulation following NSD 1015 administration

In a separate group of animals, a supramaximal dose of NSD 1015 (25 mg/kg, i.v.; Duda and Moore, 1985) was administered concurrent with the onset of electrical stimulation of the dorsal raphe nucleus, and the concentration of 5-HTP was determined in brain regions containing 5-HT nerve terminals. In the absence of NSD 1015 (i.e., at zero min) the concentration of 5-HTP was near the limits of detectability. Following the administration of NSD 1015, however, the concentration of 5-HTP increased with time in each brain region of control animals (Table 5). When stimulating current was applied for 30 min at a frequency of 5 or 10 Hz, the concentration of 5-HTP significantly increased above control levels in the nucleus accumbens; 15 min of stimulation was without effect in this region (Figure 6D). While 30 min of 10 Hz stimulation increased the 5-HTP levels in all brain regions, 15 min of 10 Hz stimulation was sufficient to significantly elevate 5-HTP concentrations in the hypothalamic nuclei (Table 5).

TABLE 3

Concentrations of 5-HT in Selected Brain Regions Following Electrical Stimulation of the Dorsal Raphe Nucleus

Region	Min of Stimulation	Rate of Stimulation		
		0 Hz	5 Hz	10 Hz
N. Accumbens	0	9.52±0.50	---	---
	15	8.93±0.55	9.04±0.49	8.30±0.29
	30	9.08±0.33	8.51±0.67	7.63±0.50
Amygdala	0	6.65±0.82	---	---
	15	6.45±0.41	5.73±0.18	5.24±0.39[a]
	30	5.84±0.56	5.32±0.36	6.07±0.34
Suprachiasmatic N.	0	7.78±0.72	---	---
	15	6.59±0.36	6.47±0.32	6.80±0.46
	30	7.12±0.52	6.55±0.34	7.02±0.48
Dorsomedial N.	0	9.01±0.40	---	---
	15	8.98±0.29	8.77±0.28	8.61±0.38
	30	8.61±0.35	8.49±0.25	8.77±0.35

Experimental procedures are as described in the legend to Figure 6B. Values represent the mean ± 1 SEM of 7-12 determinations, expressed as ng 5-HT/mg protein. [a], significantly different from 0 Hz (control) at the same time point.

TABLE 4

Ratio of the Concentrations of 5-HIAA to 5-HT in Selected Brain Regions Following Electrical Stimulation of the Dorsal Raphe Nucleus

Region	Min of Stimulation	Rate of Stimulation		
		0 Hz	5 Hz	10 Hz
N. Accumbens	0	0.82±0.02	---	---
	15	0.92±0.06	1.04±0.05	1.39±0.06[a,b]
	30	0.98±0.06	1.20±0.06	1.66±0.10[a,b]
Amygdala	0	0.82±0.11	---	---
	15	0.80±0.06	0.99±0.05	1.24±0.08[a,b]
	30	0.92±0.08	1.21±0.10	1.30±0.09[a]
Suprachiasmatic N.	0	0.59±0.03	---	---
	15	0.65±0.03	0.76±0.04	0.83±0.08
	30	0.65±0.03	0.87±0.04[a]	0.81±0.05[a]
Dorsomedial N.	0	0.83±0.03	---	---
	15	0.88±0.04	1.02±0.04	1.14±0.06[a]
	30	0.95±0.03	1.13±0.03[a]	1.25±0.04[a]

Experimental procedures are as described in the legend to Figure 6C. Values represent the mean + 1 SEM of 7-12 determinations, expressed as ng 5-HIAA/mg protein to ng 5-HT/mg protein. [a], significantly different from 0 Hz (control) at the same time point ($p < 0.05$). [b], significantly different from 5 Hz at the same time point ($p < 0.05$).

TABLE 5

Concentrations of 5-HTP in Selected Brain Regions
Following NSD 1015 Administration and Electrical Stimulation
of the Dorsal Raphe Nucleus

Region	Min of Stimulation	Rate of Stimulation		
		0 Hz	5 Hz	10 Hz
N. Accumbens	0	0.31±0.08	---	---
	15	0.88±0.08	0.96±0.08	0.88±0.09
	30	1.15±0.15	1.62±0.14[a]	1.73±0.09[a]
Amygdala	0	0.23±0.03	---	---
	15	0.62±0.06	0.85±0.09	0.82±0.10
	30	1.09±0.07	1.43±0.13	1.53±0.15[a]
Suprachiasmatic N.	0	0.30±0.07	---	---
	15	2.14±0.11	2.31±0.06	2.74±0.34[a]
	30	2.94±0.25	3.12±0.11	3.61±0.21[a]
Dorsomedial N.	0	0.40±0.16	---	---
	15	1.70±0.25	2.34±0.12[a]	2.66±0.23[a]
	30	2.70±0.21	3.31±0.17[a]	3.88±0.17[a,b]

Experimental procedures are as described in the legend to Figure 6D. Values represent the mean ± 1 SEM of 6-12 determinations, expressed as ng 5-HTP/mg protein. [a], significantly different from 0 Hz (control) at the same time point ($p < 0.05$). [b], significantly different from 5 Hz at the same time point ($p < 0.05$).

3. Measurement of the rate of 5-HT accumulation and 5-HIAA decline following pargyline administration

Pargyline is typically administered intraperitoneally at a dose of 75 mg/kg (Tozer et al., 1966), but in the present experiments the drug was administered intravenously to ensure rapid inactivation of monoamine oxidase. Therefore, a dose sufficient to elevate 5-HT and decrease 5-HIAA concentrations was first determined. Pargyline (0, 3, 10, 30 or 60 mg/kg) was injected through a femoral vein catheter into chloral hydrate-anesthetized animals, and the concentrations of 5-HT and 5-HIAA were determined 30 min later (see Figure 7). A dose of 60 mg/kg proved to be lethal when administered intravenously, but lower doses appeared to have no toxic effects. Because 30 mg/kg significantly enhanced the concentration of 5-HT in each region and decreased the concentration of 5-HIAA in most regions to levels (approximately 140 and 50 percent of control, respectively) that were not significantly different from those obtained with 10 mg/kg, 30 mg/kg was considered to be a supramaximal dose and was used in the next experiments. It should be noted that the concentrations of 5-HT and 5-HIAA obtained following 30 mg/kg, i.v., were similar to those reported following 75 mg/kg, i.p. (130-160% and 60-75%, respectively; Tozer et al., 1966; Neckers and Meek, 1976; Johnson and Crowley, 1982).

To test the hypothesis that changes in the pargyline-induced increase in 5-HT accumulation and decrease in 5-HIAA concentrations are reflective of changes in 5-HT turnover and, therefore, of changes in 5-HT neuronal activity (Tozer et al., 1966; Neff et al., 1969; Weiner, 1974; Van Loon et al., 1981), a paradigm identical to that employed in the preceding electrical stimulation studies was used. Therefore, pargyline was administered at the onset of electrical stimulation, and the concentrations of 5-HT and 5-HIAA were determined 0, 15 or 30 min later. As shown in Tables 6 and 7, the concentrations of 5-HT and 5-HIAA increased and decreased, respectively, in each brain region of

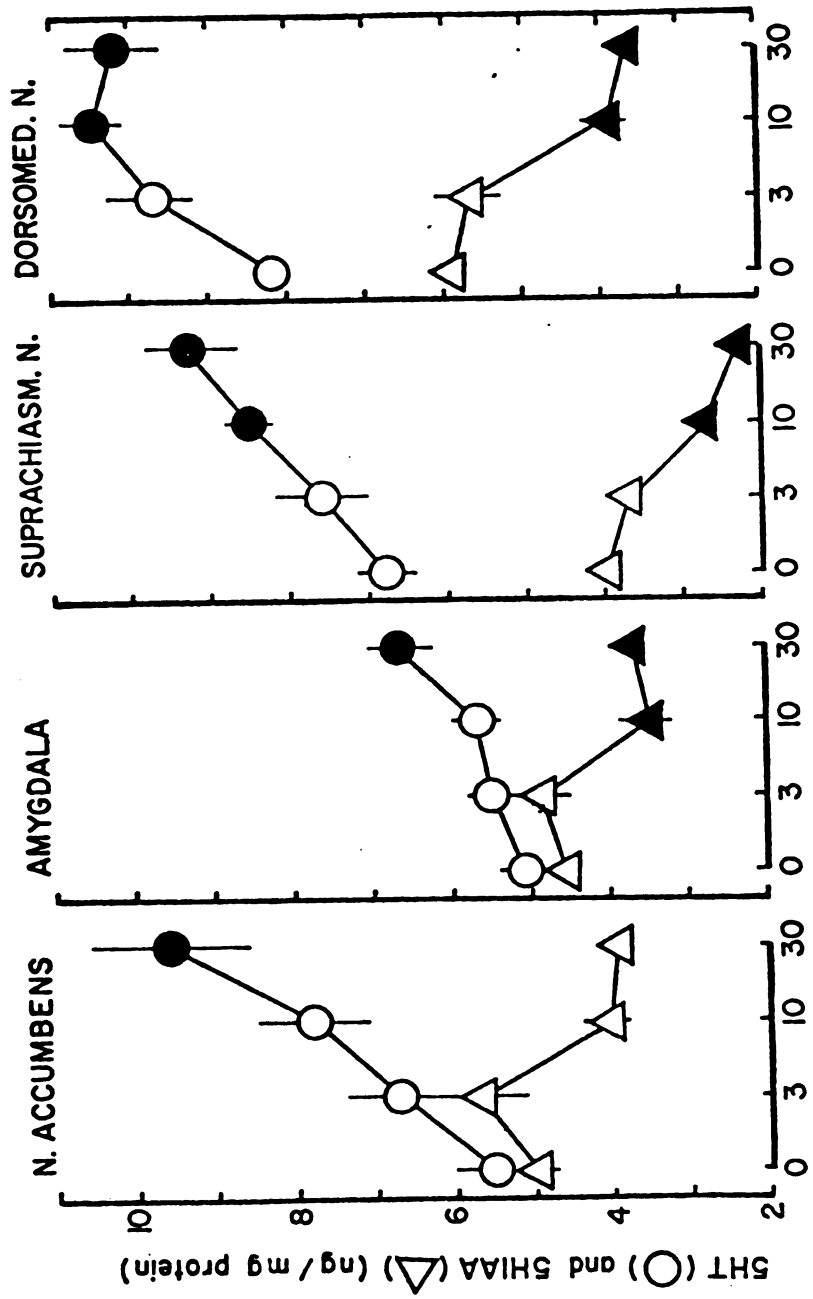

Figure 7. Concentrations of 5-HT and 5-HIAA in brain regions following intravenous administration of different doses of pargyline. Thirty min prior to sacrifice pargyline was administered to chloral hydrate-anesthetized rats via a femoral vein catheter. Symbols (○, 5-HT; △, 5-HIAA) represent the mean and vertical lines + 1 SEM of 7-9 determinations. Where no vertical line is depicted the SEM is less than the radius of the symbol. Solid symbols are significantly different from control (zero dose pargyline), $p < 0.05$.

TABLE 6

Concentrations of 5-HT in Selected Brain Regions Following Pargyline Administration and Electrical Stimulation of the Dorsal Raphe Nucleus

Region	Min of Stimulation	Rate of Stimulation		
		0 Hz	5 Hz	10 Hz
N. Accumbens	0	8.61±0.62	---	---
	15	9.58±0.55	10.51±0.63	9.88±0.52
	30	11.24±0.64[a]	11.31±0.77	10.21±0.87
Amygdala	0	5.29±0.42	---	---
	15	6.23±0.37	6.39±0.46	7.28±0.43
	30	7.42±0.69[a]	6.88±0.64	6.78±0.81
Suprachiasmatic N.	0	5.77±0.43	---	---
	15	7.80±0.43[a]	8.71±0.33	8.92±0.47
	30	8.33±0.53[a]	8.38±0.38	9.04±0.59
Dorsomedial N.	0	6.65±0.30	---	---
	15	8.11±0.50	8.29±0.36	8.95±0.28
	30	9.17±0.49[a]	9.49±0.56	9.34±0.35

Experimental procedures are as described in the legend to Figure 6E. Values represent the mean ± 1 SEM of 6-12 determinations, expressed as ng 5-HT/mg protein. [a], significantly different from 0 min values, $p < 0.05$.

TABLE 7

Concentrations of 5-HIAA in Selected Brain Regions Following Pargyline Administration and Electrical Stimulation of the Dorsal Raphe Nucleus

Region	Min of Stimulation	Rate of Stimulation		
		0 Hz	5 Hz	10 Hz
N. Accumbens	0	6.60±0.57	---	---
	15	5.55±0.24	6.50±0.51	5.91±0.35
	30	4.91±0.27[a]	4.71±0.21	5.20±0.45
Amygdala	0	3.77±0.13	---	---
	15	4.10±0.21	3.96±0.29	4.13±0.21
	30	2.95±0.16[a]	3.28±0.20	3.62±0.17
Suprachiasmatic N.	0	2.74±0.17	---	---
	15	2.21±0.14[a]	2.31±0.13	2.22±0.14
	30	1.91±0.05[a]	1.84±0.14	1.92±0.14
Dorsomedial N.	0	4.17±0.30	---	---
	15	3.64±0.18	4.01±0.27	3.85±0.17
	30	3.23±0.20[a]	3.20±0.17	3.35±0.24

Experimental procedures are as described in the legend to Figure 6F. Values represent the mean ± 1 SEM of 6-12 determinations, expressed as ng 5-HIAA/mg protein. [a], significantly different from 0 min values, $p < 0.05$.

control animals 30 min following pargyline administration. In contrast to the results obtained with other biochemical indices of 5-HT neuronal activity, electrical stimulation of the dorsal raphe nucleus did not alter the pargyline-induced increase in 5-HT or the decline in 5-HIAA concentrations (see Figures 6E and 6F and Tables 6 and 7).

To determine whether longer periods of electrical stimulation were necessary to elicit a further increase in the pargyline-induced accumulation of 5-HT and a further decrease in the pargyline-induced decline of 5-HIAA concentrations, the dorsal raphe nucleus was electrically stimulated for 60 min in groups of animals receiving either saline or pargyline and the resultant concentrations of 5-HT and 5-HIAA were compared with those of sham-stimulated controls. As shown in Table 8, electrical stimulation produced a decrease and increase, respectively, in the concentrations of 5-HT and 5-HIAA in the nucleus accumbens of saline-treated animals. Sixty min following the administration of pargyline (30 mg/kg, i.v.), the concentrations of 5-HT and 5-HIAA rose and fell, respectively, in the nucleus accumbens of sham animals. While electrical stimulation of the dorsal raphe nucleus did not alter the pargyline-induced increase in 5-HT accumulation, electrical stimulation slightly increased the concentration of 5-HIAA in the nucleus accumbens of pargyline-treated animals, possibly suggesting residual monoamine oxidase activity.

4. Summary

In this series of experiments electrical stimulation of 5-HT cell bodies in the dorsal raphe nucleus increased the concentration of 5-HIAA, the ratio of the concentration of 5-HIAA to 5-HT and the rate of 5-HTP accumulation following NSD 1015 administration, thereby indicating that each of these measurements provides a valid index of 5-HT neuronal activity. In contrast, an identical stimulation paradigm altered neither the rate of 5-HT accumulation

TABLE 8

Concentrations of 5-HT and 5-HIAA in the Nucleus Accumbens Following 60 Minutes of Electrical Stimulation of the Dorsal Raphe Nucleus

Treatment	Indoleamine	Sham (ng/mg protein)	Stimulated (ng/mg protein)
Saline	5-HT	4.77±0.35	3.20±0.34[a]
Pargyline	5-HT	6.93±0.39[b]	7.59±0.36
Saline	5-HIAA	4.79±0.32	6.39±0.38[a]
Pargyline	5-HIAA	2.14±0.16[b]	2.85±0.15[a]

Chloral hydrate-anesthetized rats were administered either saline (1 ml/kg) or pargyline (30 mg/kg) through a femoral vein catheter concurrent with the onset of 60 min of electrical stimulation of the dorsal raphe nucleus with monophasic pulses of 1 msec duration and 0.3 mA current delivered at a frequency of 10 Hz. Values represent the mean ± 1 SEM of 7-9 determinations. [a], significantly different from sham values, $p < 0.05$. [b], significantly different from saline-treated groups, $p < 0.05$.

following pargyline administration nor the rate of 5-HIAA decline following pargyline administration. The latter results demonstrate that neither of the pargyline techniques reliably estimate the activity of 5-HT neurons.

For both theoretical and methodological reasons, studies examining 5-HT projections to the pituitary gland will employ the rate of 5-HTP accumulation as an index of 5-HT neuronal activity. Because the pituitary gland is highly vascularized, measurements of neuronal 5-HIAA and 5-HT may be confounded by 5-HIAA and 5-HT in the blood. Determination of the rate of 5-HT synthesis (i.e., 5-HTP accumulation) circumvents this difficulty because blood-borne 5-HT is found in platelets which take up preformed 5-HT but do not synthesize the compound de novo (Erspamer, 1966; Douglas, 1985). That is, 5-HTP in these regions is primarily neuronal in origin, while 5-HIAA and 5-HT may be contained in the vascular compartment. In addition to this advantage, 5-HTP accumulation is the preferred method in studies of the pituitary gland because of technical limitations. As described in the introduction, 5-HT innervation of the pituitary is rather sparse and, consequently, the concentrations of 5-HT, its precursor and its metabolite are low. While the pituitary contains enough 5-HTP to be reliably measured by the HPLC-EC system employed in these studies, the pituitary content of 5-HIAA is often at the limits of detectability of the assay.

B. Effect of Precursor Loading on 5-HT Synthesis, Storage and Metabolism

The K_m for tryptophan hydroxylase is higher than tryptophan concentrations in the brain, suggesting that under normal physiological conditions the activity of this enzyme is limited by the availability of substrate (Friedman et al., 1972; Carlsson and Lindqvist, 1978). Accordingly, procedures that increase the concentrations of tryptophan within the brain produce corresponding changes in 5-HT concentrations in whole brain (Eccleston et al., 1965; Moir and Eccleston, 1968; Fernstrom and Wurtman, 1971) or in selected brain regions (Mueller et al.,

1976; Johnston and Moore, 1983; Long et al., 1983; Hutson et al., 1985). This effect of tryptophan loading may provide a useful tool to elevate the concentrations of 5-HT, its precursor 5-HTP and its metabolite 5-HIAA in regions where the concentrations of these indoleamines are low, and thus provide a means to more easily quantitate changes in the concentrations of these substances when they are to be used as indices of 5-HT neuronal activity.

1. Effect of tryptophan administration on the synthesis of 5-HT

The rates of accumulation of 5-HTP and DOPA following the administration of NSD 1015 and various doses of tryptophan (30, 100, 300 mg/kg) are summarized in Figure 8. For the sake of comparison the data are expressed as a percentage of control values which are shown in Table 9. When measured between 30-60 min after the administration of tryptophan, this amino acid precursor caused a dose-dependent increase in the accumulation of 5-HTP in all hypothalamic regions without altering the rate of DOPA accumulation. Values for 5-HTP and DOPA following the administration of 600 mg/kg tryptophan were similar to those observed after the 300 mg/kg dose (compare Table 10 with Figure 8). A time course for the accumulation of 5-HTP in selected hypothalamic regions following the administration of an intermediate dose of tryptophan (100 mg/kg) is depicted in Figure 9. In all regions except the suprachiasmatic nucleus peak 5-HTP concentrations were attained rapidly (within 30 min) and remained significantly elevated for at least 90 min after tryptophan administration. The rate of accumulation of DOPA did not change for the most part after the administration of tryptophan (Table 11).

2. Effect of tryptophan administration on the storage and metabolism of 5-HT

Since the 5-HTP formed by the oxidation of tryptophan is rapidly decarboxylated to 5-HT, it is not surprising that in addition to an increased rate of 5-HTP formation, the administration of tryptophan caused a dose-dependent

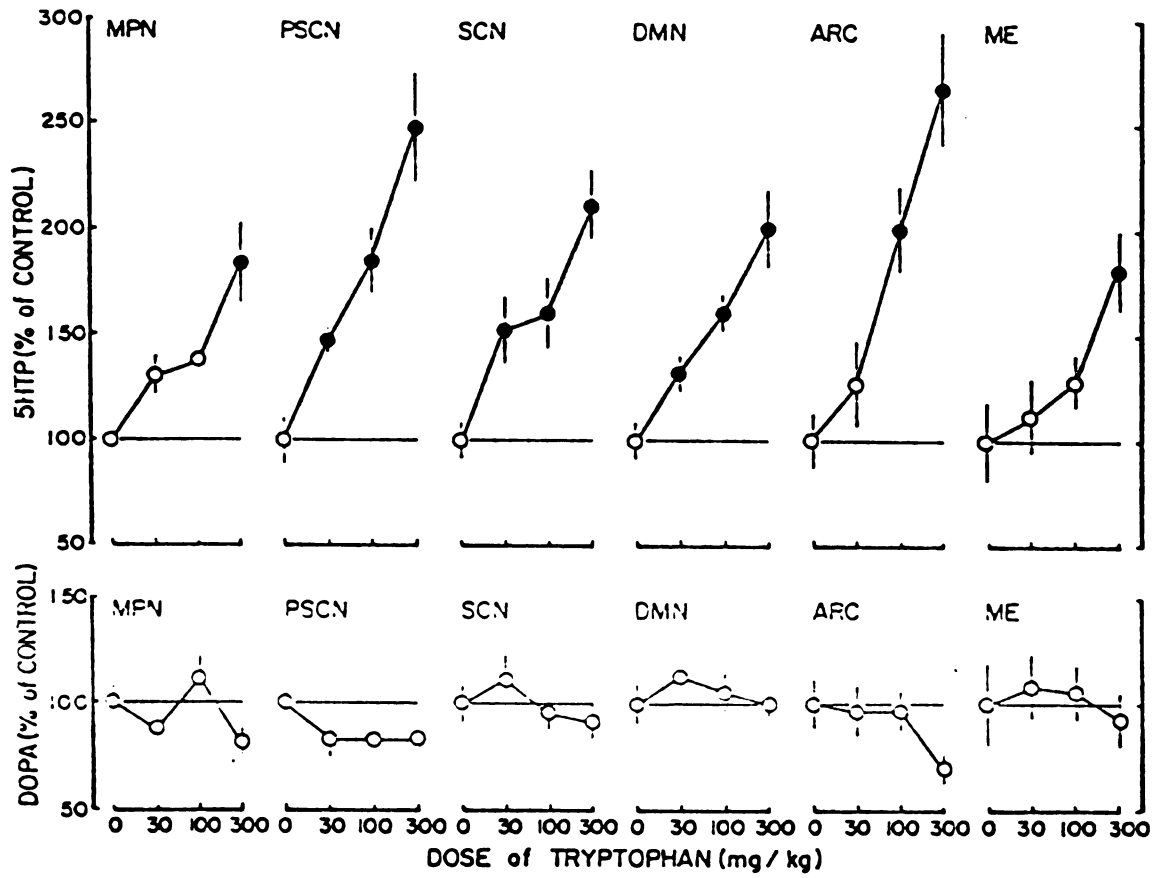

Figure 8. Effect of tryptophan administration on the accumulation of 5-HTP (top) and DOPA (bottom) in selected regions of the hypothalamus. Rats were injected with tryptophan methylester HCl (30, 100 or 300 mg/kg, i.p.) or its saline vehicle (2 ml/kg) 60 min prior to sacrifice. All rats were injected with NSD 1015 (100 mg/kg, i.p.) 30 min prior to sacrifice. Symbols represent the means and vertical lines ± 1 SEM (n = 6-8) of 5-HTP and DOPA concentrations in animals injected with tryptophan, expressed as a percentage of saline-treated controls. Mean control values are represented by the 0 mg/kg dose and the horizontal line set at 100%. The actual values for 5-HTP and DOPA concentrations in brain regions of control rats are listed in Table 9. Solid symbols represent those values that are significantly different ($p < 0.05$) from control.

TABLE 9

Concentrations of 5-HTP and DOPA in Selected Regions of the Rat Hypothalamus 30 Min After the Administration of an Inhibitor of Aromatic L-amino Acid Decarboxylase

Hypothalamic Regions	5-HTP	DOPA
Medial preoptic nucleus (MPN)	3.2±0.2	3.3±0.3
Preoptic suprachiasmatic nucleus (PSCN)	2.7±0.3	2.4±0.1
Suprachiasmatic nucleus (SCN)	2.5±0.2	2.5±0.2
Dorsomedial nucleus (DMN)	4.0±0.3	3.2±0.3
Arcuate nucleus (ARC)	1.5±0.2	3.2±0.4
Median eminence (ME)	1.7±0.3	3.4±0.6

Values (ng/mg protein) represent means ± 1 SEM (n = 6-8) of 5HTP and DOPA 30 min after the administration of NSD 1015 (100 mg/kg, i.p.).

TABLE 10

Effect of Tryptophan Administration (600 mg/kg, i.p.) on the Accumulation of 5-HTP and DOPA in Selected Regions of the Hypothalamus

Hypothalamic Regions	5-HTP	DOPA
MPN	153±13	87± 3
PSCN	233±22	75± 8
SCN	232±12	96± 8
DMN	203±13	100± 6
ARC	233±13	94± 9
ME	182±18	80±12

Each animal received tryptophan methyl ester (600 mg/kg, i.p.) 60 minutes and NSD 1015 (100 mg/kg, i.p.) 30 minutes before sacrifice. Values represent the mean ± 1 SEM of 6 to 8 determinations, expressed as a percentage of control values listed in Table 9.

Figure 9. Time course of the effect of tryptophan administration on the accumulation of 5-HTP in selected regions of the hypothalamus. Rats were injected with tryptophan methylester HCl (100 mg/kg, i.p.) 30, 60, 90 or 120 min, or saline (2 ml/kg, i.p.) 60 min prior to sacrifice. All rats were injected with NSD 1015 (100 mg/kg, i.p.) 30 min prior to sacrifice. Symbols represent means and vertical lines $\pm$ 1 SEM (n = 6-7) of 5-HTP concentrations in animals injected with tryptophan, expressed as a percentage of saline-treated control values (0 min and horizontal line set at 100%). Solid symbols indicate those values that are significantly different ($p < 0.05$) from control.

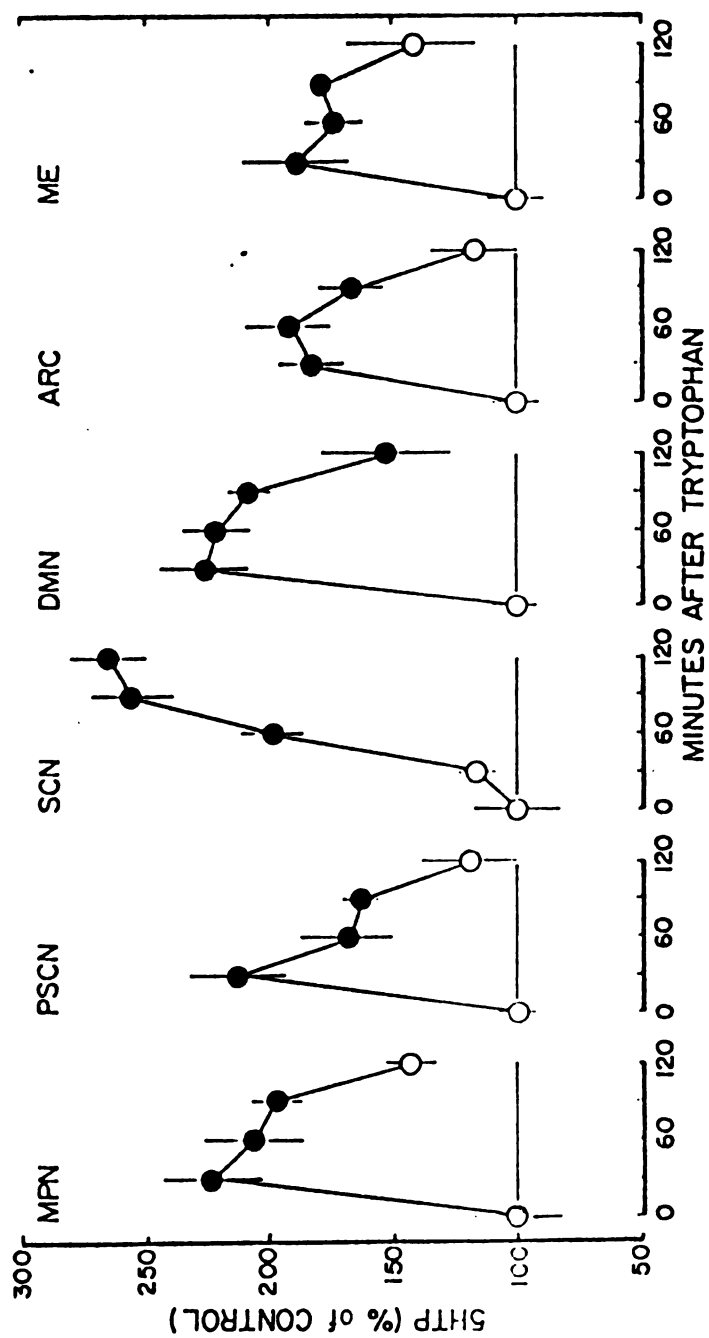
MPN
PSCN
SCN
DMN
ARC
ME
300
250
200
150
ICC
50
5HTP (% of CONTROL)
0
60
120
MINUTES AFTER TRYPTOPHAN

Figure 9

TABLE 11

Time Course of the Effect of Tryptophan Administration on the Accumulation of DOPA in Selected Regions of the Hypothalamus

Hypothalamic Region	Minutes After Tryptophan Administration 0	30	60	90	120
MPN	1.1±0.1	2.1±0.9	1.3±0.2	2.2±0.2	2.0±0.2
PSCN	4.8±0.3	4.5±0.2	3.7±0.5	3.0±0.3*	4.3±0.8
SCN	7.4±0.7	6.5±0.3	5.7±0.5	7.7±0.6	7.2±1.6
DMN	2.6±0.1	2.7±0.2	2.7±0.2	2.5±0.3	2.3±0.4
ARC	3.4±0.8	2.2±0.2	2.1±0.3	2.2±0.3	2.1±0.4
ME	5.4±0.6	5.5±0.4	5.0±1.0	6.8±0.6	6.0±1.0

The data were obtained from the same animals as in Figure 9 and are expressed, in ng DOPA/mg protein, as the mean ± 1 SEM of 4 to 7 determinations. *, significantly different from 0 minute control values ($p < 0.05$).

increase in 5-HT concentrations throughout the hypothalamus (Figure 10). This increase in 5-HT was accompanied by a concomitant increase in the concentration of 5-HIAA, and when measured 60 min after tryptophan there was an equivalent increase in both the amine and its deaminated product. Accordingly, as shown in Table 12, the 5-HIAA/5-HT ratio remained relatively constant at approximately 1.0 throughout the hypothalamus following the administration of various doses of tryptophan. These results suggest that the tryptophan-induced increase in 5-HT synthesis is accompanied by an increase in the storage and metabolism of 5-HT.

C. Comparison of newly synthesized 5-HT and previously released 5-HT as sources of 5-HIAA

The increased 5-HIAA concentrations observed following electrical stimulation of the dorsal raphe nucleus or administration of tryptophan could represent either an increased intraneuronal metabolism of newly synthesized 5-HT, or an increased release of 5-HT followed by the uptake of the released amine and its subsequent deamination by monoamine oxidase. To distinguish between these two possibilities the following studies employed fluoxetine, a drug which selectively inhibits the uptake of 5-HT from the synapse (Wong et al., 1974, 1975; Lemberger et al., 1978; Richelson and Pfenning, 1984). It was hypothesized: 1) that if 5-HIAA was derived solely from intraneuronal metabolism of newly synthesized 5-HT the administration of fluoxetine should not alter 5-HIAA concentrations, 2) that if 5-HIAA was formed exclusively from 5-HT that had been released and taken back up into the neuron before it was metabolized the administration of fluoxetine should dramatically decrease the concentration of 5-HIAA, and 3) that if 5-HIAA was derived from a combination of these two sources of 5-HT the administration of fluoxetine should slightly lower 5-HIAA concentrations.

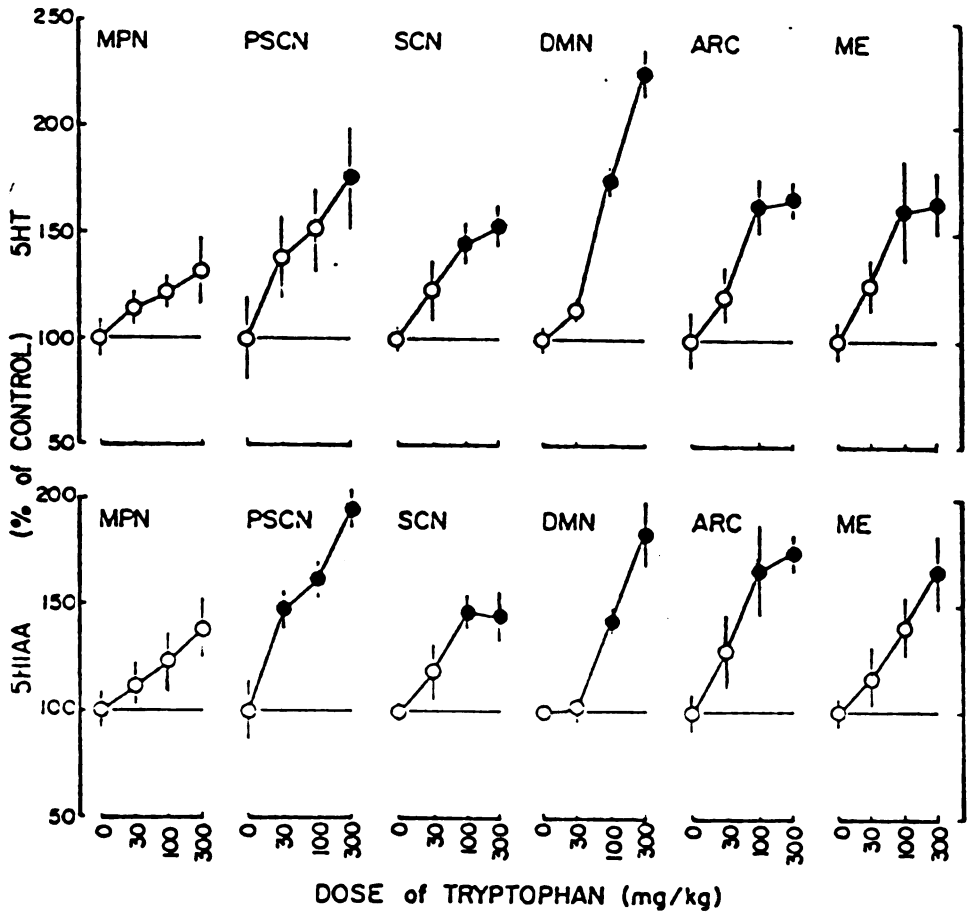

Figure 10. Effect of tryptophan administration on the concentrations of 5-HT and 5-HIAA in selected regions of the hypothalamus. Rats were injected with tryptophan methylester HCl (30, 100 or 300 mg/kg, i.p.) or its saline vehicle (2 ml/kg, i.p.) 60 min prior to sacrifice. Symbols represent the means and vertical lines $\pm$ 1 SEM (n = 7-9) of 5-HT (top) and 5-HIAA (bottom) concentrations in animals injected with tryptophan, expressed as a percentage of saline-treated controls. Mean control values are represented by the 0 mg/kg dose and the horizontal line set at 100%. The actual values for 5-HT and 5-HIAA concentrations in brain regions of control rats are listed in Table 12. Solid symbols represent those values that are significantly different ($p < 0.05$) from control.

TABLE 12

Concentrations of 5-HT and 5-HIAA in Selected Hypothalamic Regions and the Effects of Injections of Tryptophan on 5-HIAA/5-HT Ratios in These Regions

	MPN	PSCN	SCN	DMN	ARC	ME
5-HIAA (ng/mg protein)	4.4±0.4	2.1±0.3	5.1±0.2	4.6±0.2	2.4±0.2	2.9±0.2
5-HT (ng/mg protein)	3.7±0.3	2.1±0.4	5.1±0.3	3.6±0.2	2.4±0.3	3.4±0.3
5-HIAA/5-HT RATIO						
Dose of Tryptophan						
0 mg/kg	1.2±0.1	1.0±0.1	1.0±0.1	1.3±0.1	1.0±0.1	0.8±0.1
30 mg/kg	1.2±0.1	1.1±0.1	1.0±0.1	1.2±0.1	1.1±0.1	0.8±0.1
100 mg/kg	1.2±0.1	1.0±0.1	1.0±0.1	1.1±0.1	1.0±0.1	0.8±0.1
300 mg/kg	1.2±0.1	1.2±0.1	0.9±0.1	1.0±0.1	1.0±0.1	0.9±0.1

Values represent means ± 1 SEM (n = 7-9). Tryptophan methylester HCl or its saline vehicle were injected i.p. 60 min before decapitation.

1. Effect of fluoxetine administration on 5-HIAA concentrations following sham or actual electrical stimulation

Fluoxetine (10 mg/kg, i.p.) or saline (2 ml/kg, i.p.) was administered concurrent with the onset of 60 minutes of sham or actual electrical stimulation of the dorsal raphe nucleus. This dose of fluoxetine, which down-regulates 5-HT receptors and reduces food intake (Wong et al., 1985), was chosen as a supramaximal dose because it decreases 5-HIAA concentrations to the same extent as 5 or 15 mg/kg, i.p. (e.g., in the nucleus accumbens 5, 10 and 15 mg/kg, i.p. of fluoxetine reduced 5-HIAA concentrations to 82±4%, 73±3% and 78±6% of control values, respectively). In each brain region of sham animals, fluoxetine produced a consistent but small (20-30%) reduction of 5-HIAA concentrations, and the increased 5-HIAA concentrations following electrical stimulation were reduced to about the same extent (10-20%) by fluoxetine administration (Figure 11). A 2x2 factorial analysis of these data revealed no interactional effects between the two experimental factors (i.e., drug administration and electrical stimulation), thereby indicating that the proportion of 5-HIAA arising from the two diffrent sources of 5-HT is not altered by stimulation of the dorsal raphe nucleus. In general, both electrical stimulation and fluoxetine failed to alter the concentration of 5-HT in these animals (Table 13).

2. Effect of fluoxetine administration on 5-HIAA concentrations following tryptophan administration and sham or actual electrical stimulation

In a similar paradigm fluoxetine was used to determine if increased 5-HIAA concentrations following tryptophan administration resulted from the metabolism of newly synthesized 5-HT or of previously released 5-HT. At 60 minutes following the injection of tryptophan (100 mg/kg, i.p.), 5-HIAA concentrations in the brain rose to approximately 200% of control values (Figure 12). The concurrent administration of fluoxetine (10 mg/kg, i.p.) slightly

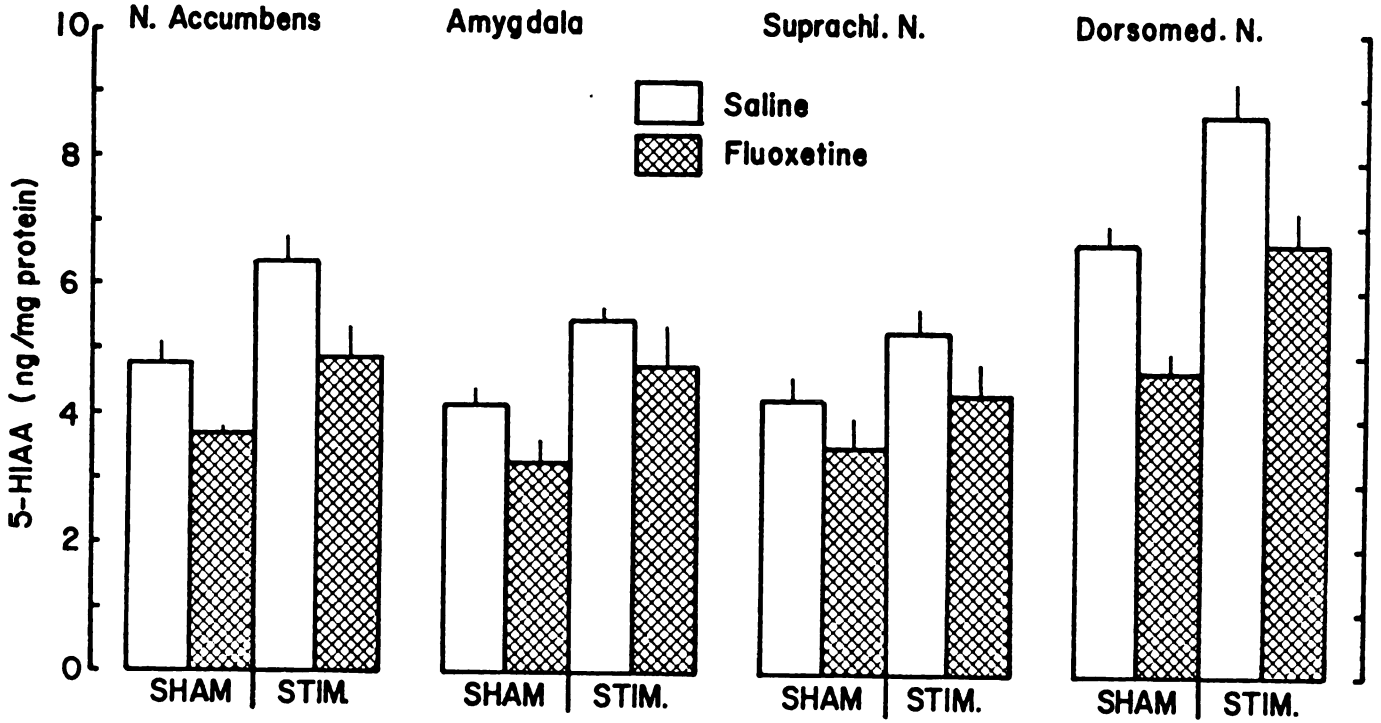

Figure 11. Effect of fluoxetine administration on the concentrations of 5-HIAA in brain regions of rats receiving sham or actual electrical stimulation of the dorsal raphe nucleus. Saline (2 ml/kg, i.p.; open columns) or fluoxetine (10 mg/kg, i.p.; hatched columns) was administered to each animal concurrent with the onset of 60 minutes of sham or actual electrical stimulation (monophasic cathodal pulses of 1 msec duration and 0.3 mA current delivered at a frequency of 10 Hz) of the dorsal raphe nucleus. Columns represent the means and vertical lines 1 SEM of 7 to 8 determinations. Electrical stimulation increased 5-HIAA concentrations in each brain region of saline-treated ($p < 0.05$ vs. sham-stimulated saline-treated group).

TABLE 13

Effect of Fluoxetine Administration on the Concentrations of 5-HT in Brain Regions of Rats Receiving Sham or Actual Stimulation of the Dorsal Raphe Nucleus

Region	Group	Treatment	
		Saline	Fluoxetine
N. Accumbens	Sham	4.77±0.35	4.78±0.46
	Stim.	3.20±0.34*	4.65±0.39
Amygdala	Sham	4.93±0.35	4.71±0.51
	Stim.	4.11±0.43	5.06±0.47
Suprachi. N.	Sham	6.17±0.31	6.14±0.54
	Stim.	6.04±0.68	6.01±0.65
Dorsomedial N.	Sham	6.85±0.24	7.71±0.42
	Stim.	6.23±0.57	6.62±0.72

The data were obtained from the same animals as in Figure 11. Values represent the mean ± 1 SEM of 7 to 8 determinations, expressed as ng 5-HT/mg protein. *, significantly different from sham-stimulated saline-treated values ($p < 0.05$).

Figure 12. Effect of fluoxetine administration on the concentrations of 5-HIAA (ng/mg protein) in brain regions of rats receiving tryptophan and sham or actual stimulation of the dorsal raphe nucleus. Tryptophan methyl ester (100 mg/kg, i.p.) and saline (2 ml/kg, i.p.; open columns) or fluoxetine (10 mg/kg, i.p.; hatched columns) was administered to each animal concurrent with the onset of 60 minutes of sham or actual electrical stimulation (monophasic cathodal pulses of 1 msec duration and 0.3 mA current delivered at a frequency of 10 Hz) of the dorsal raphe nucleus. Columns represent the means and vertical lines 1 SEM of 7 to 10 determinations. Electrical stimulation increased 5-HIAA concentrations in each brain region of saline-treated rats ($p < 0.05$ vs. sham-stimulated saline-treated groups).

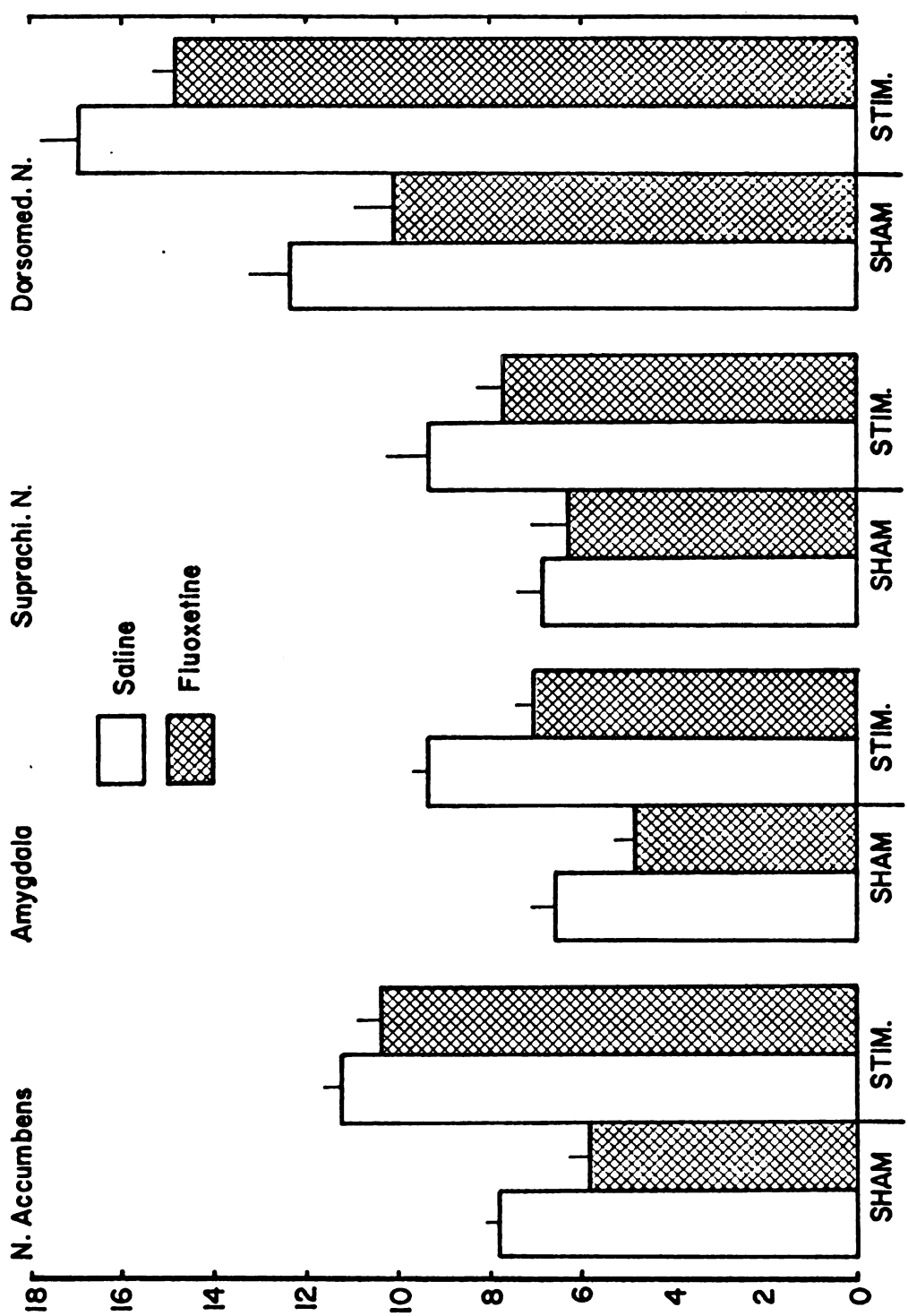
N. Accumbens
Amygdala
Suprachi. N.
Dorsomed. N.
Saline
Fluoxetine
SHAM
STIM.
0
2
4
6
8
10
12
14
16
18

Figure 12

decreased (10-25%) the concentration of 5-HIAA in each brain region of tryptophan-treated animals as compared with values from tryptophan-treated animals which did not receive the 5-HT uptake inhibitor (see "sham" values in Figure 12). Since the percent decline following uptake inhibition seen here is approximately the same as observed earlier in animals who did not receive tryptophan, these data indicate that precursor administration does not alter the proportion of the newly synthesized 5-HT that is metabolized to 5-HIAA prior to release or following release and reuptake.

It is known, however, that tryptophan administration inhibits the firing of 5-HT neurons (Aghajanian, 1972), presumably by the conversion of this precursor to 5-HT followed by feedback inhibition (Gallager and Aghajanian, 1976). Because the inhibition of neuronal firing caused by tryptophan may prevent release of the newly synthesized 5-HT resulting from precursor administration, more 5-HT may be metabolized by monoamine oxidase prior to release. Consequently, the effect of 5-HT uptake inhibition in tryptophan-loaded animals in which the firing of 5-HT neurons had been artificially maintained was determined and the results are depicted in Figure 12. Here again, fluoxetine administration only slightly reduced (10-25%) the concentration of 5-HIAA in each brain region of tryptophan-treated, electrically-stimulated animals as compared with the values obtained from animals not receiving fluoxetine. These data, compared with the "sham" data of Figure 12 and analyzed with a 2x2 factorial test, indicate that regardless of the presence of electrical stimulation, 5-HIAA in tryptophan-loaded animals is primarily derived from newly synthesized 5-HT that has undergone intraneuronal metabolism prior to release.

D. Effect of exposing brain extracts to sulfatase hydrolysis on the concentrations of "free" 5-HT and "free" 5-HIAA

A number of studies have demonstrated the presence of conjugated forms of catecholamines and of their metabolites in rat brain (Schanberg et

al., 1968; Gordon et al., 1976; Elchisak et al., 1977; Buu et al., 1981; Karoum et al., 1983; Hornsperger et al., 1984; Warnhoff, 1984; Buu, 1985). These are primarily sulfate conjugates and are synthesized by the enzyme phenolsulfotransferase (EC 2.8.2.1) with 3'-phosphoadenosine 5'-phosphosulfate (PAPS) serving as the sulfate donor. Given that the activity of rat brain phenolsulfotransferase toward 5-HT and 5-HIAA is very low (Meek and Neff, 1973), it is unlikely that significant quantities of the sulfoconjugates of these latter compounds exist in rat brain under normal physiological conditions. There is one report, however, that the sulfoconjugate of 5-HT is formed following the administration of the monoamine oxidase inhibitor pargyline (Gal, 1972). Likewise, another investigator has repored that exposure to sulfatase, an enzyme which cleaves off the sulfate moiety, increases the concentration of "free" 5-HIAA in brain tissue obtained from rats treated with probenecid (Warnhoff, 1974). Therefore, it seems reasonable to suspect that other manipulations which increase the concentration of 5-HT and 5-HIAA may also increase the concentration of the sulfated forms of these compounds. If this were the case, the concentrations of total 5-HT and 5-HIAA would be underestimated with the present analytical techniques because the sulfoconjugates of 5-HT and 5-HIAA are not detected electrochemically. To test this hypothesis, animals were subjected to pharmacological and electrophysiological manipulations designed to maximally enhance the concentrations of 5-HT and 5-HIAA, and extracts of brain tissue of each animal were then divided and half of the extract was exposed to sulfatase hydrolysis.

1. Effectiveness of the sulfatase hydrolysis system

Because neither 5-HT-sulfate nor 5-HIAA-sulfate were commercially available, p-nitrocatecholsulfate (PNC-sulfate) and 3-methoxy-4-hydroxyphenylglycol (MHPG-sulfate) were used to test the ability of the enzymatic hydrolysis system to cleave off sulfate. The unconjugated forms of these

compounds were readily detectable electrochemically, and a sample chromatogram of a PNC standard is shown in Figure 13A. When a PNC-sulfate standard was injected onto the HPLC column no peak was detected (Figure 13B). However, following 3 hours of incubation with sulfatase, the chromatogram of the PNC-sulfate standard exhibited a peak which co-chromatographed with that of PNC (compare Figures 13A and 13C). Similar results were obtained wtih MHPG-sulfate. When a time course of the sulfatase reaction was determined for each of these compounds, it was found that the reaction was essentially complete by 3 hours and that approximately 80% of the total sulfoconjugate was hydrolyzed (Figure 14). Consequently, in each of the following studies tissue extracts were incubated with sulfatase for 3 hours. In addition, 300 pg of PNC-sulfate were added to each of the samples to determine the effectiveness of the sulfatase hydrolysis.

2. Effect of sulfatase hydrolysis on 5-HT concentrations following pargyline administration and/or electrical stimulation of the dorsal raphe nucleus

To determine if the concentration of "free" 5-HT could be increased by incubating tissue extracts with sulfatase, samples of the nucleus accumbens were obtained from animals receiving saline (1 ml/kg, i.v.) or pargyline (30 mg/kg, i.v.) 30 minutes prior to sacrifice. In addition, a third group of animals received both pargyline and 30 minutes of electrical stimulation of the dorsal raphe nucleus. The latter group of animals was included to determine if the lack of effect of electrical stimulation on the pargyline-induced accumulation of 5-HT discussed above resulted from the shunting of 5-HT into the phenolsulfotransferase pathway.

As shown in Table 14, the concentration of 5-HT was elevated in the nonhydrolyzed tissue extracts from pargyline-treated animals as compared with that of saline-treated animals, and, as observed previously, electrical

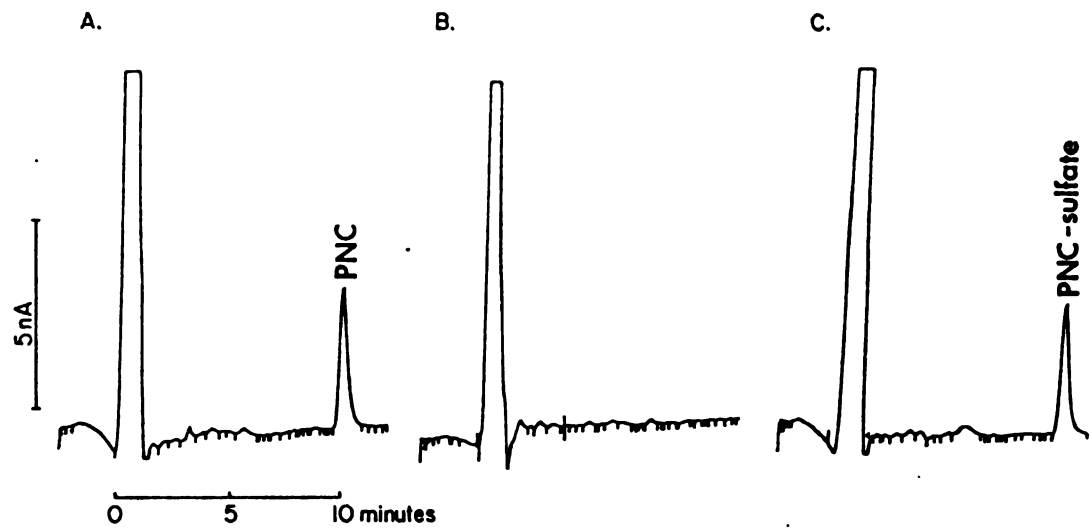

Figure 13. Sample HPLC-EC chromatograms obtained following the injection of PNC (p-nitrocatechol) and of PNC-sulfate before and after incubation with sulfatase. A 1 ng standard of PNC following 3 hours of incubation with sulfatase (A), a 1 ng standard of PNC-sulfate before incubation with sulfatase (B), and a 1 ng standard of PNC-sulfate following 3 hours of incubation with sulfatase (C) were injected onto an HPLC-EC column. Details of the sulfatase incubation and the HPLC-EC system are described in the "Materials and Methods" section.

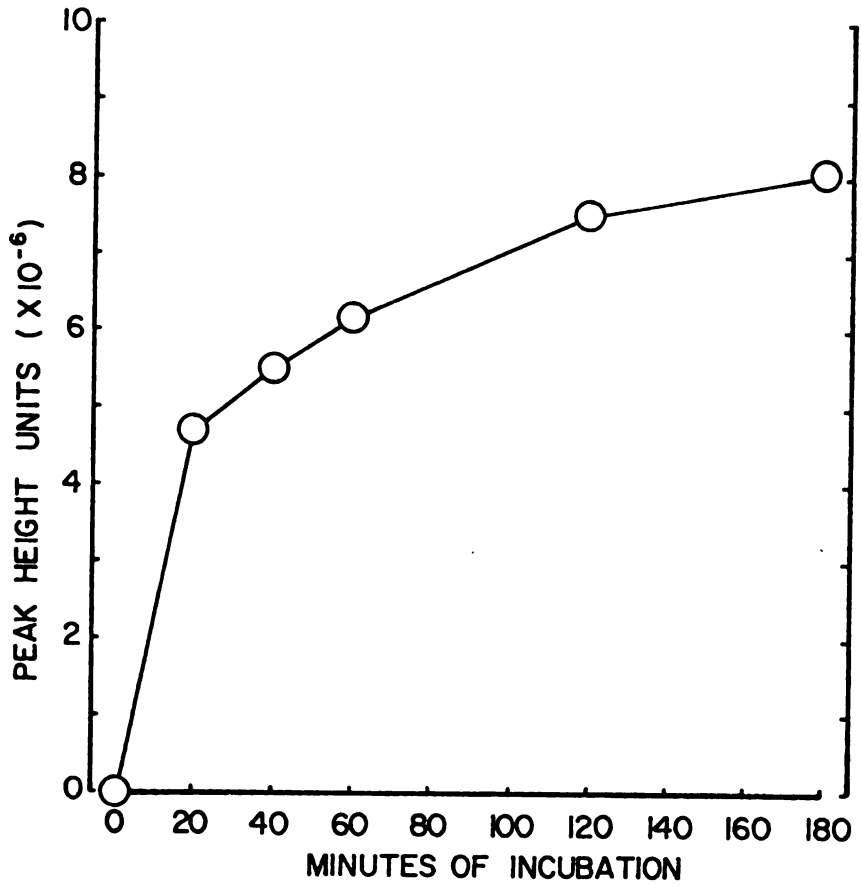

Figure 14. Time course of the effect of incubation with sulfatase on the deconjugation of PNC-sulfate. The peak heights obtained following HPLC-EC analysis of 1 ng PNC-sulfate standards incubated with sulfatase (details described in the "Materials and Methods" section) for various times up to 180 minutes are plotted. When a 1 ng PNC standard was exposed to the same incubation conditions a peak height of approximately 950,000 units was obtained for each time point.

TABLE 14

Effect of Sulfatase Hydrolysis on the Concentration of 5-HT in the Nucleus Accumbens of Rats Receiving Saline, Pargyline and/or Electrical Stimulation of the Dorsal Raphe Nucleus

Treatment	5-HT (ng/mg protein)	
	Non-Hydrolyzed	Hydrolyzed
Saline Shams	6.58±0.97	8.11±1.42
Pargyline Shams	8.45±0.54*	9.25±1.09
Pargyline + Stimulation	9.12±0.84	9.54±1.22

Chloral hydrate-anesthetized rats received an injection of saline (1 ml/kg, i.v.) or pargyline (30 mg/kg, i.v.) concurrent with the onset of 30 minutes of sham or actual electrical stimulation of the dorsal raphe nucleus (monophasic pulses of 1 msec duration and 0.3 mA current delivered at a frequency of 10 Hz). Values represent the mean ± 1 SEM of 8 to 9 determinations. *, significantly different from non-hydrolyzed saline-treated sham values ($p < 0.05$).

stimulation of the dorsal raphe nucleus did not alter the pargyline-induced accumulation of 5-HT. The concentrations of 5-HT in tissue extracts incubated with sulfatase were never significantly different from those of non-hydrolyzed tissue (Table 14), even though the PNC-sulfate added to each sample was hydrolyzed by this exposure to sulfatase. These data indicate that 5-HT is not sulfated to an appreciable extent in control animals or in animals with increased concentrations of 5-HT due to pargyline administration. Furthermore, these results suggest that electrical stimulation of 5-HT cell bodies in pargyline-treated animals does not redirect 5-HT along the phenolsulfotransferase pathway.

3. Effect of sulfatase hydrolysis on 5-HIAA concentrations following pharmacological manipulations and/or electrical stimulation of the dorsal raphe nucleus

The next set of experiments were designed to determine if manipulations which enhance the formation of 5-HIAA also result in the sulfoconjugation of this metabolite. Animals received saline (2 ml/kg, i.p.), tryptophan (100 mg/kg, i.p.), probenecid (250 mg/kg, i.p.) or tryptophan plus probenecid 60 minutes before sacrifice. While the concentration of 5-HIAA in non-hydrolyzed nucleus accumbens samples from these animals was significantly enhanced in each of the drug-treated groups as compared with that of the saline controls, exposing tissue extracts to sulfatase hydrolysis did not significantly alter the concentration of "free" 5-HIAA above the non-hydrolyzed levels (Figure 15). In contrast, the PNC-sulfate added to each of the samples was hydrolyzed by the sulfatase incubation.

Since electrical stimulation of 5-HT cell bodies has been shown to increase 5-HIAA concentrations in terminal regions, the effect of sulfatase hydrolysis on nucleus accumbens extracts from stimulated animals was determined. Because, as noted earlier, the rate of 5-HIAA formation may be indirectly limited by the availability of tryptophan, this precursor was administered to a

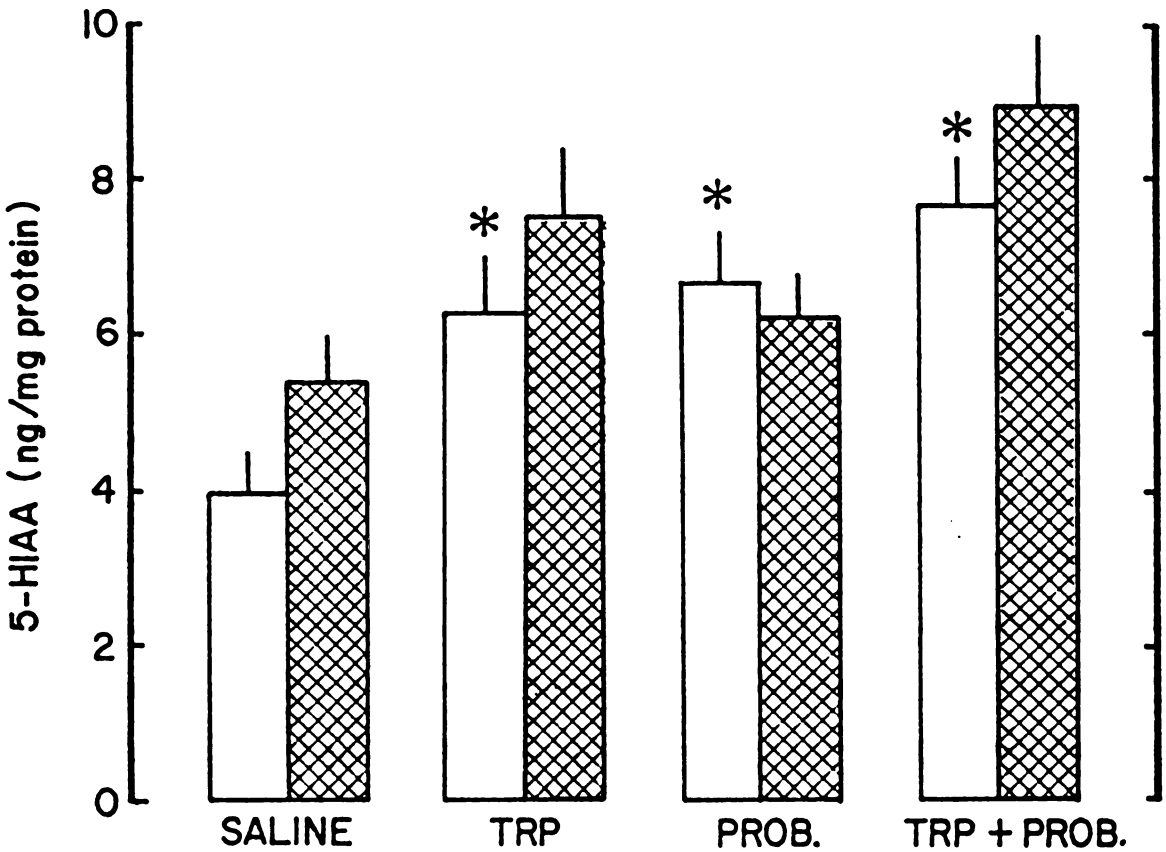

Figure 15. Effect of sulfatase hydrolysis on the concentrations of 5-HIAA in the nucleus accumbens of rats following various pharmacological manipulations. Animals received an intraperitoneal injection of saline (2 ml/kg), tryptophan methyl ester (100 mg/kg), probenecid (250 mg/kg) or tryptophan methyl ester plus probenecid 60 minutes before sacrifice. Extracts of each nucleus accumbens were divided, and half was incubated with sulfatase ("hydrolyzed", hatched columns) while half was not ("non-hydrolyzed", open columns). Columns represent the means and vertical lines 1 SEM of 7 to 9 determinations. *, significantly different from non-hydrolyzed saline-treated values, $p < 0.05$.

group of stimulated animals. In both saline- and tryptophan-treated animals 60 minutes of electrical stimulation of the dorsal raphe nucleus increased 5-HIAA concentrations above sham values (Figure 16). Again, incubation with sulfatase hydrolyzed PNC-sulfate in each sample but did not alter "free" 5-HIAA concentrations as compared with non-hydrolyzed values (Figure 16). These results indicate that even after extensive measures were taken to elevate 5-HIAA concentrations, 5-HIAA did not appear to be significantly converted to 5-HIAA sulfate.

II. CHARACTERIZATION OF 5-HT NEURONS TERMINATING IN THE NEURAL AND INTERMEDIATE LOBES OF THE PITUITARY GLAND

Low concentrations of 5-HT have been quantified in the neural and intermediate lobes of the rat pituitary gland using a radioenzymatic technique (Saavedra et al., 1975), and a small number of 5-HT nerve fibers have been identified in these regions using immunocytochemical procedures (Steinbusch and Nieuwenhuys, 1981; Westlund and Childs, 1982; Friedman et al., 1983; Léránth et al., 1983; Payette et al., 1985). It has recently been reported, however, that the 5-HT content of the intermediate lobe, but not of the neural lobe, may represent the accumulation from blood of preformed 5-HT rather than amine that has been synthesized in neurons (Saland et al., 1985). Therefore, the purpose of the present neurochemical experiments was to determine if 5-HT synthesis could be quantified in the neural and intermediate lobes of the pituitary gland and if, as in other 5-HT neurons, the synthesis rate is altered by precursor availability and neuronal impulse traffic. Since the biochemical dynamics of DA neurons terminating in the neurointermediate lobe have been previously characterized (for review see Holzbauer and Racké, 1985), the rate of DA synthesis in this region was also measured for comparison.

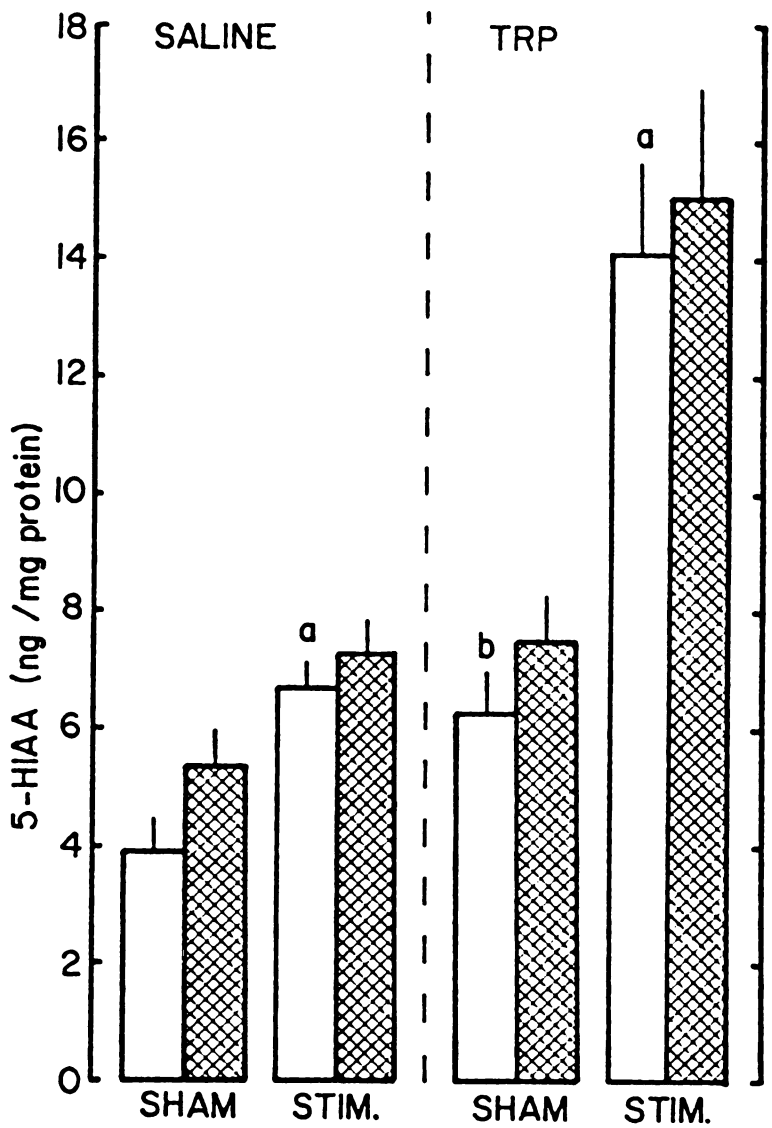

Figure 16. Effect of sulfatase hydrolysis on the concentrations of 5-HIAA in the nucleus accumbens of saline- or tryptophan-treated rats following sham or actual stimulation of the dorsal raphe nucleus. Concurrent with the onset of 60 minutes of sham or actual stimulation of the dorsal raphe nucleus (monophasic cathodal pulses of 1 msec duration and 0.3 mA current delivered at a frequency of 10 Hz) animals received an injection of either saline (2 ml/kg, i.p.) or tryptophan methyl ester (100 mg/kg, i.p.). Extracts of each nucleus accumbens were divided, and half was incubated with sulfatase ("hydrolyzed", hatched columns) while half was not ("non-hydrolyzed", open columns). Columns represent the means and vertical lines 1 SEM of 8 to 10 determinations. [a], significantly different from the appropriate sham-stimulated non-hydrolyzed group, $p < 0.05$. [b], significantly different from sham-stimulated, non-hydrolyzed saline-treated group, $p < 0.05$.

A. Effect of NSD 1015 administration on 5-HTP accumulation in the neurointermediate lobe

5-HT biosynthesis is the result of a sequential process in which tryptophan is hydroxylated by the rate-limiting enzyme tryptophan hydroxylase forming 5-HTP, and the latter compound is rapidly decarboxylated by the ubiquitous enzyme ALAAD to produce 5-HT. The accumulation of 5-HTP in brain regions following the administration of an ALAAD inhibitor has been used as an *in vivo* index of tryptophan hydroxylase activity (Carlsson *et al.*, 1972). Consequently, in order to determine if tryptophan hydroxylase is active in the rat neurointermediate lobe *in vivo*, the ALAAD inhibitor NSD 1015 was administered and 5-HTP accumulation was measured. For comparison, the concentration of DOPA was determined in the same animals since DOPA, synthesized from tyrosine by tyrosine hydroxylase and metabolized by ALAAD to form DA, is synthesized in DA neurons terminating in the rat neurointermediate lobe. In the absence of NSD 1015 the concentrations of 5-HTP and DOPA were essentially zero (at the limits of sensitivity of our assay) in both the neurointermediate lobe and in the nucleus accumbens, a brain region receiving 5-HT (Azmitia, 1978) and DA (Moore and Bloom, 1978) neuronal projections. Thirty minutes following the administration of NSD 1015 both 5-HTP and DOPA accumulated to the concentrations shown in Table 15.

B. Effect of manipulations known to increase the rate of 5-HTP accumulation in the brain on the rate of 5-HTP accumulation in the neurointermediate lobe

As demonstrated in the previous sections, the administration of the 5-HT precursor tryptophan increases the rate of 5-HTP accumulation but does not alter the rate of DOPA accumulation in the brain. If the 5-HT containing neurons of the pituitary gland are similar to 5-HT neurons of the brain, analogous results would be predicted following the injection of tryptophan (100 mg/kg, i.p.) 30 minutes prior to NSD 1015 administration. As shown in Table 16, a significant

TABLE 15

Concentrations of Monoamines and the Accumulation of their Precursors in the Neurointermediate Lobe of the Pituitary Gland (NIL) and in the Nucleus Accumbens (NA) of the Chloral Hydrate-Anesthetized Rat

Region	5-HT (ng/mg protein)	5-HTP (ng/mg protein)	DA (ng/mg protein)	DOPA (ng/mg protein)
NIL	8.56±0.93	0.89±0.14	15.8±0.85	0.34±0.04
NA	6.42±0.34	1.59±0.11	65.8±3.50	5.77±0.56

Male rats were anesthetized with chloral hydrate and the concentrations of 5-HT and DA were determined in animals administered saline (1 mg/kg, i.p.) 30 min prior to sacrifice, whereas the concentrations of 5-HTP and DOPA were determined in another group of animals administered NSD 1015 (100 mg/kg, i.p.) 30 min prior to sacrifice. Values represent the mean ± 1 SEM of 5 to 10 determinations.

TABLE 16

Effect of Tryptophan Administration on the Accumulation of 5-HTP and DOPA in the Neurointermediate Lobe

Treatment	5-HTP (ng/mg protein)	DOPA (ng/mg protein)
Saline	0.89±0.14	0.34±0.04
Tryptophan	2.16±0.24*	0.36±0.05

Male rats were anesthetized with chloral hydrate and were administered tryptophan methyl ester (100 mg/kg, i.p.) or its saline vehicle (2 ml/kg, i.p.) 60 min prior to sacrifice. Each animal was injected with NSD 1015 (100 mg/kg, i.p.) 30 min prior to sacrifice. Values represent the mean ± 1 SEM of 5 to 7 determinations. *, significantly different from saline group ($p < 0.05$).

increase in 5-HTP accumulation was observed in the neurointermediate lobe while the concentration of DOPA in tryptophan-treated animals did not differ from control values.

Since these results indicate that 5-HT synthesis occurs in the neurointermediate lobe and is increased by precursor administration in a fashion similar to that reported for brain regions containing 5-HT nerve terminals, it was of interest to determine if 5-HT synthesis in the neurointermediate lobe also responds to changes in neuronal impulse traffic. Previous studies have demonstrated that, following ALAAD inhibition, electrical stimulation of 5-HT and DA neurons results in an increase in the rate of 5-HTP (Bourgoin et al., 1980; Boadle-Biber et al., 1983, 1986; Duda and Moore, 1985; Section I.A. of "Results") and DOPA accumulation (Murrin and Roth, 1976), respectively, in regions containing terminals of these neurons. Therefore, nerve fibers coursing through the pituitary stalk were activated by electrical stimulation, and 5-HTP and DOPA accumulation were measured. As shown in Figure 17, electrical stimulation significantly enhanced the accumulation of 5-HTP and DOPA in the neurointermediate lobe following NSD 1015 administration, but did not alter the accumulation of either of these precursors in the nucleus accumbens.

C. Effect of repeated fluoxetine administration on the concentrations of 5-HT in the neural and intermediate lobes of the pituitary gland

While conclusions regarding the neurointermediate lobe as a whole may be drawn from the preceding experiments, they do not address the issue of possible regional differences between 5-HT containing fibers in the two divisions of this lobe. Therefore, in the following experiment the concentration of 5-HT was determined in the neural and intermediate lobes separately following repeated injections of fluoxetine, a selective 5-HT uptake inhibitor (Wong et al., 1975; Richelson and Pfenning, 1984), in order to determine if the 5-HT content of either of these divisions could be attributed to the uptake of blood-borne 5-HT.

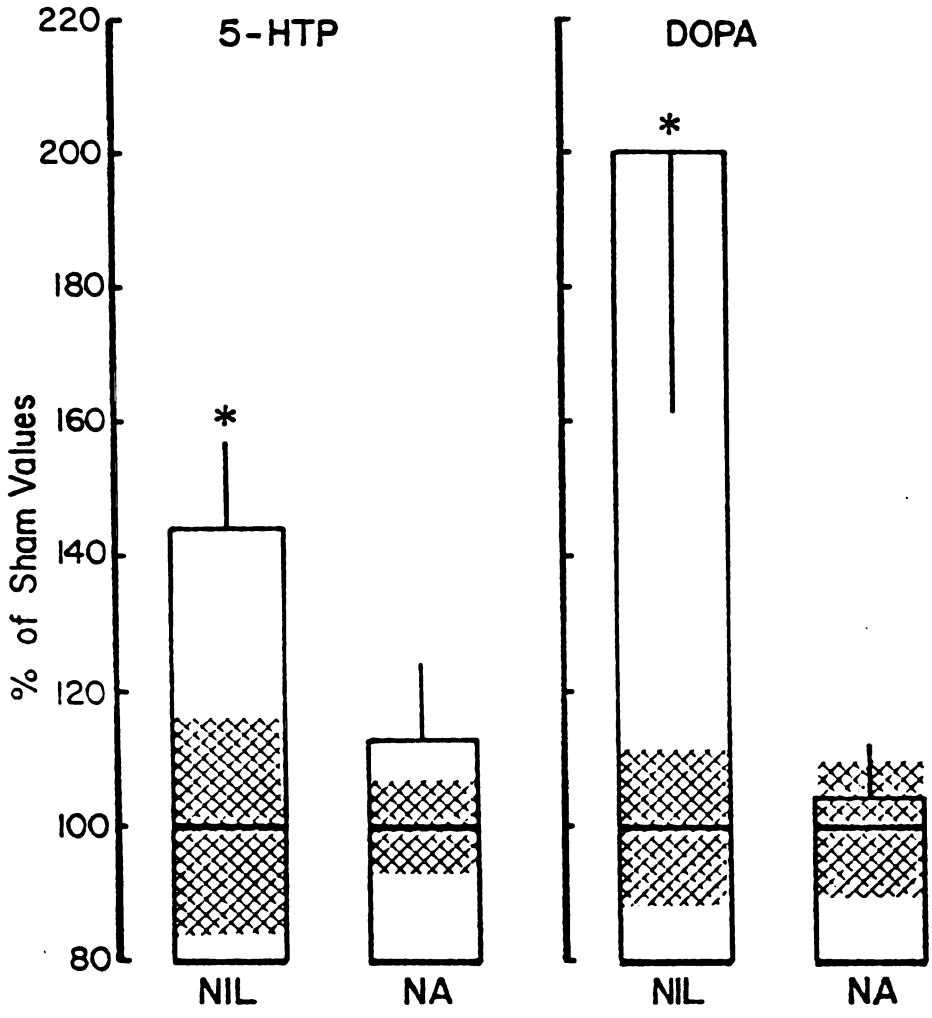

Figure 17. Effect of electrical stimulation of the pituitary stalk on 5-HTP and DOPA accumulation in the neurointermediate lobe (NIL) and in the nucleus accumbens (NA). NSD 1015 (100 mg/kg., i.p.) was administered to each animal concurrent with the onset of 30 min of sham or actual electrical stimulation (monophasic cathodal pulses of 1 msec duration and 0.3 mA current delivered at a frequency of 10 Hz) of the pituitary stalk. Columns represent the means and vertical lines 1 SEM of 5 to 10 determinations, expressed as a percentage of sham control values (listed in Table 15 and represented by horizontal lines with the hatched areas representing $\pm$ 1 SEM). *, Significantly different from sham values ($p < 0.05$).

Fluoxetine was administered at a dose (10 mg/kg, i.p.) which, when administered chronically, down-regulates 5-HT receptors and reduces food intake (Wong et al., 1985). Animals were sacrificed by perfusion of the vasculature with ice-cold saline introduced through the left ventricle of the heart in order to avoid the possible confounding effect of blood-borne 5-HT on determinations of tissue 5-HT. Measurement of 5-HT concentrations in platelets, cells which possess a high affinity 5-HT uptake mechanism but are incapable of synthesizing 5-HT de novo (Douglas, 1985), showed that repeated injections of fluoxetine decreased the concentration of 5-HT in these cells to 39% of control values (Figure 18A), and indicated that the regimen of fluoxetine administration employed in this study effectively blocked 5-HT uptake. In contrast to the results obtained in platelets, fluoxetine administration did not elicit a change in the concentration of 5-HT in the neural lobe, intermediate lobe or nucleus accumbens of the same animals (Figure 18B). This finding indicates that the concentrations of 5-HT in both the neural and intermediate lobes of the pituitary gland, as in the nucleus accumbens, do not simply result from the uptake of 5-HT from the blood but rather from the intraneuronal synthesis of this amine.

III. DETERMINATION OF THE ORIGIN OF 5-HT INNERVATION OF THE NEURAL AND INTERMEDIATE LOBES OF THE PITUITARY GLAND

To date, a definitive study using the technique of double-labeling has yet to be performed to determine the cell bodies of origin of 5-HT fibers in the neural and intermediate lobes of the pituitary gland. The few experiments that have been performed give conflicting results. Mezey et al. (1984) have found that midcollicular knife cuts reduce 5-HT concentrations in the intermediate lobe by 25% as do lesions of the dorsomedial nucleus of the hypothalamus. When knife cuts and lesions were combined, a 50% drop in 5-HT concentrations in the intermediate lobe was noted. The authors conclude that both the midbrain raphe

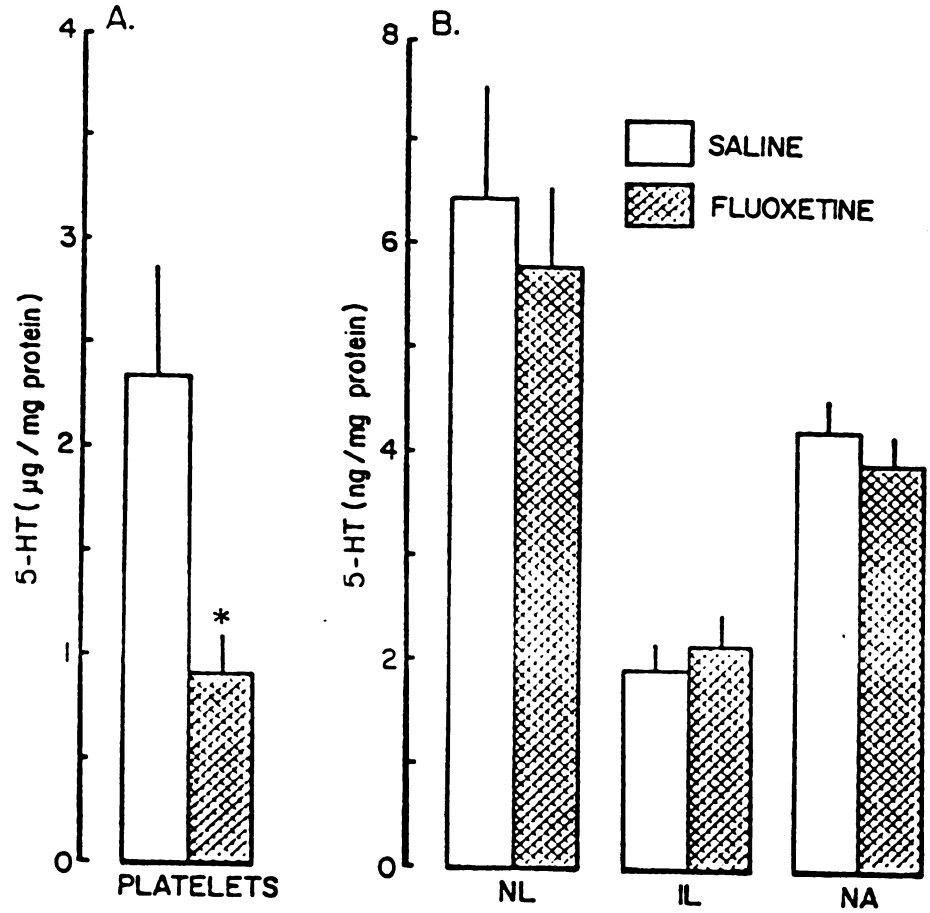

Figure 18. Effect of repeated fluoxetine administration on the concentrations of 5-HT in platelets and in selected pituitary and brain regions. Animals were administered either saline (2 ml/kg, i.p., open columns) or fluoxetine (10 mg/kg, i.p., hatched columns) every 12 hours for a total of 7 injections. The animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and were sacrificed by intracardiac perfusion with ice cold saline 60 min following the last fluoxetine injection. The concentration of 5-HT was subsequently determined in a sample of platelets (panel A) and in the neural (NL) and intermediate lobes (IL) of the pituitary gland and in the nucleus accumbens (NA) (panel B). Columns represent the mean and vertical lines 1 SEM of 9 to 10 determinations. *, Significantly different from saline group ($p < 0.05$).

nuclei and the dorsomedial nucleus send 5-HT projections to the intermediate lobe, but find no evidence for 5-HT innervation of the neural lobe. In two other studies the retrogradely transported tracer horseradish peroxidase (HRP) was injected into the neurointermediate lobe of the pituitary gland (Sherlock et al., 1975) or into the blood (Broadwell and Brightman, 1976) for easy access to the neural lobe which is outside of the blood-brain barrier (Weindl, 1973). In contrast to the work of Mezey et al., these investigators did not observe HRP labeling of cells in the dorsomedial nucleus but did see labeling of cells in other hypothalamic nuclei (supraoptic, paraventricular, arcuate). While the brainstem raphe nuclei were not examined in the HRP labeling studies, the results in the hypothalamus suggest that the dorsomedial nucleus does not project to the neural or intermediate lobe.

In order to resolve this conflict concerning 5-HT innervation of the intermediate lobe and to determine the cell bodies of origin of 5-HT fibers in the neural lobe, a series of experiments were conducted using electrical stimulation and electrolytic or chemical lesioning. Previous studies (Bourgoin et al., 1980; Duda and Moore, 1985) have shown that, in animals pretreated with NSD 1015, electrical stimulation of 5-HT cell bodies increases 5-HTP accumulation in regions receiving projections from these cells but not in regions which do not receive 5-HT innervation from the stimulated cells. Consequently, 5-HT-containing cell groups were stimulated and 5-HTP accumulation was measured in the neural and intermediate lobes to see if the stimulation produced an increase in the concentration of this compound. Conversely, 5-HT-containing nuclei were lesioned electrolytically or chemically with the 5-HT neurotoxin 5,7-DHT to determine if such lesions would decrease 5-HTP accumulation in the neural or intermediate lobe.

A. Electrical Stimulation and 5,7-DHT Lesions of the Brainstem Raphe Nuclei

The dorsal and median raphe nuclei project throughout the brain (Azmitia, 1978). Consequently, it is possible that one or both of these nuclei also send 5-HT projections to the neural and intermediate lobes of the pituitary gland. To test this hypothesis, animals received an injection of NSD 1015 (100 mg/kg, i.p.) concurrent with the onset of 30 minutes of sham or actual stimulation of the dorsal and median raphe nuclei. As shown in Figure 19, 5-HTP accumulation was significantly enhanced in both the neural and intermediate lobes of stimulated animals when compared with values from sham animals. These results suggest that 5-HT cell bodies residing in either the dorsal and/or median raphe nuclei project to the neural and intermediate lobes of the pituitary gland. An alternative explanation for the data is that fibers of 5-HT cell bodies located caudal to but projecting through the dorsal and median raphe nuclei were stimulated in this experiment and that it is the more caudal cell bodies which actually project to the pituitary gland. In any event, these results are compatible with the idea that the 5-HT innervation of the pituitary gland arises, at least in part, from 5-HT perikarya in the brainstem.

In an effort to confirm the results of the stimulation study, an experiment involving chemical lesioning of the raphe nuclei was performed. 5,7-DHT was injected intracranially so as to lesion 5-HT cell bodies in the dorsal and median raphe nuclei. Ten days following the injection and 30 minutes after NSD 1015 administration, accumulation of 5-HTP dropped to 16% of control values in the nucleus accumbens, a region receiving 5-HT projections from the midbrain raphe (Azmitia, 1978), but 5-HTP accumulation was not altered in either the neural or intermediate lobes of the pituitary gland (Figure 20). Because the administration of NSD 1015 does not significantly alter the concentration of 5-HT in brain regions 30 minutes following the injection of the drug (Table 17), 5-HT

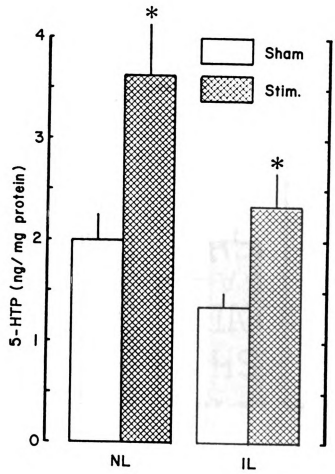

Figure 19. Effect of electrical stimulation of the dorsal and median raphe nuclei on 5-HTP accumulation in the neural (NL) and intermediate (IL) lobes of the pituitary gland. NSD 1015 (100 mg/kg, i.p.) was administered to each animal concurrent with the onset of 30 minutes of sham (open columns) or actual (hatched columns) stimulation (monophasic cathodal pulses of 1 msec duration and 0.3 mA current delivered at a frequency of 10 Hz) of the dorsal and median raphe nuclei. Columns represent the means and vertical lines 1 SEM of 6 to 7 determinations. *, significantly different from sham values. $p < 0.05$.

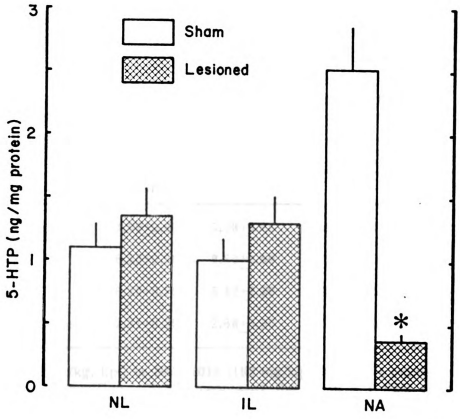

Figure 20. Effect of 5,7-DHT lesions of the dorsal and median raphe nuclei on 5-HTP accumulation in the neural (NL) and intermedite (IL) lobes of the pituitary gland and in the nucleus accumbens (NA). A total of 60 µg of 5,7-DHT was injected into the dorsal and median raphe nuclei of "lesioned" animals (hatched columns) 10 days prior to sacrifice. Sham surgery (open columns) consisted of exposing and drilling through the skull. Each animal received NSD 1015 (100 mg/kg, i.p.) 30 minutes prior to sacrifice. Columns represent the means and vertical lines 1 SEM of 6 to 9 determinations. *, significantly different from "sham" values, $p < 0.05$.

TABLE 17

Effect of NSD 1015 Administration on the Concentration of 5-HT and DA in Selected Brain and Pituitary Regions

Region	5-HT (ng/mg protein)		DA (ng/mg protein)	
	Saline	NSD 1015	Saline	NSD 1015
N. Accumbens	4.99±0.61	5.26±0.65	60.0±3.74	60.1±2.31
Dorsomedial N.	8.23±0.50	8.89±0.59	3.78±0.30	3.43±0.31
Neural Lobe	6.20±0.79	5.42±0.30	7.61±0.54	8.74±0.94
Intermediate Lobe	2.31±0.35	2.68±0.30	13.4±1.46	12.6±1.02

Saline (1 ml/kg, i.p.) or NSD 1015 (100 mg/kg, i.p.) was administered 30 minutes before sacrifice. Values represent the mean ± 1 SEM of 7 to 8 determinations.

concentrations were also determined in the 5,7-DHT lesioned animals. Again, 5,7-DHT lesions of the dorsal and median raphe nuclei markedly reduced 5-HT concentrations in the nucleus accumbens to 15% of control values but did not alter the concentration of this amine in either the neural or intermediate lobe (Table 18).

In light of the stimulation data, these results are rather surprising. They are consistent, however, with the alternative hypothesis advanced for the stimulation data. That is, while electrical stimulation of the dorsal and median raphe nuclei may have also activated 5-HT fibers of passage, these fibers may have been spared from the mechanical and chemical damage caused by the 5,7-DHT injection. These results, therefore, would be predicted if 5-HT innervation of the neural and intermediate lobes of the pituitary gland originated in cell bodies located caudal to the dorsal and median raphe nuclei. To test this hypothesis, the nuclei raphe pontis and raphe magnus were lesioned with 5,7-DHT. In contrast to the more rostral injections, 5,7-DHT injected into the nuclei raphe pontis and raphe magnus decreased the accumulation of 5-HTP in both the neural and intermediate lobes to 66% and 63% of control values, respectively (Figure 21). By way of comparison, 5-HTP accumulation decreased to 42% of control values in the thoracic spinal cord (Figure 21), a region which receives 5-HT innervation, in part, from the nucleus raphe magnus (Willis, 1984). 5-HT concentrations were also determined in these animals (Table 19), and the pattern of these values was similar to that of 5-HTP.

In summary, the combined results of the stimulation and lesioning experiments suggest that 5-HT projections to the neural and intermediate lobes of the pituitary gland arise, at least in part, from cell bodies residing in the brainstem. 5-HT perikarya in the dorsal and median raphe nuclei probably do not contribute to this innervation, but rather 5-HT cells in the nuclei raphe pontis

TABLE 18

Effect of 5,7-DHT Lesions of the Dorsal and Median Raphe Nuclei on the Concentrations of 5-HT in the Neuronal and Intermediate Lobes of the Pituitary Gland and in the Nucleus Accumbens

Region	5-HT (ng/mg protein)	
	Sham	Lesioned
Neural Lobe	6.23±0.90	5.83±0.36
Intermediate Lobe	3.35±0.24	3.61±0.40
N. Accumbens	5.83±0.34	0.90±0.16*

The data were obtained from the same animals as in Figure 20. Values represent the mean ± 1 SEM of 6 to 9 determinations. *, significantly different from sham values ($p < 0.05$).

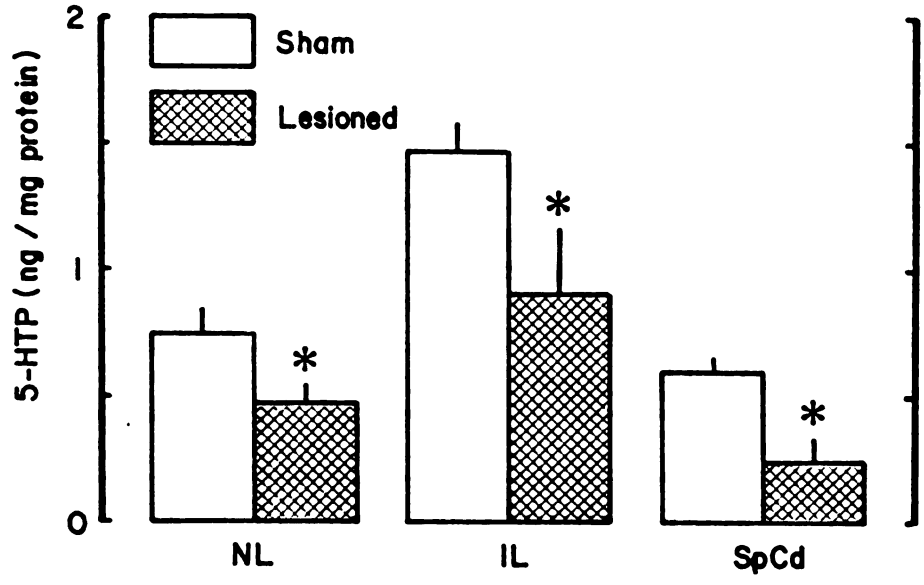

Figure 21. Effect of 5,7-DHT lesions of the nuclei raphe pontis and raphe magnus on 5-HTP accumulation in the neural (NL) and intermediate (IL) lobes of the pituitary gland and in the thoracic spinal cord (SpCd). A total of 120 µg of 5,7-DHT was injected into the nuclei raphe pontis and magnus of "lesioned" animals (hatched columns) 10 days prior to sacrifice. Sham surgery (open columns) consisted of exposing and drilling through the skull. Each animal received NSD 1015 (100 mg/kg, i.p.) 30 minutes prior to sacrifice. Columns represent the means and vertical lines 1 SEM of 6 to 10 determinations. *, significantly different from "sham" values, $p < 0.05$.

TABLE 19

Effect of 5,7-DHT Lesions of the Nuclei Raphe Pontis and Raphe Magnus on the Concentrations of 5-HT in the Neural and Intermediate Lobes of the Pituitary Gland and in the Thoracic Spinal Cord

Region	5-HT (ng/mg protein)	
	Sham	Lesioned
Neural Lobe	4.32±0.35	2.30±0.38*
Intermediate Lobe	2.87±0.32	1.66±0.29*
Spinal Cord	2.28±0.08	0.84±0.18*

The data were obtained from the same animals as in Figure 21. Values represent the mean ± 1 SEM of 6 to 10 determinations. *, significantly different from sham values, $p < 0.05$.

and/or raphe magnus or perhaps in surrounding areas appear to send axons to the neural and intermediate lobes.

B. Electrical Stimulation and Electrolytic Lesions of the Dorsomedial Nucleus of the Hypothalamus

5-HT-containing cell bodies have been reported in the dorsomedial nucleus of the hypothalamus (Fuxe and Ungerstedt, 1968; Chan-Palay, 1977; Beaudet and Descarries, 1979; Frankfurt et al., 1981; Steinbusch et al., 1982; Frankfurt and Azmitia, 1983; Sakumoto et al., 1984). Furthermore, there is one report (Mezey et al., 1984) that lesions of the dorsomedial nucleus reduce the concentration of 5-HT within the intermediate lobe. The authors interpret this finding as evidence that the putative 5-HT cell bodies in the dorsomedial nucleus project to the intermediate lobe. In order to confirm these results and to determine if the neural lobe receives 5-HT projections from this nucleus, the dorsomedial nucleus of NSD 1015-treated animals was stimulated for 30 minutes. While this manipulation increased 5-HTP accumulation in the dorsomedial nucleus itself, it did not alter 5-HTP accumulation in either the neural or intermediate lobe (Figure 22). Likewise, electrolytic lesions of the dorsomedial nucleus altered neither 5-HTP accumulation nor 5-HT concentrations in the neural and intermediate lobes (Table 20). These results indicate that the dorsomedial nucleus does not send 5-HT projections to either the neural or intermediate lobe of the pituitary gland.

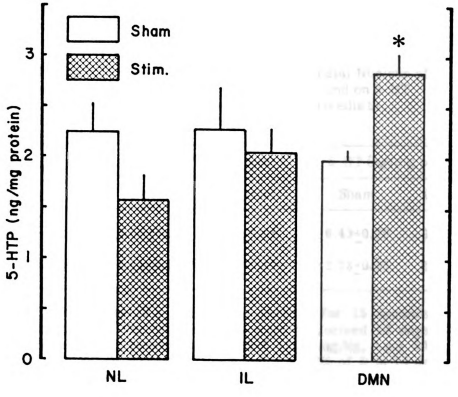

Figure 22. Effect of bilateral electrical stimulation of the dorsomedial nucleus of the hypothalamus (DMN) on 5-HTP accumulation in the neural (NL) and intermediate (IL) lobes of the pituitary gland and in the DMN. NSD 1015 (100 mg/kg, i.p.) was administered to each animal concurrent with the onset of 30 minutes of sham (open columns) or actual (hatched columns) electrical stimulation of the DMN (cathodal monophasic pulses of 0.3 mA current and 1 msec duration delivered at a frequency of 10 Hz). Columns represent the means and vertical lines 1 SEM of 5 to 12 determinations. *, significantly different from "sham" values, $p < 0.05$.

TABLE 20

Effect of Electrolytic Lesions of the Dorsomedial Nucleus of the Hypothalamus on 5-HTP Accumulation and on 5-HT Concentrations in the Neural and Intermediate Lobes of the Pituitary Gland

Region	5-HTP (ng/mg protein)		5-HT (ng/mg protein)	
	Sham	Lesioned	Sham	Lesioned
Neural Lobe	0.58±0.04	0.59±0.06	6.43±0.70	6.69±0.61
Intermediate Lobe	1.11±0.08	1.22±0.18	2.73±0.35	2.41±0.17

Electrolytic lesions (0.5 mA anodal current for 15 seconds) of the dorsomedial nucleus of the hypothalamus were performed 10 days prior to sacrifice. Each animal received NSD 1015 (100 mg/kg, i.p.) 30 minutes before sacrifice. Values represent the mean ± 1 SEM of 8 to 14 determinations. In no instance were lesioned values significantly different from sham values ($p < 0.05$).

DISCUSSION

I. Biochemical Indices of 5-HT Neuronal Activity

Although these studies were initially conducted in order to simply determine which biochemical estimate of 5-HT neuronal activity was most appropriate for use in experimentation on the neural and intermediate lobes of the pituitary gland, these investigations have yielded results which are significant in their own right and which can be widely applied to 5-HT neuronal systems. Each section of the results will be discussed separately.

A. Comparison of Biochemical Indices of 5-HT Neuronal Activity Following Electrical Stimulation of the Dorsal Raphe Nucleus

In agreement with previous reports (Aghajanian et al., 1967; Sheard and Aghajanian, 1968; Kostowski et al., 1969; Sheard and Zolovick, 1971; Curzon et al., 1978), increased 5-HT neuronal activity elicited by electrical stimulation of 5-HT cell bodies in the midbrain raphe resulted in frequency- and time-dependent increases in the concentration of 5-HIAA and in the ratio of the concentrations of 5-HIAA to 5-HT. In contrast to previous studies which show a decline in 5-HT concentrations following electrical stimulation of midbrain raphe neurons (Aghajanian et al., 1967; Sheard and Aghajanian, 1968; Kostowski et al., 1969), only a slight, non-significant decrease in 5-HT concentrations was observed. This discrepancy between the present study and previous reports may have resulted from differences in the site of stimulation (dorsal raphe nucleus vs. median raphe nucleus) or in the region of 5-HT measurement (discrete nuclei vs. whole brain or forebrain). Nevertheless, the inability of stimulation to alter the concentration of 5-HT could be explained by an increased rate of 5HT synthesis following dorsal

raphe stimulation. Since electrical stimulation produced an increase in the rate of 5-HTP accumulation following ALAAD inhibition, an index of the rate of 5-HT synthesis in vivo (Carlsson et al., 1972), the 5-HT released and metabolized following activation of 5-HT neurons appears to be replaced by newly synthesized 5-HT. Thus, this study confirms that measurement of 5-HT metabolism (the concentration of 5-HIAA and the ratio of the concentrations of 5-HIAA to 5-HT) and determination of the rate of 5-HT synthesis (the rate of accumulation of 5-HTP following ALAAD inhibition) are valid indices of 5-HT neuronal activity.

No marked differences among these measurements in the magnitude of response to changes in the activity of 5-HT neurons were observed. In some regions (nucleus accumbens, amygdala) 15 min of electrical stimulation significantly increased 5-HIAA concentrations and 5-HIAA/5-HT concentration ratios but not the rate of 5-HTP accumulation; in other regions (suprachiasmatic nucleus, dorsomedial nucleus) all three techniques showed significant effects at 15 min. These differences may have resulted from variations in the activity of tryptophan hydroxylase in hypothalamic vs. non-hypothalamic regions. That is, under non-stimulated conditions the rate of 5-HTP accumulation following NSD 1015 administration is much greater in hypothalamic nuclei than in areas outside of this brain region, reflecting the higher concentration of tryptophan (Knott and Curzon, 1974), the greater rate of uptake of this substrate (Denizeau and Sourkes, 1977) and the higher rate of tryptophan hydroxylase activity (Kuhar et al., 1972) in the hypothalamus as compared to other brain regions. Therefore, tryptophan hydroxylase in the hypothalamus may also be more responsive to the effects of electrical stimulation. In any event, the measurement of 5-HIAA concentrations or 5-HIAA/5-HT concentration ratios is more suited to estimating 5-HT neuronal activity over short periods of time in non-hypothalamic regions than is the determination of the rate of 5-HTP accumulation.

Measurement of 5-HIAA concentrations or of 5-HIAA/5-HT concentration ratios holds an additional advantage over the 5-HTP method in that no drug administration is required. Since ALAAD is common to the biosynthetic pathways of 5-HT and catecholamines, NSD 1015 inhibits the formation of both 5-HT and catecholamines (Carlsson et al., 1972). Therefore, some functions of catecholaminergic neurons, which preferentially release newly synthesized neurotransmitters (Kopin et al., 1968), may be altered by NSD 1015 and may, in turn, affect 5-HT neuronal activity. Conversely, the determination of the rate of 5-HTP accumulation following NSD 1015 administration may be preferable in certain instances. For example, biochemical estimation of the rate of 5-HT neuronal activity in the neural and intermediate lobes of the pituitary gland could best be accomplished with the 5-HTP technique since the concentrations of 5-HIAA in these regions is at the limits of the sensitivity of our assay. Likewise, determination of the rate of 5-HTP accumulation may be a more accurate index of 5-HT neuronal activity in highly vascularized regions, such as the neurointermediate lobe of the pituitary, because blood-borne 5-HT is found in platelets which take up preformed 5-HT but do not synthesize the compound de novo (Erspamer, 1966). That is, 5-HTP in these regions is primarily neuronal in origin, while 5-HT and 5-HIAA may be contained in the vascular compartment.

In contrast to the responsiveness of the above three estimates of 5-HT neuronal activity to increases in impulse traffic in 5-HT neurons, no alterations in the rate of 5-HT accumulation or of 5-HIAA decline following pargyline administration was observed after 30 min of electrical stimulation of 5-HT neurons, while 60 minutes of stimulation actually elevated the concentration of 5-HIAA in pargyline-treated rats. These results are in variance with those that would be predicted by the model of Tozer and coworkers (1966), which states that, following monoamine oxidase inhibition, an increase in 5-HT turnover produced by

an increased rate of 5-HT nerve firing should be reflected by an increase in the rate of 5-HT accumulation and of 5-HIAA decline. On a theoretical basis, however, it is difficult to imagine how the latter would occur. Once monoamine oxidase is blocked by pargyline, it would appear that the rate of 5-HIAA decline no longer reflects the rate of 5-HT synthesis, as it does under steady state conditions, but rather may be indicative of the activity of the acid transport system. Therefore, the inability of 30 min of stimulation to alter the pargyline-induced decline in 5-HIAA is not unexpected. Furthermore, the model may involve unjustified assumptions (see Tozer et al., 1966; Neff et al., 1969; Weiner, 1974). For instance, while the pargyline model assumes that 5-HT is metabolized to only 5-HIAA, under some circumstances such as pargyline or probenecid administration a sulfoconjugate of 5-HT may be formed (Gál, 1972; Warnhoff, 1984). Thus, the predicted increase in 5-HT concentrations in pargyline-treated animals following electrical stimulation of 5-HT neurons may not have been observed if 5-HT was directed along an alternate metabolic pathway. In light of these findings, a reinterpretation of studies employing either of the pargyline methods for estimating 5HT neuronal activity would appear to be in order.

B. Effect of Precursor Loading on 5-HT Synthesis Storage and Metabolism

The results from this study demonstrate that exogenous tryptophan administration produces a dose-dependent increase in the synthesis of 5-HT in the rat hypothalamus. These results are consistent with previous reports of increased rates of 5-HTP accumulation in the hypothalamus following tryptophan administration (Johnston and Moore, 1983; Long et al., 1983) and indicate that the tonic activity of tryptophan hydroxylase in hypothalamic 5-HT neurons can be accelerated by increasing the availability of its substrate. As a consequence of an accelerated rate of 5-HT synthesis following tryptophan, there is a concurrent increase in the storage and metabolism of 5-HT as indicated by an increase in

brain concentrations of 5-HT and 5-HIAA, respectively. Since there is no change in the 5-HIAA/5-HT ratio following tryptophan despite marked elevations in both the amine and its deaminated metabolite, the storage rate of newly synthesized 5-HT is apparently less than the rate of synthesis and significant amounts of cytoplasmic 5-HT become available for metabolism by mitochondrial monoamine oxidase.

Since tryptophan and the catecholamine precursor tyrosine compete for a common transport system at the blood-brain barrier, exogenous tryptophan administration could decrease the availability of tyrosine in the brain (Fernstrom, 1983). Furthermore, tryptophan has been shown to moderately inhibit tyrosine hydroxylase activity in vitro (Zhelyaskov et al., 1968). Thus, tryptophan administration could potentially decrease the synthesis of catecholamines. In the present study, however, tryptophan failed to alter the rate of DOPA accumulation in regions of the hypothalamus containing predominantly noradrenergic or dopaminergic neurons. These data suggest that the administration of tryptophan in the present regimen (i.e., 100 mg/kg) does not limit the availability of tyrosine or impede the synthesis of catcholamines in hypothalamic neurons.

Therefore, since tryptophan administration appears to selectively alter 5-HT metabolism, the precursor may be a useful tool in studying these neurons. Since an increase in tryptophan hydroxylase activity induced by an increase in 5-HT release during neuronal activation is not accompanied by an increase in the intracellular availability of tryptophan (Elks et al., 1979), neuronal activity as reflected by 5-HT synthesis may be underestimated. Likewise, measurement of 5-HIAA concentrations and of the ratio of the concentrations of 5-HIAA/5-HT may be secondarily affected by tryptophan availability. Providing 5-HT neurons with exogenous tryptophan would circumvent these limitations and would allow for easier biochemical measurements of 5-HT neuronal activity in regions with a

sparse 5-HT innervation. Furthermore, tryptophan administration may be helpful in experiments utilizing drugs which bind to plasma proteins. Since tryptophan itself is highly bound to plasma albumin (Chaouloff et al., 1985, 1986), these drugs could compete for binding sites, dislodge the amino acid and increase the availability of free tryptophan and thereby increase 5-HTP accumulation and 5-HIAA concentrations in the brain without necessarily altering 5-HT neuronal activity (e.g., Fuller et al., 1976; Neckers et al., 1977; Van Wijk et al., 1979). These confounding effects could be eliminated by the prior administration of tryptophan to each experimental group.

While tryptophan loading could be theoretically useful in experiments biochemically estimating 5-HT neuronal activity in which 5-HT concentrations are low or in which drugs that bind to plasma proteins are administered, this tool is actually limited in its application by at least two factors. First, it must be shown that the administration of tryptophan does not increase the release of 5-HT. This will be considered in the next section of the "Discussion". Secondly, the usefulness of tryptophan may also be limited by the fact that its administration decreases the firing of 5-HT neurons (Aghajanian, 1972; Gallager and Aghajanian, 1976). This observation was made in anesthetized rats and since it is clear that anesthesia alters the effect of a number of drugs on the firing rate of 5-HT neurons (Trulson and Trulson, 1983; Heym et al., 1984) the results must be replicated in unanesthetized animals. If similar effects of tryptophan are observed, tryptophan loading should be limited to situations such as electrical stimulation studies in which 5-HT neuronal activity is artificially maintained.

C. Comparison of newly Synthesized 5-HT and Previously Released 5-HT as Sources of 5-HIAA

Fluoxetine selectively inhibits the uptake of released 5-HT (Wong et al., 1974, 1975; Lemberger et al., 1978; Richelson and Pfenning, 1984). The resultant increase in synaptic 5-HT content following this drug and similar 5-HT

uptake inhibitors prolongs the stimulation of both pre-and postsynaptic 5-HT receptors and, through a mechanism mediated by inhibitory neuronal feedback circuits and/or presynaptic 5-HT autoreceptors, decreases the rates of firing of 5-HT neurons (Gallagher and Aghajanian, 1975; Willner, 1985). This decrease in 5-HT neuronal activity is associated with a decrease in 5-HT synthesis (Carlsson and Lindqvist, 1978; Marco and Meek, 1979) and a decrease in spontaneous 5-HT release (Gallagher and Aghajanian, 1975). In the present study fluoxetine was utilized to determine the relative contributions of unreleased 5-HT and recaptured 5-HT to the total pool of 5-HT metabolized to 5-HIAA in brain tissue of normal and tryptophan-treated rats which received sham or actual electrical stimulation of the dorsal raphe nucleus. Since the inhibitory effects of fluoxetine on the activity of 5-HT neurons is dependent upon the presence of released 5-HT in the synapse, and tryptophan does not alter 5-HT release unless metabolism of newly synthesized 5-HT is blocked (Grahame-Smith, 1971; Elks et al., 1979; Trulson, 1985), we reasoned that this drug should act similarly in normal and tryptophan-treated rats. These assumptions seem warranted since uptake inhibitors have been shown to produce similar decreases in 5-HTP accumulation in normal and tryptophan-treated rats (Carlsson and Linqvist, 1978). Likewise, we assumed that 5-HT neuronal activity maintained by electrical stimulation of 5-HT cell bodies would not be inhibited by increased synaptic 5-HT produced by fluoxetine administration.

In the present study, fluoxetine produced a decrease in 5-HIAA concentrations in brain regions of normal rats of only about 20-30% of saline control values. These results are consistent with the effects of fluoxetine in whole brain (Reinhard and Wurtman, 1977) and with the findings of Wolf et al. (1985) which suggest that 5-HT need not be released from neurons before it is metabolized to 5-HIAA, and they support the idea that the majority of 5-HIAA measured in brain

tissue reflects metabolism of intraneuronal 5-HT rather than released 5-HT. Although the possibility that an increase in intraneuronal metabolism of 5-HT following application of uptake inhibitors cannot be ruled out, there is an accompanying decrease in 5-HT synthesis following inhibition of 5-HT uptake (Carlsson and Lindqvist, 1978; Marko and Meek, 1979; Johnston and Moore, 1983), and any elevation in intraneuronal metabolism of 5-HT under these conditions would for the most part be negligible. Given that fluoxetine produces a similar decrease in 5-HIAA concentrations in brain regions of rats receiving electrical stimulation of the dorsal raphe nucleus it appears that, under both basal and elevated activity of 5-HT neurons, 5-HIAA concentrations reflect intraneuronal metabolism of 5-HT prior to its release. While these results discredit the commonly held assumption that the 5-HIAA measured as an index of 5-HT neuronal activity results from metabolism of released 5-HT (e.g., Aghajanian et al., 1967; Sheard and Zolovick, 1971), they do not invalidate the use of this biochemical index of the activity of 5-HT neurons. Rather it appears that 5-HIAA concentrations, like 5-HTP accumulation, may indirectly reflect the synthesis of 5-HT.

Since inhibition of 5-HT uptake in tryptophan-treated rats produces a similar decrease in 5-HIAA concentrations as that observed in normal rats, be they sham or electrically stimulated animals, tryptophan administration apparently does not produce a marked change in 5-HT release. If, on the other hand, the metabolism of newly synthesized 5-HT is blocked by the administration of a monoamine oxidase inhibitor, postsynaptic receptor-mediated effects are observed following tryptophan suggesting that 5-HT release is enhanced (Grahame-Smith, 1971; Willner, 1985). Taken together these results demonstrate the importance of intraneuronal metabolism in eliminating excess nonvesicular 5-HT

from the cytoplasm and suggest that increasing 5-HT synthesis does not alter spontaneous 5-HT release in the brain regions.

Although tryptophan has been previously reported to increase the concentrations of 5-HT (Mueller et al., 1976; Johnston and Moore, 1983; Long et al., 1983) and 5-HIAA (Johnston and Moore, 1983) in the hypothalamus, Knott and Curzon (1974) observed that a single injection of 50 mg/kg tryptophan fails to alter hypothalamic 5-HT or 5-HIAA levels. On the other hand, in 5-HT-depleted animals, the same dose of tryptophan selectively increases 5-HT, but not 5-HIAA concentrations in the hypothalamus (Curzon and Marsden, 1975; Curzon et al., 1978). Evidently the storage pool of 5-HT will determine the disposition of newly synthesized 5-HT. In the case of normal 5-HT storage pools, a tryptophan-induced increase in 5-HT synthesis results in a concurrent increase in 5-HT storage and intraneuronal metabolism. If the amount of 5-HT available for release is low, newly synthesized 5-HT is preferentially stored. If, on the other hand, the stores of 5-HT are normal and the metabolism of 5-HIAA is blocked, there is an increase in cytoplasmic 5-HT and this "leaks" into the synapse where it is available to act at pre- and postsynaptic 5-HT receptors (Elks et al., 1979).

D. Effect of Exposing Brain Extracts to Sulfatase Hydrolysis on the Concentrations of "Free" 5-HT and "Free" 5-HIAA

No evidence could be found in the present experiments of significant quantities of either a 5-HT or a 5-HIAA sulfoconjugate in the nucleus accumbens despite rather drastic measures taken to elevate the concentrations of these indoleamines and to block their further metabolism and their transport out of the brain. These results are in accord with the predictions of the in vitro work by Meek and Neff (1973) which demonstrates a very low affinity of rat brain phenolsulfotransferase toward 5-HT and 5-HIAA. Likewise, the present findings are consistent with those of Korf and Sebens (1970) who could not detect an increase of "free" 5-HT following acid hydrolysis of brain homogenates of control

rats or of rats treated with a monoamine oxidase inhibitor, but differ from those of Gál (1972) who demonstrated the presence of 5-HT-sulfate in rat brain following pargyline administration. The reasons for these discrepancies concerning 5-HT-sulfate are unclear but are also seen with studies on 5-HIAA-sulfate. That is, in the present study as well as in a report by Hutson et al. (1984) who exposed rat cerebrospinal fluid to acid hydrolysis no evidence for 5-HIAA-sulfate could be found, yet Warnhoff (1984) reports a slight increase in "free" 5-HIAA following incubation of mediobasal hypothalamus samples of probenecid-treated rats with sulfatase. Perhaps the mediobasal hypothalamus possesses greater phenosulfotransferase activity and/or sulfoconjugates are rapidly transported out of the latter structure and out of the cerebrospinal fluid.

In any event, it is doubtful that sulfoconjugates of 5-HT or 5-HIAA, if they are produced by the brain, would be of any consequence in studies of 5-HT neurons in the pituitary gland since phenolsulfotransferase activity in this region is very low when compared with that of most brain regions (Foldes and Meek, 1974). Similarly, other forms of 5-HT or 5-HIAA conjugates formed in the periphery (e.g., 5-HT-glucuronate; Keglevic et al., 1959; Weissbach et al., 1961) are probably negligible in the pituitary gland since non-specific acid hydrolysis does not appear to elevate concentrations of "free" indoleamines in brain or cerebrospinal fluid (Korf and Sebens, 1970; Hutson et al., 1984).

II. Characterization of 5-HT Neurons Terminating in the Neural and Intermediate Lobes of the Pituitary Gland

The accumulation of 5-HTP in the neurointermediate lobe following the administration of NSD 1015 observed in this study clearly demonstrates the in vivo activity of tryptophan hydroxylase in this region. These data are consistent with reports of tryptophan hydroxylase and ALAAD activity in neurointermediate

lobe preparations in vitro (Renson, 1973; Saavedra et al., 1975; Johnston et al., 1984). While 5-HTP accumulation in the neurointermediate lobe may have been due, in part, to synthesis within non-neuronal tissue (i.e., mast cells), the rapid rate of 5-HTP accumulation measured in this lobe is not consistent with the low rate of 5-HT turnover in mast cells (Erspamer, 1966).

Further evidence of intraneuronal synthesis of 5-HT in the neurointermediate lobe is provided by the increased rate of 5-HTP accumulation following either the administration of tryptophan or the electrical stimulation of the pituitary stalk. In other 5-HT neuronal systems the activity of tryptophan hydroxylase is limited by substrate availability, so that administration of tryptophan increases both the rate of 5-HT synthesis, as estimated from the rate of 5-HTP accumulation, and the concentration of 5-HT (for review see Sved, 1983). Likewise, electrical stimulation of 5-HT neurons projecting to either the brain or the spinal cord increases 5-HTP accumulation in regions innervated by the stimulated fibers (Bourgoin et al., 1980; Boadle-Biber et al., 1983, 1986; Duda and Moore, 1985; Section I.A. of "Results"). The inability of electrical stimulation to increase 5-HTP accumulation in regions not receiving input from the stimulated neurons (i.e., stimulation of the pituitary stalk did not alter 5-HTP accumulation in the nucleus accumbens) indicates that this is a specific effect which occurs only in terminals of neurons which have been electrically stimulated. These data demonstrate, therefore, that at least a portion of 5-HT in the neurointermediate lobe is synthesized intraneuronally.

That this conclusion could be extended to each of the two divisions of the neurointermediate lobe was ascertained by the experiment involving repeated fluoxetine injections. Here again, the neural and intermediate lobes responded to a pharmacological manipulation in a manner identical to that of the nucleus accumbens, another region receiving 5-HT innervation: repeated injections of a

5-HT uptake inhibitor did not alter the concentration of 5-HT. Since 5-HT levels were maintained in the presence of uptake inhibition, the amine must have been synthesized intraneuronally in each of these regions. While this conclusion is not consistent with that of Saland et al. (1985), the discrepancy in the findings of these two studies may be due to the greater sensitivity of biochemical measurements compared with immunocytochemical techniques, as suggested by Friedman et al. (1983)

The use of an HPLC-EC system in this study permitted concurrent measurement of DOPA accumulation following NSD 1015 administration as an index of DA neuronal activity. As previously reported for other regions, tryptophan administration had no effect on DOPA accumulation in the neurointermediate lobe. Electrical stimulation of the pituitary stalk did, however, increase the accumulation of DOPA in this region. These results are consistent with the in vitro findings of Holzbauer et al. (1983).

III. Determination of the Origin of 5-HT Innervation of the Neural and Intermediate Lobes of the Pituitary Gland

It has been shown that electrical stimulation of nuclei containing 5-HT cell bodies increases 5-HTP accumulation in regions receiving projections from these neurons but not in other brain and spinal cord regions (Bourgoin et al., 1980; Duda and Moore, 1985). A similar effect of electrical stimulation of 5-HT fibers of passage was demonstrated in section II of the "Results". When electrical stimulation was employed in the present study it was found that stimulation of the dorsal and median raphe nuclei increased 5-HTP accumulation in the neural and intermediate lobes of the pituitary gland, while stimulation of the dorsomedial nucleus of the hypothalamus did not alter 5-HTP accumulation in these regions. These results indicate, therefore, that at least a portion of 5-HT innervation of the pituitary gland arises from cell bodies or axons which were stimulated by

electrodes implanted in the dorsal and median raphe nuclei. Furthermore, these results suggest that the putative 5-HT-containing cell bodies in the dorsomedial nucleus do not send axons to the neural and intermediate lobes of the pituitary gland.

Lesions of the brainstem raphe nuclei and of the hypothalamic dorsomedial nucleus support these contentions. Destruction of 5-HT cell bodies in the dorsal and median raphe nuclei with the neurotoxin 5,7-DHT altered neither 5-HTP accumulation nor 5-HT concentrations in the neural and intermediate lobes, but 5,7-DHT injections into the nuclei raphe pontis and raphe magnus decreased the concentration of 5-HT and its precursor in both of these pituitary divisions. In contrast, discrete electrolytic lesions of the dorsomedial nucleus did not alter 5-HTP accumulation or 5-HT concentrations in these regions. The combined results of these stimulation and lesioning experiments indicate that the 5-HT innervation of the neural and intermediate lobes of the pituitary gland arise, at least in part, from cells in the brainstem located caudal to the dorsal and median raphe nuclei and perhaps in the nucleus raphe pontis, raphe magnus or other caudal raphe nuclei and that this innervation does not originate in the dorsomedial nucleus of the hypothalamus.

The present findings regarding the brainstem as a source of 5-HT innervation of the pituitary gland are consistent with the results obtained by Mezey et al. (1984) in the intermediate lobe. The observation by those investigators that lesions of the dorsomedial nucleus of the hypothalamus produce a decline in 5-HT concentrations in the intermediate lobe, however, could not be confirmed in the present study. Given that axons of putative 5-HT-containing cell bodies in the dorsomedial nucleus appear to course through this nucleus in a ventrolateral direction (Beaudet and Descarries, 1979; Frankfurt and Azmitia, 1983) rather than ventromedially toward the pituitary gland and that the administration of HRP into

the pituitary gland does not result in labeled cell bodies in the dorsomedial nucleus (Sherlock et al., 1975; Broadwell and Brightman, 1976), it is unlikely that this nucleus contributes to the 5-HT innervation of the pituitary gland.

While the results in Section II demonstrate that 5-HT is synthesized in neurons of the neural and intermediate lobes of the pituitary gland, the findings of other investigators show that 5-HT is released from the pituitary gland following electrical stimulation of the pituitary stalk (Holzbauer et al., 1985) and the present studies indicate that these fibers originate in the brainstem, little is known of the function of 5-HT innervation of the pituitary gland. Some in vitro pharmacological studies of the intermediate lobe have shown that 5-HT stimulates adrenocorticotropic hormone (ACTH) and α-melanocyte-stimulating hormone (αMSH) release (Kraicer and Morris, 1976; Randle et al., 1983), while other in vitro investigations found no evidence for the influence of 5-HT on the release of ACTH, αMSH and β-endorphin (Voigt et al., 1978; Briaud et al., 1979; Jackson and Lowry, 1983; Randle et al., 1983; Stoll et al., 1984). Investigations into a physiological role for 5-HT in the release of these peptides at the level of the pituitary gland is yet to be undertaken.

There is some evidence, however, for a role of 5-HT in the control of vasopressin release. Studies demonstrating that lesions of the brainstem raphe nuclei induce polyuria and polydipsia in rats (Tangapregassom et al., 1974) and investigations suggesting that electrical stimulation of these region releases vasopressin (Sharpless and Rothballer, 1961) are consistent with this hypothesis but certainly not conclusive. Likewise, the increased levels of plasma vasopressin observed following administration of various 5-HT agonists and the blockade of increased vasopressin release in response to dehydration produced by lesions of 5-HT neurons indicate that 5-HT may regulate vasopressin release but say nothing of the brain or pituitary level at which this occurs. Correlative studies showing

decreased 5-HT concentrations in the neural lobe following dehydration (Piezzi and Wurtman, 1970) and elevated 5-HIAA concentrations in the neurointermediate lobe following exsanguination of ether-anesthetized animals (Holzbauer et al., 1985) are more suggestive of a role of pituitary 5-HT neurons in the regulation of vasopressin release. Clearly, however, further studies need to be undertaken before this function of 5-HT is firmly established.

SUMMARY AND CONCLUSIONS

Pharmacological manipulations, electrical stimulation studies and electrolytic and chemical lesioning experiments were performed in an effort to evaluate biochemical indices of 5-HT neuronal activity and to begin to characterize the 5-HT-containing nerve fibers of the neural and intermediate lobes of the pituitary gland. The significant findings and conclusions of these studies are summarized below.

A) Electrical stimulation of 5-HT cell bodies caused a frequency- and time-dependent increase in the concentration of 5-HIAA, in the ratio of the concentration of 5-HIAA to 5-HT and in the rate of accumulation of 5-HTP following NSD 1015 administration. In contrast, electrical stimulation of 5-HT cell bodies altered neither the rate of 5-HT accumulation nor the rate of decline of 5-HIAA following pargyline administration. These results indicate that while the first three methods serve as valid indices of 5-HT neuronal activity, the pargyline-dependent techniques are not responsive to changes in the rate of 5-HT nerve firing. In light of these findings, a reinterpretation of studies employing either of the pargyline methods for estimating 5-HT neuronal activity would appear to be in order.

B) Tryptophan produces a dose- and time-dependent increase in 5-HTP accumulation following NSD 1015 administration, in 5-HT concentrations and in 5-HIAA concentrations but does not alter the rate of DOPA accumulation following ALAAD inhibition. These results indicate that tryptophan selectively increases the synthesis, storage and metabolism of 5-HT and suggest that the technique of

tryptophan loading might provide a useful tool in some investigations of brain and pituitary regions with a sparse 5-HT innervation.

C) Inhibition of 5-HT synaptic uptake by fluoxetine only slightly reduced the concentrations of 5-HIAA in brain regions of control or electrically stimulated animals. This indicates that under "basal" conditions the majority of 5-HIAA is formed from intraneuronal metabolism of 5-HT prior to release and reuptake and that the increased 5-HT release produced by electrical stimulation of 5-HT cell bodies does not alter the proportion of 5-HT that is deaminated intraneuronally. This suggests that when 5-HIAA concentrations are determined as an index of 5-HT neuronal activity they, like the rate of 5-HTP accumulation, may indirectly reflect 5-HT synthesis rather than directly reflect 5-HT release. Similar results obtained in tryptophan-loaded animals suggest that the increase in 5-HT synthesis following precursor loading is accompanied primarily by an increase in the intraneuronal metabolism of 5-HT.

D) Brain extracts were exposed to sulfate hydrolysis by sulfatase in an effort to determine if possible sulfoconjugation of 5-HT and 5-HIAA led to underestimation of the concentrations of these compounds by HPLC-EC. No increases in "free" 5-HT or "free" 5-HIAA concentrations were observed following sulfatase incubation even in situations in which brain tissue was obtained from animals in which various manipulations (pargyline, tryptophan and/or probenecid administration, electrical stimulation) designed to maximally enhance 5-HT or 5-HIAA concentrations were performed. These results indicate that 5-HT and 5-HIAA do not appear to be conjugated with sulfate to any significant extent in the brain.

E) 5-HTP accumulation was detected in the neurointermediate lobe of the pituitary gland 30 minutes following the administration of NSD 1015, and the rate of this accumulation was increased by the administration of tryptophan and

by electrical stimulation of the pituitary stalk. In addition, repeated injections of fluoxetine induced a marked depletion of platelet 5-HT but did not alter the concentration of 5-HT in either the neural or intermediate lobe of the pituitary gland. Taken together these results indicate that much of the 5-HT in the neurointermediate lobe of the pituitary gland does not represent 5-HT taken up from the blood, but rather the amine is synthesized in neurons projecting to this region. Furthermore, the rate of 5-HT synthesis in nerve terminals in the neurointermediate lobe, as in other 5-HT neurons, is dependent upon both precursor availability and neuronal impulse traffic.

F) The application of stimulating current to electrodes implanted in the dorsal and median raphe nuclei increased 5-HTP accumulation in the neural and intermediate lobes of the pituitary gland of NSD 1015-treated animals. 5,7-DHT lesions of these nuclei did not alter 5-HTP accumulation or 5-HT concentrations in the neural or intermediate lobe, but similar lesions of the nuclei raphe pontis and raphe magnus decreased both the concentration of 5-HT and the accumulation of its precursor in these pituitary regions. Taken together these results suggest that electrical stimulation activated 5-HT fibers of passage which project to the neural and intermediate lobes and which originate in cell bodies caudal to the dorsal and median raphe nuclei. These perikarya may reside in the nucleus raphe pontis and/or raphe magnus. In contrast, neither electrical stimulation nor electrolytic lesioning of the dorsomedial nucleus of the hypothalamus altered 5-HTP accumulation in either the neural or intermediate lobe indicating that this nucleus does not contain the cell bodies of origin of 5-HT fibers in the pituitary gland.

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