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THE HYPOTHALAMIC SEROTONERGIC SYSTEM: EFFECTS OF CHEMICAL NEUROTOXINS IN THE RAT

City University of New York PH.D. 1983

THE HYPOTHALAMIC SEROTONERGIC SYSTEM: EFFECTS OF CHEMICAL NEUROTOXINS IN THE RAT

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A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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ABSTRACT

THE HYPOTHALAMIC SEROTONERGIC SYSTEM:

EFFECTS OF CHEMICAL NEUROTOXINS IN THE RAT

by

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The hypothalamic serotonergic system of the rat was studied following intracerebral injection of 5,7-dihydroxtryptamine (5,7-DHT). Changes in serotonin (5-HT) immunocytochemical staining, ^{3}H5-HT uptake and 5-HT levels in the hypothalamus and lordosis behavior were assessed over time.

Three days after unilateral injection of 5,7-DHT (3 ug free base) into the dorsolateral hypothalamus 5-HT immunoreactive-(IR) fibers were swollen and darkly stained in the ipsilateral medial forebrain bundle (MFB). In the contralateral hypothalamus some degeneration was apparent, however this was generally restricted to the area adjacent to the fornix. There was a gradual decrease in the 5-HT-IR fiber density in the ipsilateral MFB 3-19 days post lesion. The ipsilateral periventricular and medial hypothalamic areas contained virtually no 5-HT-IR fibers 7-30 days post lesion. Sprouting 5-HT-IR fibers were abundent 12-19 days post lesion and 30 days post lesion there was a normal or supranormal density of 5-HT-IR fibers in the MFB, however other areas were not reinnervated. Fifty days post lesion there was an apparent hyperinnervation of 5-HT fibers ipsilateral and contralateral to the injection of 5,7-DHT as compared to sham injected animals.

The morphological data are supported by changes in ^{3}H5-HT uptake and 5-HT. 5-HT levels and ^{3}H5-HT uptake are lowest 7 days post lesion and there is a gradual recovery in both ^{3}H5-HT uptake and 5-HT levels 30-50 days post lesion.

Unilateral injection of 5,7-DHT (3 ug free base) into the dorsolateral hypothalamus resulted in the removal of a group of 5-HT-IR cells which had been observed in initial immunocytochemical studies. The injection of 6-hydroxydopamine (3 ug free base) into the dorsolateral hypothalamus or 5,7-DHT into the rostral midbrain had no effect on the staining of this cell group. Furthermore, a patch of thin 5-HT-IR fibers ventrolateral to the 5-HT-IR cells was apparent after removal of the ascending 5-HT fibers by injection of 5,7-DHT into the rostral midbrain.

Lordosis behavior was rapidly facilitated following bilateral injection of 5,7-DHT (5 ug free base) into the dorsolateral hypothalamus. The facilitation observed continued until 42-46 days post lesion. Preliminary immunocytochemical evidence indicates that the transplantation of fetal raphe tissue results in a rapid reversal of the facilitation in lordosis observed after 5,7-DHT.

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INTRODUCTION

I. Plasticity in the Central Nervous System

Plasticity in the central nervous system (CNS) of adult mammals was thought to be virtually non-existent until recently. From the early 1900's it was believed, largely due to the work of Ramon Y Cajal (1959), that although regeneration of damaged nerve fibers could occur, it was abortive. Ramon Y Cajal demonstrated that axotomized neurons in the dog spinal cord were capable of sprouting new fibers. These new fibers were, however, resorbed within several weeks. It was concluded from these studies that in adult mammals neuronal development was static.

It was not until the 1950's that some progress was made in this regard. In 1940, Sugar and Gerard demonstrated that regeneration of fibers could occur in young rats following spinal cord transection. In these animals the glial scar that was formed was less dense than in adult animals. This prompted Windle and coworkers to search for ways in which scar formation could be reduced. In studies with adult cats that were treated with bacterial pyrogens to reduce scarring, Windle and Chambers (1950) demonstrated that intraspinal axons were able to regenerate following spinal cord transection and that regenerating fibers could traverse the lesion. Furthermore, if scarring was reduced by the administration of pyrogens or adrenocorticotropin (ACTH), transplanted peripheral nerves grew in the cat CNS (Clemente and Windle, 1954; Clemente, 1964). Thus, if conditions were favorable (i.e. little glial scarring) regeneration of axotomized fibers in the adult mammalian CNS could occur.

The first evidence for plastic changes in the CNS other than regeneration came from a study by Liu and Chambers (1958) in adult cats. These workers demonstrated that partial denervation of the spinal cord resulted in sprouting of intact fibers (collateral sprouting). Collateral sprouting has since been shown in many different CNS systems such as the septal nuclei (Raisman, 1969; Raisman and Field, 1973; Moore et al., 1971), hippocampus (Azmitia et al., 1978), dentate gyrus (Lynch et al.,1972) and hypothalamus (Silverman and Zimmerman, 1982). At the ultrastructural, level Raisman and Field (1973) have shown that collateral sprouting in the adult rat septal nuclei results in the formation of new synapses.

A. Monoaminergic Systems

1) Mechanical and Electrolytic Lesions

Since monoamines (MA) exposed to formaldehyde gas become fluorescent, it became possible to trace MA containing fiber systems with relative accuracy using fluorescence histochemistry (Dahlstrom and Fuxe, 1964). Therefore, the response of a particular MA neuronal system to axotomy could be studied.

Following mechanical (Bjorklund et al.,1971) or electrolytic (Katzman et al.,1971) lesions in the CNS, there is an interruption of axonal transport and a build up of MA in nerve fibers. Therefore, MA fibers are easily visualized by fluorescence histochemistry. Within several days of the lesion MA fibers are observed to sprout vigorously from the stumps of degenerating fibers. There is abundant fiber growth into the necrotic zone caused by the lesion and into the neuropil proximal to the necrotic zone. However, as noted by Ramon Y Cajal

(1959), the regenerative sprouts are unable to traverse the scar formed in response to the lesion and gradually disappear.

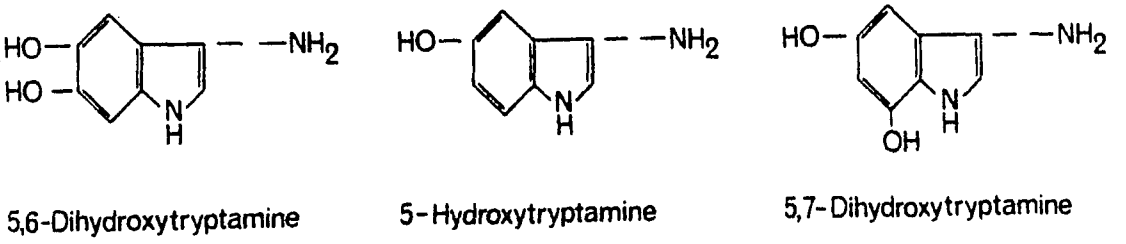
Regenerative sprouting of MA containing neurons has also been demonstrated following transplantation of denervated peripheral tissue into the CNS (Bjorklund and Stenevi, 1971; Bjorklund and Stenevi, 1979). Transplantation of an iris into the caudal diencephalon transects the ascending path of NE fibers. Within several days of transplantation, NE fibers are seen, by histochemical fluoresence, to sprout. The sprouts elongate and grow into the iris, become branched, and within several weeks of transplantation, normal patterns of innervation are observed in the iris. Transplantation of irides into the midbrain has been shown to result in regeneration of transected 5-HT fibers if the catecholamine (CA) systems are removed (Svengaard et al., 1975).

2) Chemical Lesions

The introduction of chemical neurotoxins as denervation tools has radically expanded the possibilities for studying regenerative phenomena because their use does not result in the formation of a dense glial scar. The compounds used include 6-hydroxydopamine (6-OHDA), which destroy dopamine (DA) and norepinephrine (NE) containing fibers and 5,6- and 5,7-dihydroxytryptamine (5,6- and 5,7-DHT) which destroy serotonin (5-HT) containing fibers. The latter compounds are specific for MA because as structural analogs of the MA (Figure 1) they are substrates for the high affinity uptake system of MA containing cells (Baumgarten et al., 1977). Although 5,7-DHT is a serotonergic neurotoxin, it will destroy NE containing neurons unless it is used in

conjunction with a NE uptake inhibitor, such as desmethylimipramine (Baumgarten, et al., 1977). Once inside the cell the mechanism by which the cell is killed is thought to involve the generation of reactive compounds such as superoxide and hydroxyl free radicals which are cytotoxic (Jonsson, 1980).

Figure 1: Structure of 5,6- and 5,7-dihydroxytryptamine (5,6- and 5,7-DHT) as compared to 5-HT. Adapted from Baumgarten et al. (1977).



5,6-Dihydroxytryptamine 5-Hydroxytryptamine 5,7-Dihydroxytryptamine

The intraventricular administration of 6-OHDA has been shown to result in degeneration and regeneration of NE fibers in the spinal cord (Nygren and Olson, 1977). The ability of NE fibers to regenerate depended on the dose of 6-OHDA used. Following injection of 50 ug of 6-OHDA into the lateral ventricle the bulbospinal NE system regenerated, whereas if higher doses of 6-OHDA (250-500 ug) were administered regeneration was not apparent.

Bjorklund and Lindvall (1979) have also demonstrated the regenerative capacity of NE fibers in the adult rat brain after 5,7-DHT injection (150 ug free base, without a NE uptake blocker) into the lateral ventricle. This injection resulted in a large decrease in NE content, ^{3}HNE uptake, and fluorescent histochemically demonstrable NE fibers in the medulla and forebrain within 2 weeks of the injection. Five days after the injection of 5,7-DHT, fine sprouts were observed coming from the swollen, degenerating NE fibers in the major ascending NE pathways. Within several months (4-6), the medulla, hypothalamus and sensorimotor cortex were substantially reinnervated as shown by an increased number of NE fluorescent fibers, ^{3}HNE uptake, and NE levels. In addition, the pattern of reinnervation resembled the normal pattern. This was particularly evident in the anterior hypothalamus where the pattern of decussating NE fibers was reestablished.

Regeneration in the CNS following chemical axotomy has been extensively studied in the adult rat 5-HT system. After intraventricular administration of 5,6-DHT, regeneration of 5-HT fibers was shown to occur by fluorescence histochemistry in the spinal cord (Nobin et al., 1973; Nygren et al., 1974; Wiklund and Bjorklund, 1980)

medulla, hypothalamus, septum and striatum (Bjorklund et al., 1973; Wuttke et al., 1977; Bjorklund and Stenevi, 1979).

Several phases of regenerative growth can be distinguished. A detailed study of the morphological (Wiklund and Bjorklund, 1980) and biochemical (Bjorklund and Wiklund, 1980) correlates of 5-HT fiber regrowth in the adult rat bulbospinal system illustrates this. As shown by fluorescence histochemistry, ^{3}H5-HT uptake and 5-HT levels there is an almost complete denervation of the spinal cord and a partial denervation of the brainstem nuclei within 1 week of 5,6-DHT (75 ug free base) injection into the lateral ventricle. Morphologically, the earliest phase of regrowth was characterized by the presence of 5-HT fibers sprouting in the caudal medulla within 1 week of 5,6-DHT injection. In the next phase (2-4 weeks), the sprouts were seen to proliferate and grow caudally to reinnervate the brainstem nuclei (facial nucleus, inferior olivary nucleus) and the cervical spinal cord. Finally, 7-19 months post lesion, the regenerating 5-HT fibers had grown great distances and were seen to partially reinnervate the thoracic and lumbar spinal cord. Normal patterns of reinnervation were established in the brainstem and spinal cord with the exception that certain areas of the brainstem, in particular the inferior olivary nucleus were hyperinnervated.

The morphological observations of a gradual reinnervation of the bulbospinal 5-HT system are supported by gradual increases in specific ^{3}H5-HT uptake and 5-HT levels (Bjorklund and Wiklund, 1980). An additional finding of the latter study was that an inverse relationship existed between the density of innervation and the turnover of 5-HT.

Serotonin turnover was assessed by measuring the in vitro synthesis of ^{3}H5-HT (from ^{3}H-tryptophan) and breakdown of ^{3}H5-HT to ^{3}H-5-hydroxyindoleacetic acid (^{3}H5-HIAA), which is the major degradation product. Six to eight months after 5,6-DHT injection, 5-HT turnover was decreased in hyperinnervated areas (caudal medulla) and increased in hypoinnervated areas (spinal cord). Bjorklund and Wiklund (1980) suggest, on the basis of these findings, that the density of innervation is compensated by alterations in turnover.

3) Functional Restoration

It has become clear, therefore, that in the MA fiber systems regeneration can occur after axotomy, and in the case of neurotoxin induced axotomy, regenerating fibers are able to grow long distances to reinnervate denervated areas. Whether this results in functional connections is yet to be established. There are, however, examples of apparent functional recovery paralleling regeneration of 5-HT fibers after axotomy. Using the hindlimb-extensor reflex, it has been shown that there is a return of function in the rat spinal cord that correlates with 5-HT return (Nygren et al., 1974). Following 5-HT denervation by 5,6-DHT, 5-HT receptors are supersensitive and the hindlimb extensor reflex in increased in response to 5-HT agonists. The progressive increase in 5-HT innervation, as determined by fluorescence histochemistry and ^{3}H5-HT uptake, is paralleled by a decreased extensor reflex. Functional return has also been demonstrated in the neuroendocrine system. Two weeks after 5,7-DHT administration into the lateral ventricle (100 ug free base), ^{3}H5-HT uptake and 5-HT synthesis in the hypothalamus are reduced to

20-35% of control values. At this time luteinizing hormone (LH) release from the pituitary was 50% of control. Two months after 5,7-DHT when 5-HT synthesis and ^{3}H5-HT uptake in the hypothalamus returned to normal, LH release from the pituitary had also returned to normal (Wuttke et al., 1977). Finally, 2 weeks following 5,6-DHT injection, tyrosine hydroxylase (TH) activity in the locus coeruleus (LC) is increased. Within 2-4 months, when 5-HT innervation in the LC had returned, TH activity was back to normal levels (McRae-Daguerce et al., 1981b).

4) Collateral Sprouting

Regeneration of 5-HT fibers does not occur in certain 5-HT fiber systems. For example, Wiklund and Mollgard (1979) have observed that in the rat subcommissural organ (SCO), destruction of the 5-HT innervation by 5,6-DHT results in reinnervation of the SCO by non-monoaminergic fibers. Three to eight months after the intraventricular injection of 5,6-DHT, no 5-HT fibers were evident in the SCO (with fluorescence histochemistry). At the ultrastructural level it was observed that the boutons present were larger, contained more mitochondria and a different population of synaptic vesicles than the 5-HT boutons they replaced. Moreover, 5,6-DHT caused an increase in the secretory activity of the SCO that remained high despite reinnervation, indicating that the new fibers were incapable of substituting for 5-HT fibers functionally.

While this finding appears to contradict what is known about the regenerative capacity of 5-HT fibers, Wiklund and Mollgard suggest that the mode of termination of 5-HT fibers may influence their regenerative

ability. It has been observed that in many CNS areas 5-HT fibers do not make classical synapses in that their junctions lack pre- and post-membrane specializations (Beaudet and Descarries, 1981). These have been termed non-junctional synapses. Classical 5-HT synapses have been observed in the suprachiasmatic nucleus (Noyjo and Sano, 1978) and in the SCO (Mollgard and Wiklund, 1979). Moreover, it has been demonstrated that 5-HT fibers do not regenerate in the suprachiasmatic nucleus (Bjorklund et al., 1973) and the SCO (Wiklund and Mollgard, 1979). In the inferior olive, which is known to contain primarily non-junctional 5-HT synapses, 5-HT regeneration has been shown to occur (Wiklund et al.,1981). On the basis of these observations Wiklund et al. have suggested that regeneration of 5-HT fibers occurs in areas where non-junctional synapses are made and vice versa. Further ultrastructural studies of 5-HT synapses are required to ascertain whether the mode of termination of 5-HT fibers plays a role in regeneration.

B. Transplantation of CNS Tissue

1) Growth

Transplantation of CNS tissue into the adult mammalian brain has also been used to study neuronal plasticity. Both MA and peptide containing neurons have been successfully transplanted. It has been demonstrated by histochemical fluorescence that CA neurons transplanted to the CNS are capable of survival and differentiation. The resulting patterns of innervation resemble the normal pattern of CA fibers (Stenevi et al., 1976, Bjorklund et al., 1976). Stenevi et al. (1976) have demonstrated that good survival of transplanted neurons was

obtained only from fetal tissues (vs newborn or adult) that were placed in contact with vessel-rich tissue, such as pia.

Fetal 5-HT neurons have been sucessfully transplanted into the lateral ventricle of adult rats (McRaé-Degueurce et al.,1981a) and the hippocampus of adult mice (Azmitia et al., 1981). Using 5-HT immunocytochemistry, Azmitia et al. (1981) demonstrated that following transplantation of fetal raphe tissue into the adult mouse hippocampus 5-HT neurons were able to survive and differentiate into large multipolar neurons. 5-HT fibers from the transplanted 5-HT neurons were seen to hyperinnervate areas of the hippocampus that normally contained 5-HT fibers.

2) Functional Restoration

Functional deficits resulting from genetic abnormalities have been reversed in rodents by transplantation of the appropriate neuronal tissue into the brain. Brattleboro rats are congenitally deficient in vasopressin and therefore have polyuria and polydipsia. Gash et al. (1980) have demonstrated that transplantation of fetal anterior hypothalamic area (which contains vasopressin neurons) into the median eminence of these rats could result in a dramatic decrease in water consumption. Vasopressin neurons were shown, by immunocytochemistry to have survived (although the rate of survival was poor) and processes from these neurons were seen to extend to the median eminence. Transplantation of fetal preoptic area, which contain LH-releasing hormone (LH-RH) neurons, have been shown to reverse congenital hypogonadism in male mice (Krieger et al., 1982). Following transplantation of fetal preoptic area into the third ventricle,

hypogonadism was reversed as assessed by increases in gonadal hormones, LH-RH levels and testicular weight. LH-RH neurons were demonstrated by immunocytochemistry to have developed and LH-RH fibers could be traced to the median eminence.

Functional deficits induced in the nigrostriatel system have also been ameliorated by transplantation of the appropriate neurons. Following denervation of the striatum by unilateral injections of 6-OHDA into the nigrostriatal tract, rats have pronounced behavioral and motor deficits. These can be partially reversed by transplantation of fetal substantia nigra (SN), the area of DA containing cell bodies. Spontaneous or drug induced turning behavior and T-maze asymmetries can be compensated for by the transplantation of fetal SN into a cortical cavity overlying the striatum (Bjorklund et al., 1981; Dunnett et al., 1981a; Perlow et al., 1979). Fluorescence histochemical studies demonstrate that the transplanted SN cells have reinnervated the dorsal portion of striatum (Dunnett et al., 1981b; Freed et al.,1980). Furthermore, the behavioral data obtained in this model are supported by the biochemical data of Schmidt et al. (1982). In the latter study, it was found that the metabolism of DA and the utilization of glucose in the transplanted SN cells was similiar to that of intact SN cells.

II. Serotonin Innervation of the Rat Hypothalamus

Serotonin has been shown by biochemical assay to be present in relatively high quantities in hypothalamic nuclei (Saavedra et al., 1974). The rate limiting enzyme in 5-HT biosynthesis, tryptophan hydroxylase (Figure 2), has also been demonstrated in the hypothalamus

(Kizer et al., 1975; Keller et al., 1977). The presence of a specific high affinity uptake mechanism for 5-HT in the hypothalamus indicates that there are many 5-HT terminals in this area (Kuhar et al., 1972; Keller et al., 1977).

Figure 2: Diagram indicating the synthesis and degradation of 5-HT. Adapted from Cooper et al. (1978).

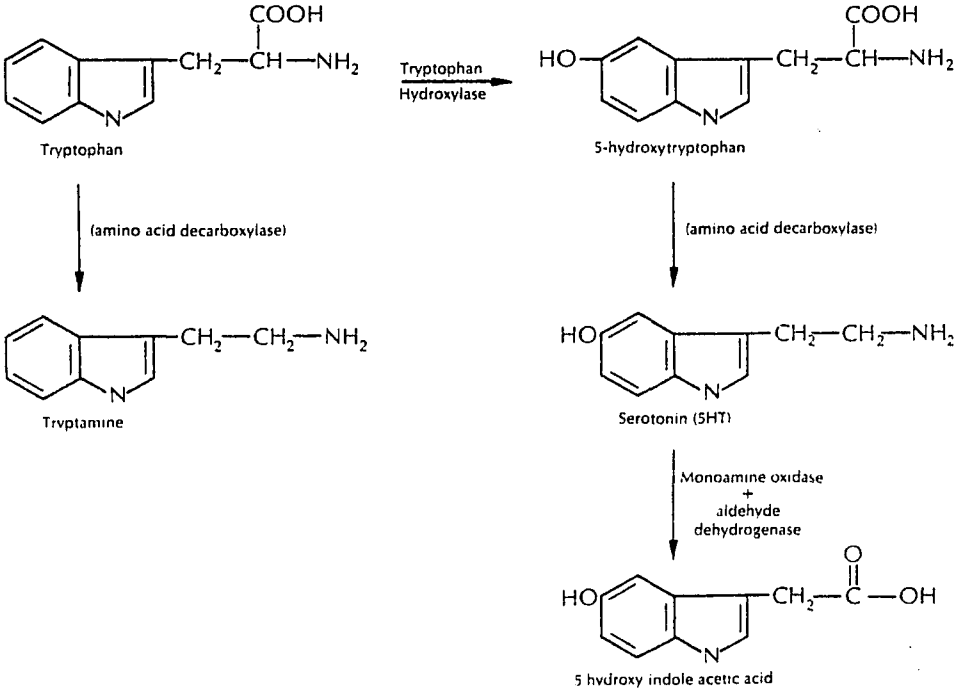
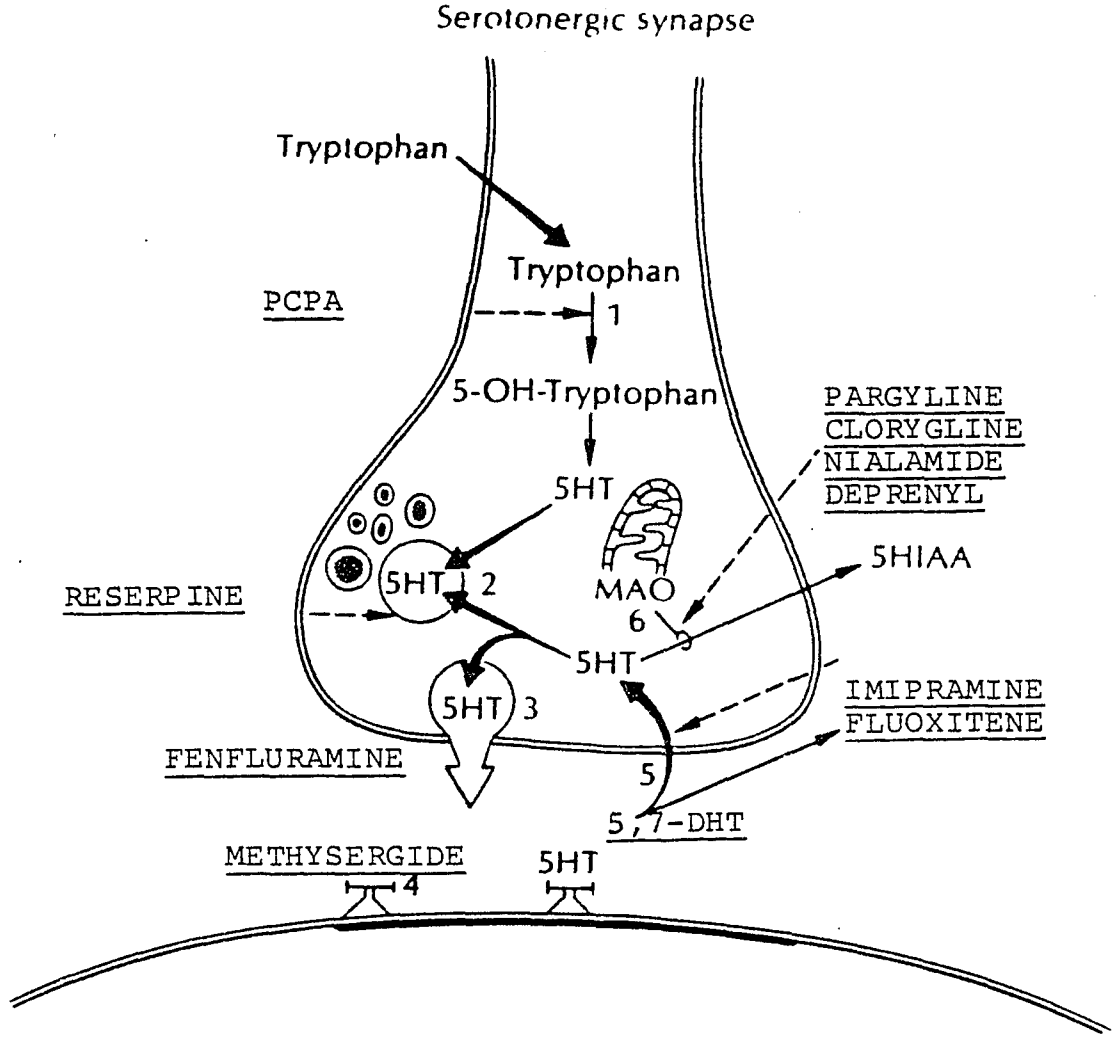


Figure 3: Diagram of a typical 5-HT synapse indicating where drugs interact. 1. Parachlorophenylalanine (PCPA) inhibits tryptophan hydroxylase. 2. Reserpine inhibits the uptake of 5-HT into storage vesicles. 3.Fenfluramine causes the release of 5-HT. 4. Methysergide is a post-synaptic receptor antagonist. 5. 5,7-DHT is a neurotoxin that is a substrate for the reuptake mechanism of the cell. Both imipramine and fluoxitene inhibit the reuptake mechanism. 6. Pargyline, deprenyl, clorgyline and nialamide are monoamine oxidase inhibitors. Adapted from Cooper et al. (1978).



A. Morphology

1) Ascending Pathways

Morphological localization of 5-HT in the brain has been achieved by three techniques: histochemical fluorescence, radioautography and immunocytochemistry. The initial localization of 5-HT in the rat brain was achieved by Dahlstrom and Fuxe (1964) using fluorescence histochemistry. These workers described the presence of nine cell groups in the rat brainstem which contained 5-HT. The majority of 5-HT containing cell bodies were located in the dorsal and median raphe nuclei (B-7 and B-8, respectively, in the nomenclature of Dahlstrom and Fuxe) of the brainstem. It was also demonstrated that there were 5-HT terminals throughout the hypothalamus, particularly in the suprachiasmatic nucleus (Fuxe, 1965).

Indirect evidence for the existence of 5-HT projections from the brainstem raphe to the hypothalamus came from studies in which the medial or dorsal raphe (or both) were destroyed. This resulted in a large decrease in hypothalamic 5-HT (Geyer et al., 1976; Keller et al., 1977; Moore et al. 1978; Palkovits et al., 1977). More direct evidence was obtained from fluorescence histochemical studies that were combined with lesions or pharmacological manipulations. Increasing 5-HT levels by the administration of reserpine, which inhibits uptake of MA's into synaptic vesicles, or parygline, which inhibits monoamine oxidase (MAO, the major degradative enzyme for 5-HT) or axon transection, which causes a pile up of transmitter, allowed for better visualization of 5-HT by histochemical fluorescence. Using this method a system of 5-HT fibers that ascended from the midbrain raphe to the

hypothalamus via the medial forebrain bundle (MFB) was described. (Anden et al., 1966; Fuxe and Ungerstedt, 1968; Ungerstedt, 1971a). The MFB is a complex bundle of ascending and descending fibers that courses through the lateral hypothalamus.

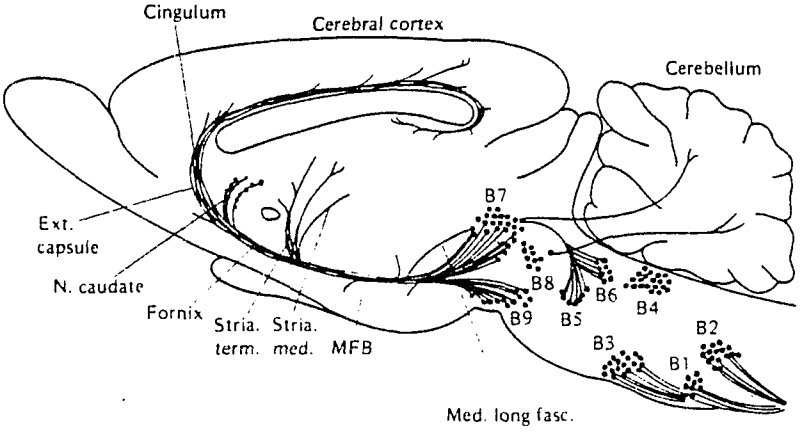
Radioautography of anterogradely transported tritiated amino acids has demonstrated ascending projections to the hypothalamus from the midbrain raphe nuclei (Conrad et al., 1974; Azmitia and Segal, 1978; Bobillier et al., 1979). When radioautography of tritiated amino acids is used in conjunction with 5,6- or 5,7-DHT lesions, 5-HT projections can be differentiated from non-5-HT projections. Azmitia and Segal (1978) have identified six ascending 5-HT pathways from the midbrain raphe of which four were shown to innervate the hypothalamus. The median raphe nucleus was shown to project to the lateral hypothalamus via the ventromedial part of the MFB. In addition, the hypothalamus was shown to receive 5-HT innervation from tracts which did not ascend in the MFB. The dorsal raphe periventricular tract originates in the the dorsal raphe nucleus and ascends along the midline to innervate the periventricular nuclei and median eminence of the hypothalamus. A second tract originating in the dorsal raphe nucleus and ascending outside the MFB, the dorsal raphe arcuate tract, was shown to innervate the suprachiasmatic nucleus. Lastly, a small projection that arises from the dorsal and median raphe nucleus, the raphe medial tract, and innervates the mamillary body was described. Figure 4 summarizes the ascending 5-HT pathways.

Radioautography following the intraventricular infusion of ^{3}H5-HT has also been done. In a recent study, Parent et al.

(1981) confirmed the presence of a periventricular 5-HT system in addition to the fibers that ascend in the MFB.

More recently, the technique of 5-HT immunocytochemistry has been used to trace pathways and describe fiber distribution. It has been demonstrated that virtually all hypothalamic nuclei contain some 5-HT fibers (Steinbusch, 1981; Lidov and Molliver, 1982).

Figure 4: Diagram of a mid-sagittal section of the rat brain illustrating ascending 5-HT pathways originating in the brainstem raphe nuclei (B7, dorsal raphe; B8, median raphe) MFB, medial forebrain bundle ascends in the lateral hypothalamus. Adapted from Cooper et al. (1978).



2) Hypothalamic 5-HT Cell Bodies

Although the majority of 5-HT containing cell bodies have been shown to be located in the brainstem raphe nuclei (Dahlstrom and Fuxe, 1964; Steinbusch et al., 1981; Parent et al., 1981) there is increasing evidence for the presence of an endogenous source of 5-HT within the hypothalamus. High concentrations of 5-HT and tryptophan hydroxylase are present in the surgically isolated mediobasal hypothalamus (Popova et al., 1972; Brownstein et al., 1976). In a fluorescence histochemical study, Fuxe and Ungerstedt (1968) noted that a group of cells in the dorsomedial nucleus of rats treated with nialamide (an MAO inhibitor, Figure 3), exuded a faint yellow fluorescence (indicative of 5-HT). Kent and Sladek (1978) reported the presence of 5-HT cell bodies in the arcuate nucleus of the hypothalamus following pargyline, which is another MAO inhibitor (Figure 3) and L-tryptophan pretreatment. Two groups have reported the presence of 5-HT concentrating cells in the hypothalamus using radioautography after intraventricular infusion of ^{3}5-HT. Chan-Palay (1977) reported the presence of 10 groups of cells (including the DMN, arcuate and periventricular nuclei) in the hypothalamus which concentrated 5-HT after infusion of 10^{-5}M ^{3}H5-HT with 10^{-4}M NE. Beaudet and Descarries (1979), using the same technique and the same concentration of ^{3}H5-HT, however, found only a single cluster of 5-HT concentrating cells in the ventral portion of the DMN. In the latter study, increasing the concentration of ^{3}H5-HT to 10^{-4}M resulted in the labelling of cells in the arcuate and periventricular nuclei. The cells in the arcuate and periventricular nuclei were no longer labelled if 10^{-3}M NE was added to the infusion indicating that

these cells were CA cells capable of taking up 5-HT by a low affinity mechanism. However, no 5-HT positive cells were observed in the hypothalamus in a recent immunocytochemical study of pargyline and colchicine pretreated rats (Steinbusch and Nieuwenhuys, 1979). It was suggested that the cells thought to contain 5-HT were either dopaminergic cells taking up exogenous 5-HT or were cells that contained an indolamine other than 5-HT.

B. Function

Hypothalamic 5-HT has been implicated in a variety of physiological functions such as thermoregulation (Myers, 1981), sexual behavior (Foreman and Moss, 1978; Luine and Fischette, 1982) control of pituitary secretions (Wuttke et al., 1977; Kordon et al., 1980; Kordon et al., 1981) and neuroendocrine rhythms (Kordon et al.,1981; Williams et al., 1983). There are conflicting data on the role of 5-HT in controlling basal secretions of pituitary hormones. Increasing hypothalamic 5-HT, by injection of nialamide into the mediobasal hypothalamus, has been reported to decrease LH secretion (Kordon, 1969). However, Wuttke et al.(1977) have reported that decreased LH can be correlated to decreases in hypothalamic 5-HT following 5,7-DHT injection into the lateral ventricle. Furthermore, van de Kar et al. (1980) have shown that bilateral injections of 5,7-DHT into the ventromedial hypothalamus result in a significant decrease (46%) in serum LH. Hypothalamic 5-HT has been reported to inhibit thyrotropin (TSH) secretion by inhibiting the release of thyrotropin releasing hormone (TRH) from hypothalamic synaptosomes (Bennett et al., 1975) and hypothalamic fragments (Grimm and Reichlin, 1973). Tuomisto et al.,

(1975) have shown that the cold stress induced rise in TSH secretion is reduced by 5-hydroxytryptophan (5-HTP, Figure 2) which is the presursor of 5-HT. Recently, however, Smythe et al. (1982) reported that increased turnover of 5-HT was associated with increased TSH secretion in the hypothyroid rat and that there was a linear correlation between serum TSH and the turnover of 5-HT in the euthyroid rat.

The role of 5-HT in mediating certain neuroendocrine reflexes is more clearly understood. The suckling induced rise in prolactin (PRL) secretion that occurs in lactating rats appears to depend on hypothalamic 5-HT. Inhibition of 5-HT synthesis by parachlorophenylalanine (PCPA, Figure 3) abolishes the rise in suckling induced PRL and administration of the 5-HT precursor, 5-HTP, restores it (Kordon et al., 1973). Administration of methysergide (Figure 3), a 5-HT receptor blocker abolishes the suckling induced rise in PRL (Gallo et al., 1975). Mena et al. (1976) have shown that suckling increases the turnover of 5-HT in the hypothalamus and that this coincides with increased PRL levels.

Hypothalamic 5-HT appears to be involved in the activation of the pituitary-adrenal axis during stress. Administration of 5-HT to hypothalamic fragments has been shown to block release of ACTH from pituitary cells in vitro (Vermes et al., 1972). The stress induced rise in corticosterone secretion is inhibited by implantation of 5-HT crystals into the mediobasal hypothalamus (Vermes and Telegdy, 1972). PCPA (Figure 3), has been reported to increase the amplitude of the ACTH response to stress (Berger et al., 1974).

The ability to maintain circadian rhythmicity appears to be

dependent on the hypothalamic 5-HT system. Removal of 5-HT has been reported to reduce or abolish the circadian rhythm of prolactin, TSH and LH secretion (this has been recently reveiwed by Kordon et al., 1981). Recently, Williams et al.(1983) have demonstrated that bilateral microinjection of 5,7-DHT into the suprachiasmatic nuclei of the rat disrupted the circadian rhythm of corticosterone release.

Conversely, hypothalamic 5-HT has been shown to be involved in neuroendocrine feedback. Estrogen and progesterone administration has been reported to increase 5-HT turnover in the median eminence (Crowley et al., 1979) and increase MAOA (which degrades 5-HT) in the basomedial hypothalamus (Luine and McEwen, 1977). Levels of hypothalamic 5-HT have been reported to fluctuate during the estrous cycle (Kueng et al.,1976). The uptake of ^{3}H5-HT by hypothalamic synaptosomes has been reported to increase when ovariectomized (OVX) rats are given estradiol (Cardinali and Gomez, 1977). Estrogen administration has also been reported to increase the level of 5-HT receptors in certain hypothalamic nuclei (Biegon et al., 1982). In this study, increases in 5-HT receptor levels were only found in nuclei known to selectively concentrate estrogen (McEwen et al., 1980) such as the anterior hypothalamus and the arcuate nucleus-median eminence. These areas are known to be involved in the control of ovulation and therefore an alteration in 5-HT receptor number by estrogen may represent a step in this control.

III. 5-HT and Lordosis

Female mammals are sexually receptive just prior to ovulation. Sexual receptivity includes the lordosis reflex, which is characterized

by arching of the back and lifting of the tail, as well as other soliciting behavior (Hardy and Debold, 1971; Crowley and Zemlan, 1981). The lordosis reflex is often used as a reliable method to quantify female sexual behavior.

Both estrogen and progesterone are involved in controlling female sexual behavior (Whalen, 1974; McEwen and Parsons, 1982). It has been previously demonstrated that OVX rats trated with both estrogen and progesterone display maximal lordotic responding (Hardy and Debold, 1971). The mechanism of this regulation is presumably through feedback of these compounds at hypothalamic levels. Estrogen and progesterone have been shown to have high affinity binding sites in the hypothalamus (McEwen, 1980; Pfaff and McEwen, 1983) and are able to induce biochemical events subsequent to binding (McEwen and Parsons, 1982). In the latter studies, implantation of estradiol into the ventromedial hypothalamic nucleus, an area kown to accumulate estrogen (McEwen, 1980), has been reported to induce lordosis behavior in rats (Davis et al., 1979; Rubin and Barfield, 1980). Implantation of estradiol into nuclei other than the VMN in the hypothalamus did not result in the facilitation of lordosis. The facilitation of lordosis by estrogen and progesterone is thought to occur by modification of neurotransmitter metabolism, primarily the MA's, within the hypothalamus (Crowley and Zemlan, 1981).

There is increasing evidence for the role of 5-HT in mediating sexual behavior in the female rat. Indirect evidence that 5-HT tonically inhibits lordosis comes from studies of systemically administered drugs. MAO inhibitors and antidepressants (such as

imipramine, Figure 3), which increase 5-HT, decrease the lordotic response (Meyerson, 1964; Meyerson,1966). Although these drugs also increase CA levels, serotonin levels have been shown to correlate better with behavioral changes. Lordosis was facilitated in estrogen-treated OVX rats in which 5-HT biosynthesis was inhibted by PCPA administration. (Meyerson and Lewander, 1970; Everitt et al., 1974). Zemlan et al.(1973) administered 5-HT antagonists, such as methysergide (Figure 3) systemically as well as by implanting them directly into the hypothlamus. Both treatments significantly enhanced lordosis behavior in estrogen-treated OVX rats.

The administration of fenfluramine, a drug which causes release of 5-HT (Figure 3) lowered estrogen-progesterone induced receptivity in female rats (Everitt et al.,1974). Intravenous administration of MAO inhibitors, clorgyline and deprenyl, inhibited lordosis in estrogen-progesterone primed OVX rats (Luine and Paden, 1982). In addition, in the latter study, it was demonstrated that the greater the preoptic-hypothalamic 5-HT levels, the lower the lordosis response. Implantation of pargyline crystals into the ventromedial nucleus (VMN) or DMN of the hypothalamus inhibited lordotic behavior in estrogen-progesterone OVX rats (Luine and Fishette, 1982). This effect was seen 5-7 h after implantation into the VMN and 29-31 h after implantation into the DMN. Taken together these data suggest that the both the VMN and 5-HT play an important role in modulating lordosis behavior in the female rat.

IV. Experimental Design and Rationale

In the present study the hypothalamic 5-HT system was manipulated

by intracerebral injection of 5,7-DHT in order to:

A) further characterize the group of 5-HT-immunoreactive cells in the DMN of the hypothalamus that had been observed in preliminary immunocytochemical studies. In order to assess whether these cells were sensitive to either 5,7-DHT or 6-OHDA, injections of these neurotoxins were made into the dorsolateral hypothalamus and the cells subsequently immunostained for 5-HT. In addition, the possibility that these cells were sequestering 5-HT was considered and adjacent 5-HT terminals removed by injection of 5,7-DHT into the rostral midbrain.

B) ascertain the time course of degeneration and regeneration of 5-HT fibers in the hypothalamus. Unilateral intracerebral injections of 5,7-DHT were made into the hypothalamus in order to induce specific and localized denervation. This allowed for comparison between ipsilateral and contralateral hypothalami in 5,7-DHT animals as well as between 5,7-DHT animals and sham injected controls. Patterns and density of innervation were studied with 5-HT immunocytochemistry. The functional status of 5-HT fibers was determined by specific ^{3}H5-HT uptake and 5-HT levels.

C) assess the effect of 5,7-DHT on lordosis behavior since there was increasing evidence for hypothalamic 5-HT inhibiting lordosis behavior. In the first of these experiments, bilateral 5,7-DHT injections were made and lordosis behavior examined on subsequent days. In the second of these experiments, fetal raphe neurons were transplanted into the hypothalamus in an attempt to reverse the facilitation observed after 5,7-DHT. Determination of 5-HT depletion was done by specific uptake of ^{3}H5-HT into hypothalamic

synaptosomes and 5-HT immunocytochemistry at the end of behavior testing. Examination of lordosis behavior after intrahypothalamic 5,7-DHT also provided a functional parameter to complement the degeneration and regeneration studies.

MATERIALS AND METHODS

I. Drugs

Pargyline hydrochloride, 5-hydroxytryptamine creatinine sulfate, dopamine, norepinephrine, tryptamine, tryptophan, 5,7-dihydroxytryptamine creatinine sulfate, 6-hydroxydopamine hydrochloride, dihydroxybenzylamine, and colchicine were obtained from the Sigma Chemical Co., St. Louis, MO. Other drugs were purchased from the following companies: fluoxitene, Eli Lilly and Co., Indianapolis, IN; Ketalar (Ketamine hydrochoride), Parke Davis, Detroit MI; Rompun (Xylazine), Haver Lockhardt, Shawnee KA; Desipramine hydrochoride, Merrill Labs, Cincinnati, OH; Neosporin Aerosol, Burroughs Wellcome, Research Triange Park, NC.

II. Animals

Male albino rats (Sprague Dawley) weighing 200-220 g at the time of surgery were purchased from Perfection Breeders, Douglassville, PA. Female albino rats (220-250 g) were obtained from Charles River, Wilmington, MA. Pregnant rats (350 g, Sprague Dawley) were obtained from Zivic Miller, Allison Park, PA. Prior to surgery animals were housed 3-5 to a cage. Following surgery animals were housed individually in a temperature-controlled room with a 12h lights on/off cycle. Purina rat chow and water were available ad libitum.

III. Stereotaxic Surgery

A. Neurotoxin Injections

To determine the coordinates used for intracerebral stereotaxic

injections, trial injections of the dye, trypan blue, were made into the particular brain area. Following intracerebral trypan blue injection, animals were perfused intracardially with 10% formalin for 30 min. Brains were removed, postfixed in the same fixative for 3 h and 100 um sections cut on a freezing microtome. Sections were mounted onto glass slides and stained with cresyl violet for microscopic examination.

For intracerebral injections of 5,7-DHT, rats received an intraperitoneal (i.p.) injection of 10 mg/kg desipramine HCl (DMI) in 1.0 ml 0.9% sterile saline 45 min prior to surgery. DMI inhibits the uptake of 5,7-DHT into NE fibers and therefore 5,7-DHT injection results in damage to 5-HT neurons only. Rats were anesthetized with 30 mg/kg Ketalar i.p. followed 5 min later with an intramuscular (i.m.) injection of 12 mg/kg Rompun.

The skin overlying the cranium was shaved free of fur and the animal placed in a stereotaxic apparatus with earbars positioned to perforate the animal's eardrums and the incisor bar set to 3.2 mm below the interaural line. An incision was made in the skin after swabbing with alcohol. A surgical scalpel was used to remove the underlying fascia. With the aid of an operating microscope, an opening in the skull was made using a dental drill. The dura was then pierced with a fine syringe tip.

For the injection of neurotoxins, a hand driven glass micropipette (diameter 70-100 um) was used (Figure 5). Neurotoxins were made up in 0.9% saline containing 0.2 mg/ml ascorbic acid. Three ug 5,7-DHT (free base) in 250 nl was injected into the dorsolateral hypothalamus

(coordinates from lambda suture: 4.5 mm anterior, 0.6 mm lateral and 8.5 mm ventral, Figure 6). The 5,7-DHT solution was injected at the rate of 50 nl/min for 5 min and the micropipette left in place for another 3 min. For sham injections, 250 nl of vehicle solution was injected into the dorsolateral hypothalamus over the same period of time. Three ug (free base) of 6-OHDA was injected into the dorsolateral hypothalamus as described above for 5,7-DHT with the exception that the animals did not receive DMI pretreatment.

Figure 5: Diagram of the micropipette system used to deliver nl quantities of neurotoxic drugs. Pipette tip = 50-80 um.

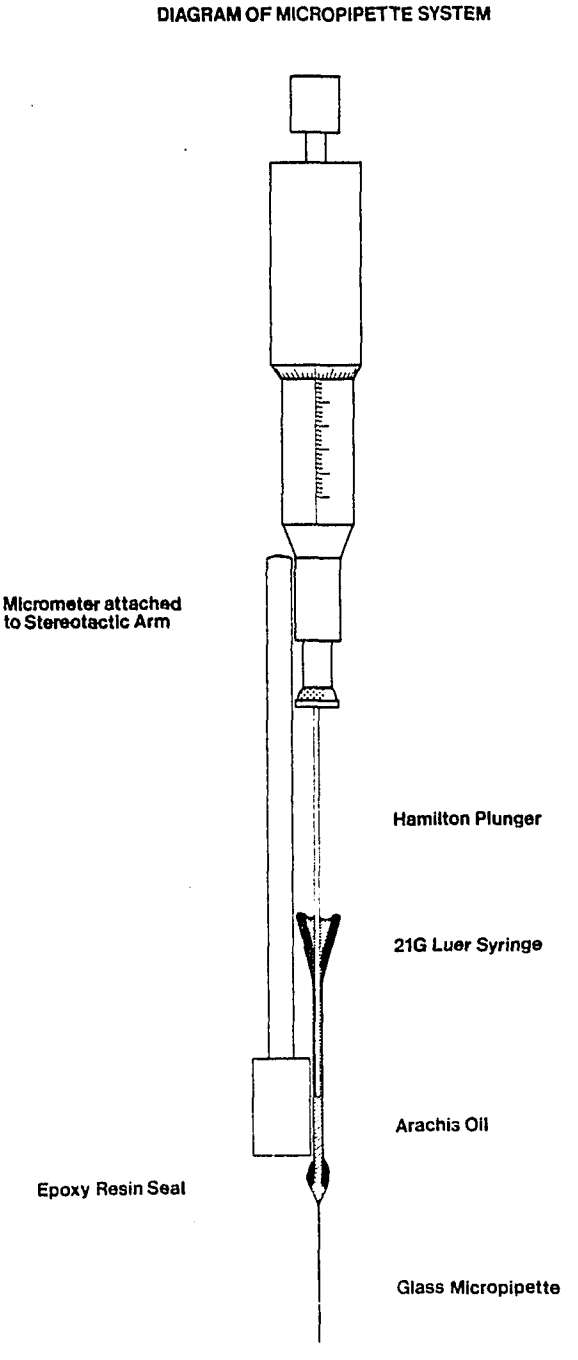
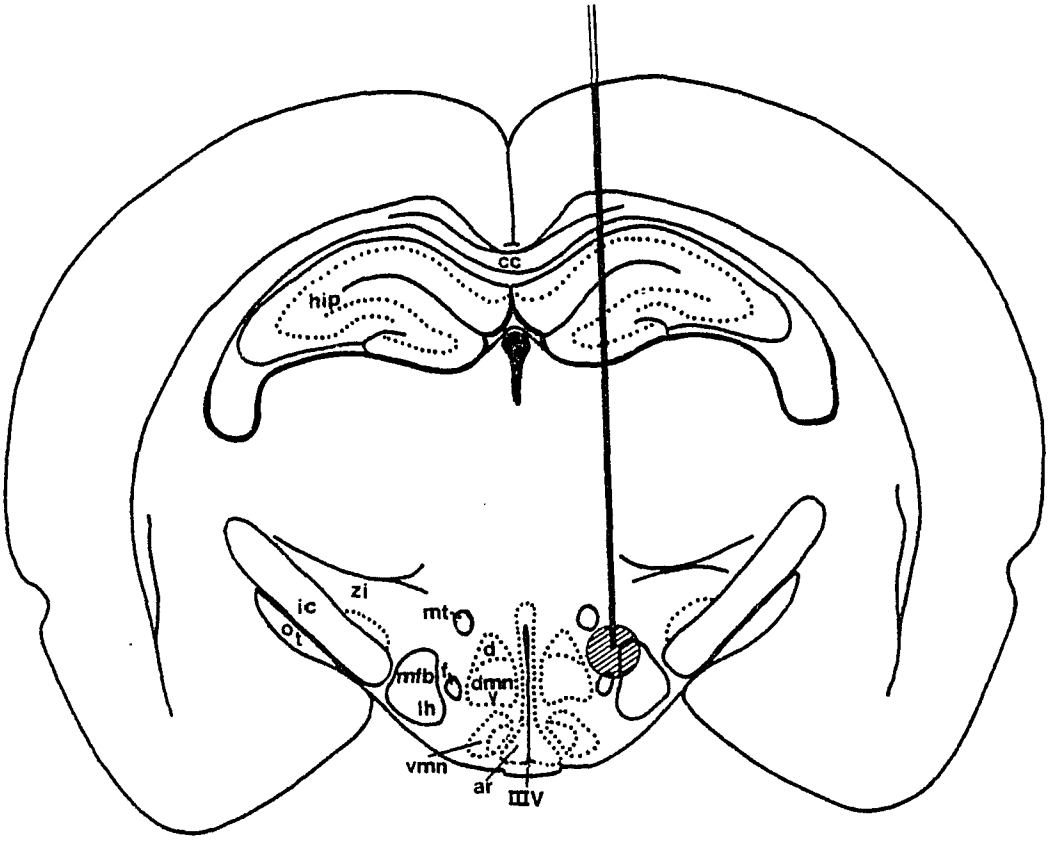


Figure 6: Diagram of injection site in the rat dorsolateral hypothalamus. ot, optic tract; ic, internal capsule; zi, zona incerta; mfb, medial forebrain bundle; lh, lateral hypothalamic area; mt, mamillothalamic tract; f, fornix; dmn, dorsomedial nucleus, pars ventralis (v) and dorsalis (d); vmn, ventromedial nucleus; ar, arcuate nucleus; IIIv, third ventricle. Adapted from Konig and Klippel (1963).



Unilateral and bilateral 5,7-DHT injections were made into the rostral midbrain (coordinates from lambda suture: 1.8 mm anterior, 1.6 mm lateral and 7.8 mm ventral). These animals were injected with 5 ug 5,7-DHT (free base) in 400 nl at the rate of 50 nl/min for 8 minutes and the micropipette was left in place for an additional 3 min.

Five ug of 5,7-DHT (free base) in 400 nl, at the rate of 50 nl/min, were injected bilaterally into the dorsolateral hypothalamus of ovariectomized (7 days prior to stereotaxic surgery) female rats used for the lordosis study. Coordinates used were the same as those for unilateral injection into the dorsolateral hypothalamus. Sham injected animals received injections of vehicle over the same time period.

Animals were ovariectomized one week prior to neurotoxin injection. Silastic capsules containing 10% estrogen/90% cholesterol were implanted one week after 5,7-DHT and lordosis behavior was assessed on subsequent days. Ovariectomies, silastic capsule implantation and behavior testing were done by Dr. V.N. Luine at the Rockefeller University.

B) Transplanation of Fetal Raphe Tissue

Fetal raphe tissue was transplanted bilaterally into the hypothalami of rats that had been bilaterally denervated (of 5-HT fibers) 6 days earlier in an attempt to reverse the effect observed following 5,7-DHT injection. Transplantation of fetal raphe tissue was done according to the method of Azmitia et al., (1981). Pregnant animals (14-16 days gestation) were killed by decapitation after being anesthetized with ether and their placentae placed in a petri dish on

ice. Brains were removed to sterile ice-cold Hanks balanced salt solution (GIBCO,NY) containing 1% glucose. Under a dissecting microscope, a strip of tissue containing the raphe nuclei was dissected from between the mesencephalic and pontine flexures and minced into small pieces. A suspension of raphe pieces was drawn up into a glass pipette (tip diameter 300 um) and 1 to 2 ul of this suspension injected stereotaxically into the dorsolateral hypothalamus (as above) or ventrolateral hypothalamus (coordinates from lambda suture: 4.5 mm anterior, 0.7 mm lateral and 9.1 mm ventral) at the rate of 200nl/min. Animals were then treated as described above for lordosis behavior testing.

IV. 5-HT Immunocytochemistry

A. 5-HT Antiserum

The 5-HT antiserum used in this study was the generous gift of Dr. J. Lauder. It was prepared according to the method of Wallace et al. (1982). 5-HT creatinine sulfate (Sigma Chemicals) was dissolved in 1.2 M acetate buffer at a concentration of 6.7 mg/ml. Limulus hemocyanin (HC, horseshoe crab-type VIII, Sigma Chemicals) was dissolved separately in the same buffer at a concentration of 20 mg/ml. The 5-HT and HC solutions were mixed with a 3% formaldehyde solution (1:1:2). This reaction mixture was placed overnight on a shaker at room temperature and then dialyzed against distilled water for 3 days at $4^{o}C$, changing the water dialy. Following dialysis, phosphate buffered saline (PBS, pH 7.4) was added to the dialysate so that the final volume contained a concentration of 5-HT-HC equivalent to 2 mg/ml. One ml of this mixture was combined with 2.5 ml complete

Freund's adjuvant and brought to a final volume of 4 ml with PBS. The HC conjugate was then emulsified and injected subcutaneously at 3-4 sites on the back of a rabbit. This procedure was repeated two additional times at one month intervals and blood was collected from an ear vein 7 days after the third injection. Blood was kept in an incubator for 1h at 37°C and then stored overnight at 4°C. The serum was separated by filtration and stored frozen (-20°C).

B) Drug Pretreatment

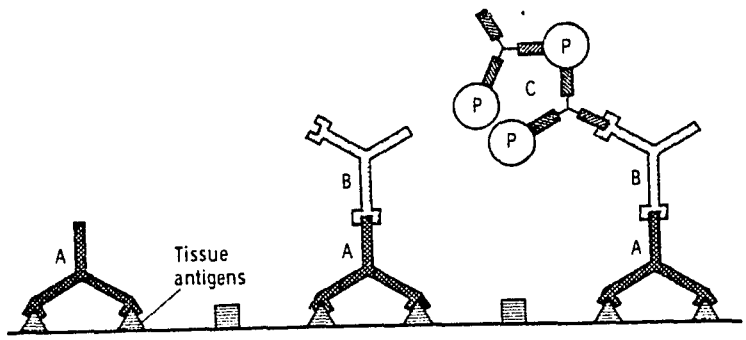
As endogenous levels of 5-HT levels are low in fibers, several pretreatments were used to enhance staining of 5-HT fibers (Figure 2). Pargyline (200 mg/kg) which inhibits MAO, was injected two hours prior to perfusion. Pargyline (200 mg/kg) and L-tryptophan (200 mg/kg), the precursor to 5-HT, were injected i.p. 1.5 and 1 h, respectively, prior to perfusion. Colchicine (50 or 100 ug) a drug which inhibits axoplasmic transport, was injected into the third ventricle (coodinates from bregma suture: 1.5 mm posterior, 0.0 lateral and 8.5 mm ventral) 24 or 48 h prior to perfusion. Rats that received no prior treatment were also used.

To assess the effect of DMI on pargyline and L-tryptohan pretreatment DMI (1mg/kg or 10mg/kg) or 0.9% saline was injected i.p. into rats 7 days prior to perfusion for 5-HT immunocytochemistry.

C) Immunocytochemical Procedure

5-HT immunocytochemistry was done using the indirect antibody enzyme technique (Figure 7) of Sternberger et al. (1970) with minor modifications.

Figure 7: Diagram of the peroxidase anti-peroxidase (PAP) immunocytochemical method. A. Tissue is incubated with rabbit antiserum directed against antigen (5-HT). B. Anti-rabbit IgG produced in sheep or goat. C. rabbit PAP. Adapted from Pickel (1981).



In establishing the technique of 5-HT immunocytochemistry in this laboratory the following variables were tested using sections of midbrain containing the dorsal raphe nucleus: 1) time of post fixation, 2 or 18 h; 2) different concentrations of 5-HT antiserum (1/500, 1/1000, 1/1500, and 1/2000) for either 18 or 48 h; 3) staining obtained with TBS buffer and PBS buffer; 4) 0.06% protease incubation prior to 5-HT antibody incubation; 5) 0.1% and 0.2% Triton X-100 in all antibody reagents; 6) diaminobenzidine hydrochloride (DAB) reaction for 1,2,5 and 10 min.

Rats were killed by transcardiac perfusion 3,5,7,12,19,30 and 50 days after 5,7-DHT injection. Animals were anesthetized with ether and perfused intracardially for 5 min with ice cold 0.9% saline containing 0.1% $MgSO_4$ followed by ice cold 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) containing $MgSO_4$ for 30 min. The brains were removed, postfixed in the same fixative for 18 h at 4°C and then rinsed several times in 0.1M phoshate buffer containing 0.9% saline (PBS pH 7.4).

A hypothalamic block was prepared by making cuts at the optic chiasm rostrally, the interpeduncular fossa caudally, the hypothalamic sulci laterally and the lateral ventricle dorsally. The midbrain block consisted of the area between the superior and inferior colliculi dorsally and the rostral edge of the pons and the interpeduncular fossa ventrally.

Serial sections of the midbrain and hypothalamus were cut in the coronal plane on a vibratome (Oxford Instruments) and collected in ice cold PBS. Sections were rinsed 3 times (5 min per rinse) in ice cold

PBS, 3 times in ice cold 0.1M Tris buffer containing 0.9% saline (TBS, pH 7.4) and then incubated in the following sequence: (1) 18 h in 5-HT antiserum (1:2000) at 4°C; (2) 30 min in sheep anti-rabbit antiserum (1:100, Antibodies Inc., Davis, CA) at room temperature; (3) 60 min in rabbit peroxidase anti-peroxidase (1:100, Miles, Elkhart,IN) at room temperature; (4) 5 min in 0.05% DAB in TBS containing 0.006% H_2O_2 (prepared from 3% stock solution). DAB was made and filtered immediately before use. All antibody reagents were made up in TBS containing 1.0% normal sheep serum (Antibodies, Inc.) and 0.2% Triton X-100. Between incubations sections were rinsed 3 times (5 min per rinse) in TBS. Following the immunocytochemical procedure, sections were mounted on glass slides from a water bath containing albumin and dried in a 45°C oven for 18 h. Slides were counterstained with methyl green, coverslipped and viewed with a Leitz Orthoplan microscope under bright and darkfield illumination.

To demonstrate specificity, sections of midbrain containing the dorsal raphe nucleus were incubated with 5-HT antiserum (1:2000) that had been preabsorbed with various concentrations of 5-HT, NE, and DA and tryptamine (10^{-5}-10^{-2}M). An aliquot of antiserum was incubated with the given antigen for 18 h, the mixture was then centrifuged at 14,000 x g for 15 min (Sorvall RC2-B, Norwalk, CN) and the supernatant used to stain the sections. Areas known to contain dopaminergic cells, such as the substantia nigra and the hypothalamic arcuate nucleus were also used as 5-HT immunostaining controls.

D) Photography

A Leitz light microscope with 2.5x, 6.3x, 10x, 25x, and 63x

planapochromatic objective lenses was used with a 35 mm photographic attachment and Panatomic-X film (32 ASA, fine grain panchromatic). All black and white photographs were printed on Ilfospeed paper using Dektol (Kodak) as developer and fixed with Kodak Rapid Fixer.

V. Synaptosomal 5-HT Reuptake

Serotonin fibers have a high affinity reuptake mechanism for 5-HT. Therefore it is possible to assess the number of viable 5-HT terminals in a given area by incubating synaptosomes, which are pinched off nerve terminal endings, with low concentrations of ^{3}H5-HT. Intracerebral injections of 5,7-DHT were performed as described and animals were killed by 7, 30 and 50 days post lesion. The specific high affinity uptake into hypothalamic synaptosomes was determined according to the method of Azmitia et al.(1983).

A) Preparation of Synaptosomes

Rats were killed by decapitation between 8:00 and 10:00 h and the brains placed in ice cold 0.32M sucrose. Left and right hypothalami were dissected (from the optic chiasm rostrally to mammillary bodies caudally) and homogenized using a glass-teflon homogenizer (clearance 0.1mm to .15mm) in 1 ml 0.32M sucrose at 1000 rpm. Homogenates were centrifuged (all centrifugations performed in a Sorvall RC2-B centrifuge, Sorvall, Hamden,CT) at 1000 x g for 10 min at 2°C, the supernatants decanted and saved and the pellets resuspended in 1 ml 0.32M sucrose and centrifuged again as above. Supernatants from the two centrifugations were combined and centrifuged at 14,000 x g for 15 min at 2°C. The resulting pellets (P_2) were used for the

actual uptake. This fraction, the crude mitochondrial fraction, has previously been shown to contain synaptosomes as well as mitochondria and pieces of myelin (Whittaker, 1965; Kuhar et al., 1972).

B) ^{3}H5-HT Uptake into Hypothalamic Synaptosomes

Uptake was performed in modified Krebs Ringer bicarbonate buffer (KR buffer) pH 7.4, which consisted of 114 mM NaCl, 4.6 mM KCl, 1.2 mM $CaCl_2 \cdot 6H_2O$, 1.2 mM KH_2PO_4, 1.14 mM $MgSO_4 \cdot 7H_2O$. A solution of neutralized $NaCO_3$ was added to this (28.7 mM final concentration) and the resulting solution was gassed for 20 min with 95% O_2, 5% CO_2 while on ice. Finally, pargyline, ascorbic acid and dextrose (10^{-4}M, 10^{-3}M, 10^{-2}M final concentration, respectively) were added.

The P_2 fraction was resuspended in 10 volumes (original wet weight) modified KR buffer. Synaptosomal uptake was performed in triplicate in multi-well tissue culture plates (Linbro, Hamden, CT) containing 285 ul KR buffer, 15 ul P_2 suspension and 5 x 10^{-8}M ^{3}H5-HT (final concentration, New England Nuclear, Boston,MA specific activity 22-33 Ci/mmol) at 37^{o}C for 3 min. Nonspecific uptake for each sample was determined by incubating triplicate aliquots as described above in the presence of 10^{-5}M unlabelled 5-HT. The reaction was terminated by filtering the incubation medium through Whatman filter paper (GF-B) and washing for 15 sec with ice cold PBS using a Titertek cell harvester (Flow Laboratories, Rockville,MD). Filters were dried in a 45^{o}C oven for 30 min, cut into scintillation vials containing 10 ml of scintillation fluid (Econofluor, New England Nuclear) and counted for 5 min in a Beckman

Scintillation counter (Beckmann Instruments). Counting efficiency of the scintillation fluid was determined to be 50%. The uptake was expressed as pmol/gram wet weight/3 min by the following calculation:

Counts per minute (CPM) x efficiency = disintegrations per minute (DPM); DPM ÷ 2.22 x 10^{12} disintegrations per curie ÷ specific activity ÷ tissue weight = pmol/gram

C) Specifity of 5-HT uptake into rat hypothalamic synaptosomes

Specificity of 5-HT uptake was ascertained by incubating 5 x 10^{-8}M ^{3}H5-HT in the presence of various concentrations of unlabelled 5-HT (10^{-10}M to 10^{-3}M). 5-HT uptake was also performed in the presence of the 5-HT uptake inhibitor fluoxitene (concentration of 10^{-7}M to 10^{-3}M). This compound has previously been shown to be a highly selective 5-HT uptake inhibitor. (Wong et al., 1973). High affinity uptake of both ^{3}H5-HT and ^{3}H3-NE (both used at a final concentration of 5 x 10^{-8}M) was done in rat hypothalami that had been used in the lordosis experiment.

Linearity with concentration was assessed by incubating hypothalamic synaptosomes with 5,10,15 and 20 ul aliquots of P_2 suspension. The effect of time (0.5 to 3 min) on uptake of ^{3}H5-HT was also assessed.

VI. 5-HT Levels, High Performance Liquid Chromatogragphy

Animals were lesioned as described above and killed by decapitation (between 8:00-10:00 h) 7,30 and 50 days post lesion.

Brains were removed, placed in ice cold 0.9% saline and the left and right hypothalami dissected as for synaptosomal uptake and immediately frozen on dry ice. Tissue was stored at -70°C until 5-HT determinations were carried out. Tissue was never frozen more than 30 days, as it has been determined that 5-HT levels are stable only up to this time (Wagner et al., 1982). 5-HT levels were measured by a modification of the method of Zaczek and Coyle (1982) using high performance liquid chromatography (HPLC) with electrochemical detection in the laboratory of Dr. V.N. Luine with the aid of Dr. K. Renner at the Rockefeller University.

A model 6000-A-HPLC pump in conjunction with a model 710-B automatic injector and a Waters Bondupac C-18 chromatographic column preceded by a guard column (Waters Associates, Waltham, MA) were used. An LC-4 electrochemical detector (Bioanalytical Systems, West Lafayette, IN) with a glassy carbon electrode (detector +0.55 volts; sensitivity: 1nA) was connected with a Waters 730 Data Module. The mobile phase consisted of 82.9 mM sodium acetate, 269 um EDTA and 4.5 mM heptone sulfonate (Eastman Kodak, Rochester,NY). The pH of this solution was adjusted to 3.5 using acetic acid and 20 mls of acetylnitrile were added. The solution was then filtered through a 0.2 um Millipore filter and degassed under vacuum. Serotonin standard was prepared as 0.5 uM solution in 0.1M perchloric acid.

Tissue was weighed while frozen, homogenized in 10 vol (frozen weight) 0.1M perchloric acid containing 0.5 uM dihydroxbenzylamine (internal standard). Homogenates were centrifuged at 16,000 x g for 15 min at 2°C. The supernatant was decanted into vials that were

placed in the automatic injector. No more than 12 samples were placed in the system at one time. Following every fifth sample the standard solution of 5-HT was injected. A volume of 20 ul of either sample or 5-HT standard was injected onto the column. The flow rate through the chromatographic column was 1ml/min.

Results were calculated from the peak heights corrected by an internal standard correction factor and are expressed as ng 5-HT/g weight.

VII. Statistical Analysis

Data were analyzed by three way analysis of variance (ANOVA) and levels of significance assessed by multiple t-tests. Data from the lordosis study were analyzed using the student's t test.

RESULTS AND DISCUSSION

Part I: Methodology

Results

A. Immunocytochemical Staining

Of the various concentrations of antibody used, it was found that good, clean specific staining with low background was obtained using the 5-HT antibody at 1/2000 for 18 h (Figure 8a). Higher concentrations of 5-HT antibody resulted in high background staining with no obvious increase in specific staining. There was no apparent difference in the staining obtained after 18h or 48h incubation. The 5-HT immunocytochemical reaction done in TBS buffer resulted in cleaner staining than that done in PBS buffer. Preincubation of sections in 0.06% protease resulted in improved staining however, the staining was never as good as that obtained when all antibody reagents were made up in 0.1 or 0.2 % Triton X-100. Of the various times of DAB exposure tested, 5 min (at room temperature) was found to be optimal. Shorter periods of time resulted in light staining whereas longer periods resulted in over-stained sections. Postfixation of tissue for 18 h (as opposed to 2 h) did not alter the staining obtained, but post fixation of tissue for 18h facilitated vibratome sectioning.

1) Specifity of 5-HT Immunocytochemical Reaction

Antibody specifity was assessed using sections of midbrain which included the dorsal raphe nucleus. Increasing concentrations of 5-HT (10^{-5}M to 10^{-2}M) resulted in decreased staining and no staining

was apparent above 10^{-3}M 5-HT in the dorsal raphe nucleus (Figure 8b). Tryptamine (10^{-3}M) also greatly decreased the staining observed in the dorsal raphe nucleus. DA reduced the intensity but not the number of stained cells in the dorsal raphe nucleus at high concentrations (Figure 8c). NE, at a concentration of 10^{-3}M decreased 5-HT staining somewhat but never abolished it. In the hypothalamus, 5-HT immunostaining of fibers and cells was abolished when sections were stained with 5-HT antiserum (1/2000) that had been preabsorbed with 10^{-2} 5-HT (Figure 9).

In the substantia nigra, which is known to contain the majority of dopamine cell bodies, there was moderate staining of cell bodies in animals that received both pargyline and L-tryptophan pretreatment. However, the intensity of staining was low and in no way resembled that observed in cells of the dorsal raphe nucleus. In the arcuate nucleus of the hypothalamus, where there are also dopaminergic cell bodies, no staining was observed.

2) Drug Pretreatment

In rats that did not receive any pretreatment, 5-HT immunostaining was apparent in 5-HT cell bodies and large fibers near the cell bodies. However, fibers in terminal areas were poorly stained. Pargyline pretreatment improved the staining of 5-HT fibers. The combination of pargyline and L-tryptophan pretreatment resulted in even better staining of 5-HT fibers throughout the brain. Under these conditions varicose fibers were clearly visualized throughout the brain. Intraventricular injections of colchicine markedly enhanced the staining of fibers throughout the brain. In these sections varicosites

on 5-HT fibers were bulbous and darkly staining.

Various concentrations of DMI and the injection of saline had no effect on the 5-HT immunostaining observed following pargyline and L-tryptophan pretreatment.

B) Synaptosomal Uptake

The high affinity uptake of ^{3}H5-HT into hypothalamic synaptosomes was found to be linear with respect to time between 1.0 and 3.0 min (Table I). Increasing concentrations of tissue (5-20 ul of P_2 suspension) resulted in a linear increase in high affinity uptake (Table II).

Specific uptake was never less than 50% of total uptake. The high affinity uptake of ^{3}H5-HT into hypothalamic synaptosomes was inhibited by increasing concentrations of unlabelled 5-HT (Table III). Above 10^{-5}M 5-HT, there was a 66% inhibition. Fluoxitene resulted in a marked inhibition of ^{3}H5-HT uptake into hypothalamic synaptosomes above a concentration of 10^{-6}M (Table IV).

Time (min)	pmol ^{3}H5-HT/g wet weight/3 min
0.5	48
1.0	49
2.0	99
3.0	142

Table I: Effect of time on the specific high affinity uptake of ^{3}H5-HT into rat hypothalamic synaptosomes. Results are mean of 6 determinations.

Concentration (ul)	pmol ^{3}H5-HT/g wet weight/ 3 min
5	22
10	55
15	74
20	119
40	334

Table II: Effect of concentration (P_2) on the specific high affinity uptake of ^{3}H5-HT into rat hypothalamic synaptosomes. Results are the mean of 3 determinations.

Concentration	Inhibition (%)
10^{-10}M	4.2
10^{-9}M	2.2
10^{-8}M	20.2
10^{-7}M	39.2
10^{-6}M	66.1
10^{-5}M	65.6
10^{-4}M	61.6

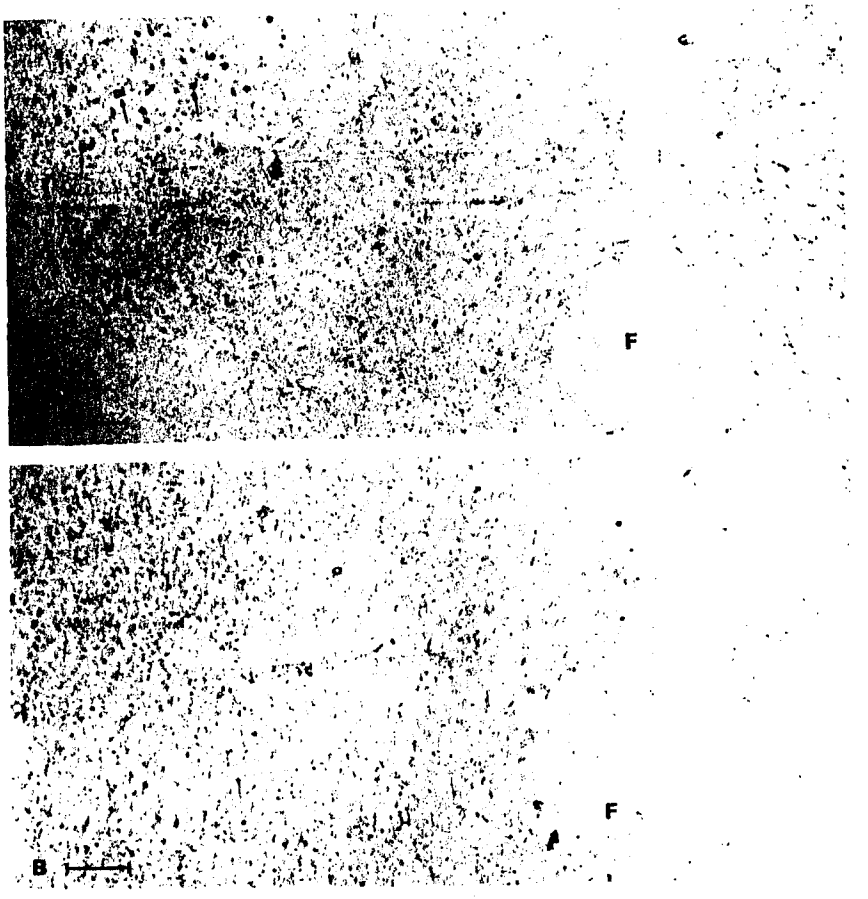
Table III: Effect of various concentrations of unlabelled 5-HT on the total uptake of ^{3}H5-HT into rat hypothalamic synaptosomes. Results are the mean of 3 determinations.

Concentration	Inhibition (%)
10^{-7}M	39
10^{-6}M	60
10^{-5}M	68
10^{-4}M	70.1
10^{-3}M	76.3

Table IV: Effect of various concentrations of fluoxitene on total ^{3}H5-HT uptake into rat hypothalamic synaptosomes. Results are the mean of 3 determinations.

Figure 8: Photomicrograph of serial coronal sections (50 um) of rat dorsal raphe nucleus immunostained for 5-HT. a) 5-HT antiserum (1/2000). b) 5-HT antiserum (1/2000) preabsorbed with 10^{-2}M 5-HT for 18 h at 4°C. c) 5-HT antiserum (1/2000) preabsorbed with 10^{-2}M DA for 18 h at 4°C. Scale bar = 100 um. IV, fourth ventricle; MLF, medial longitudinal fasciculus.

Figure 9: a) Photomicrograph of a 50 um coronal section of medial hypothalamus immunostained for 5-HT. Stained cells and fibers are present on the far left hand side. b) Section adjacent to (a) in which 5-HT antiserum used was preabsorbed with 10^{-2}M 5-HT for 18 h at 4^{o}C. Cells and fiber staining are no longer apparent. Scale bar = 100 um. F, fornix.



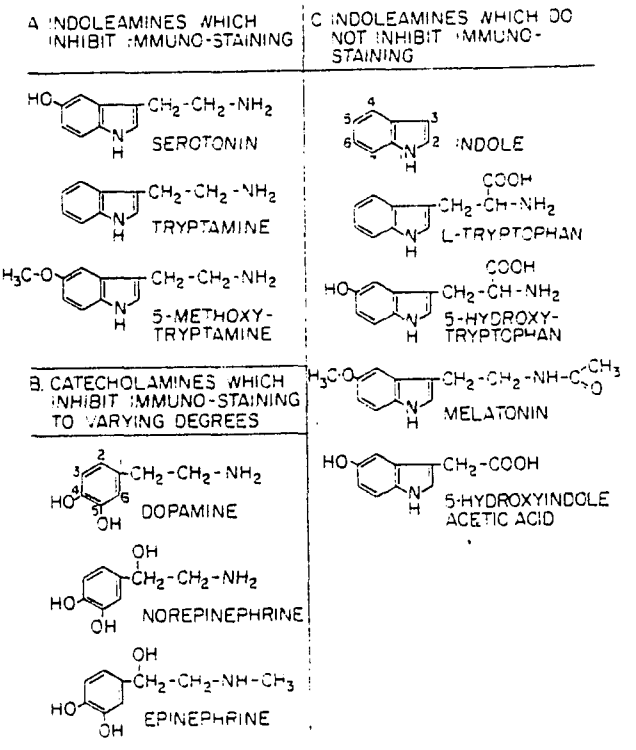
Discussion

Because 5-HT is a small molecule it is necessary to conjugate it to a protein in order to raise an antiserum to it. Formaldehyde has been used to conjugate 5-HT to bovine serum albumin (BSA) (Grota and Brown, 1974; Steinbusch et al., 1978) and to the invertebrate protein hemocyanin (HC) (Wallace et al.,1982). The 5-HT-HC antiserum has been shown to have a higher titre of 5-HT antibodies than the 5-HT-BSA antiserum (Wallace et al., 1982). This is probably because HC is more immunogenic than BSA in rabbits. Nevertheless, all the 5-HT antisera have similiar cross-reactivity characteristics. Both tryptamine and 5-methoxytryptamine show a high degree of cross-reactivity with the 5-HT-HC antiserum used in the present study, whereas DA only has a moderate cross-reactivity (Figure 10). This is in agreement with previous studies (Steinbusch et al, 1978). Recently, a monoclonal antibody has been made to 5-HT which has been shown to have similiar cross reactivity (Consolazione et al., 1981). Based on these studies it can be deduced that the major antigenic determinant on the 5-HT molecule involves binding to the side chain. This explains the cross reactivity with other indoleamines and DA which have the same side chain and the fact that increasing substitution on the side chain decreases cross reactivity (Wallace et al., 1982).

Although the 5-HT antiserum used in the present and previous studies appears specific for 5-HT it must be remembered that cross reactivity studies have been done with soluble antigens whereas in the tissue these antigens may be altered (as is 5-HT) by fixation. Recently, a method has been developed to test cross reactivity which

takes this into account. Schipper and Tilders (1983) have used gelatin molds in which the antigen has been mixed to test immunostaining of a 5-HT antiserum. The gelatin molds were "fixed" with formaldehyde, sliced and stained. It was found that the 5-HT antiserum had a higher affinity for 6-hydroxy-tetrohydro-B-carboline (6-OH-BC), which is known to be formed from formaldehyde and 5-HT (Corrodi and Jonsson, 1967) , than for 5-HT. Therefore, it appears that the antiserum was raised to 6-OH-BC rather than 5-HT. It is likely that fixation of 5-HT with formaldehyde causes the formation of 6-OH-BC which is linked to tissue proteins in a way that resembles the 5-HT-BSA or 5-HT-HC conjugate. If this is the case for all 5-HT antisera, then the specificity of the antiserum lies in localization of 6-OH-BC in brain. As Shipper and Tilders (1983) point out this compound has not been measured in the brain and therefore the 5-HT antisera can still be assumed to be relatively specific.

Figure 10: Structure of compounds tested in immunoabsorption experiments. A. totally inhibit. B. partially inhibit (dopamine > norepinephrine > epinephrine). C. do not inhibit. Adapted from Wallace et al. (1982).



Part II: 5-HT Immunostaining in the Hypothalamus

Results

Following pargyline and L-tryptophan pretreatment, there was moderate to heavy staining of fibers in all areas of the hypothalamus. Fibers were relatively thin with many varicosities along their length. Areas of particularly dense innervation included the suprachiasmatic nuclei, the periventricular hypothalamus and the lateral hypothalamus in the area of the MFB. 5-HT-immunoreactive (5-HT-IR) fibers could clearly be seen to extend into the third ventricle. The DMN, posterior hypothalamic nucleus and mammillary nuclei contained fewer fibers. In the median eminence fibers were observed in the internal and external layers. In the ventrolateral part of the DMN the 5-HT-IR fiber pattern differed from that seen in the MFB and other hypothalamic areas. These 5-HT-IR fibers and their varicosities were very fine (approximately one third the size of those seen elsewhere) and formed a dense fiber network around the cells in this part of the DMN. The ventromedial hypothalamic area contained relatively few 5-HT-IR fibers.

In addition to the fiber staining in the hypothalamus, a group of cell bodies were immunostained for 5-HT in animals that had been pretreated with both pargyline and L-tryptophan. These cells were not observed under any other conditions (colchicine or pargyline pretreatment). The majority of these cells were located adjacent to the third ventricle, primarily in the ventral part of the DMN (Figure 11). These 5-HT-IR cells, scattered among non-5-HT-IR cells, were about 9 um along their longitudinal axis and had a slightly ovoid shape. The nucleus filled most of the cell leaving a narrow band of

cytoplasm. Often a single process and occasionally 2 processes could be seen emanating from the long axis of the cell (Figure 12). The number of cells was estimated with the use of the following formula: total # of cells = # counted x tissue thickness/tissue thickness + cell diameter (Abercrombie, 1946). It was estimated that there were approximately 350 cells on each side of the third ventricle. The rostro-caudal extent of the cell group was about 900 um anterior to the inter-thalamic adhesion (Figure 13). Aside from this group, no other positively stained cells were seen in the hypothalamus. The thin fibers in the DMN were located ventrolateral to the 5-HT-IR cells in the DMN.

Figure 11: a) Photomicrograph of a 50 um coronal section of medial hypothalamus immunostained for 5-HT. Cells in the ventral part of the DMN are shown by arrows. Scale bar = 100 um. b) higher magnification of left side of (a). Scale bar = 50 um; V, third ventricle.

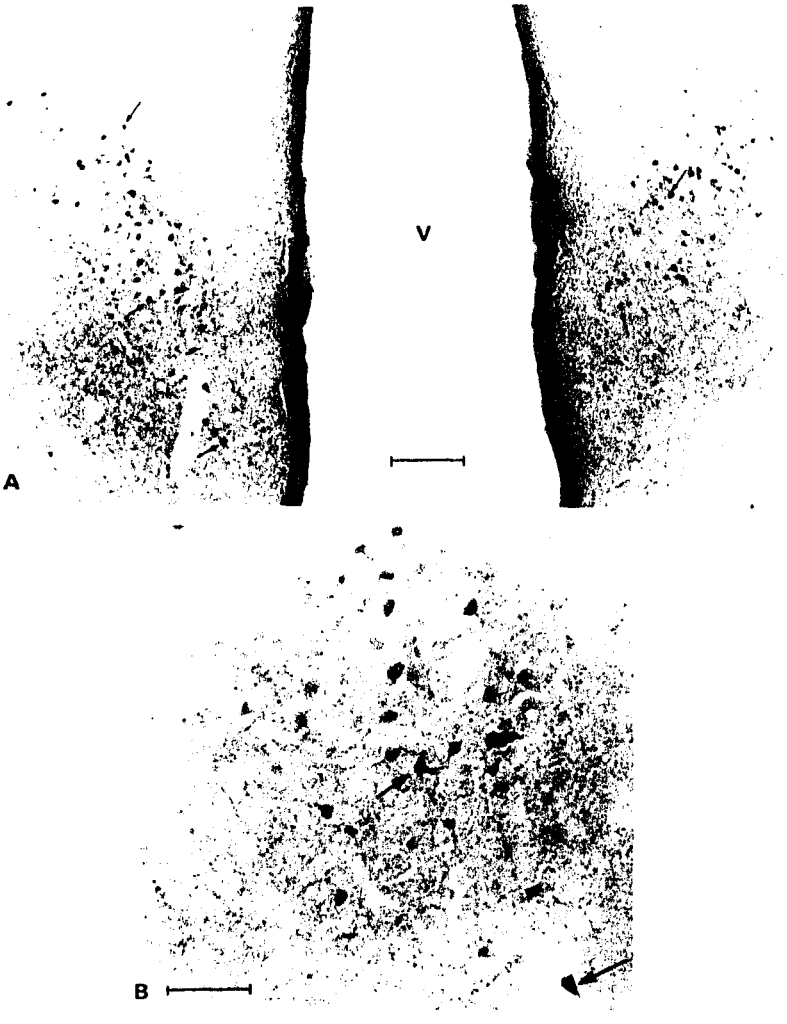


Figure 12: a and b) High power photomicrograph of the 5-HT-IR cells in the DMN of the hypothalamus. Note processes emanating from the cells (arrows). Scale bar = 10 um.

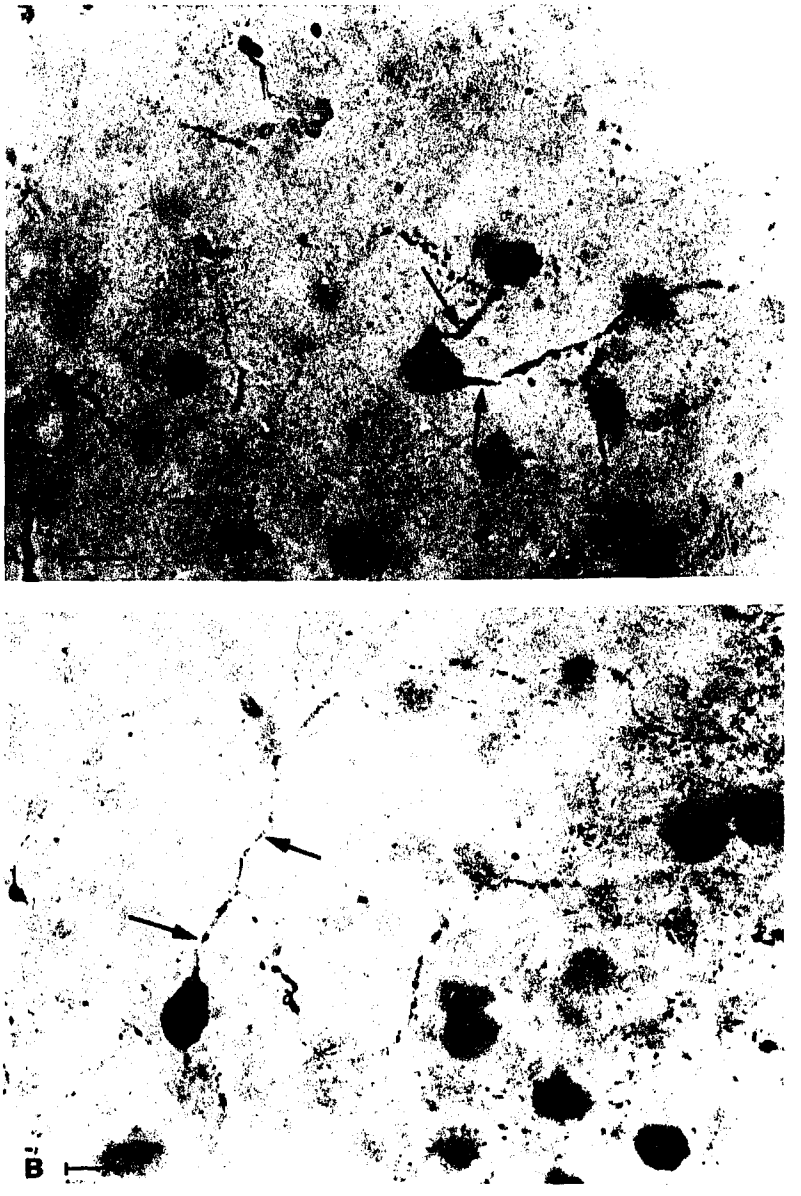
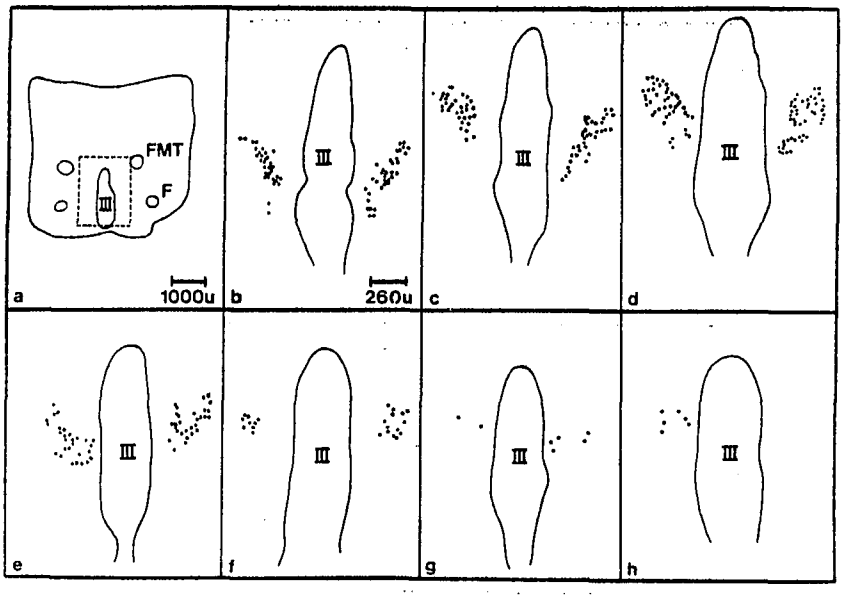


Figure 13: a) Diagram of a hypothalamic section midway between the suprachiasmatic nuclei and the interthalamic adhesion. Hatched area enlarged in b-h. b-h) 50 um coronal sections through medial hypothalamus (100 um apart) showing the rostro-caudal extent of the 5-HT-IR cells. III, third ventricle, FMT, mammillothalamic tract; F, fornix.



Discussion

In initial immunocytochemical studies it was found that many 5-HT-IR fibers were present throughout the hypothalamus. The distribution of 5-HT-IR fibers in the hypothalamus is in agreement with previous studies (Azmitia and Segal, 1978; Parent et al., 1981; Steinbusch, 1981). The presence of 5-HT fibers extending into the third ventricle has been noted in the past (Lorez and Richards, 1982).

In addition to the 5-HT-IR fibers in the hypothalamus, a group of 5-HT-IR cells was localized in the DMN. This result extends the finding of Beaudet and Descarries (1979) of a group of neurons in this area that selectively concentrates exogenous ^{3}H5-HT. The location, distribution, number and size of the 5-HT-IR cells in the DMN is similiar to the cells described by these workers. This finding may explain the observation that surgical isolation of the hypothalamus results in only a 50% decrease in the 5-HT and tryptophan hydroxylase content of the DMN (Brownstein, et al., 1976). Moreover, 5,7-DHT injections into the dorsal or median raphe nuclei deplete many hypothalamic nuclei of 5-HT with the exception of the DMN (van de Kar et al., 1980). The lack of positively stained cells in the DMN of rats that were untreated or colchicine-pretreated is in agreement with the study of Steinbusch and Nieuwenhuys (1979). Furthermore, Steinbusch and Verhofstad (1982) have recently reported the presence of 5-HT-IR cells in the DMN in nialamide and L-tryptophan treated rats.

The 5-HT-IR neurons in the DMN of the hypothalamus differ from 5-HT neurons in the brainstem raphe nuclei. The raphe cells are large (17-30 um), fusiform, multipolar and have a well developed cytoplasm

(Beaudet and Descarries, 1981). In contrast, the 5-HT-IR cells in the DMN are small, ovoid, uni- or bipolar, and have a small amount of cytoplasm. The general characteristics of the 5-HT-IR cells in the DMN are those of immature 5-HT neurons (Loizou, 1972; Lauder et al., 1982). The latter are, however, easily visualized by fluorescence histochemistry (Loizou, 1972) and immunocytochemistry (Lauder et al., 1982) without pargyline or tryptophan pretreatment.

Part III: Neurotoxin Induced Axotomy: Effect on 5-HT-IR Cells

Results

In order to establish if the 5-HT-IR cells in the DMN were sensitive to the 5-HT neurotoxin, injections of 5,7-DHT were made adjacent to the DMN. Within 5 days, the majority of 5-HT-IR fibers in the ipsilateral medial hypothalamus were no longer visible. The 5-HT-IR cells in the DMN were also not visible (Figure 14). In the contralateral hypothalamus the 5-HT-IR cells and the normal complement of 5-HT-IR fibers were stained. This pattern occurred throughout the rostro-caudal extent of the 5-HT-IR cell group.

To ascertain whether the 5-HT-IR cells in the DMN were sequestering 5-HT released from adjacent terminals, 5,7-DHT injections were made into the rostral midbrain to interrupt the raphe-hypothalamic fibers. This resulted in ipsilateral denervation 5,7 and 12 days post lesion. Degeneration was apparent on the injected side 5 and 7 days post lesion, in the MFB and in the region of the substantia nigra. In the hypothalamus, the loss of 5-HT-IR fibers was not apparent until 12 days post lesion. At this time there was a marked decrease in the number of 5-HT-IR fibers in both the medial and lateral ipsilateral hypothalamic areas. There were also some degenerating 5-HT fibers contralateral to the injection, scattered throughout medial and lateral areas. The 5-HT-IR cells in the DMN were clearly stained on both sides of the hypothalamus (Figure 15a). In addition the group of thin 5-HT-IR fibers ventrolateral to the cells was also present on both sides of the hypothalamus (Figure 15b).

It has been suggested that these cells are actually dopaminergic cells capable of taking up exogenous 5-HT (Steinbusch and Nieuwenhuys, 1979). If this were the case 6-OHDA, a CA neurotoxin, should destroy these cells. However, 5 days after unilateral injection of 6-OHDA into the dorsolateral hypothalamus the 5-HT immunostaining of the cells in the DMN and the fibers throughout the hypothalamus were unaffected.

Figure 14: Photomicrograph of a 50 um coronal section of medial hypothalamus immunostained for 5-HT 5 days after unilateral injection of 5,7-DHT (3 ug free base) into the dorsolateral hypothalamus. Side ipsilateral to the injection (right) contains few 5-HT fibers and cell bodies. On the contralateral side both 5-HT-IR cell bodies and fibers are present. Scale bar = 100 um. V, third ventricle.

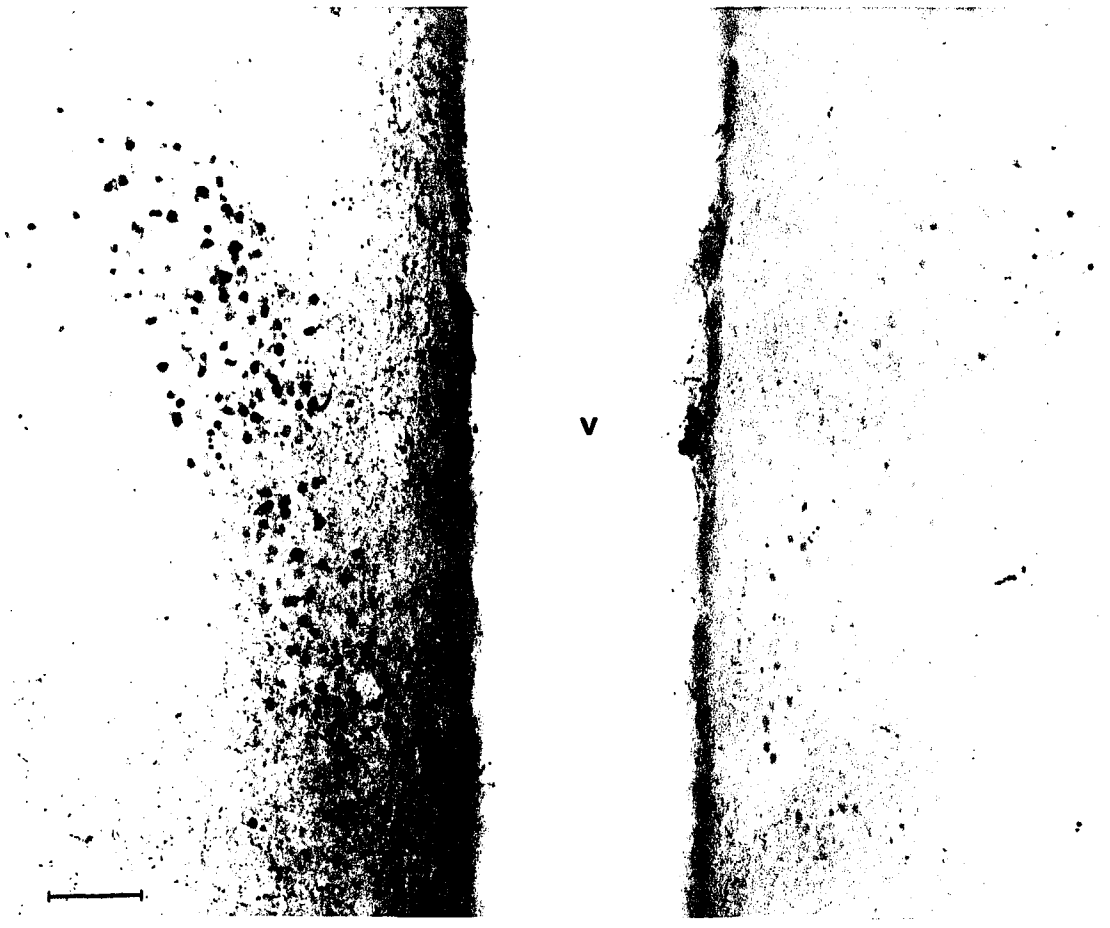


Figure 15: a) Photomicrograph of a 50 um coronal section of medial hypothalamus immunostained for 5-HT 12 days after unilateral injection of 5,7-DHT (5 ug free base) into the rostral midbrain. 5-HT-IR cell bodies are present on both the injected and control sides. b) same section photographed under dark-field illumination. Note that the side of the hypothalamus ipsilateral to the injection (right) contains relatively few fibers in the DMN but that there is a group of fine fibers in the ventrolateral DMN (hatched area). Scale bar = 100 um. V, third ventricle.

Discussion

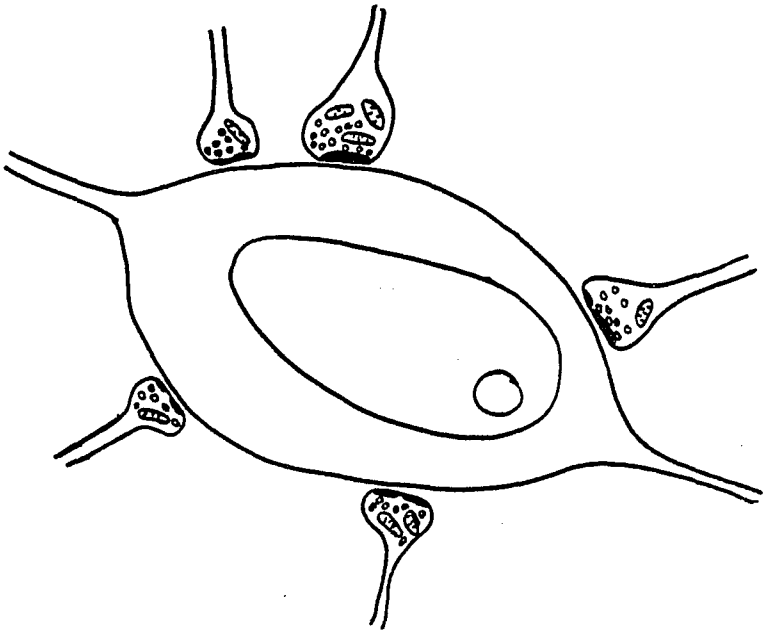
The effect of 5,7-DHT and 6-OHDA injections on the 5-HT-IR cells in the DMN provides further evidence for the serotonergic nature of this cell group. If the 5-HT-IR cells in the DMN are actually serotonergic they should be destroyed by 5,7-DHT, which is a selective 5-HT neurotoxin when used in conjunction with DMI (Baumgarten et al., 1977; Azmitia et al., 1978). As the 5-HT-IR cells in the DMN were no longer stained after 5,7-DHT it appears that the cells took up the toxin and were destroyed by it.

It has been demonstrated that dopaminergic neurons in the arcuate and periventricular nuclei of the hypothalamus can take up 5-HT by a low affinity uptake system (Lichtensteiger et al., 1967). In order to determine whether the 5-HT-IR cells were CA cells capable of taking up 5-HT, as has been suggested by Steinbusch and Nieuwenhuys (1979), 6-OHDA injections were made into the dorsolateral hypothalamus. Intracerebral injection of 8 ug/4 ul 6-OHDA into the left hypothalamus has been shown to result in an almost total lack of CA fluorescence ipsilateral to the injection (Understedt, 1971b). This neurotoxin had no effect on the 5-HT-immnuostaining of cells in the DMN, indicating that the cells are not catecholaminergic.

It has also been shown that certain cells which do not appear to be synthesizing 5-HT possess a specific, saturable, high affinity uptake system for ^{3}H5-HT. This is true for the anterior pituitary (Nunez et al., 1981; Johns et al.,1982) and the retina (Ehinger and Floren, 1980). Thus, it seemed possible that the 5-HT-IR cells in the DMN were sequestering 5-HT released from adjacent terminals (Figure

16). Therefore unilateral injections of 5,7-DHT were made into into the rostral midbrain in order to remove extra-hypothalamic 5-HT afferents to the DMN.

Figure 16: Drawing illustrating the possible relationship between the 5-HT-IR cells in the DMN and the 5-HT fibers adjacent to them. 5-HT terminals may synapse on 5-HT-IR cell bodies and release 5-HT (in vesicles, clear circles) which is sequestered by 5-HT-IR cell.



This resulted in a marked decrease in the number of 5-HT-IR fibers seen in the ipsilateral hypothalamus. However, the 5-HT-IR cells in the DMN and the small patch of fibers ventrolateral to these cells were unaffected. This suggests that the 5-HT-IR cells in the DMN are synthesizing 5-HT rather than sequestering it from adjacent 5-HT terminals. However, since the 5-HT-IR cells are only apparent after pargyline and tryptophan pretreatment it must be concluded that they contain low levels of 5-HT and that their synthesis of 5-HT may be limited by the availability of substrate.

From preliminary evidence, it appears that the 5-HT-IR cells in the DMN give rise to a group of fine fibers that extend ventrally and laterally. This patch of fibers was seen contralateral to the 5,7-DHT injection in the hypothalamus but never ipsilateral to it. Interruption of the ascending 5-HT afferents to the hypothalamus by the injection of 5,7-DHT into the rostral midbrain had no effect on this patch of fibers. Therefore, the patch of fibers ventrolateral to the 5-HT-IR cells in the DMN could arise from cells in the medial hypothalamus. This case would be similiar to the local innervation provided by the intrahypothalamic dopamine system decribed by Bjorklund et al. (1975). Another possiblity that cannot be overlooked is that this patch of fibers arises from a small projection from the midbrain raphe that is not damaged by the 5-HT injection into the rostral midbrain, such as the periventricular tract (Azmitia and Segal, 1978). However, as the cells in the DMN and the fibers ventrolateral to them take up intraventricularly administered ^{3}H5-HT after midbrain transection (A. Beaudet, personal communication) and 5-HT-IR fibers are not observed adjacent to the third ventricle after 5,7-DHT

injection into the rostral midbrain, this would appear unlikely.

Part IV: Neurotoxin Induced Axotomy: Degeneration of 5-HT Fibers

Results

The time course of 5-HT fiber degeneration in the hypothalamus was examined after unilateral injection of 5,7-DHT into the dorsolateral hypothalamus by 5-HT immunocytochemistry, specific high affinity uptake of ^{3}H5-HT into hypothalamic synaptosomes and 5-HT levels. Lordosis behavior after bilateral hypothalamic injections of 5,7-DHT was also assessed.

Within 3 days of the unilateral injection of 5,7-DHT into the dorsolateral hypothalamus there were signs of neurotoxin induced damage (Figure 17). Degenerating fibers appeared swollen and darkly stained for 5-HT in all hypothalamic areas ipsilateral to the injection. In the contralateral hypothalamus some degenerating fibers were evident in the medial areas, generally restricted to the areas around the fornix. Degeneration was apparent only very close to the injection site (300 um). Sham injections did not result in any obvious changes in 5-HT-IR staining.

In the ipsilateral hypothalamus there was a gradual decrease in the number of 5-HT-IR fibers that were present in the MFB following 5,7-DHT injection. Swollen proximal stumps of 5-HT fibers were numerous 3-12 days post lesion. Medial and periventricular hypothalamic areas contained essentially no 5-HT-IR fibers in the ipsilateral hypothalamus after 5 days (Figure 18,19). In the contralateral hypothalamus few changes were apparent.

Following unilateral injection of 5,7-DHT into the dorsolateral

hypothalamus there was no apparent decrease in the number of 5-HT stained cells in the dorsal and median raphe nuclei at any time post lesion. The fiber staining observed in the midbrain region was not decreased at any time post lesion.

From the morphological data it appeared that following 5,7-DHT injection into the dorsolateral hypothalamus there was marked degeneration in the ipsilateral hypothalamus and some degeneration in the contralateral hypothalamus. As high affinity synaptosomal uptake has previously been shown to correlate well with density of innervation (Jonsson, 1976), synaptosomal uptake of ^{3}H5-HT was performed in order to quantitate the decrease in 5-HT terminals. Seven days after unilateral injection of 5,7-DHT (3 ug free base) into the dorsolateral hypothalamus there was a significant decrease ($p < 0.001$) in both ipsilateral (23% of sham) and contralateral (53% of sham) sides of the hypothalamus with respect to sham (Table V, Figure 20). In the ipsilateral hypothalamus ^{3}H5-HT uptake was significantly lower than the contralateral hypothalamus ($p < 0.01$). It must be noted, however, that the high affinity uptake in this sham group (7 days post lesion) was significantly higher than the sham groups throughout the study ($p < 0.001$). When sham injected animals were compared to uninjected animals (7 days post lesion) there was no statistical difference between the two groups.

Serotonin levels were also determined 7 days after unilateral 5,7-DHT injection. Results are given in Table VI and Figure 21. At this time 5-HT levels were significantly decreased on both sides of the hypothalamus with respect to sham. In the ipsilateral hypothalamus

5-HT levels were 45% of sham ($p < 0.001$) and in the contralateral hypothalamus 5-HT levels were 69% of sham ($p < 0.01$). The ipsilateral hypothalamus was significantly lower than the contralateral hypothalamus ($p < 0.001$) and the sham groups were not significantly different over time.

A) Effect of 5,7-DHT on Lordosis

In order to assess the effect of hypothalamic 5-HT on lordosis behavior bilateral 5,7-DHT injections were made into the dorsolateral hypothalamus of OVX female rats. Lordosis quotients (L/Q = lordosis/mounts x 100) were determined from 3 days after 5,7-DHT injection.

Following bilateral injection of 5,7-DHT into the hypothalamus there was a relatively rapid facilitation of lordosis behavior in estrogen primed rats. Nine days post lesion, 5,7-DHT animals displayed a significantly higher L/Q ($p < 0.05$) than sham injected animals (Figure 22). Levels of response at 11 and 14 days post lesion were greater than 80, a level rarely seen unless progesterone is given in addition to estradiol. In sham injected animals the L/Q rarely exceeded 20. At the end of behavioral testing all rats were given 500 ug of progesterone subcutaneously and tested for L/Q 3-5 h later. This dose of progesterone in combination with the estrogen results in maximal lordosis responding (Hardy and Debold, 1971). All animals given this dose had L/Q of > 90 indicating that stereotaxic surgery did not damage brain areas necessary for lordosis. Rats were killed following the last behavioral test (25 days post lesion) and the high affinity specific uptake of ^{3}H5-HT and ^{3}H-NE into hypothalamic

synaptosomes was assessed. In animals that received 5,7-DHT, ^{3}H5-HT uptake was significantly decreased by 46% whereas ^{3}HNE uptake showed a non-significant decrease of 16% as compared to sham injected animals.

Figure 17: a) Photomicrograph of a 50 um coronal section of medial hypothalamus immunostained for 5-HT 3 days after the unilateral injection of 5,7-DHT (3 ug free base) into the dorsolateral hypothalamus. Ipsilateral to the injection (right) fibers in the medial and lateral hypothalamus are swollen and darkly stained. b and c) higher magnification of the contralateral and ipsilateral sides, respectively. V, third ventricle; F, fornix; MT, mammillothalamic tract.

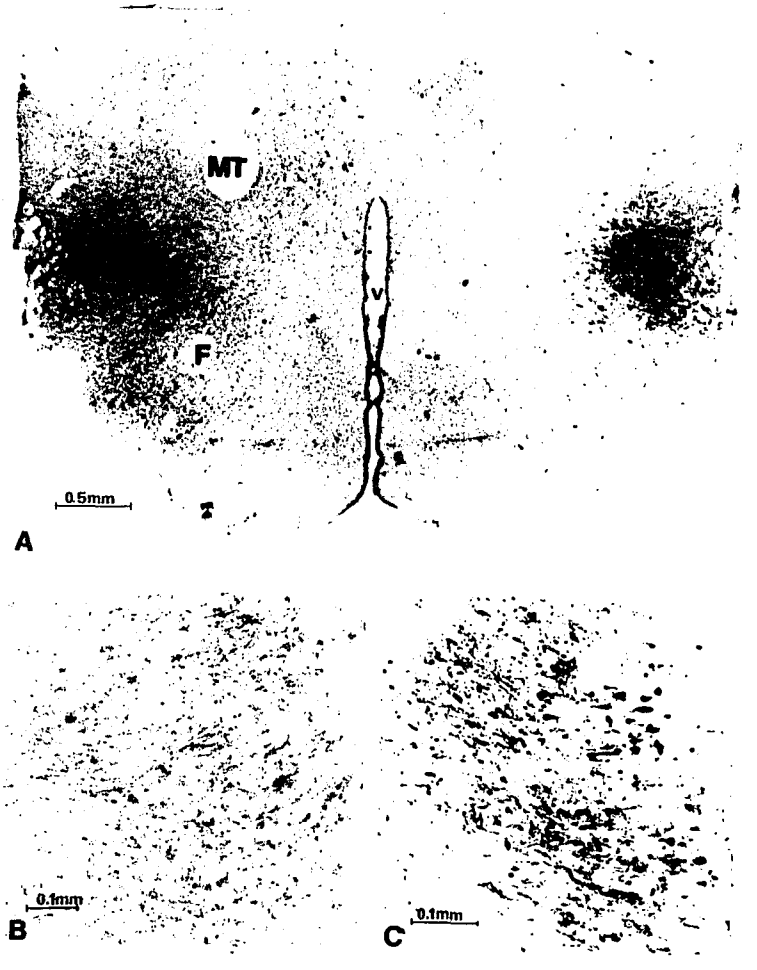


Figure 18: Photomicrograph of a 50 um coronal section of anterior hypothalamus 7 days after the unilateral injection of 5,7-DHT (3 ug free base) into the dorsolateral hypothalamus. Ipsilateral to the injection (left) there is a decrease in the density of swollen 5-HT-IR fibers in the MFB and virtually no 5-HT-IR fibers in the medial hypothalamus. Fibers are present in the medial and lateral contralateral hypothalamus. Scale bar = 500 um. F, fornix.

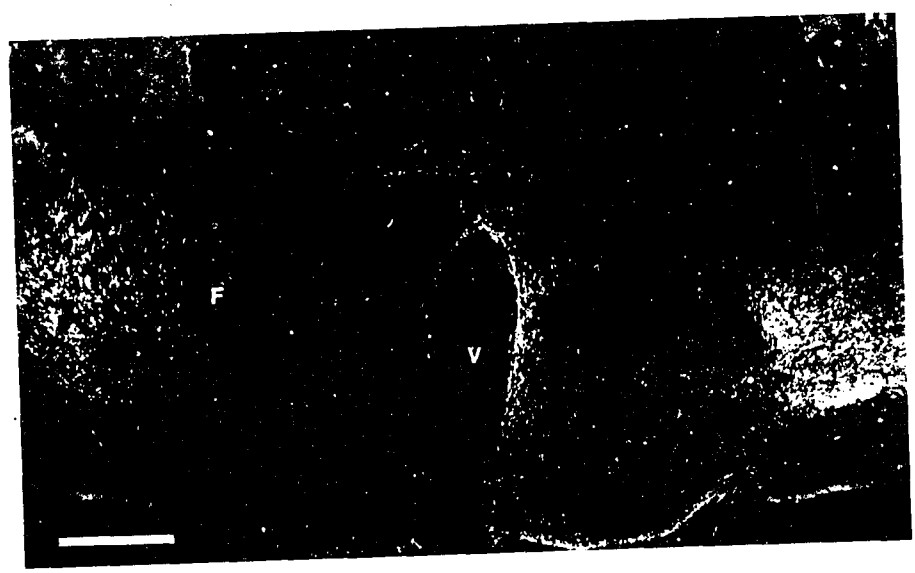
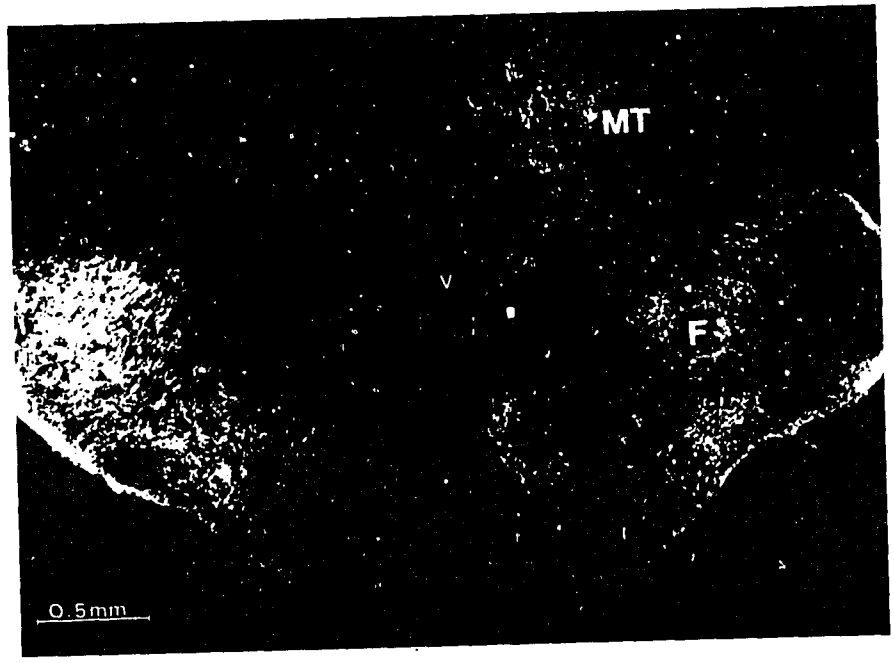


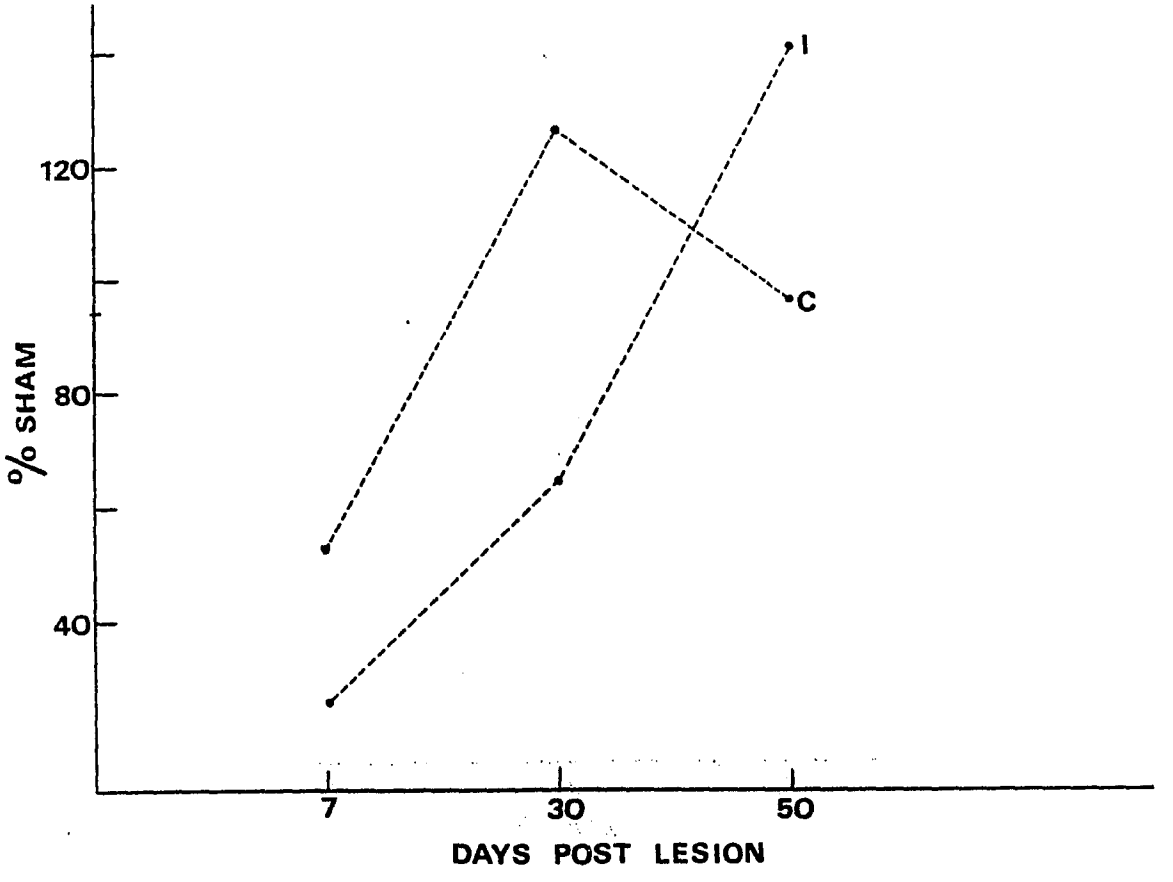
Figure 19: Photomicrograph of a 50 um coronal section of medial hypothalamus immunostained for 5-HT 12 days after the unilateral injection of 5,7-DHT (3 ug free base) into the dorsolateral hypothalamus. Ipsilateral to the injection (left) fibers in the lateral hypothalamus are swollen and darkly staining. In the medial hypothalamus there are few 5-HT-IR fibers. V, third ventricle; F, fornix; MT, mammillothalamic tract.



DAYS	SIDE	SHAM	5,7-DHT
7	I	125.6 ± 11.2 (8)	33.8 ± 6.6 (10)*
7	C	139.2 ± 10.7 (8)	73.7 ± 9.6 (10)*
30	I	82.0 ± 12.5 (6)	53.9 ± 12.0 (7)
30	C	63.8 ± 6.8 (6)	81.0 ± 11.1 (7)
50	I	63.1 ± 10.6 (7)	89.0 ± 10.3 (8)+
50	C	77.0 ± 13.7 (7)	73.6 ± 11.7 (8)

Table V: Effect of unilateral 5,7-DHT injection on the specific high affinity uptake of ^{3}H5-HT into rat hypothalamic synaptosomes 7, 30 and 50 days post lesion. Values are expressed as pmol ^{3}H5-HT/ g wet weight/ 3 min (mean ± S.E.M.) * $p < 0.001$ as compared to corresponding sham group. + $p < 0.001$ as compared to 7 days post lesion. Number of animals is given in parentheses. I, ipsilateral C, contralateral.

Figure 20: Same data as in Table V expressed as percent of corresponding sham.



DAYS POST LESION	SIDE	SHAM	5,7-DHT
7	I	1354 ± 122 (8)	616 ± 73 (7)***
7	C	1503 ± 106 (8)	1038 ± 121 (7)**
30	I	1445 ± 142 (11)	965 ± 107 (8)**
30	C	1373 ± 124 (11)	1053 ± 83 (8)*
50	I	1667 ± 83 (6)	1111 ± 132 (9)**
50	C	1608 ± 104 (6)	1238 ± 102 (9)*

Table VI: Effect of unilateral 5,7-DHT injection (3 ug free base) on 5-HT levels in the hypothalamus 7, 31 and 50 days post lesion. Values are expressed as ng 5-HT/ g wet weight (mean ± S.E.M.). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to corresponding sham group. Number of animals is given in parentheses.

Figure 21: same data as in Table VI expressed as percentage of corresponding sham.

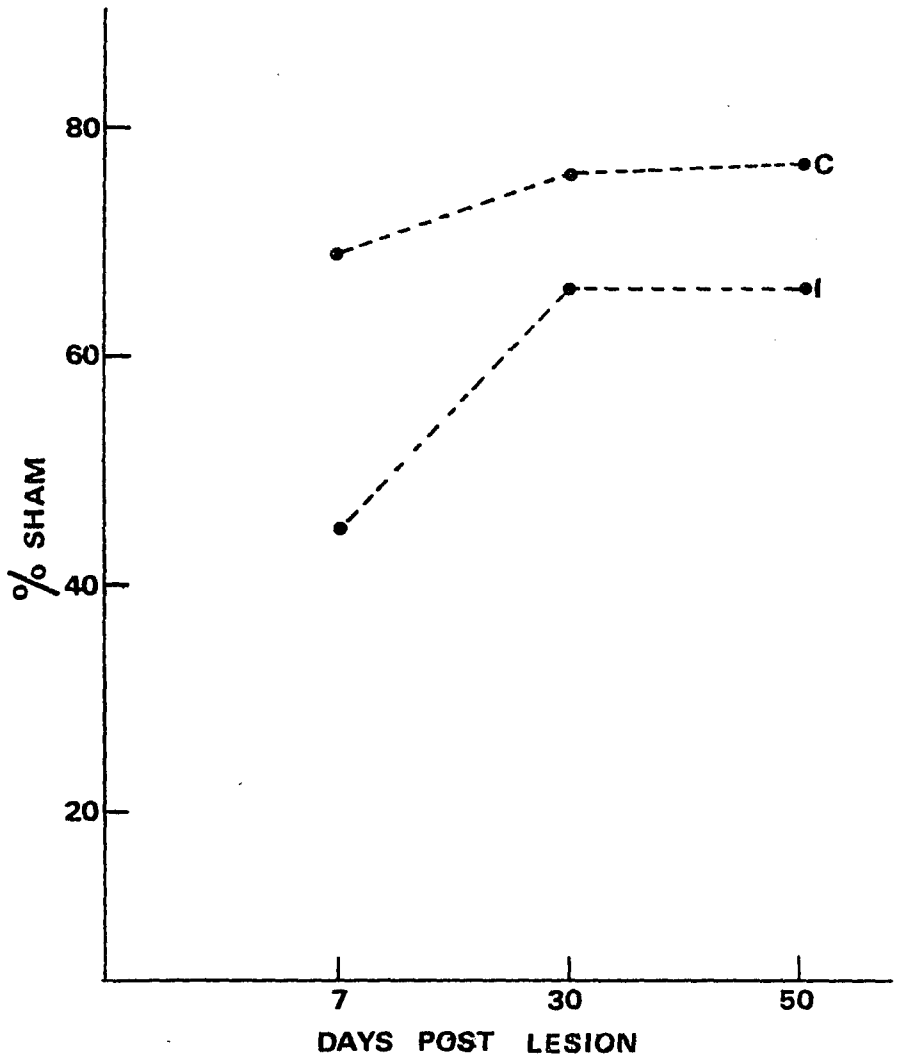
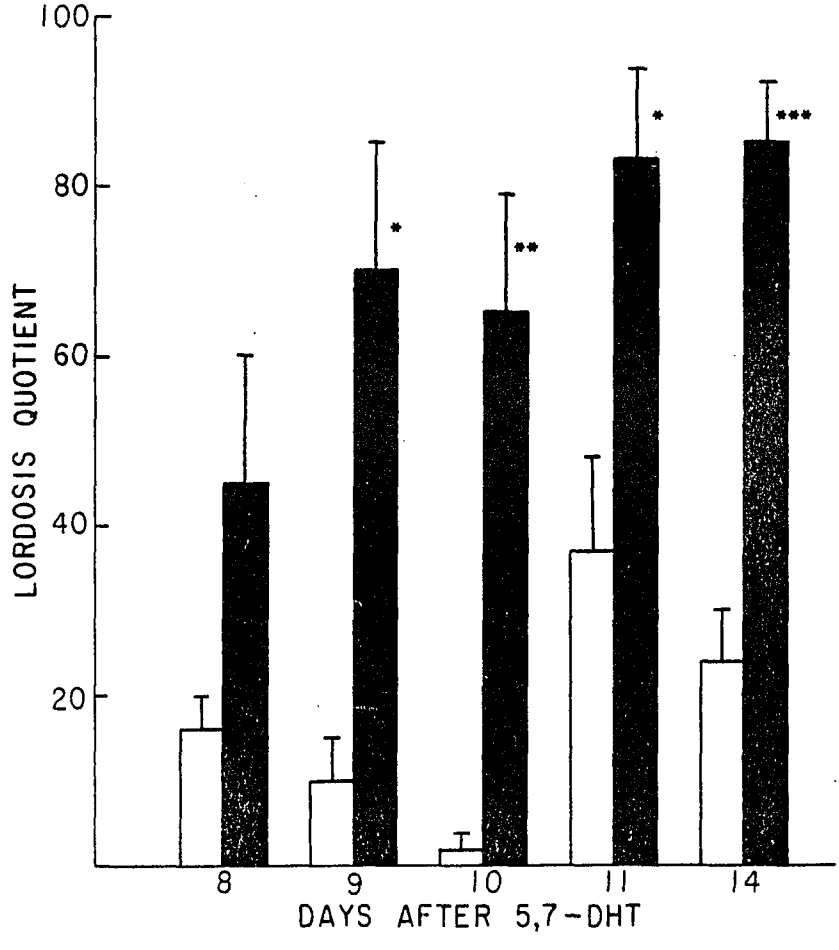


Figure 22: The L/Q in sham treated (open bars) and 5,7-DHT treated (closed bars) female rats is shown at various days after stereotaxic surgery. Data pooled from two experiments. Group 1 (3 sham, 3 5,7-DHT) was tested 9 and 11 days after surgery and group 2 (5 sham, 4 5,7-DHT) was tested 8,10 and 14 days after surgery. Results are expressed as mean ± S.E.M. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as determined by student's T-test.



Discussion

Intrahypothalamic injections of 5,7-DHT result in marked morphological, biochemical and behavioral changes. Following unilateral injection of 5,7-DHT into the dorsolateral hypothalamus fibers appear swollen and darkly stained as has been previously described for degenerating axons (Bjorklund and Stenevi, 1979). After axotomy there is a gradual decrease in 5-HT fiber density in the ipsilateral hypothalamus due to anterograde and retrograde degeneration. The morphological observations made with 5-HT immunocytochemistry are paralleled by the decreases seen in ^{3}H5-HT uptake and 5-HT levels in the ipsilateral hypothalamus. The time course of the decreases observed in these parameters following neurotoxin lesions is similiar to that demonstrated in other studies. Kuhar et al. (1972) have demonstrated that after electrolytic lesions of the raphe nuclei forebrain levels of 5-HT and ^{3}H5-HT uptake were lowest between 8-14 days post lesion. The same is true for the forebrain NE system after 5,7-DHT administration (Bjorklund and Lindvall, 1979) and the bulbospinal 5-HT system (Bjorklund and Wiklund, 1980; Wiklund and Bjorklund, 1980). However, as these determinations were made after intraventricular injection of 5,6- and 5,7-DHT, one side of the brain could not be compared to the other. The denervation observed on the contralateral side of the hypothalamus in this study could be due to diffusion of the toxin or to the interruption of 5-HT fibers known to cross the midline in the posterior hypothalamus (Moore et al, 1978; Azmitia and Segal, 1978).

Bilateral hypothalamic 5,7-DHT injections result in significant

changes in lordosis behavior in OVX-estrogen primed female rat that can be correlated with decreased ^{3}H5-HT uptake by hypothalamic synaptosomes. Previous studies (see Introduction) demonstrated that 5-HT had a role in inhibiting lordosis behavior. However, injection of 4 ug 5,7-DHT into the ascending 5-HT pathways in the midbrain has been reported to produce only modest facilitation of lordotic behavior (Everitt et al., 1976) or none at all (Sodersten et al., 1978). The present study indicates that it is necessary to inject 5,7-DHT directly into the hypothalamus to achieve maximal facilitation of lordosis. The period of enhanced lordosis response correlates well with the time that the hypothalamus is maximally depleted of 5-HT (7-19 days, as shown by immunocytochemistry).

Part V: Neurotoxin Induced Axotomy: Regeneration of 5-HT Fibers

Results

Following denervation, the regeneration of 5-HT fibers in the hypothalamus was assessed by 5-HT immunocytochemistry, ^{3}H5-HT uptake, 5-HT levels, and lordosis behavior. In addition, lordosis behavior was ascertained after bilateral transplantation of fetal 5-HT neurons into the denervated medial hypothalamus.

Twelve days after 5,7-DHT injection, the first sprouts were seen to emerge from the swollen proximal stumps of 5-HT fibers. The majority of sprouting fibers were observed along the medial border of the MFB in the caudal hypothalamus (Figure 23). Swollen fibers were often seen to have numerous sprouts (Figure 24). Sprouts were generally smooth with occasional varicosities. Sprouting fibers were abundant 12-19 days post lesion, however 19 days post lesion there were few swollen fibers in the MFB. Few sprouting fibers were seen in the contralateral hypothalamus.

The reinnervation of 5-HT fibers in the hypothalamus occurred gradually from caudal to rostral hypothalamus. It was initially apparent in the ipsilateral lateral hypothalamic area 30 days post lesion. At this point, substantial areas contained a normal or supra-normal density of 5-HT fibers, whereas the medial and periventricular hypothalamic areas remained sparsely innervated as compared to shams (Figure 25).

The apparent hyperinnervation was more evident 50 days post lesion. At this time, the lateral hypothalamus, both ipsilateral and

contralateral to the injection of 5,7-DHT, was more densely innervated than sham injected controls (Figure 26). Moreover, there was a partial reinnervation of the medial and periventricular hypothalamic areas. The ipsilateral dorsomedial hypothalamic area also appeared to be hyperinnervated. In this area the number of fibers extending into the third ventricle was greater than in sham injected animals. However, even 50 days post lesion the ventromedial hypothalamic area remained poorly innervated.

As the morphological data indicated that at 30 days there was a return to normal fiber density and at 50 days a hyperinnervation in the hypothalamus, ^{3}H5-HT uptake was examined at these times. Data from synaptosomal reuptake 30 and 50 days post lesion support morphological observations of regeneration (Table V, Figure 20). There was a gradual increase in ^{3}H5-HT uptake in both ipsilateral and contralateral sides of the hypothalamus. In the ipsilateral hypothalamus, ^{3}H5-HT uptake increased to 54% of sham from 23% of sham by 30 days post lesion. Fifty days post lesion there was a significant ($p < 0.001$ as compared to 7 days) increase in ^{3}H5-HT uptake in the ipsilateral hypothalamus (140% of sham). In the contralateral hypothalamus the ^{3}H5-HT uptake was somewhat greater than sham (126% of sham) values at 30 days post lesion and was approximately the same as sham (96%) 50 days post lesion.

Serotonin levels also increase with time after 5,7-DHT injection. Thirty days post lesion 5-HT levels rose to 66% of sham in the ipsilateral hypothalamus (Table VI, Figure 21) from a low of 45% of sham at 7 days post lesion. In the contralateral hypothalamus 5-HT

levels rose to 76% of sham. Levels of 5-HT 50 days post lesion did not differ significantly from those 30 days post lesion. The increase from 7 to 50 days post lesion was significant in the ipsilateral hypothalamus ($p < 0.01$) but not in the contralateral hypothalamus.

A) Lordosis Behavior

There was a gradual decrease in lordosis behavior with time from 30 days after bilateral injection of 5,7-DHT into the dorsolateral hypothalami of female rats (Figure 27). Lordosis behavior was no longer facilitated 45-50 days post lesion. This indicated that regeneration of 5-HT fibers had occurred and therefore 5-HT was again inhibiting lordotic behavior. Examination of hypothalamic sections that had been immunostained for 5-HT 50 days post lesion gave equivocal results. Of the four animals examined, there was substantial bilateral reinnervation as compared to sham in only two animals. In these animals the lateral hypothalamus was densely innervated and the medial hypothalamus was partially innervated. In addition, sprouting fibers were observed in the medial portion of the MFB on both sides of the hypothalamus. The hypothalami of the other two animals contained few 5-HT fibers.

The effect of bilateral transplantation of fetal raphe tissue into the denervated hypothalamus on the enhanced lordosis seen after 5,7-DHT, was difficult to assess. The L/Q with respect to time in individual animals is shown in Table VII. With the exception of 2 animals (of 8) there was no difference in the time course of facilitation between the two groups. In two animals (4,6) facilitation of behavior was no longer evident 20 days after lesion. In all other

animals, facilitation was present until 30-34 days post lesion. Examination of 5-HT immunocytochemistry revealed that one animal (4) had one transplant in the third ventricle and the other in the VMN and the second animal (6) had bilateral transplants in the VMN. However all the other transplant were dorsal to the VMN.

The transplanted neurons, as seen by 5-HT immunocytochemistry, were able to grow and develop processes (Figure 28). In some, but not all, of the transplant animals there was substantial reinnervation of 5-HT fibers.

Figure 23: a) Photomicrograph of a 50 um coronal section of medial hypothalamus immunostained for 5-HT 19 days after the unilateral injection of 5,7-DHT into the dorsolateral hypothalamus. In the MFB ipsilateral to the injection there are no longer many swollen 5-HT-IR fibers. Sprouting fibers are seen primarily in the medial portion of the MFB. Scale bar = 500 um b) Higher magnification of the area lateral to the fornix (F). Sprouting fibers are denoted by arrows. Scale bar = 100 um. V, third ventricle; MT, mammillothalamic tract.

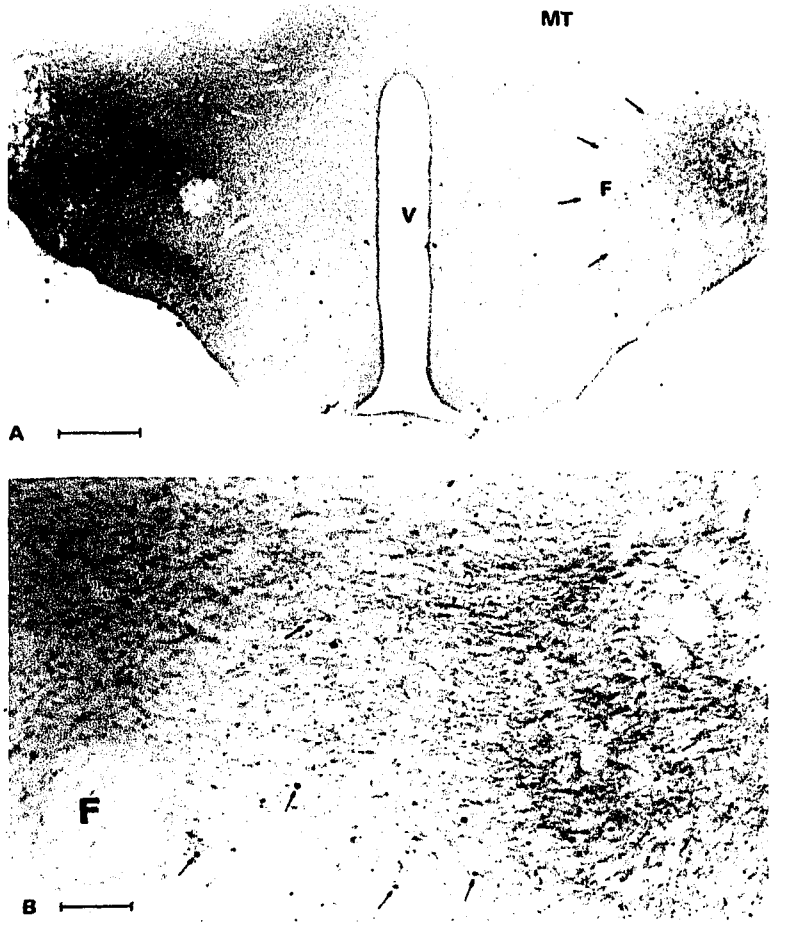


Figure 24: a and b) High magnification of sprouting fibers observed 19 days after the unilateral injection of 5,7-DHT (3 ug free base) into the dorsolateral hypothalamus. Note multiple sprouts emanating from one swollen proximal stump (arrows).

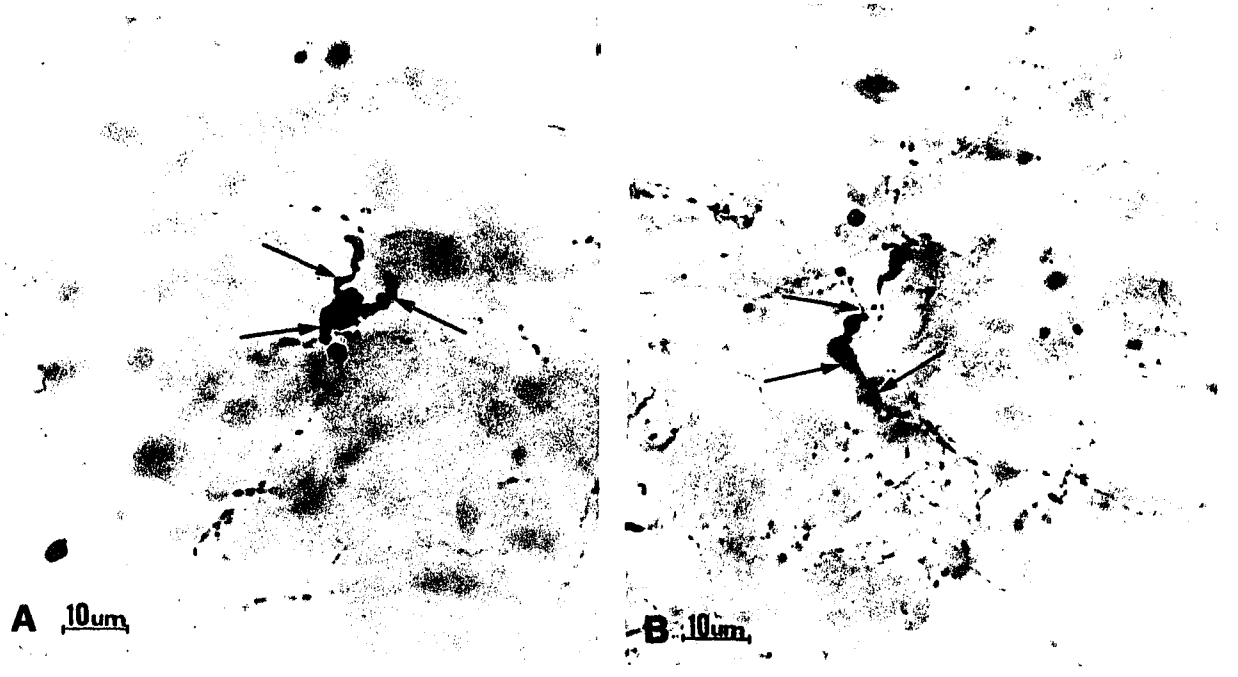


Figure 25: Photomicrograph of a 50 um coronal section of medial hypothalamus immunostained for 5-HT 30 days after the unilateral injection of 5,7-DHT into the dorsolateral hypothalamus. In the MFB ipsilateral to the injection there is a normal density of fibers, however medial areas of the hypothalamus remain denervated at this time. Scale bar = 500 um. V, third ventricle.; MT, mammillothalamic tract.

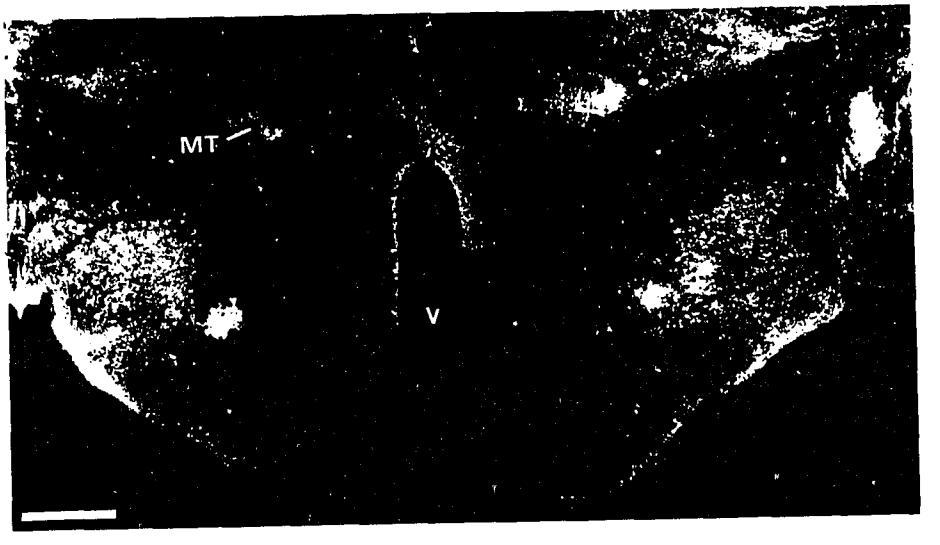


Figure 26: a) Photomicrograph of a 50 um coronal section of medial hypothalamus immunostained for 5-HT 50 days after the unilateral injection of 5,7-DHT (3 ug free base) into the dorsolateral hypothalamus. On both sides of the hypothalamus there is considerable hyperinnervation as compared to sham injected rats (b). Ipsilateral to the injection (left) there is hyperinnervation in the lateral and dorsomedial hypothalamic areas while the ventromedial area remains devoid of fibers. In the contralateral side the hyperinnervation is most pronounced in the lateral hypothalamus.

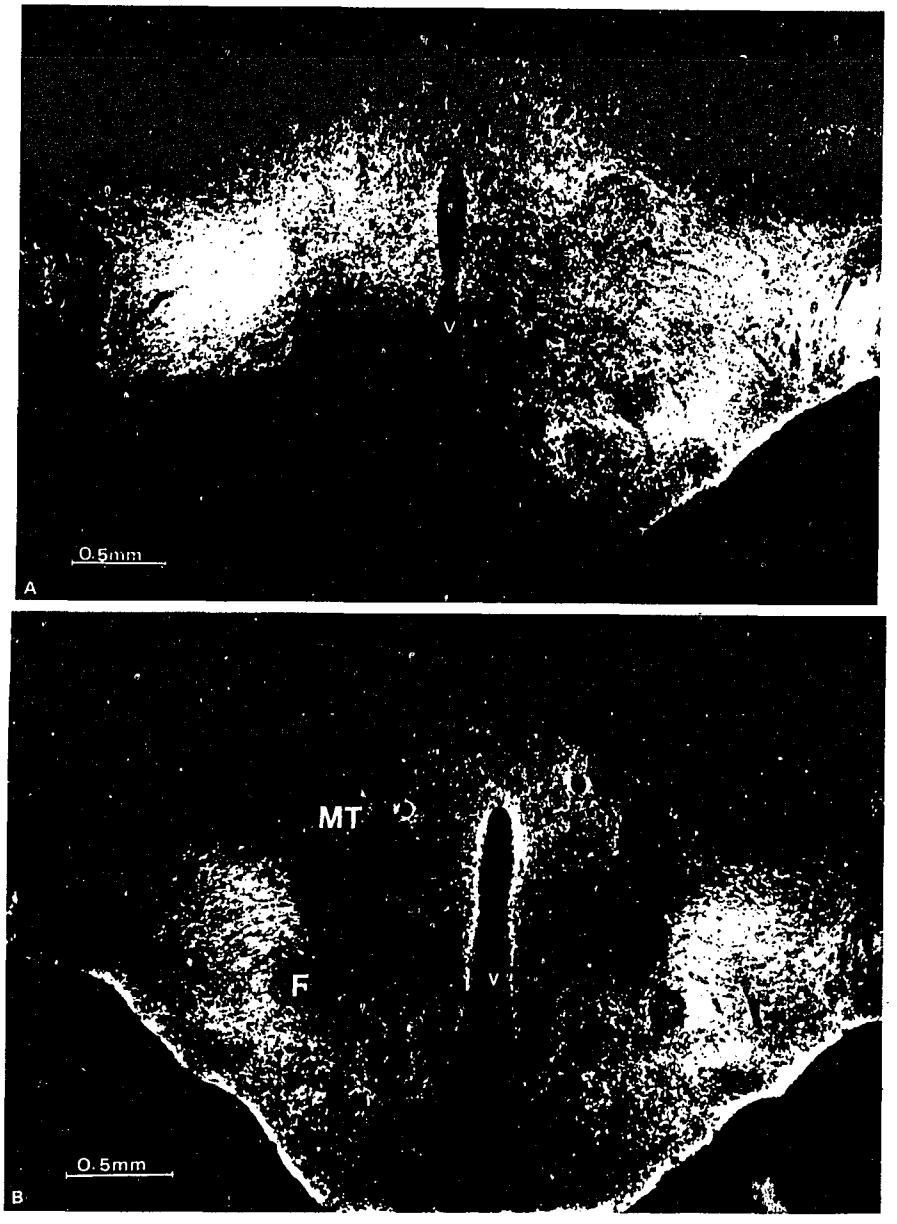


Figure 27: Graph of L/Q with time after 5,7-DHT. Data are mean values ± S.E.M. for 4-5 animals.

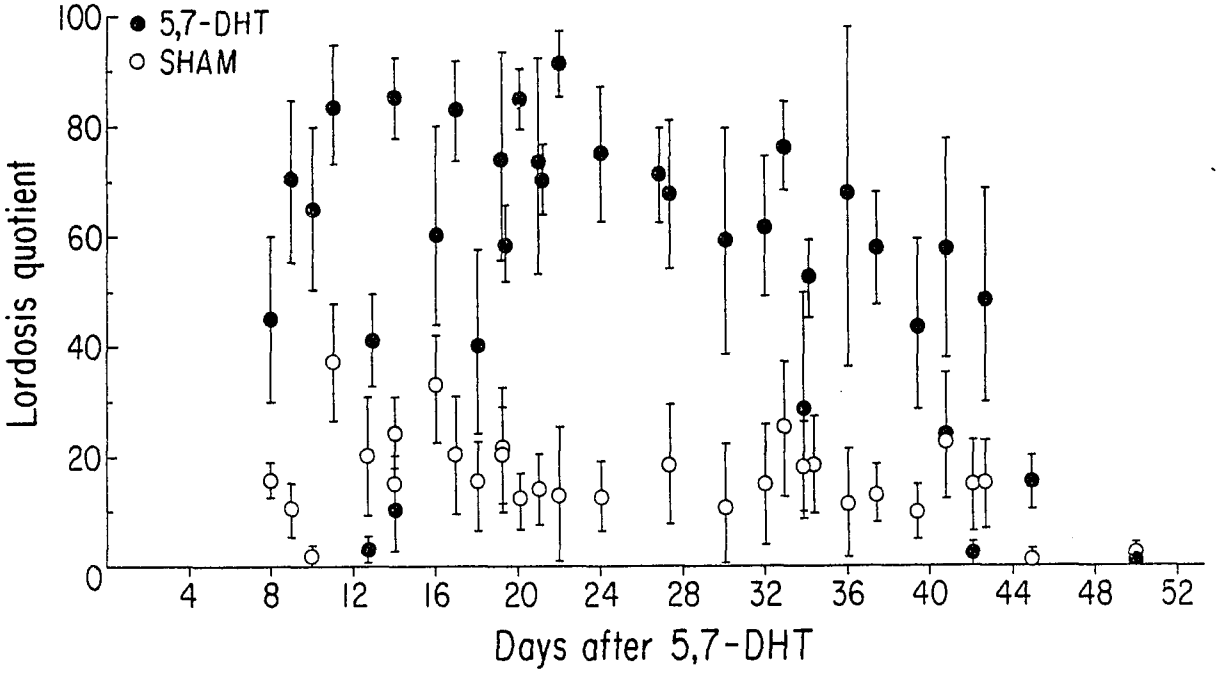


Figure 28: a) Photomicrograph of a 50 um coronal section of medial hypothalamus immunostained for 5-HT 45 days after the transplantation of fetal raphe tissue. Area of transplant is denoted by arrows. Scale bar = 500 um. V, third ventricle; F, fornix; MT, mammillothalamic tract. b) and c) higher magnification of raphe transplant. b) Scale bar = 100 um c) Scale bar = 50 um.

Day	5,7-DHT	T1	T2	T3	T4	T5	T6	T7	T8
13	42 + 9	90	40	40	30	90	50	100	20
14	10 + 6	50	20	30	60				
15						100	30	100	90
16						90	10	90	64
19	74+20	40	50	50	30				
22		20	60	60	0	80	0	80	100
23						80	0	90	90
27	72 + 20	80	100	50	10				
30						30	0	80	20
32	64 + 14	20	80	40	0	20	0	70	10
34	29 + 20	0	80	100	0				
41	24 + 12	10	10	80	0				
42	2 + 2	0	10	0	0				

Table VII: The effect of fetal raphe transplantation on the L/Q obtained 14-42 days after bilateral 5,7-DHT injection into the hypothalamus. Animals 1-4, 5-8 are from separate experiments. Values given for 5,7-DHT animals (without transplants) are the mean ± S.E.M. for 5 animals. T = transplant.

Discussion

The denervation observed in the hypothalamus is followed by a relatively rapid reinnervation. The presence of sprouting fibers 12-19 days post lesion is in agreement with previous studies (Daly et al., 1973; Bjorklund and Lindvall, 1979; Wiklund and Bjorklund, 1980). Daly et al. (1973) observed sprouting 5-HT fibers 14 days after 5,6-DHT was injected into the raphe area. Sprouts arising from damaged fibers proliferate and grow along the existing path of the MFB to reinnervate many hypothalamic areas by 30-50 days post lesion. The time course of regeneration is similar to that of Wiklund and Bjorklund (1980) in their study of the bulbospinal 5-HT system. In the latter study, sprouts were first seen in the caudal medulla 7 days post lesion, a return to normal innervation occurred 1 month post lesion and hyperinnervation was apparent several months post lesion.

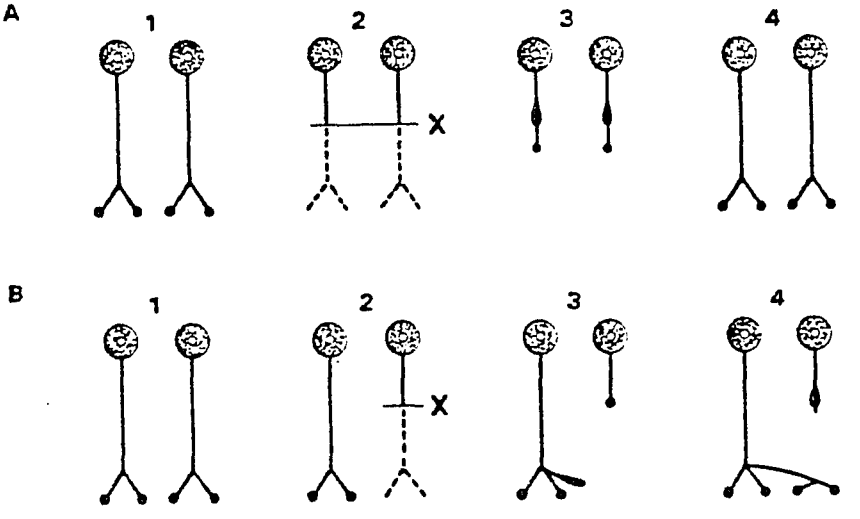
In the hypothalamus the pattern of reinnervation resembled normal with two exceptions. Firstly, 50 days post lesion, there was a hyperinnervation in both lateral and dorsomedial hypothalamic areas and secondly, the ventromedial hypothalamic area was poorly reinnervated. The results obtained in the present study with 5-HT immunocytochemistry have been confirmed by radioautography in rats that received unilateral 5,7-DHT injections in the hypothalamus 50 days prior to the intraventricular administration of ^{3}H5-HT (A. Beaudet, personal communication).

Abnormal hyperinnervation of MA containing fibers has been observed in the CNS of adult and developing rats following neurotoxin-induced axotomy. The density of NE fibers was shown, by

fluorescence histochemistry, to be greater than normal in the anterior hypothalamic area 7 months after intraventricular administration of 5,7-DHT (Bjorklund and Lindvall, 1979). Wiklund and Bjorklund (1980) have observed, with fluorescence histochemistry, that there is a hyperinnervation of 5-HT fibers in the caudal medulla 7 months after intraventricular administration of 5,6-DHT. The fibers appear to be permanent as they were still present 19 months post lesion. Further work at the ultrastructural level has been done by Wiklund et al. (1981) in a study of the rat dorsal accessory olive (DAO) after intraventricular 5,6-DHT administration. Using quantitative radioautography these workers found that 5 days after 5,6-DHT the number of labelled 5-HT varicosities was reduced to 1/10 of normal. Two months after 5,6-DHT, the number of labelled varicosities in the DAO had returned to normal, and by 6 months there was approximately a 3-fold increase in the number of labelled varicosities in the DAO.

The mechanism underlying regrowth, whether due to regeneration or collateral sprouting is poorly understood (Figure 29). However, several principles will be reviewed here for the purpose of discussing the hyperinnervation in the present and previous studies. First, it is known that partial denervation of any kind is a potent stimulus for axonal sprouting (Azmitia et al., 1978; Bjorklund and Stenevi, 1979; Cotman, 1981). This may be due to the denervated target directing growth towards itself or the need of the partially denervated neuron to regain a given number of synapses.

Figure 29: Diagram illustrating two different responses to denervation (A) A damaged neuron regenerates to reinnervate denervated area. B) An intact neuron sprouts new fibers to reinnervated denervated area (collateral sprouting). Adapted from Moore (1975).



There is much evidence for the denervated target directing growth toward itself. In developing animals, removal of target areas results in incorrect fiber growth (Veraa et al., 1979). Patterns of reinnervation, following partial denervation, have been shown to be highly specific and resemble those observed normally. The regeneration of NE and 5-HT fibers has been shown to restore normal innervation patterns in the anterior hypothalamus and spinal cord, respectively (Bjorklund and Lindvall, 1979; Wiklund and Bjorklund, 1980). Azmitia et al., (1978) have demonstrated that the sprouting of intact 5-HT fibers in rat hippocampus, following partial denervation, results in reinnervation patterns that resemble those normally observed. Lastly, in vitro studies support these findings. Retinal explants grown between retinal and tectal cells grow preferentially towards the tectal cells which are their target in vivo (Bonhoeffer and Huf, 1980). diPorzio et al. (1980) have demonstrated that mesencephalic cells (which contain SN cells) are stimulated by the presence of striatal cells (SN target) when they are grown together.

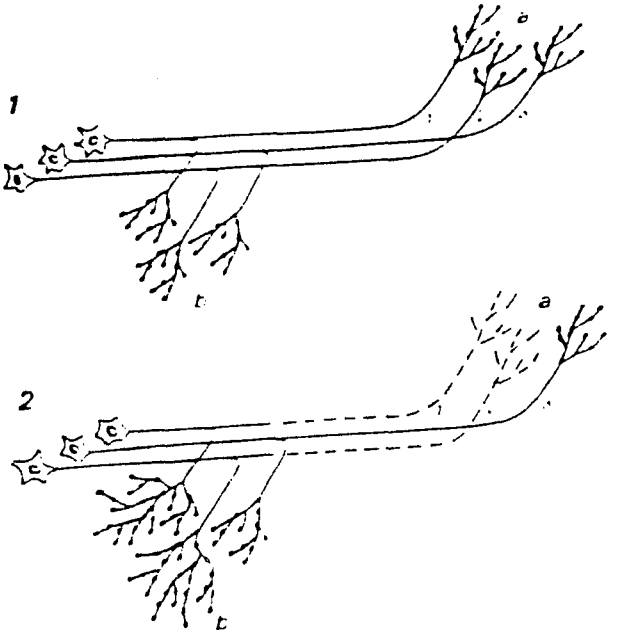
Target directed growth may be mediated by the release of trophic factors. This has clearly been shown to occur in the peripheral nervous system (PNS) (reviewed by Cotman et al., 1981). Nerve growth factor (NGF) has been shown to be an absolute requirement for the outgrowth of sympathetic ganglia (Varon, 1978). Whether NGF has a physiological role in the CNS is not clear. It has been isolated from brain tissue but exists in relatively small quantities in the brain (Freed, 1976). Bjerre et al. (1973) have shown that the administration of NGF can stimulate growth of MA fibers into transplanted tissue. In animals that received homologous iris

transplants into the caudal diencephalon, NE fiber growth into the iris was facilitated when NGF was injected intraventricularly and intracerebrally (Bjerre et al., 1973, Stenevi et al., 1974). It was found that NGF was most effective when administered at the time of transplantation. Furthermore, Bjerre et al. (1974) found that administration of anti-NGF antiserum intracerebrally blocked sprouting of NE fibers into the iris transplants.

Another possible mechanism underlying reinnervation is the need of the damaged neuron to reestablish a given number of terminal arborizations. This effect was first described by Schneider (1973) who observed sprouting of optic tract fibers in areas that do not normally contain these fibers (such as the lateral posterior nucleus of the thalamus) after lesions in the superior colliculus. In addition, he noted that the fewer fibers observed in the superior colliculus, the greater the number of fibers in the lateral posterior nucleus. Schneider suggested that more than one part of the neuron's axon tree was sprouting in response to denervation and termed this effect "pruning" (Figure 30). This explains the observations made by other workers of aberrant sprouting in various CNS areas. Pickel et al. (1974) noted that following lesions of the fibers to the cerebellum there was an increase in the number of fibers in the hippocampus. It has also been demonstrated that neonatal administration of 5,7-DHT to rats results in a reorganization of 5-HT fibers (Sachs and Jonsson, 1975; Ponzio and Jonsson, 1978). Within several weeks there is a hyperinnervation in the brainstem and a hypoinnervation in the cortex. The authors attribute this to pruning, as it is assumed that fibers innervating the cortex and the brainstem arise from the same cells in

the midbrain raphe nuclei. The hyperinnervations observed in the medulla and anterior hypothalamus (Wiklund and Bjorklund, 1980; Bjorklund and Lindvall, 1979) may also be due to pruning.

Figure 30: Pruning response to denervation. Following damage to part of a neurons axon tree (1a) a different part of that neuron's axon tree sprouts (2b). Adapted from Jonsson (1976).



The reinnervation observed in the hypothalamus after 5,7-DHT treatment is probably due to both target directed growth and pruning. The fact that normal patterns of innervation are reestablished indicates that the growing axons are directed in some way by the target. Whether this involves trophic factors remains to be seen.

The hyperinnervation observed in the present study may be due to pruning. As 5,7-DHT injections into the hypothalamus destroy 5-HT fibers in the MFB, areas anterior to the hypothalamus must be largely denervated. It has been demonstrated that intracerebral injection of 5,7-DHT results in great decreases in cortical 5-HT (Daly et al., 1973; Hole et al., 1976). Although areas anterior to the hypothalamus were not examined in the present study, it can be assumed that they remain largely denervated 50 days post lesion. Therefore, the hyperinnervation in the hypothalamus may be a compensation for the lack of terminals in distal areas, such as the cortex. This finding is similiar to that described for 5-HT in the brainstem (Ponzio and Jonsson, 1978; Wiklund and Bjorklund, 1980).

The presence of a bilateral hyperinnervation may result from several factors. As relatively few fibers were observed to sprout in the contralateral hypothalamus, it appears that reinnervation may be due to sprouting of intact collateral 5-HT fibers. There is evidence for the presence of bilaterally projecting 5-HT cells in raphe nuclei (Azmitia, 1981) and therefore it is possible that pruning is occurring in the contralateral as well as ipsilateral hypothalamus. Another possibility is that information to sprout is being passed from one cell to another in the raphe nuclei. In the locus coeruleus, subtotal

destruction of NE containing fibers by intraventricular injection of 6-OHDA has been shown to increase the firing rate of LC cells four-fold (Chiodo et al., 1983). Furthermore, there is electrophysiological (Wang and Aghajanian, 1982) and anatomical (Felten and Harrigan, 1980) evidence that the 5-HT cells in the raphe communicate. Therefore, it appears plausible that a stimulus to sprout is passed from one cell to the next and as many midbrain raphe cells lie on the midline, sprouting on both sides of the hypothalamus is apparent.

The specific high affinity uptake of ^{3}H5-HT has been shown to correlate well with density of innervation (Jonsson, 1976). In the study of the 5-HT bulbospinal system by Bjorklund and Wiklund (1980) specific uptake of ^{3}H5-HT paralleled the observed hyperinnervation in slices of caudal medulla. Two weeks after 5,6-DHT, specific uptake of ^{3}H5-HT was 36% of control. Two months later, specific uptake had increased to 136% of control. In the present study ^{3}H5-HT uptake in the ipsilateral hypothalamus, 50 days post lesion, was greater than sham values, but this was not statistically significant. This is probably due to the lack of reinnervation in the ventromedial area of the hypothalamus.

The data related to 5-HT levels after 5,6- and 5,7-DHT treatment are more complicated. Bjorklund and Wiklund (1980) found that in the spinal cord, although levels of 5-HT increased significantly 7 months after 5,6-DHT, they never were greater than 33% of normal. Different results were obtained in Bjorklund and Lindvall's (1979) study of the NE system after 5,7-DHT. Normal levels of NE had returned to the forebrain several months after 5,7-DHT injection. The data in the

present study are similiar to those of Wuttke et al. (1977). In the latter study levels of 5-HT in the hypothalamus were approximately 60% of normal 55 days post lesion, although specific uptake had returned to normal.

The discrepancy between ^{3}H5-HT uptake and 5-HT levels may be partially explained by the studies of Grafstein and McQuarrie, (1978). These workers have found, in their studies of regeneration in the goldfish retinal ganglion, that following axotomy there is a shift in protein synthesis. During regeneration, the synthesis of cytoplasmic proteins occurs first and the synthesis of secretory proteins is secondary. This is consistent with the finding of the present and previous studies (Wuttke, et al., 1977; Bjorklund and Wiklund, 1980) that regenerating 5-HT neurons regain their high affinity uptake capacity before having normal transmitter levels. The critical question that remains is whether, as has been shown (Bjorklund and Wiklund, 1980; Wuttke et al, 1977), the turnover of 5-HT compensates for the levels of 5-HT. In these studies, areas which contained fewer 5-HT fibers and low levels of 5-HT were shown to have a greater turnover of 5-HT. This is similiar to the situation observed in development of the 5-HT system. Hamon and Bourgoin (in press) have shown that the turnover of 5-HT and the activity of MAO is greater in neonatal animals than in adults.

It is not clear from the present study whether the reinnervating 5-HT fibers in the hypothalamus restore function. The facilitation of lordosis behavior seen after 5,7-DHT decreased at approximately 30 days post lesion, but it this was not clearly due to hypothalamic 5-HT. It

is possible that the function of 5-HT fibers is taken over by another system, particularly NE and DA which appear to have a role in mediating lordosis behavior (Crowley and Zemlan, 1981). Therefore, studies relating MA levels in discrete hypothalamic nuclei to lordosis behavior after 5,7-DHT are in progress.

Transplantation of fetal tissue has been previously demonstrated to restore function in animals with certain denervations (see Introduction). In the present study the apparent failure of transplanted raphe cells to reverse the effects of 5,7-DHT may be due to the fact that the majority of transplants were located dorsal to the VMN and therefore the reinnervating fibers were unable to reach the proper target cells. Although the 5-HT cells survived transplantation they were not as large nor did they have as many processes as those seen in the hippocampus (Azmitia et al., 1981), and therefore the possibility that they were incapable of restoring function must be considered.

GENERAL DISCUSSION

In the present study a method for manipulating the hypothalamic 5-HT system using the specific 5-HT neurotoxin, 5,7-DHT, is described. This can be used to study the role of 5-HT in mediating hypothalamic functions, such as control of pituitary secretions and sexual behavior.

The advantage of direct injection of 5,7-DHT into the hypothalamus as opposed to intraventricular injection of 5,7-DHT or destruction of the ascending 5-HT pathways is evident from the results obtained on lordosis behavior. It was previously demonstrated that systemic administration of anti-5-HT drugs facilitated lordosis behavior (Meyerson, 1970) whereas administration of 5,7-DHT into the ascending 5-HT pathways had little (Everitt et al., 1976) or no effect (Sodersten et al., 1978). However, in this study it was clearly demonstrated that direct application of 5,7-DHT to the mediobasal hypothalamus results in a rapid facilitation of lordosis behavior. The reason for this apparent discrepancy is not clear. One possibility, however, is that the 5-HT-IR cells in the DMN give rise to fibers which are involved in mediating the lordosis response. These cells would be affected by systemic administration of anti-5-HT drugs and unaffected by destruction of the ascending 5-HT pathway.

Another example of this kind of discrepancy concerns the secretion of LH. Systemic administration of PCPA completely abolishes the circadian rhythm of LH, whereas raphe ablation only disrupts it (Kordon et al., 1981). Again, Kordon et al. suggest that the presence of an intrahypothalamic 5-HT cell group may account for this discrepancy. Clearly, the use of intrahypothalamic injections of 5,7-DHT to assess

the function of the hypothalamic-5-HT system circumvents this problem.

The hypothalamic-5-HT system also lends itself to the study of neuronal plasticity. Intrahypothalamic injections of 5,7-DHT result in specific denervation within several days which is followed by relatively rapid reinnervation of 5-HT fibers in the hypothalamus. As is evident from 5-HT immunocytochemistry, the 5-HT fibers which reinnervate the hypothalamus do so with relative accuracy. Furthermore, the morphological changes observed in the hypothalamus correlate with biochemical changes in ^{3}H5-HT uptake and 5-HT levels. However, most importantly, the extensive involvement of the hypothalamic 5-HT system in neuroendocrine and behavioral events (see Introduction) provides a framework in which functional restoration can be studied.

In the present study, the facilitation that was observed in lordosis behavior paralleled the degeneration of 5-HT fibers in the hypothalamus, as shown by a significant decrease in ^{3}H5-HT uptake by hypothalamic synaptosomes. Unfortunately, it was not clear if the subsequent decrease was due to regeneration of 5-HT fibers in the hypothalamus. Further work is required to assess whether lordosis behavior is a good functional parameter for monitoring 5-HT regeneration.

A number of other hypothalamic functions that are mediated by 5-HT could be used as indices of 5-HT regeneration. For example, the secretion of TSH (Smythe et al., 1982) or LH (Wuttke et al., 1977) in response to denervation and reinnervation could be examined. In this context it must be stressed that intrahypothalamic injections of

5,7-DHT would probably clarify some of the conflicting data on the 5-HT. control of pituitary secretions (see Introduction). Circadian rhythmicity of pituitary hormones, such as LH and ACTH (Kordon et al., 1981; Williams et al., 1983) may also provide a good index of hypothalamic-5-HT function.

The hypothalamic 5-HT system provides a good model for the study of gonadal steroid effects on neuronal plasticity. Neonatal estrogen administration has been shown to increase the number of mature synapses in the rat arcuate nucleus (Arai and Matsumoto, 1978). In adult rats estradiol treatment promotes synapse formation in the arcuate nucleus following surgical isolation of the mediobasal hypothalamus (Matsumoto and Arai, 1979). In hypothalamic explants of newborn mouse hypothalamus neurite outgrowth is stimulated in the presence of estrogen (Toran-Allerend, 1980). It was demonstrated by ^{3}H-estradiol radioautography that neurites came from areas of explant that accumulated estrogen. It has been suggested, therefore, that gonadal steroids influence brain development during sexual differentiation (McEwen, 1980; Toran-Allerand, 1980). This is further supported by anatomical studies which demonstrate that there exist sexually dimorphic nuclei in the hypothalamus and preoptic area of several mammalian species (Bleier et al, 1982).

A role for estradiol in development and aging is suggested by the work of Schipper et al. (1981) who found that the rate of degeneration in the female mouse arcuate nucleus, as judged by astrocytic and microglial activity, was decreased by ovariectomy. More recently, it has been shown that constant exposure to estradiol over a period of 12

weeks resulted in the degeneration of neurons in the arcuate nucleus of the female rat (Brawer et al., 1983).

The mechanism by which estrogen effects changes in the hypothalamus is not understood. However, it has been shown that subsequent to binding estrogen can induce changes in the hypothalamus by altering membrane permeability and by effecting the genome (McEwen and Parsons, 1982). Therefore, it is possible that normal estrogen feedback phenonmena may be the result of growth and developmental changes. Given the extensive interaction between estrogen and 5-HT in the hypothalamus (see Introduction) and the finding that 5-HT turnover increases with age in the rodent hypothalamus (Simpkins et al., 1978), it is possible that 5-HT could be involved in this type of modulation. Future studies, utilizing the system described in the present study, would help determine whether this type of modulation occurs.

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