HISTOCHEMICAL AND ELECTROPHYSIOLOGICAL STUDIES ON THE ORIGINS AND SITES OF TERMINATION OF THE SPINAL SEROTONERGIC PATHWAY

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by

JOHN E. MENZIES

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ABSTRACT

In an effort to determine the distribution of 5-HT terminals in the feline spinal cord, cats were injected intraspinally at the thoraco-lumbar junction with 6-hydroxydopamine in order to deplete spinal noradrenaline levels. The results of these experiments indicate that the majority of 5-HT terminals are located in the ventro-medial aspect of the ventral horn in Rexed's lamina VII, the dorso-medial part of lamina VIII, and lamina IX. Many terminals were also seen in the more ventro-lateral area of the ventral horn encompassing the lateral lamina IX, and in lamina X in the area of the central canal. Few terminals were found in lamina II and III in the dorsal horn, with very few in the intermediate zone.

In order to measure the conduction velocities of the 5-HT fibers projecting to the spinal cord, the antidromic responses to stimulation of the spinal cord at L_1 were made in the raphé nuclei of the caudal pons and upper medulla. The results revealed that the conduction velocities of bulbospinal serotonergic fibers ranged between 17.3 and 120 m/sec. Such values were far greater than those expected for small unmyelinated fibers (2.5 - 3.5 m/sec).

In order to explain the results, sagittal sections of thoracic spinal cord were examined in chronic spinal cats for fibers containing 5-HT. These sections were counterstained with Luxol Fast Blue and examined for myelin sheaths.

The results indicated that at least a portion of these fibers were surrounded by a myelin sheath, and may be capable of conducting at the observed conduction velocities. These results may indicate the presence of a fast bulbospinal serotonergic pathway.

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INTRODUCTION

Interest in the possibility that serotonin (5-hydroxy-tryptamine - 5-HT) may be a neurotransmitter in mammalian central nervous system was initially provoked by the discovery of its presence in the brain by Twarog and Page (1953) and Amin et al (1954). Since then numerous investigations have been concerned with mapping out the locations of 5-HT containing cell bodies and terminals in various parts of the brain and spinal cord. Using the then recently developed technique for the demonstration of fluorescent compounds of monoamines (Falck, 1962; Falck et al, 1962), Dahlstrom and Fuxe (1964) succeeded in demonstrating the presence of 5-HT containing cell bodies in the raphé nuclei of the lower brainstem of the rat. More recently it has been confirmed microspectrofluorimetrically (Jonsson et al, 1975) that the majority of cell bodies in these nuclei are capable of synthesizing, storing and metabolizing 5-HT. Such findings are consistent with a role for 5-HT as a CNS transmitter, showing that it fulfills many of the established criteria for transmitters (cf., Werman, 1966; Phillis, 1970).

Since the early demonstration of the presence of 5-HT in cells of the raphé nuclei, several investigators have attempted to delineate both biochemically and histochemically, the distribution of this monoamine in various other CNS regions. Fluorescence histochemical studies (Carlsson et al, 1962; Anden et al, 1965; 1966 a,b; Fuxe, 1965) have shown the presence of

5-HT in various diencephalic and telencephalic loci, where it is confined to small (.3 - 1.0 um) beadlike "varicosities" which, in many cases, were seen to make close contact with neuronal perikarya. These varicosities were presumed to be the terminals of 5-HT containing axons, and the localization of serotonin to such terminals strengthened the notion that it may be involved in transmission at serotonergic synapses. Further work by these investigators (Carlsson et al, 1964; Fuxe, 1965) also demonstrated similar tryptamine containing varicosities in the spinal cord of both mouse and rat, a finding confirmed by Anderson and Holgerson (1966). These studies have shown the largest numbers of these terminals to be located in the sympathetic lateral column and the ventrolateral and dorsolateral motor groups of the anterior horns in the cervical and lumbar enlargements. At these levels the presence of 5-HT containing varicosities in the medial motor group is less than in the corresponding motor group at thoracic levels. Scattered terminals are distributed throughout the dorsal parts of the posterior horn at all levels, with somewhat fewer of them in the remaining areas of this horn. In the intermediolateral column it appears that most of the nerve cells are surrounded by terminals containing 5-HT, and it has been reported that some of the 5-HT varicosities in the anterior horns of cervical and lumbar enlargements make "intimate contact" with dendrites and cell bodies of motoneurones. On the other hand the cells of the ventral area of the dorsal horn

seem to have fewer contacts with 5-HT terminals with even less cellular contact by terminals in the superficial aspects of the dorsal horn (Carlsson et al, 1964).

In addition, several biochemical investigations have confirmed and extended the findings referred to above. Anden (1964) showed that there is usually two to four times as much 5-HT as noradrenaline (NA) at all locations in the cord, with more in the gray matter of the lateral horns than the anterior and posterior horns and very low levels in the white matter. Recently, Zivin et al (1975) have confirmed this distribution for rabbit and rat spinal cords in a more precise fashion through the use of microdissection techniques, and have shown that a substantial amount of 5-HT may also be seen in the area of the central canal.

Of central importance in this discussion is the question of whether or not the 5-HT terminals observed by fluorescence microscopy originate from the cell bodies of the raphe shown to contain this amine. Correspondingly, investigations aimed at resolving this issue have dealt with the biochemical and histochemical distribution of 5-HT after lesions in the brain and spinal cord which are thought to interrupt monoamine pathways. Caudal to a transection in the mid-thoracic region of rabbits, 5-HT levels fell to a minimum after seven to nine days, while there was a slight rise in cord 5-HT content rostrally (Anden et al, 1964b).

Fluorescence histochemical studies (Carlsson et al, 1964; Dahlstrom and Fuxe, 1964; 1965) have also shown that there is a significant loss of monoamine fluorescence caudal to a spinal transection even after treatment with agents known to enhance visualization of fluorescent products. Rostral to the transection however, there is improved visibility of fluorescence due to accumulation of amines in the end of the axons above the transection, and it has also been reported that following administration of the monoamine oxidase inhibitor Nialamide (which improves fluorescence due only to 5-HT) it was possible to trace axons containing 5-HT from the site of the transection up to 5-HT containing neurone soma in the medulla oblongata of the rat (Dahlstrom and Fuxe, 1964).

In cross sections of these spinal cords above the transection it was possible to visualize a build-up of 5-HT in non-terminal axons and to discern their position of travel in the spinal cord. The fibers were seen to be very fine with small varicosities (Carlsson et al, 1964), while the sizes of the fibers themselves have been reported to be between 1 - 2 μm (Dahlstrom and Fuxe, 1965) and were unmyelinated. This latter suggestion is based on the findings of Dahlstrom and Fuxe, (1965) that fluorescent axons in "about the same location" as those showing a build-up of amine after transection of the cord did not show the weak brown fluorescence rings characteristic of

myelin. It has also been suggested that the failure of Heller et al (1966) to trace degeneration of nerve fibers to neocortex in cats after destruction of the median forebrain bundle is explainable because the 5-HT fibers ascending in this pathway are not myelinated and, hence, would not appear in Nauta stained material.

It was thus demonstrated that the descending fibers travel in the dorsolateral, lateral, and anterior funiculi near the exterior surface of the spinal cord and give off collaterals that enter the gray matter at all levels. This distribution within the white matter of 5-HT axons in the rat has also recently been confirmed for the cat by Coote and Macleod (1974).

After hemisection of the spinal cord, the reduction in fluorescent terminal density was less than after complete transection, and it could be seen that many terminals both contralateral as well as ipsilateral to the lesion remained. This is suggestive of crossed and uncrossed pathways from the raphe. (Carlsson, etal, 1963)

The findings presented thus far are strongly suggestive of a function of 5-HT as a neuro-transmitter originating from the brainstem and having both ascending and descending projections. Further support for this idea is provided by the findings that electrical stimulation of the mesencephalic

raphé nucleus in rats can cause release of 5-HT and 5-hydroxindol - 3 - ylacetic acid from cerebral cortex of rats (Eccleston et al, 1969), depletion of spinal cord 5-HT (Dahlstrom et al, 1965) and release of 5-HT but not acetylcholine (ACh) into the lateral ventricle of cats (Ashkenazi et al, 1972). In addition, Anden et al (1964a)have shown release of 5-HT into a perfusion solution of isolated frog and mouse spinal cords by electrical stimulation of the upper end of the cord, and Dahlstrom et al (1965) have demonstrated a decrease in both the number and intensity of fluorescent terminals remaining after 1 - 2 hours of tetanic electrical stimulation of the medullary raphé nuclei in rats.

So far the evidence is suggestive for 5-HT as a neurotransmitter in mammalian CNS,and although its exact role in the spinal cord has not yet been elucidated, it is thought to play a role in modulation of both motor and sensory functions at the spinal level.

Intravenous administration of the 5-HT precursor, 5-hydroxytryptophan (5-HTP) (100 mg./kg.),into cats spinalized at the first cervical level has been shown to increase the L_7 monosynaptic reflex (MSR) by up to 300 percent of control values (Anderson and Shibuya, 1966). Such an increase in the monosynaptic reflex was also accompanied by an increase in alpha motoneuronal discharge measured in ventral root filaments. Such findings were described earlier by Anden et al (1964c) and

Lundberg (1965). In addition to these findings, Anden et al and Lundberg have shown that intravenous 5-HTP in acute spinal cats causes marked depression of transmission in the pathway from flexor reflex afferents (FRA) to alpha motoneurones. After administration of a decarboxylase inhibitor there is no effect from large doses of 5-HTP, and this has led to the conclusion that 5-HTP works entirely through formation and liberation of 5-HT from nerve terminals (Anden et al, 1964c).

After administration of monoamine oxidase inhibitors, the MSR height was selectively increased, whereas there was no significant change in the polysynaptic potentials (Anderson et al, 1967). This increase in MSR could not be prevented by the use of the peripheral noradrenergic alpha receptor blocker phenoxybenzamine, although the facilitation of the MSR due to intravenous administration of the NA precursor, L-3, 4 - dihydroxyphenylalanine, was effectively antagonized by phenoxybenzamine. Banna and Anderson (1968), however, have shown that cinanserin, methysergide and d-lysergic acid diethylamide (LSD-25) can block the 5-HTP-induced facilitation of the MSR without altering the inhibitory effects of 5-HTP on polysynaptic reflexes, indicating that the facilitatory, but not the inhibitory, effects of 5-HTP on spinal reflexes may be mediated by serotonin released from nerve terminals of a descending pathway.

In addition to the effects on alpha motoneurones, there has also been shown an effect of intravenous 5-HTP on

gamma (γ) motoneuronal discharge. Dixon et al (1969) have shown that the discharge of single fusimotor fibers to semitendinosus muscles in decerebrate spinal rabbits is made "regular" after i.v. 5-HTP or LSD-25, but such effects are not seen in chronic spinal rabbits. Further to this, Ahlman et al (1971) have shown the occurrence of a tonic stretch reflex after 5-HTP, probably mediated by the concomittant increase in the discharge rate of static gamma motoneurones. Later studies by Ellaway et al (1973) and Ellaway and Trott (1975) disagree with the interpretation of Ahlman et al regarding the basis for the facilitation of the tonic stretch reflex. They did confirm, however, the finding that gamma motoneurone discharge is increased after 5-HTP.

Such findings are of considerable interest in view of the role of 5-HT in locomotor behavior of rabbits. Viala and Buser (1969, 1971, 1974; Viala et al, 1974) have shown that 5-HTP administration in acute spinal rabbits can cause rhythmic flexion and extension of the hind limbs, an action reminiscent of that of L-DOPA in acute spinal cats (Anden et al, 1964c). While 5-HT does not cause such obvious effects in acute spinal cats, Anderson and Shibuya (1966) have reported "integrated flexion and extension" and exaggerated flexion reflexes after 5-HTP administration in cats spinalized at the first cervical levels, although they made no attempt to study this finding

directly. The results are nevertheless highly suggestive of a possible involvement of 5-HT in mammalian locomotor behavior.

Because there is much evidence for a descending tryptaminergic pathway, but only scant reason to suspect segmental 5-HT containing neurons (Bjorklund et al, 1970), several investigations have employed electrical stimulation of the raphé nuclei to activate the terminals of this pathway in the spinal cord. Thus, several studies were able to demonstrate either bulbospinal inhibition (Clineschmidt and Anderson, 1969) or a time dependent facilitation and depression of the segmental MSR (Clineschmidt and Anderson, 1970; Proudfit and Anderson, 1973). Because the bulbospinal inhibitory effects were antagonized by various 5-HT antagonists, it was concluded that a 5-HT neurone was interposed in the bulbospinal inhibitory pathway (Clineschmidt and Anderson, 1970). If such a view were correct, then an excess of 5-HT at the synaptic terminal should potentiate the effects of stimulation of the raphé nuclei. Sinclair and Sastry (1974 a,b), however, have shown that bulbospinal inhibition is blocked, not enhanced, by imipramine, desipramine and pargyline, drugs shown to prevent re-uptake of 5-HT, and on the basis of these results they suggested a tryptaminergic pathway that exerted inhibitory control over the bulbospinal inhibitory pathway, the locus of this control being uncertain (Sinclair and Sastry, 1974a). In addition, these authors have suggested that a serotonergic pathway may enhance the NA mediated tonic inhibition

of the recurrent inhibition of quadriceps monosynaptic reflex (Sinclair and Sastry, 1974b). Such a proposal is, therefore, not in agreement with the earlier work of Clineschmidt and Anderson (1970).

In addition to the involvement of 5-HT in spinal reflexes, recent interest has focused on a possible involvement in pain, and several investigators have assigned a role for 5-HT in analgesia and the effects of morphine. It was initially shown by Liebeskind et al (1973) that analgesia to peripheral noxious stimuli could be produced by stimulation of the periaqueductal gray matter in cats. Later investigations showed that the evoked firing of certain lamina V interneurones to a peripheral noxious stimulus was depressed by electrical stimulation of the raphe nuclei (Oliveras et al,1974) and a suppression of this inhibitory effect could be produced by LSD-25 (Guilbaud et al, 1973). Samanin and Valzelli (1971) concluded that dorsal raphe stimulation could increase the effectiveness of a sub-analgesic dose of morphine, while Sinclair (1973) suggested a blockade of bulbospinal inhibition of the MSR by i.v. morphine. At a cellular level, Randic and Yu (1975) have described depression of nociceptive cells in dorsal horn lamina I neurones following iontophoretically administered 5-HT. Such a finding is somewhat surprising, however, since this area has been shown to contain very few 5-HT terminals (Carlsson et al, 1964; Fuxe, 1964; Dahlstrom and Fuxe, 1965). Proudfit and Anderson (1973) have

suggested a reduction in the dorsal root potential in Group II and Group III afferents after stimulation of the raphé nuclei, an interesting finding in view of the fact that some of Group III afferents may carry "pain information" (Iggo, 1959, 1960).

In addition to the studies employing administration of 5-HT precursors or antagonists, or electrical stimulation of the brainstem to investigate 5-HT effects in the spinal cord, other studies have investigated the effects of 5-HT administered to single spinal neurones by microiontophoresis (Curtis, 1964). In barbiturate anaesthetized cats, Curtis et al (1961) failed to find any spinal neurones sensitive to 5-HT, but such results are different from those obtained in unanaesthetized preparations. Engberg and Ryall (1966) examined the effects of 5-HT on spinal alpha-motoneurones, Renshaw cells, and interneurones of cat ventral horn, and showed that 5-HT reduced the firing of 13 of 48 interneurones excited by D, L-homocysteic acid (DLH), but was without effect on motoneurones or the remaining interneurones. This lack of effect on motoneurones is somewhat surprising in view of the increase in motoneuronal discharge seen after i.v. infusion of 5-HTP or l-tryptophan (Anderson and Shibuya, 1966). 5-HT did, however, reduce the acetylcholine (ACh) or DLH-induced firing of some Renshaw cells, a finding later confirmed by Jordan and McCrea (1976). Weight and Salmoiraghi (1966) further showed that cat

spinal interneurones may respond with either facilitation or depression of firing to iontophoretic 5-HT, in some instances with an initial depression followed by facilitation for a single neurone. Tebecis and Phillis (1967), however, described mainly potent excitatory actions of 5-HT on isolated perfused toad spinal cord accompanied by an increased excitation of motoneurones, but, in experiments performed on cats, Phillis, Tebecis and York (1968) concluded that 5-HT was probably an inhibitory transmitter. This conclusion was based on the findings that 5-HT, like NA, depressed the invasion of antidromic action potentials into motoneurone somas, as well as caused an increase in membrane polarization and a reduction in the amplitudes of both excitatory and inhibitory postsynaptic potentials. Such findings are similar to those of NA on spinal neurones, in the latter case being accompanied by a decrease in membrane conductance (Engberg and Marshall, 1971). Such an action is suggestitive of a role for both NA and 5-HT as inhibitory neurotransmitters in mammalian spinal cord.

While the evidence presented thus far is supportive of a role for 5-HT as a spinal cord transmitter, there are still several features of the tryptaminergic pathway which are somewhat unclear. Because of the nature of the fluorescent histochemical technique, there is the inherent possibility that many of the sites of termination of 5-HT fibers have not been described since (a) the 5-HT varicosities are very fine and

smooth and, at least in the hypothalamus, may be submicroscopic (Carlsson et al, 1962), (b) tryptamine fluophors are more labile under ultraviolet light than are those due to NA, hence substantial amounts of 5-HT fluorescence may have faded before being adequately determined (Fuxe et al, 1970), and (c) several authors have warned that it is possible, in some instances, to find it difficult to distinguish between 5-HT and NA fluorescence found in the same tissue (Corrodi et al, 1966). For these reasons the distribution of 5-HT terminals were examined in the spinal cords of cats depleted of their NA content.

An equally important limitation in knowledge regarding the effects of 5-HT in the spinal cord is that while the anatomical evidence summarized above suggests a monosynaptic pathway to certain spinal neurones, no direct knowledge is available concerning the conduction velocities of these descending fibers. Based on the reported size of 5-HT fibers (1-2μm) and the fact that they are reputedly unmyelinated, the expected conduction velocity would be in the range of 2 - 3.5 meters per second. Several investigations (Proudfit and Anderson, 1973; Clineschmidt and Anderson, 1970) have circumstantially supported this idea based on their findings concerning the time interval needed between brainstem stimulation and segmental afferent stimulation of the spinal cord to observe an effect at the spinal level. The time course for these effects (30 - 40 milliseconds for facilitation and 50 -

60 milliseconds for inhibition of the MSR) may represent conduction through a "slow" monosynaptic pathway or faster conduction through a pathway of an unknown number of synapses.

This thesis was undertaken in order to provide additional information regarding the distribution of 5-HT terminals in the lumbar cord of cats depleted of spinal NA as well as to provide direct evidence of the conduction velocities of these 5-HT fibers by recording antidromic responses of single raphé units to stimulation of the spinal cord. Such information is an essential prerequisite to the understanding of the role played by 5-HT in spinal cord neurotransmission.

METHODS

Fluorescent Histochemical Experiments:

In order to accurately assess the sites of termination of the 5-HT fibers in the lumbar cord, spinal NA was first depleted by intraspinal injection of 6-hydroxydopamine (6-OHDA) at the thoraco-lumbar (T-L) junction. This substance has been shown to cause fairly specific lesioning of catecholamine fibers (Malmfors and Sachs, 1968). Adult cats were anaesthetized with 35 mg./kg. sodium pentobarbital (Nembutal) intraperitoneally (i.p.) and the skin over the T-L junction was excised. The spinal cord was exposed, the dura mater was cut, and the animal was rigidly held in a spinal frame by clamps attached to spinal vertebrae. The 6-OHDA was dissolved in deoxygenated 0.9% saline with 1.0% ascorbic acid and injected into the spinal central gray at the T-L junction through a 30 gauge needle. The injections were performed stereotaxically at a rate not exceeding 1 microliter (ul) per minute and the needle was left in place for several minutes following injections. The spinal cord was then covered with gelfilm, the skin sutured and a topical antibacterial spray applied. In addition, 50 mg. ampicillin was administered intramuscularly. Survival times following the surgical procedure varied from 7 - 12 days before the spinal cords were removed for histochemical and biochemical analyses. Under halothane-nitrous oxide anaesthesia, a dorsal laminectomy was performed, the dura resected and small pieces of lumbar spinal cord were rapidly dissected out, coated with talc and quickly frozen in liquid nitrogen. Larger sections used for biochemical deter-

minations of lumbar monoamine levels were frozen in liquid nitrogen and stored at -80°C pending analysis. The biochemical analysis of spinal cord monoamine levels was carried out as reported by Jordan et al (in preparation) according to the method of Shellenberger and Gordon (1971) and the results expressed in nanograms of monoamine per gram tissue weight (ng./gm.).

To control for the nonspecific damage that might have occurred to the spinal cord due to the injection procedure, "sham" injections were also performed in control cats. The injection procedure as outlined above for 6-OHDA was identical in sham animals except that an equal volume of 0.9% saline with 1.0% ascorbic acid was administered. Pieces of tissue for biochemical analysis were removed from these animals in the same manner as for the 6-OHDA treated cats and levels of 5-HT and NA determined by the same procedure as for the experimental animals. This procedure allowed for an evaluation of the amount of both NA and 5-HT depletion caused by 6-OHDA.

The sections used for fluorescence histochemistry were removed from the liquid nitrogen and freeze dried in an Edwards-Pearse tissue dryer under vacuum with phosphorous pentoxide for 4 - 6 days at -40°C. At the end of this time, the tissues were removed from the freeze-dryer, exposed to

formaldehyde gas at 70% relative humidity at 80°C for 80 minutes (Dahlstrom and Fuxe, 1964; Marsden and Kerkut, 1969) and embedded in paraffin. Tissue sections 10 µm in thickness were cut, mounted on albuminized slides, examined for monoamine fluorescence with a Zeiss fluorescent microscope, and photographs were taken of fluorescent terminals using high speed colour and black and white film. Under ultraviolet light, fluophors due to indolealkylamines (5-HT) appear yellowish, while those due to the presence of catecholamines (NA,DA) give a greenish colour (Falck, 1962; Fuxe and Jonsson, 1973). Hence the presence of different monoamines is discernible on the basis of the colour of the fluorescent product. A further differentiation is possible since the fluophors of 5-HT are more labile under ultraviolet light than are those of NA or DA (Dahlstrom and Fuxe, 1965). Thus the rapidity of fading of fluorescent products can be used as an indicator of the presence or absence of 5-HT.

The distributions of the 5-HT terminals in the lumbar gray matter were plotted on cross-sectional drawings of several spinal cord sections and a composite drawing was made showing the distribution in both ventral and dorsal horns. In addition, the relative frequency of occurrence of these terminals was determined by assigning to them a value on a scale of 1 to 5 (Fuxe, 1965) for the following areas of the spinal cord: (a) dorsal horn, (b) area of central canal, (c) medial

areas of anterior horn, (d) lateral area of anterior horn and (e) intermediate zone, and the data was represented on composite drawings of cross sections of the spinal cord. Photomicrographs were also made of the above areas of the cord in an effort to illustrate the nature of 5-HT terminal distribution in the lumbar cord.

In a separate series of experiments aimed at determining whether 5-HT containing axons in the white matter of the spinal cord possess myelin sheaths, 2 cats were spinalized in the upper lumbar region under Nembutal anaesthesia and allowed to recover for 5 days before the lumbar cord immediately above the transection was removed and subjected to the freeze-drying procedure outlined above. Four hours prior to removal of the spinal cord tissue, the cats were administered 100 mg./kg. Nialamide (pH 7.0) intraperitoneally, in order to enhance the build-up of 5-HT in axons central to the transection (Dahlstrom and Fuxe, 1964). Following examination of the fluorescent tissue, the sections were counterstained with Luxol Fast Blue to demonstrate myelin sheaths and the counterstained material photographed at the same locations as in the fluorescent tissue. These photographs were then compared and the axons which had previously contained 5-HT fluorescence located. The counterstained sections were then viewed under a microscope and the presence or absence of the myelin sheath noted.

Electrophysiological Experiments:

Twelve successful experiments were carried out on adult cats anaesthetized either with alpha-chloralose (80 mg./kg. intravenously), sodium pentobarbital (35 mg./kg.i.p.) or halothane in 70% nitrous oxide (N_2O), and 30% oxygen (O_2). Both carotid arteries were ligated and the right carotid was cannulated for continuous monitoring of arterial blood pressure. Mean arterial blood pressure was maintained above 75 mm. Hg. by intravenous injection of dextran when necessary. Body temperature was controlled automatically by means of a heating pad, a rectal thermistor probe and a feedback circuit, and maintained at 37.5°C. Animals were paralyzed by continuous intravenous infusion of 0.08% succinylcholine and artificially respired. Expired air pCO_2 was monitored and maintained between 4.5 and 5.5%. In some cases a bilateral pneumothorax was performed to aid in the stability of recording and, in such cases, respiratory parameters were adjusted to give pCO_2 values obtained prior to this procedure.

The spinal cord was exposed by dorsal laminectomy from T_{12} to L_3 and transected at L_1 - L_2. The animals were then placed in a Transvertex spinal frame and firmly held by clamps onto spinal vertebrae. The skin was tied to the sides of the spinal frame and the spinal cord covered by mineral oil maintained at 37.5°C by a thermistor probe and a heating

lamp. The head was rigidly held in a stereotaxic head frame.

The central end of the cut spinal cord was mounted on an electrode array that consisted of a thin silver wire in the area of the central canal and two small silver ball-tipped electrodes placed on the surface of the cord bilaterally near the lateral funiculus. Two additional silver ball-tipped electrodes were placed on the anterior funiculus near the anterior median fissure. Bipolar stimulation was accomplished by switches which allowed stimulation between the centrally placed wire and any one of the four surface electrodes. Stimulation was effected through a Grass S88 square wave stimulator, stimulus isolation units and constant current device. Square wave pulses of 0.5 millisecond duration and from 10 microamperes to greater than 1 milliampere could be delivered in this fashion.

In order to record antidromic responses to stimulation of the spinal cord, a craniotomy was performed over the cerebellum, the cerebellum was removed using a blunt spatula, and the floor of the fourth ventricle was exposed. Bleeding was controlled by packing the edges of the rhomboid fossa with Surgicell ® (Johnson & Johnson).

Glass microelectrodes for extracellular recording (tip diameter = 1.5 μm) were routinely filled with 3M Na Cl and, in some cases, electrodes were filled with 3M Na Cl and a supersaturated solution of fast green dye (FGF, Matheson Coleman and Bell). After extracellular recordings had been

made, a maintained negative current (-15 μA for 20 min.) was passed through the electrode and the dye ejected in the vicinity of the unit from which the recording had been obtained. In subsequent histological treatment of the tissue it was possible to verify the position of the unit to within a fairly localized area (50 - 100 μm.).

Single unit action potentials were monitored after amplification by a WP Instruments model M-4A precision electrometer on an oscilloscope and Grass audio monitor, and traces from a Tektromix 565 cathode ray oscilloscope (CRO) were photographed on Kodak RAR 2495 film. Latency measurements were made from the beginning of the stimulus artifact to the onset of the single unit response and they were computed by both direct inspection from the CRO and checked by later measurement directly from the photographic film.

In order to separate recordings obtained from axons from those obtained from cell bodies, intracellular recordings were made in as many instances as possible. A unit was classified as a cell body and not a fiber by the presence of an inflection on the rising phase of the action potential either spontaneously or in response to intracellular application of hyperpolarizing current (Eccles, 1955), and the presence of an afterhyperpolarization. The presence of an inflection is taken as evidence

that the recording is from a cell body since it indicates that the action potential is composed of two temporally distinct components arising from two different parts of the neurone. Since the initial segment of the axon is considered to be the site of impulse generation for both antidromic (Brock et al, 1952) and orthodromic (Araki et al, 1953) responses, the action potential recorded from the neurone soma consists of the temporally earlier initial segment response (IS) and the later soma-dendritic (S-D) component.

Mapping of the distribution of antidromically activated units in the raphé nuclei was accomplished using microelectrode penetrations made in the medial brainstem from 4.0 to 7.5 mm. anterior to the obex along the midline. This area has been shown to contain the largest numbers of descending projections from the raphé to the spinal cord (cf introduction).

Units were classified as antidromic if they showed (a) a constant latency which did not change with an increase in the frequency or intensity of stimulation of the spinal cord, (b) the ability to follow repetitive stimulation of the spinal cord at frequencies greater that 200 Hz, and (c) collision of action potentials evoked from the cell soma and the site of stimulation in the cord. Collision was demonstrated in 10 cells with conduction velocities ranging from 53.2 to 115 m/sec. In this latter event, an antidromic response will not occur to stimulation of the spinal cord if an action potential has been

generated by prior intracellular stimulation at the cell soma at a minimum time interval of twice the latency of response plus the refractory period of the cell. In the case of an orthodromic response capable of following at high frequencies, the minimal time period between stimuli necessary to prevent an action potential is the sum of the synaptic delay, the refractory period of the cell, plus the conduction time of the action potential. Thus by comparison of the minimal time period needed for collision to occur, it is possible to decide whether or not a unit is antidromically or orthodromically activated.

Following completion of the experiments, the brainstem was removed and prepared for histological analysis by one of two methods. The tissue was either processed by the Falck - Hillarp fluorescence histochemical method as outlined previously, or cut at 30 μm on a Vibratome® (Oxford Instruments) (Hokfelt et al, 1974), placed on albuminized slides, exposed to paraformaldehyde gas at 70% relative humidity and later examined for monoamine fluorescence with a fluorescence microscope. In cases where fast green dye had been injected (-15 μA for 20 min.) sections were examined for fluorescent cell bodies as previously described, and then counterstained with thionine for cell bodies to determine the relative numbers of both 5-HT and non 5-HT containing cells in the vicinity of the electrode tip.

After termination of the experiment, the entire spinal cord was exposed and the length of the cord measured post mortem by placing a piece of string along the dorsal surface of the cord from the obex to the positions of the stimulating electrodes. Such measurements were used to compute the conduction velocities (CV) of antidromically activated units, based on the formula CV = Distance (meters/time (sec.)).

RESULTS

Distribution of 5-HT Fibers in Lumbar Cord

In order to accurately determine the distribution of 5-HT terminals in a feline spinal cord, it was essential to establish that the observed fluorescence was due to 5-HT and not NA. For this reason, several criteria were met before the identity of the fluorescence could be satisfactorily established. In addition to removing pieces of tissue for fluorescence histochemistry, pieces of tissue were simultaneously taken from adjacent regions of the spinal cord, frozen in liquid nitrogen and stored at -80°C pending biochemical analysis for both the NA and 5-HT content. Results of this procedure revealed that after 6-OHDA treatment, spinal cord NA levels were reduced to 57 to 13.7 ng. per gram tissue weight as compared to a mean of 194.3 ng./gm. for control cats. The mean level of spinal cord 5-HT was 852 ng./gm. tissue in control animals as compared to 751.3 ng./gm. in 6-OHDA treated animals. These results provide a high degree of certainty that the fluorescent terminals seen were due to 5-HT and not NA. Secondly, almost all of the terminal varicosities were observed to show a yellowish colour indicative of the presence of 5-HT (Falck, 1962) while very few showed the greenish fluorescence characteristic of NA (Falck, 1962; Fuxe and Jonsson, 1973). Thirdly, during the course of examination of the tissue, the fluorescence faded very quickly and it was essential that the examination and photography be carried out within 1 to 2 minutes.

Such fading indicates that the fluorescence is due to the presence of 5-HT since NA does not fade nearly so rapidly under exposure to ultraviolet light (Dahlstrom and Fuxe, 1965). On the basis of these criteria, it was possible to establish that the fluorescent terminals remaining after 6-OHDA treatment were those of 5-HT containing axons. Only in very rare cases were terminals with a greenish colour observed, and these were considered to contain NA.

The relative density of the 5-HT terminals in various areas of the lumbar spinal cord is presented in Table 1 and schematically represented on the right side of Figure 1. Numerous fine yellow terminals were seen in the ventral horn and area of the central canal, with fewer in the dorsal horn and very few in the intermediate zone. In the gray matter of the ventral horn many yellow terminals were found which were distributed in a band just dorsal to the underlying white matter of the ventral funiculi. This band extends medio-laterally across the gray matter with the greatest concentration of terminals along the medial border of the ventral horn (Figure 2). In this region it was consistently observed that there were many terminals distributed throughout the cell bodies located herein (Figure 3), and in some cases it was possible to observe fine yellow varicosities in close proximity to either soma

AREA of LUMBAR CORD	TERMINAL DENSITY
DORSAL HORN	2
CENTAL CANAL	3
INTERMEDIATE ZONE	1
VENTRAL HORN	
LATERAL	3
MEDIAL	4

TABLE 1: The density of the 5-HT terminals innervating various regions of the gray matter of the lumbar spinal cord. The innervation density is on a scale of 1 to 5.

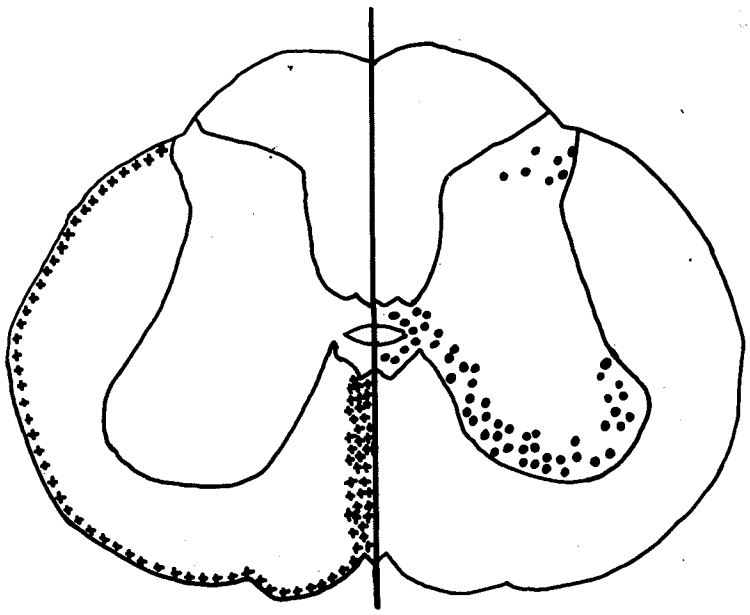
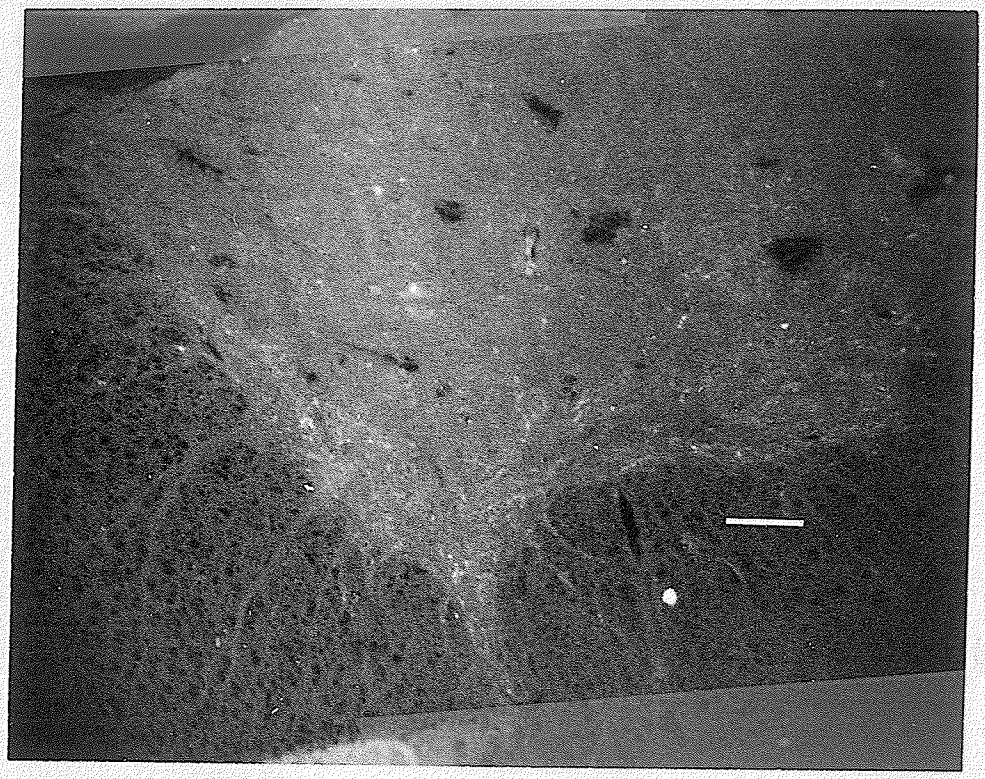


FIGURE 1: A schematic representation of a cross section of cat lumbar spinal cord showing the distribution of both terminal(●) and non-terminal (+) 5-HT containing fibers.



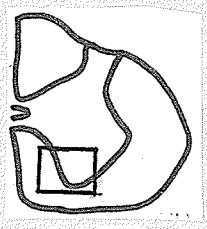
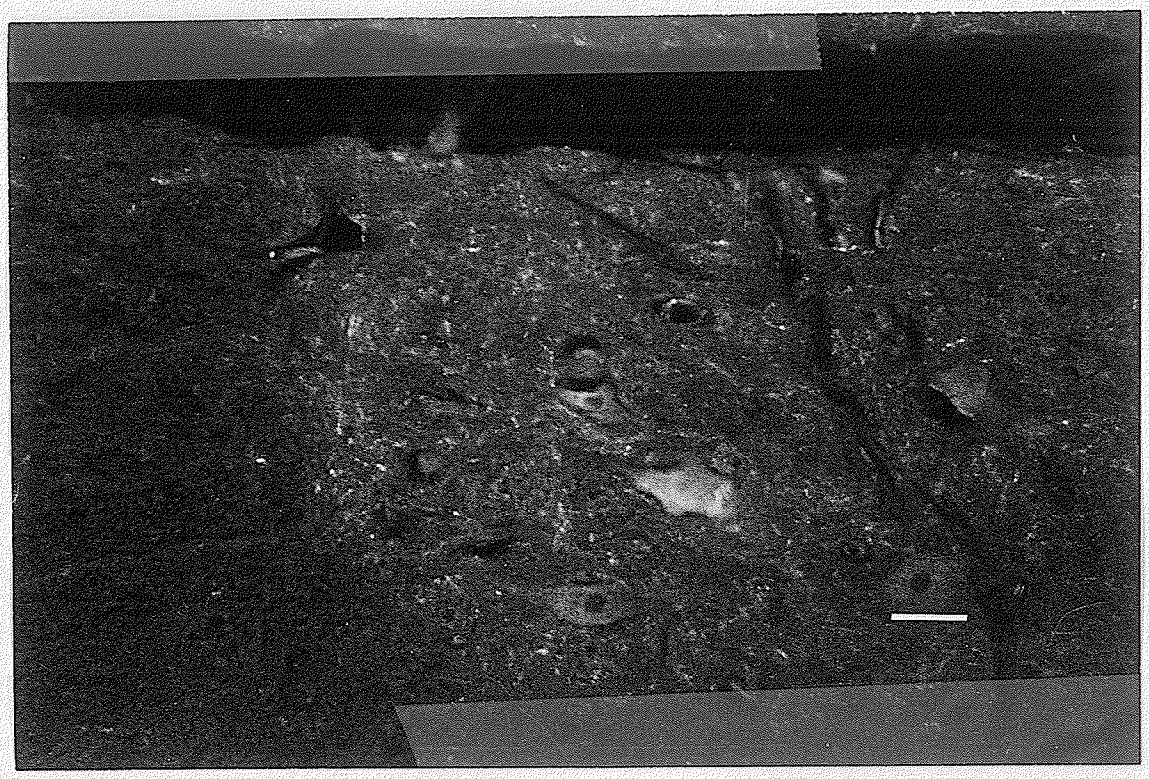


FIGURE 2: A low power photomicrograph of the distribution of 5-HT terminals within the lumbar cord of a 6-OHDA treated cat. The medial side of the ventral horn is to the left in the photo. Calibration bar represents 125µm. The figure accompanying the caption indicates the location in the spinal cord at which the photograph was taken.



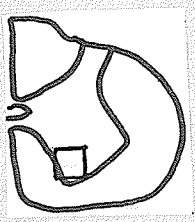


FIGURE 3: A high power photomicrograph of the medial aspect of the anterior horn in a 6-OHDA treated cat spinal cord. Calibration: 50μm. The figure accompanying the caption indicates the location in the spinal cord at which the photograph was taken.

(Figure 4) or dendrites (Figure 5) of anterior horn cells. These were presumed to be alpha motoneurones on the basis of their large size and location. These yellow terminals were never observed to be in close contact with small ventral horn cells; however, since large alpha motoneurones are more visible in fluorescent tissue than are small cells, there is a definite bias in favour of seeing fluorescent terminals close to alpha motoneurones but not to smaller cells.

In the area of the central canal there were many fine yellow terminals (Table 1 & Figure 6), some of which appeared to decussate in both the dorsal and ventral commissures. Because it was possible to follow many of these terminals for short distances only, it is difficult to conclude the proportion of crossing vs. non-crossing fibers. Just ventral to the area of the central canal along the medial wall of the ventral horn, there were numerous yellow terminals similar in size and colour to those described for other areas of the ventral horn and central canal. Many of the fibers in this region appeared to be coursing through this area and were not seen in close association with cell bodies. However, the absence of such associations does not mean that they do not exist, and since no attempt was made to examine counterstained material of the fluorescent tissue, it cannot be concluded that such terminals are merely passing through

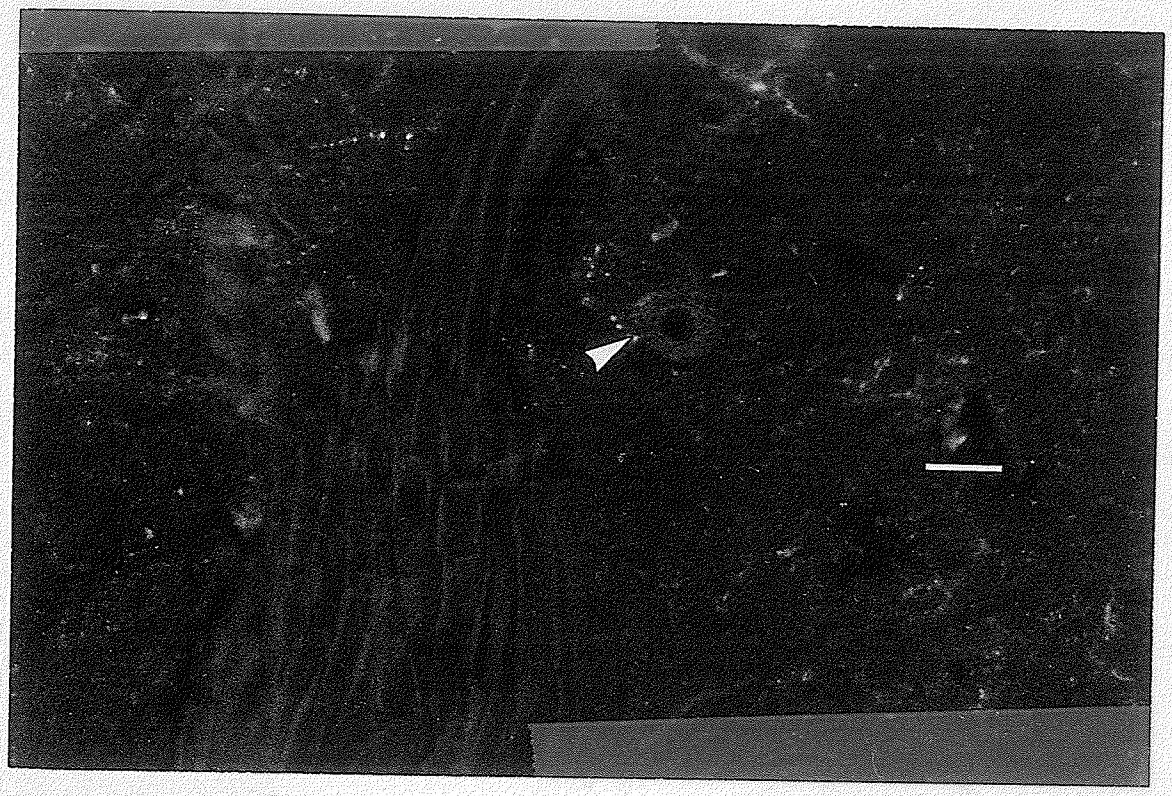
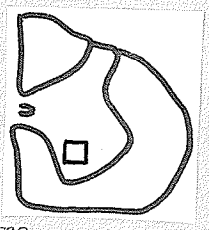


FIGURE 4: A high power photomicrograph from the ventro-medial area of the anterior horn showing the close proximity of fluorescent 5-HT terminals to the soma of a large alpha-motoneurone. Calibration: 50µm. The figure accompanying the caption indicates the location in the spinal cord at which the photograph was taken.



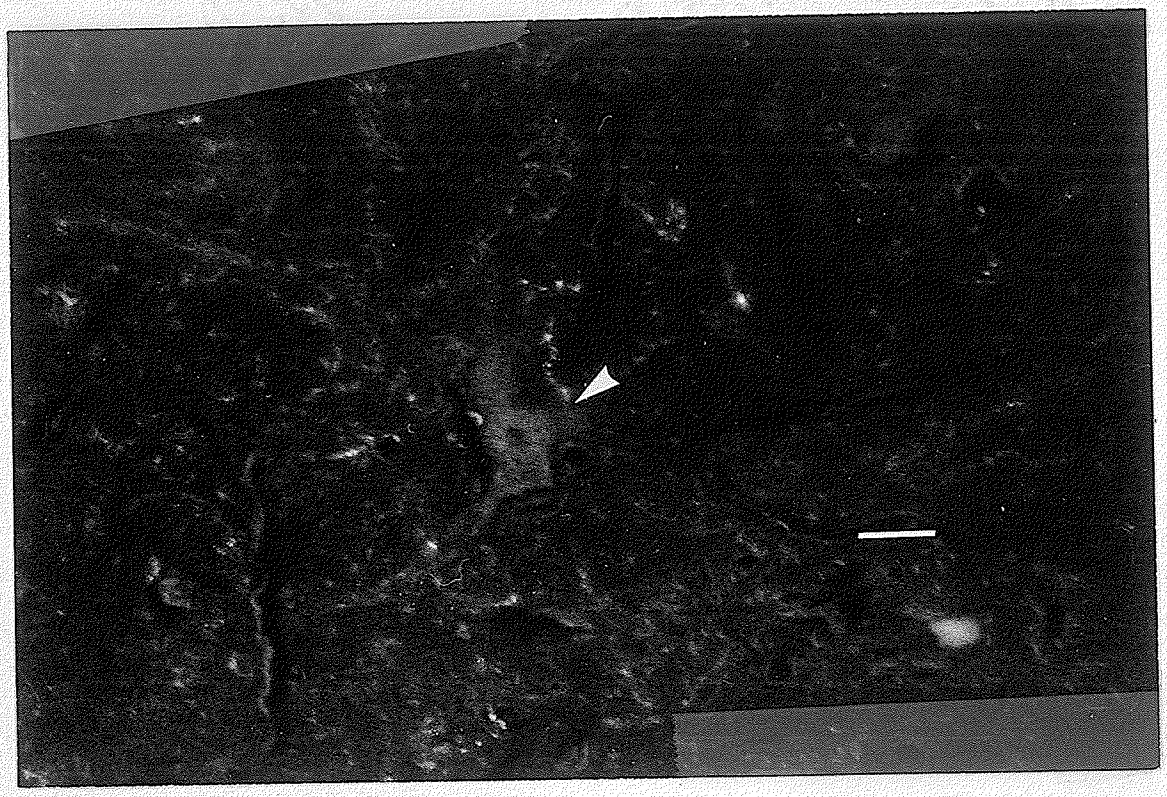
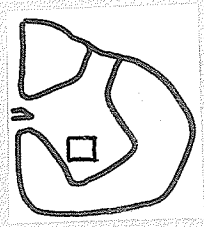


FIGURE 5: A high power photomicrograph of the ventral horn of a 6-OHDA treated cat showing the close proximity of fluorescent 5-HT terminals to dendrites of a large alpha-motoneurone. Calibration: 34μm. The figure accompanying the caption indicates the location in the spinal cord at which the photograph was taken.



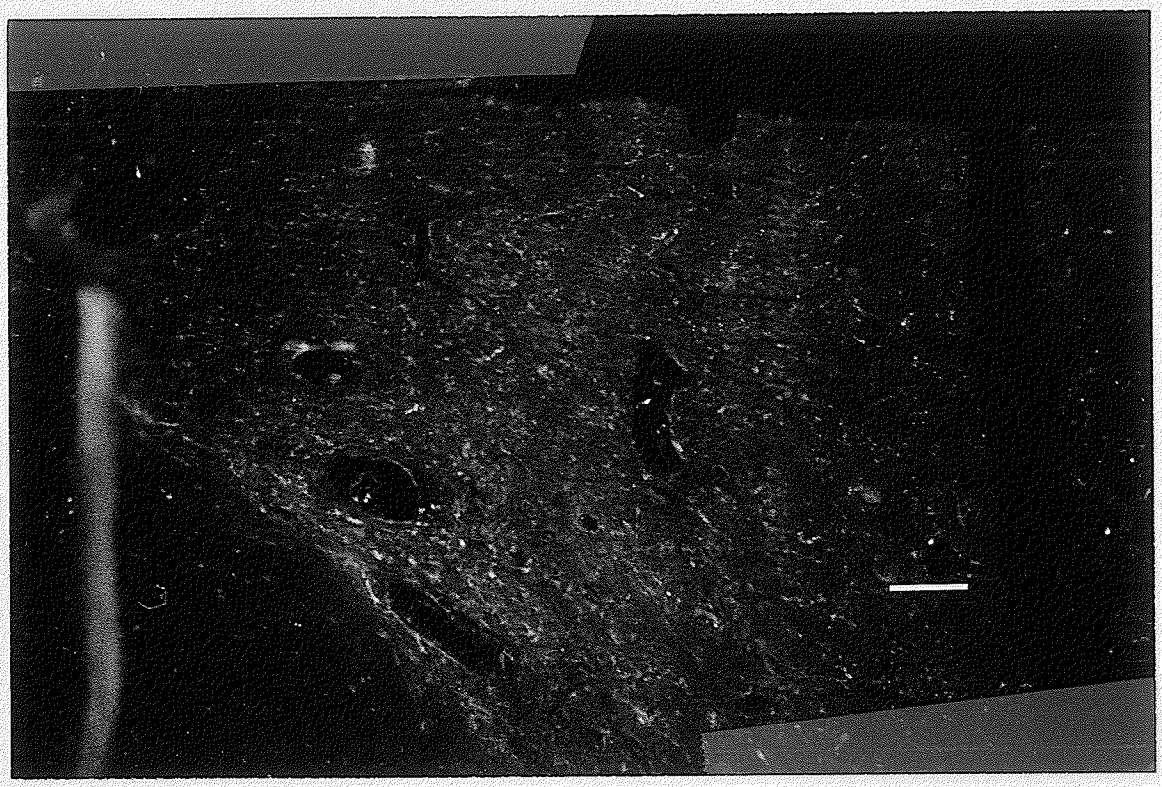
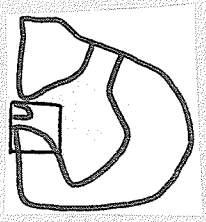


FIGURE 6: A high power photomicrograph of the area of the central canal and upper medial aspect of the ventral horn in the lumbar spinal cord of a 6-OHDA treated cat. Calibration: 75µm. The figure accompanying the caption indicates the location in the spinal cord at which the photograph was taken.



this area and do not contact cell bodies.

Only very few terminals were seen in the dorsal horn with somewhat more of them in the upper, but not the more ventral layers. This absence of terminals in the ventral aspect of the dorsal horn is illustrated in Figure 7. These fibers were similar in colour and shape to those found in other areas of the lumbar cord; however, in view of the limited number of terminals observed in this horn, it is not possible to decide the extent to which they make contact with cells of this region.

The distribution of 5-HT fibers in the white matter of the spinal cord was investigated in two cats spinalized at the thoraco-lumbar junction five days prior to removal of spinal cord sections rostral to the transection. The tissue was processed according to the Falck-Hillarp fluorescence histochemical method (cf Methods). The distribution of non-terminal containing 5-HT axons of the thoracic level is shown in the left side of Figure 1. This distribution of fibers was discerned on the basis of examination of many cross sections of thoracic spinal cord and the composite result presented in Figure 1. Because in chronic spinal animals there is a build-up of amine proximal to the transection, localization of fibers is easier than in non-spinal animals. However, there is concommittant swelling and distortion of the axons (Dahlstrom and Fuxe, 1965), hence valid decisions regarding

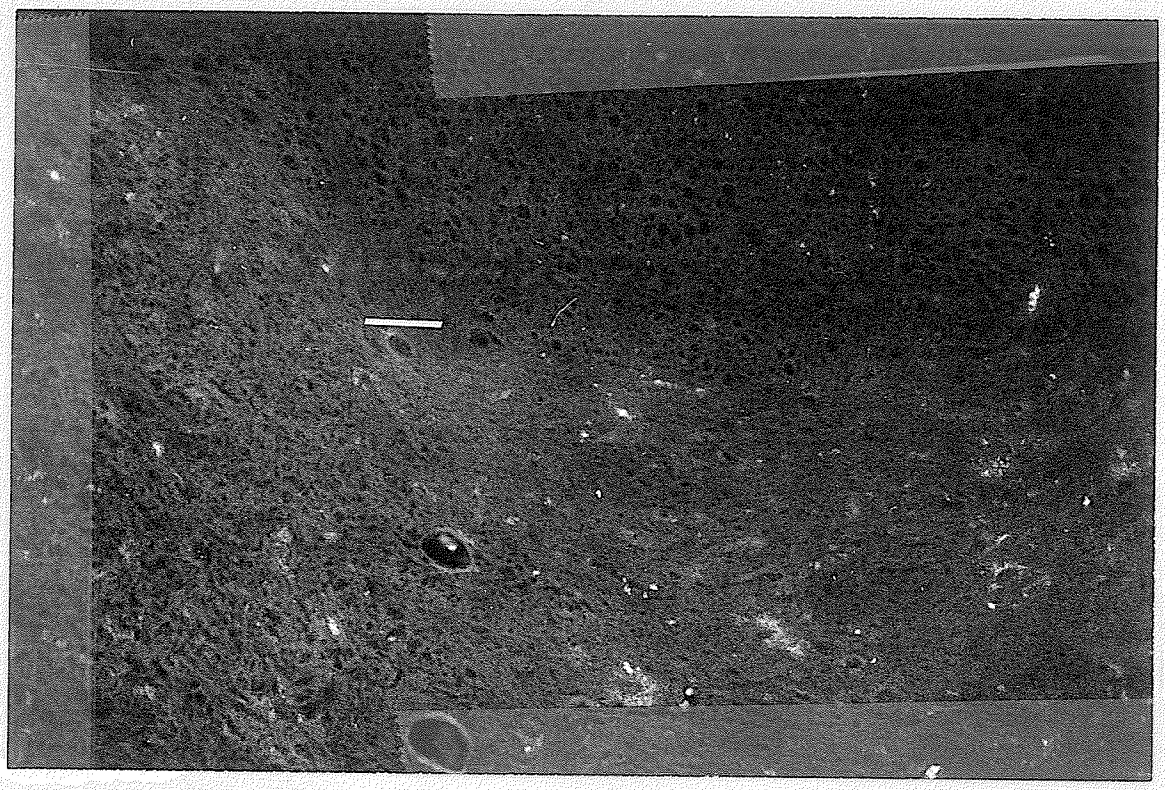
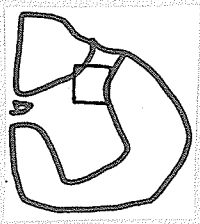


FIGURE 7: A high power photomicrograph of the dorso-medial area of the dorsal horn. Calibration: 60µm. The figure accompanying the caption indicates the location in the spinal cord at which the photograph was taken.



the diameters of these fibers are not possible. Because of the easier localization of these fibers, it was possible to examine the position of axons in the fluorescent material. Some of the fibers could be followed for some distance in sagittal sections and were seen to be smooth, although many beaded fibers were also observed (Figure 8). In some cases, the fibers could be seen travelling beneath the outer surface of the spinal cord, in general, confirming the pattern seen in cross section.

ELECTROPHYSIOLOGICAL STUDIES

Properties of Antidromically Activated Units

In confirmation of earlier anatomical studies (Brodal et al, 1960; Kuypers and Maiskey, 1975) showing that raphé neurons project to the spinal cord, stimulation of the ventral and lateral funiculi at the first lumbar level resulted in antidromic activation of raphé neurons. In all, 95 units were antidromically activated in 12 successful experiments. Of these, 48 were intracellular and 37 were extracellular recordings. Extracellular recordings (Figure 9) were considered to be antidromic if they showed a constant latency to repetitive stimulation of the spinal cord and were capable of following at high frequencies (>200 H_Z). The range of conduction velocities for extracellular recordings was from 18.1 to 120 meters per second

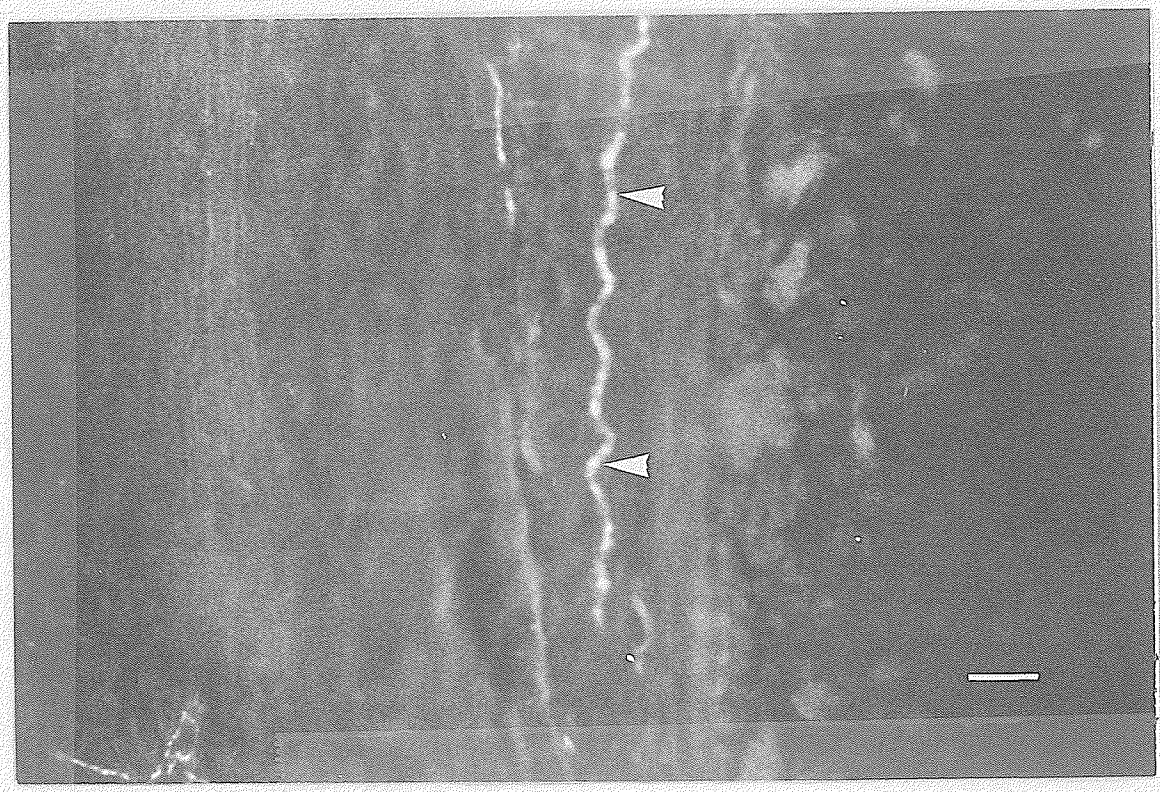


FIGURE 8: High power photomicrograph of a sagittal section of thoracic spinal cord from a chronic spinal cat transected at the thoraco-lumbar junction and treated with Nialamide (100 mg./kg.) 4 hours prior to removal of the tissue. The arrows indicate the presence of a 5-HT containing axon running just beneath the external surface of the cord. Calibration: 30μm.

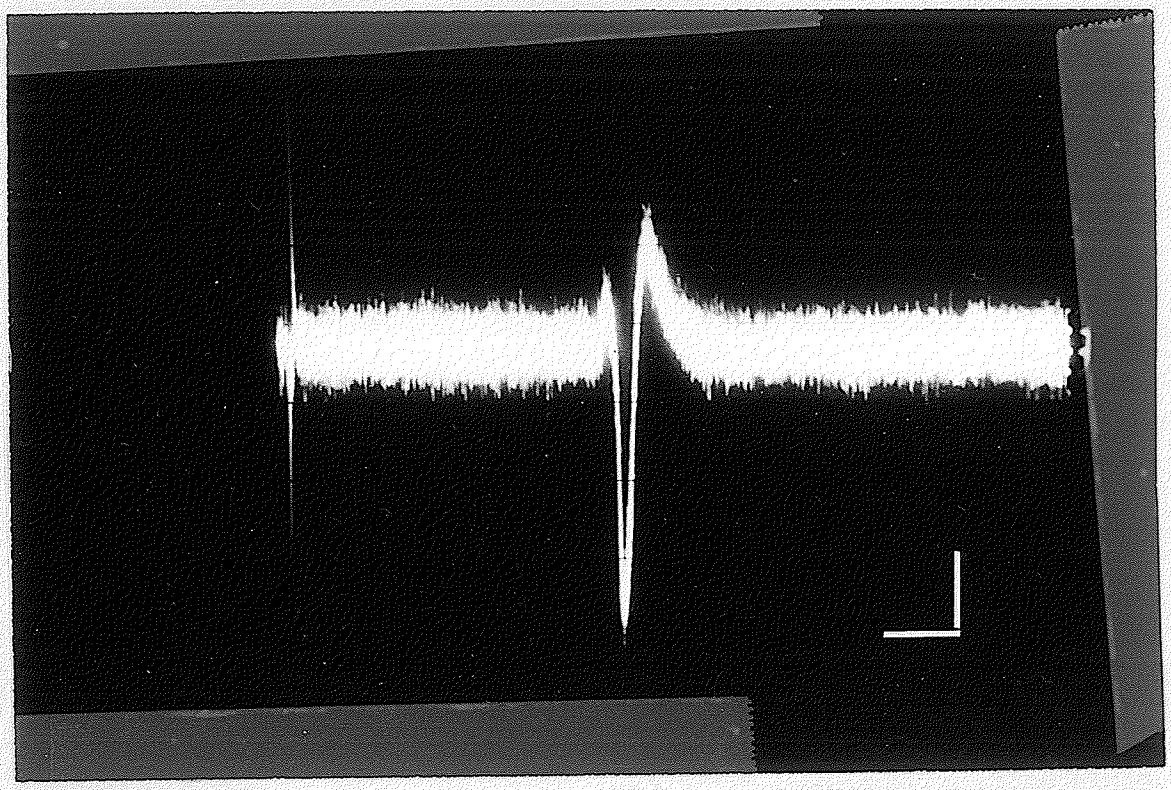


FIGURE 9: Medial brainstem neuron fired antidromically by stimulation of the anterior funiculus at the first lumbar level. Calibration: 1 msec and 0.5 mV.

(Figure 10). Since it was not possible on the basis of extracellular recordings to ascertain whether or not the recording was from an axon or cell body, intracellular records were obtained as well. Recordings were considered to be from cell bodies if the following criteria were met: (a) the presence of an afterhyperpolarization (Figures 11 and 12), (b) the presence of an inflection on the rising phase of the spike (Figure 12) and (c) collision of action potentials can be demonstrated (Figure 13). Collision was tested in 10 cells with conduction velocities ranging from 53.2 to 115 m/sec and was demonstrated by first firing the cell by intracellular injection of the depolarizing current through the recording electrode followed by stimulation of the spinal cord at varying intervals. If a unit is being activated antidromically, then the minimum time interval for its appearance after previous activation by the intracellular current is equal to twice the antidromic conduction time plus the refractory period of the cell (Jankowska and Roberts, 1972). In the examination shown in Figure 13, the action potential due to intracellular depolarizing current (second arrow in traces A, B and C) is superimposed on the current pulse (onset indicated by first arrow). In trace A, the interspike interval is 8.0 msec and there is a consistent response to both intracellular and spinal cord stimulation. In trace B, however,

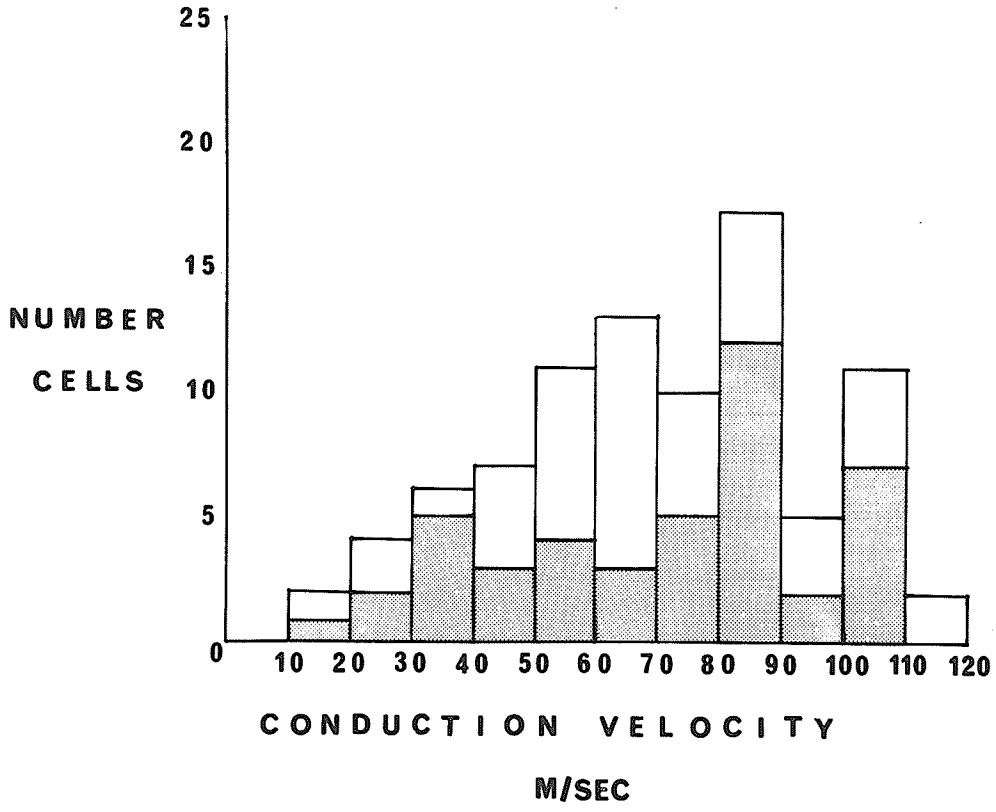


FIGURE 10: Frequency distribution of the conduction velocities of antidromically activated units in the medial brainstem of caudal pons and upper medulla. Stippled portions of the bars refer to cells recorded intracellularly and the clear portions represent cells from which extracellular recordings were obtained.

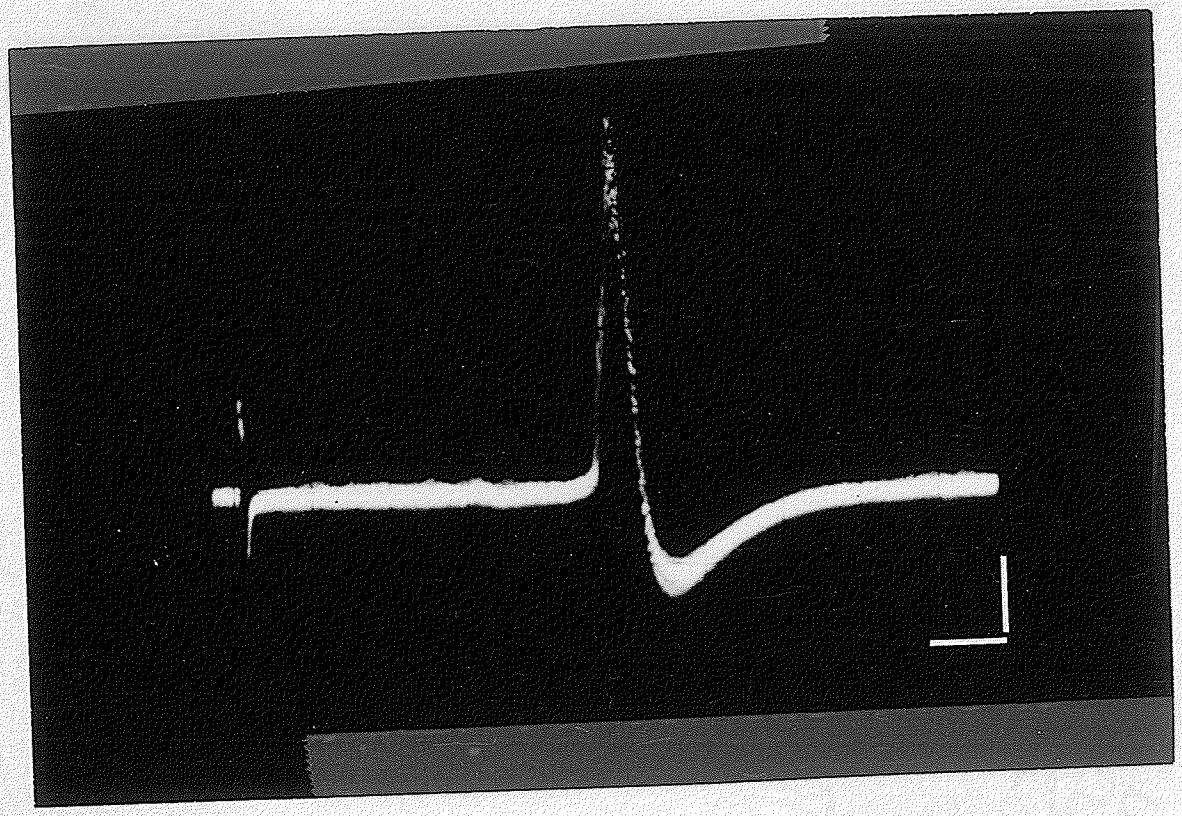


FIGURE 11: Intracellular recording from a single unit in the medial brainstem showing a spike followed by a large afterhyperpolarization. The cell was fired by stimulation of the spinal cord at L_1 anterior funiculus at 2.5 H_Z. Calibrations: 1 msec and 2 mV.

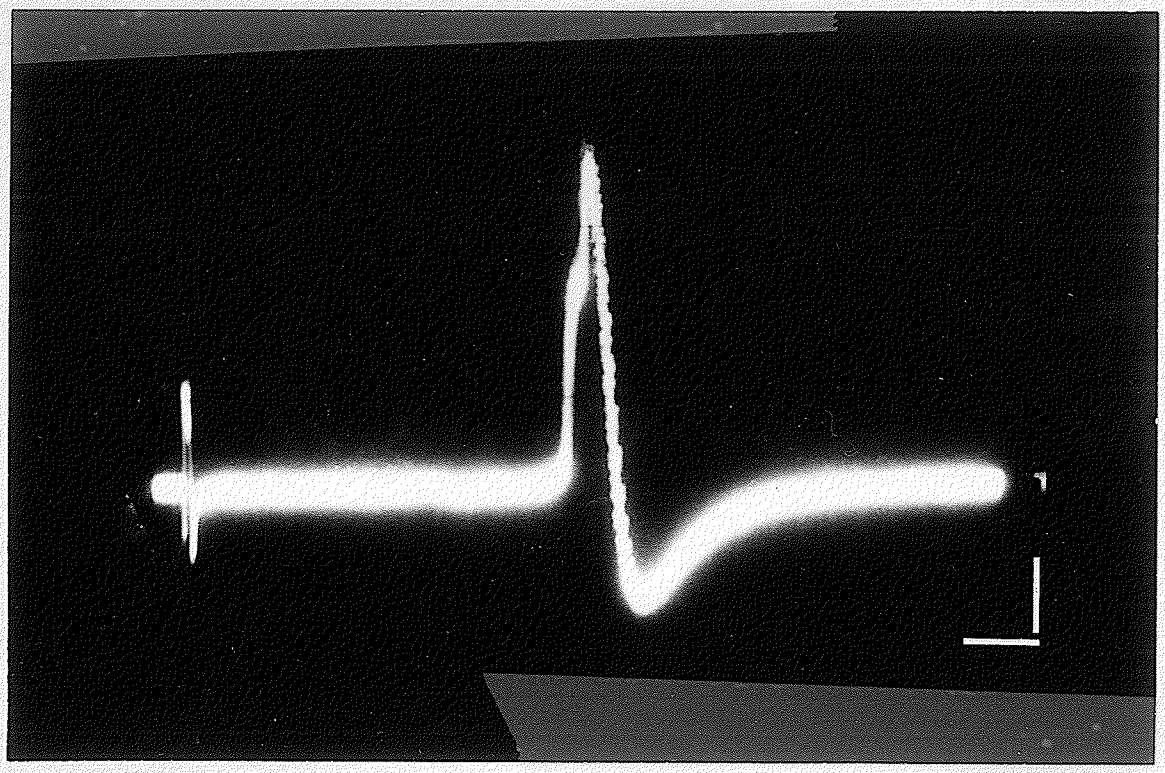


FIGURE 12: Intracellular recording from the same unit as shown in figure 11 at 10 H_Z stimulation. Note the presence of an inflection on the rising phase of the action potential. Calibration: 1 msec and 2 mV.

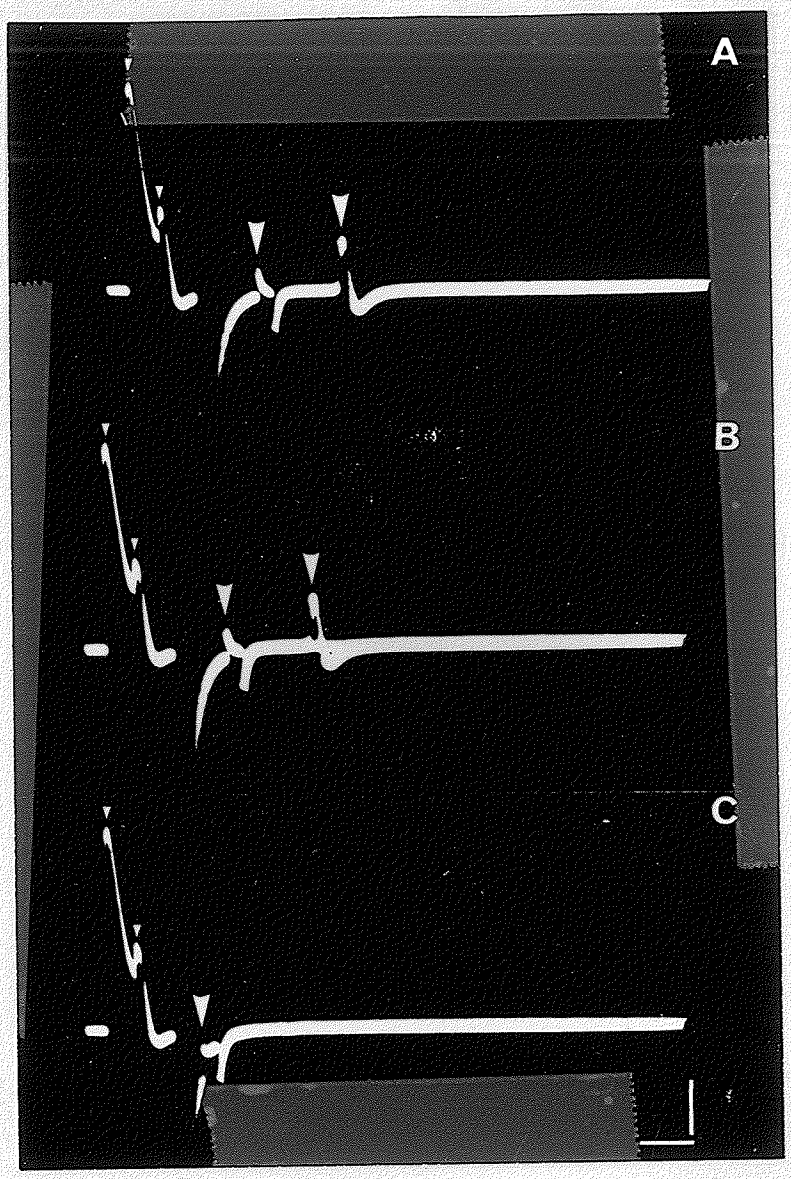


FIGURE 13: Intracellular recording showing collision. In trace A, the intracellular and spinal cord stimuli are separated by 8.0 msec and the spike consistently occurs in response to both pulses. The stimulus artifact from the intracellular current injection is shown by the first small arrows and the spike due to this pulse by the second small arrows in traces A, B and C. The stimulus artifact from stimulation of the spinal cord and the spike due to this stimulus are indicated by large arrows in all traces. In trace A at an interspike interval of 8.0 msec, the cell responds consistently to both stimuli. As the interval shortens

... Cont'd

to 6.0 msec there is partial occlusion (B) and when the interspike interval is less than 6.0 msec (C) there is complete occlusion of the response to spinal cord stimulation. Note the consistent occurrence of an action potential in response to intracellular depolarizing current application. In order to visualize the response to intracellular stimulation, the traces have been filtered. (Calibration: 1.5 msec and 10 mV.

the action potentials are separated only by 6.0 msec and collision occurs some of the time. At short intervals (trace C) there is consistent response to intracellular current but no response to spinal cord stimulation. The latency of this cell was 2.3 msec, hence the interval in trace B is approximately 1.2 msec greater than twice the antidromic time and such a value is consistent with the refractory period for other reticulospinal neurones (Magni and Willis, 1963b).

It was possible in most cases to identify units as cells by both the criteria (a) and (b) above; however, in several instances hyperpolarizing current applied through the recording electrode was necessary to demonstrate the IS-SD separation. Such a separation is indicative of a recording in the soma near the axon initial segment. Since the axon initial segment is considered to be the site of origin of the propagated spike for antidromic activation of a neurone (Eccles, 1955), the inflection on the rising phase of the action potential indicates that the initial segment is firing first followed by discharge of the soma-dendritic membrane. By thus noting the presence or absence of an IS-SD separation (either spontaneously or by intracellular hyperpolarizing current injection) it is possible to decide whether or not the recording is from a cell body or fiber.

The range of conduction velocities observed for intracellular recordings was from 17.3 to 110 meters per second, and from examination of the frequency distribution of conduction velocities (Figure 10) it can be seen that the distribution is very similar for both intracellular and extracellular recordings. This suggests that both types of recordings were obtained from similar populations of neurones.

Because the range of conduction velocities encountered were inconsistent with the values expected for small unmyelinated fibers (2.5 - 3.5 m/sec), it was necessary to explore the possibility that the 5-HT fibers were myelinated. Correspondingly in sagittal sections of thoracic cord from 2 chronic (5 days) spinal cats, the positions of non-terminal 5-HT containing fibers were located and photographed. After these sections had been counterstained with Luxol Fast Blue, it was possible to determine the corresponding location of the fluorescent fiber. By using this method, it was possible to observe that some of the axons shown to contain 5-HT (Figure 14A) were also surrounded by a myelin sheath (Figure 14B). Because of the swelling and distortion of axons central to a transection (Dahlstrom and Fuxe, 1965), no valid conclusions can be reached regarding the sizes of 5-HT axons, however, the fact that at least some of the axons appear

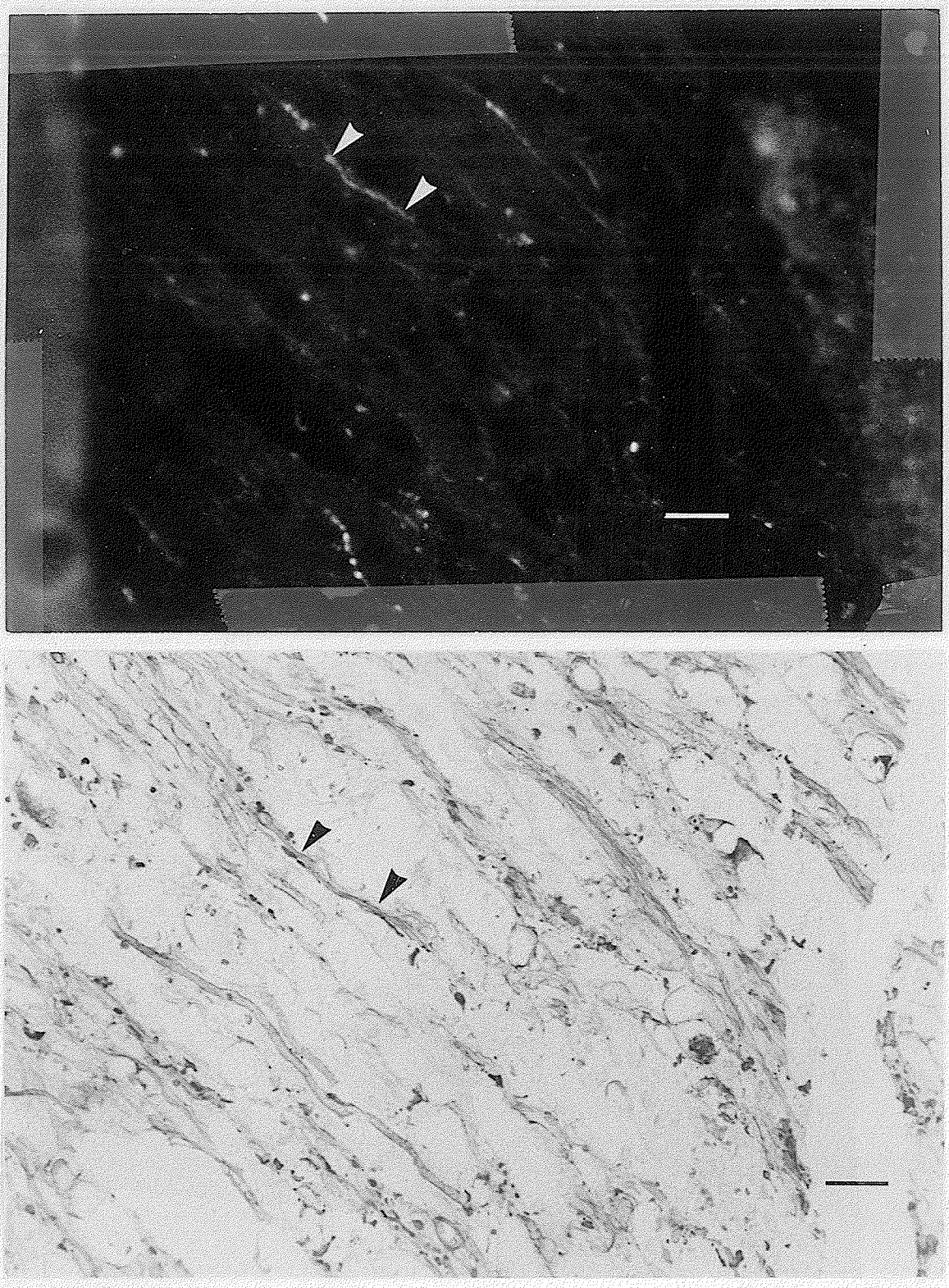


FIGURE 14: High power photomicrographs of 5-HT axons in the white matter of thoracic cord of a chronic spinal cut in fluorescent tissue (A) and Luxol Fast Blue (B) stained material. The same axon in both photographs is marked by arrows. Calibration: 41µm. for photgraph (A) and 23µm for photograph (B).

myelinated may aid in explaining the discrepancy between the predicted values for conduction velocity and those obtained in the present study.

Intracellular recordings revealed that the cells in this area varied greatly in their ability to follow repetitive stimulation of the spinal cord. For many units there was a clear separation of IS and S-D components at high frequencies (eg. 250 H_Z), although for other units no such separation was observed. Rather, there was a partial failure in these units characterized by the consistent appearance of the IS component but only intermittent appearance of the SD spike. For other units, however, no separation was seen even at frequencies above 300 H_Z (Figure 15). The rise times of these units were typically very fast with a duration in the order of 1 msec as measured from the onset of the rising phase of the spike to the peak of the afterhyperpolarization. Excitatory inhibitory postsynaptic potentials were never seen to be associated with the antidromic response, findings consistent with those of Magni and Willis (1963, a,b.)

Units were frequently encountered which did not follow high frequency stimulation of the spinal cord and which showed a variable latency to both threshold and supra-threshold stimulation. These were classified as orthodromically activated units and no attempt was made to classify their properties. It was noted, however, that these responses would

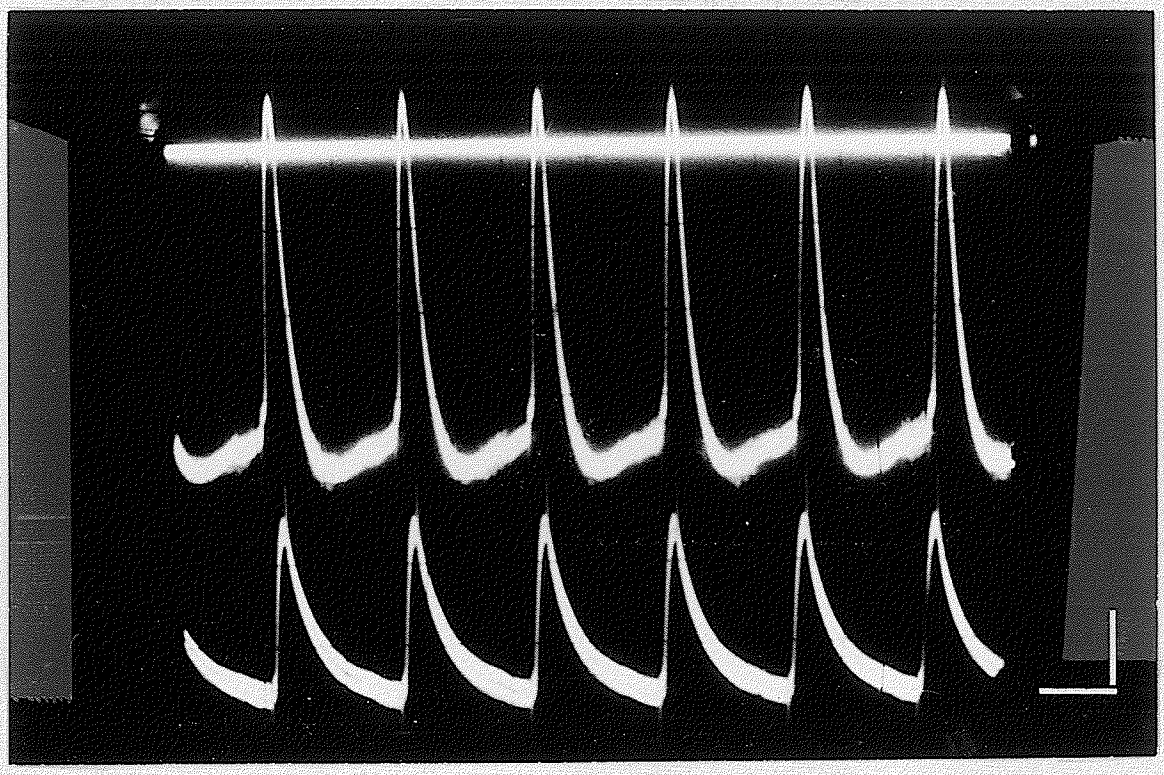


FIGURE 15: Intracellular recording showing the response of a neurone to stimulation of the spinal cord at 300 H_z. Calibration marks represent 2 msec and 5 mV for the lower trace and 2 msec and 2.5 mV for the middle trace. The upper trace is current monitor for intracellular current injection. Bottom trace is D.C. coupled, the middle trace is capacity coupled.

not usually follow stimulation of the spinal cord at frequencies greater than approximately 30 H_z and none followed at frequencies greater than 100 H_z.

In an effort to determine whether or not the neurones in the medial brainstem could be grouped according to the threshold current required to activate their axons, the threshold values (milliamperes) obtained were compared with the depths at which the units were encountered (Figure 16), the distance of the units rostral to the obex (Figure 17), and their conduction velocities (Figure 18). There was no clear relationship between threshold current and any of the other three variables, although there was a slight trend for the units between 1.5 and 4.5 μm below the floor of the fourth ventricle to show an increased threshold with increased depth, and for a group of neurones of slow to intermediate conduction velocity (up to 50 m/sec) to have thresholds between 0.175 and 1.290 mA. Such a group seems to be a separate population from the rest of the units in this figure; however, the significance of such a population is not clear.

Distribution of Antidromically Activated Units

Recordings from single units were obtained between 4.5 and 7.5 mm. rostral to the obex with the majority of the responses being between 5.0 and 5.5 mm. rostral to the obex (Figures 19 and 20). Units were encountered from 0.22 to 7.5 mm. below the floor of the fourth ventricle along the

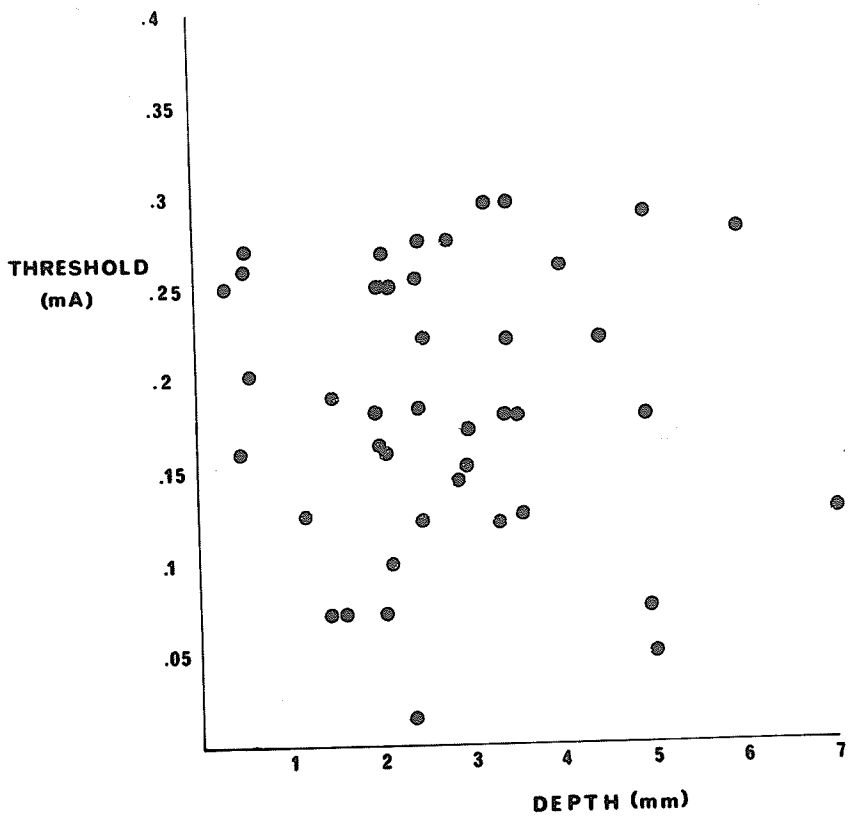


FIGURE 16: The relationship between threshold (mA) and depths of units below the floor of the fourth ventricle.

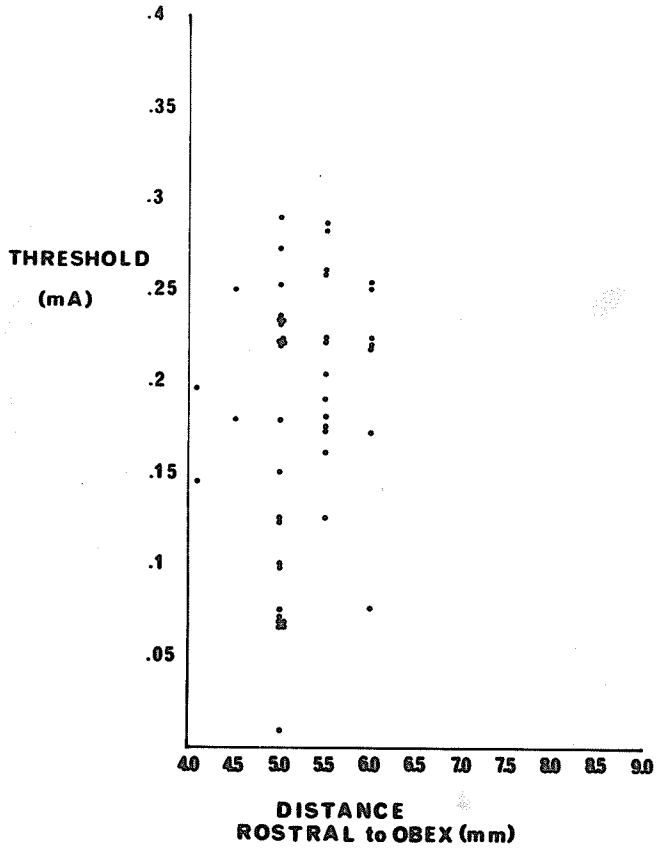


FIGURE 17: The relationship between threshold (mA) and distance of antidromic units rostral to the obex.

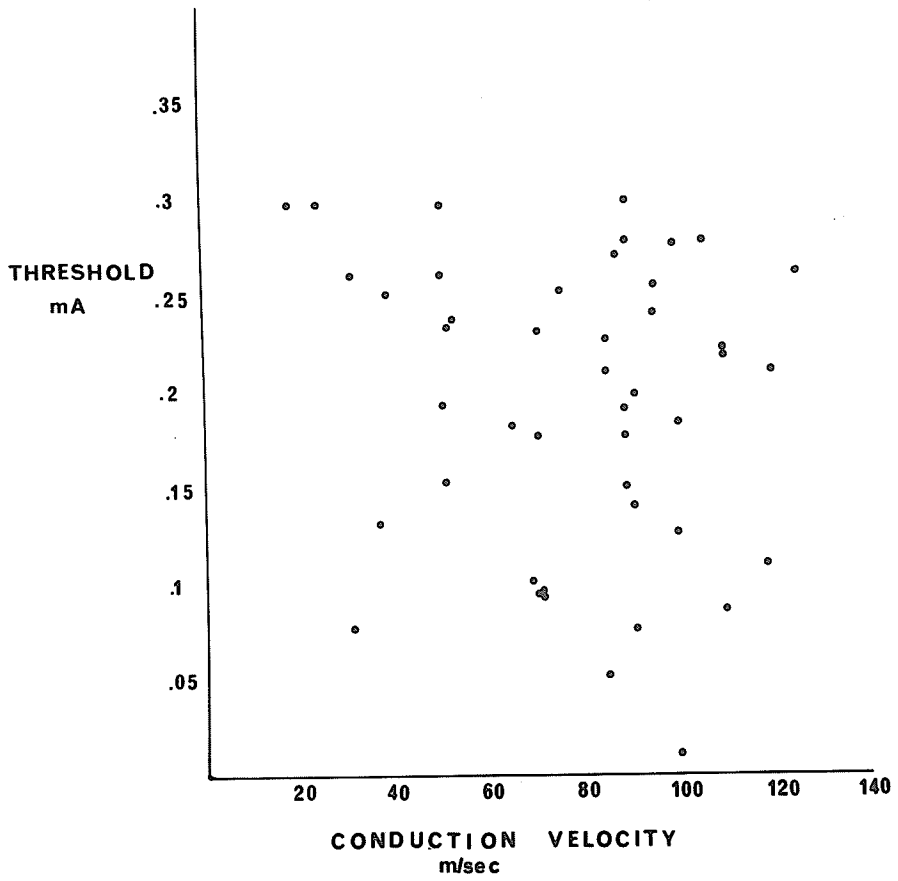


FIGURE 18: The relationship between threshold (mA) and conduction velocity (m/sec).

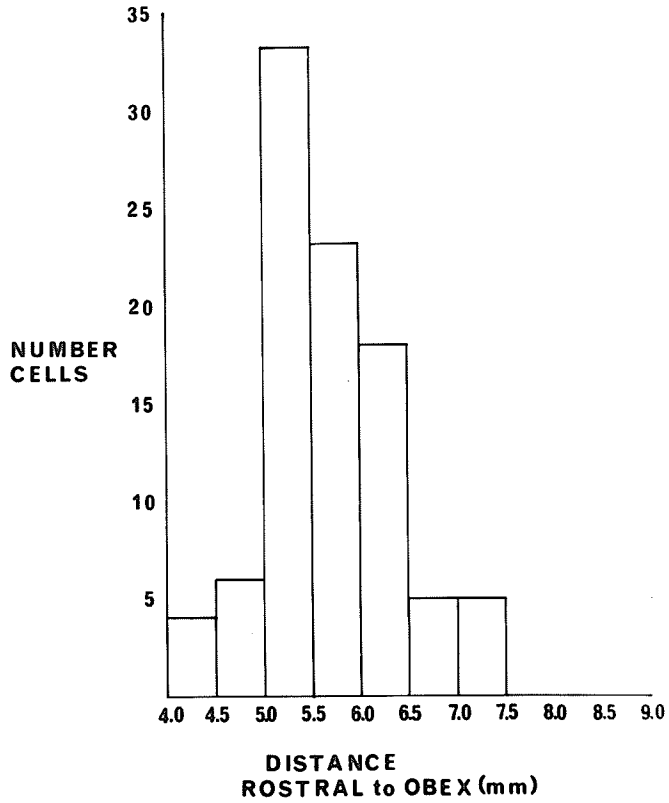


FIGURE 19: Frequency distribution of the cells encountered at various distances rostral to the obex.

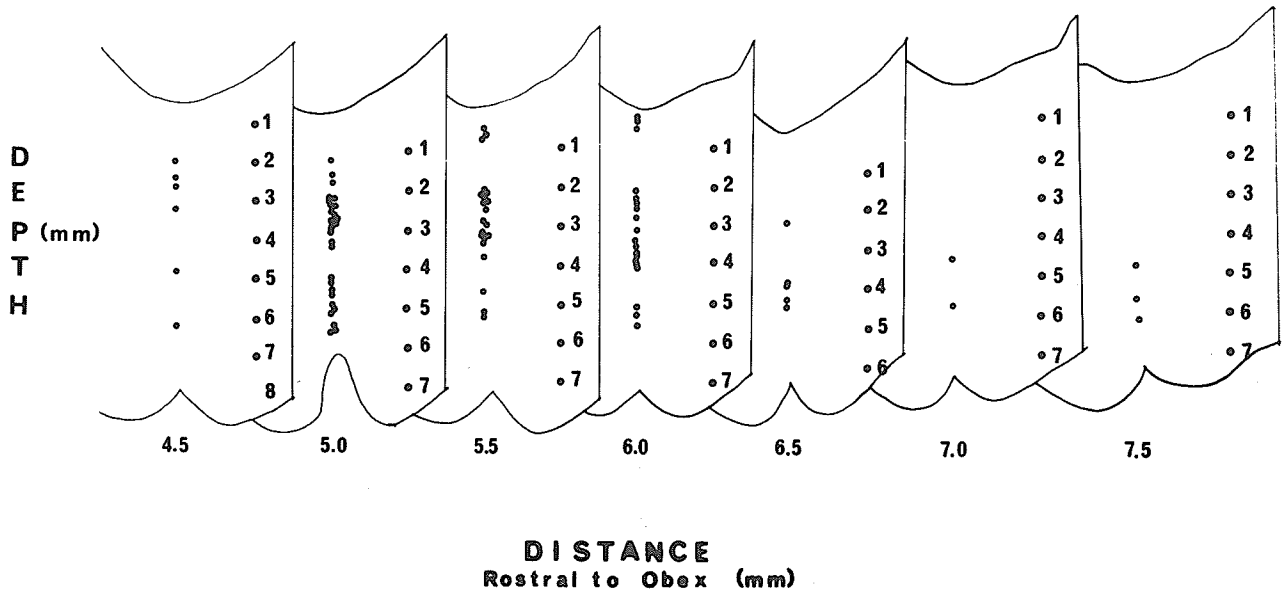


FIGURE 20: A schematic representation of cross sections of the caudal brainstem at different distances rostral to the obex and the depths (●) at which antidromic units were encountered. The numbers on the right of each cross section represent the depth for that section only.

midline (Figure 21) with the majority lying in the vicinity of 2 - 3 mm. beneath the surface. It should be pointed out that the distribution of units with respect to depth beneath the floor of the brainstem (Figure 20) refers only to the depth at which antidromic units were encountered and it should not be construed from this figure that all of the recordings were made from units exactly along the midline. Such is probably not the case.

In addition to careful placement of the electrode according to easily identifiable landmarks, electrode tracks were confirmed histologically in a few cases. Examples of this are shown in Figure 22 in which the track of an electrode through a region of yellow fluorescent cell bodies is indicated (arrows). In a different animal (Figure 23), fast green dye was ejected from the recording electrode to mark the location of the electrode tip and was subsequently localized to a region of yellow cell bodies. This section was subsequently counterstained with thionine to demonstrate cell bodies and the location of the fast green mark found (arrow) (Figure 24). Since the fast green dye had diffused considerably during the histological and histochemical treatment, it is not possible to locate cell bodies at the electrode tip, but yellow cell bodies are seen at 100 μm from the tip.

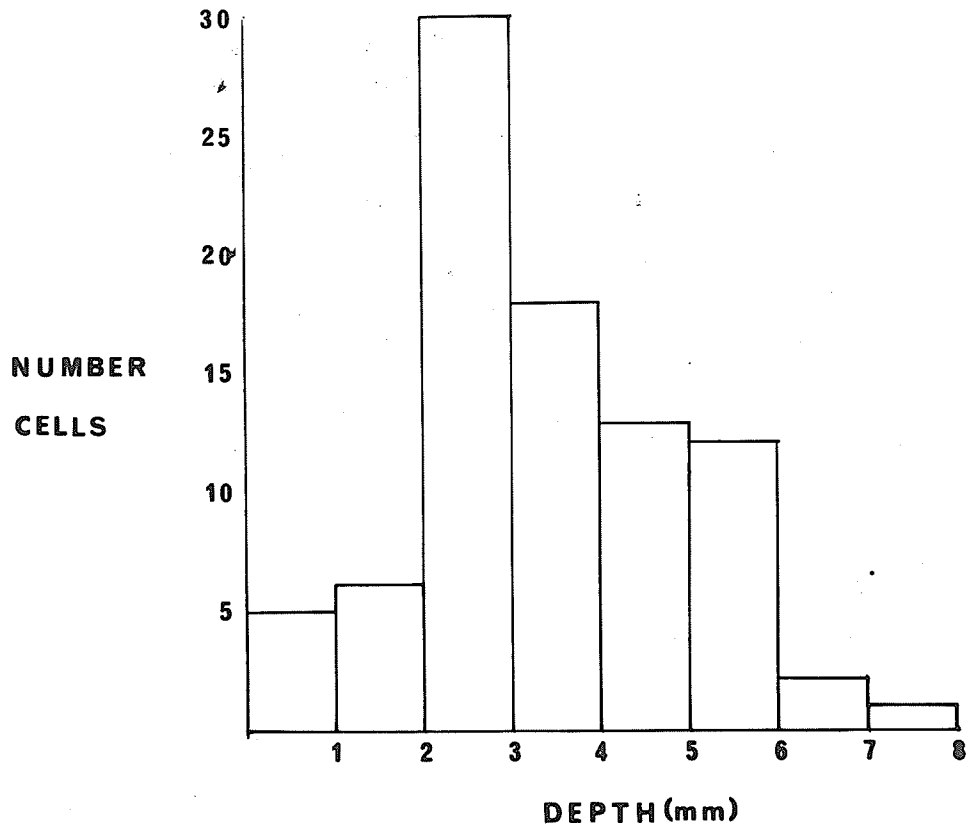


FIGURE 21: Frequency distribution of the cells encountered at various depths from the floor of the fourth ventricle.

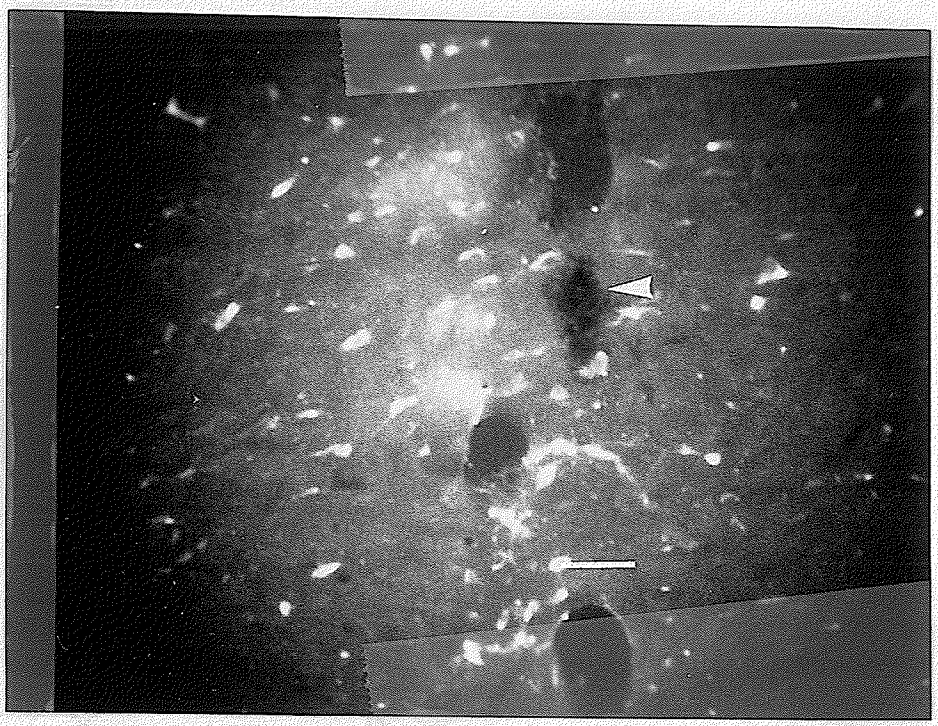


FIGURE 22: A low power photomicrograph of the medial brainstem showing an electrode track (arrows) passing through an area of yellow cell bodies. Calibration bar: 80µm.

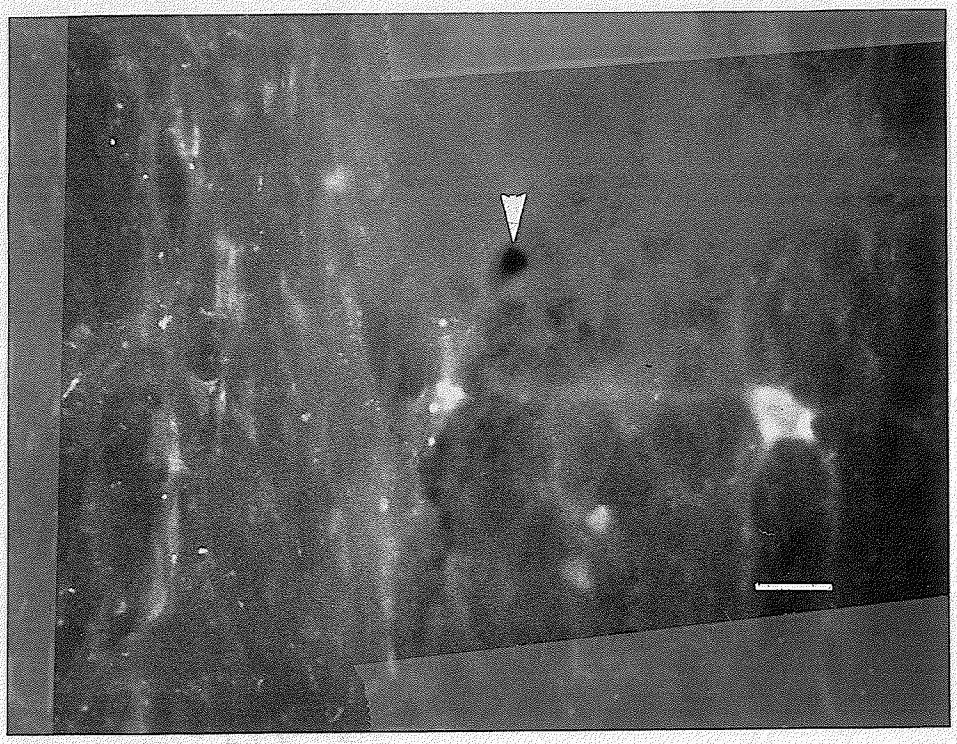


FIGURE 23: High power photomicrograph of the medial brainstem showing the location of fast green dye ejected from the recording micropipette (arrow). Calibration: 125 μm.

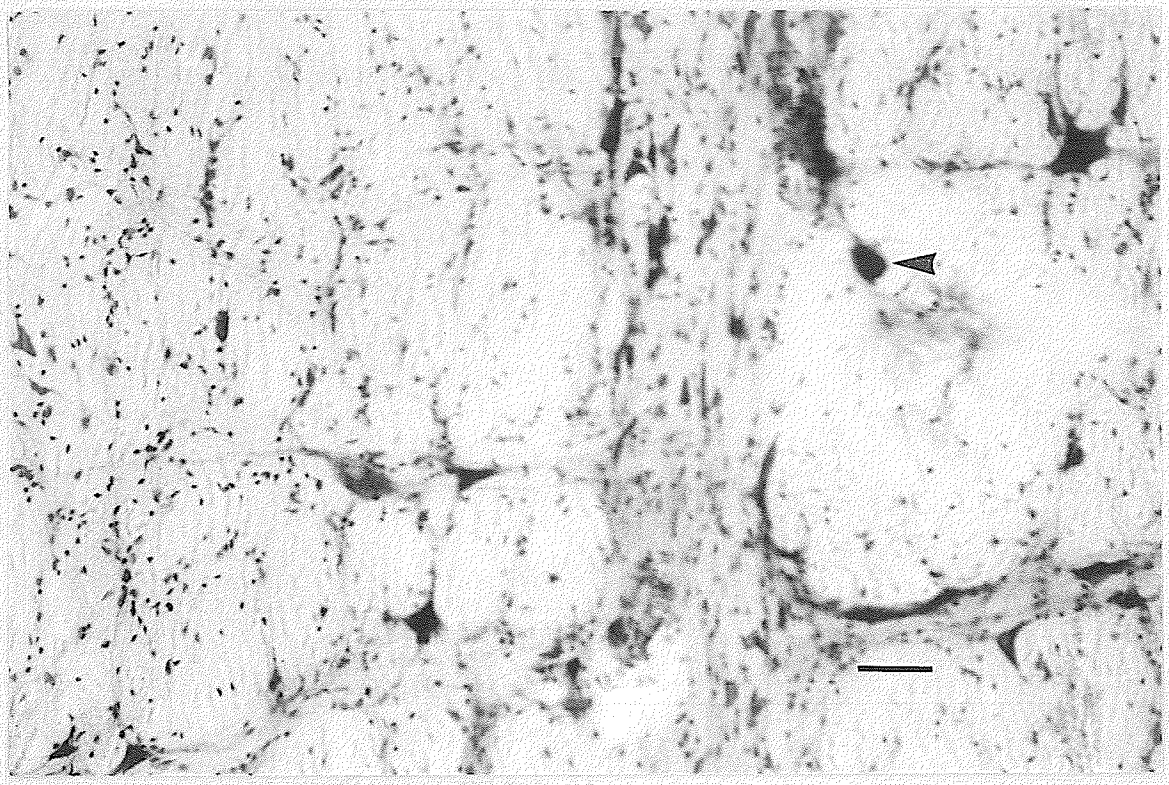


FIGURE 24: The same section as figure 21 after thionine staining. The position of the fast green dye is indicated by an arrow. Calibration: 100 µm.

To rule out the possibility that recordings were made from non-5-HT containing cells in this area, the numbers of both 5-HT and non-5-HT containing cells were counted in a separate series of tissues. The number of cells showing yellow fluorescence was first ascertained by counting them from negatives of this area of the medial brainstem, followed by counting the total numbers of cells in this tissue after thionine counterstaining. Of a total of 125 cells from 2 cats, 123 could be identified as being present in the fluorescent tissue, making it highly probable that the majority of antidromic responses obtained from this region were from 5-HT containing cell bodies. The area of the medial brainstem from which the cells were counted extended approximately 1.0 mm. on either side of the midline. In instances in which electrode tracks were located, they deviated from the midline in 3 out of 6 cases, but not by more than 0.2 mm. The fact that all of the electrode tracks fell within the area which contained such a high proportion of 5-HT containing cells tends to support the conclusion that the majority of antidromic responses were from 5-HT containing cell bodies.

DISCUSSION

I. The Distribution of 5-HT Terminals in the Lumbar Gray Matter.

Early investigations employing the Falck - Hillarp (1962) fluorescent histochemical technique have demonstrated the presence of both NA and 5-HT terminals in the lumbar cord of the rat; however, since 5-HT fluorescence is extremely labile under exposure to ultraviolet light (Fuxe et al, 1970) and since NA terminals may be mistaken for those containing 5-HT (Corrodi et al, 1966) when the two are present in the same tissue, it was important that the distribution of 5-HT terminals be carried out in cats depleted of spinal NA. The first aim of this thesis was, therefore, to investigate the distribution of 5-HT containing varicosities in the lumbar cord of 6-OHDA treated cats, since such treatment has been shown to cause fairly specific lesioning of catecholamine pathways (Malmfors and Sachs, 1968).

The results indicate that the majority of 5-HT terminals that could be observed were located in the medial areas of the ventral horn corresponding to the ventromedial portion of Rexed's (1952, 1954) lamina VII, the medial part of lamina VIII and the medial lamina IX. A less extensively innervated area was the lateral motor group of the ventral horn, and substantial numbers of terminals were also seen in lamina X in the area of the central canal where some of the fibers appeared to cross. The upper regions of the dorsal horn (lamina II, III, and perhaps the upper aspect of lamina IV) contained few terminals, while very few were

seen in the intermediate zone (lamina V, VI and the lateral parts of lamina VII). Such a distribution is felt to be an accurate one since the NA content of the spinal cords examined had been depleted from 73 to 98% and greenish fluorescent terminals were rare, and since 5-HT content was unchanged. The locations of these terminals are of some interest, therefore, in view of current notions regarding the role of 5-HT in spinal neuronal organization.

Investigations have revealed behavioral analgesia upon stimulation of the raphé nuclei and periaqueductal gray matter in normal cats but not in cats administered with the serotonin synthesis inhibitor parachlorophenylalanine (pCPA)(Liebeskind, 1973) or with LSD (Guilbaud et al, 1973) and it has been inferred from these findings that the analgesia is mediated via a descending serotonergic pathway acting at a spinal level. In addition, since the demonstration that cells responding to different forms of cutaneous afferent input (Wall, 1967; Pomerantz et al, 1968) are located in lamina IV and V interest has centered upon the responses of "nociceptive" lamina V interneurones to stimulation of the midbrain raphé nuclei (Oliveras et al, 1974). Stimulation at sites which yielded behavioral analgesia also inhibited interneurones in lamina V which fired in response to pain, without having any effect on the spontaneous activity of these cells. The conclusions of Oliveras et al (1974) that the bulbospinal serotonergic system directly inhibits lamina V interneurones is interesting since analgesia is only produced by stimulation of

the dorsal raphé nuclei which has not been demonstrated to project to the spinal cord (Brodal et al, 1960), and since the results of this investigation reveal that 5-HT terminals were rarely encountered in this region of the spinal cord gray matter. Once alternative is that stimulation of the raphé nucleus may in turn activate other pathways known to descend to the lamina V. Examples of such pathways are the cortico spinal (Nyberg - Hansen and Brodal, 1963) and rubrospinal (Nyberg-Hansen and Brodal, 1964) systems. Another alternative is that the descending 5-HT fibers may presynaptically inhibit some of the afferent fibers known to terminate in lamina V as well as the upper lamina II - IV. Such evidence was indirectly obtained by Proudfit and Anderson (1973), by monitoring the dorsal root discharge and the length constants of afferents showing a dorsal root potential obtained by stimulation of raphé nuclei. Their findings indicated that electrical stimulation of the raphé nuclei caused a depolarization in afferent fibers of small diameter. This was an interesting finding in view of the earlier work by Anden et al (1964c), who demonstrated a depression of transmission in flexion reflex afferents by intraperitoneal administration of 5-HTP. Further work by Randic and Yu (1975) showed a depression of certain neurones in lamina I by iontophoretically administered 5-HT, but since few 5-HT terminals were found in this region, in the current study, these results are difficult to interpret.

The predominance of terminals observed in the ventral horn, both in the medial and lateral motor group, is of considerable interest in view of the apparent involvement of 5-HT in locomotor behavior, and in the alteration of spinal reflexes. Intravenously administered 5-HTP has been shown to result in an increase in the amplitude of the L_7 MSR (Anderson and Shibuya, 1966) in animals spinalized at C_1, thus suggesting an action at the spinal level. Concommittant with the increase in the MSR there is an increase in spontaneous ventral root discharge presumably due to increased excitability of alpha-motoneurones, although several investigators have shown an increase in the activity of gamma motoneurones as well (Ahlman et al, 1971; Dixon et al, 1969; Ellaway et al, 1973 and Ellaway and Trott, 1975). Such a direct action of 5-HTP on anterior horn cells in perhaps possible, however, the electrophysiological evidence regarding 5-HT actions in the spinal cord suggests that the effects of 5-HT on motoneurones may be indirect. Firstly, while the effect of 5-HT is one of facilitation of the MSR and a depression of polysynaptic reflexes (Anden et al, 1964; Anderson and Shibuya, 1966; Anderson et al, 1967), electrical stimulation of the brainstem raphé nuclei results in an inhibition (Clineschmidt and Anderson 1970) or a biphasic facilitation-inhibition (Proudfit and Anderson, 1973) of the L_7 MSR without any effect on spontaneous ventral root discharge. Secondly, investigations in

which 5-HT was iontophorectically applied into motoneurones have shown that the predominant effect of 5-HT is one of depression (Engberg and Ryall, 1966; Weight and Salmoiraghi, 1966 and Phillis et al, 1968). Thirdly, the anatomical data obtained in this investigation revealed that only rarely did motoneurones seem to be in close contact to 5-HT terminals. This lack of close contact of tryptaminergic terminals to alpha-motoneurones has also been reported for NA varicosities in the lumbar cord (Jordan et al, 1976) and may represent a feature of the neuronal innervation by monoaminergic terminals in cat spinal cord.

In contrast, Barasi and Roberts (1973, a,b, 1974) have indicated a facilitation of the antidromic motoneuronal field potential by administration of l-tryptophan, the iontophonetic delivery of 5-HT or by electrical stimulation of the nucleus raphé-medianus. It is noteworthy, however, that these authors did not record directly from single motoneurones, nor could they localize the ejection of the 5-HT to the immediate vicinity of motoneurones. Thus, the possibility that 5-HT causes an increased motoneuronal excitability via inhibition of inhibitory interneurones is worthy of consideration. The results of the present investigation lend some support to this idea on anatomical grounds. Firstly, 5-HT terminals were only rarely seen in close contact with motoneurones but many were seen in areas of lamina VIII and VII, in which are found many small

cells presumed to be interneurones (Rexed, 1954) and which have intrasegmental projections. There was also substantial 5-HT innervation into lamina X and few terminals in lamina II and III. Since the large alpha motoneurones are relatively confined only to lamina IX, the results of the present study are strongly suggestive of a role for 5-HT in modulation of motoneuronal activity via interneuronal pathways.

One possible candidate for this inhibitory interneurone is the Renshaw cell. Sinclair and Sastry (1974b) have proposed tonic inhibition of the Renshaw cell by a monoaminergic system and that after administration of drugs which increase synaptic availability of 5-HT (imipramine, pargyline, desipramine) the recurrent inhibition of quadriceps MSR is blocked. The net result of this blockade of recurrent inhibition would be enhanced alpha-motoneuronal excitability. However, it cannot be concluded from such evidence that other types of interneurones inhibitory to alpha-motoneurones are not inhibited as well by increased synaptic quantities of 5-HT, nor that the serotonergic involvement in the blockade of recurrent inhibition is not via other supraspinal centers previously shown to inhibit Renshaw cells (Haase and Van Der Meulen, 1961; MacLean and Leffman, 1967). Furthermore, such release from inhibition of motoneurones by increased 5-HT may be important in "tonic" events such as decerebrate rigidity (Engberg et al, 1968). Such a suggestion receives some support

in view of the fact that excitation of motoneurones is seen in high spinal (C_1) or decerebrate cats, but not on cats with an intact neuraxis, this latter preparation being the one used by Clineschmidt and Anderson (1970) and Proudfit and Anderson (1973).

The elucidation of the role of the bulbospinal serotonergic pathway in motor control mechanisms is still in its infancy. Intravenously administered 5-HTP in acute spinal rabbits has been shown to cause rhythmic hind limb stepping (Viala and Buser, 1969, 1971, 1974; Viala et al, 1974), an action reminiscent of L-DOPA in acute spinal cats (Anden et al, 1964c). However, although 5-HTP has no such obvious actions in cats (ie. generation of rhythmic stepping) Anderson and Shibuya (1966) noted that after intraperitoneal administration of 5-HTP on C_1 spinalized cats, there occurred "integrated flexion and extension" of the limbs, although no attempt was made to study this observation in depth. Jacobs and Klemfuss (1975) have suggested that the increased hyperactivity, resting tremor, rigidity or hyperactivity, hind limb abduction and lateral head weaving seen in rats after central 5-HT levels have been increased is mediated by the caudal brainstem and spinal cord, based on the absence of the syndrome after transection of the neuraxis at various levels, and Guillemault et al (1973) have demonstrated a reduction in the symptoms of intention myoclonus due to

hypoxic encephalopathy after administration of 5-HTP in human subjects. These results are interesting in view of ascribing 5-HT a role in locomotion, and it is interesting that there are some similarities between the effects of L-DOPA and 5-HTP in locomotion. Both L-DOPA and 5-HTP have been shown to depress a short latency transmission from FRA to motoneurones (Anden et al, 1964c) and while L-DOPA, but not 5-HTP can cause stepping in acute spinal cats (Anden et al, 1964c), 5-HTP produces the same result in rabbits (Viala and Buser, 1969, 1971, 1974; Viala et al, 1974). Current notions regarding the supraspinal control of locomotion from the midbrain locomotor region (MLR) (a site in the brainstem which causes locomotion on a treadmill when stimulated in precollicular-postmamillary decerebrate cats (Shik et al, 1966, 1967; Grillner and Shik, 1973)) suggest that activation of a descending noradrenergic pathway from the MLR releases a spinal cord mechanism capable of generating locomotion. Sinclair and Sastry (1974b) have recently suggested that enhancement of central 5-HT levels may also reduce recurrent inhibition by an increase in the tonic supraspinal inhibition of Renshaw cells. However, in view of the rather limited experimental treatment of this topic, it is difficult to assess the effects of 5-HTP in locomotor behavior. Such an evaluation must, therefore, await further research.

II. Conduction Velocities of Descending Raphe Fibers.

Previous studies of the effects of electrical stimulation of the raphe nuclei on spinal neuronal events (Clineschmidt and Anderson, 1969, 1970; Barasi and Roberts, 1973 a,b, 1974; Proudfit and Anderson, 1972, 1973) have all employed a condition-test paradigm in which the brainstem stimulation precedes segmental afferent stimulation by intervals ranging from 25 to 120 milliseconds. Because the optimum interstimulus intervals for observing effects at the spinal level are so great, several of these authors (Clineschmidt and Anderson, 1970; Anderson, 1972) have concluded that the conduction velocities of the bulbospinal serotonergic pathway were less than 10 meters per second. Furthermore, in unanaesthetized decerebrate cats the 5-HT antagonists BOL or Deseril have been shown to cause partial release from the tonic descending control of transmission from the FRA (Engberg et al, 1968c) by a ventral bulbospinal pathway, and that another pathway in the dorsal part of the lateral funiculus must be involved in mediating part of this tonic inhibition. It was also concluded that this pathway could not be monoaminergic since its conduction velocity was on the order of 30 meters per second. The studies cited above have all assumed the descending serotonergic pathway to be comprised of unmyelinated axons of

small diameter, since Dahlstrom and Fuxe (1965) indicated that 5-HT axons in cross-sections of fluorescent tissues were not surrounded by a brown fluorescent ring characteristic of myelin sheaths seen under ultraviolet light.

The results of these studies must be questioned on several grounds. Firstly, since Dahlstrom and Fuxe (1965) did not counterstain their tissue to demonstrate myelin sheaths, it cannot be concluded that 5-HT containing axons are unmyelinated. Secondly, the distribution of 5-HT terminals in the lumbar gray matter of cats as reported in the results indicates that there are probably very few motoneurones innervated by 5-HT terminals, hence there is evidence that the effects of stimulation of the raphé nuclei in motoneurones is not via a monosynaptic pathway. Since the number of synapses involved in the raphé motoneurone pathway is uncertain, studies which use the condition test paradigm as an index of the conduction velocity of 5-HT fibers are misleading. Thirdly, previous studies using antidromic activation of reticulospinal neurones from the spinal cord (Magni and Willis, 1963, a, b) have reported conduction velocities ranging from 90 to 120 m/sec. It is interesting to note that some of their electrode penetrations were in the caudal brainstem along the midline, suggesting that cells on the raphé nuclei may be capable of conducting at high speeds. Fourthly, Foote et al (1974) have demonstrated

short latency (approximately 7 msec) effects in the lateral geniculate body by single shock stimulation of the dorsal raphe nucleus, and Proudfit and Anderson (1973) have reported similar latencies for effects at the spinal level. Based on the fact that these latter investigators could not block the short latency effects by serotonin antagonists, it was concluded that they were not mediated by 5-HT, but that longer latency effects (approximately 40 msec) blocked by these antagonists were due to tryptaminergic pathways. The validty of conclusions based on results using 5-HT antagonists has recently been seriously questioned, since the inhibitory effects of 5-HT in brain areas receiving a prominent serotonergic input were not blocked cinanserin, methysergide, cyproheptadine, metergoline, or methiothepin, compounds which do block the effects of 5-HT in the peripheral nervous system (Haigler and Aghajanian, 1974).

In view of the above evidence, it was deemed important to measure the conduction velocities of 5-HT fibers directly, by recording the antidromic responses of raphe neurones to stimulation of the spinal cord. The results revealed no conduction velocities in the range expected for unmyelinated fibers, the slowest conducting fiber being 17.3 m/sec. Since the track of the microelectrode was shown to have passed through regions of cell bodies containing yellow fluorescent

cells and since most (123/125) of the cells in the area of electrode penetrations exhibited yellow fluorescence, it seems reasonable to conclude that at least some proportion of the responses were obtained from serotinin-containing cell bodies. Furthermore, electrode penetrations were made in the region of the raphé nuclei known to project to the spinal cord (Brodal et al, 1960; Kuypers and Maisky, 1975) strengthening the argument that raphé units are capable of conducting at fast conduction velocities.

While it seems clear that raphé axons are not slowly conducting, the significance of the range of conduction velocities and the lack of a relationship between conduction velocity and the distance rostral to the obex, or the depth at which the units were encountered is not so clear. One explanation for these findings is that the bulbospinal serotonergic pathway does not arise from an anatomically or electrophysiologically distinct population of brainstem units. Such a conclusion seems worthy of consideration in view of the wide spread involvement of the raphé nuclei in various CNS activities such as sleeping and dreaming (Cohen et al, 1973); behavioral analgesia (Liebeskind, 1973); modulation of visual input to the lateral geniculate body (Humpherys, PhD thesis); control of spinal reflexes (Anden et al, 1964c; Lundberg, 1965; Banna and Anderson, 1966; Proudfit and Anderson, 1973; Clineschmidt

and Anderson, 1970); and locomotion (Viala and Buser, 1969, 1971, 1974; Viala et al, 1974), and excitation of sympathetic preganglionic neurones by iontophoretically administered 5-HT (DeGroat and Ryall, 1967; Ryall and DeGroat, 1972).

Such a conclusion is not consistent with other evidence concerning the effects of stimulation of the medial brainstem on transmission through spinal interneuronal pathways and primary afferent terminals. Carpenter et al (1966) were able to demonstrate dorsal root potentials (DRP) in L_6 dorsal rootlets by stimulation of the medial caudal brainstem, the latency of these effects being shorter for stimulation sites 1 mm. below the floor of the fourth ventricle than for more ventral positions, and these effects were not evoked from more rostral brainstem levels. Furthermore, effects from the more dorsal regions were seen in Ia, Ib and cutaneous afferents and were mediated through ventral spinal pathways. Subsequently, Lundberg and Vyklicky (1966) demonstrated inhibition in primary afferent fibers with an absence of a DRP suggesting an effect at an interneuronal level. These results were obtained by stimulation of the caudal medial brainstem via pathways in the ipsilateral ventral quadrant. Evidence for a dorsal spinal pathway mediating inhibition of transmission in FRA and Ib pathways but not in Ia or recurrent IPSP's nor on Ia EPSP's (Engberg et al, 1968a) was shown to occur in fibers with conduction

velocities of at least 20 m/sec, hence these authors have concluded that the effects cannot be mediated via 5-HT containing axons. Such conclusions were also arrived at by these authors (Engberg et al, 1966) with respect to the bulbospinal inhibition of IPSP's and EPSP's in interneurones receiving direct FRA input.

The results of these studies were often obtained with caudal brainstem stimulation 6 mm. rostral to the obex along the midline but at varying depths. It has been previously shown that this area of the brainstem raphé nuclei projects to the spinal cord (Brodal et al, 1960) and since most of the cells in this region have been shown to contain 5-HT, there is reason to believe that some of the effects are mediated by a bulbospinal 5-HT pathway. Furthermore, since previous investigations (Dahlstrom and Fuxe, 1965; Coote and McLeod, 1974) have shown that the descending 5-HT axons travel in both lateral and anterior funiculi, and since this has been confirmed in the present investigation, it must be considered feasible that some of the effects observed on transmission through primary afferent pathways and interneurones could be mediated by a bulbospinal serotonergic system.

In addition, the range of conduction velocities seen in the present study encompasses that reported by Engberg et al (1966) and since they used a condition-test paradigm to provide data regarding conduction velocities, it is likely that their

values (20-30 m/sec) will be under rather than overestimates. It is, therefore, conceivable that the bulbospinal serotonergic system may, in fact, be functionally and anatomically differentiable, at least with respect to their position in the white matter of the spinal cord, but that such organization is absent in the cells of origin of these fibers. It is worth noting, however, that since the numbers of slowly conducting units (less than 40 m/sec) was small (n = 12/95) the possibility that there are anatomically and physiologically distinct populations of raphe units at the brainstem level cannot be entirely discounted. Obviously, further research is needed to clarify this issue.

In view of the conduction velocities encountered, and the certainty that some proportion of these responses were from 5-HT containing neurones, it was considered crucial to examine 5-HT containing axons for the presence of myelin sheaths. Such information is important since, whereas unmyelinated fibers have been shown to have conduction velocities proportional to the square root of axon diameter, the speed of conduction for myelinated axons is proportional to approximately six times the fiber diameter (Rushton, 1951). Based on the findings that unmyelinated fibers are not found in CNS larger than 1 um (Vizoso and Young, 1948) it might be expected that such fibers could conduct no faster than 6 m/sec ($6 \times \sqrt{1\ \mu m}$). The results

of this study have indicated that the slowest conduction velocity encountered was 17.3 m/sec, approximately threefold faster than the fastest unmyelinated fibers. Correspondingly, 2 cats spinalized at the thoraco-lumbar junction were allowed to survive for 5 days before processing pieces of thoracic cord by the Falck-Hillarp fluorescence histochemical method to demonstrate monoamine fibers. Since there is build-up of the amines in the central ends of cut fibers, but concommittant distortion of the axon, it is possible to reach conclusions regarding the presence or absence of myelin sheaths only, with valid decisions regarding fiber size being impossible. Although examination of serotonergic fibers for myelin sheath has been carried out only on a limited number of axons, it was possible in several instances to make positive localization of a myelin sheath around these fibers. Such results are completely contrary to the widely held notion that CNS monoaminergic fibers are unmyelinated, hence slowly conducting, and they provide, for the first time, an anatomical basis for many of the observed short latency effects evoked from stimulation of the ventromedial brainstem. Even more interesting is the evidence that the saltatory conduction in central myelinated fibers may be faster than six times fiber diameter. Such a suggestion is based on the finding that the rise time for the action potential at the central nodes of Ranvier may be greater than for peripheral nervous system (Waxman and Bennet, 1972), hence overall conduction time will be

increased. This implies that unmyelinated fibers of smaller diameter may be able to conduct at speeds approaching those for larger central myelinated fibers. Furthermore, there is some evidence that fibers as small as 0.2 μm in the central nervous system may be myelinated (Waxman and Pappas, 1971; Adinolfi and Pappas, 1968), hence implying that the slowest conduction velocity for a central myelinated fiber may be in the order of 1 m/sec. Since none of the conduction velocities observed by antidromic activation of raphé units was anywhere near this value, two explanations are possible. Firstly, it must be considered that the current strengths employed were inadequate in activating the 5-HT containing axons, or that the efferent information in the raphé spinal pathway is carried by myelinated fibers considerably larger than 0.2 μm. Because of the failure to find any units activated by very high current strength (greater than 1 milliampere), and the consistent finding of both slowly and rapidly conducting fibers over a wide range of threshold currents, this explanation does not seem to be adequate to explain the results. It is, therefore, proposed that the effects of the bulbospinal serotonergic pathway are mediated through myelinated axons of at least 3 μm in size. As stated previously, such measurements were not possible in this investigation, however, such efforts are forthcoming.

It is also possible that the judgements about the absence of myelin sheaths around central 5-HT axons are in fact partially correct. Recent evidence (Waxman, 1972) suggests that an axon may be capable of transforming the pattern of nerve impulses along its course, so that the temporal pattern initiated at the axon initial segment differs from that at axon terminals. Such transformation would be possible if the safety factor for axonal transmission changed at different points along the axon, or if axon collaterals lost their myelin sheath. Both alternatives have received experimental support (Parnas et al, 1969; Waxman, 1972), and it is possible, (in fact highly probable) that the axon terminals observed by Dahlstrom and Fuxe (1965), in spinal cord gray matter are really unmyelinated. Such information should not be construed to mean, however, that the entire course of the axon lacks a myelin sheath.

III. General Summary.

The aims of this thesis have been to demonstrate the distribution of 5-HT terminals in the lumbar spinal cord of the cat, and to measure the conduction velocities of descending 5-HT containing axons. The fluorescent histochemical results indicate substantial 5-HT innervation in the ventral horn and

central canal, but only scattered terminals in the dorsal horn and intermediate zone. These findings have provided an anatomical basis for an evaluation of the role of 5-HT in spinal neuronal activities.

Antidromic activation of raphé units from the L_1 spinal cord revealed conduction velocities ranging from 17.3 to 120 m/sec, a finding which is not consistent with current notions regarding conduction speeds of central monoaminergic pathways. Such results are explained on the basis of finding myelin sheaths around several 5-HT containing axons, although the sizes of these fibers could not be measured. It is, therefore, concluded that the raphé nuclei of the caudal brainstem may give rise to a fast descending pathway which may be involved in such activities as modulation of afferent input, alterations of spinal reflexes and locomotion.

SUMMARY AND CONCLUSIONS

1. The distribution of 5-HT terminal varicosities investigated in cats depleted of spinal NA reveals that the majority of these terminals are in the ventral horn with more in the medial as opposed to the lateral motor group. Fewer terminals were found in the area of the central canal with only sparse innervation in the dorsal horn and few, if any, in the intermediate zone.

2. In the white matter of the thoracic spinal cord in chronic spinal cats it was found that the 5-HT fibers travel in a continuous band just below the external surface of the cord from the dorsal region of the lateral funiculus to the walls of the anterior median fissure where they are relatively more dense than remaining areas of the white matter.

3. The conduction velocities for these descending tryptaminergic axons ranged from 17.3 to 125 meters per second. Such a range has not been previously reported for CNS monoaminergic fibers, the accepted values being in the order of 2.5 - 3.5 meters per second.

4. Subsequent investigations revealed that at least a portion of the descending serotonergic fibers are myelinated, this being the first direct evidence that central

nervous system fibers may be capable of conducting impulses at speeds faster than previously believed.

5. It is concluded that at least a portion of the descending fibers from the pontine and medullary raphé nuclei are myelinated suggesting the existence of a "fast conducting" bulbospinal serotonergic pathway.

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