THE MESENCEPHALIC LOCOMOTOR REGION IN THE DECEREBRATE CAT: PROJECTIONS AND THE ROLE OF NORADRENALINE

by

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"As to what nervous mechanism it is which, present in the decerebrate preparation and absent from the spinal, contributes so importantly to reflex standing and to the extensor phase of the step, and tends to convert alternating reflexes into tonic postures by suppressing refractory phase, a main portion of it clearly lies between the levels of anterior collinculus and hinder edge of pons."

(Sherrington, 1910, p. 116)

Abstract

Studies in decerebrate cats have demonstrated that repetitive stimulation of a discrete area below the inferior collinculus, the mesencephalic locomotor region (MLR), gives rise to locomotion, even in the deafferented state. However, very little is known about the axonal projections of the neurons within the radius of effective current spread from the MLR stimulating electrode. Previous work has shown that acute low spinal, deafferented cats are able to perform stepping movements with the hindlimbs in response to an i.v. injection of L-3, 4-dihydroxyphenylalaine (L-DOPA), a precursor of noradrenaline (NA), and there is evidence for the direct activation of NA neurons which are found in close proximity to the MLR.

The present study examined the axonal projections of neurons corresponding to the MLR, the cross-sectional areas of the spinal cord that are essential for MLR evoked locomotion, and the possible role of NA in the control of locomotion. By employing the method of autoradiography the fiber pathways originating from the MLR were delineated. No direct projections were found to the spinal cord but projections were found to the gigantocellular and magnocellular reticular formation of the pons and medulla from which locomotion can also be evoked in decerebrate cats. Projections were also seen to other areas of the brainstem such as the subthalamic nucleus, dorsal, lateral, and posterior nuclei of the hypothalamus, intralaminar nuclei of the thalamus, ventral tegmental area of Tsai, superior colliculus,

pontine reticular nuclei, and periaqueductual gray region. Stimulation of some of these areas will also evoke locomotion in decerebrate cats.

By selectively disrupting restricted areas of the spinal cord at the high cervical level in a MLR evoked locomoting cat, it was found that the only cross-sectional area of the cord which could not be disrupted was the ventrolateral funiculus. One of the main descending pathways coursing through the ventrolateral funiculus is the reticulospinal pathway originating from the gigantocellular and magnocellular reticular formation. Therefore, it would appear that one of the essential and perhaps common pathways for the activation of spinal stepping mechanisms is the pathway originating in the gigantocellular and magnocellular reticular formation, which descends through the spinal cord within the ventrolateral funiculus. Nevertheless, the initiation of locomotion by supraspinal structures is probably mediated by several descending systems, any one of which is not sufficient by itself to activate spinal stepping mechanisms.

To determine the importance of a descending NA system in the initiation and maintenance of locomotion in the decerebrate cat, specific pharmacological interventions were employed. An 80% depletion of NA by the neurotoxin 6-hydroxydopamine (6-OHDA) did not alter locomotion in the decerebrate cat. Nor did the tyrosine hydroxylase inhibitor, α-methyltyrosine (AMT) which reduced NA levels by 80%, and also blocked de novo NA synthesis. Finally, it has been reported that the α-adrenergic blocking agent, phenoxybenzamine, (POB) inhibits MLR evoked locomotion.

Yet the administration of POB in the present study did not effect MLR initiated locomotion. Therefore, a descending NA system does not appear to be essential for the initiation of locomotion by supraspinal structures.

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Introduction

Locomotion is defined, simply, as movement from one place to another. The basic underlying mechanisms of vertebrate locomotion are the coordinated limb movements, which are controlled by the alternate contractions of antagonistic limb muscles. However, very little is known about the nature of the neuronal networks which control the muscles.

Graham Brown (1911) discovered that stepping could be evoked in the bilaterally deafferented hindlimbs of acute, decerebrate, unanesthetized cats by transection of the low thoracic spinal cord. Graham Brown concluded that the lumbosacral cord contained local centers (stepping generator) capable of coordinating muscle activity within a limb and between limbs, which did not require peripheral input for the initiation or maintenance of the stepping movements. More recently, Grillner and Zangger (1974) have confirmed that the transected and deafferented lumbosacral cord can generate rhythmic alternating stepping movements. After spinalizing kittens at the thoracolumbar level of the cord they cut the dorsal roots (L_3 - S_4) of both hindlimbs. Stimulation of the dorsal columns in the caudal portion of the transected cord evoked rhythmic alternating activity in knee flexors and extensors. Similar results were obtained when recording from peripheral nerve filaments in curarized preparations. The stimulus for the lumbosacral stepping generator could be either activation of descending fivers or antidromically stimulating ascending fibers which, via collaterals, activate the generator.

Several other studies, using pharmacological agents, suggest that the neuronal organization subserving rhythmic alternating movements is intrinsic to the spinal cord. Budakova (1973) showed that acute spinal cats, which exnibited no locomotor activity prior to treatment, commenced stepping on a treadmill after intravenous (i.v.) infusion of L - 3, 4 - dihydroxyphenylalanine (L-DOPA). L-DOPA is thought to act through the release of noradrenaline (NA) from descending fibers, which then acts on the NA receptors of spinal neurons (Anden, et al, 1964, 1966b). Forssberg and Grillner (1973) have also shown that i.v. infusion of Clonidine, which is thought to directly stimulate central - adrenergic receptors (Anden et al, 1970), enables acute spinal cats to walk on a treadmill. Finally, after bilateral deafferentation or curarization of acute spinal cats, rhythmic alternating activity in peripheral nerves to antagonistic hindlimb muscles can still be obtained after i.v. L-DOPA or Clonidine (Grillner and Zangger, 1974). The results of these studies are interesting based on the findings of earlier fluorescence histochemical work which showed that there are no intraspinal neurons containing NA, and all the noradrenergic fibers visualized within the spinal cord originate in the brainstem (Carlsson, et al, 1964; Dahlstrom and Fuxe, 1965). Furthermore, the possibility that this locomotion is due to effects mediated by dopamine (DA) is not considered likely, since spinal cord DA has never been biochemically measured in appreciable amounts (Anden, 1965; Rawe et al 1977a).

It may be inferred from these studies that stimulation of noradrenergic receptors or release of NA from descending noradrenergic fibers in the spinal cord activates intrinsic neuronal mechanisms which give rise to the rhythmic alternating locomotor movements. Indeed, Anden et al (1966a) and Jankowska et al (1967 a, b) have shown that intrinsic spinal neuronal

circuits which could give rise to locomotion are activated after an i.v. injection of L-DOPA.

Since proprioceptive and exteroceptive afferent input are not necessary for the initiation and maintenance of stepping in the spinal animal, then what are the central nervous system (CNS) mechanisms responsible for the activation of the spinal stepping generator? The use of decerebrate animal preparations has aided investigators in their attempts to answer this question.

In an early pioneering study, Sherrington (1910) reported that acute intercollicular decerebrated cats were capable of standing and reflex walking. The ability of decerebrate cats to stand, as opposed to acute spinal cats which are unable to stand, was suggested to be due to the rigidity present within the limb extensors of the decerebrate preparation. The reflex walking evoked in Sherrington's decerebrate preparations was more effective than that witnessed in the high spinal (decapitate) preparations. He attributed the difference to an unknown nervous mechanism situated between the levels of the superior colliculi and the caudal edge of the pons.

Subsequently, several investigators (Hinsey et al, 1930; Bard and Macht, 1958; Villablanca, 1966) have studied the locomotor capabilities of decerebrate cats. The extent and effectiveness of locomotion depends on the level of transection (see Figure 1), whether the preparation is chronic or acute, and on the amount of background decerebrate rigidity (cf. Wetzel and Stuart, 1976). The capability for spontaneous locomotion is retained in premammillary preparations, where the level of transection extends along a plane

from the rostral border of the superior colliculi dorsally, to the anterior margin of the mammillary bodies ventrally (Hinsey et al, 1930). Such preparations are capable of spontaneous righting from a prone position, standing, and aimless walking after a survival period of only 1 day. If, however, the plane of transection is altered so that ventrally the transection extends to the caudal border of the mammillary bodies (postmammillary preparation) then the cat is unable to exhibit any spontaneous righting, standing, or walking until approximately 2 weeks after the transection (Bard and Macht, 1958; Villablanca, 1966). These experiments indicated the importance of structures within the brainstem which are necessary for the expression of locomotion. Many studies have been undertaken to elucidate these essential brainstem structures.

Waller (1940) discovered that stimulation of the subthalamic region in lightly anaesthetized animals evoked locomotion. More recently, acute premammillary preparations have been made to walk on a treadmill by stimulation of this subthalamic locomotor region (SLR) (Orlovsky, 1969a). The profound differences in locomotor ability between an acute premammillary and an acute postmammillary cat might be explained by the presence of the SLR in the former but not the latter preparation. Bilateral destruction of the SLR, in otherwise intact animals, inhibits voluntary locomotion for approximately two weeks; nevertheless during the interim period, stimulation of the mesencephalic locomotor region (MLR) enables the cat to perform coordinated locomotion (Sirota and Shik, 1973).

Therefore, the discovery of Shik et al (1966a) is of particular interest. They decerebrated cats at the postmammillary level and suspended the animals over a treadmill. As mentioned above, such postmammillary cats are unable to spontaneously step in an acute state. However, when they stimulated a discrete area below the inferior colliculus, the MLR, with a weak repetitive stimulation ($\sim$60 Hz) the cats began to walk. Increasing the strength of stimulation and/or the treadmill speed resulted in faster stepping and sometimes a conversion from an "out of phase" bilateral step mode characteristic of walking, to an "in phase" bilateral step mode characteristic of galloping. They concluded that the locomotion evoked in the postmammillary cat is identical to that of an intact cat walking in a straight line. A subsequent study showed that an acute postmammillary cat, stimulated in the MLR, can walk unassisted across a floor providing there are no obstacles in its path (Sirota and Shik, 1973).

The locomotion evoked in postmammillary preparations by stimulation of the MLR is not dependent on direct peripheral afferent input. Grillner and Zangger (1975) deafferented both hindlimbs of such a preparation and showed that the locomotor pattern evoked by MLR stimulation was unchanged from that observed in a cat having an intact himdlimb afferent input. However, it is possible that propriospinal influences arising in the cervical cord facilitate locomotion in the hindlimbs, and mask the effects of bilateral hindlimb deafferentation. This possibility seems unlikely, since lumbar ventral root filament activity is identical both before and after paralysis in MLR evoked locomotion (Jordan et al, 1979).

In intact cats, under chronic conditions, MLR stimulation also induces a cat to walk (Sirota and Shik, 1973). The locomotion induced by MLR stimulation is greatly improved after bilateral lesions of the SLR or the centrum medianum-parafascicular nucleus complex of the thalamus (Sirota and Shik, 1973). The interactions between these areas are poorly understood and will require further investigations before the identity of their relationship can be established. Bilateral lesions of the MLR in the intact cat result in an inability of the animal to run. The hindlimb movements also become uncoordinated after such lesions.

The first suggestion of the anatomical identity of the MLR was also proposed by Shik et al (1967). On the basis of histological verification of the sites of the MLR stimulating electrodes, they stated the MLR corresponds to the caudal division of the cuneiform nucleus (CNF). A fundamental question that arises from these findings is whether it is the neurons of the cuneiform nucleus or axons passing through this region, but originating elsewhere, which are responsible for evoking locomotion due to MLR stimulation.

Several studies have provided indirect evidence that it is the neuronal cell bodies, situated within the effective radius of current spread from the MLR stimulating electrode, that are responsible for initiating locomotion in the postmammillary cat. Bilateral lesions of the red nuclei (Shik et al, 1968), or removal of the superior and inferior colliculi bilaterally (Shik et al, 1967) does not alter MLR evoked locomotion. Therefore, they concluded that the synaptic activation of rubrospinal and tectospinal neurons is not necessary

for locomotion evoked by MLR stimulation. Also, if the ventral border of the transection is moved approximately 3 mm caudal to that in the postmammillary preparation (i.e. behind the exit of the 3rd cranial nerve) then MLR stimulation becomes ineffective (Shik et al, 1967). Such a small shift would not have such a decisive effect if the locomotion, evoked by MLR stimulation, depended on direct excitation of axons descending from more rostral areas.

Another important question is whether the MLR activates the spinal stepping generator directly or indirectly. Orlovsky (1969b) did not find any monosynaptic connections to the spinal cord from the MLR. However, monosynaptic responses were evoked in reticulospinal neurons of the dorsal medial pons and medulla by stimulation of the MLR (Orlovsky, 1970a). This finding prompted an extensive investigation into the activities of single neurons of the reticulospinal, vestibulospinal, and rubrospinal tracts, which are well known fast-conducting, descending pathways.

Orlovsky (1970b, 1972b,c) found that vestibulospinal neurons fire rhythmically during locomotion and are maximally fired during the stance (extensor) phase of the hindlimb step cycle, whereas the reticulospinal and rubrospinal neurons are rhythmically active during the swing (flexor) phase of the step cycle. He also noted that stimulation of each of these descending pathways during locomotion resulted in activation of the same appropriate hindlimb motoneurons, as would accur during stimulation when the cat was at rest (orlovsky, 1972a). However, the synchrony between the activity in a descending pathway and that in a corresponding motoneuron does not mean that these descending pathways are responsible for the initiation and control of stepping movements.

Orlovsky noted that the rhythmic modulation of activity in these descending pathways ceases if one of the hindlimbs is temporarily arrested. Also, stimulation of the descending pathways during locomotion does not affect the frequency of stepping or the duration of the stance and swing phases of the step cycle. It has been suggested that these fast-conducting descending pathways only serve a modulatory role in the control of locomotion, and their rhythmic activity is dependent on ascending influences from the spinal cord directly or indirectly via the cerebellum (cf. Shik and Orlovsky, 1976).

In acute premammillary cats, removal of the cerebellum results in the disappearance of rhythmic discharges in reticulospinal, vestibulospinal and rubrospinal neurons during locomotion evoked by SLR or MLR stimulation (Orlovsky, 1970c, 1972b, c). Also, the fact that locomotion can be evoked in a decerebellate premammillary or postmammillary cat provides further evidence against the involvement of these fast-conducting descending pathways and the cerebellum in the initiation of locomotion.

It has been suggested that MLR stimulation evokes locomotion via the excitation of the descending noradrenergic system (cf. Grillner, 1975). Since stimulation of the MLR enables a postmammillary cat to walk, it is reasonable to hypothesize that MLR stimulation "releases" the intrinsic spinal stepping generator in a manner similar to that observed in acute spinal cats after i.v. infusions of L-DOPA or Clonidine. An investigation by Grillner and Shik (1973) revealed that stimulation of the MLR produced changes in spinal cord reflexes and polysynaptic pathways which could be the underlying mechanism for locomotion.

Stimulation at a strength that evoked walking, prior to curarization, induced a depression of inhibitory short-latency reflex effects to α- motorneurons from cutaneous and high threshold muscular afferents without changing the direct excitability of the α-motorneurons. At the same time, long-lasting discharges having a long central delay could be evoked from peripheral nerves during MLR stimulation. There is a strong resemblance between these discharges and those evoked in the spinal cat after an i.v. infusion of L-DOPA, a precursor of NA which is able to cross the blood-brain barrier (Jankowska et al, 1967a, b). This could come about if the MLR was a source of descending NA-containing fibers terminating in the spinal cord. Alternatively, stimulation of the MLR might cause indirect activation of NA- containing neurons located in brainstem areas outside the confines of the MLR. In order to test the former suggestion, Steeves et al (1975) looked for the presence of catecholamine (CA)- containing cell bodies within the confines of the functionally effective MLR. They found CA-containing neurons, of the nucleus locus coeruleus, within 50μm of the electrode tip position. On the basis of quantitative evaluations of the spread of current from an electrode tip through CNS tissue (Stoney et al, 1968; Wise, 1972; Bagshaw and Evans, 1976), the MLR stimulus (20-190μA, 30 Hz) necessary to evoke locomotion would directly activate those CA- containing cells. Subsequent studies (Kuypers and Maisky, 1975, 1977) have demonstrated that the nucleus locus coeruleus projects fibers to the spinal cord. Together these studies suggest that MLR stimulation results in direct activation of descending NA fibers and subsequently their spinal terminals, thereby activating

the spinal stepping generator which gives rise to locomotion.

However, it is unlikely that this descending NA system alone is responsible for the initiation of locomotion since selective destruction of lumbosacral NA terminals by intraspinal injection of, the CA- specific neurotoxin, 6-hydroxydopamine (6-OHDA) does not alter MLR evoked locomotion in post-mammillary cats (Jordan and Steeves, 1976).

Most recently, the autoradiographic and horseradish peroxidase techniques for tracing neuronal pathways have demonstrated the existence of many new brainstem descending pathways (cf. Kuypers and Maisky, 1975, 1977; Basbaum et al, 1978; Castiglioni et al, 1978). Possibly some of these projections are related to the initiation of MLR evoked locomotion. As a final introductory note, a group of investigators have recently shown that it is possible to evoke locomotion in a post-mammillary cat by weak stimulation of a number of areas in the reticular formation, throughout the pons and medulla (Mori et al, 1977, 1978; Shik and Yagodnitsyn, 1977). As yet, they have been unable to determine whether these locomotor areas are axonal tracts arising from cells originating in the MLR, or are "columns" of inter-connected cells (polysynaptic pathways).

Therefore, our present understanding of the role of supraspinal structures in locomotor control is very incomplete. We know that weak stimulation of a circumscribed area beneath the inferior colliculus, the MLR, initiates a complex behavior, locomotion, in a post-mammillary cat. We do not know for certain if the MLR projects fibers directly to the spinal cord, or whether the activation of the spinal stepping generator is via a descending polysynaptic pathway. There is uncertainty

surrounding the importance of a descending NA system in the initiation and maintenance of locomotion. Finally, there is only limited information available as to how the MLR interacts anatomically or physiologically with other CNS areas implicated in motor performance.

The proposed research attempts to answer some of the questions outlined above. With the use of autoradiographic methods, the axonal projections of neurons activated by MLR stimulation will be determined. This experimental procedure (see methods) will enable us to determine whether the MLR projects directly to the spinal cord, as well as revealing what other areas of the brainstem receive projections from neurons corresponding to the MLR.

The basis of the autoradiographic method relies on the incorporation of a tritium labelled amino acid into protein by cell bodies in close proximity to the injection site, the MLR, and its subsequent movement, via axoplasmic transport, towards the axon terminals (cf. Cowan and cuenod, 1975). Ultrastructural studies indicate that the sites of protein synthesis for neurons, the ribosomes, are concentrated within the cell body and large dendrites, where they are found as elements of rough endoplasmic reticulum (or Nissl bodies) (Droz, 1967). Ribosomes have never been observed within the axons of neurons (Palay and Palade, 1955). Heuser and Miledi (1970) have shown that labelled leucine and glutamate, whether applied externally or injected intra-axonally, are not incorporated into protein by the squid giant axon. Finally, Cowan et al (1972) have injected labelled amino acids into the corpus callosum, a known axonal pathway connecting the two cerebral hemispheres, which does not contain any neuronal cell bodies. They did not find any

radioactive label in either hemisphere, indicating that axons are unable to incorporate and transport labelled amino acids. Therefore, by using autoradiography, an injection into the MLR will only label axons arising from cell bodies within the MLR. The labelled amino acid will not be incorporated or transported by fibers passing through the MLR. Although it has been shown that axon terminals are capable of taking up labelled amino acids, only a small amount of protein synthesis accurs in the terminals (Cotman and Taylor, 1971). Also, Cowan et al (1972) have found no morphological evidence for a significant retrograde transport of tritiated amino acids. Thus, any label found overlying a CNS structure will have been transported in an orthograde direction from the cell bodies of origin at the injection site.

A second aspect of this thesis will attempt to isolate the spinal funicular trajectories of descending supraspinal signals necessary for MLR initiated locomotion in the cat. In acute spinal animals, the best cross-sectional areas of the cord to stimulate, so as to evoke locomotion, are the dorsal columns or the dorsolateral funiculus (Sherrington, 1910; Grillner and Zangger, 1974). Under chronic conditions, if a small portion of the ventromedial funiculus is left intact at the low thoracic cord level, then the rest of the cross-sectional cord can be cut and the cat will still be able to walk (Afelt, 1974; Windle et al, 1958). The conclusion that the walking by these chronic cats is due to the few intact fibers in the ventromedial funiculus is suspect, since complete transected spinal cats and dogs are known to engage in stepping movements and sometimes even walk (Hart, 1971; Shurrager and Dykman, 1951). Therefore, it would be interesting to study which cross-

sectional areas of the cord are functionally necessary for MLR evoked locomotion.

The third and final part of this thesis involves a series of experiments designed to investigate the role of descending NA systems in the initiation and control of locomotion. As outlined above, there is reason to believe that the descending NA system is not necessary for the activation of the spinal stepping generator, even though there has been agreat deal of compelling indirect evidence favoring its involvement.

The first series of experiments involves a continuation of previous work in this laboratory using the specific CA neurotoxin 6-OHDA (Jordan and Steeves, 1976). It was Tranzer and Thoenen (1968) who discovered that 6-OHDA caused an actual destruction of the terminal endings of sympathetic neurons. Subsequently it has been shown that 6-OHDA selectively destroys CNS CA - containing neurons, which results in a marked reduction of CA levels (See Kostrzewa and Jacobowitz, 1974). Therefore, 6-OHDA will be injected into a lateral ventricle of a cat to maximally destroy all CNS CA cells and fibers. Then, the animal will be decerebrated and tested to see whether locomotion can be evoked by MLR stimulation. The percentage reduction of NA by 6-OHDA will be determined by the Schellenberger and Gordon (1971) method for assaying monoamines. To assess the selectivity of 6-OHDA, 5-hydroxytryptamine (5-HT) will also be measured.

The second series of experiments will involve the injection of α-methyltyrosine (AMT) prior to MLR stimulation. Spector et al (1965) have shown that AMT reduces tissue NA levels by the inhibition of

tyrosine hydroxylase, an enzyme involved in the formation of NA (see figure 2). Tyrosine hydroxylase is the rate-limiting step in NA biosynthesis, converting dietary tyrosine to L-DOPA (Nagatsu et al, 1964). Once tyrosine hydroxylase is inhibited, tissue NA levels fall at a rate determined by the normal turnover of NA in the tissue (Udenfriend et al, 1966). In the cat, maximal depletion of CNS NA by AMT occurs after 24 hr (Rawe et al, 1977b). However, maximal inhibition of tyrosine hydroxylase activity in brain occurs after only 4 hr. (Udenfriend et al, 1966). Therefore, to assure that NA will not be present in the CNS, this study will utilize two injections of AMT. The first injection will be given 24 hr. prior to the experiment, to ensure maximal depletion of NA stores; the second will be given 4 hr. prior to the experiment, to prevent the de novo synthesis of NA during the MLR stimulation trials.

Finally, one argument for the involvement of a descending NA pathway in the initiation of locomotion has been that the α-adrenergic blocking agent, phenoxybenzamine (POB) (cf. Marley and Stephenson, 1972), inhibits MLR initiated locomotion (see Grillner, 1973, 1975). Due to the lack of experimental details in Grillner's reports, and the apparent discrepancy between the effects of POB and intraspinal 6-OHDA, it would be interesting to re-examine the effects of POB on MLR evoked locomotion.

Methods

All experiments were performed on adult cats ranging in weight from 2.0 kg to 5.0 kg. Anaesthesia was maintained throughout all surgical procedures using a mixture of halothane, nitrous oxide and oxygen. Asceptic surgical technique was used in every instance that required the survival of the animal for a period longer than 24 hr. In experiments involving the recovery of consciousness, every effort was made to minimize the discomfort resulting from surgery with the use of analgesics (eg. meperidine HCl). A regimem of antibiotics and corticosteroids, to reduce the degree of infection and inflammation, respectively, were also used for every cat maintained longer than 24 hr. after surgery.

Locomotion Preparation

Anaesthesia was induced in a closed box containing 4% halothane. After induction the cat was initially maintained at approximately 2% halothane with the aid of a face mask. Then a tracheostomy was performed and a T-tube was inserted into the trachea to maintain an adequate airway for the subsequent delivery of the anaesthetic.

The left common carotid was ligated, while the right common carotid was cannulated for the purpose of recording blood pressure. One of the femoral veins was also cannulated for intravenous (iv.) administration of fluids as necessary. In most animals, thin enamelled copper wires (diameter = 0.1 mm) were implanted, with the aid of a hypodermic needle, in the iliopsoas muscle (hip flexor) and the adductor femoris

muscle (hip extensor). These electrodes were used for electromyographic (EMG) recordings during locomotion.

The animal was then placed in a stereotaxic device which was positioned above a treadmill of our own design. The hindquarters of the cat were suspended above the treadmill by a strap running underneath the abdomen. The cat was kept warm by an overhead heat lamp. A bilateral craniotomy was performed. Then the cat was decerebrated with a spatula along a plane extending from the rostral border of the superior colliculus dorsally to a point just caudal to the mamillary bodies ventrally, for the production of a postmammillary preparation which does not spontaneously initiate locomotion (Hinsey et al, 1930; Grillner and Shik, 1973). The portions of brain rostral to the site of decerebration were then removed and any bleeding was stopped with the assistance of an adsorbable hemostat (Surgicel®). Immediately after the completion of the decerebration, anaesthesia was discontinued, and at least two hours were allowed for recovery from the anaesthetic before MLR stimulation was begun.

In preparation for stimulation, an insulated monopolar stimulating electrode (Kopf SNE 300, exposed tip = 0.25 mm, diameter = 0.1 mm), or electrode-needle assembly in experiment 1B (see below), was positioned 4.0 mm lateral to the midline, 1.0 mm caudal to the division between the superior and inferior colliculi, and lowered to an initial point of 4.0 mm below the surface of the inferior colliculus. For convenience, most of the preparations were stimulated on the left side of the mesencephalon. However, identical results were obtained in experiments

employing stimulation of the right side MLR (Shik, et al, 1966; Steeves, et al, 1975).

The treadmill belt was always set in motion prior to the onset of stimulation to verify that the cat could not walk without stimulation. Constant current stimulation was in the form of square wave pulses with a duration of 0.5 msec and a frequency of 30 Hz. The strength of the current never exceeded 200 μA. The time of each trial never exceeded 30 sec, with the intervals between trials being at least 3 min. The electode was lowered in successive 0.5 mm steps, with a number of trials at different current strengths at each depth to establish the optimal electrode tip position at which the lowest stimulation strength (threshold) could evoke coordinated walking.

EMG recordings were performed differentially between the wires implanted in each muscle. The signals were fed to amplifiers and then to an analogue chart writer. Additionally, the position of each hindlimb throughout the step cycle was monitored by means of a string attached to the ankle at one end, with the other end attached to a potentiometer. The voltage representing a particular position of the limb was then fed to the analogue chart writer.

The experimental set-up is graphically illustrated in Figure 3. An example of the locomotion evoked by MLR stimulation is provided by Figure 4, which illustrates one complete step cycle by the cat.

Autoradiography Procedure

The preparations and procedures for the injections of the tritiated amino acids are outlined in experiment 1 (see below). The following

methodology for processing CNS tissue for light microscopic autoradiography was modified from the technique of Cowan, et al (1972).

After an appropriate survival period (up to 10 days), the cat was anaesthetized with halothane and sacrificed by perfusion with an oxygenated Ringer's solution via the left ventricle of the heart. This was followed by perfusion with a 10% neutral buffered formalin solution to fix the CNS (McManus and Mowry, 1960). The CNS was then removed and further dissected to obtain manageable pieces of tissue. The tissues were fixed for a further 7 days in 10% neutral buffered formalin, and then dehydrated through a series of graded alcohols. The tissues were embedded in paraffin and serial sections were cut on a microtome at an approximate thickness of 10 μm. Every fifth section was placed on the surface of a warm (50^{o}C) water bath in order to flatten the section. The sections were then mounted on 0.5% gelatin - 0.05% alum coated slides (subbed slides). The slides were then deparaffinized in xylene for a few hours and then hydrated through a series of graded alcohols.

In preparation for coating the slides with a photographic emulsion, the emulsion (Kodak, NTB2) was melted in total darkness and then diluted with distilled water 1:1 and maintained in a temperature regulated water bath at 40^{o}C. The slides were then dipped once in the emulsion and set to dry in a high humidity oven at 25-30^{o}C for 2 hours. The high humidity prevents the emulsion from cracking while drying. The slides were then packed in light-proof containers and placed in a refrigerator for exposure to the β - particles emitted by the ^{3}H - amino

acid. Exposure times varied but the best results were obtained after 4 weeks incubation. Blank test slides were dipped in the emulsion and developed to ascertain the quality of the emulsion. Emulsion yielding background radiation levels of greater than 20 grains throughout the entire high dry objective field was discarded. The slides were developed in Kodak D-19 developer for 2 min., rinsed in distilled water, and fixed in rapid fix (without hardener) for 4 minutes. The developer, distilled water, and fixer should be maintained at 15^{o}C since higher temperatures markedly increase the background activity. It is also important to remember that until the emulsion is fixed it must be handled in complete darkness; no light emitting sources (including safelights) are advised since their presence will increase background activity.

After fixing, the slides were washed for approximately 20 min. and then counter-stained. For the purposes of detailing the cytoarchitectonic structure, a 0.1% cresyl violet stain was used. Subsequently, the slides were dehydrated through a graded series of alcohols and cover-slipped in the usual manner.

The slides were then examined, using a Zeiss photomicroscope, under both brightfield and darkfield illumination, for the presence of labelled fibers and terminals, correlating the pattern of activated silver grains with the underlying neuroanatomical structures. A structure was considered to be labelled if the label was present in adjacent sections and if the structure was labelled in a number of injected animals. A structure was not considered to be labelled if the concentration of

silver grains overlying the structure was not considerably more dense than that of the background (see Figure 5). Composite drawings of representative levels of the neuraxis were made detailing the sites of the labelled axons and their terminals.

Monoamine Assay

At the conclusion of MLR stimulation trials on animals in experiment 3 (see below), the decerebrated cats were reanaesthetized with a very low level of halothane (0.2-0.8%). A complete lumbar and cervical laminectomy was performed, as well as a complete removal of the parietal, temporal, and occipital bones. The following areas of the CNS were then rapidly removed and immediately frozen in liguid nitrogen: mesencephalon, pons, medulla oblongata, cervical cord enlargement (C_4 - C_8), lumbar cord enlargement (L_4 - L_7), and sacral cord. The tissue was then labelled and stored in a freezer maintained at -80^{o}C.

The tissue pieces were assayed for NA and 5-HT content using the Schellenberger and Gordon (1971) assay procedure. Tissue samples (approx. 1g) were homogenized in 0.4N perchloric acid ($HClO_4$) using Duall tissue grinders. The acid extract was then buffered to a pH of approximately 7.8 using Tricine. The catecholamines were then selectively absorbed onto 0.3g of aluminum oxide (Al_2O_3). The supernatant, containing the 5-HT, was then stored in a refrigerator overnight. The catecholamines were subsequently eluted from the Al_2O_3 using 0.05N $HClO_4$. The conversion of NA to a fluorescent thihydroxindole derivative was accomplished by oxidation of the NA with the addition of 0.1N iodine. The samples were heated in an oven at 100^{o}C for 3.5 min., allowed to cool, and the

fluorescence was read on an Aminco-Bowman spectrophotofluormeter. The excitation and emission wavelength settings were 382 mμ and 479 mμ, respectively.

The following day the 5-HT was assayed. The method involved solvent extraction of 5-HT into heptanol from a salt saturated solution. The 5-HT was then eluted from the heptanol by a 0.05 M neutral phosphate buffer. The fluorescence was developed by the addition 0.1M ninhydrin. The samples were heated in an oven at 100°C for 25 min. and then brought to room temperature during the next hour. The fluorescence readings were done on an Aminco-Bowman spectrophotofluormeter; excitation 383 mμ, emission 486 mμ. Internal and external standards were done with each assay, as well as tissue blank readings for each sample.

Experiment 1 - Autoradiography

A. Chronic intact series

Each animal was anaesthetized with an intraperitoneal (ip.) injection of sodium pentobarbital (35 mg/kg). The animal was then placed in a stereotaxic device. The cat was kept warm by a heating pad.

After removal of a portion of the parietal bone on one side of the cranium, the occipital lobe was gently retracted forward to reveal the dorsal surface of the mesencephalon (superior and inferior colliculi). Any small amounts of bleeding were controlled with an adsorbable hemostat.

The following ^{3}H-amino acids, obtained from New England Nuclear, were used:

Leucine, L- [4,5-^{3}H(N)] ~ 60Ci/mmol, 1mCi/ml

Proline, L- [2,3-^{3}H(N)] ~ 20Ci/mmol, 1mCi/ml

Leucine, L- [3,4,5-^{3}H(N)] ~ 110Ci/mmol, 1mCi/ml

Proline, L- [2,3,4,5-^{3}H(N)] ~ 110Ci/mmol, 1mCi/ml

In preparation for the injection, 0.5mCi of either the ^{3}H-proline, ^{3}H-leucine, or a mixture of the two was dryed under a stream of nitrogen to vaporize the solvent, 0.1 N HCl. The dried material was then re-dissolved in sterile Ringer's solution to a final concentrated solution of 25-50 μCi/ul (Cowan et al., 1972). A 1 μl syringe (Hamilton 7001-SN, 25 gauge needle) was then filled with the ^{3}H-amino acid.

In accordance with the experience of several groups of investigators (cf. Grillner, 1975) the stereotaxic coordinates for the MLR are P_2, L_4, H_0. On the bais of over a hundred experiments in our laboratory utilizing MLR stimulation to evoke locomotion; we have confirmed that the most predominant effective MLR stimulation site corresponds to 4 mm lateral to the midline, 1.0 mm caudal to the division between the superior and inferior colliculi, and 5.5-6.0 mm below the surface of the inferior colliculus. Therefore our injection needle was positioned accordingly. The ^{3}H-amino acid was then injected at a rate of 10 nl/5 min until a specified volume ranging between 50-200 nl had been injected. The needle was left in place for an additional 15 min following the injection. Control injections into brainstem areas outside the confines of the MLR were also performed in a couple of cats utilizing identical

injection procedures.

The retracted occipital lobe was then positioned in its proper place overlying the mesencephalon, and a protective covering of Surgicel® was placed over the exposed cortex. After suturing the skin, the animal was removed to a cage to recover from the anaesthetic. During the recovery period the cat was kept warm by a heat lamp. After recovery from the anaesthetic the cat was maintained in a healthy state for a period of up to 10 days. None of the animals in this experiment showed any deleterious effects due to the surgery. Maintaining an injected cat for a period of time was necessary to allow the uptake of the ^{3}H-amino acid by the cell bodies in the immediate vicinity of the site of injection, and its subsequent transport to the axon terminals (cf. Cowan et al, 1972).

After the appropriate survival period the animal was sacrificed and the CNS tissue was processed for the autoradiographic demonstration of axonal projections as outlined in methods above.

B. Chronic decerebrate series

Cats were prepared for MLR evoked locomotion as outlined in the methods above, except for performing a tracheostomy. Instead, a small endotracheal tube was passed through the epiglottis into the trachea with the aid of a laryngoscope. The endotracheal tube was removed upon completion of the surgery, so that during the subsequent survival period the amount of evaporative water loss from the respiratory system would be reduced.

One other modification involved fixing the stimulating electrode alongside the needle barrel of the 1 μl syringe. Only ^{3}H-proline was used in this series and it was prepared according to the procedures outlined in experiment 1A (see above). The syringe needle was filled with the ^{3}H-proline prior to the placement of the electrode-needle assemble in the MLR for the stimulation trials.

After establishing the optimal electrode tip position at which coordinated locomotion could be evoked with the lowest stimulation strength (never exceeding 100 μA; cf. Steeves et al, 1975), the ^{3}H-proline was injected from the 1 μl syringe. The volume injected ranged from 50-100 nl with the rate of injection being 10 nl/5 min. The electrode-needle assembly was left in place for an additional 15 min following the completion of the injection.

The cat was then removed from the stereotaxic device and the cranial cavity was filled with a sterile 4% agar solution. Before suturing the skin together, a protective covering of acrylic was applied to the top of the skull. The animal was then removed to a cage where its vital functions were maintained for periods up to 7 days.

Great effort was made to maintain the animal in a healthy homeostatic state during this survival period. To this end, the rectal temperature was monitored by means of a rectal probe, and any variance from the normal body temperature of 38^{o}C was corrected for by adjusting the height of a heat lamp above the cat. Likewise, the urine output and the specific gravity were also measured, to ascertain the condition of the kidneys and make sure the cat was not being administered excessive

amounts of fluids. Normally the animal was fed iv. with 5% dextrose in ½ normal Ringer's, approximately 100-200 ml/day. At a minimum of twice a day, 1ml of venous blood was withdrawn in order to measure serum electrolyte levels. Any deviation from the normal state were corrected for by changing the electrolyte levels of the iv. fluids. Approximately every hour, the cat's heart rate, respiration rate, and temperature were checked. The animal was also turned over every two hours. All normal physiological parameters were obtained from Altman and Dittmer (1961). As stated in experiment 1A, the animals were sacrificed after an appropriate survival period and their CNS tissues were subsequently processed for autoradiography.

Experiment 2 - Subtotal Spinal Cord Transections and Locomotion

Cats were prepared for MLR evoked locomotion as outlined in the methods above, except that a high cervical laminectomy was performed prior to decerebration. The laminectomy involved the reflection of the neck muscles and removal of the first and second cervical vertebrae. After establishing the optimal electrode tip position in the MLR for coordinated locomotion at the lowest stimulation strength, a longtitudinal incision was made along the dura mater of the cervical cord at the C_1 - C_2 level. The C_1 - C_2 cord was then cooled by applying small blocks of frozen Ringer's solution directly to the cord. The cord was cooled until the extensor rigidity of the fore and hindlimbs disappeared and breathing ceased (apnea), which signalled the blocking of spinal cord transmission, protecting the cat against spinal shock due

to the following surgery.

Subsequently, various combinations of partial transections of the spinal cord at the C_1-C_2 level were performed. The partial transections of the cord were accomplished with the use of micro-dissecting scissors, except for sectioning of the ventral funiculus. To section the ventral funiculus a sharp probe was inserted through the dorsal columns to the ventral funiculus and the lesion was then made. Lesions of some areas of the cord resulted in a cessation of respiration. When this occurred, the animal was immediately respired artificially with the aid of a respirator. End tidal CO_2 was monitored with a CO_2 analyzer (Beckman, model 16) and kept within 3.5%-5% by adjustment of the respirator tidal volume and rate.

If the cat was able to walk in response to MLR stimulation after a partial transection of the cord, then further sections of the cord were performed until locomotion could not be evoked by MLR stimulation. At the end of the stimulation trials the cervical spinal cord was removed and cooled in a freezer. Serial cross-sections of the C_1-C_2 cord were cut on a cryostat (American Optical) at approximately 30μm and mounted on subbed slides. The sections were stained with 0.1% cresyl violet stain using standard histological technique and coverslipped. The sections were then examined under the Zeiss photomicroscope to determine the extent of the lesions. Composite drawings of representative cross-sections of the cervical cord were made detailing the sites of the lesions.

Experiment 3 - Catecholamines and Locomotion

A. Catecholamine depletion

Each animal was anaesthetized with an ip. injection of sodium pentobarbital (35 mg/kg). The animal was then placed in a stereotaxic device. The cat's temperature was maintained by a heating pad.

In preparation for inserting a permanent indwelling cannula into one of the lateral ventricles, a small hole was drilled through the cranium at the stereotaxic coordinates A_{13},L_3. These coordinates correspond to the largest portion of the body of the lateral ventricle (Snider and Niemer, 1961). The cannula was constructed from a 23 gauge needle, cut to an approximate starting length of 16 mm. To prevent clogging of the cannula a protective cap was made from the bottom portion of a plactic Luer-lock 5 cc syringe, which was filled with acrylic. Extending downward from the acrylic was a cleaning wire. Before implanting the cannula, the cap was fastened to the top of the cannula and the cleaning wire was cut so that the tip of the cleaning wire exceeded the cannula length by 0.5mm. The cannula, with affixed cap and cleaning wire, was then inserted through the hole in the cranium. Proper placement of the cannula in the lateral ventricle was confirmed by a continuous outflow of cerebrospinal fluid (CSF) from the cannula when the cap and cleaning wire were removed. Additionally, a few cannula placements were made during fluoroscopic examination. By injecting a small quantity of a radio-opaque fluid (iophendylate) it was possible to adjust the cannula placement so that an outline of the lateral ventricle could be visualized (see Figure 6). The radio-opaque

fluid was then withdrawn, and the cannula fixed in place with dental acrylic and finally capped. The animal was then allowed to recover for 3 days before commencing the 6-OHDA (Labkemi AB, Stockholm, 6-hydroxydopamine HCl) injections.

Injections of 6-OHDA were usually carried out at two different times with an interval of 5 days between each injection. The dose for each injection was never greater than 2500 ug administered at a rate of 10 ul/min with the aid of a Harvard infusion pump. The total for each animal varied from 2500 ug to 5000 ug. The 6-OHDA was dissolved in deoxygenated normal Ringer's solution, to which 0.2 mg/ml of ascorbic acid was added to prevent the rapid oxidization of the 6-OHDA (cf. Kostrzewa and Jacobowitz, 1974). The final concentrations of the 6-OHDA solutions were 5 ug/ul or 6 ug/ul.

Seven days after the final injection, the cat was prepared for MLR stimulation as outlined in the methods above. At the conclusion of the stimulation trials the CNS was removed and assayed for monoamine content using the procedures outlined in the methods above. Corresponding CNS tissue was also removed from untreated controls and assayed for monoamine content.

B. Catecholamine synthesis inhibition

One day (24hr) prior to MLR stimulation, the cat was initially anaesthetized in a closed box with 4% halothane, and subsequently maintained at approximately 2% halothane with the aid of a face mask. One of the femoral veins was cannulated for the purpose of iv. administration of the tyrosine hydroxylase inhibitor, AMT (Labkemi AB, Stockholm,

dl-α-methyltyrosine-methylester HCl). The dose of AMT administered was 250 mg/kg made up in a concentration of 20mg/ml in normal Ringer's solution. The AMT was delivered at a rate of 1 ml/min with the aid of a Harvard infusion pump. Upon completion of the injection the cannula was removed and the anaesthesia was discontinued.

The following day, 4 hr prior to MLR stimulation, the animal was given another injection of AMT identical to that administered 20 hr previously. Surgery was then begun to prodece a decerebrate MLR stimulated preparation. After completion of the stimulation trials the CNS tissue was removed and the monoamine content was assayed according to the procedures outlined above. An untreated control group was also assayed for monoamine content.

C. Catecholamine receptor blockade

Each cat was prepared for MLR evoked locomotion as outlined in the methods above. The optimal electrode tip position at which coordinated locomotion could be evoked with the lowest stimulation strength was established. Subsequently, the α- adrenergic blocking agent, POB (Smith, Kline and French, Montreal, phenoxybenzamine HCl) was infused iv. The total dose of POB was 20 mg/kg dissolved to a concentration of 3 mg/ml in normal Ringer's solution, and infused at a rate of 10 ml/min.

The MLR stimulation trials were then repeated for over two hours. During the trials the quality of locomotion was determined with the use of the methods outlined above. At the end of the experiment, the animal was sacrificed by iv. injection of a lethal dose of sodium pentobarbital.

Abbreviations

AHY	adenohypophysis
AL	ansa lenticularis
Al_2O_3	aluminum oxide
AMT	∝methyltyrosine
AQ	adqueduct
BC	brachium conjunctivum
BP	brachium pontis
CA	catecholamine
CB	cerebellum
CCU	constant current unit
CM	nucleus centrum medianum
CNF	cuneiform nucleus
CNS	central nervous system
CO_2	carbon dioxide
CP	cerebral peduncle
CU	cuneate nucleus
DA	dopamine
EMG	electromyogram
EPSPs	excitatory postsynaptic pontentials
FF	fields of Forel
FN	fastigial nucleus
FTC	central tegmental field
FTG	gigantocellular tegmental field

FTL	lateral tegmental field
FTM	magnocellular tegmental field
FTP	paralemniscal tegmental field
GR	gracile nucleus
$HClO_4$	perchloric acid
HLA	lateral hypothalamic nucleus
HDA	dorsal hypothalamic nucleus
HM	medial habenular nucleus
HPA	posterior hypothalamic nucleus
IC	inferior colliculus
IOD	dorsal accessory nucleus of the inferior olive
IOMC	medial accessory inferior olive, caudal division
IOMR	medial accessory inferior olive, rostral division
IN	interpositus nucleus
IP	central interpeduncular nucleus
LC	locus coeruleus
L-DOPA	L-3,4 - dihydroxyphenylalanine
LGD	dorsal nucleus of the lateral geniculate body
LLD	dorsal nucleus of the lateral lemniscus
LLV	ventral nucleus of the lateral lemniscus
LR	lateral reticular nucleus
MLB	medial longitudinal bundle
MLR	mesencephalic locomotor region
MM	mammillary bodies
NA	noradrenaline
NHY	neurohypophysis

OT	optic tract
P	pyramidal tract
PAG	periaqueductal gray
PBL	lateral parabrachial nucleus
PBM	medial parabrachial nucleus
PC	posterior commissure
PF	parafascicular nucleus
PG	pontine gray
PLR	pontine locomotor region
POB	phenoxybenzamine
PR	paramedian reticular nucleus
R	red nucleus
RB	restiform body
RM	red nucleus, magnocellular division
RMN	raphé magnus
RP	red nucleus, parvocellular division
S	solitary tract
SC	superior colliculus
SCL	sub coeruleus
SG	substantia gelatinosa
SLR	subthalamic locomotor region
SN	substantia nigra
SNC	substantia nigra, compact division
SNR	substantia nigra, reticular division
SOL	lateral nucleus of the superior olive
SOM	medial nucleus of the superior olive
SUB	subthalamic nucleus

TB	trapezoid body
TRC	tegmental reticular nucleus
V3	third ventricle
V4	fourth ventricle
VH	ventral horn
VLD	lateral vestibular nucleus, dorsal division
VLV	lateral vestibular nucleus, ventral division
VIN	inferior vestibular nucleus
VMN	medial vestibular nucleus
VSCT	ventral spinocerebellar tract
VSN	superior vestibular nucleus
VTA	ventral tegmental area of Tsai
ZI	zona incerta
3	oculomotor nucleus
^{3}H	tritiated
4N	trochlear nerve
5-HT	5-hydroxytryptamine
5M	motor trigeminal nucleus
5ME	mesencephalic trigeminal nucleus
5N	trigeminal nerve
5P	principal sensory trigeminal nucleus
5PV	principal sensory trigeminal nucleus, ventral division
5SL	laminar spinal trigeminal nucleus
5SM	alaminar spinal trigeminal nucleus, magnocellular division

5ST	spinal trigeminal tract
6	abducens nucleus and/or abducens nerve
6-OHDA	6-hydroxydopamine
7	facial nucleus
7N	facial nerve
8N	stato-acoustic nerve
12	hypoglossal nucleus

Results

Axonal Projections from Neurons Within the Confines of the Mesencephalic Locomotor Region

Sites of injection

The most effective MLR stimulation point corresponds to the stereotaxic coordinates P2.0, L4.0, H-1.0 (Shik et al., 1966a, 1967; Steeves et al., 1975). On the basis of histological examination of the sites of MLR stimulation electrodes, Shik et al (1967) suggested that the MLR corresponds to the cuneiform nucleus (CNF). Their findings are confirmed and extended in the present study. The neurons corresponding to the MLR and their axonal projections were studied in two series of autoradiographic experiments.

In one series of autoradiographic studies, injections were made in postmammillary cats. After establishing the optimal MLR stimulation site from which locomotion could be evoked, the ^{3}H-amino acids were slowly infused into the CNS tissue surrounding the MLR electrode tip. Subsequently, every effort was made to maintain the animal for as long as possible, before removing the brainstem and spinal cord and processing the tissue for autoradiography.

A total of 10 cats were started in this series. In 6 of the 10 animals, locomotion was evoked by MLR stimulation. The threshold current intensities required to evoke locomotion ranged from 25μA to 100μA at 30 Hz. The quality of the evoked locomotion was excellent, and similar to that shown in Figure 4.

Of these 6 cats, 4 survived in a chronic decerebrate state for 4 to 7 days. This period of survival is considered sufficient for the labelling of axon terminals as well as pre-terminal axon fibers over distances as far away as the lumbar spinal cord (see Cowan et al, 1972). The other 2 animals died within one day and were excluded from auto-radiographic analysis since there would have been very little labelling of axons or their terminals during such a short time span.

The sites of injection were visible in the 4 surviving animals, after their brainstems and spinal cords had been processed for auto-radiography. In all four cases the injection site was found to center on an area between the ventral border of the inferior colliculus (IC) and the dorsal margin of the brachium conjunctivum (BC) (see Figure 7). According to Berman (1968) and Taber (1961), this area corresponds to the caudal part of the CNF. Unfortunately, only 1 cat showed any uptake of the ^{3}H-amino acids into neuronal cell bodies and subsequent labelling of axonal projections. The other 3 animals did not exhibit any uptake or labelling of axons and axon terminals. In the one cat that could be evaluated, any ascending projections to rostral mesencephalon were obscured by widespread necrosis extending caudally from the plane of transection. The descending projections to the pons and medulla did not appear to be affected.

Even though this series of experiments was less than successful, the data on the site of injections alone reconfirmed that functionally identified MLR corresponds to the anatomically defined CNF. Knowing that the MLR corresponds to the caudal CNF provided an easily identifible target

which could be injected with stereotaxic methods in intact cats.

Injections of ^{3}H-amino acids, using the stereotaxic coordinates of the MLR, were successful in 5 intact cats. All of the animals were maintained in a healthy state for 7 to 10 days and were then sacrificed. In 4 of the cats, the ^{3}H-amino acid was taken up by the neuronal cell bodies of the caudal CNF. As shown in Figure 8, the site of injection was restricted to an area between the ventral border of the IC and the dorsal margin of the BC (compare with Figure 7). The injection sites for the other 3 animals were similar. There was no uptake of the ^{3}H-amino acid at the injection site of 1 animal and consequently no labelled fibers or terminals could be visualized.

As a control, 1 cat was injected at a slightly more rostral level. The injection site was centered within the main "body" of the CNF. Recently, Edwards (1975) and Edwards and de Olmos (1976) have traced the axonal projections from this portion of the CNF. By replicating their work, it was possible to compare and contrast the pattern of projections emanating from a more rostral part of the CNF with those originating from the caudal CNF which corresponds to the MLR.

Finally, the axonal projections observed in the 1 chronic decerebrate cat were similar to those visualized in the intact series of animals. Therefore, any projections outlined in the results below occurred in every cat examined unless otherwise stated.

Descending projections (Pons and Medulla)

Fibers descending from the caudal CNF are loosely organized and mainly confined to the ipsilateral side of the brainstem. The labelled fibers were best visualized in parasagittal sections. Figures 9 and 10 outline the distribution of the descending projections.

The initial course of most of the fibers was in a ventromedial direction passing through the paralemniscal tegmental field (FTP) into the gigantocellular tegmental field (FTG). Enroute a number of labelled axons turned in a ventral direction and penetrated the dorsal half of the tegmental reticular nucleus (TRC). Within the dorsal TRC there was also a moderate to heavy dispersion of silver grains (see Figure 9a). At the light microscopic level, labelled fibers accompanied by a dense scattering of silver grains suggests that axon terminations are occurring (Cowan et al, 1972; Edwards, 1975).

At more caudal levels, the loosely knit projection continued along its longitudinal path maintaining a ventromedial position within the ipsilateral brainstem. The labelled fibers descended as far caudally as the magnocellular tegmental field (FTM). Throughout their course, the fibers gave off what appeared to be collaterals of terminal branches perpendicular to the longitudinal path of the main axons. Associated with the labelled axons in FTG and FTM were dense dispersions of silver grains. Figure 11 shows the heavily labelled axons in FTG, while Figure 12 shows the labelled fibers within FTM. The dense scattering of silver grains in FTG and FTM were confined to the ventromedial reticular formation overlying or just caudal to the trapezoid body (TB). The rostrocaudal

extent of this suggested terminal field was approximately P6.0 to P8.0, while the lateral coordinates extend ipsilaterally from L2.5 through the midline to L1.5 contralaterally.

In transverse sections, some of labelled fibers within FTG and FTM pierced the raphé magnus (RMN), where a moderate dispersion of silver grains was observed. Other fibers passed through RMN to terminate within the medial portions of the contralateral FTG and FTM.

There were only a few labelled fibers which descended below P8.0 and these were confined to the medial limits of the ipsilateral FTG and FTM. It should also be mentioned that a few scattered descending fibers from the caudal CNF passed through the lateral tegmental field (FTL) just medial to the trigeminal nuclei. No site of termination was discerned for this light scattered projection.

There was no evidence of projections to the cerebellum. In one cat labelled fibers were visualized within the upper cervical regions of the spinal cord. However, the injection site of this animal was slightly larger than in the other cats and labelled some cells within the locus coeruleus (LC), which is known to project to the spinal cord (cf Kuypers and Maisky, 1975). Therefore, there was no conclusive evidence for a direct descending projection to the spinal cord from the MLR.

Ascending projections (Mesencephalon and Diencephalon)

The ascending projections from the caudal CNF were much more extensive than the descending projections. The fibers travelled on the ipsilateral side of the brainstem and entered the rostral mesencephalon

and caudal diencephalon as a broad radiation. Part of the projection was composed of loosely organized fibers which spread directly forward from the caudal CNF. A second component of the ascending radiation formed a more distinct aggregation of axons which passed into the diencephalon via the ventrolateral central tegmental field (FTC) and fields of Forel (FF).

As in the case of the descending projections, the ascending projections are best appreciated in parasagittal sections. Figures 13 and 14 detail the ascending projections from both the transverse and parasagittal perspective.

A. Projections to Mesencephalic Structures

In the parasagittal plane, large numbers of labelled fibers radiate rostrally in a fan-like array. A number of axons project into the deep and intermediate layers of the IC. However, the labelling of this structure is probably due to the slight diffusion of the ^{3}H-amino acids up the needle tract during the injection.

Another group of axons projected rostrally in a dorsal direction into the deep and intermediate layers of the ipsilateral superior colliculus (SC). The labelled axons within these layers of the SC were accompanied by a considerable dispersion of silver grains, suggesting that numerous terminations had occurred (see Figure 13b, c).

Other diffusely organized fibers projected directly forward into the ipsilateral FTC, as well as the rostral CNF (Figure 14b). The dispersion of silver grains within these structures suggested that terminations were occurring enroute, since the labelled axons continued

through these structures to more rostral areas. Some of the labelled fibers within the FTC turned tangentially to enter the ipsilateral periaqueductal gray (PAG), sometimes referred to as the central gray. These fibers then ascended within the ipsilateral PAG to the level of the posterior commissure (PC). Along the way there were numerous terminations, as evidenced by the great dispersion of silver grains that surrounded the labelled axons.

The initial course of the more ventrally radiating fibers followed the ventrolateral margin of the FTC, maintaining a path just dorsal to the BC as it extends toward the red nucleus (R). As this fiber bundle passed by the dorsal margin of the substantia nigra (SN) a few fibers turned ventrally to enter both the reticular and compact divisions of the SN (Figure 14c). As the fibers approach the R they diverge to pass either dorsally to enter the FF, or project ventrally to enter the ventral tegmental area of Tsai (VTA). There were never any labelled fibers or terminals within either the magnocellular or parvocellular divisions of R. Accompanying the labelled axons within the ipsilateral VTA were numerous dispersions of silver grains which indicated the presence of axon terminations (Figures 13a, b). The labelled fibers within the VTA then continued forward into the caudal diencephalon.

In general, there was a distribution of label in the appropriate corresponding nuclei on the contralateral side. Contralateral projections to SC via the commissure of the superior colliculi were unequivocal. The contralateral CNF was also labelled, especially the

caudal division (see Figure 13d), which corresponds to the contralateral MLR. This site of termination is shown in Figure 15. There was also a light projection to the contralateral VTA. All the projections to the contralateral side appeared to be via labelled axons which crossed over within the FTC.

B. Projections to Diencephalic Structures

The results presented below were obtained from the intact series of autoradiographic experiments, since the diencephalon was removed in preparing a chronic decerebrate cat. At the mesodiencephalic junction there is a considerable divergence of the ascending fibers over a large cross-sectional area. However, for convenience, the ascending fibers can be divided into a dorsomedial group and a ventral group. The dorsomedial fibers are the axons which have ascended along a paramedial trajectory within the FTC and now penetrate the intralaminar nuclei of the thalamus. Meanwhile, the ventral fibers arrive in the caudal diencephalon via the FF and spread into the zona incerta (ZI), the subthalamic region (SUB), and caudal nuclei of the hypothalamus.

The heaviest areas of termination for the labelled axons entering the intralaminar nuclei are the parafascicular nucleus (PF) and the nucleus centrum medianum (CM). There was a great density of dispersed silver grains within the PF, as shown in Figure 16. The labelling in CM was not quite as dense. The only other distinct heavy labelling was observed in the rostral extent of the ipsilateral PAG, as mentioned above.

The broad ventral radiation enters the diecephalon via the FF, where the more lateral ascending fibers continue into the ZI (Figure 14).

There were particularly dense dispersions of silver grains in the ZI, suggesting axon terminations. At more rostral levels a small number of fibers turned ventrolaterally from the ZI, passed beneath the entopeduncular nucleus, and then ascended through the ansa lenticularis (AL).

Within more medial regions of the diencephalon, the labelled axons ascending within the FF distributed heavily labelled terminations to the medial SUB region and the lateral hypothalamic nucleus (HLA). The dense labelling within the SUB region is shown in Figure 17. Further forward, the label was distributed over the dorsal hypothalamic (HDA) and the posterior hypothalamic (HPA) nuclei. In more rostral areas the number of labelled fibers and the density of labelling surrounding them declined dramatically, and no projections were found rostral to the anterior commissure.

In general, the contralateral projections were very light. At the mesodiencephalic junction, some fibers crossed in the PC to distribute amongst the intralaminar nuclei, especially the contralateral PF and CM nuclei. Similarly, a few labelled fibers were seen within the contralateral SUB, HPA, HLA, and AL.

No projections were seen to any of the specific thalamic nuclei. In only two animals was there any labelling of the ipsilateral ventral lateral geniculate nucleus or pretectal nuclei, as mentioned by Edwards and de Olmos (1976). There was a light labelling of the ipsilateral thalamic reticular nuclei; however, this observation was not consistently made in all the animals.

Projections from the rostral cuneiform nucleus (Control)

The distribution of projections in the control cat was similar to that reported by Edwards (1975) and Edwards and de Olmos (1976). The most prominent projection observed in this animal, which was not observed in the other autoradiographic animals, was a labelled bundle of fibers that descended through the contralateral pons and medulla. Edwards (1975) termed this projection the ventral tegmental bundle. The contralateral FTG and ipsilateral lateral reticular nucleus were distinctly labelled sites of termination in the control cat, but not in the MLR injected animals. However, the distinct labelling of the contralateral facial nucleus as reported by Edwards (1975) was not observed. Panneton and Martin (1978) stated that the labelling of the facial nucleus is probably due to the spread of the injection material into the ventral PAG in Edwards, (1975) study.

The ascending projections from the rostral and caudal CNF were similar, except that the ipsilateral thalamic reticular nucleus was distinctly labelled. At more rostral levels, the central lateral and central dorsal nuclei were also labelled, confirming the observations of Edwards and de Olmos (1976). Projections to the central lateral and central dorsal nuclei were not seen in caudal CNF injected cats.

Therefore, there were significant differences between the projections from the rostral and caudal CNF. These differences might be the anatomical basis of a subnuclear compartmentalization for physiological function and indicate why only stimulation of the caudal portion of the CNF will evoke locomotion in a decerebrate cat.

Effect of Subtotal Spinal Cord Transection on Controlled Locomotion

An insight into which spinal cord pathways are necessary for MLR evoked locomotion might be achieved by evaluating the locomotor capabilities of postmammillary cats after various partial transections of the C_1-C_2 cord. The partial transections were done at the C_1-C_2 level of the spinal cord, rather than between the cervical (C_5-C_8) and lumbar (L_4-L_7) enlargements, so that propriospinal influences arising in the cervical enlargement would not facilitate locomotion in the hindlimbs.

Figure 18 summarizes the various subtotal transections and the corresponding effect or lack of effect on MLR initiated locomotion. After comparing the various lesions it is evident that the only restricted area of the white matter that by itself is essential for MLR evoked locomotion is the ventrolateral quadrant. This statement does not mean that other cross-sectional areas of the white matter are not involved in the initiation and coordination of locomotion. However, transection of the dorsolateral quadrants, dorsal columns or ventral funiculi does not effect locomotion evoked by MLR stimulation. The following sections provide the details of the subtotal spinal cord transection experiments, which are summarized in Figure 18.

A. Transection of the dorsolateral quadrants

The dorsolateral quadrants were cut in 2 postmammillary cats to ascertain the result of such a transection on MLR evoked locomotion. Figure 20 shows that there were no variations in the locomotion from that obtained during a control situation (see Figure 19 in the same animal).

Each cat, in these experiments, provided its own control records which were obtained prior to sectioning the cord. However, it would be very redundant to include control records of the walking for each animal prior to transection. It is sufficient to say that there were no observable differences in the control records obtained from the cats in this study. The locomotion illustrated in Figure 20 was obtained with an increase in the stimulation strength of 25μA above that necessary to evoke locomotion prior to the transection. This slight increase is not thought to be significant, since there are often times when the threshold current strengths will fluctuate by 25μA or more during control circumstances.

Histological verification of the transection revealed that the damage was restricted to the dorsolateral quadrants, as shown in Figure 20. Thus the patency of the pathways that course through the dorsolateral portions of the spinal white matter are not essential for locomotion in postmammillary cats.

B. Transection of the dorsal columns

Figure 21 shows the EMG and limb movement records from a cat in which the dorsal columns of the C_1-C_2 cord were cut. The locomotion initiated by MLR stimulation was identical to the controlled locomotion prior to sectioning the dorsal columns, with no change in the MLR stimulation strength. Examination of the C_1-C_2 cord confirmed that only the dorsal columns were destroyed, as shown in Figure 21.

Cutting the dorsal columns in a second animal did inhibit MLR evoked locomotion. Even using MLR stimulation currents as great as 500μA

proved unseccessful in initiating locomotion. Examination of the site of sectioning revealed that there was no observable damage to any cross-sectional area other than the dorsal columns. This result may have been due to local edema or to direct trauma, during the cutting of the cord, which affected the unsevered parts of the cord. For these and other possible complications, positive evidence of an effect on MLR initiated locomotion by an acute subtotal lesion is less interpretable than negative evidence.

C. Transection of the ventral funiculi

Three experiments were done in which the ventral funiculi (bilaterally) were destroyed. In all three cases there were no changes in the locomotion activated by MLR stimulation, as shown in Figure 22. Comparing Figure 19 with Figure 22 reveals the respective similarities, in EMG and limb movement records, between ventral funicular transected and control cats. Two of the three cats did not require any increase in the MLR stimulation current to evoke locomotion, whereas an increase of 50μA over that required before cutting was necessary to initiate locomotion in the third.

Figure 22 also illustrates the extent of the lesion inflicted on the ventral funiculi. The lesions in the other two experiments were similar, as confirmed by histological examination. Thus, it would appear that the integrity of the ventral funiculi are not necessary for the initiation of locomotion by MLR stimulation in postmammillary preparations.

D. Transection of the dorsolateral quadrants and the ventral funiculi

Combined disruption of the fiber pathways within both dorsolateral quadrants and the ventral funiculi does alter MLR evoked locomotion. In the two cats examined, high strength MLR stimulation evoked weak alternating stepping movements by the hindlimbs but no movement by either forelimb. In both animals the MLR stimulating current was doubled beyond what was required to initiate locomotion, prior to the transections (eg. 125μA increased to 250μA). It was not possible to obtain satisfactory EMG and limb movement records due to the deficient nature of the hindlimb stepping and because the evoked hindlimb stepping was only sustained for short periods (approximately 5-15 sec). Histological examination showed that the transections were restricted to the target areas.

Severing the fiber pathways that course through the dorsolateral quadrants does not alter MLR evoked locomotion, nor are the fiber pathways that run through the ventral funiculi essential for controlled locomotion (see Results above). However, combining the two types of subtotal transections in the same animal does alter MLR initiated locomotion, even though the basic spinal mechanisms for stepping can be activated.

E. Transection of the dorsal cord

Cutting the entire dorsal cord resulted in the animal requiring artificial respiration. Even though the animals could not breath spontaneously, their blood pressures and percent respired CO_2 were not appreciably different from cats that did not require artificial respiration.

Only one of the two animals was capable of any MLR evoked movement, and the weak alternating hindlimb stepping seen in this cat was only in

response to high strength MLR stimulation. The other cat did not respond to MLR stimulation, except for increases in blood pressure while the MLR stimulus was turned on. In both animals only the dorsal cord was damaged.

The fact that both animals required a respirator and consequently might have been incapacitated can not be overlooked. However in one cat, the MLR stimulation did activate the lumbosacral stepping generator by pathways other than those running through the dorsal half of the cord.

F. Transection of the ventrolateral quadrants

Disruption of the fiber pathways coursing through the ventrolateral quadrants of the C_1-C_2 cord had a profound and consistent effect on MLR evoked locomotion. Lesions of the type illustrated in Figure 23 inhibited locomotion due to MLR stimulation in all 5 cats examined. Prior to cutting the ventrolateral quadrants every animal was capable of coordinated walking by all four limbs in response to MLR stimulation. After severing the ventrolateral quadrants MLR stimulation, of up to 500μA, was almost totally ineffective. In three animals, MLR stimulation did evoke weak stepping movements by the forelimbs when high strength MLR stimulation was used. However, there were never any movements evoked in the hindlimbs. It should also be mentioned that in the three animals that exhibited forelimb stepping, the cutting of the ventrolateral quadrants caused a slight increase in the decerebrate rigidity of their forelimbs.

All 5 cats were judged to be in excellent condition after the spinal cord sectioning. There was no reduction in the blood pressure due to the sectioning, nor did the animals require any artificial respiration. Each animal regained its decerebrate rigidity and spinal reflexes after recovering from the effects of the reversible cold block. During MLR stimulation trials the cat's blood pressure rose in response to the MLR stimulus being turned on, as it did during the control trials when the cat walked. Therefore, the condition of all five animals was excellent and does not offer an explanation as to why the animals did not walk in response to MLR stimulation.

The site of the ventrolateral transection was histologically examined in each cat and found to correspond to that shown in Figure 23. The results of the ventrolateral transection are positive results. Therefore the inability to evoke locomotion by MLR stimulation might be due to direct trauma of the unsevered parts of the cord. However, all the cats were judged to be excellent condition (see preceding paragraph) and showed no signs of non-specific damage to the spinal cord.

Unlike the previous positive results reported for dorsal column and dorsal cord transection, the effects of ventrolateral transection were based on a larger sample size. There was almost perfect accord in the results of each experiment. That is, there was no locomotion in response to MLR stimulation after ventrolateral transection, except for a few forelimb steps. It could be argued that the forelimb stepping was a "false positive" result. This is supported by the observation that, of the three animals exhibiting MLR evoked forelimb stepping, one cat also exhibited spontaneous forelimb stepping.

G. Transection of the ventral cord

Not surprisingly, transection of the entire ventral cord inhibited MLR initiated locomotion. It was not possible to evoke movement in 2 cats in which the ventral cord had been transected, even if the MLR stimulation currents were as high as 500μA. To accomplish the ventral cord transection both pairs of C_1 and C_2 dorsal roots had to be cut to allow rotation of the cord so that the ventral areas of the cord would be accessible for transection. Cutting the dorsal roots alone did not alter MLR evoked locomotion. Figure 18G illustrates the magnitude of the ventral cord transection which was confirmed by histological examination.

H. Transection of the cord except for the ventral funiculi

Finally, one animal had the entire cord transected except for the ventral funiculi. MLR stimulation was totally ineffective and no movements were evoked in any of the limbs. The animal did not exhibit any deleterious effects due to the large extent of the transection (see Figure 18H).

Effects on Controlled Locomotion Due to Diminution of CNS Catecholaminergic Function

A. Effect of 6-OHDA

As shown by Jordan and Steeves (1976), thoracolumbar intraspinal injections of 6-OHDA will deplete lumbosacral NA levels by as much as 99%, but this large reduction does not alter MLR-evoked locomotion in postmammillary cats. These findings have been confirmed and extended

in the present investigation. The responses to MLR stimulation were tested in 4 postmammillary cats 7 days after lateral ventricle infusion of 2500 to 5000 µg of 6-OHDA.

In all 4 6-OHDA treated animals, MLR stimulation evoked coordinated locomotion by all four limbs. Figure 24 shows the EMG records and limb movement tracings from a stimulation trial on one of the cats. Comparing this Figure with Figure 19, which illustrates the locomotion records of an untreated control animal, reveals no apparent differences between the 6-OHDA treated cat and the control. Any slight variations between Figures 19 and 24 are due to the small variabilities,from trial to trial, which occur within every experiment. Furthermore, there were no other alterations in the evoked walking of the 6-OHDA treated animals that might have been missed by the EMG and limb movement records, but would have been detected by visual observation during each trial. Finally, the MLR stimulus thresholds necessary to evoke locomotion in the 6-OHDA treated animals were within the range of current intensities required in untreated control experiments.

Additional experiments were carried out on 2 sham-injected cats. There were no locomotor differences between the sham-injected animals and the controls. At the conclusion of each MLR stimulation experiment the brainstem and spinal cord were removed and the tissues were assayed for NA and 5-HT content.

Shellenberger and Gordon (1971) reported that in their assay the calculated recovery of NA from tissue samples is approximately 85% whereas 5-HT recovery is only 70%. The present study confirms their

recovery ratios, as long as the reagents for the assay do not deteriorate. The sensitivity of this assay allows for tissue samples ranging from 100 mg to 1.0 gm in weight, from which quantities of amines ranging from 10 ng to 2 µg can be assayed (Shellenberger and Gordon, 1971). The NA control values measured in the present study agree with previously assayed NA levels from control brainstem (Jalowiec et al, 1973; Johansson et al, 1974) and control spinal cord tissues (Andén, 1964; Reis and Gutnick, 1970; Rawe et al, 1977). This indicates that the Shellenberger and Gordon (1971) assay provides a reliable means for measuring CNS NA content in both untreated and NA depleted cats.

From Figure 26 it can be seen that a significant reduction of NA content occurred in the 6-OHDA treated cats. Figure 26 shows the mean assayed levels of NA for both the 6-OHDA and control animals. The NA levels in the tissues from sham-injected animals did not differ from untreated control cats and were therefore grouped together. Some of the tissues from 6-OHDA treated animals were also assayed for 5-HT content. There were no significant differences in the 5-HT levels of 6-OHDA treated cats when compared with the 5-HT levels from untreated or sham-injected controls.

Figure 27 graphically illustrates the reduction in NA content by 6-OHDA, when expressed as a percentage of the control values. The NA content in spinal cord tissues of 6-OHDA treated cats was depleted more than the NA content in brainstem tissues. This is not unexpected since the predominant site of action of 6-OHDA is on CA terminals (cf. Kostrzewa and Jacobowitz, 1974), and the spinal cord only contains descending NA

fibers and their terminals, whereas the brainstem also contains the parent NA cell bodies of the descending NA fibers (Dahlstrom and Fuxe, 1965). In the present study the amount of NA reduction by 6-OHDA was not dose dependent, since there were no significant differences between cats administered 2500 µg of 6-OHDA or cats given 5000 µg of 6-OHDA. This finding confirms the earlier study of Petitjean et al (1972).

It should also be mentioned that observation of the 6-OHDA treated cats prior to decerebration did not reveal any abnormalities in their walking. When placed in an open field environment, the animals were capable of normal locomotion without any ataxia or equilibrium disorders. Only one animal was reluctant to engage in spontaneous motor activity and had to be coaxed to do so.

Also, 3 of the cats were more aggressive after the 6-OHDA infusions. The animals showed great fear at an approaching experimenter's hand, and could become highly aggressive in response to very innocuous stimuli like a puff of air. The behaviour of the cats could be defined as threat followed by attack and has been previously seen in 6-OHDA treated cats (Johansson et al,1974).

The results described above indicate that a substantial reduction of CNS NA has no observable effect on MLR evoked locomotion or voluntary walking in an open field environment. These findings, along with those of Jordan and Steeves (1976), suggest that descending NA fibers are not likely to be the sole means for activation of spinal locomotor systems from the MLR.

B. Effect of AMT

The responses to MLR stimulation were investigated in 5 post-mammillary cats after 250 mg/kg of AMT was infused i.v. 24 hr and 4 hr prior to decerebration. AMT was chosen because it blocks the synthesis of NA by inhibiting the activity of tyrosine hydroxylase (Nagatsu et al, 1964) resulting in the depletion of NA at a rate determined by the turnover of the stored NA. Therefore, a study utilizing AMT, rather than 6-OHDA, provides a different approach in the assessment of the effects of CA diminution on MLR initiated locomotion.

After AMT pretreatment, all 5 cats were capable of locomotion in response to MLR stimulation. As can be seen in Figure 25, there were no appreciable differences in the records of locomotion between an AMT treated cat and the records of either an untreated control (see Figure 19), or 6-OHDA treated animal (see Figure 23). In all instances, the AMT treated, 6-OHDA treated, sham-injected, and control animals exhibited identical locomotor behaviour in the open field situation and in response to MLR stimulation.

Figures 26 and 27 show that AMT significantly reduced the CNS NA levels. AMT pretreatment was significantly better than 6-OHDA in reducing the NA content of the pons and slightly superior to 6-OHDA in all other assayed tissues, except for lumbar spinal cord (see Figure 27). As was the case for the 6-OHDA treated animals, there were no significant changes in 5-HT levels due to the AMT treatment.

These results indicate that there were no changes in MLR evoked locomotion as a consequence of the considerable depletion of CNS NA content by AMT. These findings confirm and support the previous 6-OHDA

findings. One added bonus was finding that the MLR-evoked locomotion was unaltered 4 hr after the second AMT infusion, which corresponds to the time of maximal inhibition of tyrosine hydroxylase activity (Udenfriend et al, 1966), thereby reducing the possibility of de novo synthesis of NA occurring during the MLR stimulation trials. Thus, the inhibition of de novo NA synthesis further reduces the chance that MLR evoked locomotion is initiated by the release of NA from descending NA fibers.

C. Effect of POB

One of the lines of evidence supporting the theory that descending NA fiber pathways are responsible for the initiation of locomotion is the finding that POB inhibits MLR initiated locomotion (Grillner, 1973). However, due to the apparent discrepancy between the effects of POB and the above results, the present study re-examined the effect of POB on MLR evoked locomotion. Unfortunately, Grillner (1973) did not provide any details of his methodology. However, Andén et al (1974) state that 20 mg/kg i.p. of POB will selectively block central NA receptors. Therefore, 20 mg/kg i.v. of POB was administered to 5 postmammillary cats after establishing the optimal MLR stimulation site for evoking coordinated locomotion.

Figure 28 illustrates the similarity between a pre-injection EMG record and a post-injection EMG record. In this particular example there were no differences in the MLR evoked locomotion after an i.v. injection of POB, nor was there any difference in the MLR stimulation

strength required to initiate locomotion. In the present study, 2 of the 5 cats showed no locomotor differences between pre and post injection trials.

However, another 2 cats did exhibit abnormalities in their MLR evoked locomotion after infusion of POB. In both instances there was a threefold increase in the current intensity required to initiate locomotion by MLR stimulation. In one cat the MLR stimulus threshold was 75μA prior to POB injection but 250μA after the injection. When MLR stimulation did evoke walking, the excursions of the limbs throughout the swing and stance phases were greatly reduced. Also, the dorsal surface of the cat's paws contacted the treadmill surface first, rather than the more normal fashion of toe pads first. Locomotion in such a fashion must be considered deficient, even though the basic rhythmic alternating limb movements are preserved. The final cat in this study succumbed to the POB injection before any analysis could be undertaken.

The lack of effect of POB on MLR evoked locomotion in 2 of the cats provides further support for the contention that the NA system is not necessary for the activation of spinal motor mechanisms.

Discussion

Anatomical Identity of the Mesencephalic Locomotor Region

The results of the present study provide further evidence which confirms that the MLR corresponds anatomically to the cuneiform nucleus, as suggested by Shik et al (1967). It has been repeatedly shown that the optimal site for MLR stimulation centers on an area between the ventral border of the IC and the dorsal margin of the BC, at the stereotaxic coordinates P 2.0, L 4.0, H-1.0 (Shik et al, 1967; Steeves et al, 1975). However, there are several distinct anatomical structures located at these coordinates besides the CNF. As outlined by Berman (1968) and Taber (1961), the lateral parabrachial nucleus (PBL), medial parabrachial nucleus (PBM), locus coeruleus (LC), subcoeruleus (SCL), mesencephalic trigeminal nucleus (SME), dorsal nucleus of the lateral lemniscus, (LLD), inferior colliculus (IC) and brachium conjunctivum (BC) are also in close proximity to the optimal site for MLR stimulation (see Figure 7).

Shik et al (1967) found that removal of the superior and inferior colliculi did not effect the locomotion evoked by MLR stimulation. Furthermore, stimulation of the BC has never been shown to evoke locomotion (Shik et al, 1967; Steeves et al, 1975). Therefore, these areas do not correspond to the MLR. Stimulation of areas ventral to the BC does evoke locomotion, but the locomotion is invariably accompanied by interferring tegmental responses and requires stronger stimulation to initiate the locomotion (Shik et al, 1967). Thus, the SCL and PBM are probably not the optimal MLR sites. Moving the electrode medially or laterally from L 4.0 results in evoked locomotion complicated by spasticity

(Shik et al, 1967, Steeves and Jordan, unpublished observations), which suggests that the LLD and 5ME are unlikely MLR structures.

The possibility that the catecholaminergic LC or PBL (cf. Poitras and Parent, 1978) correspond to the functional MLR cannot be as easily dismissed, since there are remarkably similar effects on the same spinal neuronal circuits after iv. L-DOPA or MLR stimulation. Thus, Grillner and Shik (1973) suggested that the locomotion evoked by MLR stimulation might be mediated by a descending NA pathway. Subsequently, Steeves et al (1975) demonstrated CA neurons in close proximity to the site of MLR stimulation. It was suggested that these CA neurons were a part of the noradrenergic LC complex, which is known to project to the spinal cord (Kuypers and Maisky 1975, 1977; Hancock and Fougerousse 1976; Nygren and Olson, 1977; Commissiong et al, 1978). However, Saito et al (1977) stated that CA neurons visualized by Steeves et al (1975) are associated with the catecholaminergic PBL nucleus and not the LC.

Nevertheless, the present study has shown that significantly large depletions of NA within the brainstem and spinal cord (Figure 27) has no observable effect on locomotion evoked from the MLR. Therefore it is unlikely that the descending NA pathway, originating from the LC, is the essential pathway mediating the activation of the spinal stepping generator during MLR evoked locomotion (see Discussion below). The NA depletion studies also provide indirect evidence against the consideration of the PBL nucleus as the anatomical structure corresponding to the functional MLR.

Therefore, the substantial amount of indirect evidence suggests that the anatomical structure corresponding to the functionally defined MLR is

the caudal CNF as defined by Taber (1961), which corresponds to all of the CNF as defined by Berman (1968).

Involvement of Descending Noradrenergic Pathways in Locomotion

The experiments in the present study have shown that a substantial reduction of NA does not alter locomotion in the intact cat or in the postmammillary cat. As shown in Figure 27, 6-OHDA or AMT pretreatment reduced NA content by as much as 80% yet there were no observable differences in the MLR evoked locomotion when compared with untreated controls. Furthermore, POB did not prohibit MLR evoked locomotion as previously reported by Grillner (1973).

There are two obvious explanations for these results. First, NA may not be involved at all in the neural transmission of the behavior elicited by this type of stimulation. Alternatively, the brainstem and/or spinal cord might still have retained enough functionally active NA to have maintained the intensity of the responses elicited. Admittedly, there are various degrees of involvement lying between these two extremes.

There have been numerous reports in which similar amounts of depletion of NA have resulted in noticeable effects on sleep (cf. Jouvet, 1972), feeding (Heffner et al, 1977), aggression (Johansson et al, 1974), learning (Schoenfeld and Seiden, 1969) and many other behaviors under NA control. Similarily, doses of i.v. POB, identical to those used in the present study, have been shown to inhibit clonidine induced increases in flexor reflex activity (Anden et al, 1970) and spontaneous locomotor activity (Anden and Strombom, 1974). In the present study, intraventricular infusions of 6-OHDA did alter one form of behavior (increased aggression) but not another (locomotion) in the same animal. Thus, if

a NA fiber system were mediating MLR evoked locomotion, it would seem unlikely that the reduction of NA levels by as much as 80% or blockade of postsynaptic receptors by POB would be without effect.

Finally, 2-3 week chronic spinal cats will commence coordinated alternate stepping by both hindlimbs after 100 mg/kg L-DOPA i.v. (Steeves and Jordan, unpublished observations). It is well known that there are no viable NA fibers caudal to the transection level (Carlsson et al, 1964; Jordan and Steeves, 1976), thus the spinal stepping initiated by i.v. L-DOPA in acute spinal (Grillner, 1969; Budakova, 1973) and chronic spinal preparations might not be due to L-DOPA activating the release of NA from descending NA fibers. Possibly L-DOPA initiates stepping in chronic spinal cats by as yet unknown non-specific mechanisms, or it directly activates the NA receptors of the spinal neurons. These suggested mechanisms may also be true for the acute spinal stepping preparations.

The descending NA pathway is not likely to be the sole means for activation of spinal locomotor systems from the MLR. It is more probable that the initiation and control of locomotion involves several supraspinal systems. Considering that descending NA fibers are not essential for the initiation of locomotion, is the release of NA onto spinal neurons sufficient to activate spinal locomotor mechanisms? Preliminary results (Rossignol and Jordan, unpublished observations) have shown that subarachnoid infusions of NA in postmammillary cats does not evoke stepping movements and thus a NA system alone may not be sufficient to evoke locomotion.

Projections of the Mesencephalic Locomotor Region

The axonal projections and possible sites of fiber termination, outlined in the results of the present study, may help to elicidate the mechanisms by which MLR stimulation activates spinal locomotor systems. Although the sites of ^{3}H-amino acid injections centered on the caudal CNF, some of the cells of the PBL nucleus would have unavoidably incorporated the ^{3}H-amino acids. Therefore, the resulting distribution of labelled fivers probably represents some of the axonal projections of PBL neurons. This statement is supported by the similarity in the pattern of some of the ascending labelled fivers seen in the present study and those reported by McBride and Sutin (1976, 1977).

Although the injection site of McBride and Sutin is somewhat lateral to the injection site in the present study (see their figure 10, 1977), there is probably a degree of overlap. Thus some of their projections involve the caudal CNF as well as the PBL. Nevertheless there were a few noticeable differences between the two studies. McBride and Sutin (1977) reported distinct labelling of the ventromedial nucleus of the hypothalamus although this projection was not seen in the present study. Nor did they describe any descending projections caudal to FTP, whereas the present results showed a prominently labelled projection to ipsilateral FTG and FTM. Finally, McBride and Sutin (1976, 1977) did not observe projections to the ipsilateral SC, PAG or TRC which were also distinctly labelled in the present study. This suggests that although there are some similarities, any differences might be attributable to the more dorsomedial position of the injection site in the present study. Consequently, any unique labelled projections

probably originate from the caudal CNF rather than the PBL nucleus.

The labelled projection to the SC has been previously demonstrated to originate in part from the rostral CNF (Edwards, 1975, Grofova et al, 1978). On the basis of the present data it would appear that the deep and intermediate layers of the SC also receive input from the caudal CNF. The deep layers of the SC have been suggested to be a zone of convergence for fiber systems primarily concenred with conveying non-visual impulses (Harting et al, 1973). The functional importance of the projection to the SC may be the well known interactions between the visual and somatic motor systems (cf. Ingle and Sprague, 1975). However, in a more restricted sense, the removal of the SC does not interfere with the initiation of locomotion by MLR stimulation in postmammillary cats (Shik et al, 1967). Thus tectospinal projections do not appear to be essential for MLR evoked locomotion.

The projection to the ipsilateral PAG was not restricted to any one of the three subdivisions (dorsal, medial, and lateral nuclei) as outlined by Hamilton (1973a). Although the dorsal nucleus was not as heavily labelled as the medial and lateral subdivisions. The PAG has been shown to project to the CNF, including the caudal division which corresponds to the MLR (Hamilton and Skultety, 1970, Hamilton, 1973b). They also showed that the PAG projects to the VTA, PF, FF and HPA areas, which also receive efferent fibers from the MLR. The PAG projections exemplify a recurrent theme regarding the projections of the MLR. Briefly, many of the terminal areas of MLR projections send efferent fibers back to the MLR and also have reciprocal connections with other MLR projection areas. The significance of these

interconnections remains to be elucidated, as does a role of the PAG in locomotion.

Within the rostral mesencephalon, there were also unmistakable projections to the ipsilateral VTA, FTC, and contralateral caudal CNF. Descending spinal projections from the FTC, or rostral CNF as Taber (1961) and Edwards (1975) refer to it, have only been demonstrated in the monkey (Castiglioni et al, 1978). However, the FTC does project to the ipsilateral and contralateral FTG and FTM (Edwards, 1975), where descending spinal fibers do originate (cf. Basbaum et al, 1978). Stimulation within FTC results in disynaptic changes in muscle spindle afferent activity (Appelberg and Jeneskog, 1972) and spinal interneuron activity (Baldissera et al, 1972) which are thought to be mediated by reticular neurons of the pons and medulla. Even though FTC stimulation has never been reported to evoke locomotion in a postmammillary cat, Sterman and Fairchild (1966) have stated that stimulation of FTC in intact cats will increase the velocity of locomotion.

The VTA has been implicated in aggression and rage in cats by Bandler et al (1972), who reported that VTA stimulation evokes attack behavior by cats. Whether the VTA is primarily involved in the motor or motivational (ie. emotional) aspects of this behavior is unknown, even though the VTA projects to brainstem areas which have been implicated in locomotion including FTG, FTM and the SLR (Chi et al, 1976). Similarly, a role of the VTA in locomotion by the intact or postmammillary cat remains to be elucidated.

Shik et al (1967) have shown that if the ventral border of the transection is moved 3 mm. caudal to that in a postmammillary preparation,

then MLR stimulation becomes ineffective. They suggested that the functional integrity of some unidentified structure within the rostroventral mesencephalon must be preserved if MLR stimulation is to evoke locomotion in a decerebrate cat. Although the results of the present study might point to the VTA being the necessary structure, further investigations will be necessary to explain this phenomenon.

The projection to the contralateral caudal CNF might be thought to be important for the bilateral coordination of locomotion in the postmammillary cat. However, Shik et al (1967) have shown that destruction of one MLR does not inhibit the initiation of coordinated bilateral locomotion initiated by stimulation of the MLR on the other side of the brainstem.

In addition to the projections already noted, labelled fibers also ascend into the diencephalon. Although these projections are interrupted in the production of a postmammillary preparation and are therefore not involved in the locomotion evoked by MLR stimulation, there are still some interesting findings that may be related to locomotion in premammillary (Thalamic) or intact animals.

As outlined in the results, the diencephalic projections bifurcate into a dorsomedial and a ventrolateral bundle as they ascend through the rostral mesencephalon. Previous studies have also observed this bifurcation of reticulo-diencephalic projections, both neuroanatomically (McBride and Sutin, 1976) and electrophysiologically (Robertson et al, 1973).

The ventrolateral projection to the SUB, HLA, and HPA areas is interesting since portions of these areas constitute the underlying structures of the SLR, stimulation of which evokes locomotion in a premammillary cat (Orlovsky, 1969a). Bilateral destruction of the MLR does not alter the

locomotion evoked by SLR stimulation (Orlovsky, 1969a), even though the anatomical correlates of the SLR are known to project to the MLR (Sakai et al, 1977, Nauta and Cole, 1978). Therefore, although these two locomotor regions are reciprocally connected, they are not essential to each other for the initiation of locomotion.

In intact cats, lesions of the SLR abolishes voluntary locomotion which can only be evoked by subsequent MLR stimulation (Sirota and Shik, 1973). On this basis, it is tempting to speculate that in intact cats the SLR is primarily associated with the "motivational" aspects of voluntary locomotion whereas the MLR is primarily concerned with the activation of the "mechanistic" aspects of involuntary motor performance.

The present study has demonstrated a dorsomedial projection to the PF-CM complex of the intralaminar nuclei of the thalamus, and McBride and Sutin (1976) have also observed projections from the MLR to the PF-CM complex. Sirota and Shik (1973) noted that lesions of the PF-CM complex improves the locomotion produced by MLR stimulation in intact cats. Also, Grossman (1958) has shown that stimulation of the intralaminar nuclei of the thalamus inhibits locomotion evoked by SLR stimulation in lightly anaesthetized intact or decorticate cats. At present, the underlying mechanisms responsible for the inhibition of locomotion by the PF-CM complex can not be explained.

The descending projections to TRC, FTG, and FTM are more easily related to the possible supraspinal mechanisms involved in the activation of spinal locomotor systems than the diencephalic terminal areas which are not present in the postmammillary preparation. Edwards (1975) first showed a prominent projection from the CNF to TRC and this projection has

been confirmed in the present study. Recently Zangger and Schultz (1978) have shown identified TRC cells to be phasically modulated or tonically activated during spontaneous locomotion in the decorticate cat. They further state that the rhythmic modulation of the TRC cells occurs in a curarized state and is probably due to afferent input from phasically active cerebellar neurons. Although these TRC cells are rhythmically active during locomotion, they may also be involved in the initiation of locomotion by MLR stimulation. Locomotor movements have been initiated in a postmammillary cat by stimulation of the TRC (Mori et al, 1978).

The prominent ipsilateral projections to FTG and FTM are the most caudal projections from the MLR. The dense dispersions of silver grains surrounding the labelled fibers suggests that the fibers terminate within these areas (see Figures 11 and 12). Recently, Mori et al. (1978) have shown that stimulation within the ventral FTG and FTM will evoke locomotion in a postmammillary cat. Their sites of stimulation correspond to the areas of FTG and FTM which were distinctly labelled in the present study. Given that there are no confirmed projections to the spinal cord directly from the MLR, as reported in the present study and in a study by Orlovsky (1969b), it is tempting to suggest that locomotion due to MLR stimulation activates FTG or FTM neurons which project to the spinal cord (Torvik and Brodal, 1957; Basbaum et al, 1978) and "turn on" the spinal stepping generator. This conte tion is supported by the findings of Orlovsky (1969b, 1970a, b), who showed that reticulospinal neurons are monosynaptically activated by MLR stimulation. Approximately one-half of these reticulospinal cells also fired phasically during locomotion. Orlovsky (1970a, b) recorded from the medial reticular formation extending from

P5 to P15, which includes the site of termination of MLR projections within FTG and FTM.

A predominant projection to the pontine locomotor region (PLR), which is located within the lateral reticular formation, was not observed. This would suggest that the locomotion evoked by stimulation along the PLR strip (Mori et al, 1977; Shik and Yagodnitsyn, 1977) is not due to direct activation of descending axons originating from the MLR. The major origins of reticulospinal fibers are the medial portions of the pontine and medullary reticular formation, but there are also a small number of reticulospinal fibers emanating from the lateral reticular formation (Torvik and Brodal, 1957; Kuypers and Maisky, 1975). Therefore, the locomotion evoked from the PLR and the ventromedial reticular formation (Mori et al, 1978) may or may not be mediated by the same reticulospinal systems.

Spinal Cord Pathways Underlying the Control of Locomotion.

In acute spinal cats, the best cross-sectional areas of the cord to stimulate to evoke locomotion are the dorsal columns or the dorsolateral funiculus (Sherrington, 1910; Grillner and Zangger, 1974; Steeves and Jordan unpublished observations). However in the present study, the interruption of fiber pathways which project through the dorsal columns or dorsolateral funiculus does not effect the locomotion evoked by MLR stimulation. Thus the dorsolateral reticulospinal pathways, which have been suggested to be involved in the control of locomotion (cf. Grillner, 1975), do not appear to be essential for locomotion in a postmammillary cat.

Similarily the fiber pathways coursing through the ventral funiculi do not seem to be necessary for locomotion activated by MLR stimulation. This finding does not support the suggestions of Afelt (1974) and Windle et al (1958) that the sparing of the ventromedial funiculus is essential for locomotion. Furthermore, the ventral funiculus does not appear to be sufficient for the activation of locomotion in an acute postmammillary cat (see Figure 18H). A possible explanation for this conflict concerning the importance of the ventral funiculus to locomotion might be that the locomotion observed by Afelt (1974) and Windle et al (1958) was actually chronic spinal stepping (Shurrager and Dykman, 1951; Hart, 1971; Steeves and Jordan, unpublished observations). Alternatively, it may not be fair to compare these studies since the reports of Afelt (1974) and Windle et al (1958) utilized chronic cats with subtotal thoracolumbar transections, whereas the present study analyzed the effects of high cervical partial transections in acute postmammillary cats.

The one area of the cross-sectional spinal cord which appears to be essential for MLR evoked locomotion is the ventrolateral quadrant. In all five experiments of the present study, a bilateral lesion of the ventrolateral quadrants resulted in inhibition of locomotion. This finding is interesting since FTG and FTM project some of their descending fibers into the spinal cord via the ipsilateral ventrolateral funiculus (Basbaum et al, 1978; Kuypers and Maisky, 1977). Therefore, it is very possible that the interruption of the ventrolateral funiculi severs the reticulospinal pathways that are necessary for the activation of spinal locomotor mechanisms. Thus, the initiation of locomotion by MLR stimulation might be via a disynaptic pathway involving a projection from caudal CNF

to FTG and FTM neurons which subsequently activate the spinal stepping generator via their axonal projections through the ventrolateral funiculi.

Support for this hypothesis can be found in the work of Grillner and Lund (1968). They showed that the excitation of motoneurons by stimuli applied to the medial medullary reticular formation (4-6 mm. rostral to be obex) was mediated by axons concentrated in the ventrolateral funiculus. More recently, Jankowska et al (1974) have shown that monosynaptic EPSPs evoked in motoneurons by stimulating the caudal medulla (3 mm rostral to the obex), and presumably mediated by reticulospinal pathways, survive lesions of the ventral funiculus. Some of these monosynaptic EPSPs were mediated by axons descending via the ventrolateral funiculus.

However, the ascending ventral spinocerebellar tract (VSCT) is also situated within the ventrolateral funiculus, and VSCT neurons have been shown to be rhythmically active during locomotion even after deafferentation or removal of the cerebellum (Arshavsky et al, 1972). Thus VSCT cells send information to the brainstem and cerebellum concerning the activity of the spinal stepping generator. Whether the integrity of this pathway is essential for the initiation of locomotion in a postmammillary cat is not known. The fact that locomotion produced by MLR stimulation has a delay in onset of at least 0.5 sec (cf. Grillner, 1975) might suggest that some ascending information from the spinal cord is required before the spinal stepping mechanisms are put into action.

Finally, the ventrolateral funiculi alone are not sufficient for the initiation of locomotion by MLR stimulation. Cutting all cross-sectional areas of the cord except the ventrolateral funiculi in one cat resulted in complete inhibition of MLR evoked locomotion (Steeves

Figure 1. Schematic drawing the the cat brainstem from Berman (1968) along a parasagittal plane 4.1 mm from the midline. The dorsoventrally interrupted straight lines indicate the levels of transection for two types of decerebrate preparation. The rostral line illustrates the plane of section for a premammillary (thalamic) preparation which is capable of spontaneous locomotion. The caudal line delineates a postmammillary (mesencephalic) preparation in which locomotion is not spontaneous but can be induced by stimulation of a region centered on the cuneiform nucleus. The mammillary bodies (MB) and third cranial nerve (3N) are located medial to the brainstem plane illustrated and are indicated by dashed lines. Note: all abbreviations used in the figures are listed in the table of abbreviations (see page 30).

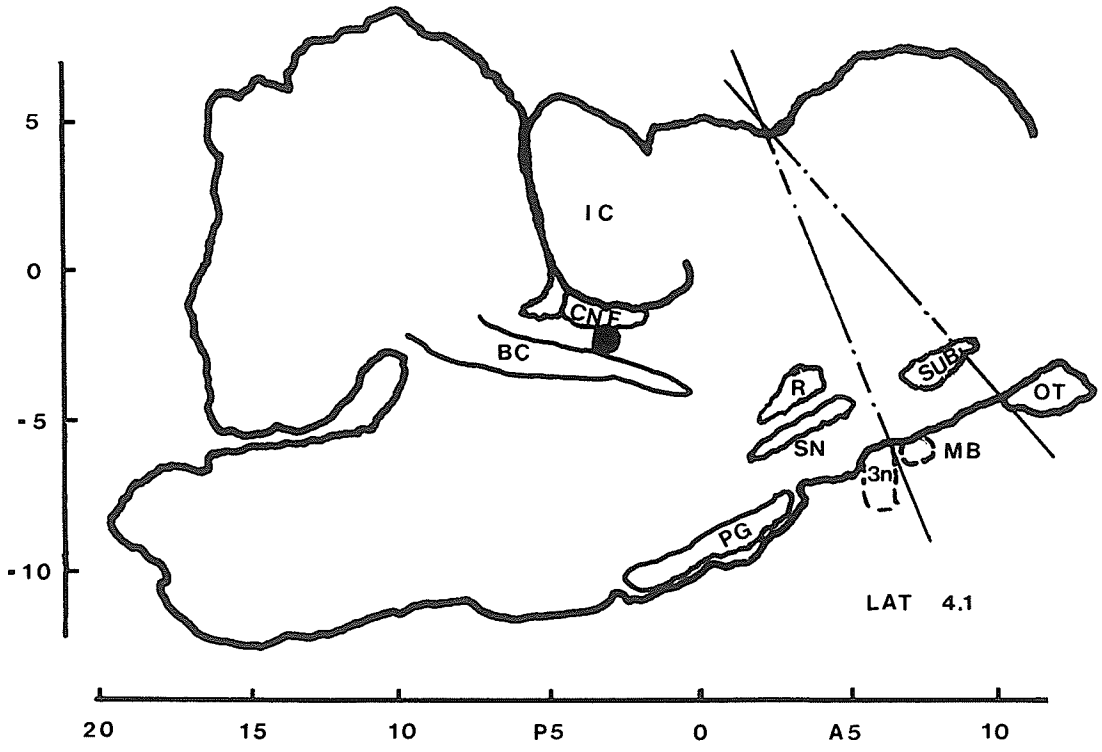

5
0
-5
-10
IC
CNF
BC
R
SUB
OT
SN
MB
3n
PG
LAT 4.1
20
15
10
P5
0
A5
10

Figure 2. Primary pathway for the biosynthesis of noradrenaline (norepinephrine) illustrating the site of α- methyltyrosine (AMT) action on the rate limiting enzyme tyrosine hydroxylase. The inhibition of tyrosine hydroxylase by AMT blocks the formation of noradrenaline, resulting in lowered noradrenaline levels.

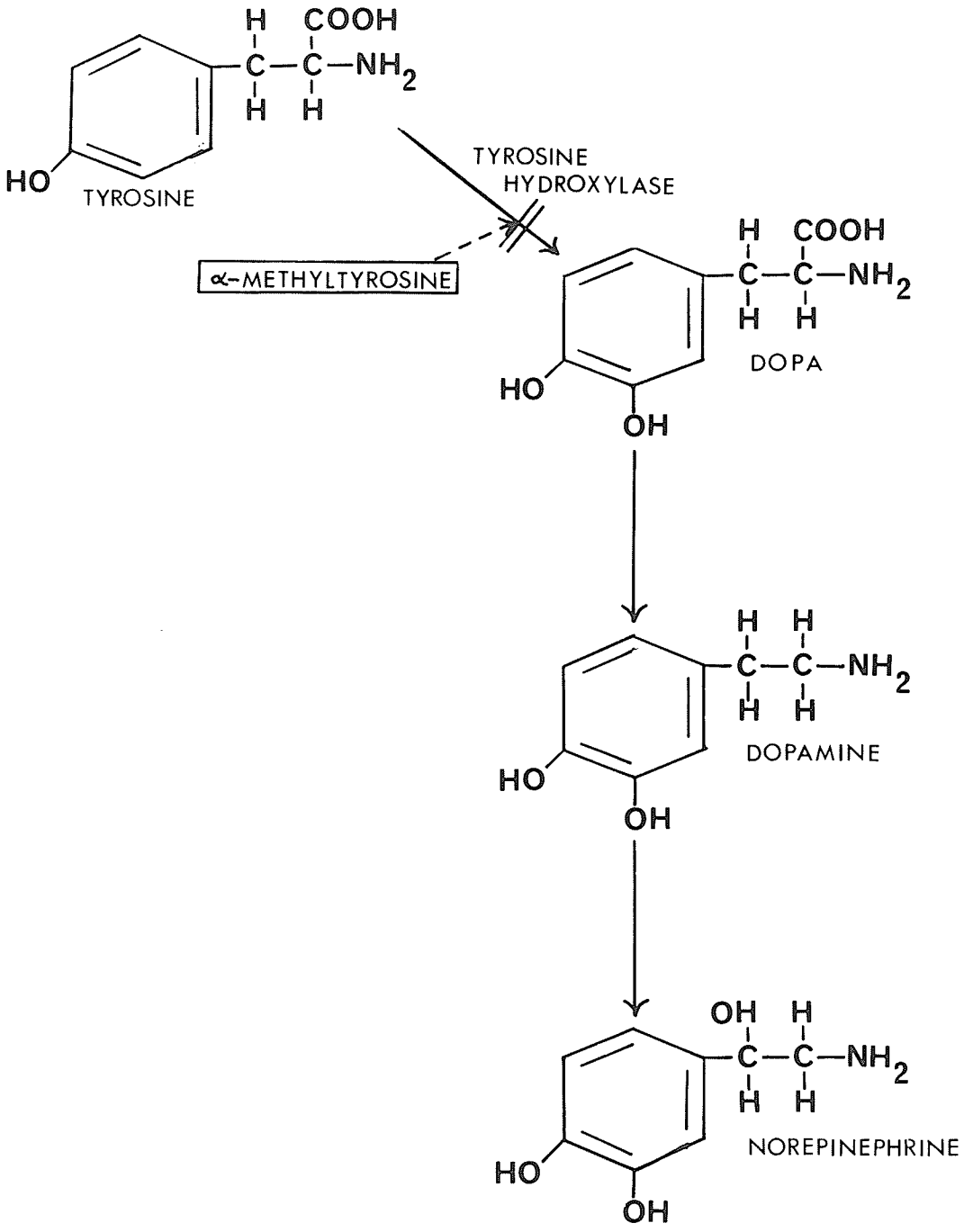

TYROSINE
TYROSINE HYDROXYLASE
α-METHYLTYROSINE
DOPA
DOPAMINE
NOREPINEPHRINE

Figure 3. Diagramatic representation of the experimental set-up for a postmammillary preparation which can be made to walk with all four limbs by stimulation of the MLR.

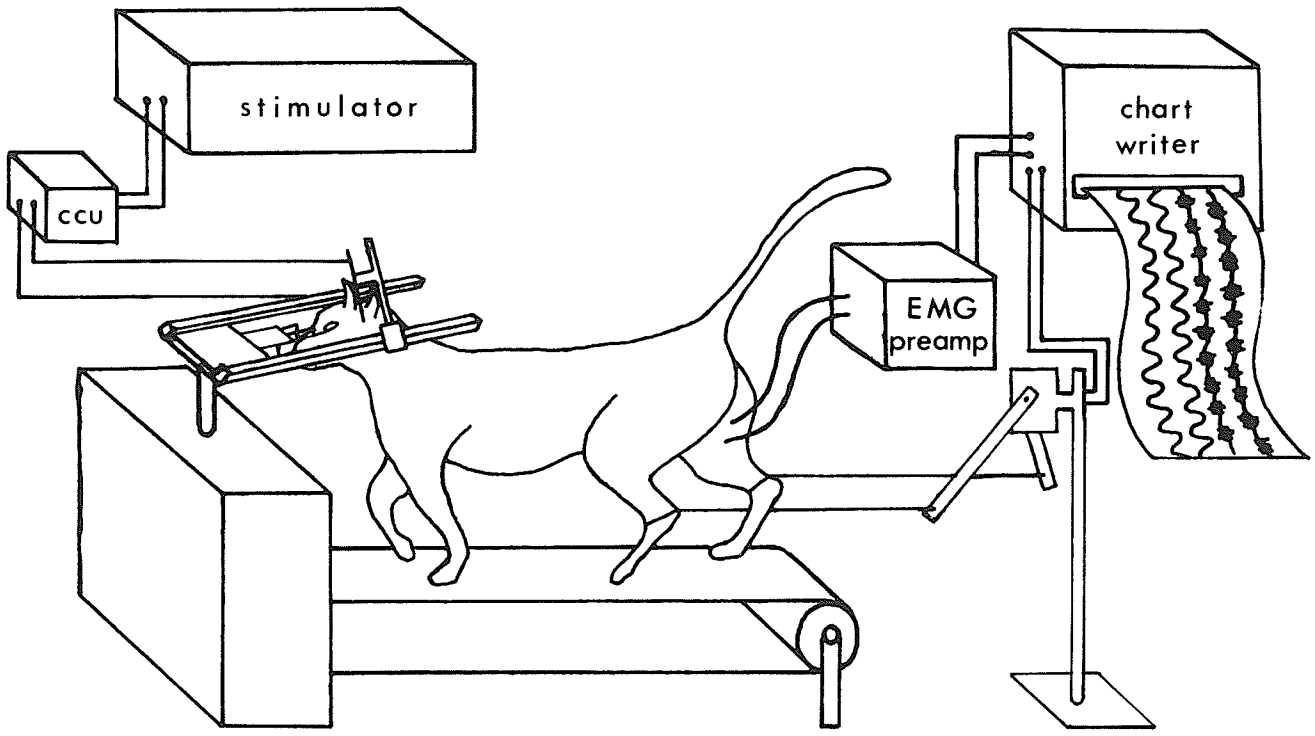

stimulator
ccu
chart
writer
EMG
preamp

Figure 4. A series of photographs taken from a 16 mm movie (24 frames/sec) of MLR evoked locomotion. One complete step cycle is illustrated in the frames a-i. The MLR stimulus parameters for this particular cat were 25μA at 30 Hz with the electrode positioned at approximately P_2, L_4, H_{-1}. This movie of the locomotion preparation was taken prior to the addition of equipment for recording EMGs and limb movements.

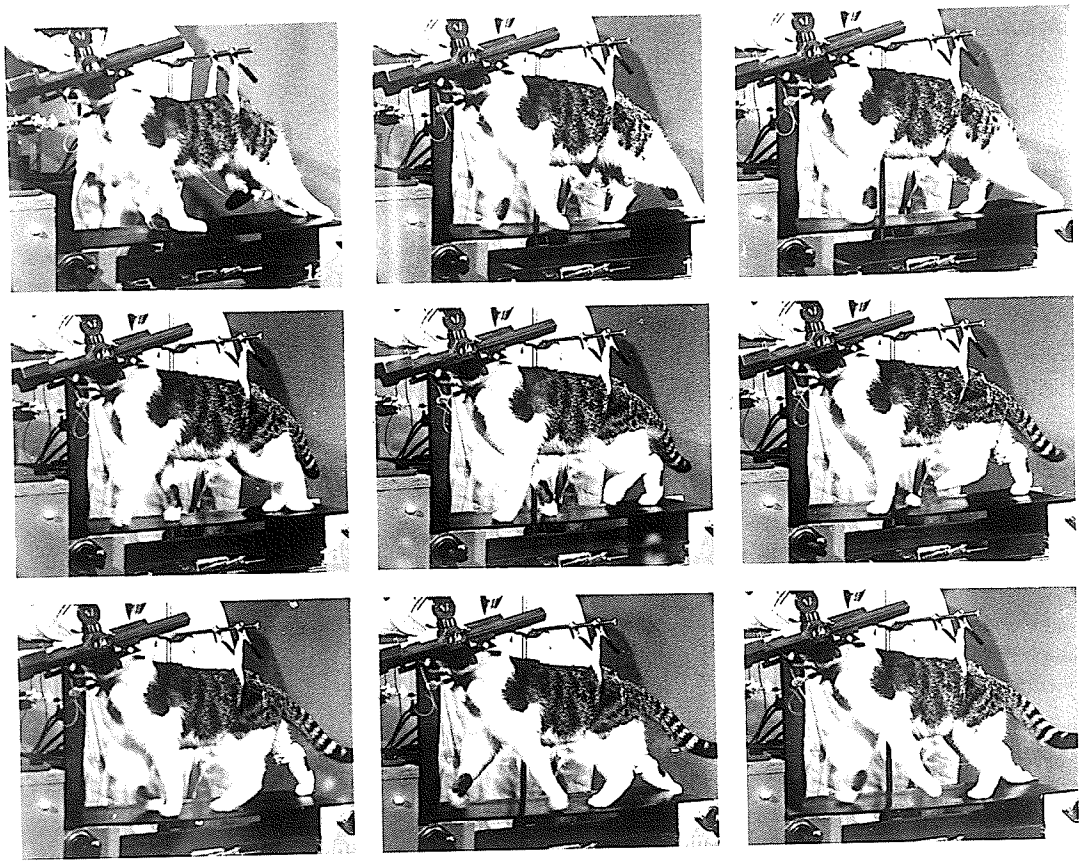
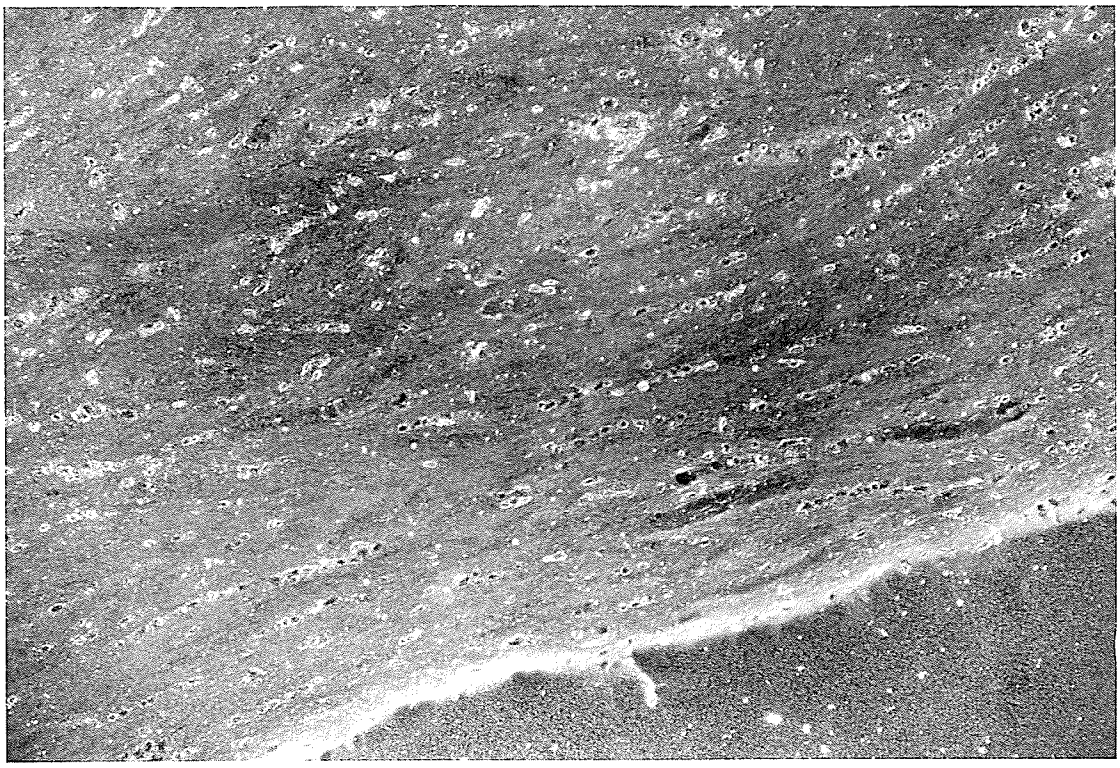


Figure 5. Darkfield photomicrograph showing a typical amount of background activity overlying a brainstem area which does not contain any labelled axons or terminals. The area shown is the pyramidal tract in the rostral medulla at a level of approximately P 7.0. At this level the pyramidal tract is ventrally adjacent to a major MLR terminal area, the FTM (see Figure 12). Note the lack of rows of white dots (silver grains), indicative of labelled axons, and the paucity in the dispersion of the silver grains in Figure 5 when compared to the adjacent terminal area shown in Figure 12.



100 µm

Figure 6. An x-ray of a cat's head from the dorsal aspect. This picture shows the filling of the ventricular system by the radio-opaque substance after it was infused into the left lateral ventricle under fluoroscopic control. Note the triangular shaped object along the midline which outlines the fourth ventricle. Distribution of the radio-opaque substance throughout the ventricular system assured that any subsequent delivery of 6-OHDA through the cannula would be dispersed to all levels of the CNS.

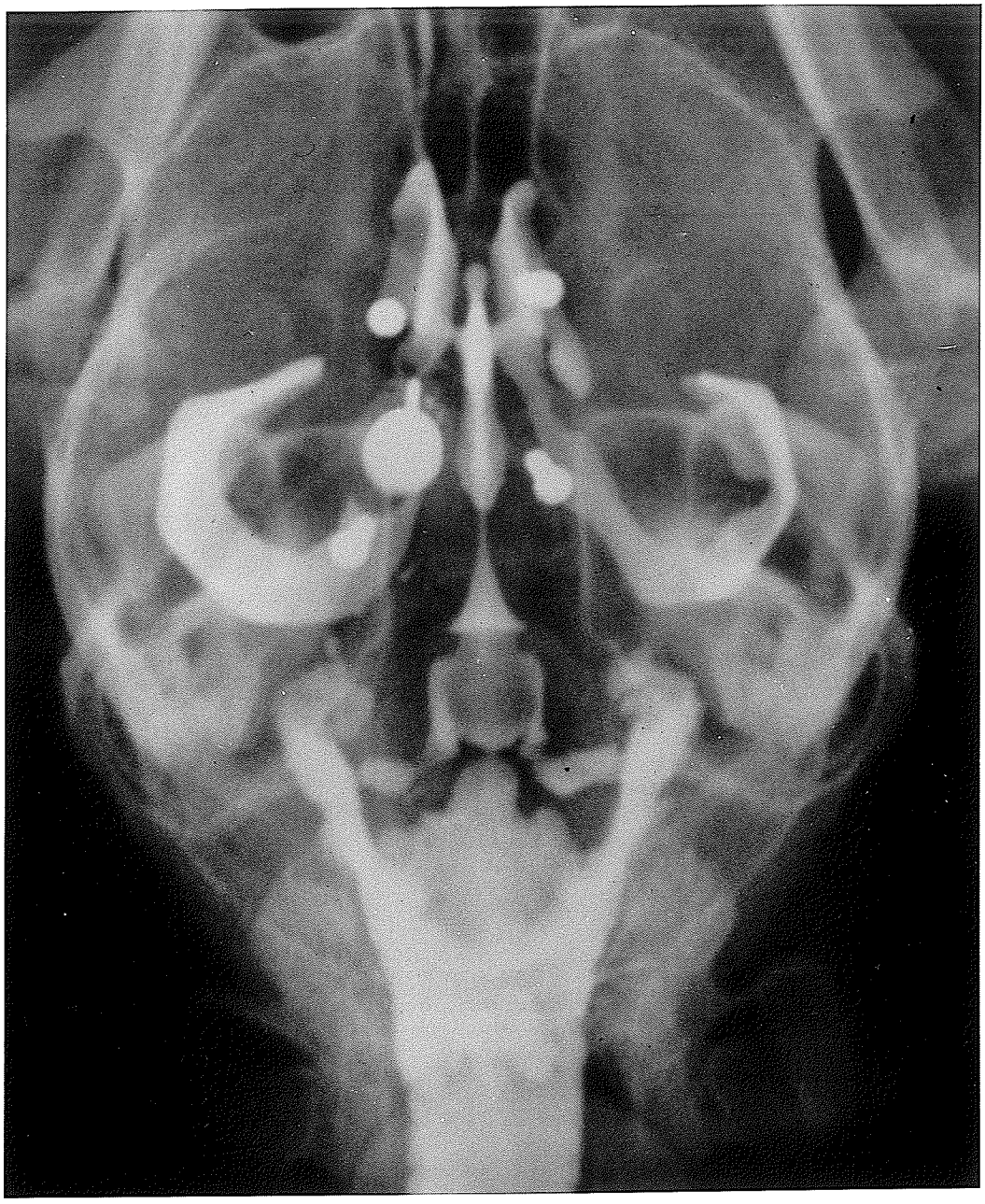
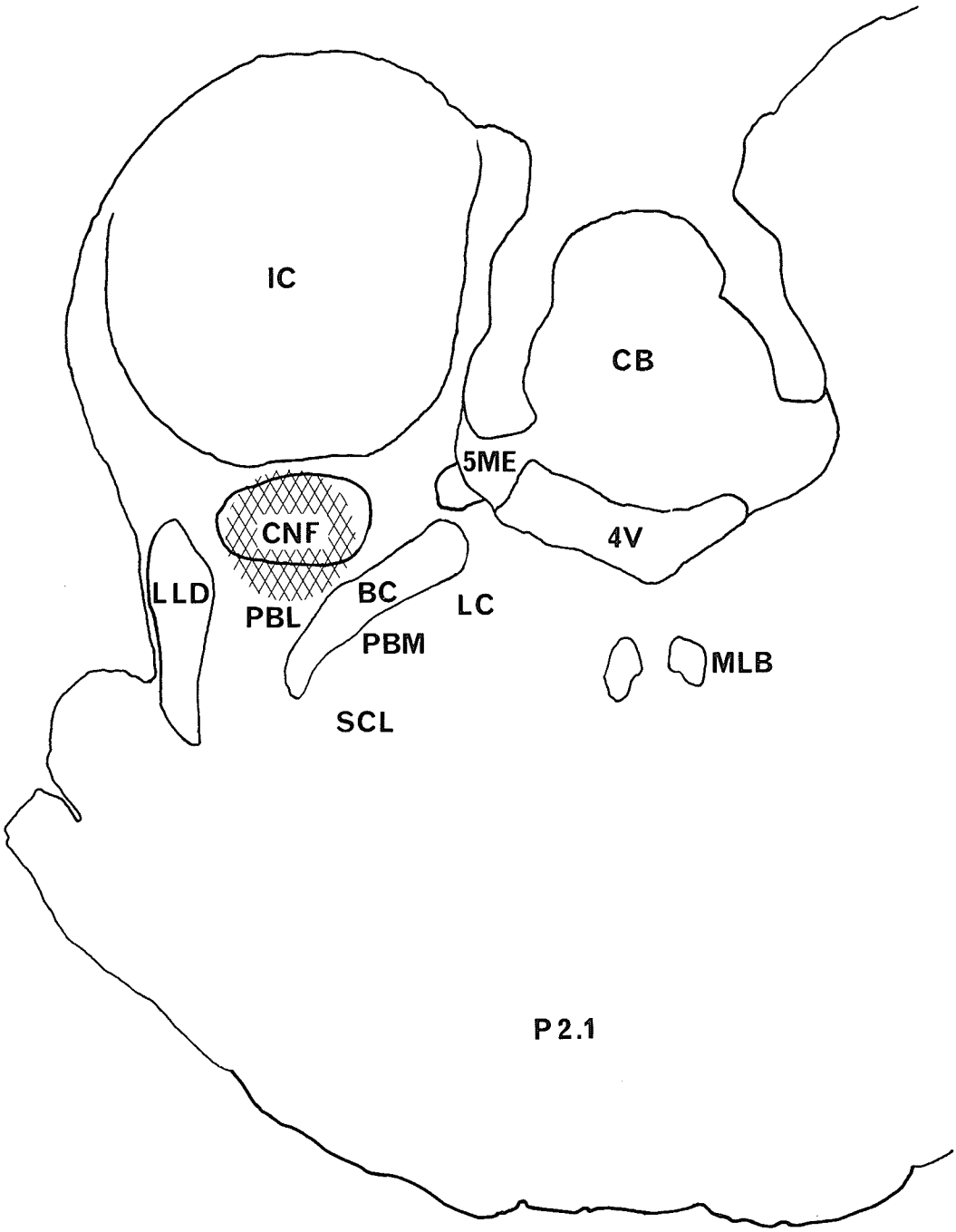
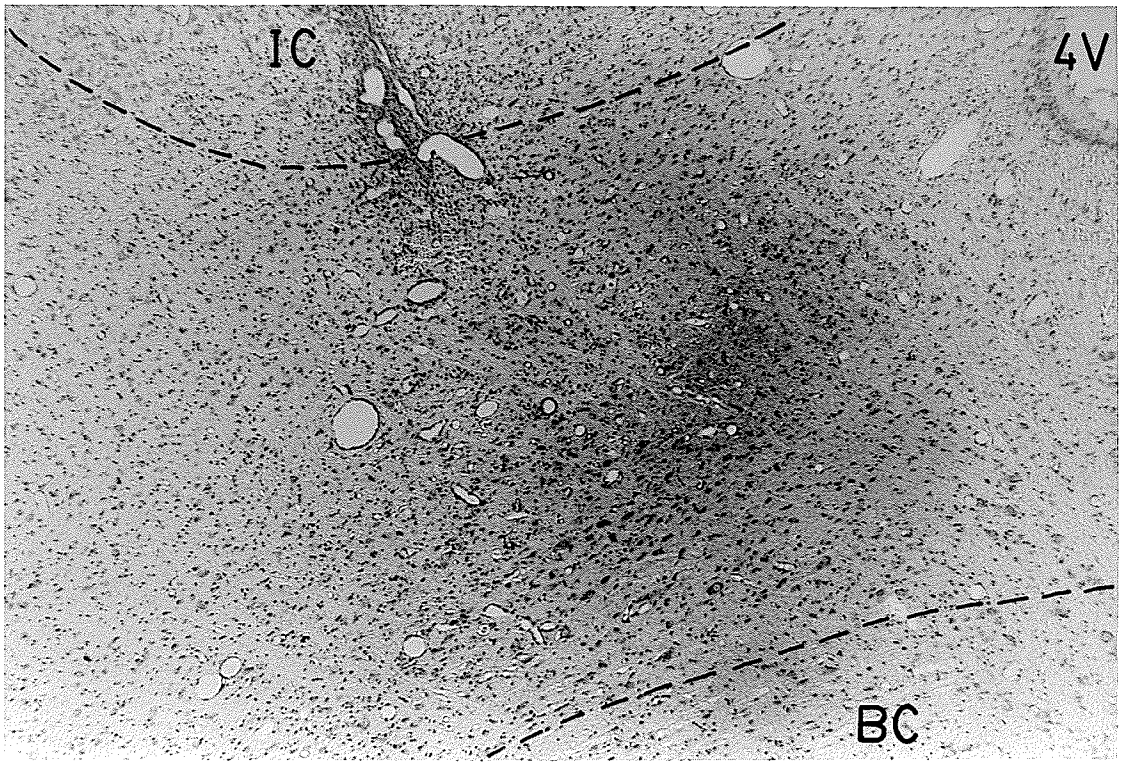


Figure 7. A transverse drawing of the cat brainstem at a level of P 2.1 according to the atlases of Berman (1968) and Taber (1961). The hatched area indicates the predominant site from which uncomplicated coordinated locomotion can be evoked using small stimulation currents. Therefore, all injections of ^{3}H-amino acids were restricted to this area.



P 2.1

Figure 8. A brightfield photomicrograph of a site of ^{3}H-amino acid injection. This picture was taken of a brainstem sectioned in the parasagittal plane. The injection site in this photomicrograph is situated between the ventral border of the inferior colliculus (IC) and the dorsal margin of the brachium conjunctivum (BC) and characterized by neurons which appear darkly stained. This photograph was taken after 6 weeks of development (incubation) of the latent image by the β- particles of the ^{3}H-amino acid.



0.5 mm

Figure 9. Drawings of selected transverse sections of the brainstem according to Berman (1968), illustrating the confirmed descending projections arising from the MLR (caudal CNF). The stippled area indicates the injection site. Dashed lines represent labelled fibers coursing in the plane of section. Dots represent regions of probable termination. The drawings are arranged rostral to caudal (A-D) with the level of each section indicated in the upper left-hand corner. Approximate lateral and horizontal coordinates have been included with the drawings. Comparing the areas of labelling with the appropriate stereotaxic coordinates will help overcome the difficulty associated with the variety of names that have been assigned to the same neuroanatomical structure.

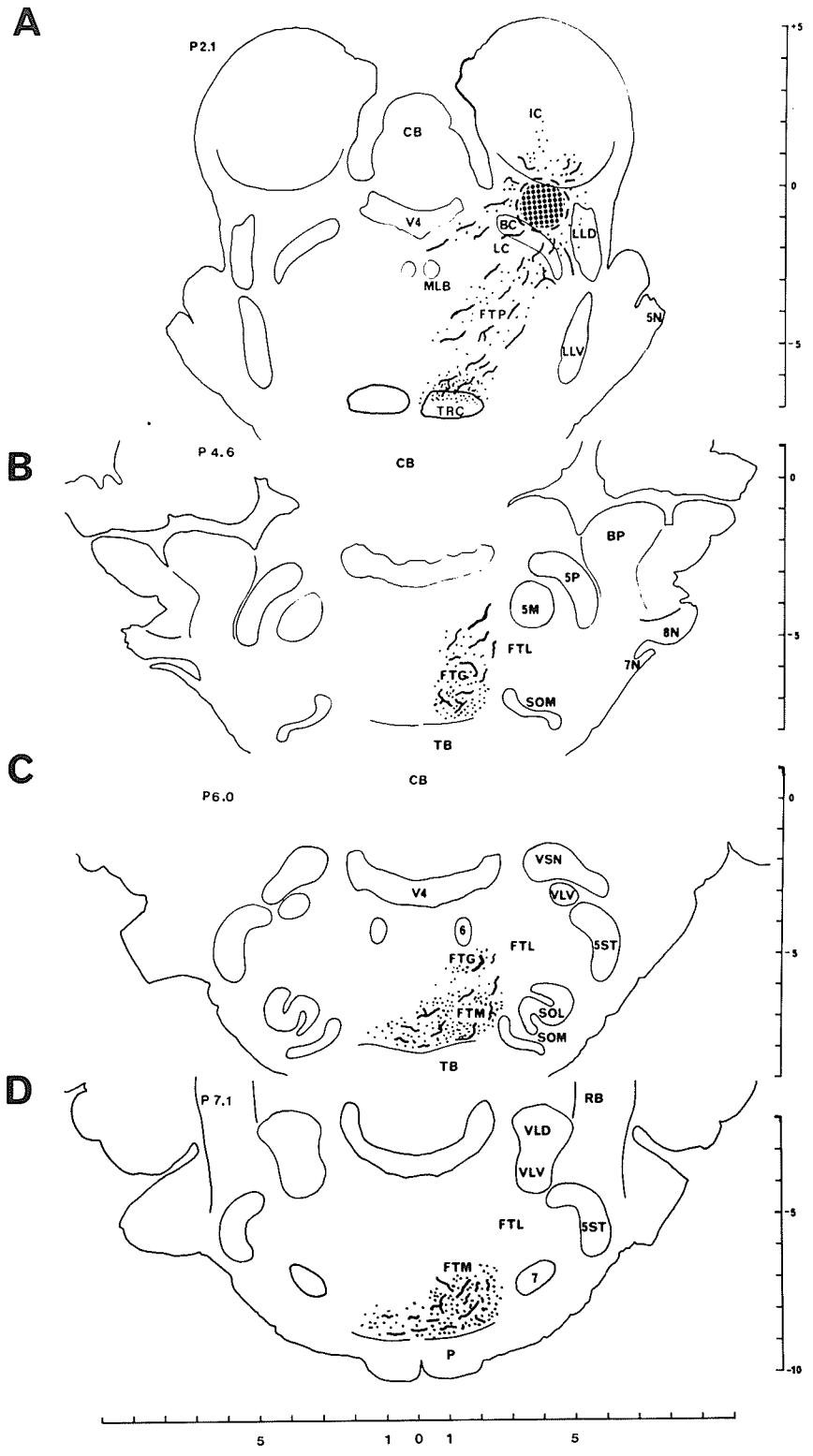

A
P2.1
CB
IC
V4
BC
LC
LLD
MLB
FTP
5N
LLV
TRC
+5
0
-5
B
P 4.6
CB
BP
5P
5M
8N
FTL
7N
FTG
SOM
TB
0
-5
C
P6.0
CB
VSN
V4
VLV
6
FTL
5ST
FTG
FTM
SOL
SOM
TB
0
-5
D
P 7.1
RB
VLD
VLV
FTL
5ST
FTM
7
P
-5
-10
5
1 0 1
5

Figure 10. Drawings of selected parasagittal sections showing the site of injection (A) and the distribution of labelled fibers and probable terminal areas on the ipsilateral side of the brainstem. The sections are arranged lateral to medial (A-C) and the plane of each drawing is indicated in the upper righthand corner. Labelled fibers and terminals are illustrated as in Figure 9.

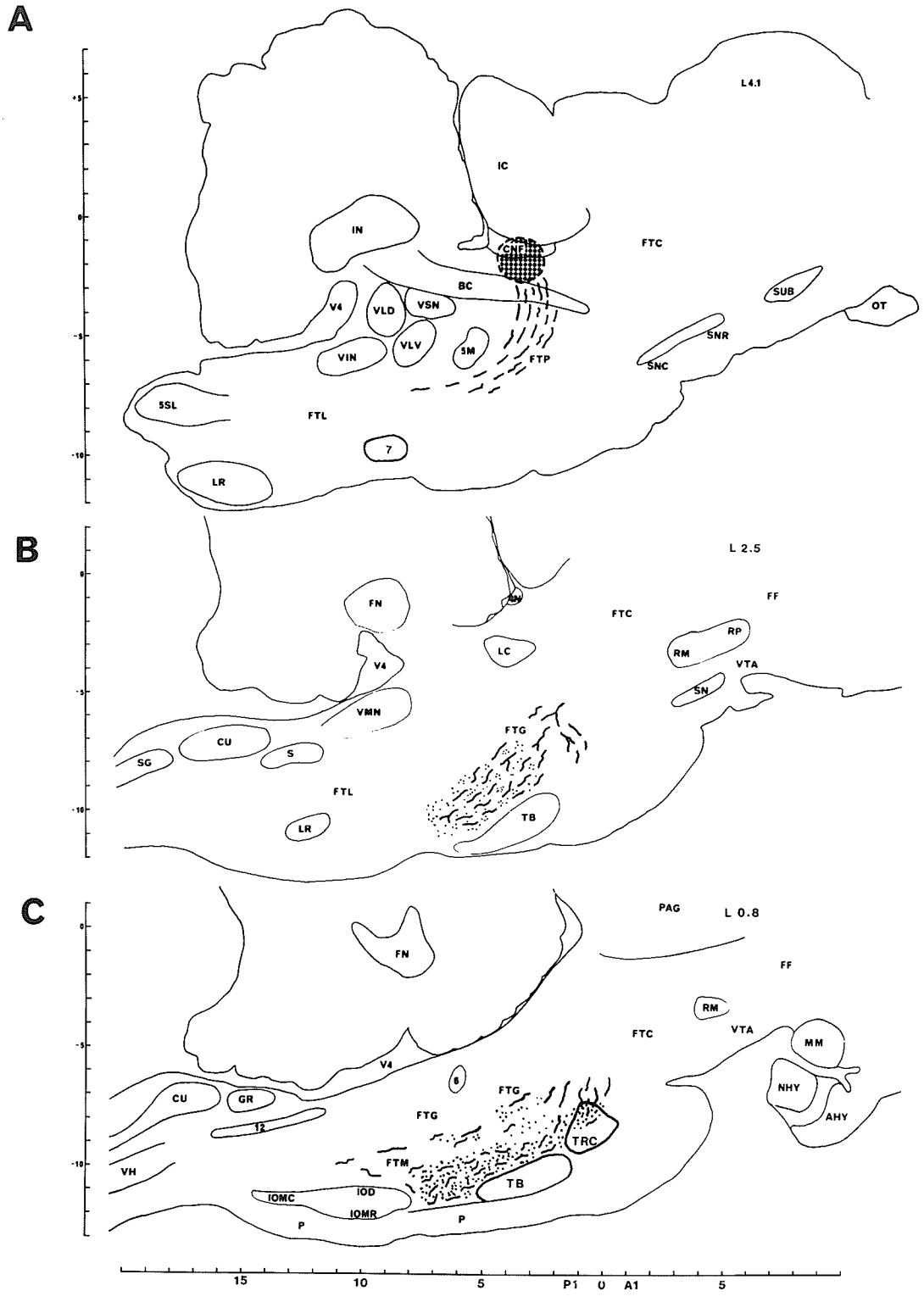
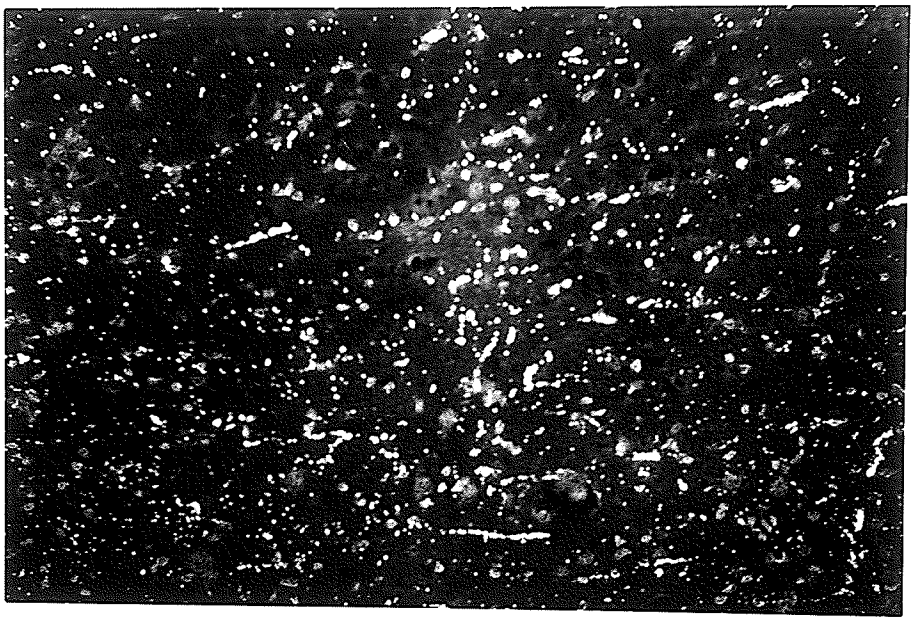

A
L4.1
IC
IN
CNF
FTC
BC
SUB
OT
V4
VLD
VSN
SNR
VLV
SM
FTP
VIN
SNC
5SL
FTL
7
LR
B
L 2.5
FF
FN
FTC
RP
LC
RM
VTA
V4
SN
VMN
FTG
CU
S
SG
FTL
TB
LR
C
PAG
L 0.8
FN
FF
RM
FTC
VTA
MM
V4
6
FTG
CU
GR
FTG
NHY
AHY
12
TRC
FTM
VH
TB
IOMC
IOD
IOMR
P
P
15
10
5
P1
0
A1
5

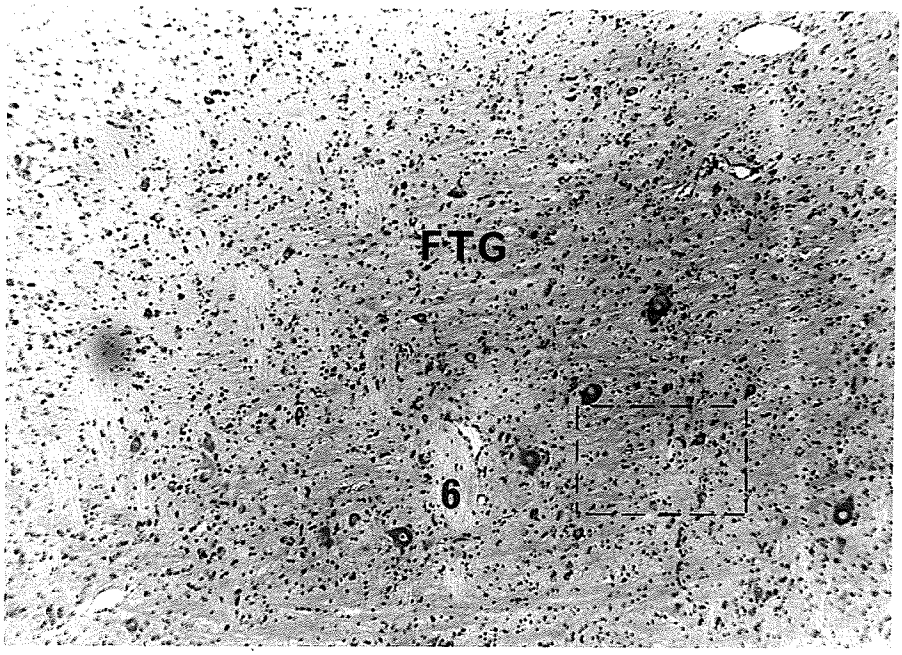
Figure 11. A: Darkfield photomicrograph showing labelled fibers within the ipsilateral gigantocellular tegmental field (FTG). The rows of white dots indicate the underlying labelled axons. B: Lower power brightfield photomicrograph of the same section and at the same coordinates as shown in A. The labelled fibers shown in A correspond to the FTG overlying the trapezoid body. A portion of the sixth cranial nerve (6) is also indicated. Several large cells characteristic of FTG are visible. The area enclosed by the dashed lines indicates the area shown in A.

A



100 µm

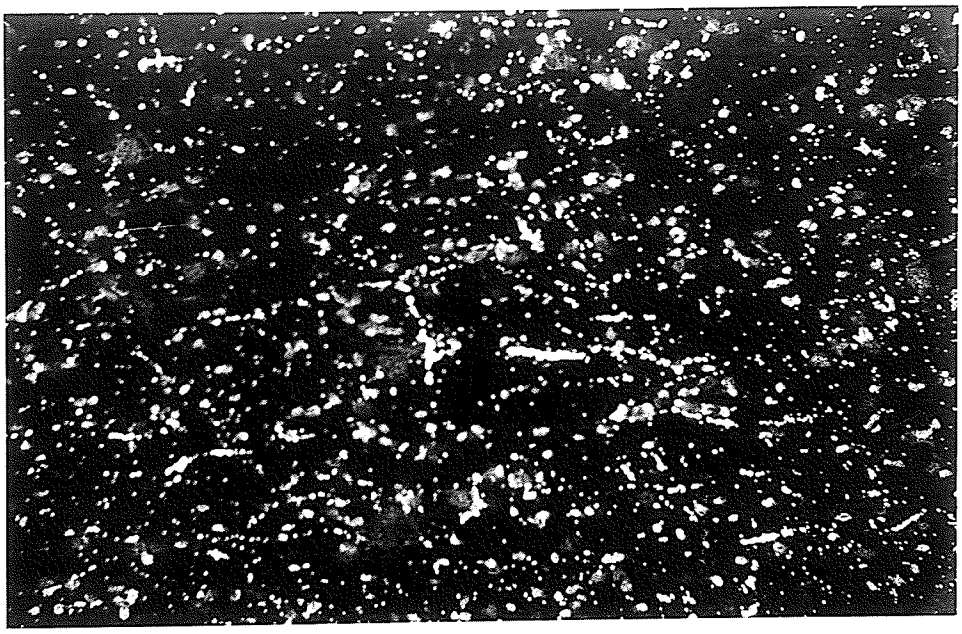
B



0.5 mm

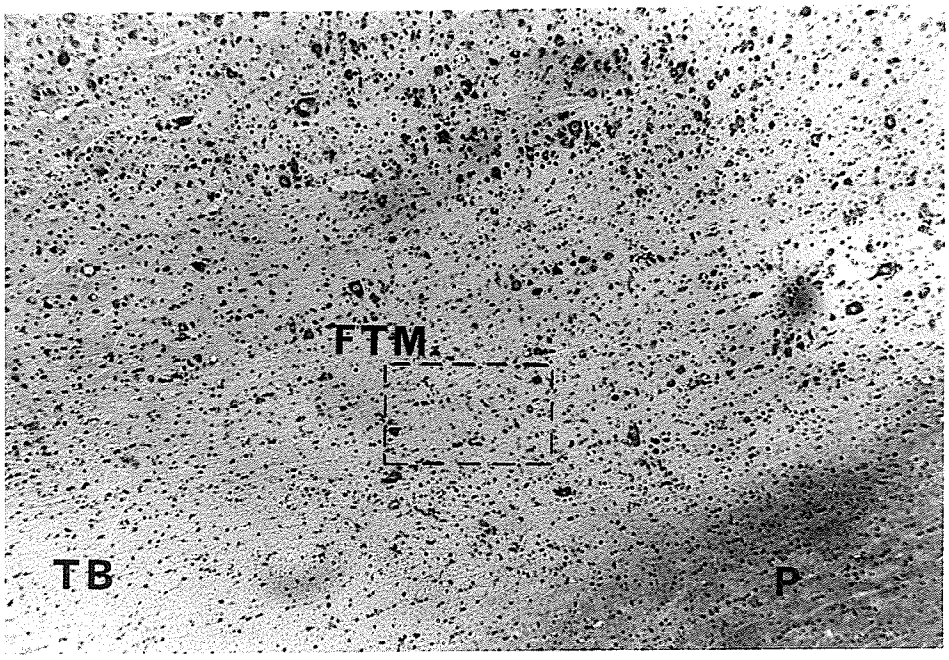
Figure 12. A: Darkfield photomicrograph showing the high density of labelled fibers and probable axon terminations. Rows of white dots indicate labelled fibers, whereas the dense dispersions of single white dots probably represent terminations. B: Lower power photomicrograph of the same section shown in A illustrating the position of the labelled fivers and terminals just caudal to the TB and dorsal to the pyramidal tract. The approximate stereotaxic coordinates of the heaviest labelling in the ipsilateral FTM are P7, L1, H-8. The area enclosed by the dashed lines corresponds to the location pictured in A.

A



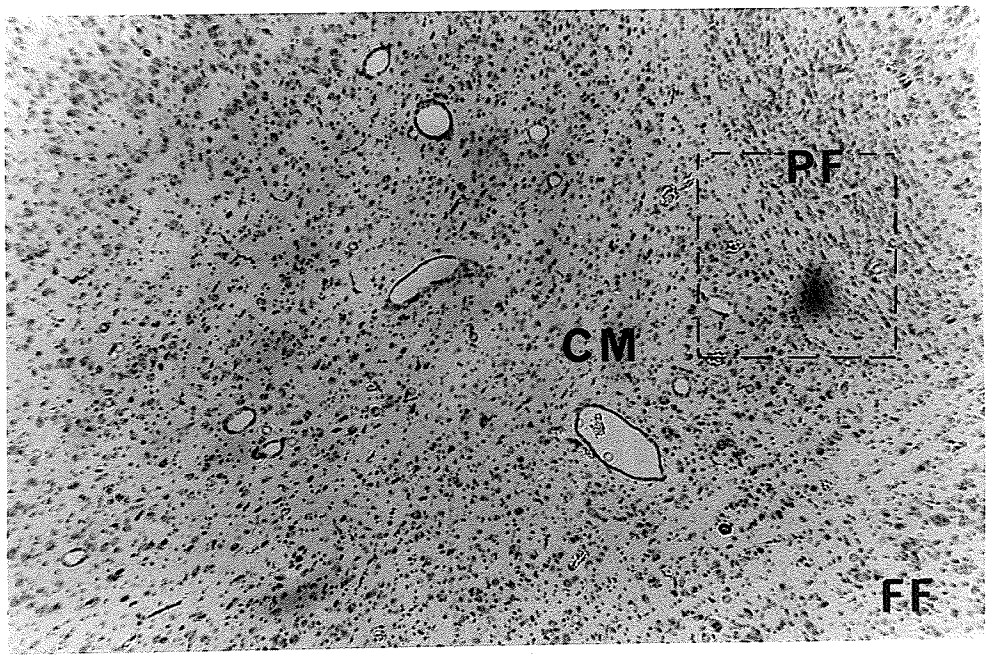
100 µm

B



0.5 mm

B



0.5 mm

Figure 13. Drawings of selected transverse sections of the brainstem illustrating the confirmed ascending projections from the MLR. The drawings are arranged rostral to caudal (A-D) and the labelled fibers and terminals are illustrated as in Figure 9.

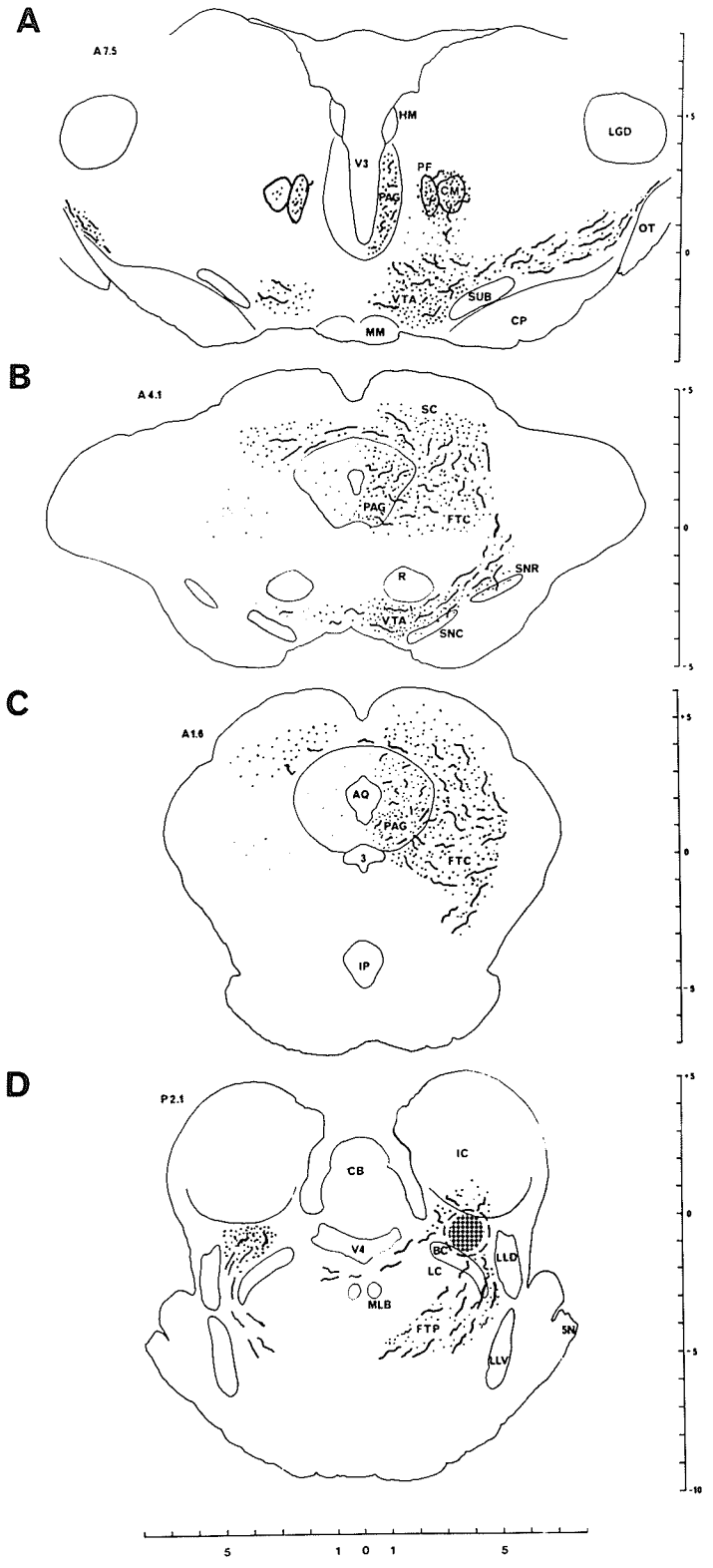

A
A 7.5
HM
LGD
V3
PF
CM
PAG
OT
VTA
SUB
CP
MM
B
A 4.1
SC
PAG
FTC
SNR
R
VTA
SNC
C
A1.6
AQ
PAG
3
FTC
IP
D
P2.1
IC
CB
V4
BC
LLD
LC
MLB
FTP
SN
LLV
+5
0
-5
-10
5
1 0 1
5

Figure 14. Selected parasagittal drawings of the brainstem illustrating the confirmed ipsilateral ascending projections emanating from the caudal CNF (MLR). The sections are arranged lateral to medial (A-E) and the labelled fibers and probable terminations are illustrated in the same manner as Figure 9.

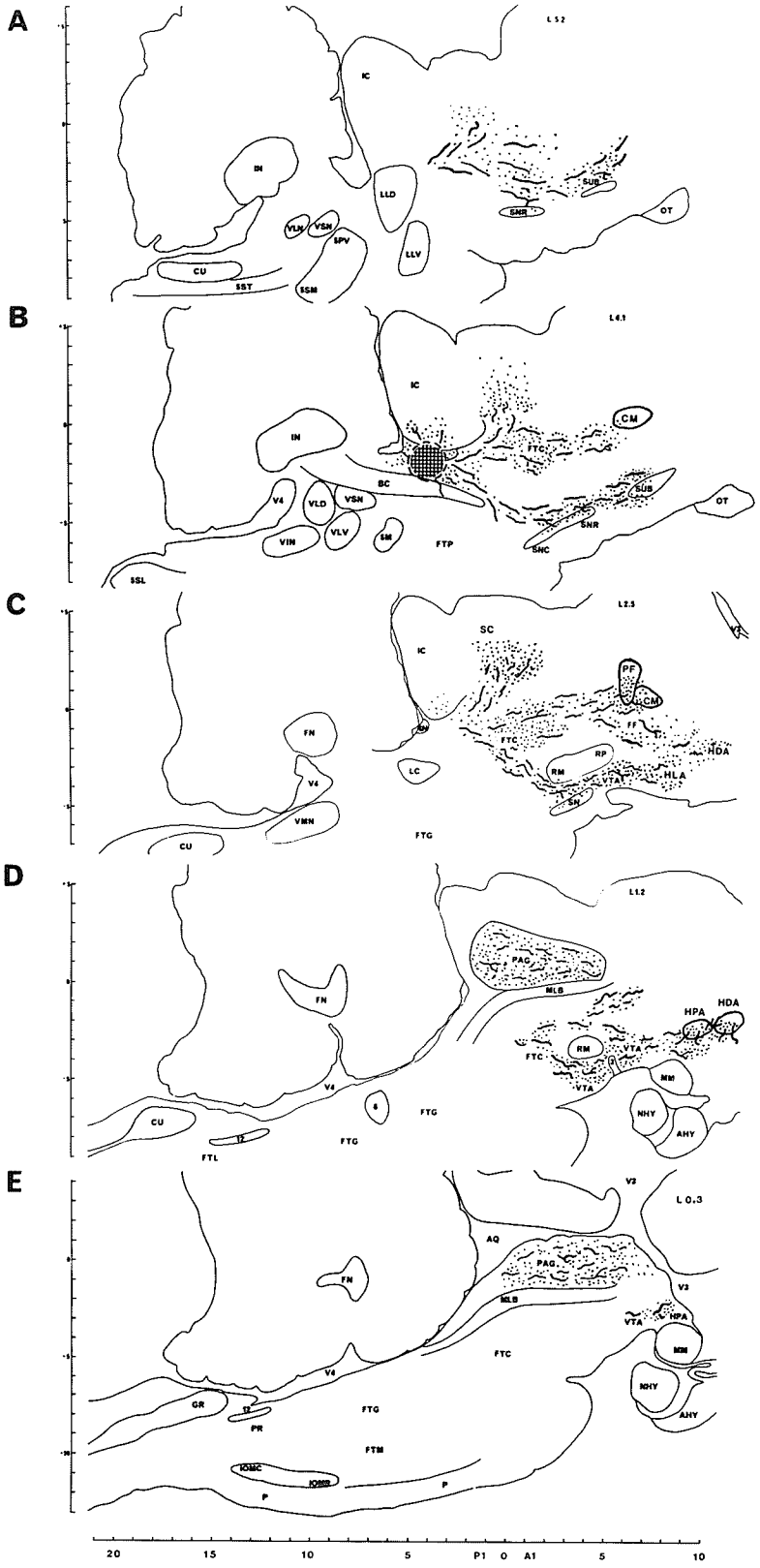
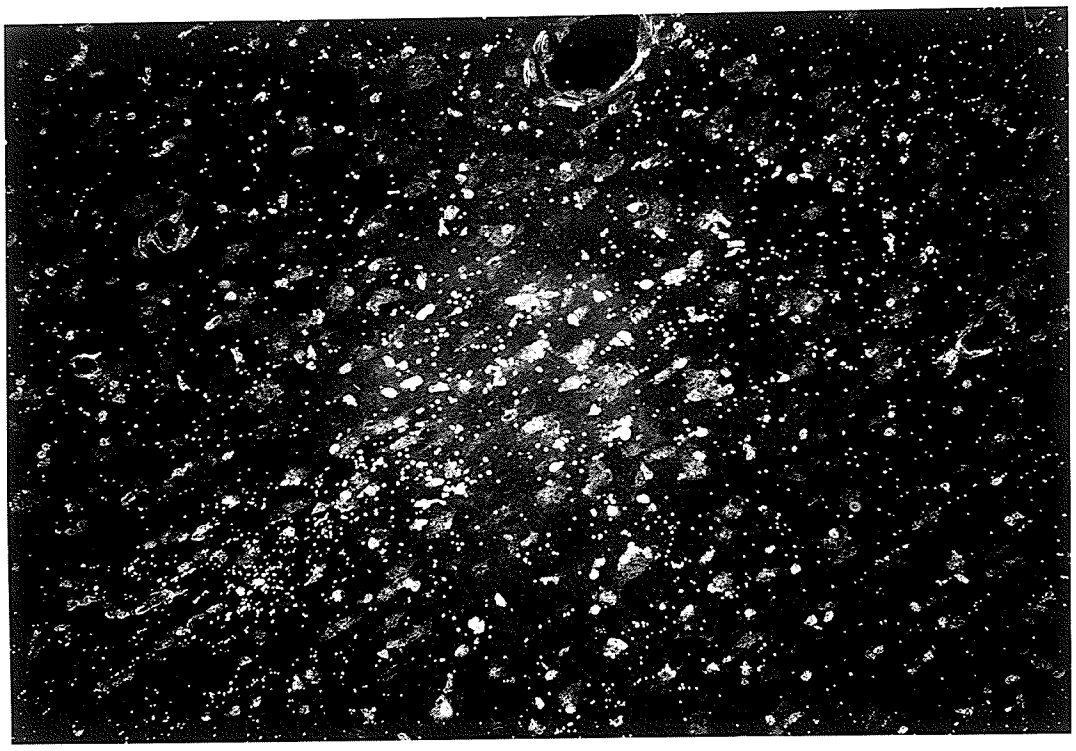

A
B
C
D
E
L 5.2
L 4.1
L 2.5
L 1.2
L 0.3
IC
IN
LLD
LLV
SUB
SNR
OT
VLN
VSN
SPV
CU
SST
SSM
CM
FTC
BC
V4
VLD
VSN
VIN
VLV
SM
FTP
SNC
SSL
SC
PF
FF
RP
HDA
HLA
LC
RM
VTA
SN
FN
VMN
FTG
PAG
MLB
HPA
MM
NHY
AHY
FTL
V2
AQ
GR
PR
FTM
KOMC
KOMB
P
20
15
10
5
P1
0
A1
5
10

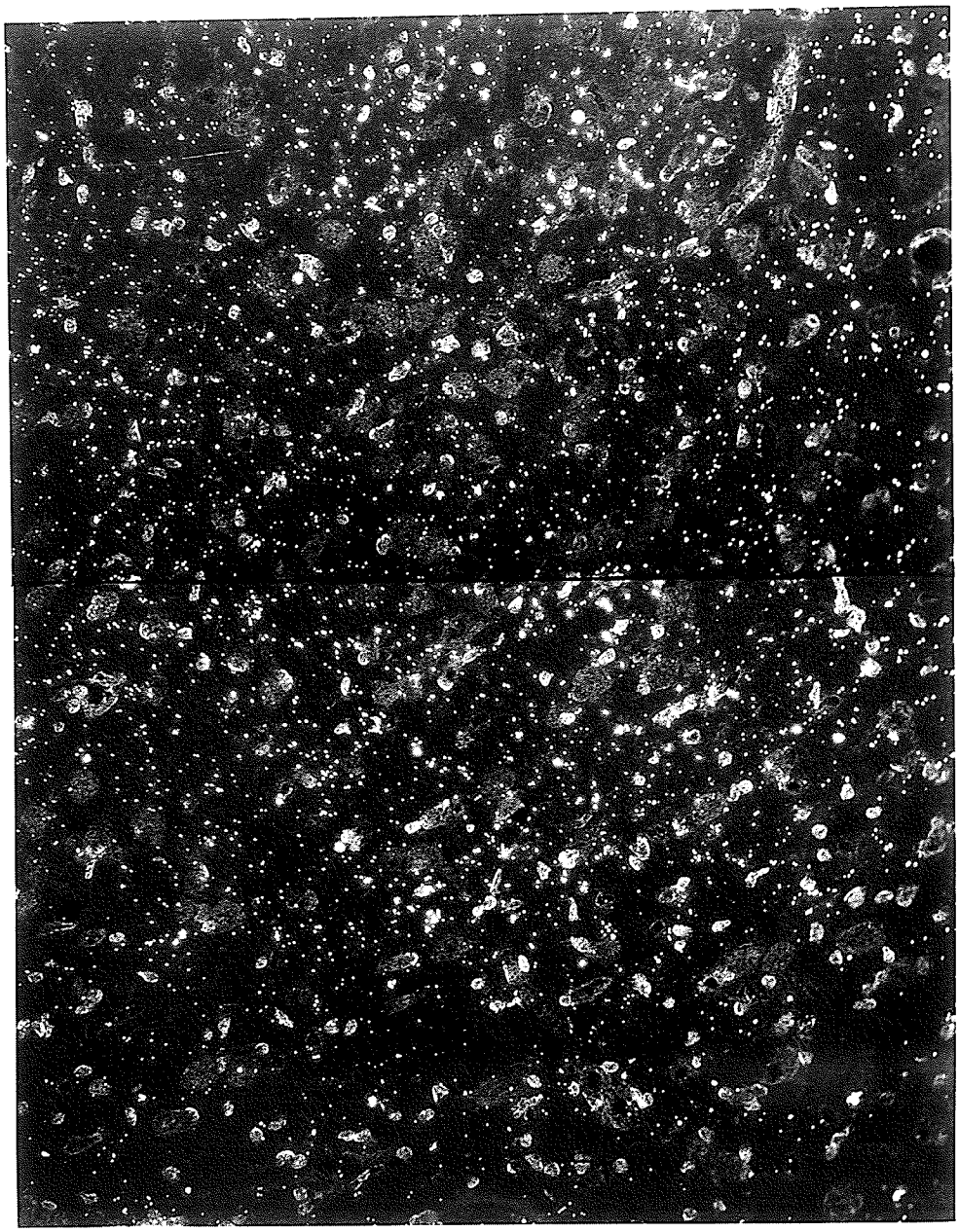
Figure 15. Darkfield photomicrograph showing the projection to the contralateral caudal CNF. The labelled fibers and probable terminals appear in the same manner as outlined in Figure 11. If a corresponding low power brightfield photomicrograph were taken of this labelled area it would be similar to that shown in Figure 8.



100 µm

Figure 16. A: Darkfield montage showing the dense labelling in the ipsilateral parafascicular nucleus (PF). The PF and nucleus centrum medianum (CM) were both prominent areas of terminations within the intralaminar nuclei of the thalamus. B: Lower power brightfield photomicrograph of the same area shown in A. The area enclosed by the dashed lines corresponds to the location of the labelling shown in A.

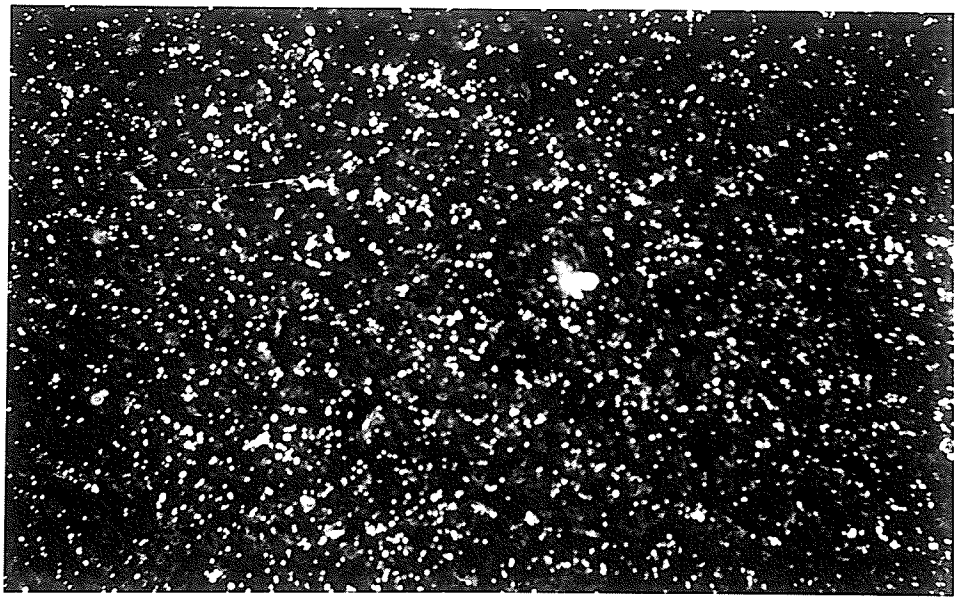
A



100 µm

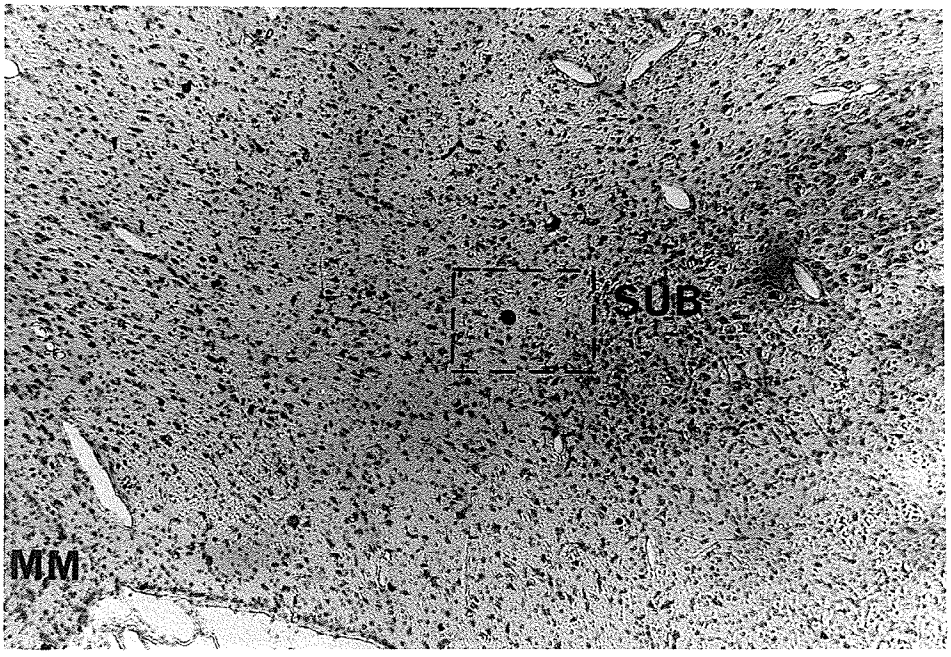
Figure 17. A: Darkfield photomicrograph illustrating the dense labelling within the subthalamic (SUB) area. B: Lower magnification brightfield picture of the SUB area shown in A. Note the ventral border of the brainstem and medially positioned mammillary bodies (MM) in relation to the labelled area. The area enclosed by dashed lines corresponds to the site of labelling pictured in A.

A



100 µm

B



0.5 mm

Figure 18. Summary of the various C_1 - C_2 spinal cord lesions and the subsequent effects on MLR activated locomotion. The single asterik indicates that the cat was only capable of hindlimb stepping and high strength MLR stimulation was necessary to evoke these movements. The double asterik indicates that 3 out of 5 animals were only capable of a few forelimb stepping movements in response to high strength MLR stimulation.

TRANSECTION		LOCOMOTION	NO LOCOMOTION
A		2	
B		1	1
C		3	
D		2*	
E		1*	1
F			5**
G			2
H			1

Figure 19. EMG and hindlimb movement records from a postmammillary preparation induced to walk on a treadmill by stimulation of the MLR. The alternating raw EMG signals from the right adductor femoris (R. hip ext.) and right iliopsoas (R. hip fl.) are shown at the top of the figure. The tracings representing the hindlimb excursions, which were recorded simultaneously with the EMGs, are shown at the bottom of the figure. Upward deflections in the tracings indicate the swing phase (flexion) of the limb. Note the onset in the right flexor EMG activity just prior to the maximal flexion of the right hindlimb. This pattern of EMG activity followed by movement in the corresponding direction is characteristic of locomotion (cf. Grillner, 1975). The stimulus parameters required to evoke locomotion in this cat were 75μA, 30 Hz at a depth of 6.0 mm below the surface of the IC.

CONTROL

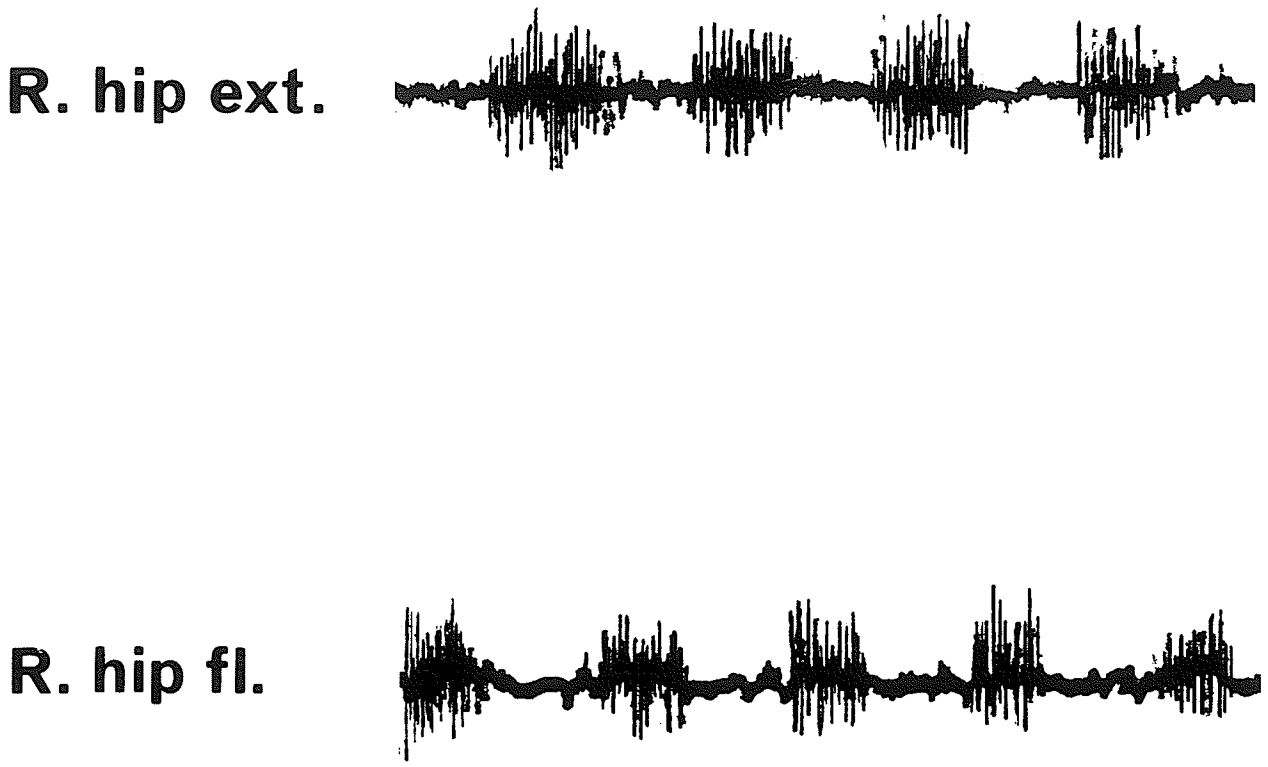
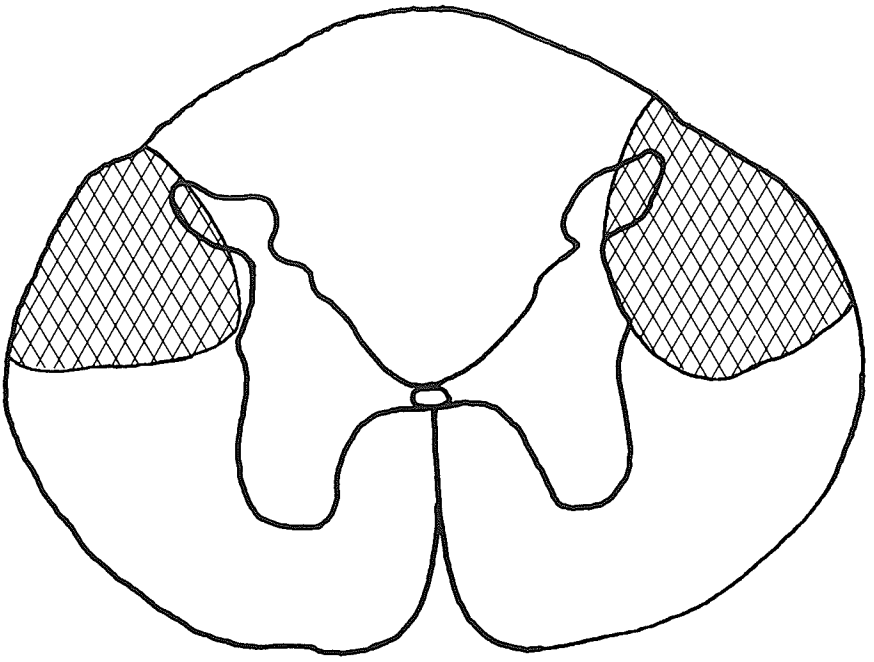


Figure 20. EMG and limb movement tracings from MLR evoked locomotion after the C_1 - C_2 dorsolateral quadrants have been lesioned. The MLR stimulus parameters prior to the transections were 125μA, 30 Hz, at 6.0 mm below the surface of the IC. After the transections a current of 150μA, 30 Hz was required to evoke locomotion with no change in the electrode position. This slight increase is probably not significant (see text). The EMG and limb movement records are shown in an identical fashion to those shown in Figure 19. There are no observable differences between the locomotion evoked in control cats (Figure 19) and dorsolateral transected preparations (Figure 20).

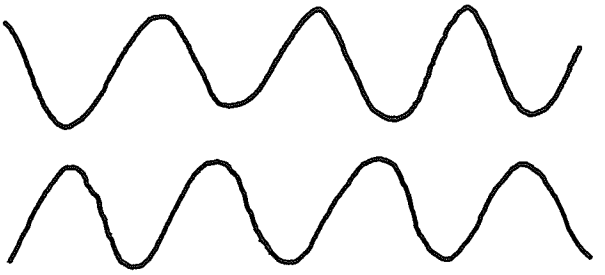


R. hip ext.

R. hip fl.

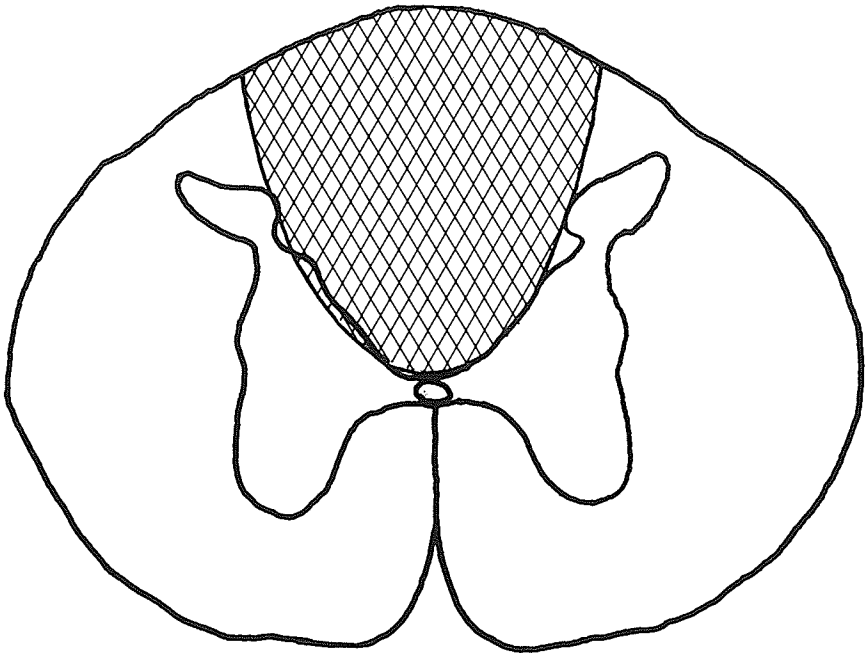
R.(flexion↑)

L.(flexion↑)



1.0 sec

Figure 21. EMG and limb movement records during MLR initiated locomotion after the dorsal columns of the C_1 - C_2 cord have been cut. The hatched area indicates the total extent of damage to the dorsal columns. The EMG and limb movement tracings are similar to those illustrated in Figures 19 and 20. The threshold stimulus current prior to the transection of the dorsal columns were 100μA and 125μA after the transection.



R. hip ext.

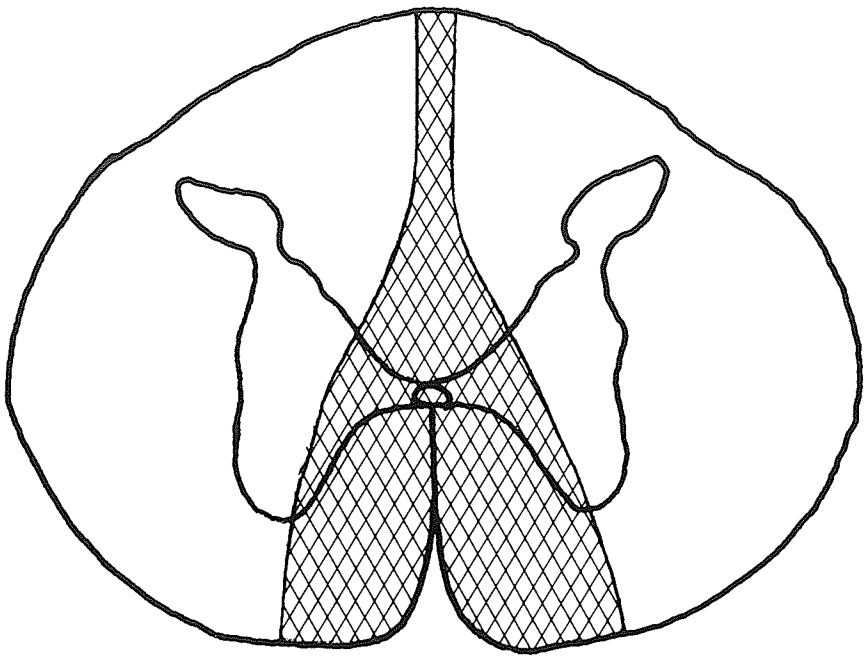
R. hip fl.

R.(flexion↑)

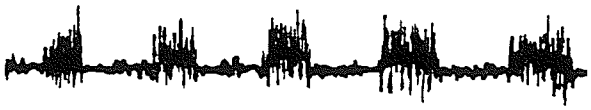
L.(flexion↑)

1.0 sec

Figure 22. EMG and limb movement tracings after a lesion of the ventral funiculi. No increase in the MLR stimulation current was necessary to evoke locomotion after the ventral funiculi were cut.



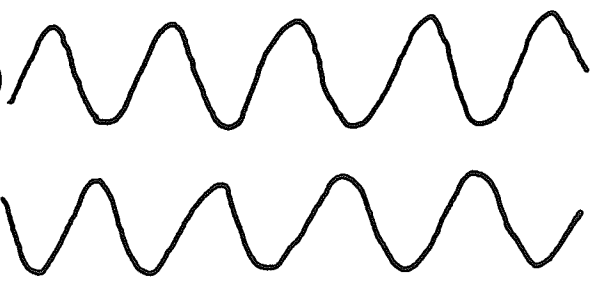
R. hip ext.



R. hip fl.

R.(flexion ↑)

L.(flexion ↑)



1.0 sec

Figure 23. A drawing of the upper cervical cord (C_1 - C_2) illustrating the full extent of the ventrolateral lesions (hatched areas).

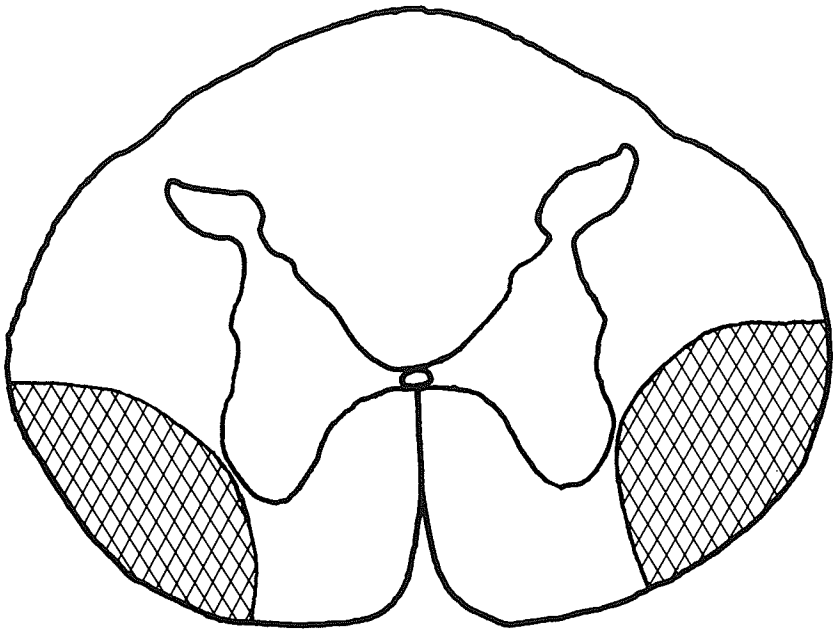


Figure 24. EMG and limb movement records of MLR evoked locomotion in a 6-OHDA treated cat. The records are illustrated in the same manner as those in Figure 19. The stimulus strength required to evoke locomotion in this animal was 60μA, 30 Hz at a depth of 5.0 mm below the surface of the IC.

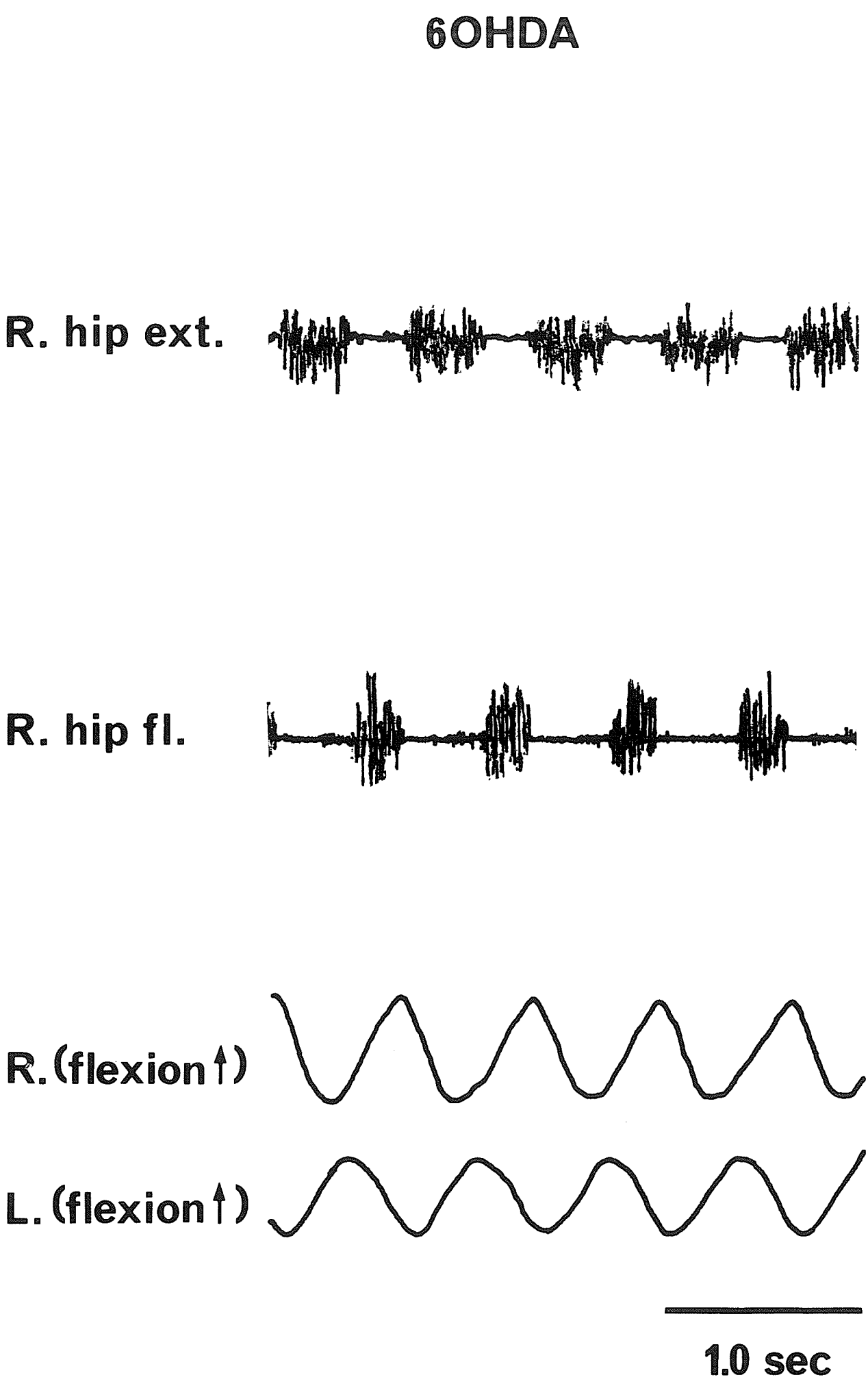

6OHDA
R. hip ext.
R. hip fl.
R.(flexion↑)
L.(flexion↑)
1.0 sec

Figure 25. EMG and limb movement records of MLR evoked locomotion in an AMT treated cat. The stimulus parameters for this animal were 75μA, 30 Hz at 5.0 mm below the surface of the IC.

AMT

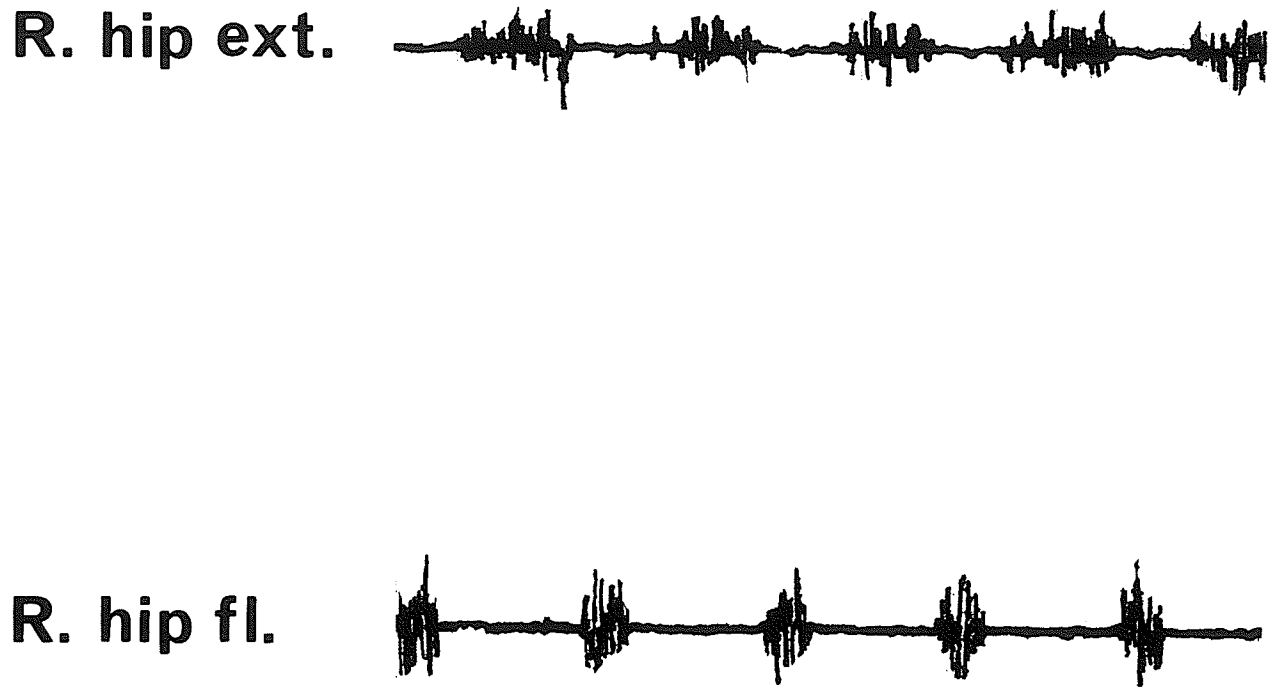


Figure 26. Mean assayed NA and 5-HT levels within various levels of the neuraxis of AMT, 6-OHDA treated and control cats. All tissues were assayed according to the Shellenberger and Gordon (1971) method for the measurement of monoamines. There were no significant differences between any of the groups for assayed 5-HT levels.

NORADRENALINE AND 5-HYDROXYTRYPTAMINE LEVELS (ng/g ± S.E.M.)

SERIES	MESENCEPHALON		PONS		MEDULLA	
	NA	5-HT	NA	5-HT	NA	5-HT
CONTROL	306±23 (n=7)	805±105 (n=3)	411±29 (n=8)	521±50 (n=3)	307±54 (n=6)	598±32 (n=7)
AMT	83±26 (n=4)	861±109 (n=3)	66±12 (n=4)	555±84 (n=3)	84±15 (n=4)	520±92 (n=2)
6-OHDA	86±11 (n=4)	745 (n=1)	234±34 (n=4)	509±30 (n=2)	115±39 (n=4)	615 (n=1)

SERIES	CERVICAL		LUMBAR		SACRAL	
	NA	5-HT	NA	5-HT	NA	5-HT
CONTROL	114±9 (n=8)	401±21 (n=9)	173±18 (n=11)	645±42 (n=9)	348±47 (n=10)	1144±130 (n=9)
AMT	35±3 (n=5)	448±25 (n=2)	32±3 (n=5)	593±46 (n=2)	61±10 (n=5)	1,205 (n=1)
6-OHDA	38±11 (n=4)	404±40 (n=2)	24±7 (n=4)	621±50 (n=3)	79±20 (n=4)	1079±81 (n=2)

Figure 27. NA levels in cats pretreated with AMT or 6-OHDA are shown as a percentage of control NA values. Using an analysis of variance (Duncan's new multiple range for one way analysis of variance) revealed a significant difference between the treated group and the control ($p < .05$).

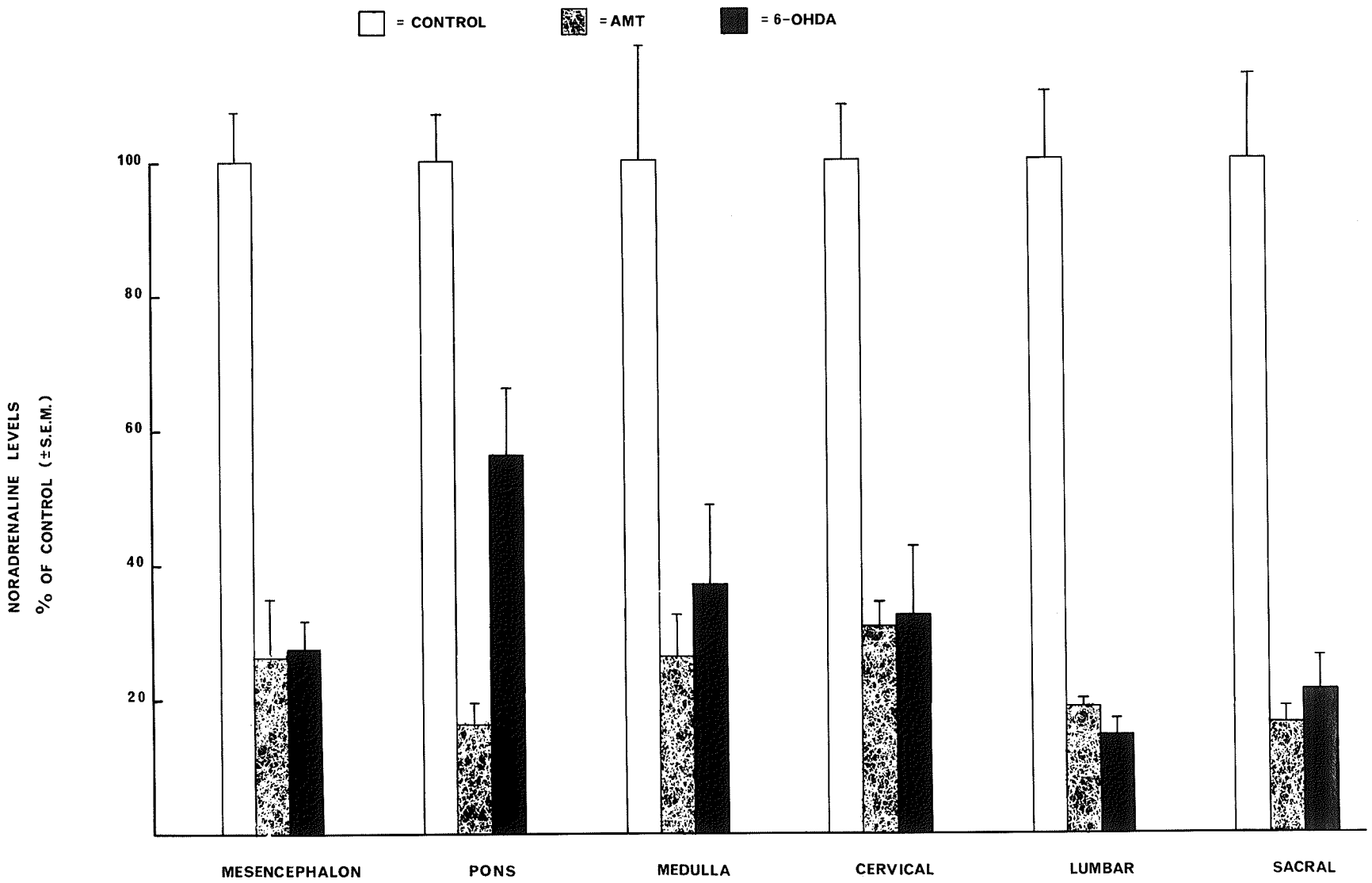

= CONTROL
= AMT
= 6-OHDA
NORADRENALINE LEVELS
% OF CONTROL (±S.E.M.)
100
80
60
40
20
MESENCEPHALON
PONS
MEDULLA
CERVICAL
LUMBAR
SACRAL

Figure 28. EMG records from a hindlimb iliopsoas (hip fl.) and adductor femoris (hip ext.) before and after the administration of POB.

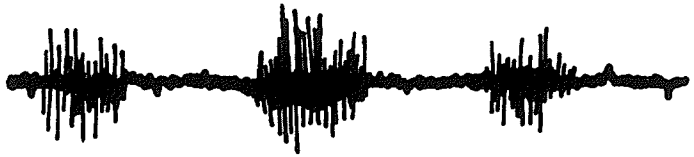
PRE-INJECTION

POB (20mg/kg)

hip fl.

hip fl.

hip ext.



hip ext.

0.5 sec

APPENDIX A

DETAILED ASSAY PROCEDURE FOR NORADRENALINE AND 5-HYDROXYTRYPTAMINE

References:

Shellenberger, M.K., and Gordon, J.H., A rapid, simplified procedure for simultaneous assay of norepinephrine, dopamine, and 5-hydroxyptamine from discrete brain areas, Anat. Biochem. 39: 356-372 (1971).

Anton, A.H., and Sayre, D.F., A study of the factors affecting the aluminum oxide-trihydroxyindole procedure for the analysis of catecholamines. J. Pharmacol. Exp. Therap., 138: 360-375 (1962).

Noradrenaline Determination (NA)

(since the centrifuge bucket has only 8 slots, only 6 samples can be done at one time)

1. Place tissue homogenizers in a bucket of ice and chill. Add 3 cc 0.4 HClO (perchloric acid) for samples $<$ 1g, or 6 cc 0.4 $HClO_4$ for samples $>$ 1g but $<$ 2g.
2. Weigh samples in a frozen state (samples should be approx. 1g); record sample number and weight, along with any other descriptive information.
3. Place weighed sample in the homogenizer; try to position the tissue along the side of the homogenizing tube in order to facilitate homogenization. Homogenize by hand until the tissue is liquidified (be sure that the tissue does not become too warm).
4. Transfer homogenate into 50cc centrifuge tubes (plastic tubes with screw tops). Let stand in ice for 10 min (this step can be shortened by placing centrifuge tubes in ice prior to transfer of homogenate).

5. Centrifuge the homogenized samples at 2°C, 20,000 G for 20 minutes (depending on centrifuge 20,000 G ≃14,000 RPM).
6. Pour supernatant into numbered beakers and store in fridge.
7. Carefully scrape pellet from bottom of centrifuge tube with a spatula and transfer to appropriate homogenizer used previously. Add 2.5 cc of 0.4 N $HClO_4$ to the centrifuge tube and swirl to remove all tiny particles. Pour the contents into the homogenizer containing the pellet (for 1-2 g samples add 5.5 cc 0.4 N $HClO_4$).
8. Rehomogenize samples and transfer homogenate back into centrifuge tubes used previously.
9. Centrifuge at 20,000 G for 15 min, as before.
10. Bring standard solutions to room temperature.
11. Add the resulting supernatant to the previous volume from the same sample and store in fridge.
12. To make internal standards add the following amounts to two labelled beakers 1.6 ml NA + 2.0 ml 5-HY + 5.4 ml .05 N $HClO_4$ = 20 ng/ml NA + 200 ng/ml 5-HT; 3.2 ml NA + 4.0 ml 5-HT + 1.8 ml .05N $HClO_4$ = 40 ng/ml NA + 400 ng/ml 5-HT (use NA standard #3, and 5-HT internal standard; see Appendix 1a for information on standards).
13. Raise pH of supernatants from samples and of standard solutions to 7.9 (exactly) using Tricine solution. Measure pH constantly while adding Tricine to supernatants being stirred on a magnetic stirrer (adjust standards last and adjust them carefully since they will become basic very quickly).
14. Add 0.3 g of aluminum oxide to empty centrifuge tubes.

15. Add pH-adjusted samples and standards to the aluminum oxide in the centrifuge tubes. Cap the tubes very tightly and rotate tubes for 25 minutes.
16. Prepare 30 (15 ml) test tubes, labelling each:

    1A 2A to 10A

    1B 2B to 10B

    1C 2C to 10C
17. Turn oven on at this time to 100°C.
18. Remove centrifuge tubes from rotator and shake to clear the cap of aluminum oxide. Centrifuge for 5 min at 1000 RPM.
19. Using a suction device pipet off the supernatant into a set of labelled centrifuge tubes and store in fridge overnight (This is the 5-HT sample).
20. Rinse the sides of centrifuge tubes and the aluminum oxide with a hard stream of deionized double distilled H_2O (D_2H_2O), repeat twice each time pipetting off top liquid with aspirator (fill approx. 1/3 full each time).
21. Centrifuge last wash for 5 min. at 5000 RPM. Aspirate.
22. Add 4.0 cc of 0.05 $HClO_4$ to aluminum oxide, cap tightly and rotate for 30 minutes.
23. Add 1.5 cc of 0.1 M phosphate-EDTA to each labelled test tube.
24. Make up external standards:

    400 λ NA std. soln. + 600 0.05 N $HClO_4$, Stir

    800 λ NA std. soln. + 200 0.05 N $HClO_4$, Stir.
25. Make up fresh 2.5% alkaline sodium sulfite sol'n by diluting stock 25% alkaline sodium sulfite sol'n with 5 N NaOH (2 ml

NA-sulfite sol'n + 20 ml 5 N NaOH).

26. Remove centrifuge tubes from rotator and pipet 1.0 ml of the appropriate sample into the corresponding series of labelled test tubes (A, B + C). Stir briefly.

27. Using a stopclock, add to parallel samples A + B:
add 0.2 ml iodine reagent, stir immediately for 10-15 sec
after 2 min add 0.5 ml Na-sulfite sol'n, stir for 10-15 sec
after 2 min add 0.4 ml glacial acetic acid, stir for 10-15 sec
(for example, refer to Appendix 1b).

28. Add to blanks ("C" tubes)
0.5 ml Na-sulfite sol'n, stir
0.2 ml iodine reagent sol'n, stir
0.4 ml acetic acid sol'n, stir
No need to use a stopwatch here since the Na-sulfite kills all fluorescence.

29. Bake all test tubes at 100° C for 3.5 min.
Remove and cool in ice water for 5 min. allow test tubes to come to room temperature.

30. Set up fluorimeter for reading NA, read values. Total time for NA determination = 7-8 hr.

5-Hydroxytryptamine (5-HT)

1. Remove the samples set aside in the fridge the day before and add 5 g NaCl. Rotate for 10 min (rotate at fastest speed).

2. Saturate borate buffer with distilled heptanol (approx. 1.0 ml of heptanol is enough for 60-80 ml of borate buffer) and shake.

There should be some heptanol visible on the surface after shaking, if the solution is saturated.

3. To a separate labelled set of centrifuge tubes add 10 ml of the borate buffer - heptanol sol'n.
4. To the centrifuge tubes on the rotator add 20 ml of distilled heptanol using a large tipped pipette. Then add 0.6 g of K_2CO_3 and hand-shake immediately. Rotate at a high speed for 10 min.
5. Centrifuge for 10 min at 8000 RPM (approx 5000 G).
6. Aspirate off the heptanol layer into the set of centrifuge tubes containing the borate buffer. Rotate at a high speed for 5 min.
7. Centrifuge for 10 min at 8000 RPM (Approx. 5000 G).
8. Add 3 cc of 0.05 M phosphate buffer to a set of empty centrifuge tubes.
9. After centrifugation in #7 remove 15 ml of the heptanol layer using a clean pipette and transfer it to the centrifuge tubes containing the 0.05 M phosphate buffer (do not contaminate with borate buffer). Rotate at high speed for 10 min.
10. Centrifuge for 10 min at 8000 RPM (approx. 5000 G).
11. After centrifugation, pour the contents of the centrifuge tubes into separatory funnels and wait for separation (use a clean glass rod to break the surface tension of the few phosphate buffer droplets floating on top of the heptanol).
12. Drain off the phosphate buffer (lower layer) into a set of labelled test tubes. Dump the heptanol into the "used heptanol" bottle for redistilling. Turn on oven to 105° C.

13. Prepare fresh ninhydrin reagent (0.1961 g of ninhydrin + 10 ml of D H_2O, use slight heat while stirring).
14. Label a rack of 20 (15 ml) test tubes, 2 test tubes for each sample.
15. Add 1.0 ml of each sample to appropriate test tube.
16. Prepare 2 external standards of 200 ng/ml

    400 ng/ml

    use fresh 5-HT external standard sol'n each time

    (Ext. std. sol'n = 400 ng/ml, to make 200 ng/ml add 2 ml of 5-HT ext. to 2 ml of 0.05 M phosphate buffer).

    Add 1.0 ml to each external standard test tube of either the 200 ng/ml or 400 ng/ml sol'n.
17. Add 0.1 ml of the fresh ninhydrin reagent to each test tube and stir immediately for 10-15 sec.
18. Bake test tubes for 25 min at 105°C.
19. Let stand at room temperature for 1 hr.
20. Set up fluorimeter to read 5-HT as outlined in Appendix C, then read 5-HT values.
21. Add 0.1 ml (100λ) of 2.5% Na-sulfate sol'n to each test tube and stir immediately for 10-15 sec.
22. Let test tubes stand for 20 min then read blank fluorescence at the same settings on the fluorimeter.

    Total time for 5-HT determination = 6-7 hours.

This outline was prepared by Barbara J. Skovgaard with subsequent slight modifications by John D. Steeves.

APPENDIX 1a (Store all standards in fridge)

To make up 5-HT Standard: (shelf life = 2 months)

Dilute .2198g 5-HT creatinine sulfate in 100 ml of 0.05 M $HClO_4$.

.2198g 5-HT creatinine sulfate - .1g 5-HT

.1g 5-HT/100ml = .001 g/ml

= 1 mg/ml (5-HT conc. stock sol.)

5-HT Internal Standard Stock: (shelf life = 2 weeks)

Dilute 40λ of 1 mg/ml 5-HT concentrated stock solution with 100 ml 0.05M $HClO_4$ to get 400 ng/ml

- i.e. 0.04 ml of 1 ng/ml stock sol. gives 0.04 mg.

0.04 mg/100 ml = 400 ng/ml.

5-HT External Standard Stock: (shelf life = 1 week)

Dilute 40λ (0.04 ml) of 1 mg/ml 5-HT conc. stock solution with 100 ml 0.05M phosphate buffer to get 400 ng/ml

- i.e. 0.04 ml of 1 mg/ml stock sol. gives 0.04 mg

0.04 mg/100 ml = 400 ng/ml.

To make up NA standards:

1. Dilute 0.0997 g NA-bitartrate in 100 ml of 0.05M $HClO^4$

0.0997 g NA-bitartrate = 0.05g NA

0.05 g/100 ml = 5×10^{-2} g/100 ml

= 5×10^{-4} g/ml

2. Dilute 1 ml of 5×10^{-4} g/ml solution (1) to 100 ml with 0.05 M $HClO_4$, to get 5×10^{-4} g/100 ml.

= 5×10^{-6} g/ml

= 5000 ng/ml (Keep as concentrated stock)

3. Dilute 1 ml of 5000 ng/ml solution (2) to 100 ml with 0.05 M $HClO^4$, to get 500 ng/100 ml.

   = 50 ng/ml

   (Use #3 as internal and external standard stocks)

   Shelf life of #3 - 1 week.

APPENDIX 1b

Solutions to be added exactly 2 min apart

A and B tubes only (parallel samples)

0.2 ml iodine reagent (in 4 tubes, sequentially)

(have 0.4 ml in pipette)

0 sec - add iodine to 1st tube, stir 5-10 sec

15 sec - add iodine to 2nd tube, stir 5-10 sec

1 min - add iodine to 3rd tube, stir 5-10 sec

1.15 min - add iodine to 4th tube, stir 5-10 sec

0.5 ml alkaline solution (use large tip or broken ended pipette)

2:00 min - 1st tube, stir as long as possible

2:15 min - 2nd tube, stir as long as possible

3:00 min - 3rd tube, stir as long as possible

3:15 min - 4th tube, stir as long as possible

0.4 ml glacial acetic acid

4:00 min - 1st tube, stir immediately

4:15 min - 2nd tube, stir immediately

5:00 min - 3rd tube, stir immediately

5:15 min - 4th tube, stir immediately

Repeat this process, doing the tubes in 4's, until all the tubes have been treated.

CHEMICAL LIST

Unless otherwise stated, keep solutions in dark bottles at room temperature.

Reagent	Preparation	Shelf Life
0.4N $HClO_4$	40.19 ml $HClO_4$ (70%) + 1.0g Na - metabisulphite + 0.5g disodium EDTA + 1000 ml D_2H_2O	Indefinite
Tricine, pH 12.5	4.47 Tricine N-tris-(hydroxymethyl) methyl glycine + 6.25g diosidium EDTA + 250 ml 0.525 N NaOH, adjust pH	2 weeks
0.525 N NaOH	21g NaOH (Solid)/l D_2H_2O or 42 ml 50% NaOH/l D_2H_2O	Indefinite
Aluminum oxide	refer to instructions in Schellenberger and Gordon (time consuming process which must be done carefully)	

Reagent	Preparation	Shelf Life
0.05 N $NClO_4$	5.02 ml $HClO_4$ (70%) + 1000 D_2H_2O	Indefinite
0.1 M Phosphate-EDTA Buffer, pH 7.0	4.76 g KH_2PO_4 + 2.135 Na_2HPO_4 + 4.5 g di-sodium EDTA + 500 ml D_2H_2O, adjust pH	1 month
Alkaline Sodium Sulfite Sol'n (25%)	5g Na_2SO_3 + 20 ml D_2H_2)	Indefinite
Alkaline Sodium Sulfite Sol'n (2.5%)	2 ml 25% Na-sulfite sol'n + 20 ml 5.0 N NaOH	1 week
5.0 N NaOH	50 g NaOH (solid) + 250 ml D_2H_2O, or 100 ml 50% NaOH/250 ml D_2H_2O	Indefinite
0.1 N Iodine Reagent	2.0 g potassium iodide + 0.5 g iodine + 40 ml D_2H_2O	Indefinite
Glacial Acetic Acid	Use full strength	Indefinite

Reagent	Preparation	Shelf Life
0.5 M Borate Buffer	95.3425 g Sodium Borate + 500 ml D_2H_2O - use heat while stirring - bring to room temperature - pour gently into clean beaker - saturate with NaCl, with stirring - pH should be 10.00, adjust with 5.0 N NaOH if necessary - filter	Indefinite
	when ready to use add approx 1 ml of distilled heptanol to 80 ml of 0.5 M borate buffer	(1 week)
Heptanol	distill in distilling apparatus at 175°C	Indefinite

Reagent	Preparation	Shelf Life
0.05 M Phosphate Buffer pH 7.0	1.35 g NaH_2PO_4 + 2.17 g Na_2HPO_4 + 500 ml D_2H_2O	Indefinite
0.1 M Ninhydrin	0.1961 g Ninhydrin (1,2,3 - idantrione monohydrate) + 10 ml D_2H_2O use heat while stirring, then bring to room temperature	
NA Standard Solution	Refer to Appendix A	
5-HT Standard Solution	Refer to Appendix A	

APPENDIX B

DETAILED PROCEDURE FOR LIGHT MICROSCOPY AUTORADIOGRAPHY

References:

Cowan, W.M., Gottlieb, D.I., Hendrickson, A.E., Price, J.L., and Woolsey, T.A., The autoradiographic demonstration of axonal connections in the central nervous system, Brain Res. 37: 21-51 (1972).

Injection

- before injection the isotope must be concentrated. Therefore evaporate in a temperature regulated vacuum centrifuge or under a stream of N_2 gas. Then redissolve the isotope to the appropriate concentration (usually 10-20 fold concentrations are most useful) in sterile 0.9% saline. In otherwords, a highly concentrated low volume injection is best.
- inject slowly over a time period of sufficient length (10 nl/5 min).
- allow the animal to survive for an appropriate time.

Perfusion

- perfuse with oxygenated Lactated Ringer (1,000 ml)
- then perfuse with 10% formalin (1,000 ml)
- remove tissue and store in 10% formalin for 5-7 days (tissue may be left longer, if desired).

Embedding

- remove tissue from the 10% formalin and wash in running tap water for 24 hr (temp of water is not important)
- then place tissue in 70% ETOH for 24 hr
- then place tissue in 80% ETOH for 8 hr
- then place tissue in 95% ETOH for 3 days (changing the 95% ETOH every day)
- then place tissue in amyl acetate, 85% for 2 days (changing the amyl acetate every day)
- then place tissue in benzene (Fisher, B-245) for 25 min
- then place tissue in fresh benzene (Fisher, B-245) for 25 min
- Using an oven maintained at 56-58°C place tissues in the following:
   1. 50% benzene/50% paraffin solution for 1 hr.
   2. 100% parrafin (wax 1) for 3 hr.
   3. 100% paraffin (wax 2) for 3 hr.
- the tissue is then placed in 100% paraffin (wax 3) in a vacuum oven maintained at 15 torr and a temp of 60°C for 45 min
- Finally embed tissue in 100% paraffin which has been "degassed" in the vacuum oven for 1 hr prior to embedding.

Sectioning

- 10-15μm serial sections are cut in the usual manner, and it is usually sufficient to use every tenth section for autoradiography.

- sections are mounted on gelatin-chrom alum slides by floating the sections on a water bath maintained at ~ 52°C and then bringing the slide up from underneath the section. It is very important to work in as clean an environment as possible. Use double distilled water for all solutions and in the water bath.
- let the slides dry for ~½ hr at room temp (make sure slides are covered by a lint free cloth to prevent dust settling on the sections).

Deparaffinization

- place slides in a fresh bath of xylene for at least 8 hr.
- then place slides in 100% ETOH for 5 min
- them place slides in fresh 100% ETOH for 5 min
- then place slides in 95% ETOH for 3 min
- then place slides in 70% ETOH for 3 min
- then place slides in 50% ETOH for 3 min
- then place slides in D_2H_2O for 1 min
- then place slides in D_2H_2O for a rinse
- let slides dry in a clean covered place before coating with emulsion.

Emulsion

- Kodak NTB2 (4 oz), store emulsion at 4°C
- the emulsion is always handled in total darkness or under a Wratten #2 safelight or a sodium vapor lamp
[glass filters = bright red and yellow (tape borders)]
- in a dark room that has a high humidity, to increase humidity

tape exhaust vents closed and use either a humidifier or turn on hot water tap.

- when ready to coat slides warm up the NTB2 to 40°C as well as D^2H_2O to 40°C. Then mix 5 parts NTB2 with 3 parts D^2H_2O (might also try 1:1)

- first do two test slides, when dipping slides be careful not to agitate the emulsion too much since this will cause air bubbles.

- take one of the two test slides into the light and leave the other in the dark room. Let both slides dry for 20 min.

- then develop the slides as per the developing instructions below except you need only wash for 5 min after the fixer instead of 50 min.

- then you take the two test slides through the following steps:

place in 50% alcohol for 3 min.

place in 70% alcohol for 3 min.

place in 100% alcohol for 5 min.

place in xylene for 5 min

- then coverslip the slides

- under 40x (high dry) (light field) any less than 10-20 grains (black) throughout the entire object means that the emulsion is satisfactory.

- if the test slides yield satisfactory background levels, then dip your slides, and dry them vertically for 2-3 hrs. in the humid dark room.

- then pack the slides in slide boxes with indicator drierite,

and then place these slide boxes in three way film boxes, always maintaining slides in an upright position.

- place these boxes in the refrigerator at 4 C. Make sure that no tritium labelled materials are close to the boxes and it is preferred that the boxes containing the slides are placed in a contamination (chemical and radioactive) free refrigerator.
- Length of exposure is variable but it is suggested that 4 weeks is satisfactory. However to check this out, develop a few test slides at various intervals after sipping to determine the best exposure time.
- Never use the NTB2 emulsion more than one time.

Developing

- temperature is very critical and all materials should be maintained at 16°C (eg. D-19, water, fixer).
- rapid fixer (without hardener) is diluted 1:1 with distilled H_2O.
- D-19 developer (full strength). Dip slides in tray for 1.5 - 2.0 min, do not agitate slides.
- then in and out of distilled H_2O bath (rinse)
- then in fixer bath for 3.0 - 4.0 min
- after fixing, place slides in H_2O for gentle wash for 20 min.
- after washing the slides, you may leave the slides to dry (dust free environment) or you may counter stain them immediately.

Staining

- D_2H_2O for approx. 1 min.
- then in 0.1% cresyl violet stain for 3-6 min.
- then in 70% ETOH for approx. 2 min.
- then in 95% ETOH for approx. 1-3 min (add a few drops of glacial acetic acid to bath for better differentiation)
- then in 100% ETOH for 5 min
- then in 100% ETOH for 5 min
- then in xylene (full strength) until you coverslip the slides.
- coverslip the slides in the usual manner
- note: other stains may be used instead of 0.1% cresyl violet, depending on the particular needs of the study.

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Vita

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