LOCALIZATION OF THE NEURONS ACTIVATED DURING LOCOMOTION IN THE SPINAL CORD AND THE BRAINSTEM USING THE C-FOS IMMUNOHISTOCHEMICAL METHOD

BY

XIAOHONG DAI

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Submitted to the Faculty of Graduate Studies
In Partial Fulfilment of the Requirements
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University of Manitoba
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ABSTRACT

This study was designed to localize the neurons in the spinal cord and the brainstem activated during locomotion.

Fictive locomotion was occurred spontaneously, or was induced by stimulation of the mesencephalic locomotor region (MLR) or by forelimb swinging. Treadmill locomotion was evoked by MLR stimulation. After bouts of locomotion during a 7-9 hour time period, the animals were perfused and the L3-S1 spinal cord segments and the brainstem were removed. Cats in the control group were subjected to the same surgical procedures but no locomotor task. The tissues were sectioned and then stained with the c-fos immunohistochemical method.

In the spinal cord, labelled cells were found concentrated in Rexed's laminae III and IV of the dorsal horn, and laminae VII and VIII of the ventral horn in the treadmill locomotion cats. Only the cells in laminae VII and VIII were labelled in the fictive locomotion cats. The result suggests that labelled cells in laminae VII and VIII are pre-motor interneurons involved in the production of locomotion, while the laminae III and IV cells are those activated during locomotion due to afferent feedback.

In the brainstem, the prominent labelling was found in the magnocellular tegmental field, the ventrolateral reticular formation, the cuneiform nucleus, the marginal nucleus of the brachium conjunctivum, the locus ceruleus and the nucleus of the solitary tract. The significance of the labelling is discussed.

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INTRODUCTION

The objective in the present study is to determine the location of the spinal cord and the brainstem neurons involved in locomotion using the c-fos immunohistochemical method. This chapter describes the c-fos gene product and why it was chosen as a marker of neuronal activity, and the rationale for embarking on this study.

Fos Expression as a Marker of Neuronal Activity

The normal cellular genes from which the virus oncogenes are derived are called proto-oncogenes. The proto-oncogene c-fos, which exists in normal cells in vertebrates, is homologous to the transforming gene of the FBJ-murine osteosarcoma virus, v-fos (Curran et al 1984). Recently, it was found that the c-fos proto-oncogene is rapidly and transiently induced by treatment of several cell types with polypeptide growth factors and other growth modulating substances (Cochran et al 1984, Muller et al 1984, Bravo et al 1985, Muller et al 1985). Therefore, a number of investigators suggested that c-fos plays a part in controlling cell cycle events. In some systems, c-fos expression has been associated with cellular differentiation (Muller et al 1985, Curran and Morgan 1985, Greenberg et al 1985). However, in addition to its induction by growth factors, c-fos has also been shown to be induced by neurotransmitters (Greenberg et al 1986, Szekely et al 1987), depolarizing conditions (Morgan and Curran 1986) and agents that provoke an influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (Morgan and Curran 1986) in cultured cells, or by pharmacological (Morgan et al 1987), electrical (Dragunow and Robertson 1987), surgical (White and Gall 1987) and physiological stimuli (Hunt et al 1987, Sagar et al 1988) in neurons of intact animals. The fact that c-fos

can be induced by various stimuli in a broad range of cells has suggested a more general role of c-fos induction, which couples extracelluar signals to alteration in gene expression (Morgan and Curran 1986).

There are three general ways in which the transcription of cellular genes can be modulated by extrinsic signals. 1) Membrane-permeant molecules such as steroids or thyroid hormones can bring about alterations in transcription rates by direct interaction with nuclear receptor proteins. 2) Stimulation agents that cannot penetrate the cell membrane can bring about direct changes in target gene transcription via changes in the level of second messenger molecules. 3) Stimuli can also bring about indirect alteration of target gene expression by rapidly activating the expression of genes that encode known or putative transcriptional regulatory proteins (Bohmann et al 1987, Franza et al 1988, Rauscher et al 1988, Cohen and Curran 1988). The extrinsic stimuli, in the third mechanism, elicit the expression of the genes whose protein products couple extracellular signals to phenotype alteration by affecting the transcription rates of target genes. These proteins should be viewed as nuclear 'third messenger' molecules in a stimulus-transcription coupling cascade (Morgan and Curran 1986). The protein product of the c-fos proto-oncogene, Fos, is considered as a potential third messenger. This is because: 1) The c-fos proto-oncogene is transcribed rapidly and transiently (Curran 1988) following stimulation. Like its mRNA, c-fos protein is relatively short-lived and thus has the characteristics of a signalling system. 2) c-fos protein has a nucleus binding property (Sambucetti and Curran 1986). Fos is translated in the cytoplasm and then translocated to the nucleus where Fos combines with another protein (Jun) to form a heterodimeric protein complex. 3) The protein complex binds to the DNA regulatory element known as the AP-1 binding site (Curran and Franza 1988). The AP-1 binding motif is a relatively common

constituent of both positive and negative transcriptional regulatory elements and is required for both basal and induced levels of gene expression (Curran and Franza 1988). Fos and Jun cooperate in the regulation of target genes that may underlie cellular adaptive responses. Therefore, c-fos protein is considered as a third messenger which couples early events associated with receptor occupation to long term changes in gene expression.

Studies on the PC12 cell line have shown that c-fos can be induced either by receptor-ligand interaction or by agents or conditions that effect voltage-dependent calcium channels. The depolarization-induced calcium influx via the voltage-dependent calcium channels appears to elicit c-fos expression by an interaction with calmodulin. However, induction of c-fos by nerve growth factor seems to be independent of extracelluar calcium (Morgan and Curran 1986).

What genes are the targets for the stimulus-transcription coupling cascade? Target genes might be different in different cells in response to different stimuli. The pentylenetetrazole seizure paradigm has proved particularly useful in the elucidation of the biochemical and molecular details of stimulus-transcription coupling in the nervous system. The c-fos and c-jun mRNAs are induced rapidly (1-2 hours) post-seizure. Previous studies of seizure models involving the hippocampus have shown effects upon the expression of a number of genes including preproenkephalin (White and Gall 1987). The preproenkephalin gene has a regulatory element, which is required for stimulation of expression by cAMP and phorbol esters, that is structurally related to the AP-1 binding site (Comb et al 1986). In this particular circumstance, Fos and Jun could play a homeostatic role in the granule cells of the dentate gyrus by increasing the expression of preproenkephalin to replenish the intracellular stores of enkephalin that are released during seizure activity. In a larger sense, this homeostatic role would be

viewed as part of a general adaptive response of the neuron to excitation.

The discovery of the immediate early gene, c-fos, has facilitated studies of the biochemical and biophysical processes that couple extracellular signals to alterations in gene expression. At the same time, the correlation between neuronal activity and the presence of Fos was noticed (Morgan et al 1987, Hunt et al 1987, Sagar et al 1988). The mapping of Fos induction could identify the cellular/neuroanatomical targets of pharmacological stimuli or the pathways involved in neurophysiological responses. Nociceptive stimulation has been shown to induce Fos expression only in certain neurons of the spinal cord. The precise pattern of Fos immunostaining in the superficial laminae of the spinal cord is dependent upon the route of stimulation (Hunt et al 1987, Bullitt 1989, Menetrey et al 1989, Williams et al 1990). Fos expression has also been shown to be induced by activation of excitatory amino acid receptors in the studies of seizure (Morgan et al 1987, Sonnenberg et al 1989), cerebral ischaemia (Jorgensen et al 1989), and trauma (Dragunow and Robertson 1988). There have been fewer studies on reflex pathways and motor control using the c-fos immunohistochemical method. At the same time that this thesis project was in progress, Barajon et al (1990) showed that c-fos protein was expressed in neurons in the intermediate zone and ventral horn where the interneurons mediating group I muscle afferent reflexes are located. It was also demonstrated that neurons in the nucleus proprius, medial Rexed's laminae V and VI and ventral horn were labelled with c-fos in rats after walking on a rotarod treadmill (Gogas et al 1990).

The c-fos immunohistochemical method has some advantages over other tracing techniques such as 2-deoxyglucose autoradiography (2-DG) and horseradish peroxidase conjugated with wheat germ agglutinin (WGA-HRP). It gives single cell resolution, and it potentially localizes all interneurons activated during certain

manipulations rather than only the last order interneurons, as is the case with WGA-HRP activity-dependent labelling. Comparisons of patterns of 2-DG uptake with Fos-like immunoreactivity have shown overlap, but there is not always an identical distribution (Sagar et al 1988). One of the contributing factors may be that autoradiographs prepared from tissue treated with 2-DG provide a measure of glucose utilization throughout the cell and may be more sensitive to alterations in functional activity in neuropil than in cell bodies (Sagar et al 1988). Another possibility suggested by Morgan and Curran (1989) is that induction of Fos expression requires the integration of various stimuli and intracellular signalling pathways, which may or may not be associated with increased metabolic activity. Therefore, Fos expression provides a unique window on neuronal physiology.

Spinal Interneurons Involved in Production of Locomotion

The ability of the spinal cord to produce complex movement has long been known based on the fact that the spinal cord is capable of producing rhythmic movement of locomotion even after transecting the spinal cord, which removes influences of the brain. In the study of the spinal mechanism of progression, Philippson first focused on the importance of the exteroceptive reflexes and later Sherrington (1910) laid stress upon the role of the proprioceptive reflexes in producing locomotion. However, Brown (1911) demonstrated that the act of progression which was evoked in the hind limbs on rapid division of the caudal thoracic spinal cord in the decerebrate cat might appear even after destruction of the proprioceptive reflexes of both hind limbs by deafferentation. Narcosis progression persisted in the cat at a depth of narcosis at which spinal reflexes were abolished (Brown 1914). The evidence suggested that the alternating rhythmic movements of the spinal animals are essentially a central spinal process. This

hypothesis was confirmed in later studies. Grillner and Zangger (1974) demonstrated that locomotor-like activity was induced in the chronic or acute spinal cats treated with dihydroxyphenylalamine (DOPA) or Clonidine even after curarization, which excludes the possibility that any phasic activity reaches the spinal cord. The cell networks in the spinal cord responsible for generation rhythmic movement are named central pattern generators (CPGs) (Grillner 1975, Grillner and Wallen 1985).

The CPGs can be activated to produce rhythmic movement by primary afferent stimulation (Sherrington 1913, Grillner and Zangger 1974), by indigenous activity in the interneurons of the CPGs induced by anaesthesia (narcosis progression) (Brown 1913, 1914) or by volleys in descending pathways. Numerous brain areas such as the mesencephalic locomotor region, the subthalamic locomotor region, or the medial reticular formation, when stimulated, can send descending volleys to the spinal cord which activate the CPGs. Stimulation of the mesencephalic locomotor region to obtain controlled locomotion is one of the most useful models in the study of locomotion, and is the model used in the present study.

A search for the locations of the spinal interneurons involved in mammalian locomotion has recently been initiated in this laboratory. The inputs, outputs and patterns of activity of several populations of spinal interneurons have been identified during locomotion. 1) The first group interneurons studied were the Ia reciprocal inhibitory interneurons and the Renshaw cells. The locations, connections and reflex functions of the Ia reciprocal inhibitory interneurons and the Renshaw cells have been well documented. The Ia inhibitory interneurons are located within a narrow zone in the Rexed's lamina VII just dorsal to the motor nuclei, while Renshaw cells are situated in the medioventral border of the motor

nuclei (Jankowska and Lindstrom 1972, 1971). The reflex functions of the Ia inhibitory interneurons and the Renshaw cells, which prevent antagonistic muscle activation (the former) or stop motoneuron firing by a negative feedback mechanism (the latter), suggested these interneurons might also be involved in the process of locomotion. With extracellular recording techniques, it has been observed that the Ia inhibitory interneurons and the Renshaw cells displayed phasic activity during fictive step cycle which is tightly coupled to the particular extensor or flexor motoneuron population with which they were associated (McCrea et al 1980, Pratt and Jordan 1987). 2) Recently a group of interneurons in the mid-lumbar segments have been identified which receive multimodal peripheral inputs from afferents in muscle (group I, II), skin and joint nerves and project to motoneurons (Edgley et al 1988, Edgley and Jankowska 1987a, 1987b). These interneurons also have been shown to receive descending input from the cuneiform nucleus, an area thought to be the MLR (Edgley et al 1988). Most of these interneurons are located in Rexed's laminae VI, VII and dorsal part of lamina VIII. The ventrally located interneurons (at depth between 2.6 and 3.6 mm from dorsal surface of the spinal cord) seem to be excited at short latencies from the MLR and to project to motor nuclei. It was proposed that these midlumbar interneurons might control motor activity dependent upon hip position and be involved in transition from the stance to the swing phase of locomotion (Edgley and Jankowska 1987b). After further investigation of the L4 interneurons, it was found that the interneurons (located between 1.7 and 2.8 mm from dorsal surface of the spinal cord) can be divided into two categories: those rhythmically active or those not active during MLR-induced fictive locomotion (Shefchyk et al 1990). All of the interneurons which are rhythmically active during fictive locomotion were found to fire action potentials during the time of activity in the ipsilateral hindlimb flexor

neurogram. This finding seems to be consistent with the above proposal. 3) The third group of interneurons which are considered to be involved in generating locomotion were also found in the midlumbar (L4/L5) segments, but in Rexed's lamina VIII (Jankowska and Noga 1990). These contralaterally projecting midlumbar interneurons showed much stronger similarities in term of afferent inputs to the ipsilaterally projecting interneurons in the intermediate zone of the same segment (described above), than to lamina VIII interneurons in the more caudal segments (Edgley and Jankowska 1987a, Edgley et al 1988). Short latency EPSPs in the lamina VIII interneurons were observed after simulation of the cuneiform nuclei, which lies within the MLR. It was proposed that the contralaterally projecting lamina VIII interneurons are involved in locomotion in addition to mediating reflex actions from muscle and tendon afferents (Jankowska and Noga 1990).

Besides those interneurons which mediate the reflex functions described above, efforts to explore all the interneurons involved in locomotion have been made. Field potential mapping of interneurons in the lumbar spinal cord activated following stimulation of the MLR indicated that such interneurons are located in the intermediate zone and ventral horn throughout the lumbosacral segments of the spinal cord, but predominantly between L4 and L6 (Fortier et al, in preparation). The attempt was also made using 2-deoxyglucose (2-DG) uptake in acute low spinal rabbits injected with nialamide and DOPA (Viala et al 1988). The isolated lumber spinal cord can generate rhythmic activities after systemic administration of DOPA (Jankowska et al 1967). The neurons activated to produce locomotion after DOPA administration or simply activated by DOPA take up 2-DG. Labelled cells were located in the intermediate part of the gray matter, which corresponds to Rexed's lamina VI and dorsolateral portion of lamina VII, extending from L6-S1 (more

rostral segments were not investigated) (Viala et al 1988). Several other studies using electrophsiological methods have shown rhythmic activity in the unidentified interneurons located mostly in the lateral part of the intermediate zone and ventral horn of gray matter during locomotion (Baev et al 1979, Orlovski and Feldman 1972).

In summary, determining the locations of interneurons comprising or activated by central pattern generators is a necessary step towards understanding the mechanism of locomotion. Only then can further studies, such as connections with other interneurons or with peripheral afferents, and the electrical and pharmacological properties of these interneurons can be efficiently carried out. At present only a small population of interneurons, with special roles in reflex functions, have been tested for their involvement in locomotion. As described above, they include neurons in Rexed's laminae VI, VII and VIII of L4/L5 segments, the Ia reciprocal inhibitory interneurons and the Renshaw cells. Anatomical localization of interneurons with 2-DG has revealed some information, but the low resolution and limited number of segments studied (only L6-S1) failed to provide a clear, complete picture of the locations of interneurons (Viala et al 1988). There are also many differences in the laminar and segmental distributions compared to the results obtained in the field potential mapping study. A better anatomical study is necessary to provide further information on the location of the interneurons activated during locomotion. As introduced earlier, the c-fos immunohistochemical method has the advantage of single cell resolution. It also has the potential to label all the neurons activated during stimulation.

Nuclei in the Brainstem Involved in Locomotion

Early studies showing that removal of the cerebral hemispheres and

diencephalon (Bard and Macht 1958, Bazett and Penfield 1922, Hinsey et al 1930, Laughton 1924) leaves animals capable of spontaneous coordinated locomotion and indicated the existence of brainstem structures responsible for the activation of the spinal cord stepping mechanisms. Shik et al (1966) were the first to show that stimulation of a discrete area below the inferior colliculus, later termed the mesencephalic locomotor region or MLR (stereotaxic coordinates P2, L4, H-1), could induce coordinated locomotion on a treadmill in acute postmammillary decerebrate cats. This finding has been confirmed in later studies. Besides the 'classical' MLR, many other structures in the mesencephalon and lower brainstem have been shown to produce locomotion when stimulated. These include the gigantocellular (FTG) and magnocellular (FTM) tegmental field, the ponto-medullary locomotor strip (PLS), the pedunculopontine nucleus (PPN), the periaqueductal gray (PAG) and the lateral parabrachial nucleus (BCM) (Shik and Yagodnitsyn 1979, Budakova and Shik 1980, Garcia-Rill 1983, Garcia-Rill et al 1981, 1983d). Anatomical (Steeve and Jordan 1984) and electrophysiological studies (Shefchyk and Jordan 1985, Edgley et al 1988) have demonstrated that the cuneiform nucleus, an area in the MLR, does not project directly to the spinal cord. The FTG and FTM in the medial pontomedullary reticular formation (MRF) but not PLS were suggested to be the relay site in the brainstem. The PLS, located in the lateral reticular formation, is suggested to be synonymous with the spinal nucleus of the trigeminal nerve. PLS-induced locomotion is thus considered analogous to sensorimotor reflex initiated locomotion (Noga et al 1988). Anatomical structures under the physiological identified MLR have been studied. The cuneiform nucleus and the pedunculopontine nucleus have both been implicated as the anatomical substrate for the MLR. A more detailed review is given below.

The medial pontomedullary reticular formation.

Various electrophysiological and anatomical studies have demonstrated that cells in the gigantocellular and magnocellular tegmental field in the medial pontomedullary reticular formation are active during locomotion. Studies also indicated that the FTG and FTM were the relay sites for MLR projecting neurons.

Orlovsky (1970a) was the first to demonstrate that stimulation of the MLR could orthodromically activate the medial reticulospinal neurons bilaterally. It was also found that the reticulospinal neurons became rhythmically active during MLR-induced locomotion (Orlovsky 1970b). Later studies have confirmed (Garcia-Rill and Skinner 1987b) and extended (Drew et al 1986, Shimamura et al 1982) the initial findings. The medial reticulospinal neurons have been shown to be rhythmically active during spontaneous locomotion in intact cats (Drew et al 1986) or in thalamic cats (Shimamura et al 1982). Furthermore, activity of many of these cells can be correlated with the excitation of specific muscle groups in one or more limbs (Drew et al 1986, Garcia-Rill et al 1983b, Shimamura et al 1982).

The first detailed anatomical study of the connections of the 'classical' MLR (L4, P2, H-1) used the autoradiographic tracing technique (Steeves and Jordan 1984). In this study, tritiated animo acids was injected into the functionally identified MLR and axons of neurons in the area. The terminal arborizations of labelled axons were confined to the ventromedial reticular formation, i.e. P6.0 to P8.0 rostrocaudally and L2.5 to contralateral L1.5 mediolaterally. This area corresponds to the caudoventral potion of the FTG and FTM. The existence of the projections from the MLR to the MRF has been confirmed in the cat (Skinner et al 1990) and rat (Garcia-Rill et al 1986).

Microinjection of neuroactive substances into the MRF has been shown to evoke episodes of locomotion in the precollicular-postmammillary transected cats

(Garcia-Rill and Skinner 1987a, Noga et al 1988) and in rats (Kinjo et al 1990). Electrical stimulation of the MRF also elicited locomotion in cats (Garcia-Rill and Skinner 1987a, Noga et al 1988, Mori et al 1978a, 1980), rats (Kinjo et al 1990) and other species (Livingston 1986, Ross and Sinnamon 1984, Steeves et al 1987). It was demonstrated that stimulation of the MRF disturbed MLR-induced step cycles and established its own (Garcia-Rill and Skinner 1987a, Drew and Rossinol 1984).

The direct evidence implying involvement of MRF cells in the mediation of MLR-evoked locomotion includes the observation that cooling restricted areas in the MRF to block synaptic transmission can reversibly abolish both spontaneous and MLR-evoked locomotion (Shefchyk et al 1984). In addition, injection of cholinergic antagonists and GABA into the MRF blocked locomotion induced by stimulation of the MLR (Garcia-Rill and Skinner 1987a).

All the evidence shown above suggested that cells in the FTG and FTM are involved in locomotor activity and are the relay sites for MLR-evoked locomotion.

A small restricted area in the medioventral medulla corresponding to the ventral part of the FTG and FTM has been studied intensively by Garcia-Rill and Skinner. In the cat, this is an area about 3 mm long and 1 mm wide, located between L1.0-L2.0 mediolaterally and P6.0-P9.0 rostrocaudally (approximately from anterior third of the inferior olive extending to posterior third of the trapezoid body). Stimulation of the area (termed MED, the medioventral medulla) could electrically and chemically elicit controlled locomotion in cat (Garcia-Rill and Skinner 1987a) and rat (Kinjo 1990). Stimulation of the MED was also capable of resetting the locomotor rhythm (Garcia-Rill and Skinner 1987a). Electrophysiological data showed that a descending MLR projection influences a large number of MED cells, some of which have direct spinal projections (Garcia-Rill and Skinner 1987b). The projection from the MLR to the MED has also been

confirmed anatomically (Skinner et al 1990). All the evidence suggests that the MED is a relay site for MLR-induced locomotion. However the possibility of relay sites in other area than the MED of the FTG and FTM can not be ruled out.

Pontomedulla locomotor strip

While the evidence summarized above supports that the MLR mediates its effects via the MRF, it has been claimed (Mori et al 1977, 1978b, Shik and Yagodnitsyn 1978) that the pontomedullary locomotor strip (PLS) was responsible for mediation of locomotion produced by stimulation of the MLR.

The PLS is a very small, discrete area (0.5-1.0 mm in diameter) (Mori et al 1978a, 1978b, Shik and Yagodnitsyn 1978) and extends throughout the lateral tegmentum of the pons and medulla just medial and ventral to the spinal nucleus of the fifth nerve (Mori et al 1977, 1978a, Shik and Yagodnitsyn 1977, 1978). However, in some experiments the PLS may be dorsolateral or lateral to the spinal nucleus (Shik and Yagodnitsyn 1977). It continues in the spinal cord in the dorsolateral funiculus (Kazennikov et al 1980, 1983a, 1983b). Based on electrophysiological recordings, it has been suggested that the lateral tegmental locomotor region is a polysynaptic pathway with the cells of origin located either within the PLS (Shik and Yagodnitsyn 1978, Selionov and Shik 1984) or located medial and ventral to the PLS in the adjacent lateral reticular formation (Selionov and Shik 1984). It is assumed that when repetitive stimulation achieves a threshold for locomotion the polysynaptic propagation occurs without decrement, and, as a result, spinal stepping generators are activated (Selionov and Shik 1984). The evidence supporting the claim that the MLR projects via the PLS comes primarily from electrophysiological data. Stimulation of rostral part of the PLS evoked field potentials in the MLR (Mori et al 1977). Subthreshold MLR stimulation together

with subthreshold PLS stimulation generated locomotion (Mori et al 1977). Stimulation of the PLS facilitated MLR-evoked locomotion (Mori et al 1978a). The best evidence came from the results presented by Mori et al (1977). They mapped the brainstem areas caudal to the MLR that could produce locomotion when electrically stimulated and concluded that the MLR was continuous with the PLS.

However, this evidence is not strong enough to make the conclusion that the PLS is the mediator of the MLR-induced locomotion. The reasons are: 1) conclusions made from the electrophysiological data were premature. For instance, although subthreshold stimulation of the MLR and the PLS will generate locomotion when delivered together, it is not necessarily true that this facilitation is due to the projection of the MLR via the lateral tegmentum; 2) the evidence was primary based on electrophysiological data. No anatomical data was provided; 3) the conclusion is in contrast with other solid evidence. For instance, synaptic responses in motoneurons produced by stimulation of the MLR occur at such a short latency that a chain of neurons at the PLS cannot be involved (Shefchyk and Jordan 1985). Cooling of the PLS does not affect the locomotion produced by stimulation of the classical MLR (Shefchyk et al 1984).

The PLS is closely related anatomically to the spinal nucleus of the trigeminal nerve since they have similar anatomical distributions and are located adjacent with each other. Noga et al (1988) recently demonstrated that they were closely related physiologically as well. It was found that not only the PLS but also the spinal trigeminal nucleus were capable of producing locomotion with the appropriated chemical stimulus. It was also observed that injection of picrotoxin into the PLS region allowed previously ineffective trigeminal field stimulation to initiate treadmill locomotion. Therefore it was suggested the PLS is closely related to or synonymous with the spinal nucleus of the trigeminal nerve and hypothesized

the PLS region contains a brain-stem system that provides a substrate for sensorimotor reflex initiation of locomotion.

Anatomical structures under the physiologically identified MLR

1. Cuneiform Nucleus (CNF)

It was first observed by Shik et al (Shik et al 1966, 1967) that repetitive stimulation of a discrete area below the inferior colliculus could induce coordinated locomotion on a treadmill in acute precollicular-postmammillary cats. The discrete area was termed the mesencephalic locomotor region (MLR). The centre of the most effective site for producing locomotion was located in an area within 1 mm radius of the stereotaxic coordinates P2, L4, H-1. The MLR was suggested to correspond to cuneiform nucleus (Shik et al 1967). The findings have now been confirmed in a number of subsequent studies (Grillner 1981, Grillner and Shik 1973, Mori et al 1977, 1978a, Steeves et al 1975, 1980).

The cuneiform nucleus is a small nucleus located in an anatomically complex area. The nucleus of inferior colliculus, the periaqueductal gray, the marginal nucleus of the brachium conjunctivum, the pedunculopontine nucleus, and the locus ceruleus are the closest neighbours. It was observed that locomotion could be induced in the area surrounding the CNF when stimulated (Steeves et al 1975, Skinner and Garcia-Rill 1984, Garcia-Rill et al 1983a). The possibility that structures other than the CNF are also responsible for initiation of locomotion exists.

2. Pedunculopontine Nucleus (PPN)

The pedunculopontine nucleus was strongly proposed to be included in the

MLR (Garcia-Rill et al 1987, Skinner and Garcia-Rill 1984). The suggestion was based on the following evidence: i) the lowest threshold locomotion inducing sites were always located in the PPN besides the CNF (Garcia-Rill et al 1985, 1987, 1990, Skinner and Garcia-Rill 1984, Coles et al 1989); ii) injection of neuroactive chemicals into the lowest threshold locomotion-inducing sites identified electrically in the PPN could evoke locomotion episodes (Garcia-Rill et al 1985, Garcia-Rill and Skinner 1987b); and iii) single unit recording has shown that neurons in the PPN are rhythmically active during spontaneous locomotion and the phasic activity is coupled with the onset or termination of cycling episodes (Garcia-Rill and Skinner 1988). In addition, neurons in the PPN were confirmed anatomically to project to the MED in the rat (Skinner et al 1990). The ascending and descending projections of the MLR match those of PPN (Garcia-Rill 1986, Garcia-Rill et al 1981, 1983c, 1983d, 1986).

Cytoarchitectonic boundaries of PPN have been difficult to determine until the recent discovery that some of the cells in the PPN contain choline acetyltransferase, which can be localized by immunohistochemical techniques (Satoh et al 1983, Kimura et al 1981, Houser et al 1983). At the level of the inferior colliculus, the location of the pars compacta of the PPN was established at the lateroventral edge (Newman 1985) of the brachium conjunctivum, with the pars dissipatus of the nucleus assuming a more ventral position at anterior levels (Newman 1985).

3. Marginal Nucleus of the Brachium Conjunctivum (BCM)

It has been shown that locomotion inducing sites were located in the dorsolateral part (above BC) of the BCM (Bayev et al 1988, Garcia-Rill et al 1983c, 1986, Steeves et al 1975) and ventral part of BCM (Steeves et al 1975). However, there is some overlap in the lateral BCM with the posterior PPN.

4. Nucleus of Locus Ceruleus (LC)

Some cells in the LC contain the catecholamine norepinephrine. It has been demonstrated that catecholamine-containing cells are close to the optimal electrode tip position which induce locomotion when stimulated (Steeves et al 1975). The close proximity of these CA cells to the optimal position suggested that these cells were activated during the stimulation that initiated coordinated locomotion.

Nuclei such as the periaqueductal gray and the mesencephalic trigeminal nucleus (5ME) were also claimed to evoke locomotion when electrically activated (Garcia-Rill et al 1983c, 1983d).

Application of the c-fos immunohistochemical method in the brainstem might provide a complete map showing the distribution of cells activated during locomotion. The results may

a) confirm the conclusions made in the previous studies. The descending pathways of the MLR induced locomotion have been studied for 20 years. Various evidence has indicated that the MRF but PLS is the relay site for MLR induced locomotion. The data obtained in present study would provide further information to confirm this conclusion.

b) help to solve uncleared questions. As discussed earlier, the MLR is a physiologically defined area, which corresponds to the cuneiform nucleus suggested in many studies. However, the cuneiform nucleus is a small nucleus located in an anatomically complex area. The surrounding nuclei might also be involved in initiating locomotion in the MLR induced locomotion. The pedunculopontine nucleus was strongly proposed to be a effective structure in the MLR. With the advantages of the c-fos technique, which are high resolution and potentially label all active cells, examining the distribution of labelled cells in the area where the

cuneiform nucleus and its surrounding nuclei located in the locomotion animals would provide the information towards the question.

METHODS

The data presented in this study was obtained from 13 cats weighing 1.9 to 2.8 kg. Four different kinds of preparations were used. They were: MLR stimulation induced treadmill locomotion (n=2), MLR stimulation induced fictive locomotion (n=2), spontaneous fictive locomotion (n=1), and forelimb swinging induced fictive locomotion (n=1). Control animals (n=6) were also set up. An additional cat was used as positive control (n=1). The animals were subjected to intubation, hindlimb nerve dissections (fictive preparations), decerebration, and locomotion (locomotion experiments). All animals were perfused at the end of experiments. The detailed procedures are described below.

Surgery Preparations and Production of Locomotion

Intubation:

Animals were initially anaesthetized with a mixture of nitrous oxide and halothane through a face mask. The trachea was intubated for direct administration of the anaesthetic and later for connection with a respiratory pump after muscle paralysis. The left common carotid artery was cannulated and connected to a pressure transducer for blood pressure monitoring. The right common carotid artery was dissected free from the surrounding tissue and a ligature was looped around it in order to permit temporary occlusion of the artery during decerebration. The right jugular vein was cannulated for all drugs and fluid administration. A 5% glucose-sodium bicarbonate buffer solution was infused to replace fluid loss and help maintain a normal PH balance in the animals through the experiments. Each animal was given 2 mg dexamethasone (Hexadrol phosphate, Organon) intravenously to reduce tissue swelling. Animal body temperature was monitored

via an esophageal temperature probe and maintained at 36-38 °C by a heating pad underneath the surgery table and a heating lamp over the animal during the surgery.

Nerve Dissection:

Branches of the sciatic nerve in the hindlimb were dissected bilaterally in order to monitor locomotion in the paralysed cats. The nerve branches include: anterior biceps, semitendinosus, lateral gastrocnemius, and tibialis anterior. The femoral nerves were cut bilaterally in order to insure symmetrical tonic afferent input to the spinal cord, which enhances the efficacy of MLR stimulation for production of fictive locomotion. The nerves were mounted on bipolar electrodes submerged in a mineral oil bath contained in custom-made plastic horizontal tray on both sides. In two experiments (MLR stimulation induced fictive locomotion and a fictive control), nerve cuff electrodes were used instead of nerve tray to simplify the procedures. The nerves chosen to monitor locomotion were the medial sartorius from the femoral nerve and gastrocnemius from the sciatic nerve in both hindlimbs. The remaining branches of the sciatic and femoral nerve branches were cut.

Decerebration:

Animals were transferred from the surgical table to a frame. In the treadmill preparations, animals were placed in a frame over a treadmill with all four limbs free to step on treadmill belt and supported by a sling under the abdomen. In the fictive preparations, animals were placed in a frame with legs pendant and supported by two vertebral clamps and pins attached to the iliac crests. The head of each animal was fixed in a stereotaxic headholder. A precollicular-

postmammillary decerebration was performed in most of cats. In the spontaneous fictive locomotion experiment and in the forelimb swinging induced fictive locomotion experiment, cats were decerebrated at the precollicular-premammillary level in order to obtain spontaneous locomotion. The anaesthesia was subsequently terminated. Up to 5 ml of oxypherol (Alpha Therapeutic Corporation), an agent capable of carrying oxygen, was given to compensate for blood lost during decerebration. Dextran 70 (Travenol) was injected after the decerebration if blood pressure did not return to at least 80 mmHg.

Maintenance:

For fictive experiments: After a 1 hour recovery period following the decerebration, animals were paralyzed with 1ml (8mg/ml) Flaxedil (Gallamine triethiodide, Rhone-Poulenc) given intravenously. Periodic injections of Flaxedil were used throughout the experiment to keep the animals in a state of flaccid paralysis. Artificial ventilation was adjusted to maintain end tidal CO_2 between 2.5% and 5%.

Body and nerve pool temperature were monitored via probes and maintained at 36-38°C by two feedback controlled heating lamps. Dextran was given if needed to maintain blood pressure at or above 80 mmHg.

Locomotion Induction:

In the MLR stimulation induced locomotion experiments, locomotion was induced by electrical stimulation (0.5-1.0 ms pulses, 15-20 Hz, 50-160 μA) of the mesencephalic locomotor region as previously described by Jordan et al (1979). In the experiment in which fictive locomotion was induced by forelimb swinging, the forelimbs were manually moved through the range of normal locomotor movement

in order to induce and maintain locomotion.

Locomotion was monitored by visual confirmation of weight support and walking on the belt of the treadmill (treadmill speed 0.46 m/s) or by electroneurograms (ENGs) for the fictive locomotion experiments. The ENGs were amplified, band-pass filtered (30-3000 Hz), rectified and integrated, then digitized on a Masscomp computer for analysis.

Because c-fos expression is activated by various stimuli (Morgan and Curran 1989), the surgery itself may induce c-fos expression in the brainstem or in the spinal cord. To eliminate fos expression induced by surgery from the analysis, a constant time interval between decerebration (final surgery) and perfusion, 8-10 hours, was established for all locomotion and control experiments. Within the time length, locomotion was tried to be obtained as much as possible.

Control Experiments:

Six cats were used in control experiments: 2 for treadmill experiments and 4 for fictive experiments. All control animals were subjected to the same amount of surgery as the locomotion test animals, and they were all decerebrated at the precollicular-postmammillary level. Those animals were not subjected to the locomotor task, however.

Two cats were neither stimulated nor had stimulating electrodes inserted. The remainder of the control animals were electrically stimulated for a short time in the early stage of the experiment to confirm that they were capable of MLR-evoked locomotion.

Positive Control Experiment:

It is important to test the quality of the c-fos antibody we were using on a

known pathway before applying it to localize interneurons involved in locomotion. The pathways involved in pain perception have been studied recently using the c-fos immunohistochemical method (Hunt et al 1987, Williams et al 1990, Bullitt 1989). Examining the pattern of Fos expression in the spinal cord after injection of a pain-producing substance would give us information on the quality of the antibody.

The cat was anaesthetized, cannulated, intubated, and decerebrated as described earlier. 5% paraformaldehyde was injected in 10 locations (each 100 μl) of the right hind leg and paw. The animal was deeply anaesthetized and perfused 3 hours after paraformaldehyde injection.

Tissue Perfusion and Procession

Perfusion:

Solutions used for perfusion were prepared the day before the experiment. They include the pre-fixative, fixative and sucrose solutions. Concentrations and ingredients in each solution are described below:

Pre-fixative solution (10% saline with $NaNO_2$ and heparin): Solution of 100 ml, 90 ml dH_2O, 10 ml 9% saline, 0.1 g $NaNO_2$, and 10 μl heparin. It was used at 0.3 ml/g of animal weight. The solution was kept at 4 °C.

Fixative solution (4% Paraformaldehyde - 0.2% picric acid): Solution of 400 ml, 16 g paraformaldehyde, 80 ml 0.5 M phosphate buffer (PH 7.4), 60 ml saturated picric acid, and dH_2O. It was used at 1 ml/g of animal weight. The solution was kept at 4 °C.

Sucrose solution (25% sucrose): Solution of 500 ml, 125 g sucrose, 50 ml glycerol, 50 ml 0.5 M phosphate buffer (PH 7.4), and dH_2O. It was kept at 4 °C.

The animal was deeply anaesthetized with sodium pentobarbital (30 mg/kg) before perfusion and quickly transferred from the frame to a tray. The thoracic

cavity was opened. A needle connected with the perfusion apparatus was inserted into the left ventricle and secured with a haemostat. The inferior vena cava was cut to allow blood and perfusion fluid out. The perfusion apparatus was then turned on carrying pre-fixative solution, followed by fixative solution. Perfusion pressure was kept at 80 mmHg.

A laminectomy was performed after the perfusion. Spinal segments (lumbar 3 to sacral 1) and the brainstem were removed and put into jars containing fixative solution for 5 hours. The tissues were then transferred to sucrose solution and stored there for at least 4 days before sectioning.

Tissue Sectioning:

A sliding microtome was used for tissue cutting. The spinal cord segments were sectioned coronally and the brainstem was sectioned sagittally. Tissue sections of 20 μm thickness were collected in 0.1 phosphate buffer solution (PBS). There were 20 sections collected from each spinal segment and 60 sections from entire brainstem. These sections were collected in even distribution from the rostral to the caudal end of each spinal segment or from the right to the left side of brainstem.

Tissue Processing:

Solutions and antibodies used:

Solution A: 0.1 M PBS - 0.3% TritonX (t-octylphenoxypoly-ethoxyethanol)

The purpose of this solution was to dissolve part of cell membrane so that the antibody could penetrate the cell membrane to bind with c-fos proteins.

Solution B: 0.2 M PBS - 0.3% TritonX - 2% BSA (Albumin, Bovine)

This solution was used as a base for incubations of primary, secondary and third antibodies.

Solution C: 0.2 M PBS - 0.3% TritonX. This solution was for a rinse, in which non-specific binding would be washed out.

Solution D: 50 mM Tris Buffer (PH 7.4). This solution was used as a base for staining.

Antibody 1: c-fos antibody (Cambridge research biochemicals inc.)

Antibody 2: Anti sheep/goat Ig, biotinylated antibody (Amersham international plc)

Antibody 3: ABC kit (Vector laboratories inc.)

Procedures:

a) Day 1: -Tissue sections were put in Solution A overnight.

b) Day 2: -Primary antibody incubation. Concentration of antibody was 1:2000=antibody 1:Solution B. Tissue sections were kept on a shaker in the cold room (4°C) for 3 days.

c) Day 5: -The tissue was washed with Solution C twice, 30 minutes each time on a shaker at room temperature.

-Secondary antibody incubation. Concentration was 1:100=antibody 2:Solution B. Incubation lasted 1.5 hours on a shaker at room temperature.

-The tissue was washed with Solution C twice, 30 minutes each time on a shaker.

-Third antibody incubation. Concentration was 1:1:100=antibody 3(Part A:B):Solution B. Incubation lasted 1.5 hours on a shaker at room temperature.

-The tissue was washed with Solution C, 30 minutes on a shaker.

-The tissue was washed with Solution D, 30 minutes on a shaker.

-Staining with DAB (3,3-diaminobenyidine tetrahydrochloride):

The tissue was immersed into the solution containing:

-100 ml Solution D

-20 mg DAB

-15 ml H_2O_2

H_2O_2 was mixed with other two ingredients just before the tissue sections were immersed. Staining was closely monitored under the microscope. When the staining was done, the tissue sections were transferred to solution D to stop further staining.

-Tissue sections were mounted on slides and dried completely (overnight).

d) Day 6: -Dehydration. Tissue slides were bathed in the following solutions:

70% alcohol 2 mins

80% alcohol 2 mins

95% alcohol 2 mins

100% alcohol 1 min

100% alcohol 1 min

xylene 3 mins

xylene 3 mins

-Cover slip with lipshaw.

RESULTS

Distribution of Labelled Cells in the Spinal Cord

Slides were examined under the light microscope, and the spinal cord and gray matter outlines, as well as locations of labelled cells, were drawn using a camera lucida. Only those neurons which obviously contained reaction product in the nucleus were considered to be labelled. Figure 1 shows examples of photomicrographs taken from the spinal cords of control (1B) and test (locomotor task) (1A) animals, and illustrates that the locomotor task produces clear nuclear labelling which is not evident in the control spinal cord. Figure 2 is a high power view showing the appearance of labelling, and intensity difference between labelled and unlabelled cells.

Positive Control Experiment:

An experiment was conducted in order to examine the pattern of c-fos expression evoked by a noxious stimulus. This was done to assure that the c-fos expression detected with the antibody which we were using was distributed in the same cell types and areas of the spinal cord as had been observed due to a noxious stimulus in other studies (Hunt et al 1987, Williams et al 1990). The locations of labelled neurons in the L6 segment from this experiment is illustrated in the camera lucida drawing shown in Figure 3. This distribution of labelling is essentially identical to that described in earlier studies using an antibody to c-fos as a marker for cells activated by a formalin injection into the paw. This data was taken as evidence that our c-fos antibody and immunohistochemical procedure was capable of detecting active neurons, and that our experiments would provide information which could be reliably compared to previously published data using the c-fos

immunohistochemical approach.

Treadmill Locomotion Experiments:

Treadmill locomotion was induced in 2 animals, and it consisted of vigorous locomotor movements of both fore- and hindlimbs and good weight support. Locomotion was repeatedly induced for periods of approximately 3 to 30 minutes duration, separated by periods in which no stimulus was applied. For treadmill locomotion cat 2 (TL-Cat2), locomotion occurred for 5 hours and 22 minutes within a 9 hour time period. For treadmill cat 1 (TL-Cat1), a period of 8 hours was allowed to elapse after the decerebration, and locomotion was induced and maintained throughout the last 2 hours before perfusion. The procedure used for TL-Cat2 may have been more effective for the production of c-fos labelling, because a total of 2737 cells were labelled in the spinal cord of this animal, while only 969 were labelled in TL-Cat1.

The distribution of labelled cells from L3 to S1 segments of TL-Cat2 is illustrated in Figure 4. A bimodal distribution of labelled cells was found among Rexed's laminae, such that labelled cells in the dorsal horn were concentrated mostly in laminae III and IV. A more ventrally located group of labelled cells was concentrated in lamina VII and VIII, with some extending into laminae VI. Labelled cells were also found in the part of lamina X which is adjacent to lamina VII. Bar graphs showing the laminar distribution of labelled cells are presented in Figure 11b.

Figures 5 and 11a illustrate the distribution of labelled cells in the spinal cord of TL-Cat1. In addition to having a smaller total number of labelled cells, fewer labelled cells were observed in lamina VII and VIII in the TL-Cat1 than in the TL-Cat2. We speculate that the difference between the two treadmill animals

was most likely due to the fact that insufficient time was allowed for the full expression of c-fos in the ventral horn neurons in TL-Cat1. Because of this, the protocol used for TL-Cat2 was adopted for subsequent experiments.

In terms of segmental distribution, the number of labelled cells started to increase at L4 or L5, reaching its peak at L6 or L7 (Figure 12a,b). Labelled motoneurons were not counted, therefore the increased number of labelled cells at L6 or L7 was not due to the appearance of labelled motoneurons.

Fictive Locomotion Experiments:

1. MLR Stimulation Induced Locomotion:

Bouts of fictive locomotion were evoked by MLR stimulation. Good locomotion was recorded in both fictive locomotion cats 1 (FL-Cat1) and 2 (FL-Cat2). Total time in which fictive locomotion was recorded was 2 hours 37 minutes in FL-Cat1 and 3.5 hours in FL-Cat2. The locations of labelled cells were similar in the two cats, as illustrated in the camera lucida drawings in Figures 6 and 7.

Most of the cells were concentrated in lamina VII and VIII (Figures 6, 7; Figure 11c,d). Interestingly, the labelling in dorsal horn cells which occurred in the treadmill locomotion animals (Figures 4, 5; Figure 11a,b) was not present in fictive locomotion animals. Only a few labelled cells were found in dorsal horn laminae after the fictive locomotion task. It was observed that the number of labelled motoneurons was much higher in FL-Cat1 than in FL-Cat2.

In terms of segmental distribution, the number of labelled cells (excluding motoneurons) in each segment started to increase at L4, and reached its peak at L6 (Figure 12c,d).

2. Spontaneous Fictive Locomotion Experiment:

Spontaneous bouts of fictive locomotion appeared at 7 hours before perfusion in this animal, and good regular locomotion occurred which was maintained for the last 3 hours prior to the perfusion.

Most of labelled cells in this spontaneous fictive locomotion cat (SFL-Cat) were located in laminae VII and VIII, and the laminar distribution of labelled cells is shown in Figure 8 and Figure 11e. As was the case for the data obtained from the MLR-induced fictive locomotion animals, only a few labelled cells were found in the dorsal horn laminae. The segmental distribution of the labelled cells (excluding motoneurons) is illustrated in Figure 12e, which shows that the number of labelled cells started to increase at the L4 segment, reaching a maximum at L7.

3. Forelimb Swinging Induced Fictive Locomotion

Locomotion was not initiated spontaneously in this animal. With swinging of the forelimbs, however, bouts of fictive locomotion were observed in the hindlimb ENG recordings. Constant forelimb swinging was necessary in order to maintain fictive locomotion. Locomotion was maintained for a total of 3 hours during an 6 hour period.

The locations of labelled cells in spinal segments L3-S1 in this forelimb swinging induced locomotion cat (FSFL-Cat) are shown in camera lucida drawings in the Figure 9 and Figure 11f. Most labelled cells were concentrated in laminae VII (especially in its medial aspect) and in lamina VIII. Some were located in laminae I and II of the dorsal horn. The segmental distribution of labelled cells is shown in Figure 12f. The largest number of labelled cells (excluding motoneurons) occurred in the L6 segment.

Control Experiments:

1. Treadmill Control Experiments:

Animals were subjected to identical surgical procedures as the treadmill locomotion animals, placed in the stereotaxic apparatus and suspended above the treadmill, but locomotion was not induced. Treadmill control cat 1 (TC-Cat1) was stimulated intermittently in the region of the MLR for approximately one hour. No consistent locomotion was obtained, and the animal was then left unstimulated for a period of 7 hours. Treadmill control cat 2 (TC-Cat2) was not subjected to any electrical stimulation. The total number of labelled cells in each cat is shown in Figure 13. It is obvious that the number of labelled cells in each of the treadmill control animals was extremely small compared to the treadmill locomotor animals.

2. Fictive Control Experiments:

Four animals were used as controls for fictive locomotion. They were subjected to the same surgical procedures as the fictive locomotion animals, including the nerve dissection, and they were paralysed with Flaxedil and artificially ventilated following the decerebration. Only fictive control cat 4 (FC-Cat4) was not electrically stimulated. The others (fictive control cats 1, 2, 3) were simulated for approximately 1 hour without good locomotion, and then left unstimulated for 5 to 6 hours. As can be seen in Figure 13, the numbers of labelled cells in the control animals were much lower than in the fictive locomotor animals.

Figure 10 illustrates the locations of labelled cells in a representative control animal (FC-Cat4). The total number of labelled cells in this animal was the third largest among the 6 control animals (see Figure 13). Labelled cells were scattered in the dorsal horn, with a few in the part of lamina VII closest to the central canal. Labelled cells in the remaining control animals had a similar distributions.

The increased numbers of cells in the fictive control animals as compared to the treadmill control animals is most likely due to the addition of surgery to expose the peripheral nerves, and the additional sensory input necessitated by the placement of the animals in the spinal frame (see Methods).

Fos-labelled Cells in the Brainstem

Sagittal sections of the brainstems from 6 fictive locomotion animals and 5 control animals were examined under the microscope, then the brainstem sections were enlarged by a factor of ten and projected onto grid paper. Locations of labelled neurons in each slide were copied to the grid paper at the coordinates which matched those on the microscope stage. Only those neurons which obviously contained reaction product in the nucleus were considered to be labelled. The mediolateral position of each section was determined on the basis of anatomical landmarks and was matched to sagittal sections in an atlas of the cat brainstem (Berman 1968). Example of neurons within selected areas of the brainstem which expressed c-fos during the locomotor task are illustrated in Figure 14.

<u>Distributions of cells in locomoting cats</u>

The distribution of labelled cells in the brainstem of FL-Cat2 is shown in Figures 15 through 25. In this animal, fictive locomotion was induced by stimulation of the MLR. The majority of labelled nuclei were found to be labelled in all of the locomoting animals. The labelling in these nuclei is described below.

Ventral Areas:

1) A large number of cells were labelled bilaterally in the ventrolateral medulla (around L4.0-L2.0) in each animal (Figures 16, 17 and 18, sections 3-7). Most cells were located in an area bounded by the facial nucleus rostrally, the

lateral tegmental field dorsally and the lateral reticular nucleus (LR) caudally (Figures 16 and 17, sections 4-5). Only a few cells were found in the LR or the spinal trigeminal nucleus (5S), especially in the more laterally located sections (Figure 16, section 3). At more medial locations, the length of this cell column shortened and was concentrated in the medulla at the ponto-medullary junction (Figures 17 and 18, sections 6-7). The labelled cells in this area overlapped the areas occupied by the nucleus ambiguus, the retrofacial nucleus, and the nucleus retroambigualis located.

2) From about L2.0-L0.8, an area caudal to the trapezoid body but rostral to the inferior olive in the ventral medulla at the ponto-medullary junction was always found to have a labelled cell cluster (Figures 18, 19 and 20, sections 8-12). The number of labelled cells in the more laterally located sections appeared greater than at more medial sites. The cells are located in the same area as the MED, bilaterally.

3) A number of labelled cells were always visible in the area from 0.8L to 0.0L just caudal to the trapezoid body (Figure 21, sections 13-14). Some labelled cells were also appeared in a area just dorsal to the trapezoid body. These midline cells were located in the region of the nucleus raphe magnus and obscurus, bilaterally.

4) The ventral tegmental area of Tsai: The ventral tegmental area of Tsai was only labelled in 2 cats (FL-Cat1 and FSFL-Cat). The nucleus was labelled on the side ipsilateral to the stimulus site in FL-Cat1, and on the right side of the brainstem in FSFL-Cat (Figure 26A). Labelling in this nucleus in the contralateral side in this animal was very difficult to examine, because the area was either too dark (due to inadequate removal of blood cells during the perfusion) to observe any labelling, or the area was not preserved during the tissue processing.

Dorsal Areas:

1) Kolliker-Fuse nucleus (KF): Labelled cells were found bilaterally in the area of KF (Figure 15, sections 1-2).

2) Cuneiform nucleus (CNF): Large numbers of cells were labelled in the CNF ipsilateral to the stimulated side (Figures 15, 16 and 17, sections 1-6). This occurred only in those animals where the CNF was stimulated to produce locomotion. The contralateral CNF was usually not labelled, unless the contralateral side had been previously stimulated in an earlier trial to locate a suitable stimulus site. In such cases, a few labelled cells were found in the contralateral CNF along the electrode track. They were considered to be labelled by direct electrical stimulation. Labelled cells were not found in the CNF in the spontaneous locomotion cat or the forelimb swinging induced locomotion cat.

3) Marginal nucleus of the brachium conjunctivum (BCM): Cells in the BCM were labelled bilaterally in all animals (Figures 15, 16 and 17, sections 1-6). On the stimulated side, the number of cells in the border area under the CNF was greater than on the contralateral side (Figures 16 and 17, sections 3-5).

4) Locus ceruleus (LC): The LC was labelled bilaterally in all locomotion cats (Figures 17, 18, 19 and 20, sections 6-11).

5) Nucleus raphe dorsalis: A number of cells were observed in the area of the nucleus raphe dorsalis bilaterally (Figures 19 and 20, sections 10-11).

6) A strip of cells located bilaterally in the dorsal medulla was labelled in all locomotion cats. Most of the cells were located in the area of the nucleus of the solitary tract and the motor nucleus of the vagus (Figures 19 and 20, sections 9-12). A few labelled cells located in the vestibular nucleus were also observed (Figure 17, sections 5-6).

7) The boundary of the pedunculopontine nucleus cannot be precisely

determined in the tissue sections without ChAT immunostaining (Garcia-Rill et al 1987). A group of labelled cells located bilaterally rostral to the BCM and KF in the more laterally located sections was observed (Figures 15 and 16, sections 1-3), but it is not clear whether these cells are part of the PPN.

8) Cuneate nucleus (CU) and Gracile nucleus (GR): The CU was labelled in TL-Cat2 (Figure 26B) and FSFL-Cat, but not in TL-Cat1. The GR contained labelled cells in only a few sections of TL-Cat2.

9) The periaqueductal gray (PAG): Numerous cells were labelled on the side of the PAG ipsilateral to the stimulus site in FL-Cat1 (Figure 26C).

Distribution of labelled cells in the control animals

The results presented here were obtained from 5 control cats, including 2 treadmill control cats and 3 fictive control cats. The brainstem of the third fictive control cat was not analyzed due to inadequate perfusion.

It was very surprising to find that almost all the nuclei (except the CNF) labelled in the locomoting cats were also labelled in the control cats. However, it seemed that the numbers of labelled cells in the nuclei of the control animals were generally lower than in the locomoting animals. Sections 1' to 22' in Figures 15 through 25 show the distribution of labelled cells in a control cat (FC-Cat4) as a example.

Comparison of the number of labelled cells in the locomotor and the control animals

1) Comparison of the total number of labelled cells: The total number of labelled cells in the brainstem of each cat is shown in Table 1. This data illustrates that the numbers of labelled cells in the MLR-stimulation induced cats (excluding

TL-Cat1, which had a different stimulation protocol from the rest of the animals - see Methods) are higher than the non-MLR-stimulation induced cats, and much higher than the control animals. The very high number of labelled cells in FL-Cat1 is due to the large number of cells labelled in the PAG, which did not occur in the other locomotor cats. The number of labelled cells in the spontaneous locomotor cat is quite low as compared to the other locomotor cats. Delayed tissue processing (3.5 months after the locomotor task) may be the main factor attributing to the low number of labelled cells in this animal. The increased numbers of cells in the fictive control animals as compared to the treadmill control animals might be due to the addition of surgery to expose the peripheral nerves in the fictive preparation, or/and the delayed tissue processing in the treadmill cats (about 7-9 months, during which depletion of the fos protein might occur).

Comparisons of total numbers of labelled cells among treadmill cats and fictive cats are shown separately in bar graphs (Figure 27A and 27B), which illustrate that the total number of labelled cells in each MLR-induced locomotor animal is much higher than the control animals.

2) Comparison of the number of labelled cells in the MED: Figure 28 illustrates that the numbers of labelled cells in the MED in the MLR-induced fictive locomotor cats are higher than in the fictive control cats. Only those cells located in L2.0-L1.0 were taken into account.

3) Comparison of the number of labelled cells in the LCα: Figure 29 shows that the numbers of labelled cells on the side ipsilateral to the stimulus site in the MLR-induced fictive locomotor animals are higher than on the contralateral side, and much higher than in the control animals.

DISCUSSION

Locations of the Spinal Interneurons Involved in Production of Locomotion

Labelled cells in Rexed's laminae I-VI

Most of labelled cells in the dorsal horn (Rexed's laminae I-VI) occurred in the treadmill locomotor cats, and were not observed in the fictive locomotor cats (Figure 11). The only difference between treadmill locomotion and fictive locomotion preparations is the limb movement and repetitive stimulation of mechanoreceptors with repeated limb contact. The possibility that the difference between the two locomoting preparations could be due to the difference in the surgery for the two preparations can be ruled out on the basis of comparisons between treadmill control and fictive control animals. The distribution of labelled cells in the dorsal horn was similar for the two preparations. Therefore, it can be concluded that the labelled cells observed in the dorsal horn (Rexed's laminae I-VI) in the treadmill locomotor cats were labelled due to their activation by afferent feedback from the moving limbs.

The cells which are labelled with c-fos in the dorsal horn may belong to certain of the ascending pathways. Studies have shown that only 22% of the primary afferent axons entering the lumbosacral enlargement reach the nucleus gracilis in the medulla to transfer information on the touch-pressure and limb position; the remainder terminate within the spinal cord (Glees and Soler 1951, Horch et al 1976). Sensory information other than touch-pressure and limb position is relayed in the spinal cord, and then transferred to the brain. It is known that many ascending sensory pathways originate from neurons located in the dorsal horn. Neurons of the spinothalamic tract and the spinomesencephalic tract are partially located in the lamina I (see Willis 1986). These cells receive strong nociceptive

peripheral inputs and are considered to be involved in processing pain information. Neurons of the postsynaptic dorsal column and spinocervical tract are located in laminae III and IV of the dorsal horn (Bennett et al 1983, Brown 1981). These neurons either receive inputs from cutaneous mechanoreceptors or are activated by nociceptors (Cervero 1986, Brown 1981). The neurons which ascend in the spinocervical tract receive mechanoreceptive inputs which are predominantly from the various types of hair follicle receptors (Brown 1971). Spinomesencephalic and spinothalamic neurons involved in the pain process are also located in lamina V (Willis et al 1979). A distinct population of dorsocerebellar cells which have muscle group II input, situated caudal to the Clark's column, is located in laminae IV and V in the caudal half of the L4 segment (Edgley and Jankowska 1988, Edgley and Gallimore 1988).

In addition to the ascending neurons, interneurons are also present in the dorsal horn. It was found that interneurons which have input from both Ia and Ib afferents are located in laminae V and VI (Hongo et al 1966, Jankowska et al 1981).

The neurons which are involved in ascending sensory information and reflex function, especially those located in laminae III-VI described above, would be activated during the treadmill walking. The large number of labelled cells which were found in laminae III and IV might have been activated by skin mechanoreceptors, including hair follicle receptors. Cells in laminae V and VI might be labelled partially due to inputs from muscle afferents of the moving limbs, and are related to reflex function.

The numbers of the cells labelled in laminae I and II (related to the pain process) were low in both locomotion and control preparations, although the hindlimb nerves were fully dissected in the fictive preparation. A period of 8-10

hours allowed those c-fos proteins expressed during surgery to be metabolized and subsequently removed. A recent study showed that Fos has a half-life of approximately 2 hours (Curran and Morgan 1985).

Previous studies suggested that the cells in lamina VI (Viala et al 1988) or in the lateral part of lamina VI (Fortier et al, in preparation) might be involved in the generation of locomotion. In this study, only a few labelled cells were observed in laminae VI. In some segments more labelled cells occurred in the lateral part of lamina VI (Figure 6, L4-L5), but this was not a consistent finding.

Labelled cells in lamina VII

The same distribution of labelled cells which was observed in lamina VII in the treadmill locomotor animals was also found in the fictive locomotor animals. Neurons in lamina VII were prominently labelled in all locomotor cats. In the control animals, however, labelled cells were mostly scattered in the dorsal horn, with only a few in lamina VII. The results indicate that lamina VII contains large numbers on interneurons involved in the production of locomotion.

Lamina VII is well known to contain interneurons which mediate reflex effects on the motor nuclei (Rexed 1954). The Ia reciprocal interneurons and the Renshaw cells are located in the ventral part of lamina VII adjacent to the motor nuclei (Jankowska and Lindstrom 1972, 1971). Recently, a population of interneurons located in lamina VII of midlumbar segments has been identified. These neurons receive inputs from group II muscle afferents and from other muscle, skin and joint afferents, and project to motor nuclei (Edgley and Jankowska 1987a, 1987b, Edgley et al 1988). All three types of interneurons have been shown to be rhythmically active during MLR-induced locomotion (McCrea et al 1980, Pratt and Jordan 1987, Noga et al 1987, Shefchyk et al 1990). The neurons displayed phasic

activity during the step cycle which was tightly coupled to the extensor or flexor neurogram. Using the WGA-HRP retrograde transneuronal labelling technique, the last order interneurons participating in the production of locomotion were primarily localized in lamina VII of L5 - L7 spinal segments (Noga et al 1987). This evidence supports the results obtained in this study.

With the c-fos technique, it is impossible to know that which cells labelled are due to activation by directly descending inputs from the brainstem or by interactions with other activated interneurons. However, it is observed that some of lamina VII cells overlap those areas in which Ia inhibitory interneurons and Renshaw cells have been localized (see Figures 4, 6, and 8) (Jankowska and Lindstrom 1972, 1971).

It is noted that labelled cells in the medial portion of lamina VII close to lamina X seemed to be more numerous than in the lateral portion. Cells in this area have not been systematically investigated to determine their role in locomotion or reflex activity. Several ascending tracts (spinothalamic, spinomesencephalic tracts) originate, in part, from this area. However, the labelled cells located in the medial portion of lamina VII appeared in the fictive locomotor cats as well as the treadmill cats, and only a few appeared in the fictive control cats. Thus, if they are ascending tract cells, they must belong to a class which receives input from the central pattern generator for locomotion. The medially-located lamina VII cells were observed in large numbers in the non-MLR-stimulated locomotor cats as well. This consistent labelling during all types of locomotion investigated in this study implicates these cells as important contributors to the process of locomotion. It also can be used to rule out the suggestion that they are merely activated by the electrical stimulation in the brainstem. Recently, extracellular recordings showed that the neurons in the medial portion of lamina VII were rhythmically active

during MLR-induced locomotion (Jordan and Noga 1991). It is noted that labelled cells in lamina VII in FSFL-Cat (Figure 9) were concentrated in the medial portion, with much less labelling in the lateral portion. Further studies on the medial located lamina VII neurons are necessary and important.

Labelled cells in lamina VIII

Cells in lamina VIII were labelled in both treadmill and fictive locomotor cats. The consistent labelling in all locomotor animals but not in the control animals suggest that these cells are involved in locomotion.

Lamina VIII is known to contain neurons with axons which cross to the other side of the spinal cord. Some lamina VIII cells were reported to terminate on contralateral motoneurons (Scheibel & Scheibel 1969). A significant proportion of commissural interneurons of lamina VIII, secondarily labelled following WGA-HRP injection into a muscle nerve, have been shown to project to contralateral motor nuclei (Harrison et al 1986). These lamina VIII neurons might be involved in crossed extension reflexes (Grillner and Hongo 1972) or might mediate excitation or inhibition of contralateral motoneurons evoked by the vestibulo- and reticulospinal tracts (Sasaki et al 1962, Hongo et al 1975). Lamina VIII neurons were also recently shown to be involved in locomotion. Noga et al (1987) found that the contralaterally located last order interneurons stained during fictive locomotion were restricted to lamina VIII after injection of WGA-HRP into the anterior biceps nerve. In the midlumbar segment (L4/L5), cells in lamina VIII have been suggested to have the same type of role in locomotion as ipsilateral neurons in lamina VII of the same segment which are activated by group II afferents (Jankowska and Noga 1990). These authors also demonstrated that lamina VIII cells projecting to contralateral motor nuclei are synaptically activated

from the cuneiform nucleus. The results obtained in this study confirm and extend the previous finding that lamina VIII cells are involved in locomotion. Only segments L5-L7 were investigated in the WGA-HRP study (Noga et al 1987). In the present study, labelled cells in the lamina VIII were found in all segments from L3 to S1, but with smaller numbers in the L3 and S1 segments (Figures 4, 6-9).

Labelled cells in lamina IX

Alpha-motoneurons are located in Rexed's lamina IX. They have very large cell bodies which allow them to be distinguished easily from interneurons even in the tissue sections with c-fos immunostaining. It was observed that some of the motoneurons were labelled (Figures 1, 6). However, the numbers of motoneurons labelled varied considerably. For instance, although locomotion in treadmill locomotion cat 2 was well developed, only a few motoneurons were labelled, while a large number of labelled interneurons were observed in the same animal (Figure 4).

Previous studies have found that neurons in certain regions such as the dorsal root ganglia (Hunt et al 1987), the substantia nigra (Dragunow and Faull 1989), despite being activated, do not show Fos elevation no matter what the stimulus. It was suggested that these neurons might lack the required biochemical messengers regulating Fos activation (Dragunow and Faull 1989). However, the reason is not clear at present. Caution should be exercised in interpretation of distribution of Fos immunoreactivity. Negative results (i.e. no Fos induction) should not be automatically taken to mean that the cells have not been activated by stimulation.

Nuclei in the Brainstem Involved in Locomotion

The PLS

Cells within the PLS were not observed to be labelled with c-fos in these experiments. The PLS has been described as a very small discrete area 0.5-1.0 mm in diameter, extending throughout the lateral tegmentum of the pons and medulla just medial and ventral to the spinal nucleus of the fifth nerve (Mori et al 1978a, Shik and Yagodnitsyn 1978). The PLS was shown to contain cells that can produce locomotion with the appropriate chemical stimulus (Noga et al 1988). A column of cells along the route of the PLS might be expected to be labelled with the c-fos immunohistochemical method if the PLS cells were the relay neurons for MLR-evoked locomotion. However, labelling was not observed in this narrow column of cells extending throughout the lateral tegmentum of pons and medulla. These results, although not conclusive, are consistent with the suggestion made in previous studies that the PLS is not the mediator for MLR-induced locomotion (Shefchyk et al 1984, Noga et al 1988). However, as discussed earlier, the possibility that the failure of c-fos to label these cells (like motoneurons) can not be excluded.

There was a large number of cells labelled in the ventral medulla around L4.0-L3.0 (Figures 16, 17, and 18, sections 4-6, and 5'-7'). The cells likely to be related to respiratory and cardiovascular functions. It is known that the rhythmic respiratory drive inputs to spinal respiratory motoneurons arise from premotor neuron populations located within the medulla in the dorsal (DRG) and ventral (VRG) respiratory groups (Feldman 1986). The VRG extends from the spinomedullary junction to levels of the ventrolateral medulla just caudal to the facial nucleus. The three subdivisions of the VRG are associated caudorostrally with the nucleus retroambigualis, the nuclues ambiguus and its immediately surrounding reticular formation, and retrofacial nucleus (Feldman 1986, Merrill

1970, Bieger and Hopkins 1987). It is also known that vagal motoneurons which regulate heart output are located in the nucleus ambiguus. Respiratory and cardiovascular function are maintained in the cats after precollicular-postmammillary or -premammillary decerebration. Therefore, the cells located in the VRG should be labelled with c-fos staining.

It was also noted that the DRG, which corresponds to the nucleus of the solitary tract, was consistently labelled in all cats.

Nuclei labelled in the control cats

Before discussing any significance of labelling in the locomotor animals, it is necessary to discuss the labelling in the control animals. Unexpectedly, we found that almost all the nuclei labelled in the locomotor group were also labelled in the control group. The numbers of labelled cells were, however, less in the control cats than in the locomotor cats; this is especially evident for the MLR-induced locomotion cats (Figure 27).

Several mechanisms may have contributed to labelling in the control cats:

(1) Multifunction of some nuclei

The nuclei which are involved in locomotor activities may also contribute to other functions. For example, nuclei such as the BCM, the KF, and the nucleus raphes magnus and obscurus have been claimed to be related to respiratory function. It was demonstrated that those nuclei send projections to dorsal and ventral respiratory groups (Smith et al 1989, Holtman et al 1987, Bianchi and St.John 1982, Baker and Remmers 1982). Profound perturbations of respiratory pattern were observed with electrical stimulation in the BCM (Bertrand and Hugelin 1971, Feldman and Gantier 1976) and the nucleus raphes obscurus (Holtman et al 1987). In fact, the BCM and the KF has long been recognized as

the pontine respiratory group (see Feldman 1986). If cells in these nuclei are rhythmically active during respiration, they are expected to be labelled with the c-fos staining. Therefore, these nucleus would be labelled in both locomotor and control cats. The prediction that increased numbers of labelled cells in the locomotor cats compared to the control cats were due to locomotor task can not be established. This is because respiration during fictive locomotion would be expected faster and more intensive, which is a result of activation of more cells. It has been demonstrated that stimulation of locomotor area induces a increase in respiration (Eldridge et al 1985).

A hypothesis was proposed in recent papers (Garcia-Rill et al 1987, Garcia-Rill and Skinner 1988) that neurons in the parabrachial region, the pedunculopontine nucleus and the locus ceruleus mediate a host of rhythmic functions. This hypothesis was based on findings that these areas were not only involved in locomotion but also related to respiratory, cardiovascular, masticatory, sleep-related, vesico-motor, oculomotor, as well as more complex behaviour patterns (Garcia-Rill et al 1987, Garcia-Rill and Skinner 1988). The hypothesis is consistent with the labelling in the control cats. On the other hand, labelled cells which appeared in the control animals, especially in the BCM and the KF, provide additional evidence supporting the hypothesis.

(2) Cell labelled due to tonic activation

Mori et al (1978a) have shown that stimulation of the ventral part of the caudal tegmental field, corresponding to the MED, increased extensor tonus of the hindlimbs. It is possible that cells in the MED may mediate some of the effects of decerebrate rigidity.

Transection of the brainstem above the level of the vestibular nucleus but below the red nucleus produces decerebrate rigidity. The pontine reticular

formation and lateral vestibular nucleus are tonically active when disconnected from cerebral control (see Ghez 1991). In decerebrate but cerebellum intact animals, decerebrate rigidity is γ kind rigidity, which is produced by activation of γ-motoneurons due to tonical inputs from the reticulospinal pathways. α-motoneurons are therefore activated through α-γ loop to produce muscle tension. This mechanism in the γ rigidity has been demonstrated that the tonic muscle contraction of the decerebrate limb disappears when the dorsal roots are cut. This interrupts the stretch reflex, preventing spindle endings from providing tonic facilitation to α-motoneurons.

Decerebrate rigidity was observed in each cat after decerebration in the present study. In the fictive preparations, cat muscles were paralysed with Flaxedil, which blocks nerve-muscle junctions. Muscle paralyzation produces an equal effect as the dorsal root section. Tonic activities are ceased in spindle afferents, and therefore in α-motoneurons after muscle paralyzation. It is not known if paralyzation also stops tonic activity in the reticular neurons. However, evidence showed that the reticular neurons producing γ-rigidity can be activated by somatosensory inputs. Somatosensory inputs such as muscle spindle activations in the intercostal muscles still exist. Therefore, it is possible that these reticular neurons still might active even after muscle paralyzation in decerebrate cats.

(3) Cell labelled due to sugary (decerebration)

Similar labelling in the locomotor and control animals could be caused by the surgery for decerebration. However, this possibility seems unlikely slim. First, the locations of the most labelled cells seem reasonable and can be related to certain functions based on previous studies. Second, a time period of 8-10 hours was used to allow the c-fos expression induced by decerebration to be metabolized.

Similar locations of labelled cells exist in the locomotor and the control cats,

making the discussion of their possible involvement in locomotion very difficult. However, several conclusions are drawn as a result of the findings obtained in other studies.

The MRF

Various electrophysiological (Orlovsky 1970a, 1970b, Gracia-Rill and Skinner 1987b, Drew et al 1986, Shimamura et al 1982), anatomical (Steeves and Jordan 1984, Shefchyk et al 1984, Garcia-Rill et al 1986, Skinner et al 1990) and pharmacological evidence (Garcia-Rill and Skinner 1987a, Noga et al 1988, Kinjo et al 1990) suggests that the FTG and the FTM in the MRF are the relay sites for MLR-induced locomotion. The MED, a small area in the MRF, has been studied intensively and shown to be a relay site (Garcia-Rill and Skinner 1987a, 1987b, Steeves and Jordan 1984, Skinner et al 1990, Kinjo et al 1990). In the present study, a cluster of cells was found labelled in the area of the MED in all locomotion cats (Figures 18, 19 and 20, sections 8-12). But it was also observed in the control cats, albeit with a smaller number of cells (Figure 28). As discussed earlier, it is possible that cells in the control animals might be labelled due to activations which produce decerebrate rigidity. Evidence obtained in previous studies supports the present conclusion that the increased numbers of labelled cells in the MED in the locomotion cats result from the locomotion task.

Structures in the MLR

1) CNF: The mesencephalic locomotor region was first suggested to correspond to the cuneiform nucleus (Shik et al 1967). Subsequent studies have confirmed that the cuneiform nucleus is a component of the MLR. Locomotion has been shown to be induced by electrical stimulation (Grillner and Shik 1973, Grillner

1981, Mori et al 1977, 1978a, Steeves et al 1975, Cole et al 1989) and by chemical stimulation (Garcia-Rill et al 1983a, 1985) in the CNF. Cells in the CNF showed rhythmic activity during spontaneous locomotion on a treadmill (Garcia-Rill et al 1983). It was also demonstrated that the CNF was labelled using the 2-deoxyglucose method during MLR-induced fictive locomotion (Kettler and Jordan 1984). In the present study, the CNF was labelled only on the side ipsilateral to the stimulation. Cells in the CNF were not labelled during spontaneous fictive locomotion nor in the forelimb swinging induced fictive locomotion. Lack of activation of cells in the CNF of the non-MLR-stimulated locomotion cats does not deny the role of the CNF on the initiation of locomotion. Structures in the brainstem other than the CNF (such as the MRF) can induce locomotion when stimulated as well. Locomotion might be initiated in these structures in the spontaneous or the forelimb swinging induced locomotor cat.

2) PPN: It was strongly proposed that the PPN was a structure in the MLR, in addition to the CNF, which induced locomotion when stimulated (Skinner and Garcia-Rill 1984, Garcia-Rill et al 1985, 1987, 1990, Garcia-Rill and Skinner 1988). The boundaries of the PPN are difficult to define without immunohistochemical labelling of choline acetyltransferase (ChAT) contained in the PPN cells (Satoh et al 1983, Kimura et al 1981, Houser et al 1983). Unfortunately, the ChAT-staining was not applied to the tissue sections in the present study, and the boundaries of the PPN can not be determined in the c-fos staining. The PPN also overlaps with nuclei such as the BCM. Therefore, it is very difficult to determine whether any of the c-fos labelled cells belong to the PPN.

3) LC: Intravenous infusion of L-DOPA, which is thought to act through the release of noradrenaline from descending fibres, produces stepping movements in acute spinal cats (Grillner 1969, Goodwin et al 1973). It was demonstrated that

catecholamine(CA)-containing cells are in close proximity to sites of stimulation in the MLR which were effective for evoking locomotion. These CA cells were situated in the LC, and it is likely that a portion of these CA cells are activated during stimulation of the MLR (Steeves et al 1975). However, chemical depletion of NA in the spinal cord did not block locomotion initiated by MLR stimulation (Steeves et al 1980). Therefore, the LC cells may not be essential for initiation of locomotion. But may play some other role in locomotion. A hypothesis was proposed recently that LC may be one half of a push-pull oscillatory control system which may function in tandem with adjacent cholinergic PPN cells (Garcia-Rill and Skinner 1988). In the present study, it was found that the number of labelled cells in the LCα (only the labelled cells in the LCα were calculated because LCα has very clear boundaries) on the side ipsilateral to the stimulus site was higher than on the contralateral side in the locomotor animals and much higher than the control animals. This result suggests that the cells in the LCα are involved in locomotion.

4) BCM: Cells were labelled in the BCM in both locomotor and the control animals. Because the BCM is closely related to respiration, it is very difficulty to determine the source of their activation.

5) PAG: Stimulation of the PAG has been shown to evoke locomotion (Garcia-Rill et al 1983c). It was also observed that the MLR neurons projected to the ipsilateral PAG (Steeves and Jordan 1984). However, cells in the PAG were only labelled in one locomotor cat in the present study.

Summary

Cells in the spinal cord and the brainstem activated during locomotion were localized using the c-fos immunohistochemical method.

Labelled cells in the spinal cord:

In cats subjected to a treadmill locomotor task, a bimodal distribution of labelled cells was found among Rexed's laminae, such that labelled cells in the dorsal horn were concentrated mostly in the laminae III and IV. A more ventrally located group of labelled cells was concentrated mostly in laminae VII and VIII, with some extending to laminae VI and X. However, only the ventrally located neurons were labelled in fictive locomotor cats. Only a few labelled cells were found in the control animals. The results suggest that the ventrally located cells (e.g. in laminae VII and VIII) are involved in production of locomotion. The cells labelled in the dorsal horn were probably activated by afferent feedback from the moving limb. In terms of segmental distribution, the number of interneurons labelled starts to increase at L4, reaching a maximum at L6 or L7. The cells labelled in the medial portion of lamina VII were the most numerous, and were labelled in all types of locomoting animals.

Labelled cells in the brainstem:

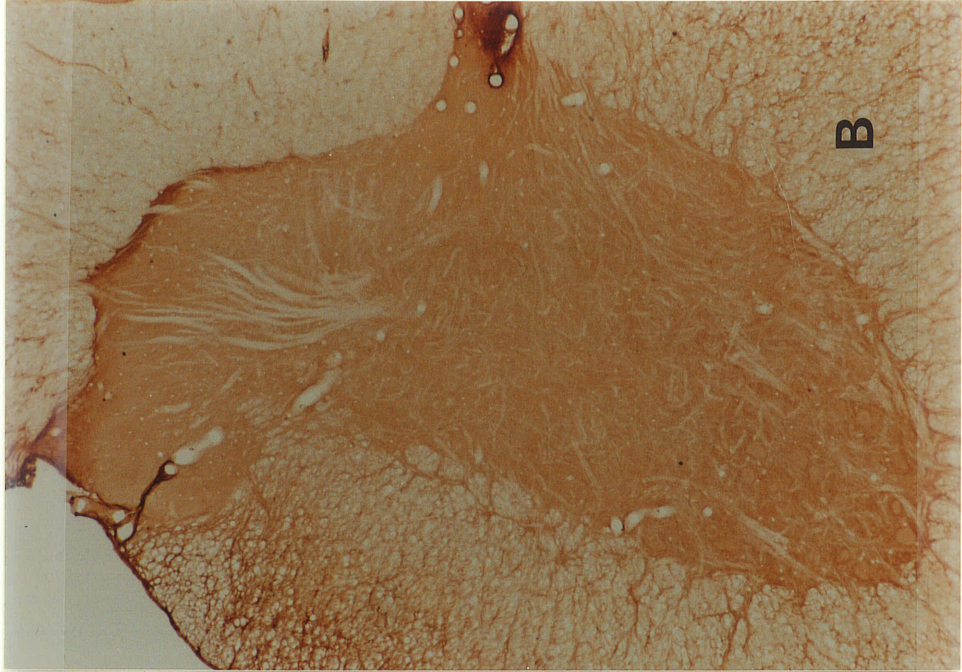
In locomoting animals, cells were found labelled in the magnocellular tegmental field, the ventrolateral reticular formation, the cuneiform nucleus, the marginal nucleus of the brachium conjuctivum, the nucleus of locus ceruleus and the nucleus of the solitary tract. However, all the nuclei (except the cuneiform nucleus) labelled in the locomotor animals were also labelled (albeit in lower numbers) in control animals. Several factors might contribute to the labelling in control cats: a) those nuclei involved in locomotion may also be involved in other functions such as respiration; b) cells in the nuclei involved in locomotion may also receive tonic activation in the absence of locomotion; and c) cells may be just labelled due to surgery for decerebration.

In the final analysis, the result shows that the CNF was labelled only in the animals with MLR-induced locomotion. The number of labelled cells in the MED and LCα were higher in locomotor cats than in the control cats. It is concluded that: 1) the labelled cells in the CNF, the MED and the LCα are involved in locomotion. 2) the PLS may not be an important relay site for the MLR-induced locomotion. 3) cells labelled in the ventrolateral reticular formation and the nucleus of the solitary tract are activated due to respiration.

Figure 1

Photomicrographs to show the appearance of labelled neurons in the spinal cord. A) A section from the L6 segment in a locomotor cat (TL-Cat2) (x36). B) A section from the L6 segment in a control cat (TC-Cat1) (x33).

Note that 2 motoneurons were labelled in the ventral horn (A).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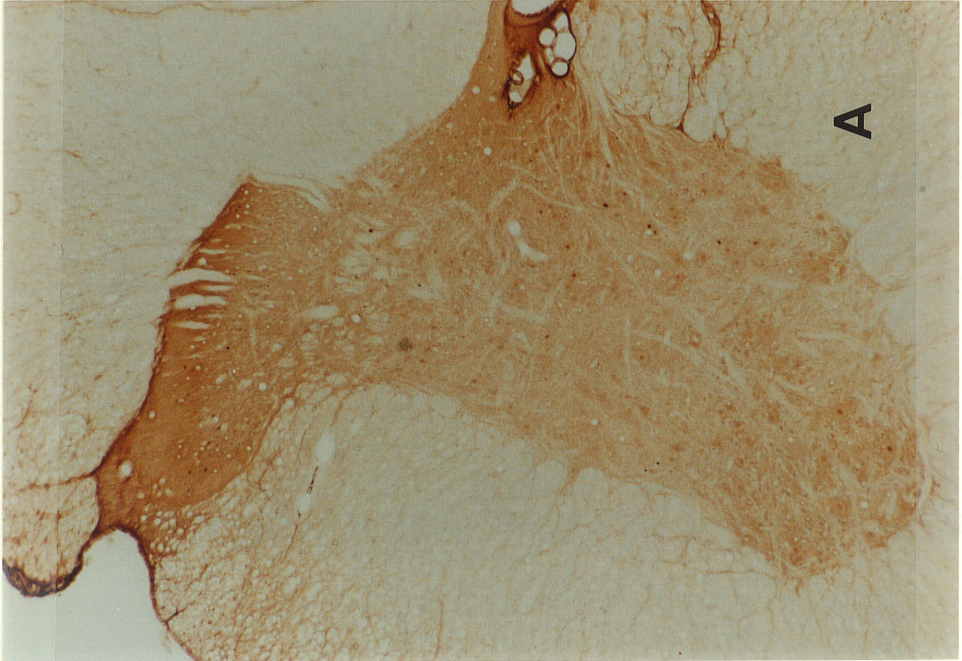

A

Figure 2

High power photomicrograph (x77) to show the difference between labelled neurons (pointed by a arrowhead) and unlabelled neurons (pointed by a arrow).

This section, which shows a medial portion of Rexed's laminae VI, VII, VIII and lamina X, is from L7 in TL-Cat2. Lamina X is situated in the far right in the photomicrograph, while laminae VI, VII, and VIII are located dorsoventrally.

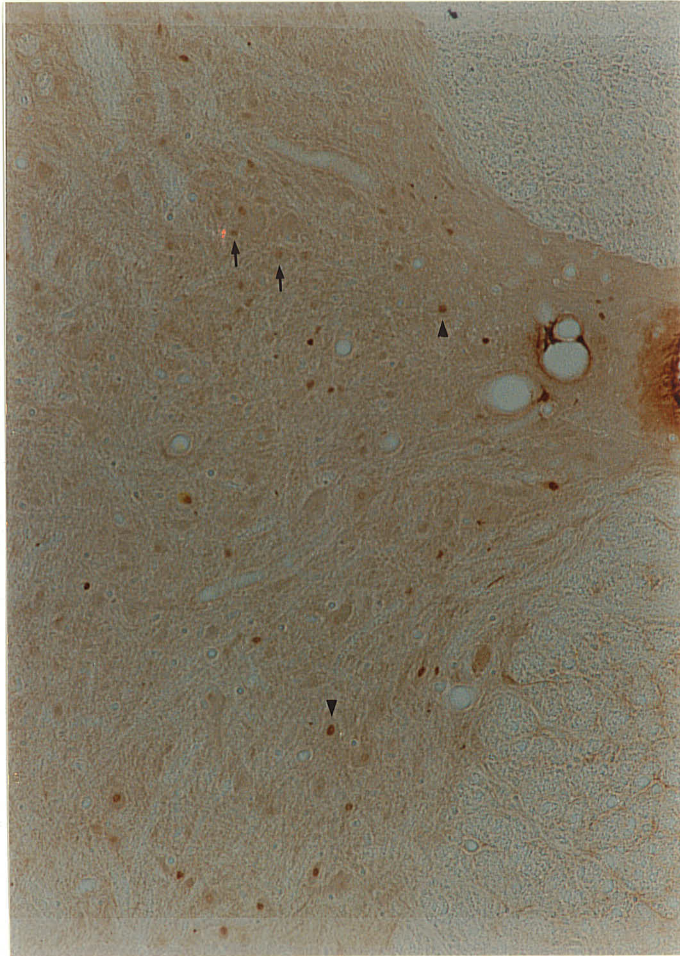
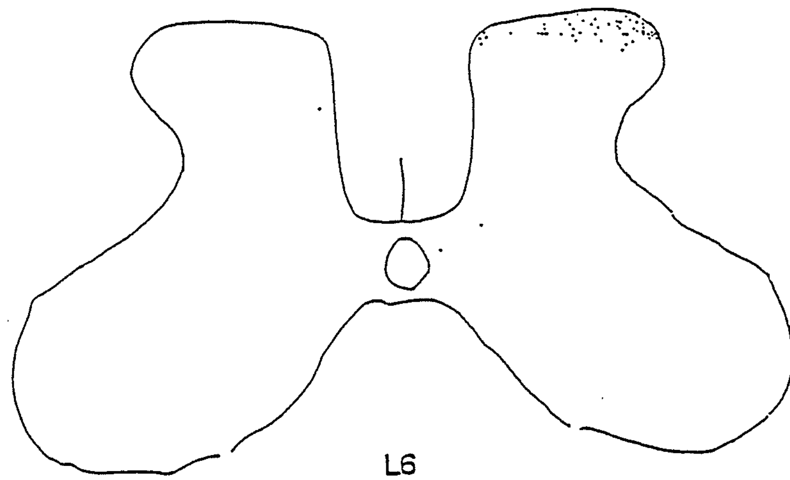


Figure 3

Camera lucida drawing to show the distribution of c-fos immunoreactive neurons in the L6 spinal segment after **injection of paraformaldehyde** in the right hind leg and paw of a cat (the upper figure). The diagram includes all labelled cells in 8 sections; each dot represents one labelled cell.

Note that most of labelled cells are concentrated in Rexed's laminae I and II in the side ipsilateral to the injection site.

The lower figure shows the division of Rexed's laminae in the L6 spinal segment.



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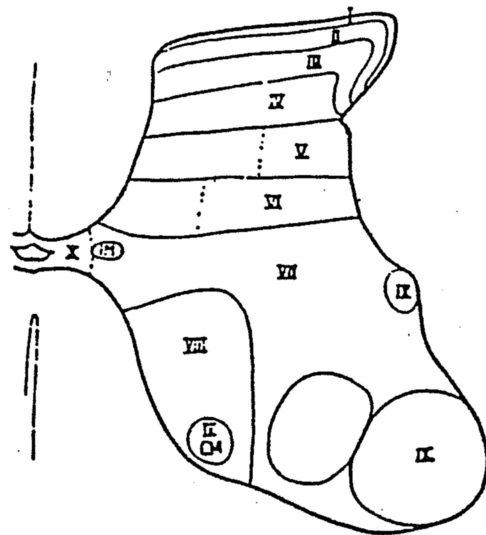
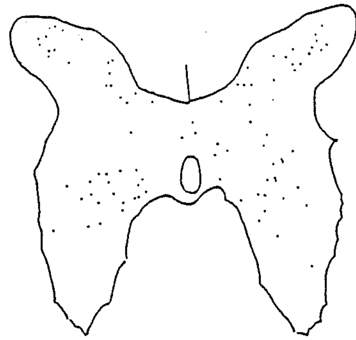


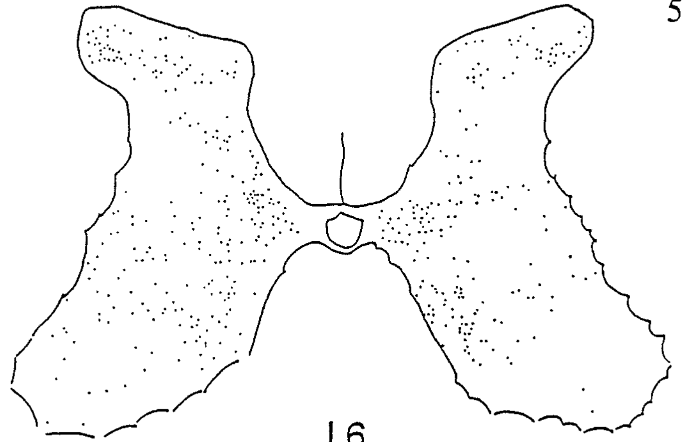
L6

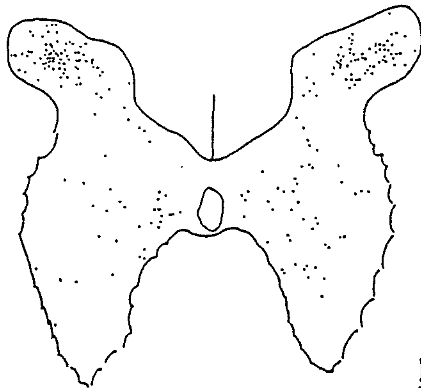
Figure 4

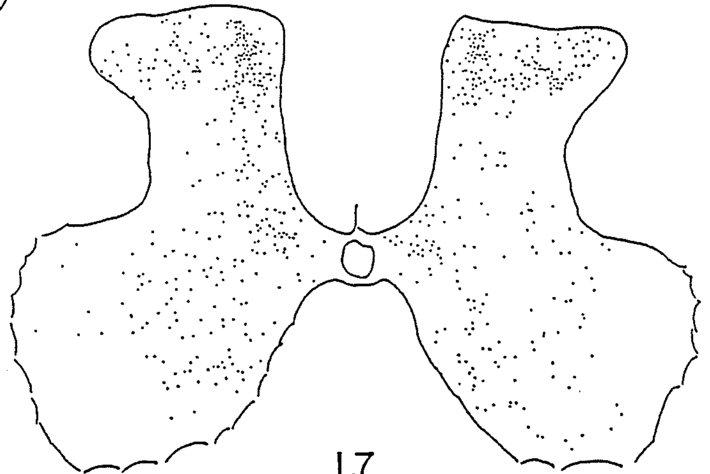
Camera lucida drawings to show the distribution of c-fos immunoreactive neurons in the spinal segments L3-S1 in TL-Cat2 which was induced to **locomote** on the **treadmill** with **MLR stimulation.** Each diagram includes all labelled cells in 16 sections of that segment; each dot represents one labelled cell.

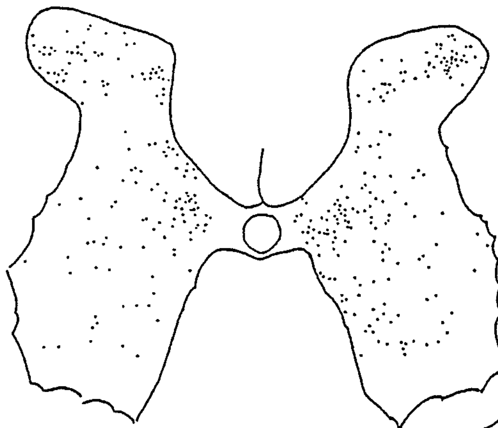
Labelled cells located in the dorsal horn were concentrated in Rexed's laminae III and IV, while those in the ventral horn centred in laminae VII and VIII with some extending into lamina VI.











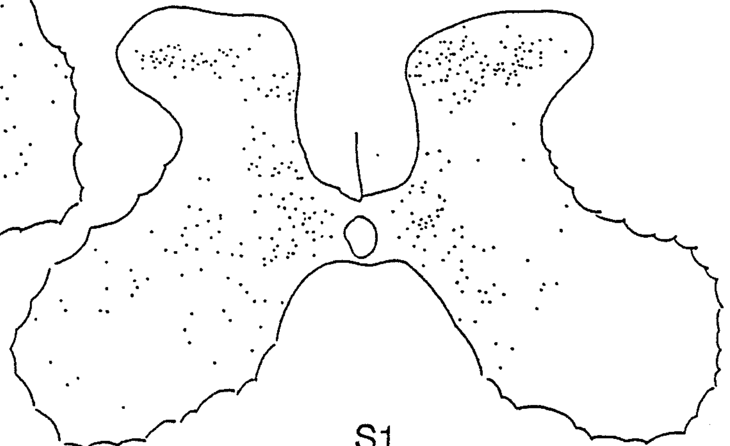


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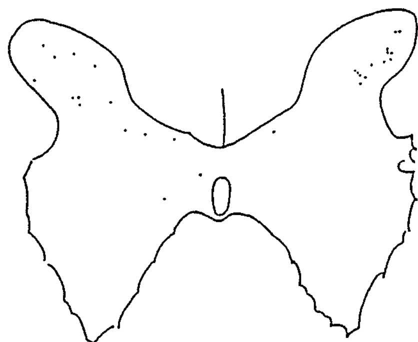
Figure 5

Camera lucida drawings to show the distribution of c-fos immunoreactive neurons in the spinal segments L3-S1 in TL-Cat1 which was induced to **locomote** on the **treadmill** with **MLR stimulation.** Each diagram includes all labelled cells in 19 sections of that segment; each dot represents one labelled cell.

Labelled cells located in the dorsal horn were concentrated in Rexed's laminae III and IV, while those in the ventral horn were in laminae VII with extension into lamina VI but much less extension into lamina VIII.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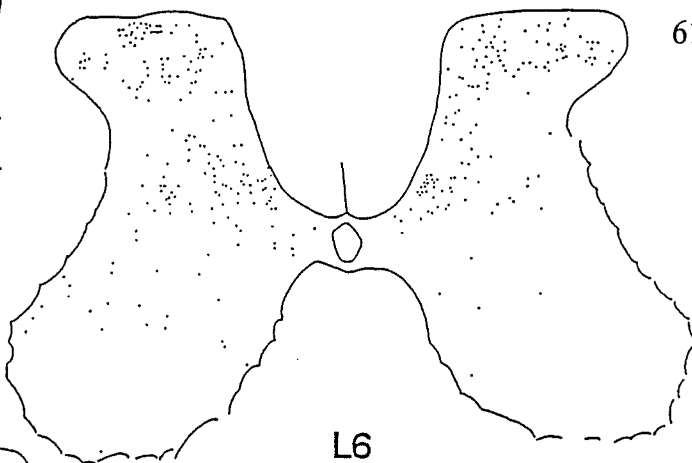
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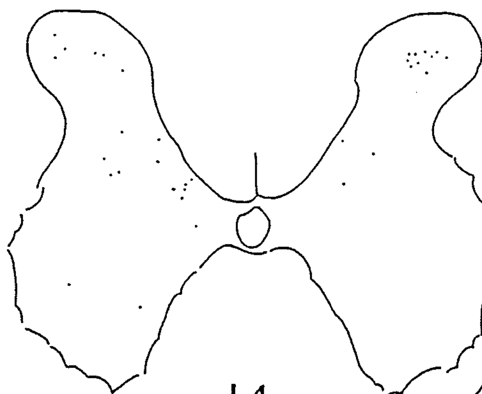
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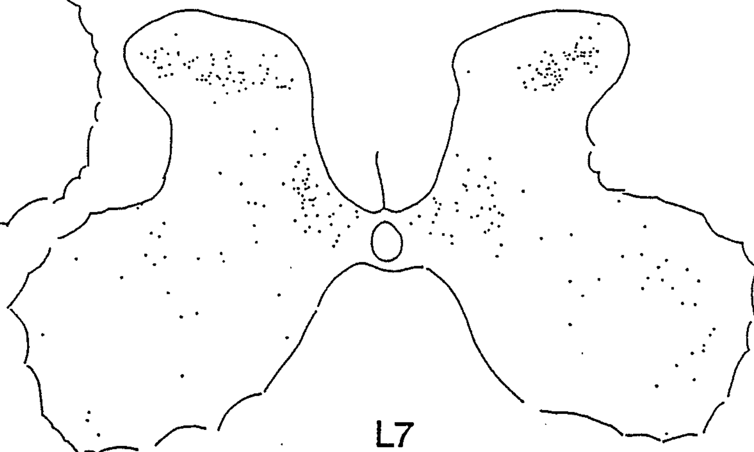
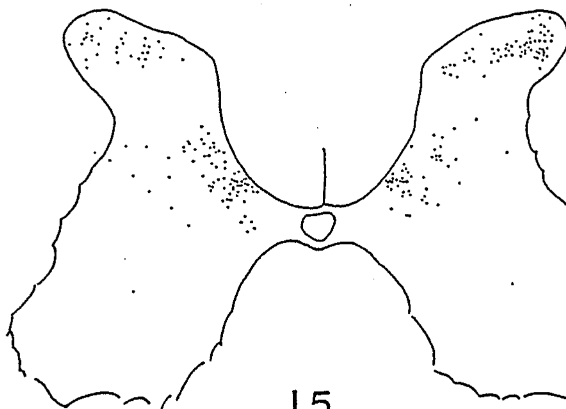

L7


L5
1 mm

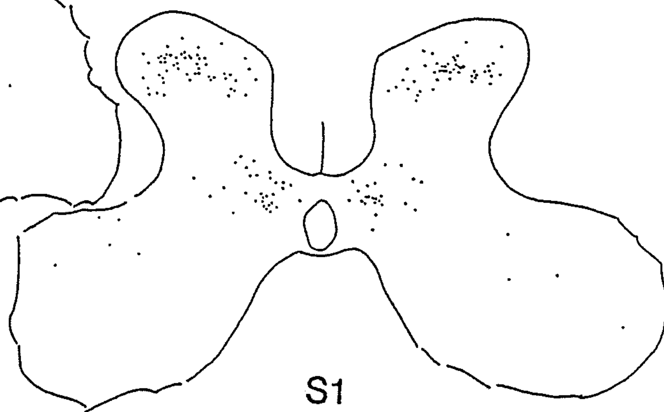
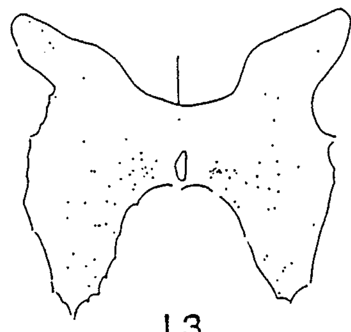
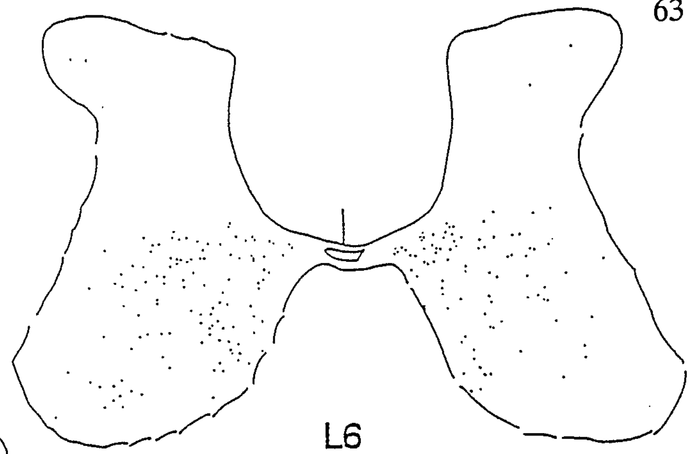

S1

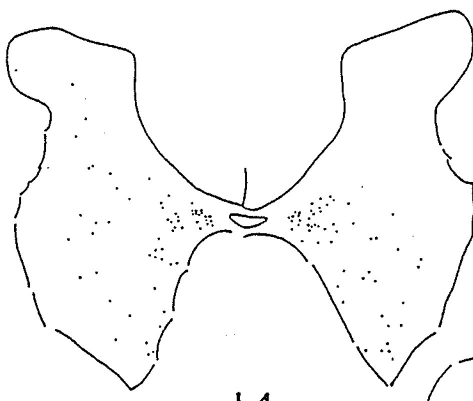
Figure 6

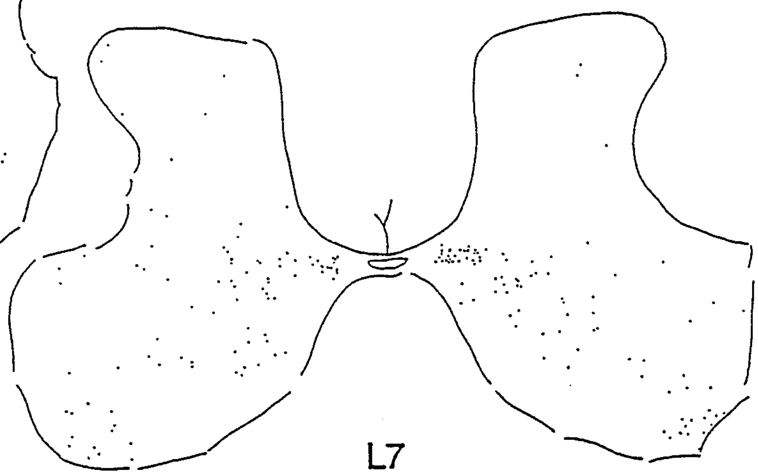
Camera Lucida drawings to show the distribution of c-fos immunoreactive neurons in the spinal segments L3-S1 of FL-Cat1 which was electrical stimulated in the **MLR** to induce **fictive locomotion.** Each diagram includes all labelled cells in 17 sections of that segment; each dot represents one labelled cell.

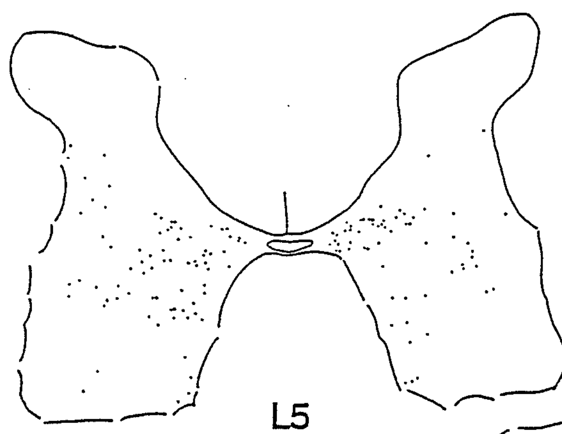
Labelled cells were mostly located in Rexed's laminae VII and VIII. Note that motoneurons were labelled.

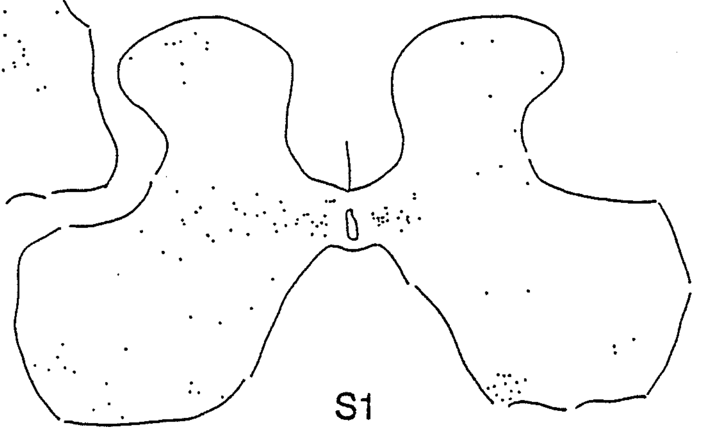










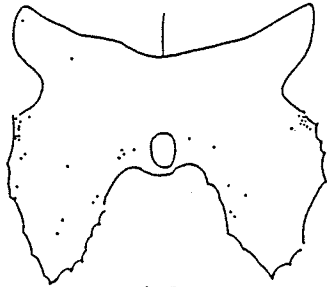


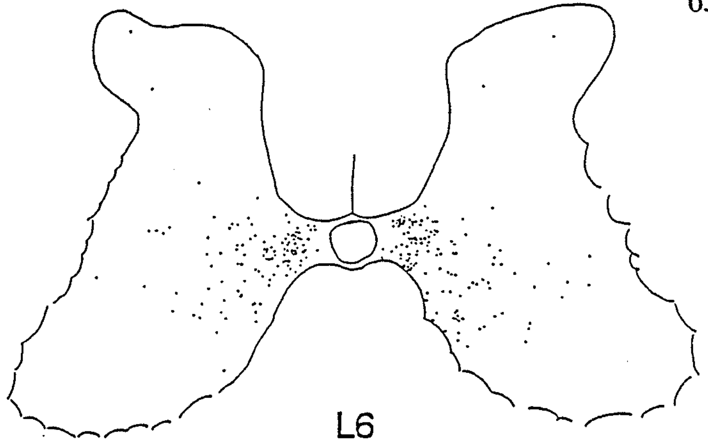
1 mm

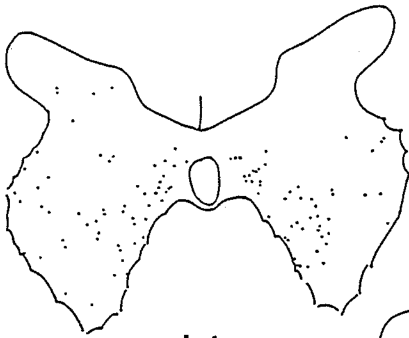
Figure 7

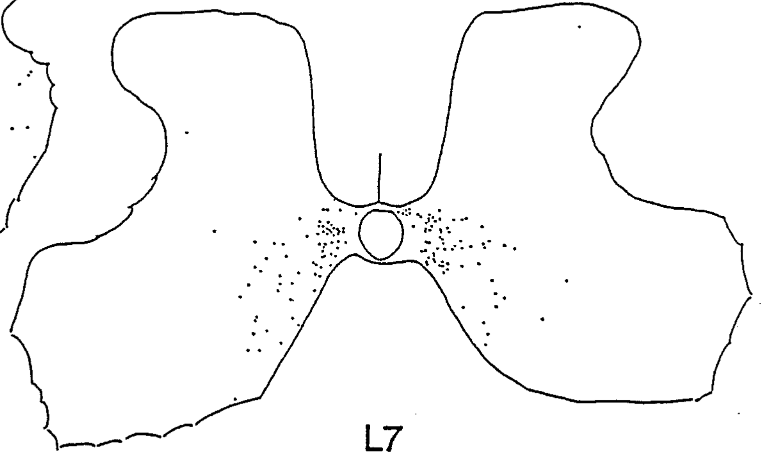
Camera lucida drawings to show the distribution of c-fos immunoreactive neurons in the spinal segments L3-S1 of FL-Cat2 which was electrical stimulated in the **MLR** to induce **fictive locomotion.** Each diagram includes all labelled cells in 19 sections of that segment; each dot represents one labelled cell.

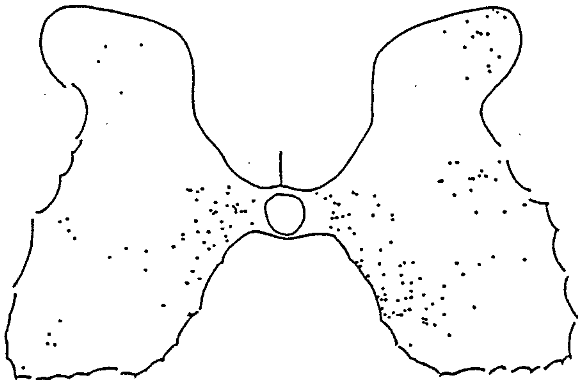
Labelled cells were mostly located in Rexed's laminae VII (especially in the medial portion) and VIII.

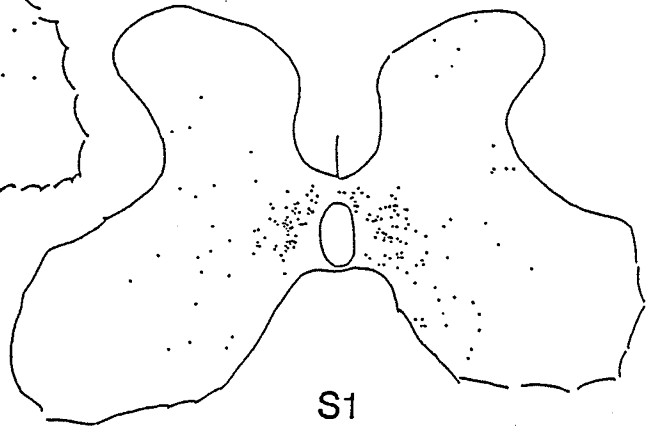










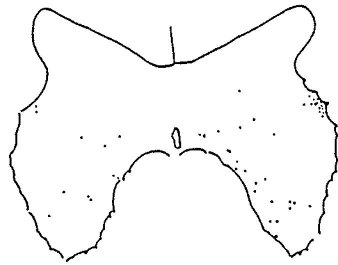


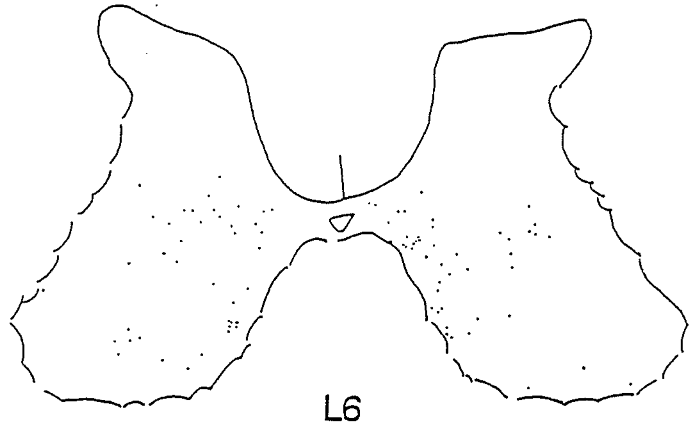
1 mm

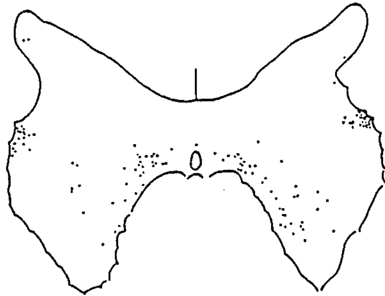
Figure 8

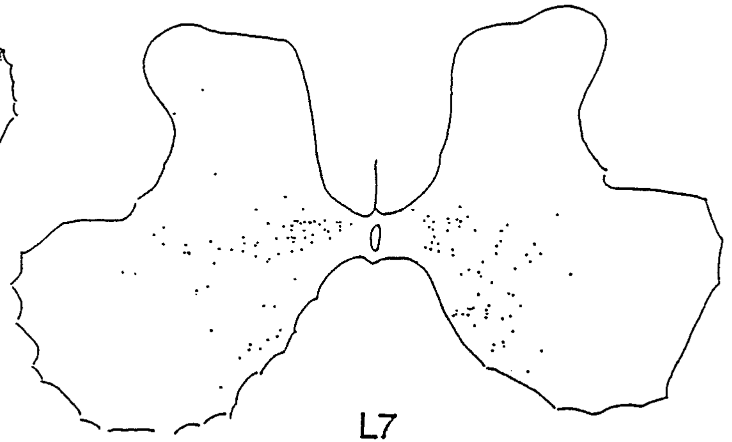
Camera lucida drawings to show the distribution of c-fos immunoreactive neurons in the spinal segments L3-S1 of **SFL-Cat**. Each diagram includes all labelled cells in 20 sections of that segment; each dot represents one labelled cell.

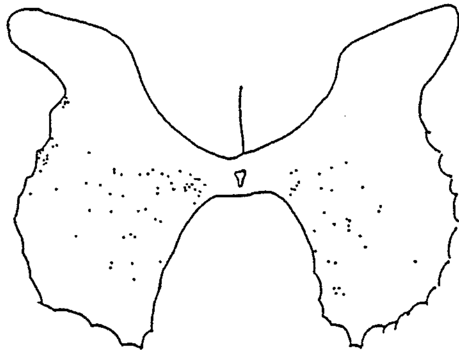
Labelled cells were concentrated in Rexed's laminae VII and VIII.

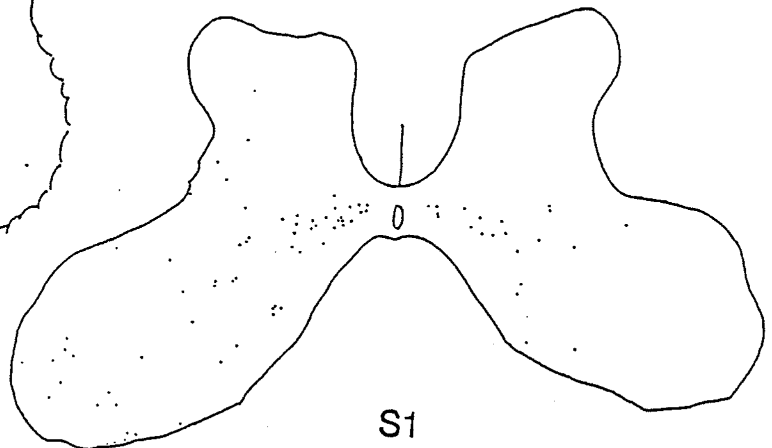










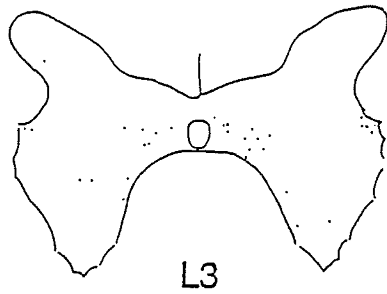


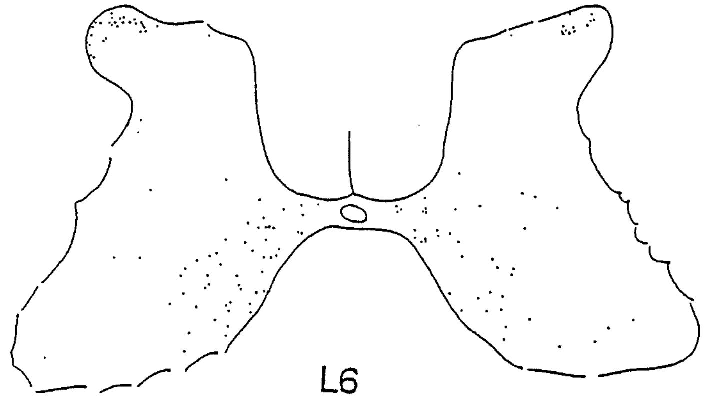
1 mm

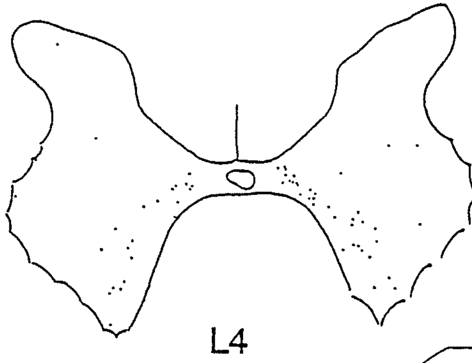
Figure 9

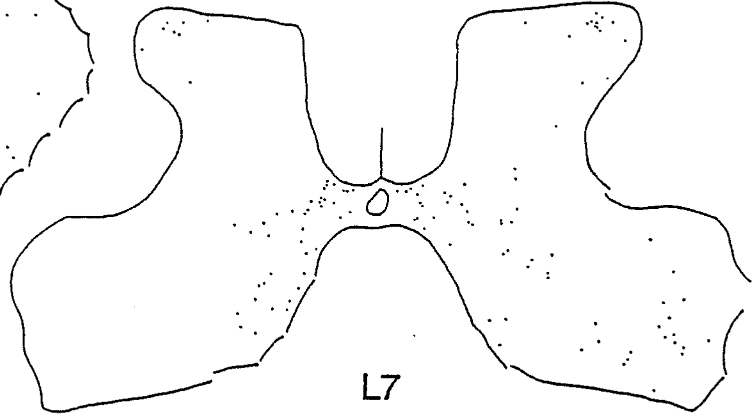
Camera lucida drawings to show the distribution of c-fos immunoreactive neurons in the spinal segments L3-S1 of **FSFL-Cat**. Each diagram includes all labelled cells in 17 sections of the that segment; each dot represents one labelled cell.

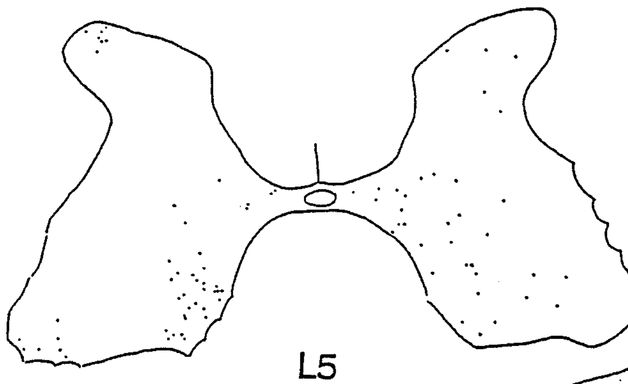
Labelled cells were concentrated in the medial portion of Rexed's laminae VII, with extending into the lamina VIII.


L3


L6


L4


L7


L5
1 mm

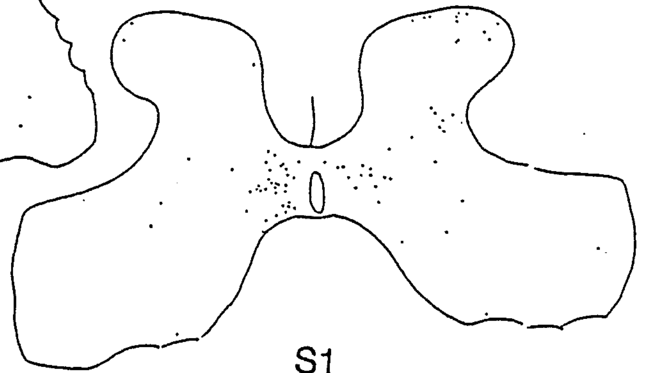
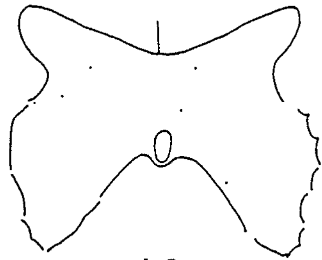
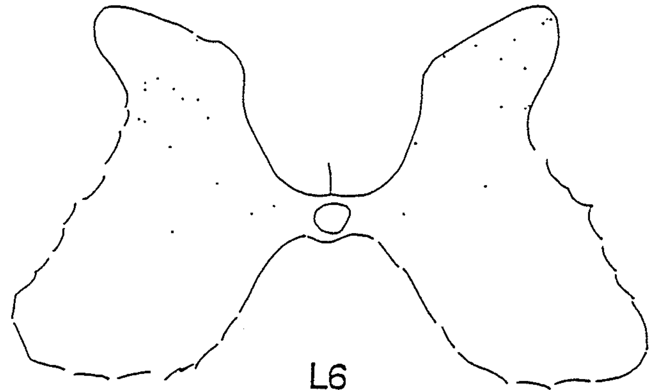

S1

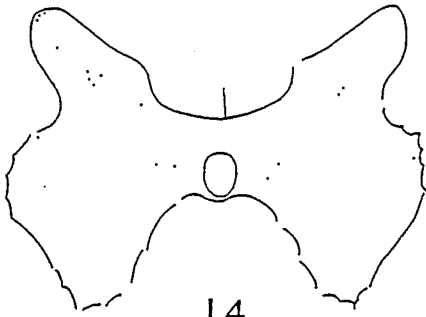
Figure 10

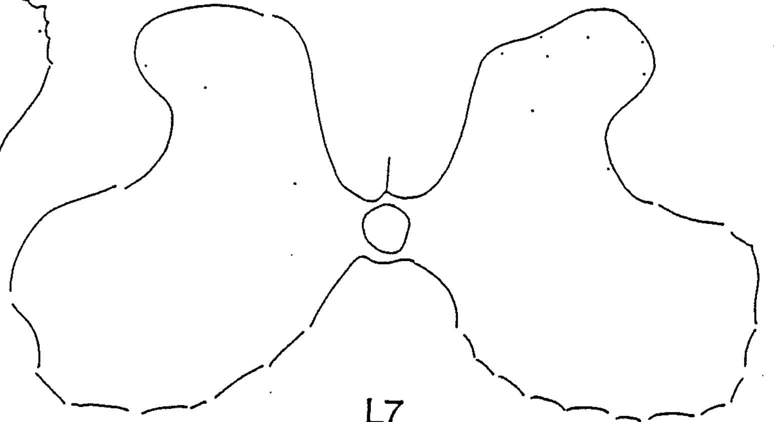
Camera lucida drawings to show the distribution of c-fos immunoreactive neurons in the spinal segments L3-S1 of a **fictive control** cat (FC-Cat4). Each diagram includes all labelled cells in 18 sections of that segment; each dot represents one labelled cell.

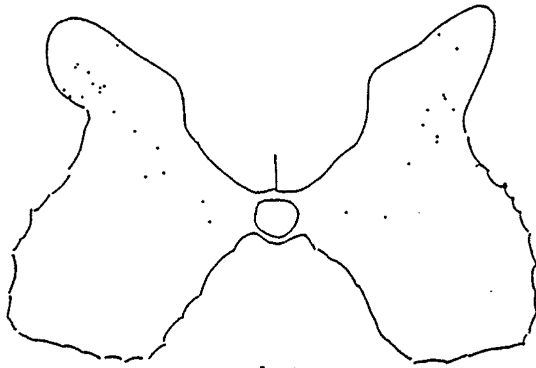
Labelled cells were scattered in the dorsal horn with a few cells in the medial portion of lamina VII.

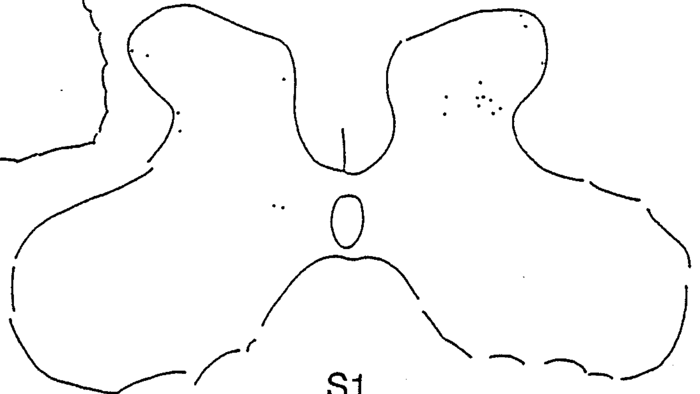












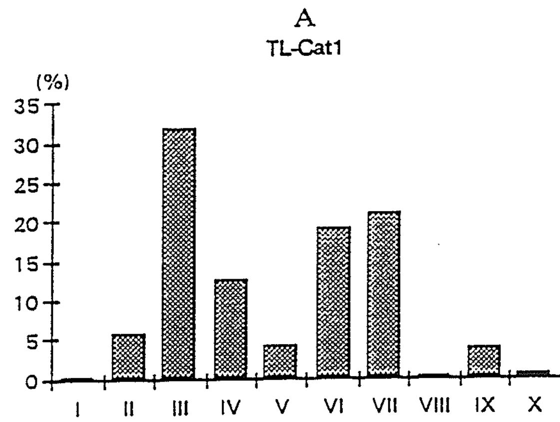
1 mm

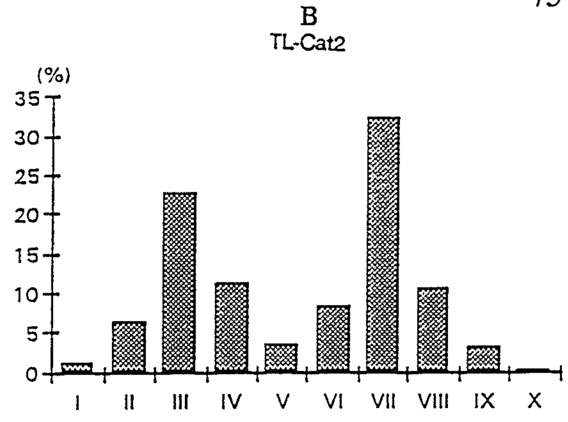
Figure 11

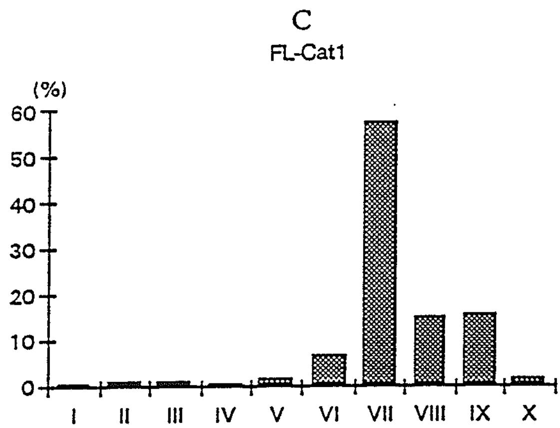
Laminar distribution of labelled cells in each locomotor cat. A & B: MLR-induced treadmill locomotion. C & D: MLR-induced fictive locomotion. E: Spontaneous fictive locomotion. F: Forelimb swinging induced fictive locomotion.

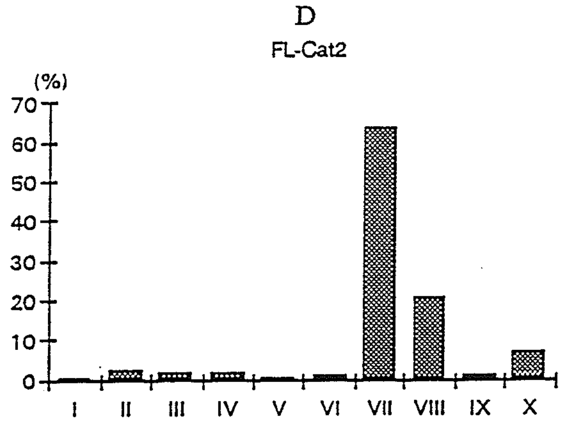
The calculation was done separately in the cats. The percentage of labelled cells (bar height) in each lamina was obtained by total number of labelled cells in that lamina from L3 to S1 divided by the total number of labelled cell in the cat.

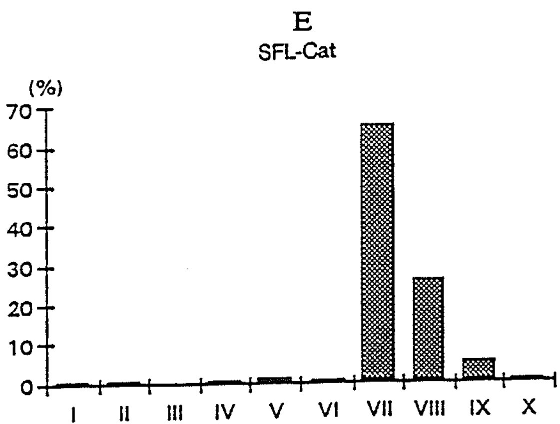
Note that the distribution of labelled cells in the dorsal horn of treadmill locomotion animals were not observed in the fictive locomotion animals.

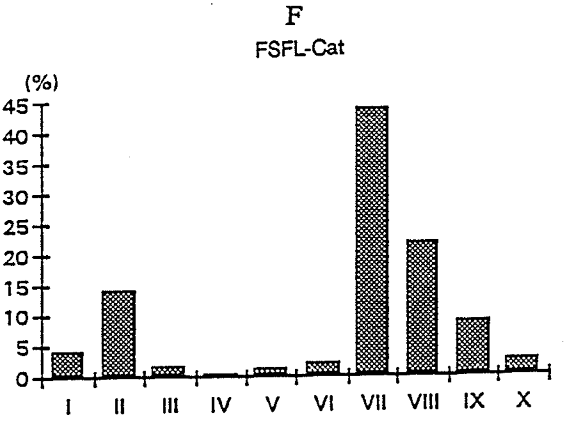












X Axis: Rexed's Laminae.
Y Axis: Percentage of Labelled Cells.

Figure 12

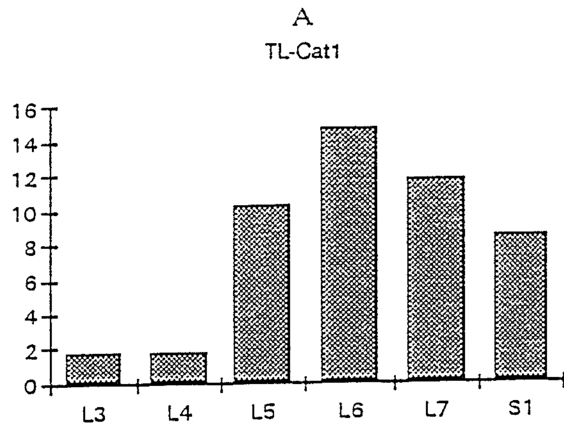
Segmental distribution of labelled cells (excluding motoneurons) in each locomotor cat. A & B: MLR-induced treadmill locomotion. C & D: MLR-induced fictive locomotion. E: Spontaneous fictive locomotion. F: Forelimb swinging induced fictive locomotion.

The calculation was done separately in the cats. The number of labelled interneurons (motoneurons were not included) per section, instead of total number of interneurons, in each segment was compared among the segments. The purpose is to correct the bias which is induced by uneven number of tissue sections collected during tissue processing.

Note that the number of labelled interneurons start to increase at L4, reaching its maximum at L6 or L7.

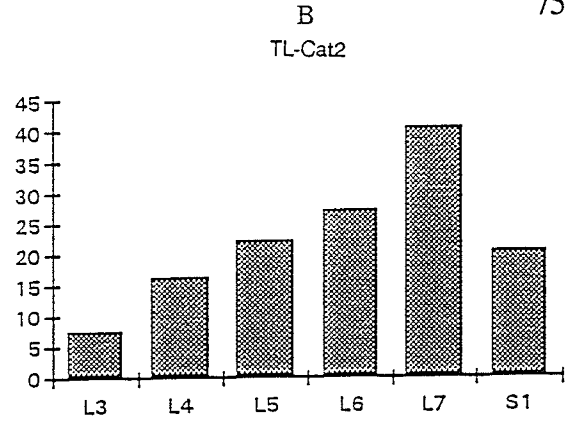
A

TL-Cat1



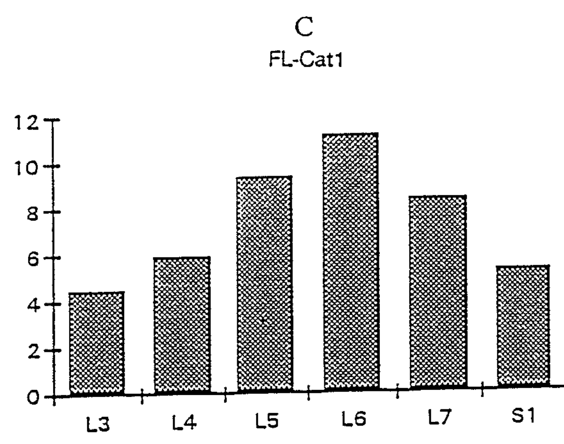
B

TL-Cat2



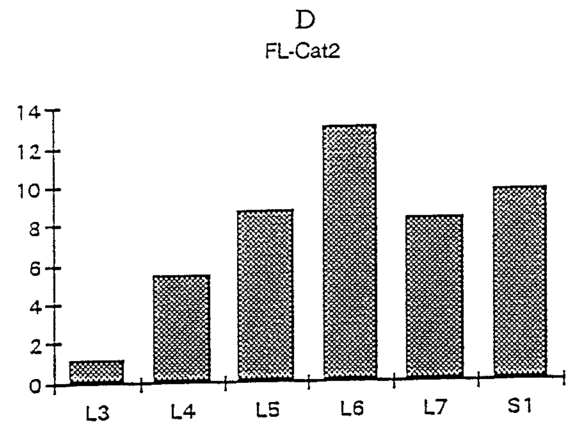
C

FL-Cat1



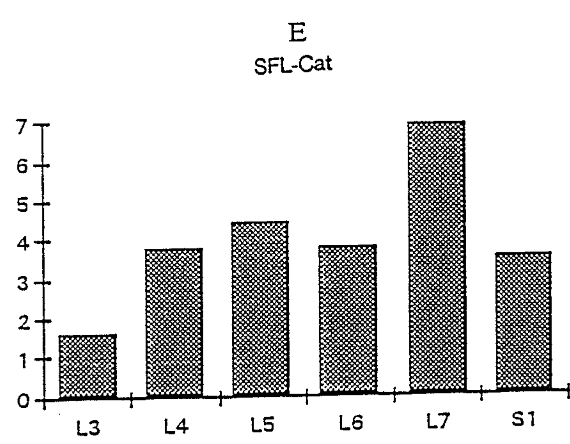
D

FL-Cat2



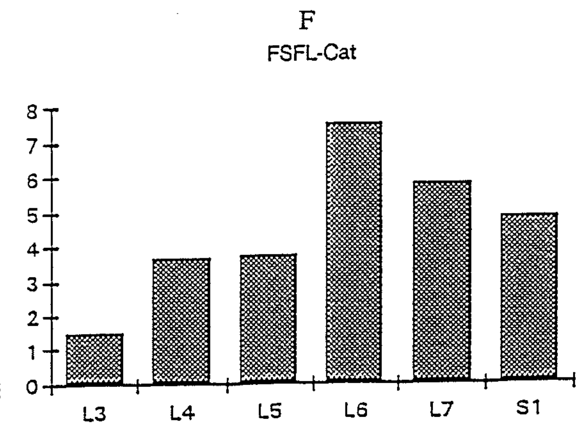
E

SFL-Cat



F

FSFL-Cat



X Axis: Spinal Cord Segments.
Y Axis: Averaged Number of Labelled Cells in Each Tissue Section.

Figure 13

Total number of labelled cells in each animal.

The total numbers of labelled neurons in the MLR-induced locomotion cats were higher than in the non-MLR-stimulated locomotion cats, and much higher than in the controls.

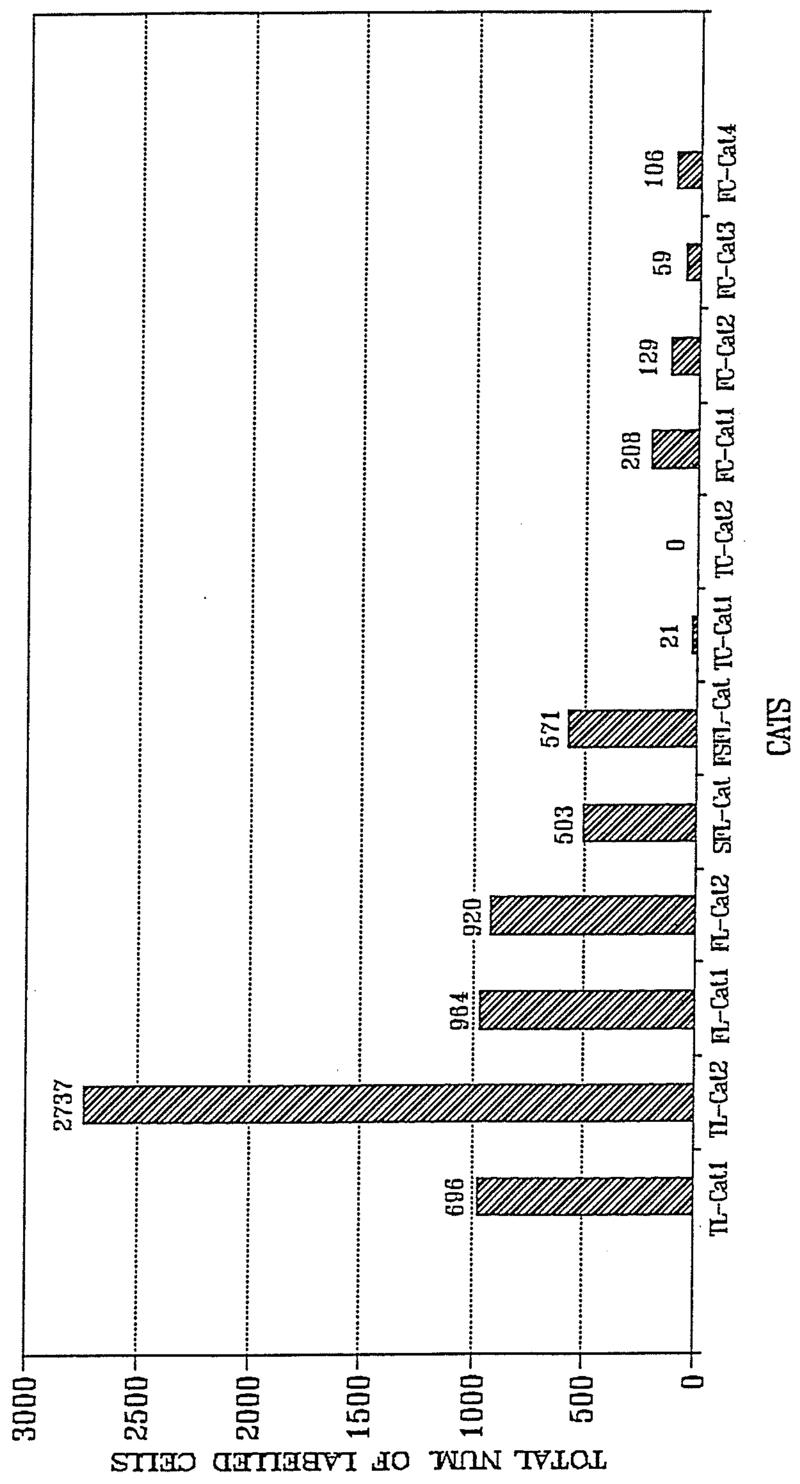

TOTAL NUM. OF LABELLED CELLS
0
500
1000
1500
2000
2500
3000
969
2737
964
920
503
571
21
0
208
129
59
106
TL-Cat1
TL-Cat2
FL-Cat1
FL-Cat2
SFL-Cat
FSFL-Cat
TC-Cat1
TC-Cat2
FC-Cat1
FC-Cat2
FC-Cat3
FC-Cat4
CATS

Figure 14

Photomicrographs (x29) to show labelled neurons in the CNF in FL-Cat2.

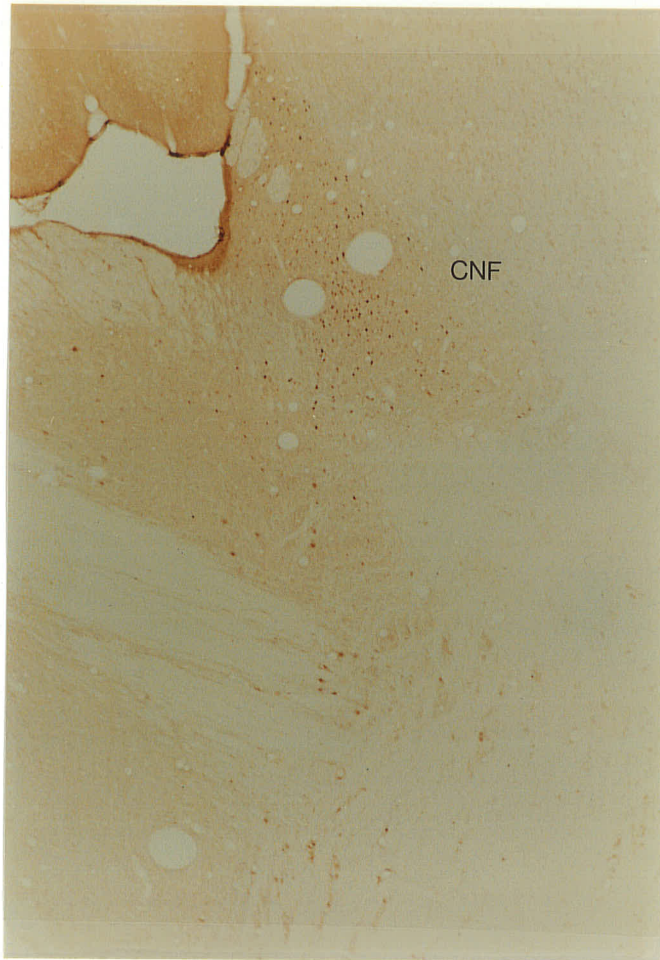

CNF

Figure 15-25

Distribution of labelled cells in a MLR-induced fictive locomotion cat (Cat2) (1-22) and a fictive control cat (Cat4) (1'-22'). Each dot represents one labelled cell.

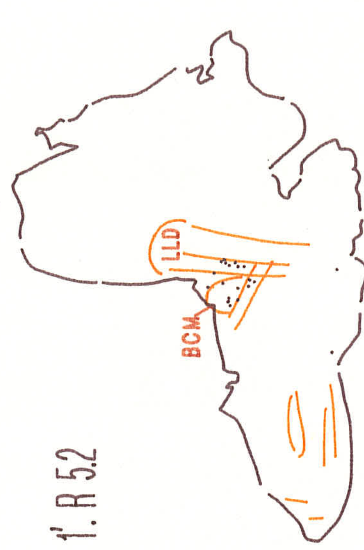
The mediolateral coordinates of ventral part of brainstem sections in this locomotor cat are about 0.4-0.6 mm more medial than in the dorsal part because the tissue was sectioned with a angle. Corrected numbers are given underneath the sections.

R: right side. L: left side. R 5.2: 5.2 mm lateral from the midline in the right side of the brainstem.

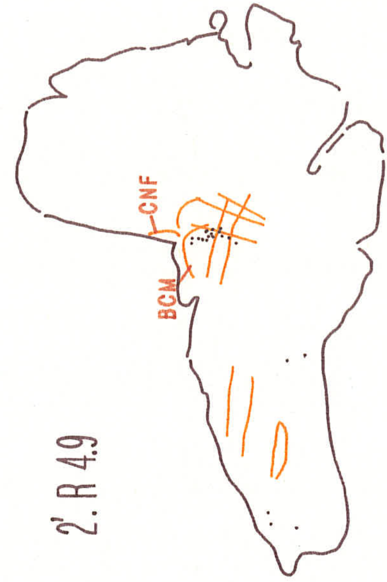
Figure 15

CONTROL

1'. R 5.2

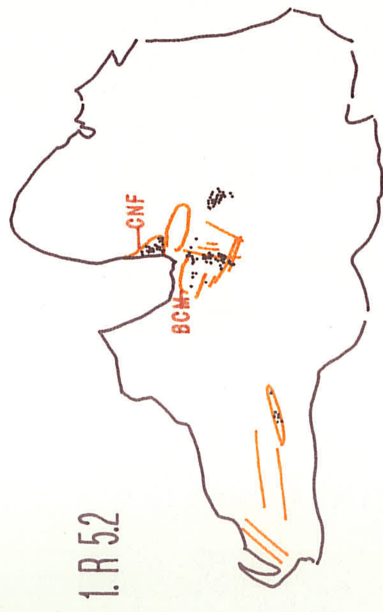


2'. R 4.9



LOCOMOTION

1. R 5.2



2. R 4.9

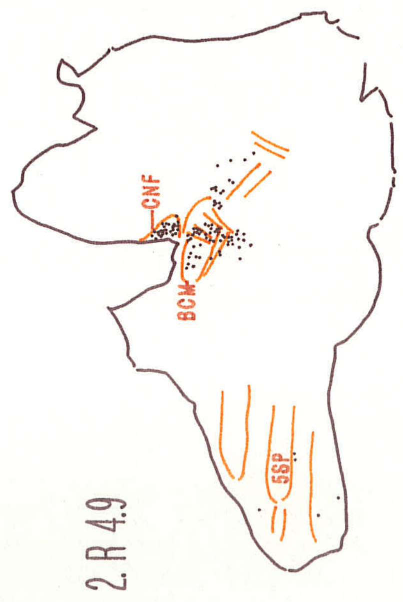
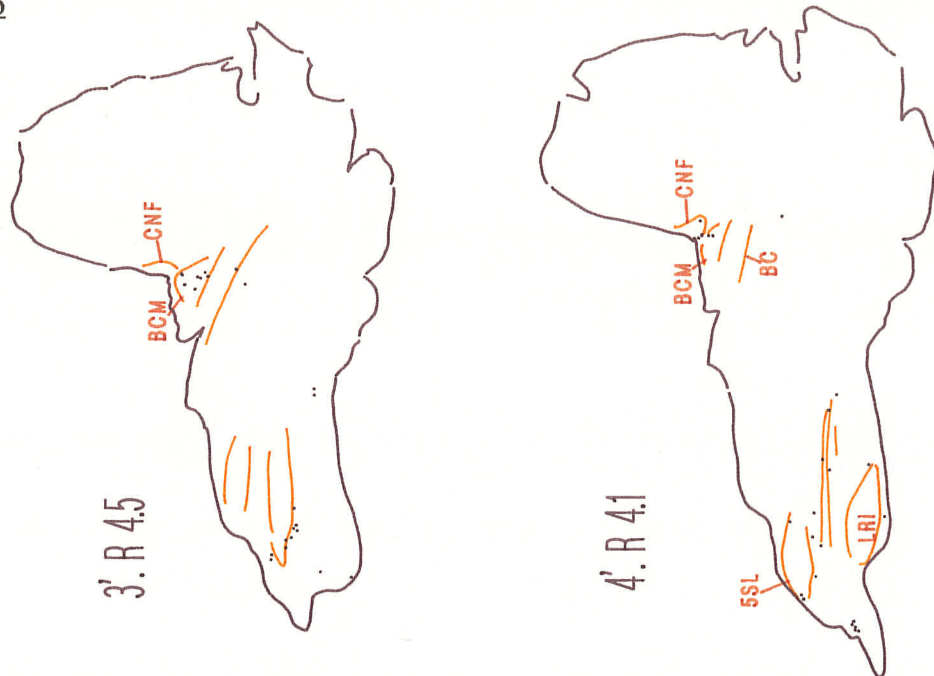


Figure 16

CONTROL



LOCOMOTION

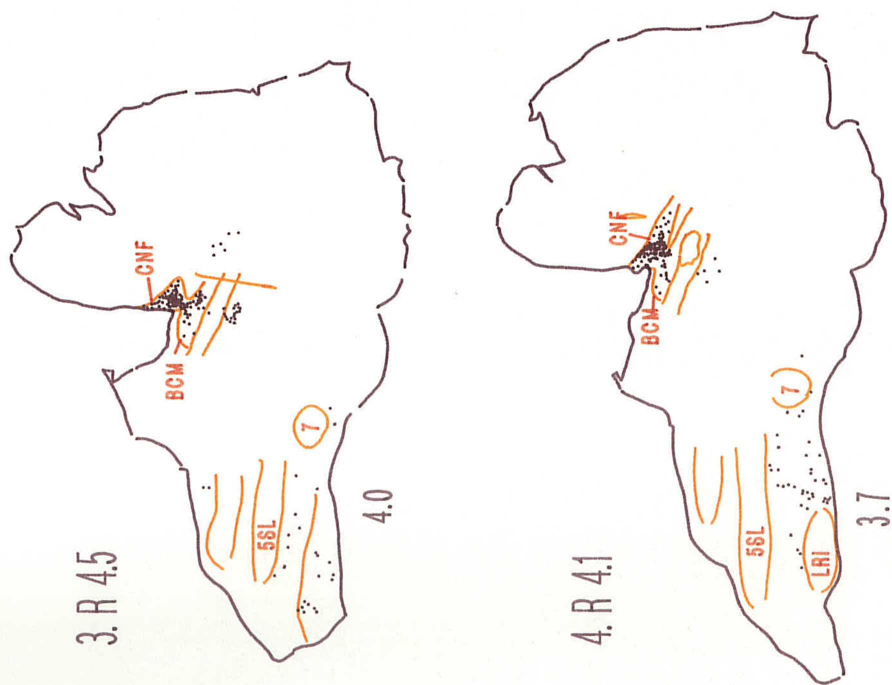
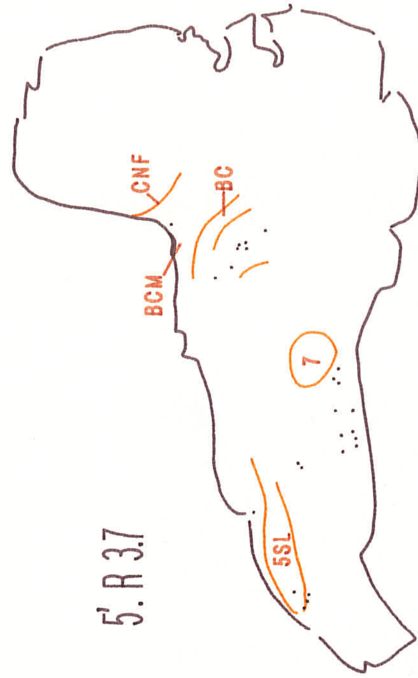
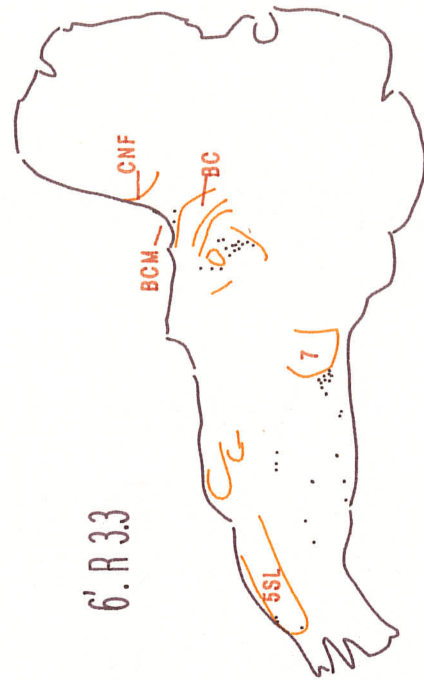


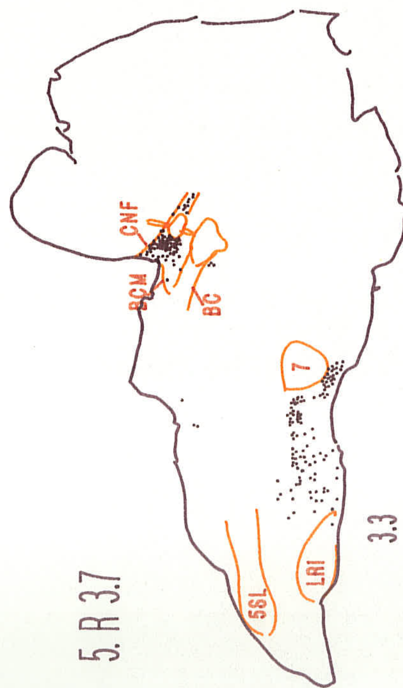
Figure 17

CONTROL





LOCOMOTION



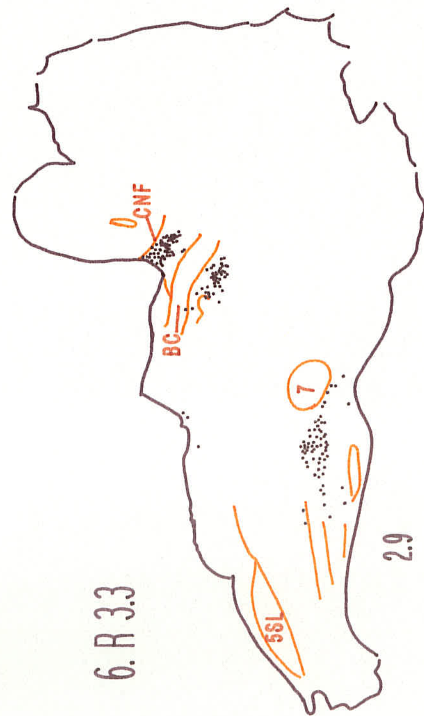
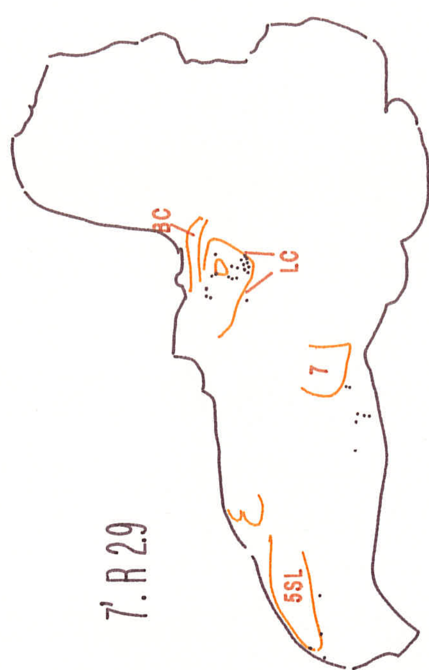
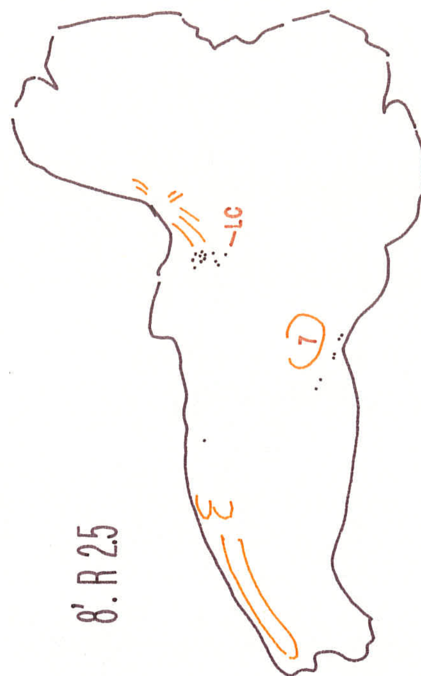


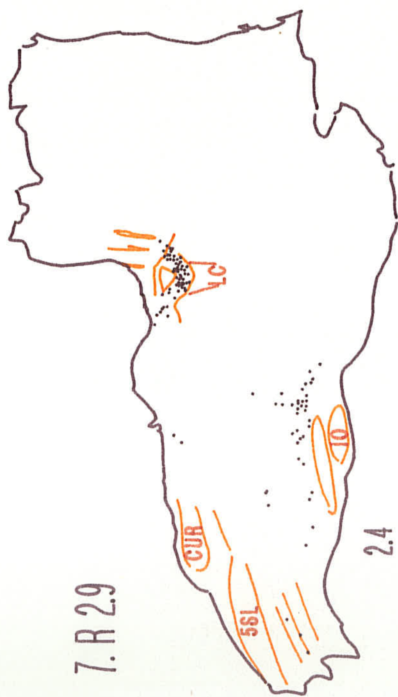
Figure 18

CONTROL





LOCOMOTION



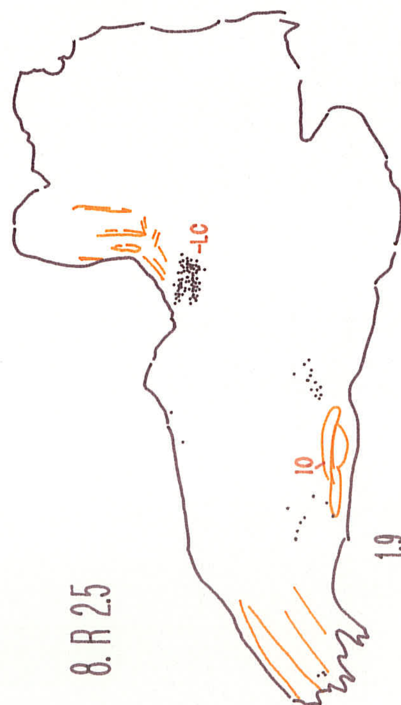
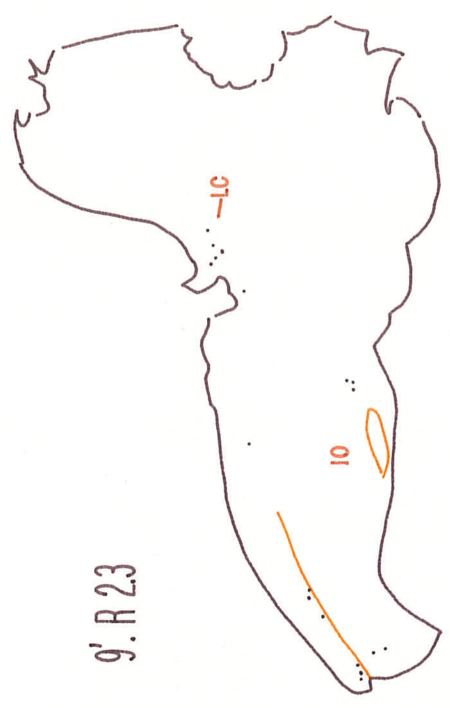
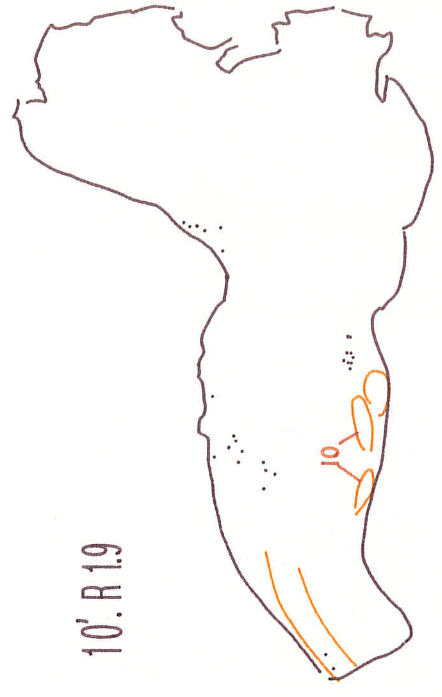


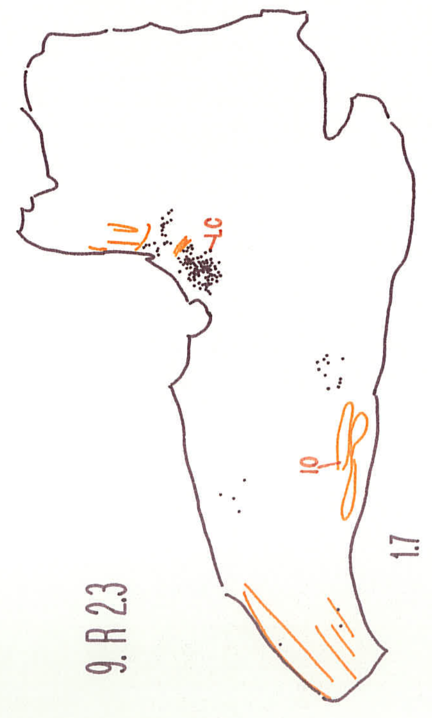
Figure 19

CONTROL





LOCOMOTION



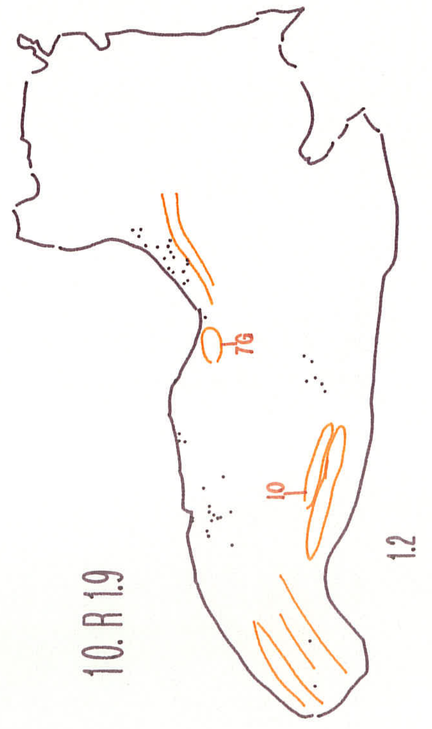


Figure 20

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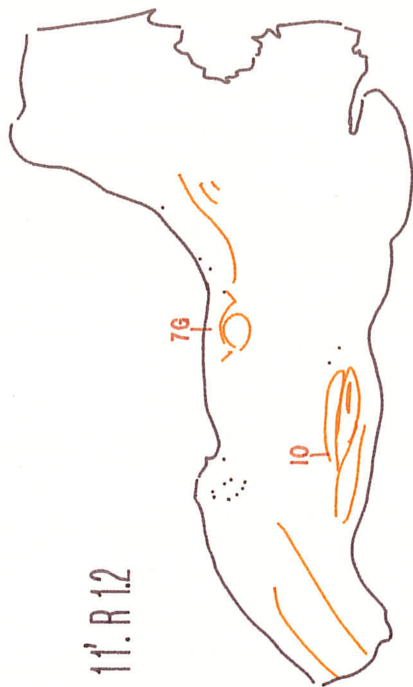

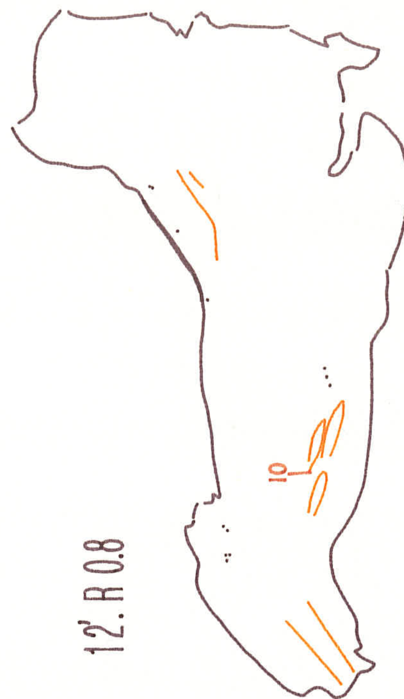

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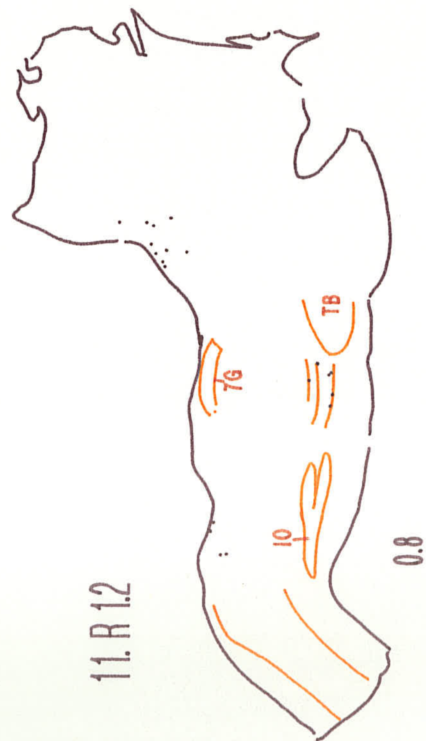

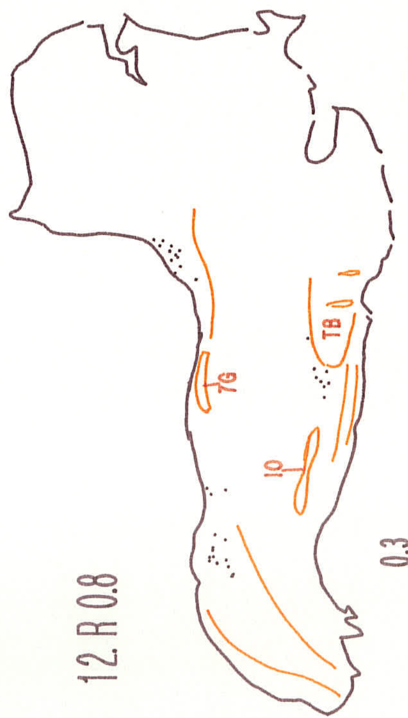

Figure 21

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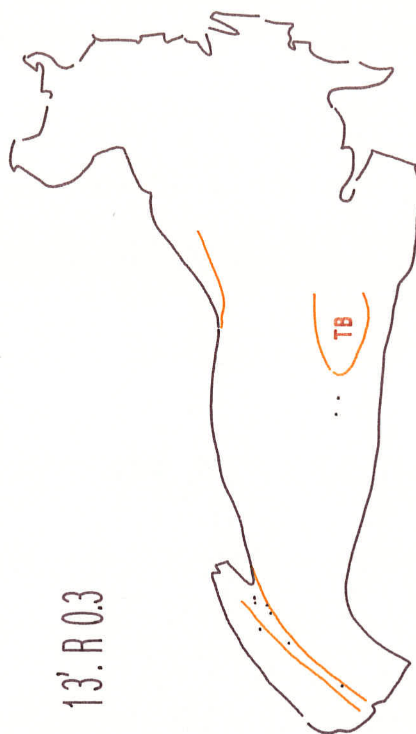

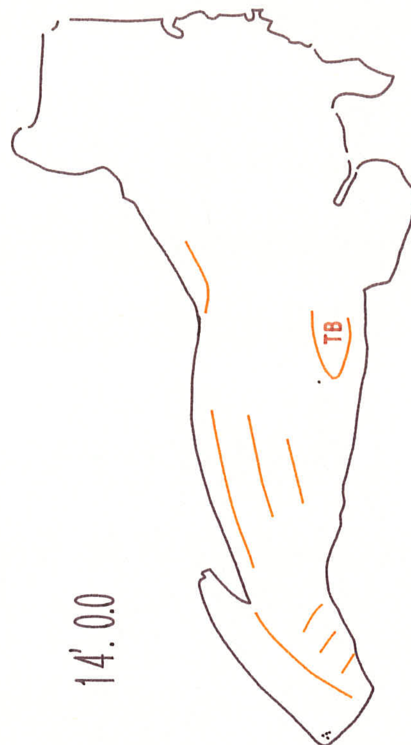

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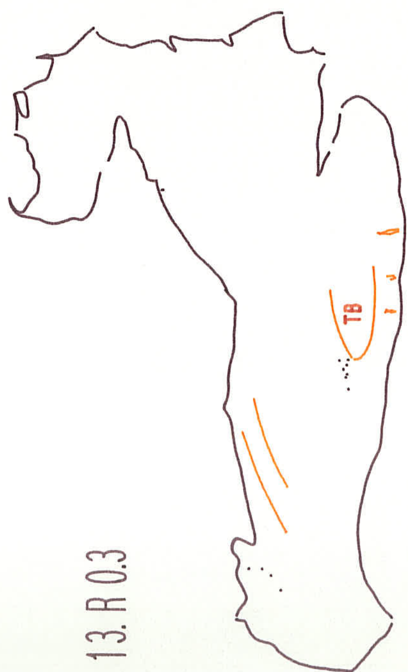

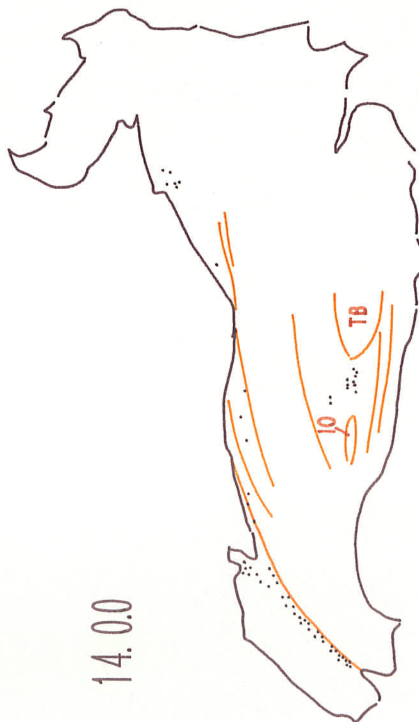

Figure 22

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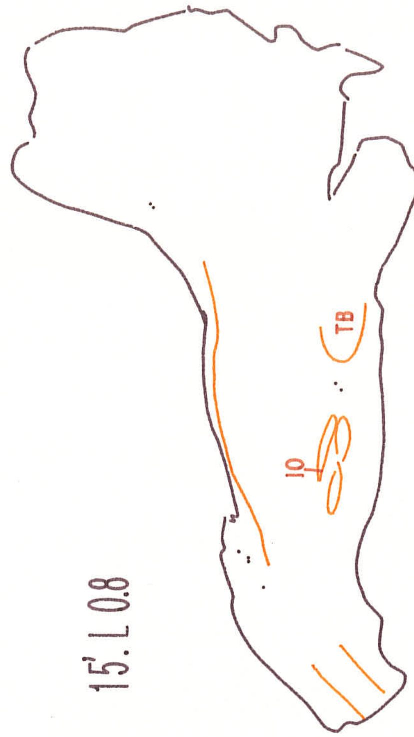

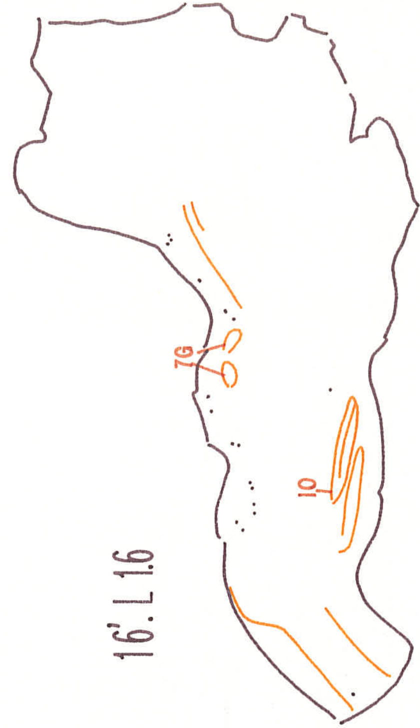

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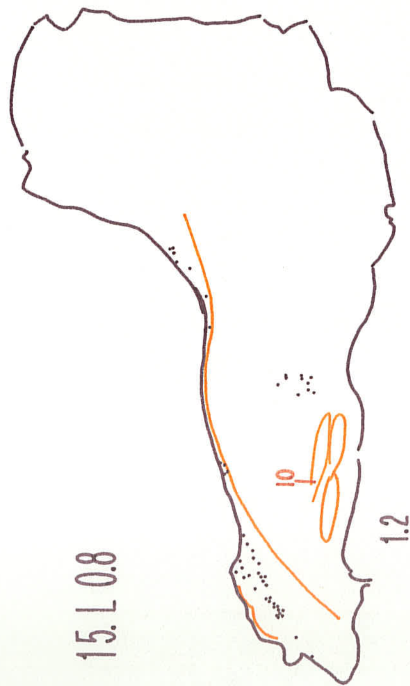

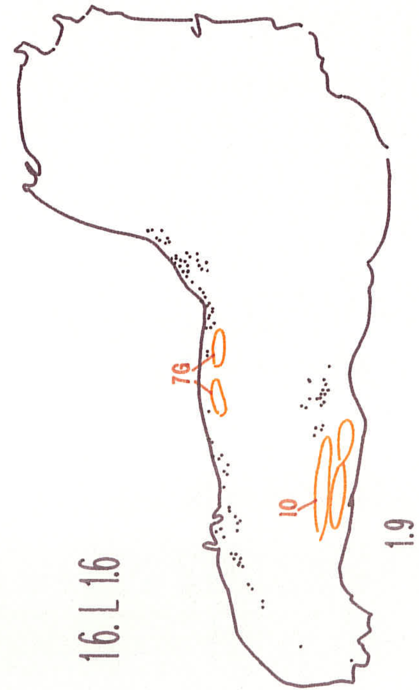

Figure 23

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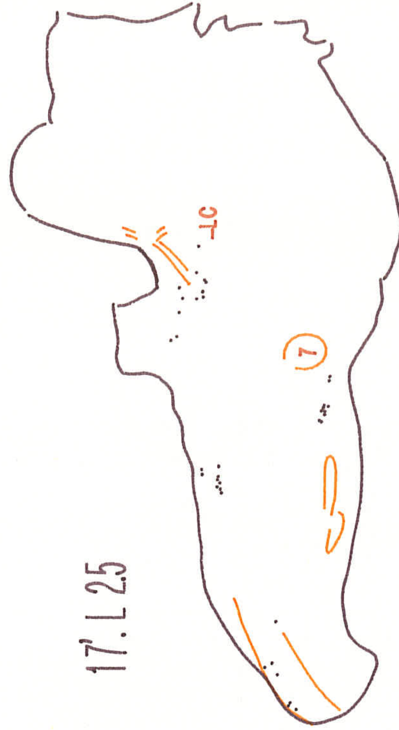

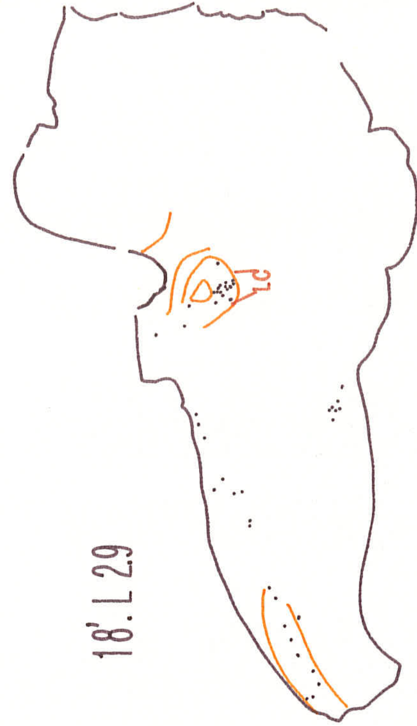

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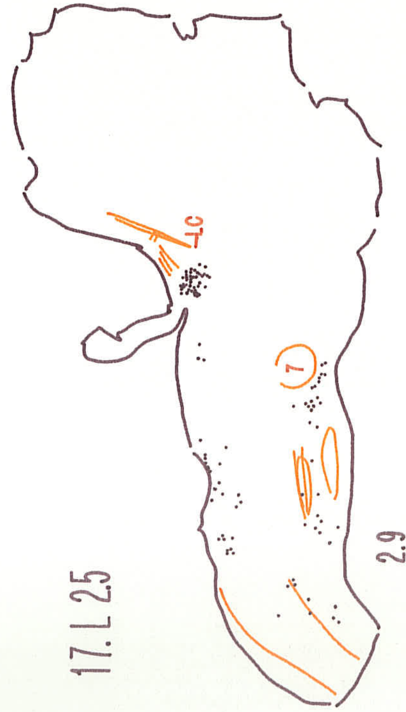

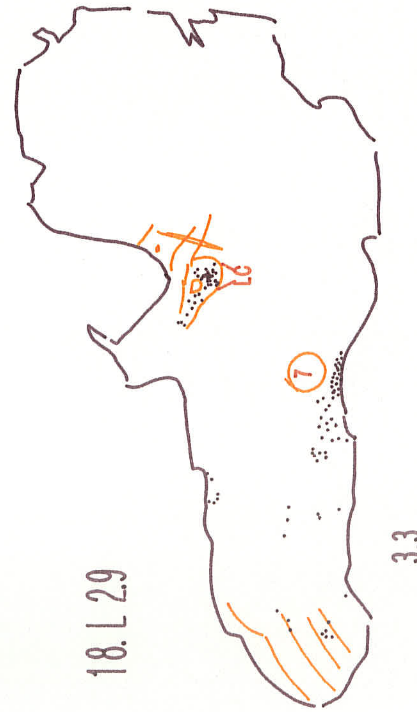

Figure 24

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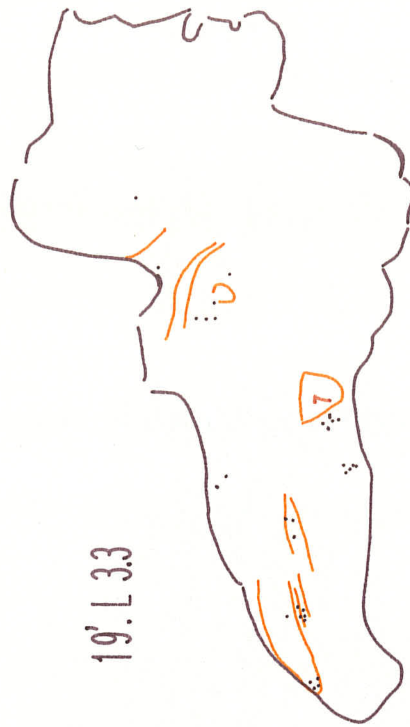

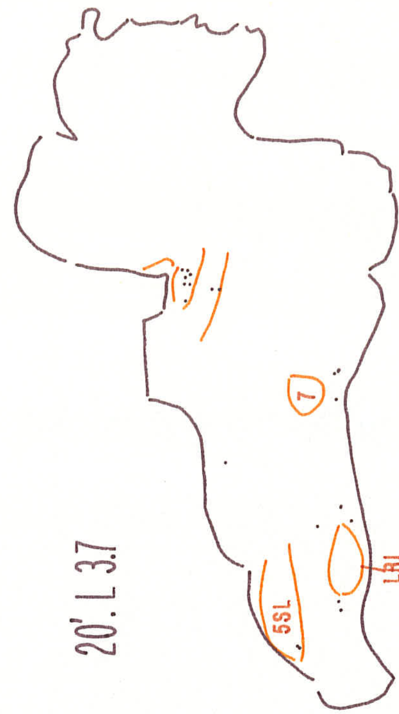

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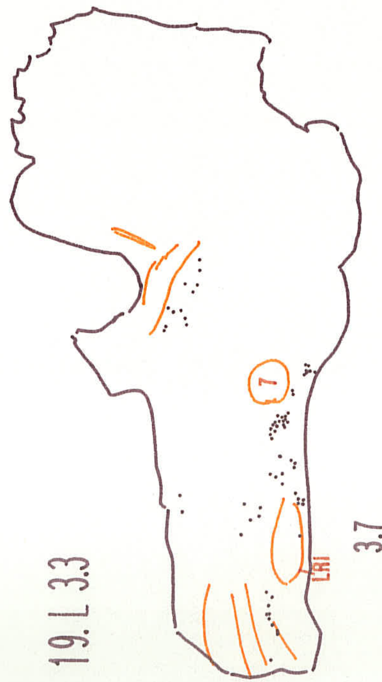


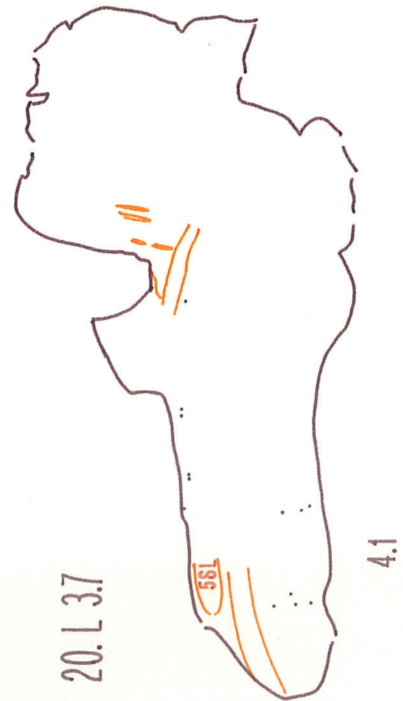
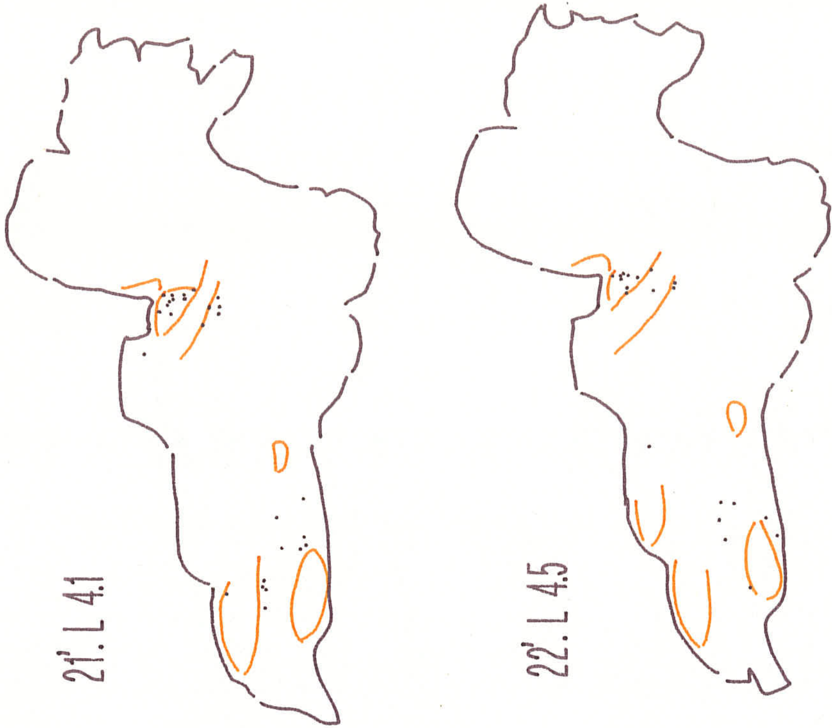


Figure 25



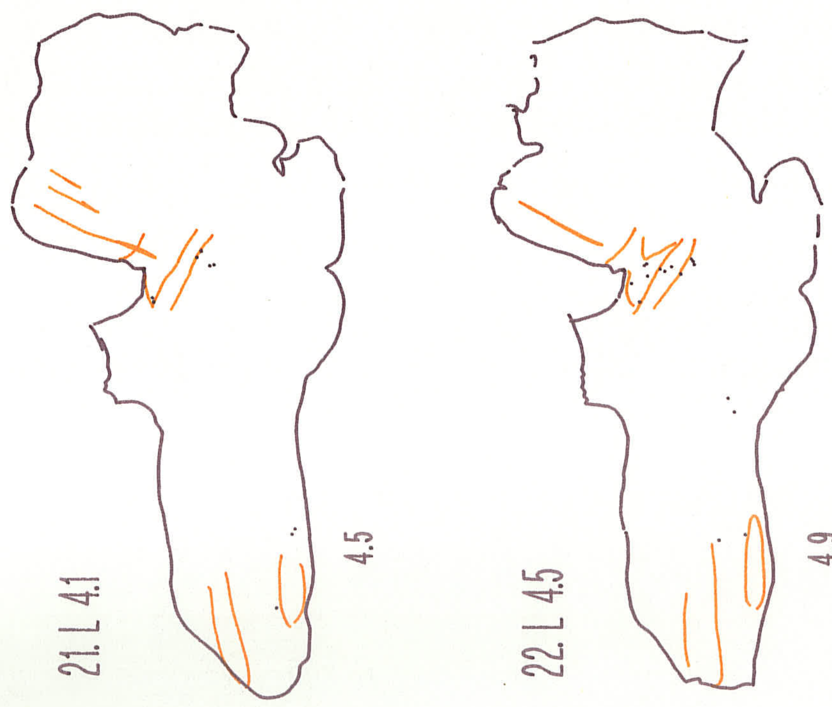
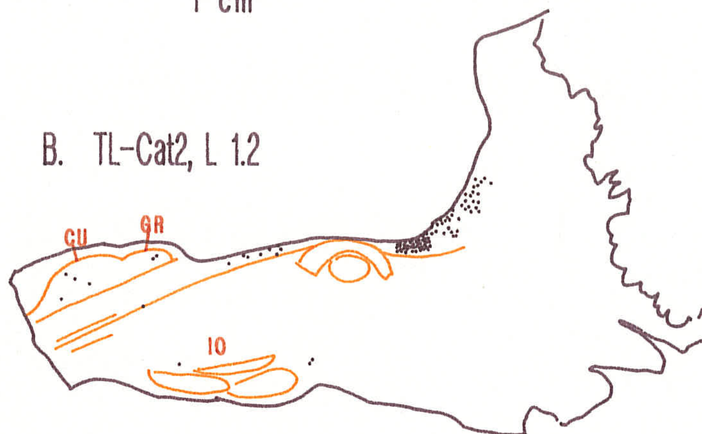


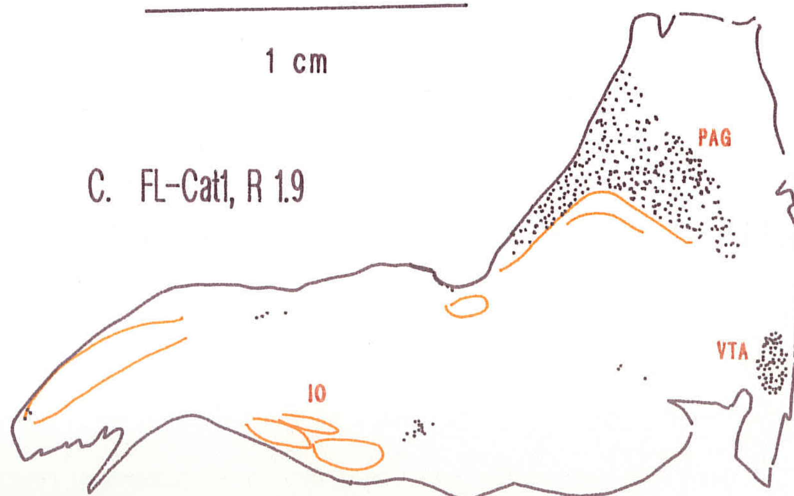
Figure 26

The schematics to show that c-fos labelled cells were observed in the VTA in the FSFL-Cat (A), in the CU and GR in the TL-Cat2 (B), and in the PAG in the FL-Cat1 (C). Labelled cells in the VTA and CU are also shown in C and B separately.

1 cm



1 cm



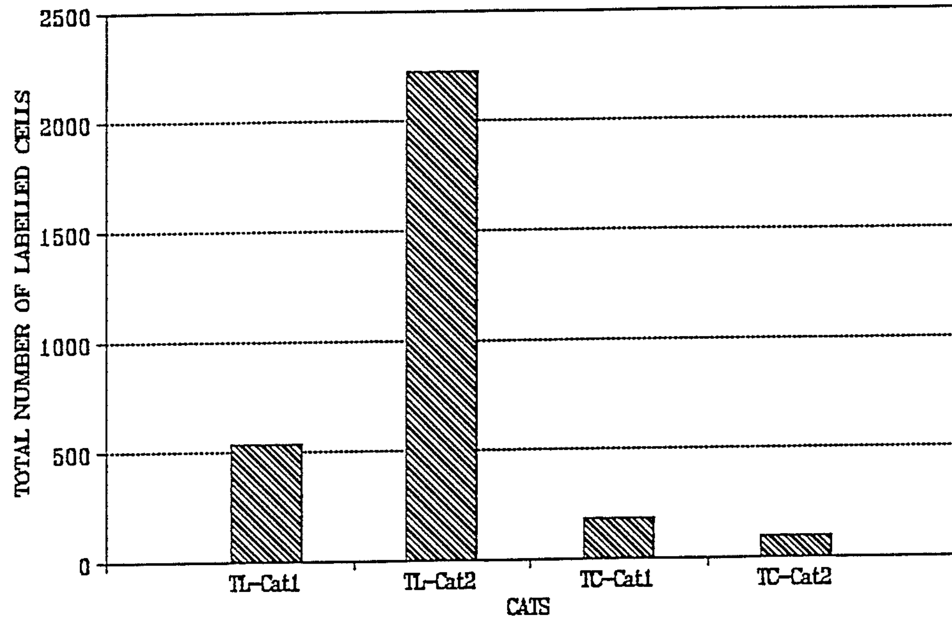
1 cm

Figure 27

Comparisons of total numbers of labelled cells among the MLR-induced locomotor cats and the control cats in the treadmill preparation (A) and in the fictive preparation (B).

The numbers of labelled cells in the locomotor cats are higher than in the control cats in both preparations.

A



B

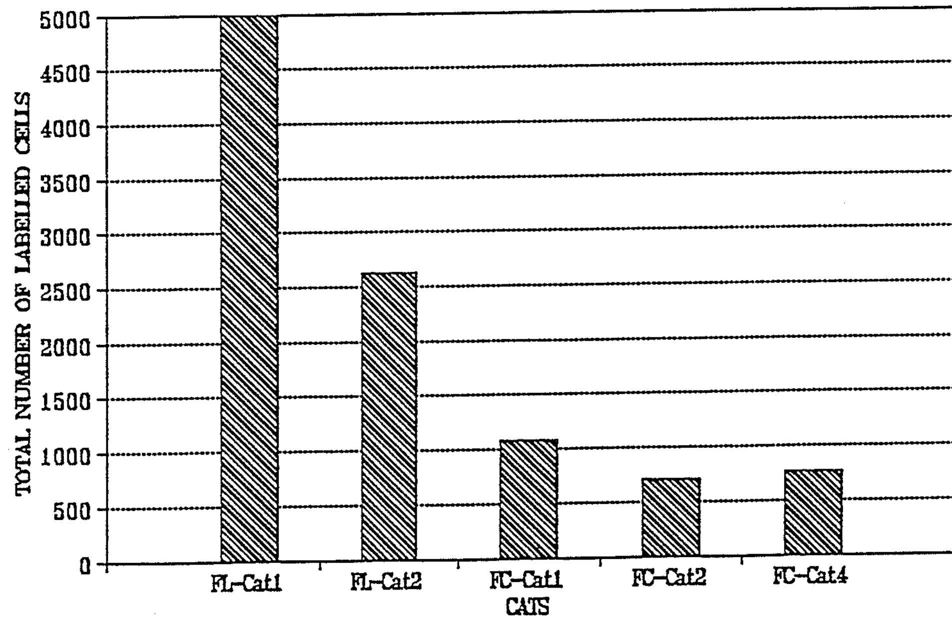
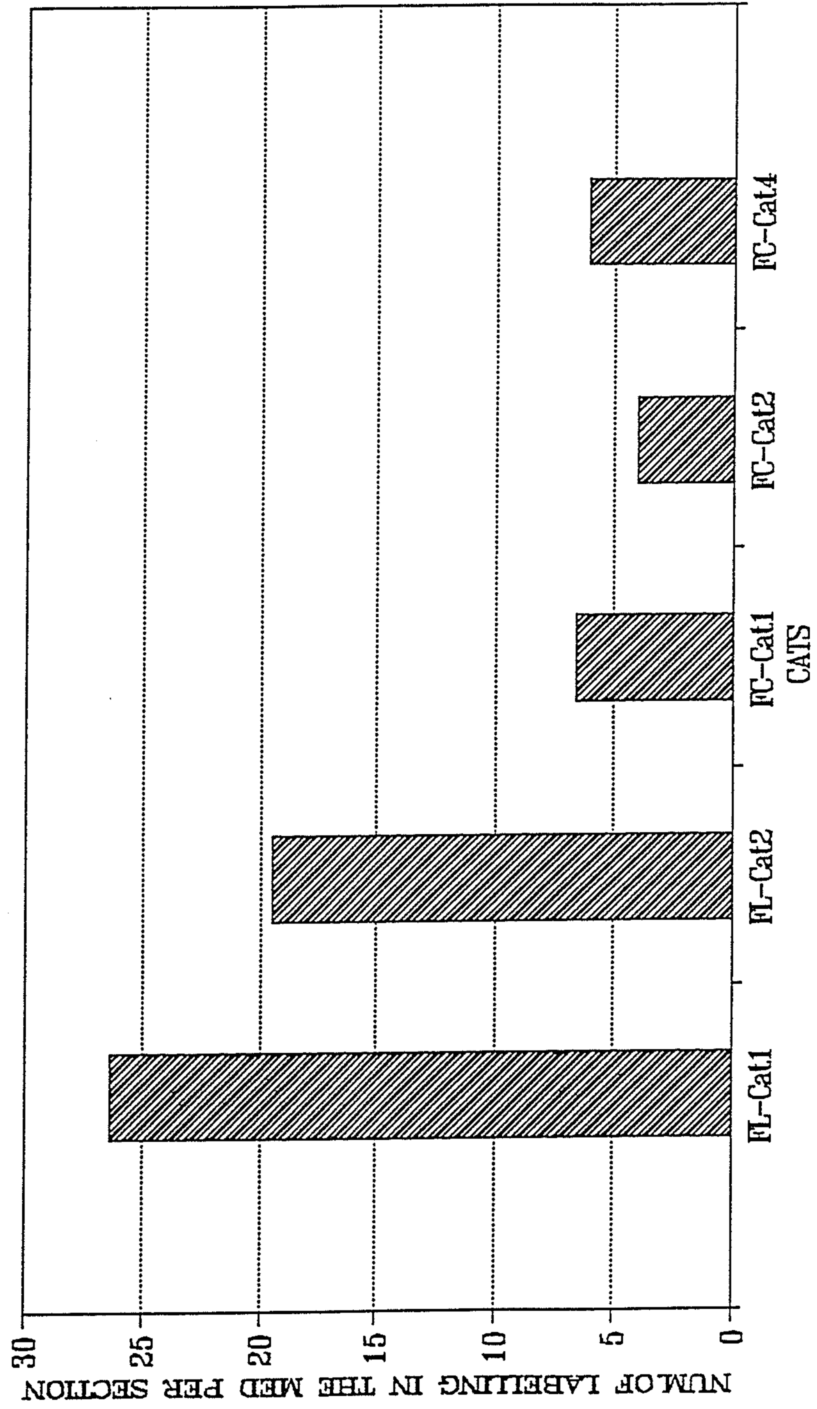


Figure 28

Comparison of the numbers of labelled cells in the MED per section among the MLR-induced fictive locomotor cats and the fictive control cats.

The numbers of labelled cells observed in the locomotor cats are much higher than in the control cats.



NUM.OF LABELLING IN THE MED PER SECTION
0
5
10
15
20
25
30
FL-Cat1
FL-Cat2
FC-Cat1
FC-Cat2
FC-Cat4
CATS

Figure 29

Comparison of the numbers of labelled cells in the LCα per section among the MLR-induced fictive locomotor cats and the fictive control cats.

The numbers of labelled cells in the LCα on the side ipsilateral to the stimulus site are higher than on the contralateral side, and much higher than in the control animals.

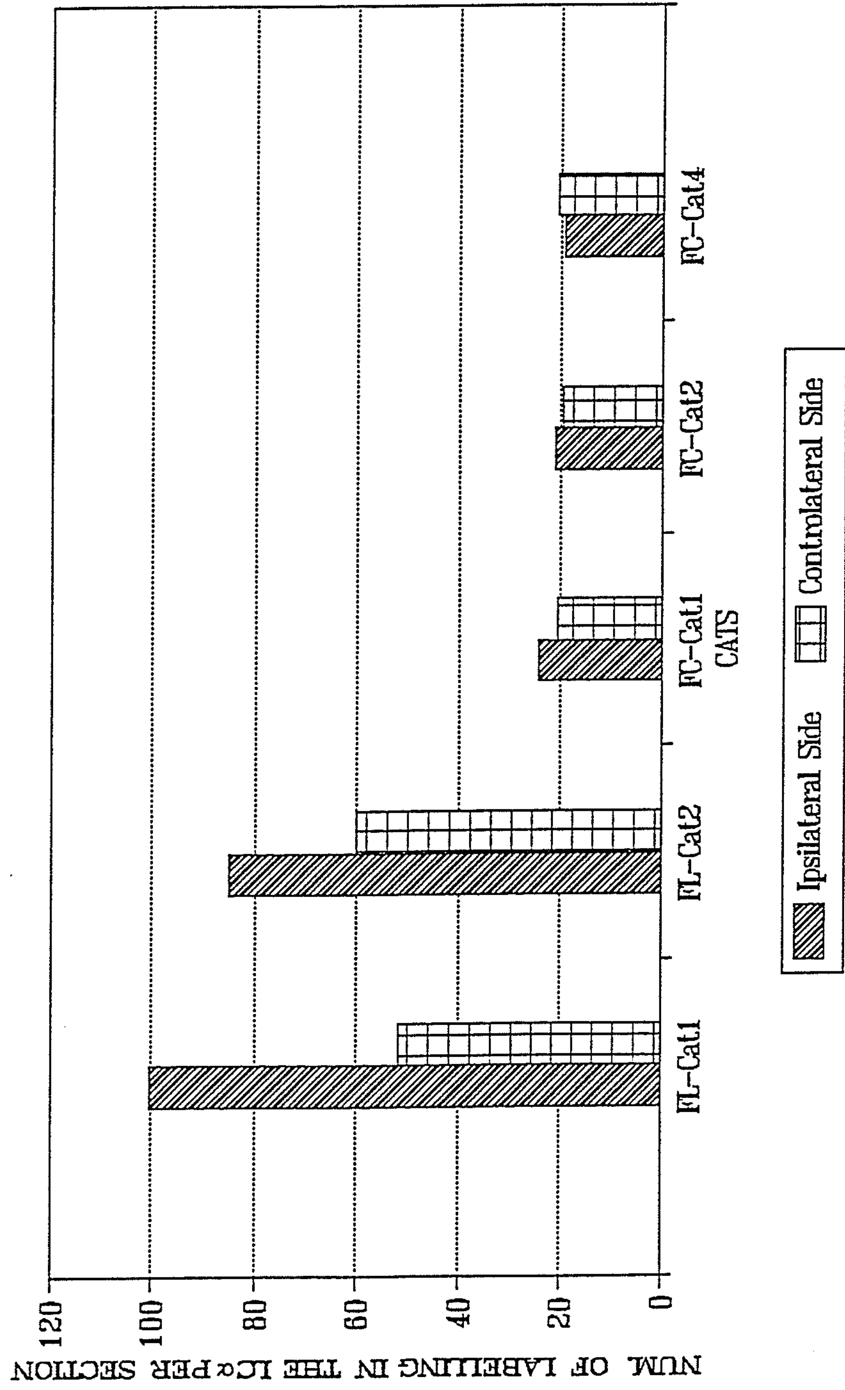

NUM. OF LABELLING IN THE LCα PER SECTION
0
20
40
60
80
100
120
FL-Cat1
FL-Cat2
FC-Cat1
FC-Cat2
FC-Cat4
CATS
Ipsilateral Side
Contralateral Side

Table 1

Total number of labelled cells in the brainstem in each animal.

LOCOMOTION		CONTROL	
Cat Names	Labelled Cells	Cat Names	Labelled Cells
TL-Cat1	593	TC-Cat1	187
TL-Cat2	2219	TC-Cat2	97
FL-Cat1	4997	FC-Cat1	1086
FL-Cat2	2643	FC-Cat2	709
SFL-Cat	458	FC-Cat4	768
FSFL-Cat	1552		

ABBREVIATIONS

5S	spinal trigeminal nucleus
BCM	marginal nucleus of the brachium conjunctivum
ChAT	choline acetyltransferase
CNF	cuneiform nucleus
CU	cuneate nucleus
FTG	gigantocellular tegmental field
FTM	magnocellular tegmental field
GR	gracile nucleus
KF	Kolliker-Fuse nucleus
LC	locus coeruleus
LR	lateral reticular nucleus
MED	medioventral medulla
MLR	mesencephalic locomotor region
MRF	medial pontomedullary reticular formation
PAG	periaqueductal grey
PLS	pontomedullary locomotor strip
PPN	pedunculopontine nucleus
VTA	ventral tegmental area of Tsai
FC-Cat	fictive control cat
FL-Cat	fictive locomotion cat
FSFL-Cat	forelimb swinging induced fictive locomotion cat
SFL-Cat	spontaneous fictive locomotion cat
TC-Cat	treadmill control cat
TL-Cat	treadmill locomotor cat

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