INPUTS TO THE VENTROMEDIAL MEDULLA FROM THE MESENCEPHALIC LOCOMOTOR REGION AND SPINAL CORD: RELATIONSHIP WITH TREADMILL LOCOMOTION INDUCED C-FOS PROTEIN

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BY

XIAO QUAN

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

The present study was designed to investigate the functional and neuroanatomical inputs to the ventromedial medulla (VMM) from the mesencephalic locomotor region (MLR) and spinal cord by using c-fos immunocytochemical and tract-tracing procedures in rats. Treadmill locomotion produces c-fos expression in neurons in the mesencephalic central gray and cuneiform nucleus (MLR) of the rat, and the MLR has reciprocal connections with the VMM. We tested whether cells in the MLR that show activation during locomotion also project to the VMM. Retrograde (Fluorogold) and anterograde tracers (rhodamine-dextran) were injected into the VMM. 7 days later, rats were placed in a treadmill for 60 min. The brain and spinal cord sections were processed for c-fos with immunofluorescence. Results showed that many retrogradely labeled cells were located in the MLR as well as numerous c-fos positive cells in the same region. Very few of these c-fos positive cells were found to project to the VMM and the greatest number of double-labeled cells were located in the rostral MLR and deep mesencephalic nucleus. Retrogradely labeled cells (spinoreticular cells) were identified in lamina V, VI, VII, VIII and X of the spinal cord. Treadmill locomotion induced c-fos protein in spinal cord neurons, but few, if any of these cells were identified as spinoreticular neurons. These results indicate that the spinal cord, MLR and adjacent mesencephalon have reciprocal connections with the VMM. The limited number of double-labeled neurons

in the mesencephalon and spinal cord suggests that the many of the cells that express c-fos protein following locomotion project to areas other than the VMM or else represent interneurons.

INTRODUCTION

1. Role of the Mesencephalic Locomotor Region (MLR), Medial Pontomedullary Reticular Formation (MRF) and Spinal Cord in Locomotion

1.1 The Mesencephalic Locomotor Region (MLR)

It has been almost 30 years since the first report that electrical stimulation of a restricted area in the posterior midbrain induced locomotion in precollicular-postmamillary decerebrate cats (Shik et al. 1966). This area was later termed the mesencephalic locomotor region or MLR and largely corresponds to the cuneiform nucleus and the pedunculopontine region (Berman 1968). MLR stimulation has also been found to produce locomotor behavior in intact cats (Sterman and Fairchild 1966). These results have been confirmed in several laboratories (Steeves et al. 1980, Grillner 1981, Eidelberg et al. 1981, Ross and Sinnamon 1984, Skinner and Garcia-Rill 1984, Coles et al. 1989, Depoortere et al. 1990). More recently, the same results were obtained in rats (Skinner and Garcia-Rill 1984, Milner and Mogenson 1988, Nicolopoulos-Stourmaras and Iles 1984, Melnikova 1975, Coles et al. 1989) and these findings imply that descending influences from the MLR can regulate the activity of locomotor central pattern generators (CPGs) in the spinal cord (Grillner and Dubuc 1988, Mori 1987). This descending influence was described as inducing "controlled locomotion", in that increasing the current

levels of stimulation to the MLR increases the frequency of stepping from a walk to a trot, and finally to a gallop (Shik et al. 1966, Grillner 1981).

It was found by Shik et al. (1967) that the center of the most effective site for eliciting locomotion in cats was situated in an area within 1 mm radius of the stereotaxic coordinates P2, L4, H-1 and this finding has been replicated in later studies (Grillner 1981, Grillner and Shik 1973, Jordan et al. 1979, Mori et al. 1977, Mori et al. 1978, Steeves et al. 1975, Steeves et al. 1980). Besides the "classic" MLR, locomotion can be evoked from stimulation of many other sites in the mesencephalon and lower brainstem. These sites include the pedunculopontine nucleus (PPN), the pontomedullary locomotor strip (PLS), the gigantocellular (FTG) and magnocellular (FTM) tegmental field, the periaqueductal gray (PAG), the lateral parabrachial nucleus (BCM) and the locus coeruleus nucleus (LC) (Shik and Yagodnitsyn 1979, Budakova and Shik 1980, Garcia-Rill 1983, Garcia-Rill et al. 1981, 1983a, Steeves et al. 1975).

It has been reported that several afferent and/or intrinsic chemical systems are involved in modulating locomotion. Infusions of GABA antagonists, biccuculline and picrotoxin (Garcia-Rilll et al. 1985) into the MLR could evoke locomotion. Administrations of muscimol or GABA blocked chemical-induced and electrical-induced locomotion. Infusions of diazepam enhanced the blockade of locomotor activity produced by GABA. Another neuroactive substance found to induce locomotion in the MLR is substance P (Garcia-Rill and Skinner 1987a). Substance P produced a dose-dependent

increase in the length of bouts of locomotion, but the stepping frequency remained the same. Thus, GABA may modulate stepping frequency whereas substance P appears to regulate stepping duration. Injections of other neurotransmitters into the MLR also modulate locomotion (Skinner and Garica-Rill 1993, Milner and Magenson 1988, Brudzynski and Magenson 1985), and together these studies indicate that neuronal elements in the MLR, and not fibers of passage, are responsible for the activation of locomotor behavior.

It has been reported that the pedunculopontine nucleus (PPN), located at the posterolateral border of the brachium conjunctivum, had the lowest threshold for electrical stimulation induced locomotion. (Garcia-Rill et al. 1985, 1987a, 1990, Skinner and Garcia-Rill 1984, Skinner et al. 1990). Neurochemical injections into the PPN modulated locomotor activity (Garcia-Rill et al. 1985, Garcia-Rill and Skinner 1987b). Similarly, bilateral injections of procaine, a local anesthetic, into the PPN decreased exploratory locomotor behavior (Mogenson et al. 1989). On the other hand, injections of glutamate, or the agonists NMDA and kainic acid, produced an increase in locomotor activity (Brudzynski et al. 1986, Milner and Mogenson 1988). The PPN contains two groups of cells with the larger cells group being cholinergic and accounting for about 50% of the cells in the PPN. The smaller cells are mostly GABAergic (Jones 1983). Cytoarchitectonic boundaries of the PPN have been determined by using immunohistochemical techniques to identify cholinergic PPN cells. The distribution of cholinergic PPN cells overlaps

completely with the distribution of effective locomotion inducing brain sites in both cats and rats. It was found that the neurons in the PPN projected to the VMM, and the ascending and descending projections of the MLR match those of the PPN (Skinner et al. 1990 Garcia-Rill 1986, Garcia-Rill et al. 1981,1983a,1983b,1986). Armstrong (1986) suggested using the term of MLR/PPN to describe the locomotor region even though the PPN is only one of several structures in and around the MLR region that may participate in locomotion.

1.2 The Medial Pontomedullary Reticular Formation (MRF)

Our understanding of the neuroanatomical pathway(s) involved in MLR-induced locomotion is not complete. One view is that reticulospinal cells in the MRF receive convergent input from the MLR and provide a descending "command" pathway for the activation of the spinal cord central pattern generators (CPGs) for locomotion (Eidelberg 1980, Eidelberg et al. 1981, Jordan 1986, Orlovsky 1969, Steeves et al., 1980). It has been suggested that this pathway is located within the ventrolateral funiculus (VLF) of the spinal cord (Orlovsky 1970, Armstrong 1986, Gelfand et al. 1988, Grillner 1981, Jordan 1986, Mori 1987).

Steeves and Jordan performed a detailed anatomical study of projection of the MLR using an autoradiographic tracing technique in cats (1984). Radiolabeled proline and leucine were injected into the functionally identified MLR. Descending projections were primarily focused in the ventromedial reticular formation (vMRF), but direct projections to the spinal

cord were not consistently observed. These projections from the MLR to the vMRF, but not to the spinal cord, has been subsequently replicated in both cats and rats (Skinner et al. 1990, Garcia-Rill et al. 1986).

Other evidence in favor of this functional pathway include: A). Stimulation of the ventral aspect of the MRF facilitates locomotion produced by MLR stimulation (Mori et al. 1978) and MRF stimulation produces locomotion in several species (Garcia-Rill and Skinner 1987c, Livingston 1986, McClellan 1986, McClellan and Grillner 1984, Noga et al. 1988, Ross and Sinnamon 1984, Steeves and Jordan 1980); B). Injections of anterograde (Garcia-Rill et al. 1983a) and retrograde tracers (Garcia-Rill et al. 1990, Skinner et al. 1990) into the vMRF region have verified that the MLR projects to the vMRF; C). Both spontaneous and MLR-evoked locomotion is abolished by cooling a restricted area in the MRF and locomotion returns after re-warming the MRF (Shefchyk et al. 1984); D). Injections of cholinergic agonists or substance P into the vMRF are able to induce locomotion whereas vMRF injections of GABA or cholinergic antagonists antagonizes MLR-induced locomotion (Garcia-Rill and Skinner, 1987); E). Lesions in the FTG (gigantocellular tegmental field) and FTM (magnocellular tegmental field) regions produce major locomotor deficits (Zemlan et al, 1983) and lesions of the reticulospinal pathway prevents MLR-evoked locomotion (Steeves et al. 1980).

Garcia-Rill et al. (1986) and Skinner (1990) have extensively studied the ventromedial medulla (VMM), a small restricted area corresponding to the

ventral part of the FTG and FTM in the vMRF. The VMM is about 3 mm long and 1 mm wide in the cat and is 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 tapezoid body). This is consistent with previous neuroanatomical findings reported by Steeves and Jordan (1984). Electrical stimulation of the VMM area produced locomotion in cats (Garcia-Rill and Skinner 1987a, Noga et al. 1988) and rats (Ross and Sinnamon, 1984, Kinjo et al. 1990) in a current-dependent manner, and stimulation of the VMM could also reset the locomotor rhythm (Garcia-Rill and Skinner 1987c). In further support of the VMM participation in locomotion, injections of cholinergic agonists, NMDA and GABA antagonists were all capable of eliciting walking (Garcia-Rill and Skinner 1987c, Kinjo et al. 1990, Noga et al. 1988). Thus, the VMM is a primary relay site for MLR-induced locomotion. These results support the view originally proposed by Orlovsky (1970): a reticulospinal system within the MRF is involved in the production of locomotion induced by stimulation of the MLR.

While it is clear that the MLR can activate locomotion via the vMRF, other evidence indicates that the pontomedullary locomotor strip (PLS) is also involved in locomotion induced by the MLR stimulation (Mori et al. 1977, 1978, Shik and Yagodnitsyn 1977). The PLS is a very small region which has been localized to the lateral tegmentum of the pons and medulla just medial and ventral to the spinal nucleus of the fifth nerve (Mori et al. 1977,1978, Shik and Yagodnitsyn 1977, 1978, Kazennikov, et al. 1979, 1983,

Budakova and Shik 1980, Selionov and Shik 1984), but it has also been suggested that the PLS may be dorsolateral or lateral to the spinal nucleus (Shik and Yagodnitsyn 1977). PLS stimulation has been shown to produce locomotion in decerebrate cats (Kazennikov, et al. 1979, 1983, Mori et al. 1977, 1980, Selionov and Shik 1984, Shik and Yagodnitsyn 1977,1978) and swimming in turtles (Kazennikov et al. 1980, Selionov and Shik 1982). Subthreshold stimulation of the MLR, when combined with subthreshold stimulation of the PLS, produced locomotion (Mori et al. 1977). Likewise, MLR-evoked locomotion was facilitated by stimulation of the PLS (Mori et al. 1978). Thus, locomotor behavior induced by PLS stimulation is similar to that observed during MLR-evoked locomotion (Mori et al. 1977). On the basis of electrophysiological recording techniques, it appears that the PLS is a polysynaptic pathway with cells located either within or medial and ventral to PLS (Shik and Yagodnitsyn 1987, Selionov and Shik 1984). Garcia-Rill et al. (1983) suggested that the PLS corresponds to Probst's tract, which is a fiber pathway descending from the mescencephalic nucleus of the trigeminal nerve (Corbin, 1942).

Direct evidence suggesting that cell bodies in the PLS are actually involved in the generation of locomotion comes from the elegant study by Shefchyk et al. (1984) showing that cooling the PLS region to temperatures that block synaptic, but not axonal transmission, reversibly abolished locomotion produced by stimulation of the medial portion of the MLR. Similarly, injections of excitatory amino acids into the PLS region produced

locomotion (Noga et al. 1988). Despite this evidence, it may be premature to conclude that the PLS is a mediator of MLR-evoked locomotion, in that the results are primarily based upon electrophysiological studies and parallel neuroanatomical data are not available. It has been suggested that the role of the PLS in activating locomotion may be related functionally and anatomically to the spinal nucleus of trigeminal nerve and they are located adjacent to each other. In support, Noga et al. (1988) reported that, same as with PLS stimulation, chemical stimulation of the spinal trigeminal nucleus could also induce locomotion. Finally, it has also been shown that locomotion produced by PLS stimulation can be blocked by reversible cooling the MRF or the VLF, verifying that the MRF/VLF pathway plays a critical role in locomotion whether evoked from the MLR or PLS (Noga et al. 1991).

Another brain area implicated in the MLR-mediated locomotion pathway is the locus coeruleus (LC). The LC is located in the pontine tegmentum at the ventrolateral border of the central gray rostrally. This nucleus consists of two major segments, the dorsal, or compact LC, and a ventral division (Swanson, 1976). Catecholamine (CA) cell bodies are localized in the LC, and it has been suggested that CA cells are activated by MLR stimulation (Steeves et al.1975, Garcia-Rilll and Skinner 1988).

1.3 Spinal Interneurons Involved in Locomotion

Supraspinal structures are capable of inducing rhythmic movement by activating spinal elements. Brown (1911) first demonstrated that the act of progression, which was evoked in the hind limbs after rapid division of the

thoracic spinal cord in decerebrate cats, appeared after deafferentation of the proprioceptive reflexes of both hind limbs. This observation suggested that the rhythmic limb movements of spinalized animals reflected a central spinal process. This hypothesis was confirmed in later studies (Grillner and Shik 1973, Grillner and Zangger 1979, 1984, Jordan et al. 1979). It has also been shown that following transection of the lower thoracic spinal cord in kittens, the hindlimbs appear to be able to generate well-coordinated stepping later in life without the need for electrical stimulation, relative to adults with spinal cord transections (Forssberg et al. 1980a, Forssberg et al. 1980b, Goldberger et al. 1986). The intrinsic neural networks in the spinal cord responsible for the generation of rhythmic movement are termed central pattern generators (CPGs) (Grillner 1975, Grillner and Wallen 1985).

Analysis of locomotor behavior in different species, and closely related motor patterns such as scratching, suggests that sensory feedback and descending pathways strongly influence the output of the CPGs (Grillner 1975, 1981, Delcomyn 1980, Jordan 1991, Rossignol 1995). The exact location and neural organization of the CPGs is still unclear. Grillner and Zangger (1979) showed that the capacity to initiate locomotion in spinal animals is lost after spinal transections below caudal L5. This corresponds to data reported by Deliagina et al. (1983) who showed that L3-L5 segments are responsible for rhythmic movements organized in the lumbar spinal cord. Noga et al. (1995) also found that interneurons in L4-L6 segments are related to the MLR-initiated locomotion as established by means of isopontential mapping.

However, it has also been shown that interneurons in the cervical cord (where all afferent input was abolished) were labeled with the fluorescent probe sulphorhodamine, an activity dependent marker, following NMDA and 5-HT induced locomotion (Kjaerulff et al. 1994). This indicates that the cervical cord is also engaged in generating a locomotor rhythm and is consistent with other findings (Zangger 1981).

A group of interneurons located in the Rexed's lamina VI,VII and dorsal part of lamina VIII of the midlumbar segments have been reported to receive descending commands from the MLR via the vMRF (Edgley et al. 1988) and are rhythmically active during fictive locomotion (Shefchyk et al. 1990). These interneurons also received multimodal peripheral inputs from afferents in muscle, skin and joint nerves, as well project onto motorneurons (Edgley et al. 1988, Edgley and Jankowska, 1987a, 1987b). Using retrograde transneuronal labeling with Wheat germ agglutinin-horseradish peroxidase (WGA-HRP) in the MLR-induced locomotion preparation, the last-order interneurons participating in locomotion were primarily localized in lamina VII of L5-L7 spinal segments (Noga et al. 1987).

A number of investigators have utilized activity-dependent markers in order to localize neurons involved in locomotion. For example, 2-deoxyglucose(2-DG), an activity-dependent metabolic marker, was used to label spinal structures during L-DOPA (dihydroxyphenylalamine)-induced fictive locomotion in low-spinal rabbits. Maximal uptake was found distributed in the intermediate gray matter of L6-S1 (Viala et al. 1988).

Utilizing a fictive locomotion model system, cells around the central canal of the spinal cord showed expression of c-fos protein, another activity-dependent cellular marker to be discussed in more detail below (Dai et al. 1995). Similarly, Jasmin et al. (1994) reported the distribution of c-fos labeled cells in rat spinal cord after walking on a rota-rod. Deafferentation to remove sensory inputs did not eliminate the labeling in the area around the central canal, but labeling in the dorsal horn and ventral horn decreased dramatically. In the in-vitro neonatal rat spinal cord preparation, cells in the medial region (around central canal and intermediate zone, lamina VI-VII and X) of the lumbar cord were labeled by the activity dependent marker sulforhodamine following a period of locomotion induced with NMDA (N-methyl-D-aspartate) and 5-HT (Kjaerulff et al. 1994). As reviewed earlier the distribution of isopotential fields in the lumbar spinal cord in cats after MLR stimulation are consistent with these findings (Noga et al. 1995).

It has been suggested that cholinergic interneurons in the spinal cord are involved in generation of locomotion in invertebrates and some simple vertebrates (Barker et al 1972, Florey 1973, Harris-Warrick RM 1988, Roberts et al. 1986, Chrachri 1990). Bath application of cholinergic durgs to the spinal cord produced rhythmic activity in in vitro brainstem-spinal cord preparation of neonatal rat (Smith et al 1986). It has been reported that a subpopulation locomotor-related commissural interneurons may be cholinergic (Carr et al. 1994). Using immunocytochemical method for c-fos and choline acetyltransferase (ChAT), the enzyme that synthesizes acetylcholine (ACh),

Huang (1995) demonstrated that most of the double labeled cells were located in medial part of lamina XII close to lamina X, and some of those cells scattered to ventral lamina VII, VIII of the lumbar cord during MLR-evoked locomotion in decerebrate cats.

In summary, interneurons in the spinal cord play an important role in MLR-induced locomotion. Identification of the locations and chemical specificity of interneurons that contribute to the CPGs is a necessary step towards understanding the neural basis of locomotion.

2. Use of C-Fos Proto-Oncogene as a Marker for Neuronal Activation

2.1 The Relationship between Gene Expression and Neuronal Activity

It is clear that synaptic activity changes postsynaptic gene expression, and the alteration in gene expression induced by neuronal activation can be divided into two general classes. The first consists of immediate early genes (IEGs) whose transcription is activated rapidly and transiently within minutes of stimulation (Greenberg et al. 1986, Morgan and Curran 1986, Bartel et al. 1989, Barzilai et al. 1989). The second consists of late-response genes whose expression is induced more slowly and over a longer period of time (hours) (Merlie et al. 1984, Castellucci et al. 1988, Goldman et al. 1988, Barzilai et al 1989, Offord and Catterall 1989, Klarsteld et al. 1989). It has been proposed that IEGs encode transcription factors and regulatory proteins that control the expression of late response genes with the late-response genes encoding differentiated neuronal products, such as neuropeptides and neurotransmitter biosynthetic enzymes.

2.2. C-Fos, a Marker of Neuronal Activity

Oncogenes were first identified as the genetic products responsible for the induction of tumors by RNA viruses. The proto-oncogene c-fos is the cellular homologue of the viral oncogene V-fos and is expressed in normal cells in vertebrates. The c-fos proto-oncogene was one of the first IEGs identified by Kelly et al. (1983), and later confirmed by Greenberg and Ziff (1984). The activation of IEGs by extracellular stimuli has been characterized as not being specific to neuronal cells. The IEGs were originally detected in growth factor-stimulated fibroblasts and this work resulted in the subsequent discovery of IEGs in neuronal cell lines (Curran and Morgan 1987, Lau and Nathans 1987). In general, IEGs, such as c-fos, share several characteristics, including that they are generally expressed at very low or undetected levels in quiescent cells, and they are rapidly induced by extracellular stimuli at the transcriptional level. This transcriptional induction is very transient (30-60 minutes) and independent of new protein synthesis. However, new protein synthesis is required for the subsequent termination of transcription and the mRNAs of IEGs are rapidly degraded (appoximately 10-15 minutes in the case of c-fos).

The c-fos gene is the best characterized IEGs at the present time. The c-fos mRNA encodes a protein product, Fos protein, to which an antibody can be produced. Fos protein is rapidly synthesized and translocated to the nucleus where it dimerizes with another protein, Jun. The heterodimeric protein Fos/Jun activates transcription of genes containing AP-1 sites and regulates the expressions of other genes. Fos, therefore has been demonstrated as a useful

marker of functional activity with resolution at the single cell nucleus level (Greenberg and Ziff 1984, Kuruijer et al. 1984, Greenberg et al. 1985, 1986, Curran and Morgan 1986, Curran and Franza 1988). In addition to its induction by growth factors, c-fos has been found to be induced by several other chemicals, including neurotransmitters (Greenberg et al. 1986, Szekely et al. 1989) and agents that cause an influx of Ca^{++} through voltage dependent Ca^{++} channels (Morgan and Curran 1986). Furthermore, c-fos can also be induced by various kinds of stimuli, including seizures (Morgan and Cohen 1987), hypertonic saline injections (Ceccatelli et al. 1989, Sharp et al. 1991), cutaneous stimulation (Hunt et al. 1987), stressful stimuli (Ceccatelli et al. 1989), lipopolysaccharide (LPS) (Wan et al. 1994) and depolarizing conditions (Morgan and Curran 1986).

An important role for c-fos in the nervous system was originally demonstrated by several studies performed in the pheochromocytoma cell line (PC12). Transcription of many IEGs, including c-fos, is dramatically induced by electrical stimulation, neurotransmitters and growth factors (Greenberg et al. 1985, 1986, Morgan and Curran 1986, Bartel et al. 1989). In the presence of nerve growth factor, c-fos transcription is markedly induced by the cholinergic agonist nicotine and by K^{+}-induced depolarization (Greenberg et al. 1985, 1986). The Ca^{++} channel agonist BAY k8644 or external Ba^{+} also rapidly induced c-fos in PC12 cells. Subsequently it has been found that c-fos can be induced in neurons 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). The induction of c-fos in response to synaptic activity was first

illustrated with convulsant drugs, such as metrazole (Morgan et al. 1987, Dragunow and Rebertson 1988, Saffen et al. 1988, Sonnenberg et al. 1989, Watson and Milbranlt 1989) and this response has been detected at both the mRNA and protein level, and can be antagonized by anticonvulsant agents such as diazepam (Morgan et al. 1987) and carbamazepine (Dragunow and Robertson 1987). Inductions of c-fos mRNA also occurs when the motor/sensory cortex is electrically stimulated (Sagar et al. 1988). These initial studies strongly indicated that synaptic activity may mediate postsynaptic gene expression. Perhaps most striking, induction of c-fos immunoreactivity was observed by Hunt et al. (1987) in spinal cord dorsal horn neurons following peripheral sensory stimulation, and by Sagar et al. (1988) in specific brain nuclei following 24 hours water deprivation. Subsequently, Bullitt (1989) reported that noxious stimuli could induce c-fos in spinal cord neurons and more recently, neurons in the brainstem and spinal cord have been shown to express c-fos protein following treadmill locomotion in cats (Dai et al. 1995) and walking on a rota-rod in rats (Jasmin et al. 1994). The evidence that c-fos can be induced by various stimuli in a broad range of neurons suggests that c-fos protein may provide a useful marker of activation of various neurons in the nervous system. However, negative results of no Fos protein production should not be automatically taken to mean that structures or neurons have not been activated by stimulation. It has been shown that although painful stimuli were very effective at inducing Fos in dorsal horn of spinal cord, there was little or no Fos expression in the primary afferent dorsal root ganglia neurons (Menetrey et al. 1989). It has been

known that biochemical messengers including cAMP, calmodulin and G proteins are required for Fos production (Morgan and Curran 1986, Szekely et al. 1987). It is possible that the reason neurons never show Fos elevation is that they lack the required biochemical messengers regulating c-fos expression in neurons.

A variety of complementary methods, including anatomical, cytochemical, physiological, metabolic, and immunohistochemical procedures have been utilized to study the central nervous system. The 2-deoxyglucose (2DG) method has been extensively used for mapping metabolic changes of glucose activity at the regional level. Virtually all types of synaptic stimulation result in increases in 2DG accumulation, one advantage of the 2DG mapping method in comparison to all other techniques currently available. Extension of this method to the cellular level, however, has been limited and interpretion of 2DG studies is severely restricted by the inability to identify individual cells.

C-fos immunostaining gives single cell resolution and it potentially identifies neurons activated by specific stimuli. It has been demonstrated that electrical stimulation of rat sensory/motor cortex induces Fos protein immunostaining in regions known to connected with motor/sensory cortex by mono- or polysynaptic pathways, and Fos immunostaining in granule and purkinje cell nuclei occured in cerebellar "microzones" which correspond to parallel results found with 2DG autoradiography (Sagar et al. 1988). These data suggest a strong correlation between the expression of c-fos and ongoing neuronal activity, and indicate that Fos immunostaining may provide a

powerful tool for mapping the pattern of postsynaptic neuronal activation with single cell resolution.

3. Anterograde and retrograde neuroanatomical tracers

Anterograde and retrograde neuroanatomical tracers have been used extensively to define the cell bodies of origin and axonal projections within the central nervous system. Tetramethylrhodamine-conjugated dextran (TMRD), an anterograde tracer, and Fluoro-Gold, a retrograde tracer, have been utilized previously to facilitate the detailed visualization and analysis of projection fibers and regions of termination after these tracers were transported from specific central injection sites (Nance and Burns, 1990, Lavail et al. 1993, Hoover and Durkovic 1992). It has been shown that TMRD was axonally transported by neurons predominantly in an anterograde direction and produced fiber and terminal labeling comparable to another anterograde neuronal tract tracer, phaseolus vulgaris leucoagglutinin (Nance and Burns 1990). Additionally, TMRD can be axonally transported over a long distance, such as from hypothalamus to spinal cord, or from visceral motor and sensory neurons peripherally to their target organs. The ability of TMRD to be fixed by formaldehyde provides flexibility not afforded by some other fluorescent tracers and provides a simple and convenient processing procedure. Free-floating sections can be stored at 4°C for several days before being floated out, counterstained and coverslipped or else processed for immuncytochemistry with minor leakage of this tracer from labeled structures. On the other hand,

Fluorogold, a retrograde tracer, has a number of characteristics that make it especially useful for tract-tracing in the central nervous system including its intense yellow/white fluorescence; its retention in cells with a variety of fixations and embedding conditions and finally there is limited tracer diffusion at the injection site with limited cell necrosis (Lavail et al. 1993). The use of these neuronanatomical tract tracers, combined with c-fos immunocytochemistry provides powerful analytical capabilities previously unattainable.

In summary, although there exists an extensive literature regarding the physiological functions of the MLR, MRF and spinal cord in the control of locomotion, our knowledge with regard to the neuroanatomical organization of locomotion still remains incomplete, and detailed information on the neuroanatomical organization of brainstem and spinal cord areas involved in locomotion has not been provided. The purpose of the present study was to provide new information on the location of brainstem and spinal cord neurons as well as the neurochemical specificity of the spinal target interneurons involved in locomotion using c-fos, ChAT immunocytochemical and tract-tracing methods. The triple-labeling technique and the rat treadmill locomotion model that has been used in the present study permitted the simultaneous analysis of complex neuronal networks involved in locomotion at the organizational, and more importantly, at the cellular level.

MATERIALS AND METHODS

Experimental Animal

Experimental animals (n=51) for the present studies were adult female and male Sprague-Dawley (S/D) rats weighing from 150 to 550 grams (Charles River Laboratories, Dorval, Que.). All animals were housed in groups of 2-3 per plastic cage in a reverse light room (12/12 lighting schedule). Tap water and Purina Rat Chow were provided *ad libitum.*

Anaesthetic

All surgical procedures were carried out under Somnotol (Sodium pentobarbitol, 60mg/kg, MTC Pharmaceutical, Cambridge, Ont.) anaesthesia. The anaesthetic was administrated intraperitoneally using a sterile tuberculin syringe.

Fluorescent Tracers

Fluoro-Gold was purchased from Fluorochrome (Englewood. CO) and Tetramethylrhodamine dextran (10,000 mol.wt, lysine fixable) was purchased from Molecular Probes (Eugene, OR). The dyes were dissolved in distilled water or 0.1M phosphate buffer (PH=7.2) and stored at 4°C.

Injection Procedure

i). vMRF (Ventromedial Reticular Formation) Injections:

1). Anesthetized rats (n=12) were placed in a Trent Wells stereotaxic apparatus with the incisor bar placed at 2.0 cm below the horizontal zero (interaural line, Paxinos and Watson 1986), 2). a sagittal incision of approximately 1.5 cm in length was made and the skin retracted to expose muscle, 3). the muscle and connective tissue were dissected. 4). the brainstem region was exposed via an incision in the atlanto-occipital membrane and dura, the glass micropipete held at 60^0 (to horizontal line) was aligned to the brainstem obex region and then lowered to the vMRF (Reticulospinal region, 3.55 mm below dura), 5). a mixture of 5% TMRD and 0.5% FG was injected iontophoretically 7 seconds on 7 second off at 8.75mA, positive current (Precision Current Source, Steoelting, Co. IL) for 60 min, and 6). the micropipette was then retracted from the brainstem, the opening packed with gel-foam and the incision closed with stainless steel woundclips.

ii) MLR (Mesencephalic Locomotor Region) Injections:

1). Anesthetised rats (n=8) were placed in the same stereotaxic apparatus with the incisor bar raised to 2.4 mm above the horizontal zero (interaural line, Paxinos and Watson 1986.), 2). a sagittal incision about 2.5 cm in length was made, the skin retracted to expose the skull, 3). the skull was scraped to remove the connective tissue, 4). the glass micropipette was then aligned with bregma, 5). and then positioned to the MLR (coordinates to

Bregma: A-P -7.0mm, L-M 1.7mm), the position was marked on the skull and a small opening in the skull was made with a dental drill. 6). the glass micropipette was lowered through the dura to the MLR (5.3mm below dura), 7). and then 0.5% FG and 5% TMRD were iontophretically (7 seconds on 7 seconds off) injected into the MLR for 60 min. 8). the micropipette was slowly removed, the opening in the skull packed with gel foam and the incision closed with stainless steel woundclips.

Treadmill Locomotion

Following one week survival periods, all rats were placed on a motorized rat treadmill (Columbus Instrument international Co. Columbus, OH.) equipped with a shock-grid at the rear. The speed was increased from 0 to 0.25 meter/sec. over a 5 min period and then held at this level for an addition 55 min. During the treadmill locomotion task, little or no signs of fatigue were observed and all animals completed the behavioral task and required very few shocks. Control animals (n=3) were either subjected to the same surgical procedure and placed in the apparatus for 60 min with the treadmill turned off or were home cage controls.

Histological Procedure

At the end of the 60 min treadmill session, animals were overdosed with sodium pentobarbital and perfused transcardially using 120ml of 1.0% sodium nitrite followed by 300-500ml of 4.0% fresh paraformaldehyde in 0.1M

phosphate buffer (PH=7.2). Brain and spinal cord were removed, post-fixed for 3-4 hours and then cryoprotected in 30% sucrose in 0.1M phosphate buffer (PH=7.2) for 48 hr. Frozen sections of brainstem/spinal cord segments were cut at 40μm coronally or horizontally on a freezing microtome, collected into 24 well culture plates containing 0.01M phosphate buffer saline (PBS) and rinsed for 30 min on an aliquot rocker (Miles). Every one in two sections were floated immediately onto subbed slides and the injection sites were verified. The remaining alternate sections were utilized for immunocytochemical procedures.

Immunocytochemical Procedure

Frozen sections through the MLR, vMRF, and three levels of the spinal cord (cervical, thoracic and lumbar) were collected, and rinsed in 0.01 M PBS three times (3x10min). Tissue sections were incubated in an affinity purified polyclonal rabbit anti-c-fos antibody (Santa Cruz Biotechnology, Inc.) which was diluted 1:5000 in 0.01M PBS+ 1.0% Triton X100 (T-octylphenoxypoly-ethoxyethanol)+ 1.0% NGS (normal goat serum, Cappel Research products) + 2% BSA (Albumin, Bovine. Sigma Chemical Co.). Sections were gently agitated on a aliquot rocker overnight (12-18 hours) at room temperature.

Subsequently, c-fos immunoreactivity was demonstrated by using one of following immunofluorescent procedures:

Procedure 1:

i) Tissue sections were rinsed three times (3x10min) in 0.01M PBS, incubated in biotinylated GAR (goat anti-rabbit IgG, Dimension Laboratory Inc.) diluted 1:150 in 0.01M PBS 1% Triton X 100 +1.0% NGS for 90 min.

ii) Sections were rinsed in 0.01 MPBS three times (3x10min) and then incubated in an avadin-fluorescein conjugate (20μg/ml, Molecular Probes Inc.) diluted in 0.01M PBS +1% Triton X 100 +1.0% NGS for 120 min.

Procedure 2:

Tissue sections were rinsed three times (3x10min) in 0.01M PBS, incubated in goat anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories Inc. PA) diluted 1:1000 in 0.01M PBS +1% Triton X 100 + 1.0% NGS for 4 hr.

All sections were then rinsed 3 times (3x10min) in 0.01M PBS, mounted onto gelatinized slides, air dried and coverslipped with fluoromount (BDH laboratory, England) after dipping them into xylene for 5 min.

For the c-fos and ChAT sequential double staining, c-fos immunoreactivity was detected using the PAP (Peroxidase-anti-Peroxidase) procedure as follows:

1. Selected sections were incubated overnight in anti-c-fos anitbody as described above, then sections were rinsed three times (3x10min) in 0.01M PBS, incubated in GAR (Cappel, Scarborough, Ont.) diluted 1:150 in 0.01M PBS+ 1% Triton X 100+1.0% NGS for 90 min.

2. Sections were again rinsed three times in 0.01M PBS and incubated for 90 min in rabbit PAP (Cappel, Scarborough, Ont.) diluted 1:300 in 0.01M PBS+ 1% Triton X 100+1.0% NGS .

3. Sections were then washed (3x10min) in 0.01M PBS and transfered to plates containing the chromogen diaminobenzidine, d-glucose, and enhancers (nickel ammonium sulphate and cobalt sulphate) for 5 min, after which glucose oxidase (Sigma, St. Louis, MO) was added to the solution. The peroxidase reaction proceeded for 30-40 min at room temperature and subsequently sections were rinsed three times in 0.01M PBS.

ChAT (choline acetyltransferase) immunoreactivity was demonstrated by the PAP procedure as follows:

1. Sections were incubated in anti-ChAT (rabbit polyclonal, Chemicon Inc.) diluted 1:1000 in 0.01M PBS+ 1% Triton X 100+ 1.0% NGS+ 2% BSA. Sections were gently agitated on a aliquot rocker overnight (12-18 hours) at room temperature.

2. Tissue sections were rinsed three times (3x10min) in 0.01M PBS, incubated in GAR (Cappel, Scarborough, Ont.) diluted 1:150 in 0.01M PBS + 1% Triton X 100 +1.0% NGS for 90 min.

3. Sections were again rinsed three times in 0.01M PBS and incubated for 90 min in rabbit PAP (Cappel, Scarborough, Ont.) diluted 1:300 in 0.01M PBS+ 1% Triton X 100+1.0% NGS.

4. Sections were then rinsed in 0.01M PBS and transfered to plates containing the chromogen diaminobenzidine and d-glucose for 5 min after

which glucose oxidase was added to the solution. The peroxidase reaction was visualized following 30-40 min and sections were rinsed, floated onto subbed slides, air dried, cleared in alcohol/xylene, and coverslipped with permount (Fisher Sci). The c-fos labeled cells showed a black cell nucleus whereas the ChAT positive cells showed a brown cytoplastmic staining.

Sections were viewed and photomicrographed with an epifluorescence/light microscope equipped with X/Y movement sensitive stage and a camera. A neurolucida image analysis system (MicroBright Field Inc. USA) was used to draw outlines of sections at low magnification and to plot retrogradely labeled neurons at high magnification.

RESULTS

Brain sections were examined with either epifluorescence or light microscopy and representative sections were selected for microphotography. Cells labeled with c-fos or FG and terminals and fibers labeled with TMRD were determined by alternate observation of the same region of the section using the appropriate filter cubes (UV, FITC and Rhodamine) for visualization. Locations of retrogradelly labeled FG neurons were drawn and plotted using a neurolucida image analysis system (Microbright Field Inc. USA).

1. vMRF Injection Experiment

Fig. 1 and Fig. 2 illustrate an injection site in the vMRF, focused in the gigantocelluar reticular nucleus, alpha (GiA) and located caudal to the trapezoid, but rostral to the inferior olive. This site is also shown in the neurolucida drawing (Fig. 3).

1.1 Distribution of FG and TMRD labeling in the MLR and spinal cord

1.1.1 MLR

The general distribution of retrogradely labeled FG cells in the brainstem mesopontine region was similar in all 12 rats with injection sites that were located in the vMRF. Numerous FG labeled cell bodies were concentrated bilaterally in the mesencephalic central gray and adjacent

cuneiform nucleus, corresponding to the MLR of the rat. As shown in Fig. 3, cells in the MLR projecting to the vMRF appear to be part of a continuous column of cells that extend rostrally to include the deep mesencephalic reticular nucleus. FG labeled cells were also observed in the lateral parabrachial n. (LPB), lateral parabrachial superior, n. (PLBs) and mesencephalic trigeminal n. (Me5). Another large group of FG labeled cells extended ventral and laterally into the ventral tegmental area. These cells were located in the pontine reticular n. oral (PnO), the pedunculopontine tegmental n. (PPT) and ventral lateral tegmental n. (VLTg). The neurolucida drawings for a representive animal illustrates the distribution of the FG labeled cells in these regions (Fig. 3). A photomicrograph of the cuneiform nucleus showed abundant white/blue cells labeled with FG (Fig.4). Fig. 5 is a double exposure photomicrograph of the MLR and illustrates anterogradely TMRD labeled fibers and terminals localized in the MLR. These fibers and terminals were observed in close proximity to FG labeled cells (white/blue), suggesting that neurons in the vMRF project onto many of the cells that project to the medulla.

1.1.2. Spinal cord

Retrogradely labeled FG cells were observed at the cervical, thoracic and lumbar levels of the spinal cord. These cells were bilaterally distributed and primarily located in laminas V, VI, VII, VIII, and X of the spinal cord. A neurolucida drawing of the distribution of these FG labeled spinoreticular

cells in the lumbar region is illustrated in Fig 6. Anterogradely TMRD labeled fibers and terminals were consistently observed in the ventrolateral funiculus (VLF) throughout the spinal cord bilaterally. Numerous TMRD labeled terminals and fibers were also observed in laminas V-X of the cervical cord, with fewer in the thoracic cord (focused in the intermediolateral region) and very few fibers observed in the lumber cord (Fig.7).

1.2 Distribution of double-labeled c-fos positive cells, FG labeled cells and TMRD labeled terminals and fibers

1.2.1 MLR

Numerous c-fos positive cells were localized in the MLR (cuneiform nucleus and adjacent central gray) following 60 min treadmill locomotion. In contrast, very few labeled c-fos positive cells were observed throughout the midbrain in rats not exercised in the treadmill (Fig 8). An example of a double exposure photomicrograph shows both retrogradelly labeled FG cells and c-fos positive cells (stained red with Cy3) in Fig. 9. C-fos protein was also detected in the locus coeruleus of the same animal (results not shown). A small proportion of these c-fos positive cells in the MLR were found to be also labeled with FG in all 12 rats following FG and TMRD injections and 60 min treadmill locomotion. Other c-fos positive cells were distributed among the numerous FG cells in the MLR, as shown in Fig 10. An increase in the number of double-labeled neurons was observed in the sections located more rostral to the cuneiform nucleus (MLR region, Bregma: -8.72 mm). This is

illustrated in the three photomicrographs taken from the caudal to the most rostral extent of the MLR. For example, percentages of the retrogradelly labeled FG cells in these photographs identified as also showing c-fos protein were 13%, 24% and 37%, respectively (Fig. 11,12,13). Thus the highest number of double-labeled neurons were located in the most rostral extent of the MLR, or deep mesencephalic reticular nucleus. Similarly, an obvious increase in the number of double labeled neurons was observed under microscopy in all 12 rats. Fig 14 is a triple-exposure photomicrograph of the MLR region and simultaneously demonstrates anterograde terminal labeling, retrograde cell bodies labeling and c-fos immunofluorecence. The red fibers represent axons and terminals from the vMRF, blue cells in the same region are FG cells retrogradely labeled from the vMRF and the c-fos nuclei are green. Note that the terminal labeling is distribuated in close proximity to both the c-fos positive cells and FG cell bodies and their process.

1.2.2. Spinal Cord

We detected a large amount of c-fos positive cells in laminae I-VI of the dorsal horn, intermediate zone (VII, X) and ventral horn (VIII, IX), bilaterally, following 60 min treadmill locomotion in 6 rats (Fig. 15). The expression of c-fos protein in the spinal cord of the control animals was minimal in comparison to the treadmill exercised rats (Fig. 16). We observed few, if any, double-labeled cells located in the medial lamina VII and X in the cervical, thoracic or lumbar cord. A double-exposure photomicrograph illustrates that

there was extensive overlap in the distribution of FG labeled spinoreticular neurons and neurons expressing locomotor-induced c-fos protein in the cord (Fig. 17). In horizontal sections, there were clusters of both FG labeled cells and c-fos positive cells located in the area around the central canal in L3-5 spinal levels (Fig 18). Anterogradely labeled TMRD fibers and terminals are illustrated in Fig. 19, and these terminals were distributed in close proximity with many c-fos positive neurons (stained red with Cy3).

1.3 Distribution of double-labeled c-fos and ChAT positive cells

ChAT positive cell bodies in coronal sections were present throughout the spinal cord with cells concentrated in the ventral horn (VIII, IX), the intermediate zone (VII, X) and some small cells scattered in the dorsal horn (III-V) in 2 rats. The double-labeled c-fos and CHAT positive cells in the cervical spinal cord are shown in Fig. 20. These cells were identified in laminae VII, VIII and X of the spinal cord, bilaterally in 2 rats.

2. MLR Injection Experiment

Fig. 21 illustrates the MLR injection site in which a mixture of 0.5% FG and 5% TMRD was iontophoreticlly injected.

2.1 Distribution of the FG cells and TMRD labelling in the medulla

The general distribution of anterograde and retrograde labeling in the brainstem was similar in 2 rats with injection sites that were located in the MLR (Interaural 1.50 mm). In caudal brainstem, anterogradelly labeled red fibers and terminals were distributed bilaterally in the medial pontomedullary RF. Numerous retrogradelly labeled cells were observed bilaterally in the gigantocellular nuclei, parvocellular reticular nuclei, gigantocellular nuclei, alpha., and pontine reticular nuclei.

2.2 Relationship with c-fos expression

Following 60 min treadmill locomotion, c-fos positive cells were detected in the vMRF, but few, if any, were double-labeled with FG and c-fos protein. Note that these c-fos positive cells were distributed around the FG labeled cells, and the TMRD labeled terminals and fibers were in close proximity to both the c-fos and FG labeled cells (Fig. 22).

In summary of the interconnections between the mesencephalon, vMRF and spinal cord, based on neuroanatomical tract tracing and activity dependent c-fos immunocytochemistry, are illustrated in Fig 23.

DISCUSSION

1. Reciprocal Connections between the MLR and vMRF in Locomotion

Shik et al. (1966) first reported that electrical stimulation of the MLR could produce locomotion on a treadmill in the precollicular-postmamillary decerebrate cats, suggesting the MLR plays an important role in mediating locomotion. Since then, many investigators have been concentrating their efforts on this area and extensive research data regarding the functional effects of stimulation of the MLR in various animal models have been reported (Sterman and Fairchild 1966, Skinner and Garcia-Rill 1984, Milner and Mogenson 1988, Coles et al. 1989). It is clear that descending projections from the MLR can modulate the activity of the CPGs in spinal cord via the vMRF (Grillner and Dubuc 1988, Mori 1987, Orlovsky 1970). One of the specific aims in the present study was to provide new evidence to further understand the neuroanatomical pathway(s) between the MLR and the vMRF in locomotion.

Numerous retrogradely labeled FG cells were found in the mesencephalic central gray and adjacent cuneiform nucleus, corresponding to the MLR, following a mixture of 0.5% FG and 5% TMRD injection into the vMRF in rats (Fig 3). It was noted that cells in the MLR projecting to the vMRF appear to be part of a continuous column of cells that extend rostrally to include the deep mecensephalic reticular nucleus. Besides the "classic MLR", many other sites such as PPN, PLS, BCM and LC could produce

locomotion when stimulated (Garcia-Rill 1983b, Garcia-Rill et al. 1981,1983d, Steeves et al. 1975). Consistent with this, we observed that FG labeled cells were also located in the PLBs, LPB, Me5, PnO, PPT and VLTg in the brain stem. Simultaneously, numerous anterogradely labeled TMRD fibers and terminals were localized in the MLR and these fibers and terminals were located in close proximity to the FG labeled cells (Fig 5). Electrophysiological evidence for monosynaptic projections from the MLR itself to the vMRF was obtained by Orlovsky (1970). Edwards (1975) showed that descending projections from cuneiform nucleus were distributed in the FTG and FTM. Following injection of anterograde tracers into the physiologically identified MLR, labeled terminals were found in the ventromedial medulla (Garcia-Rill et al. 1983c). Retrogradely labeled cells were found in the paribrachial, central gray, cuniform nucleus, PPN, and PnO following injection of flurescent dyes in the vMRF (Garcia-Rill et al. 1986). Subsequent studies confirmed these findings (Livingstone et al. 1993, Garcia-Rill et al. 1987). It has been shown that reticulospinal cells located in the vMRF project to the MLR using retrograde and anterograde tracers (Livingstone et al. 1993). The observations in the present study that numerous FG labeled cells and TMRD labeled terminals and fibers were detected in the vMRF regions in the experiments with the MLR tracers injections, and the same labeling pattern being observed in the MLR regions following the vMRF tracers injections clearly illustrated that there exist reciprocal connections between the MLR and the vMRF in rats. We also observed numerous c-fos positive cells in the MLR as well as in

the vMRF following locomotion. Previous studies in our laboratory showed that the number of c-fos positive cells in the brainstem of rats subjected to footshock was reduced in comparison to the c-fos positive cells observed in the treadmill locomotion rats (Livingstone, 1995). These data illustrated that the MLR and the vMRF are important regions in regulating locomotion activity in our intact, conscious and treadmill locomotion rat model.

2. Reciprocal Connections between the vMRF and the Spinal Cord in Locomotion

The presence of fibers from the vMRF of the brainstem to the spinal cord was established by the neuroanatomical and physiological studies. (Brodal 1957, Peterson et al. 1975, Maunz et al. 1975). Reticulospinal cells from the vMRF project to the lumbar or thoracic cord as well as give off branches to the cervical cord via the ventral and/or lateral funiculus. These findings were confirmed by other investigators using anterograde tracers (Mortin et al. 1979, Westland and Coulter 1980) and eletrophysiological techniques (Garcia-Rill and Skinner 1987). It has been shown in cats that terminations of the reticulospinal fibers in the vMRF mainly located in spinal lamina VII, VIII and IX (Nyberg-Hansen 1965, Petras 1967, Kuypers 1975). Consistent with this, we observed anterogradely labeled fibers in the ventral and lateral funiculus throughout the spinal cord and terminals in the lamina V-X of the cervical cord in rats (Fig.23). Simultaneously numerous retrogradelly labeled FG cells (spinoreticular) cells throughout the cervical, thoracic and lumbar cord were

mainly located in lamina V, VI, VII, VIII and X. It has been demonstrated that the majority of the spinoreticular fibers terminates in the vMRF (corresponding to the gigantocellularis reticularis nucleus) and the pons (pontis caudalis and oralis) in anatomical studies (Rossi and Brodal 1957). Our data indicate that the spinoreticular cells in lamina V, VI,VII,VIII and X in the cervical, thoracic and lumbar segments are responsible for the projections from the spinal cord to the vMRF. The observations of TMRD labeled terminals and fibers in the present study provide further proof of the existence of a projection pathway from the vMRF to the spinal cord. Taken together, these data strongly suggest that reciprocal connections between the vMRF and the spinal cord exist in rats.

A large number of c-fos positive cells were located in the dorsal horn (Lamina I-VI), intermediate zone (VII, X) and ventral horn (VIII, IX) bilaterally following 60 min treadmill locomotion (Fig 15). It has been recently reported that c-fos labeled cells were found concentrated in laminae III, IV, VII, VIII and X of lumbar cord in cats after treadmill locomotion (Dai et al. 1995). Walking for 1hr in a rota-rod induced c-fos expression in dorsal horn, lamina VII, X, and ventral horn in lumbar and cervical spinal cord (Jasmin et al. 1994). The present findings are in close agreement with these data and confirm that the neurons in dorsal horn, intermediate zone, and ventral horn are activated during locomotion. We anticipate that following deafferentation the c-fos positive cells in both the dorsal and ventral horns will dramatically reduce.

3. Localization of Double-Labeled Neurons (FG and C-fos)

Although the combination of applying c-fos immunocytochemistry and the anterograde and retrograde tracers techniques has numerous advantages for the characterization of neuroanatomical organization of locomotion, few studies to date have combined these methods in the same experimental preparation, especially with regard to locomotion.

As discussed above, large number of FG-labeled cells, c-fos positive cells and TMRD-labeled fibers and terminals were detected in various of regions of the MLR, the vMRF and the spinal cord. However, only a small proportion of these cells were double-labeled cells (c-fos and FG) in the above regions. In general, c-fos positive cells were distributed in close proximity to the FG-labeled cells and TMRD labeled terminals and fibers. In another word, there was extensive overlap in the distribution of the c-fos positive cells and FG-labeled cells in the MLR, the vMRF and spinal cord (Fig 9,10,14,18). A highly speculative interpretation of these data is that many of these c-fos positive cells not labeled with FG represent interneurons. For example, these interneurons in the spinal cord could send signals to the spinoreticular cells projecting to the vMRF, and/or to the motor neurons. Interneurons in the MLR, on the other hand, may modulate the activity of neurons projecting to the vMRF. Interneurons in the MLR and spinal cord have already been suggested to play an important role in regulating locomotion (Jordan 1986, Noga et al. 1987, 1995, Edgley et al. 1988, Edgley and Jankowska 1987a,1987b). The MLR has also been demonstrated to project to other brain areas, such as the motor and premotor

cortex (Garcia-Rill et al. 1981), globus pallidus (Saper and Loewy 1982), thalamus (Garcia-Rill 1983, Steeves and Jordan 1984), subthalamus and substantia nigra (Saper and Loewy 1982), all areas implicated in locomotion. Therefore, we suggest that although a substantial number of c-fos positive cells in the MLR project to vMRF, many of these c-fos labeled neurons may project to the above brain areas, instead of the vMRF. However, further experiments to investigate these regions are necessary to confirm this hypothesis.

We observed that sections located rostral to the cuneiform nucleus (MLR region) showed a systematic increase in the number of double-labeled neurons (FG and c-fos), with the maximal numbers of double-labeled neurons being detected in the deep mesencephalic nucleus (Fig 13). The percentage of the FG labeled cells which also showed c-fos activation increased (13%, 24%, 37%) from the caudal MLR to the most rostral extent of the MLR (deep mesencephalic reticular nucleus) (Fig 11,12,13). This suggests that in the rat, cells located in the deep mesencephalic reticular nucleus may play a crucial role in locomotion. The MLR may overlap rostrally with what has been termed the angular complex (Cole et al. 1989). Sinnamon et al. (1984, 1993) have consistently demonstrated that neurons located at hypothalamic and even more rostral levels are also responsible for initiation of locomotion (Epuru et al. 1995). It has been observed in previous studies in our laboratory with injections of TMRD into the MLR that the more rostral the MLR injection site, the more TMRD labeled terminals and fibers were localized in the vMRF, the location of the reticulospinal cells (Livingstone et al.

unpublished data). Therefore, further characterization of these structures rostral to the "classic" MLR would seem warranted.

The PPN has been suggested as a structure in the MLR which activates 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 can be defined by ChAT immmunocytochemical procedure. Since the ChAT immunocytochemistry was only applied in the spinal cord in the present study, we did not have data available to show whether or not the PPN is activated by locomotion. However, previous studies from our laboratory (Shojania et al, 1992) showed that PPN cells did not show c-fos expression during locomotion, indicating that the PPN may not play a major role in the regulation of locomotion. We did observed ChAT positive cells distributed throughout the spinal cord in a pattern similar to that previouly described by Huang (1995) in decerebrate cats. Likewise, the distribution of double labeled c-fos and ChAT cells in the spinal cord was also consistent with the previous findings reported by Barber et al. (1984) and Huang (1995). The results indicate that many of the c-fos labeled cells located in laminae VII, VIII and X of the spinal cord may be cholinergic interneurons engaged in locomotor generation in intact rats. It has been reported that neurons in the intermediate zone and central canal contribute to the intraspinal cholinergic circuitry which consists the main cholinergic innervation of the spinal cord and modulates the transmission of information in the cord (Barger et al. 1984, Borges and Iversen 1986). Intracellular injection of TMRD into the physiological

identified spinal neurons has suggested that many of these cells are cholinergic (Carr et al. 1994). We speculate that the cholinergic system in the spinla cord plays a critical role in generation of rhythmic activity.

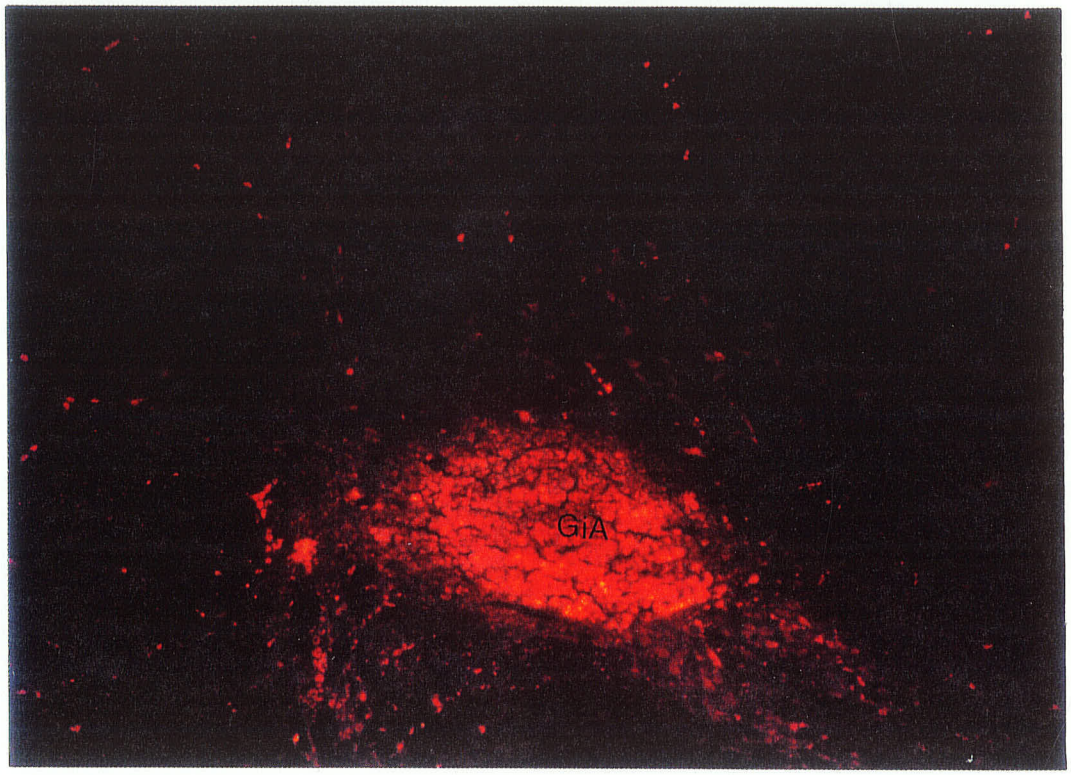
The experiments described in this thesis provide new information regarding reciprocal connections between the MLR, the vMRF and the spinal cord and indicates that although a substantial number of neurons in the rostral MLR that project to the vMRF do show locomotion induced c-fos protein, the majority of the c-fos positive cells may represent interneurons, or alternatively, neurons that project to brain areas other than the vMRF. Many of the c-fos positive cells observed in the spinal cord following 60 min treadmill locomotion were cholinergic (ChAT positive) neurons. Our current working model of this neural circuitry is summarized in Fig 24. In addition, we have identified a specific brain area located rostral to the "classic" MLR (deep mesencephalic reticular nucleus), and based upon the increase number of double-labeled neurons in this region, we suggest this area may play an important role in regulating locomotion in the rat.

FIGURE 1.

Photomicrograph of the vMRF (visualized with rhodamine filter) of a rat injected with FG and TMRD. The injection site is focused in the gigantocelluar reticular nucleus, alpha. (GiA).

L: Lateral

M: Medial



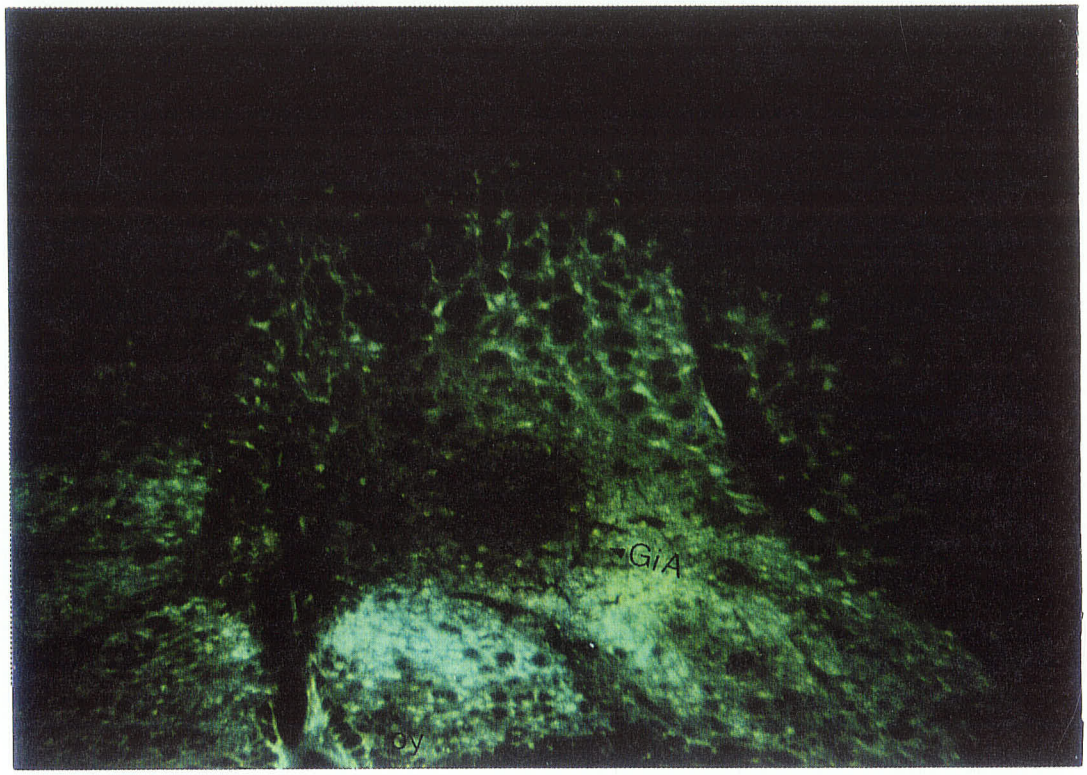
Bregma – 11.60mm

FIGURE 2.

Photomicrograph of the vMRF (visualized with UV filter) of a rat injected with FG and TMRD. The injection site is focused in the gigantocelluar reticular nucleus, alpha. (GiA).

L: Lateral

M: Medial



M L

Bregma −11.60 mm

FIGURE 3.

Neurolucida drawings illustrating the location of a FG+TMRD injection site in the vMRF and the distribution of retrogradely labeled neurons in the mesencephalon. Each dot represents a FG labeled neuron.

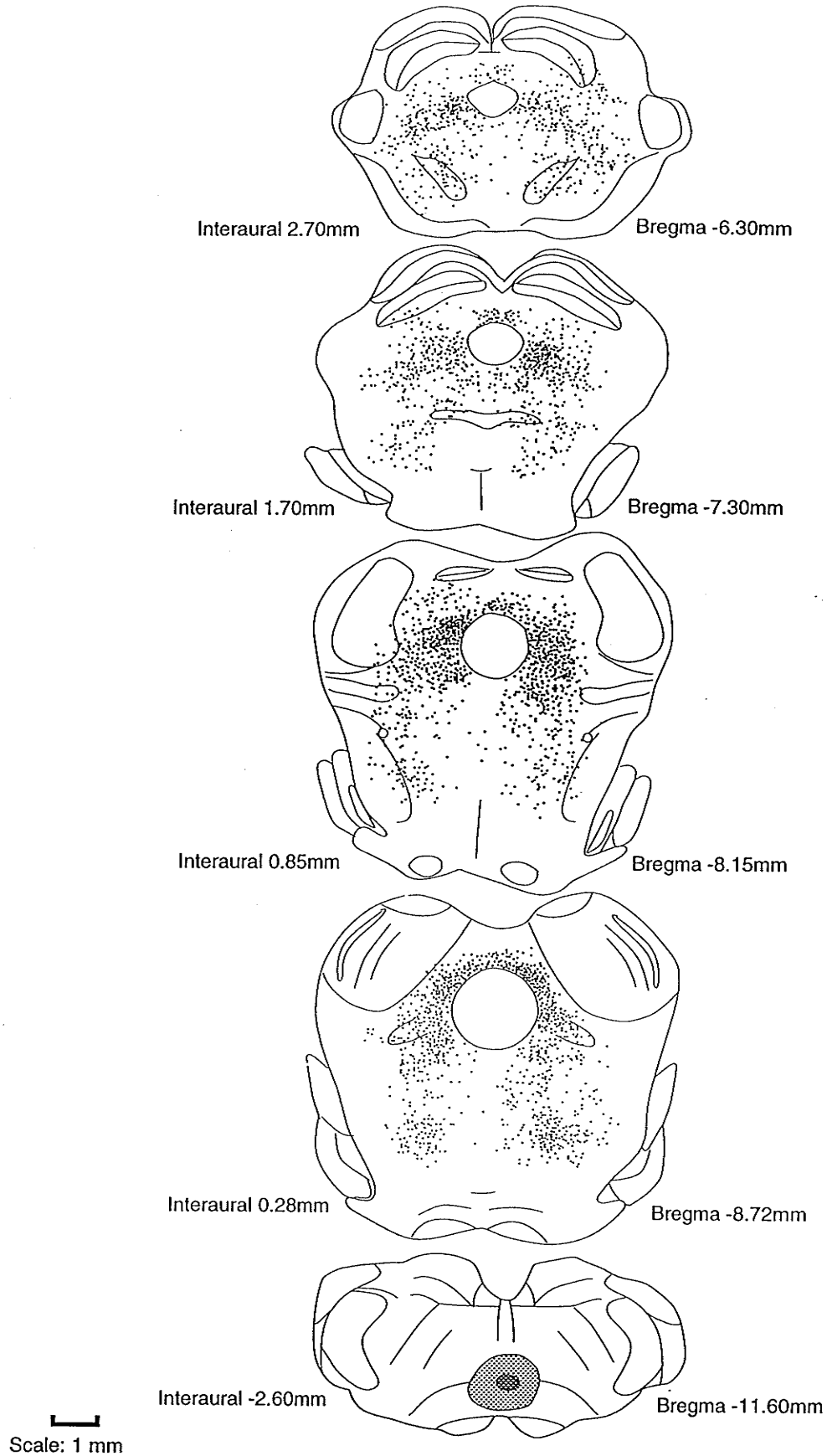
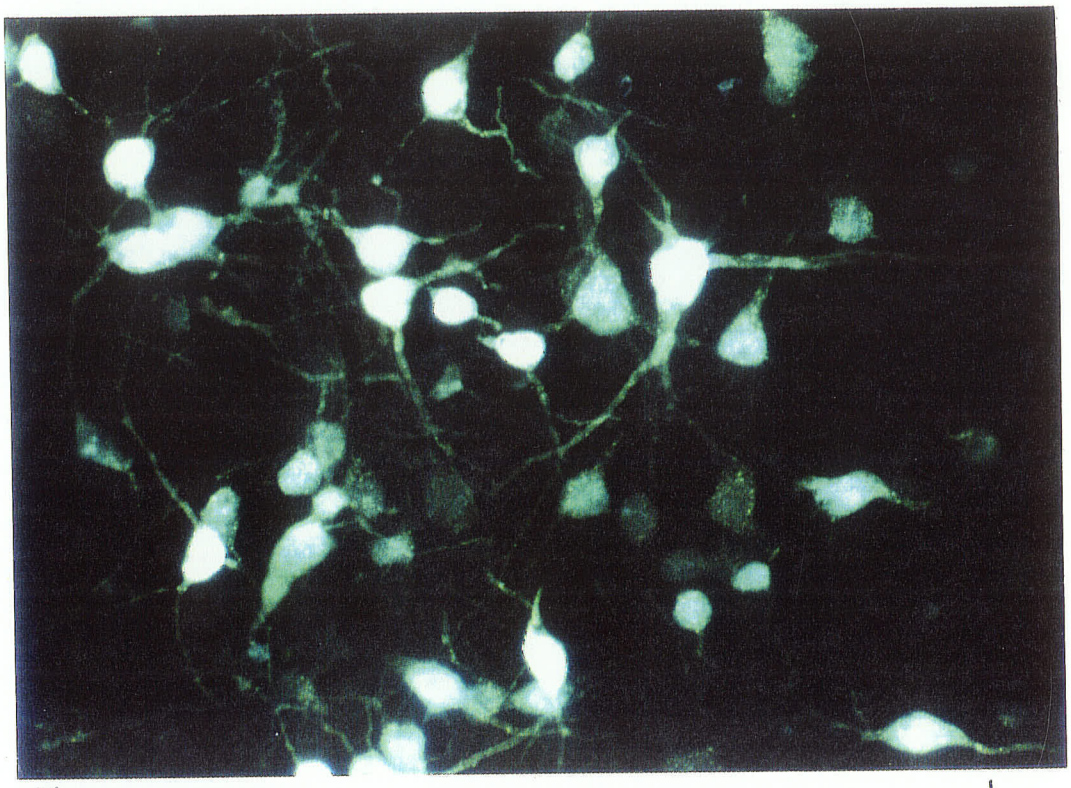

Interaural 2.70mm
Bregma -6.30mm
Interaural 1.70mm
Bregma -7.30mm
Interaural 0.85mm
Bregma -8.15mm
Interaural 0.28mm
Bregma -8.72mm
Interaural -2.60mm
Bregma -11.60mm
Scale: 1 mm

FIGURE 4.

Photomicrograph of the cuneiform nucleus showing the white/blue cells labeled with FG following the FG+TMRD injection in the vMRF.

L: Lateral

M: Medial



M L

Bregma -8.72 mm

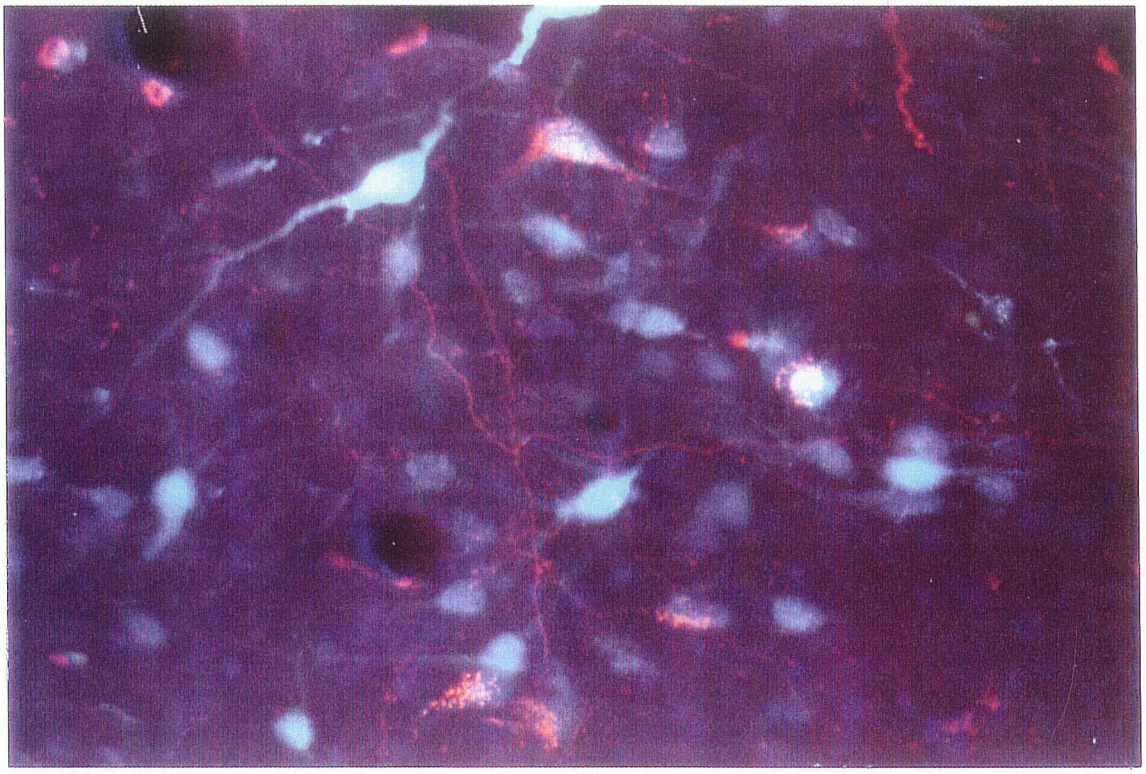
100 um

FIGURE 5.

A double exposure photomicrograph of the MLR showing anterograde (TMRD/red) and retrograde (FG/white) labeled fibers and cell bodies following a TMRD+FG injection into the vMRF.

L: Lateral

M: Medial



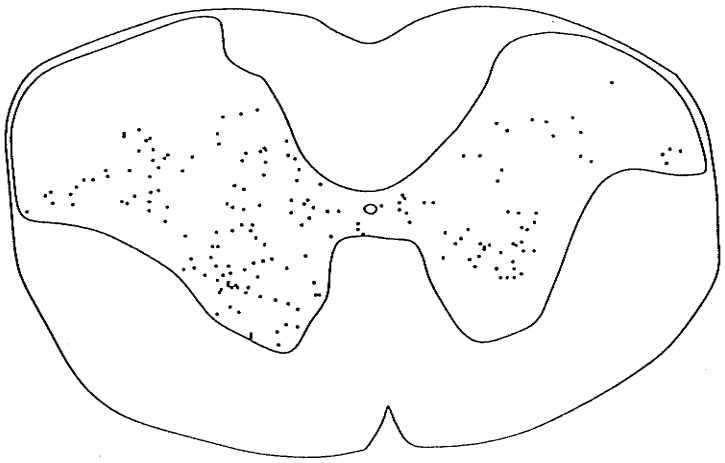
M L

100 um

Bregma -8.72 mm

FIGURE 6.

Neurolucida tracing of the FG labeled spinoreticular cells in L5 segment of a representative animal. Each dot represents one neuron.



Scale: 0.5mm

FIGURE 7.

Diagram of transverse sections of the cervical, thoracic and lumbar cord of the rat injected with FG and TMRD in the vMRF, showing the positions of the descending fibers (/) and the sites of terminations (Δ) on one side of the cord.

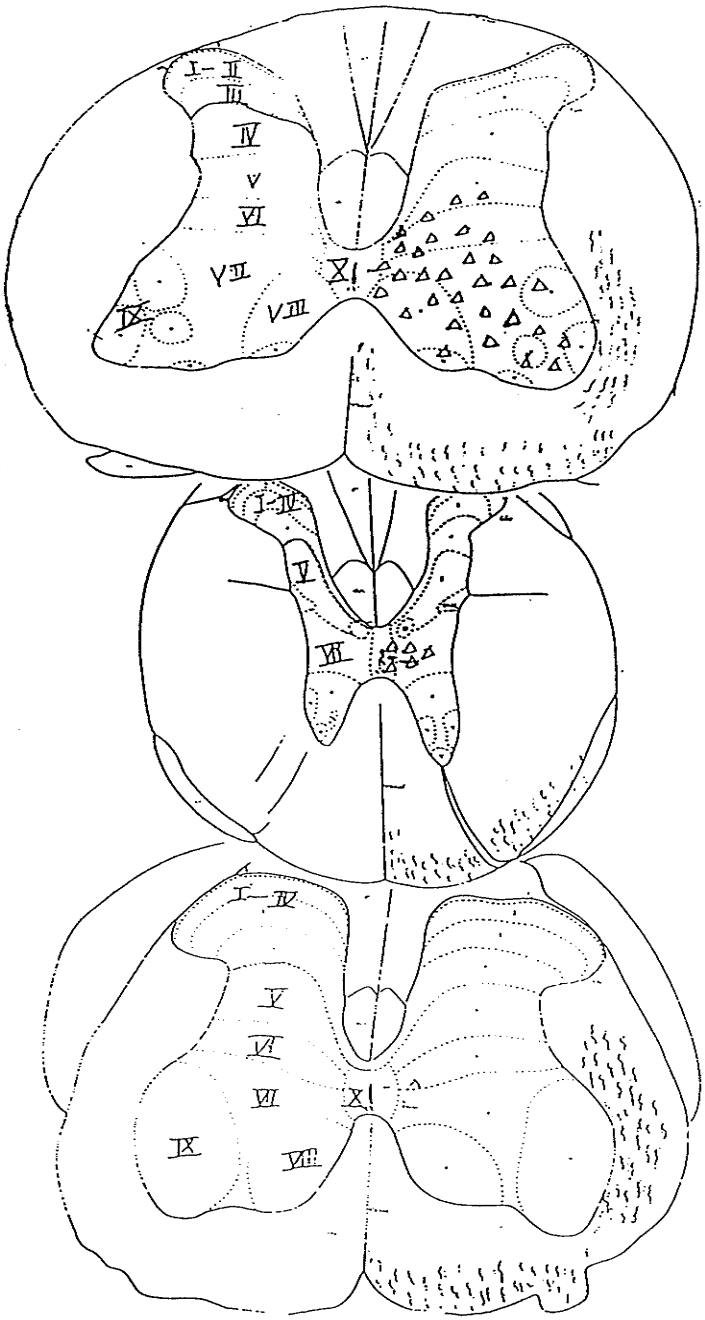
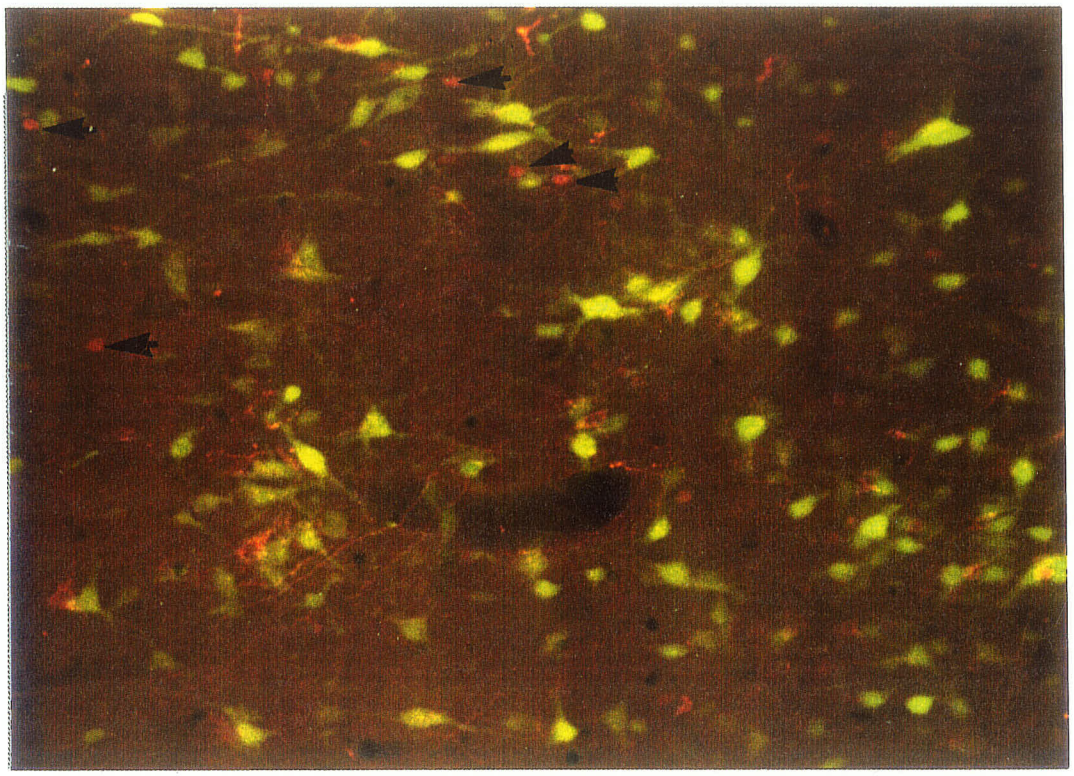

I–II
III
IV
V
VI
VII
VIII
IX
X
XI
C5
I–IV
V
VII
T8
I–IV
V
VI
VII
IX
X
XI
L5
1.5 mm

FIGURE 8.

A double exposure of photomicrograph taken from the MLR of a control animal (non-exercised) following an injection of FG+TMRD in the vMRF showing very few c-fos positive cells (red dots pointed by arrows) and numerous FG labeled cells (green/yellow) and TMRD labeled fibers (red).

L: Lateral

M: Medial



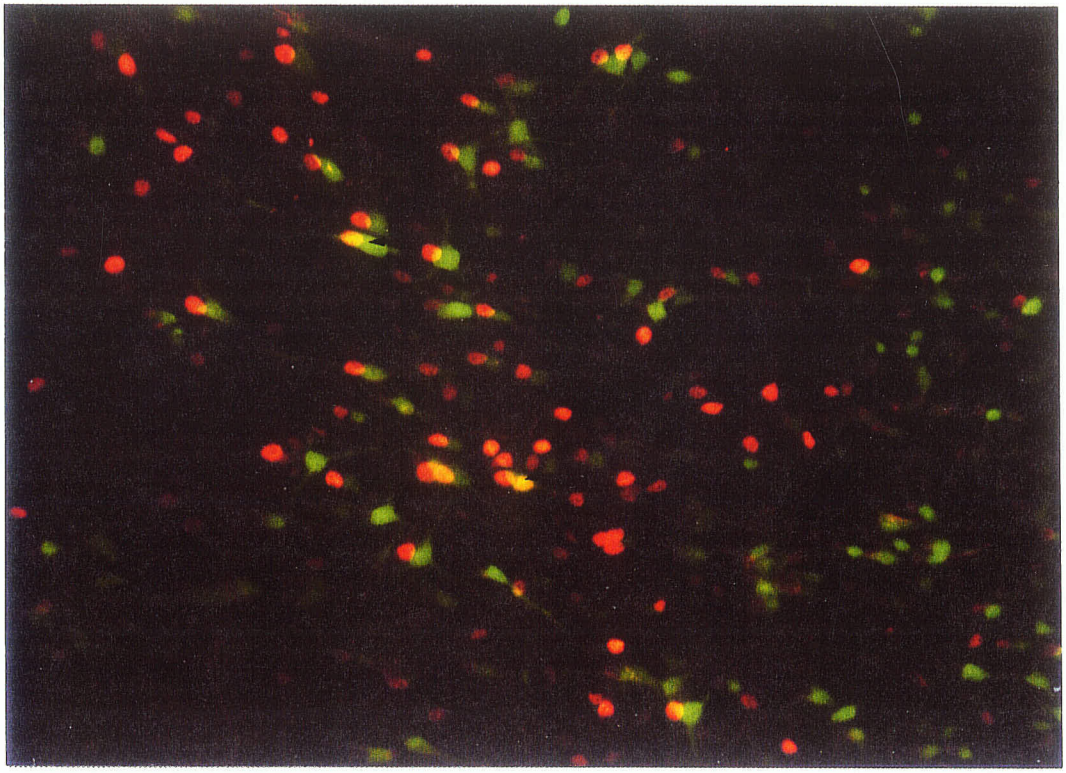
M L

100 μm

Bregma: -8.72mm

FIGURE 9.

A double exposure photomicrograph of the MLR of a rat injected with FG+TMRD in the vMRF and exercised in a treadmill. Retrogradely labeled FG cells are green and c-fos positive cells are red. Some cells demonstrate double labeling (indicated by arrows).



M L

100 µm

Bregma -8.72mm

FIGURE 10.

Two double exposure photomicrographs of the MLR (Bregma: -8.72 mm) showing that the FG labeled cells (yellow) are in close proximity to the c-fos positive cells (green) following FG+TMRD injection in the vMRF and 60 min treadmill locomotion.

L: Lateral

M: Medial

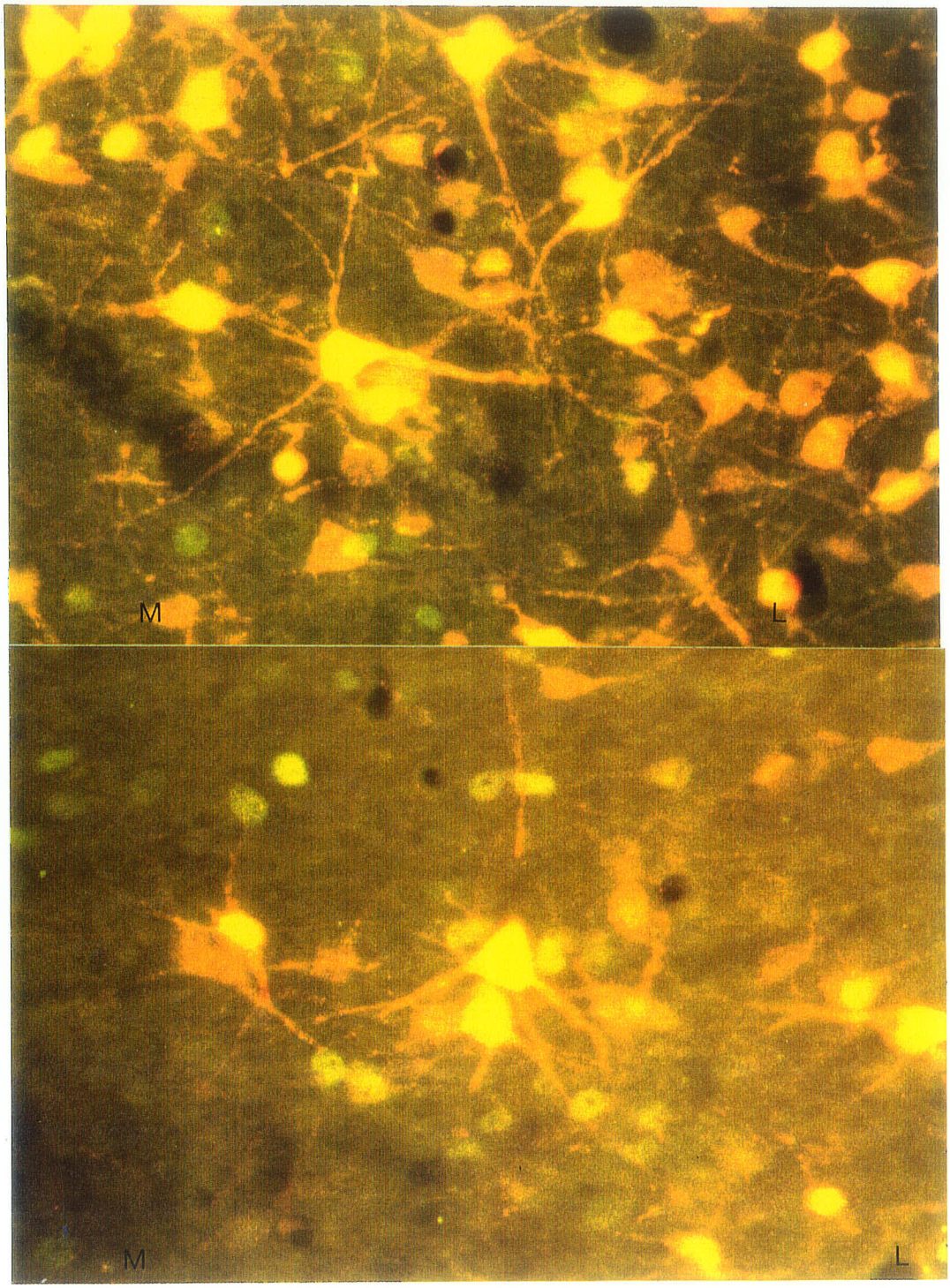
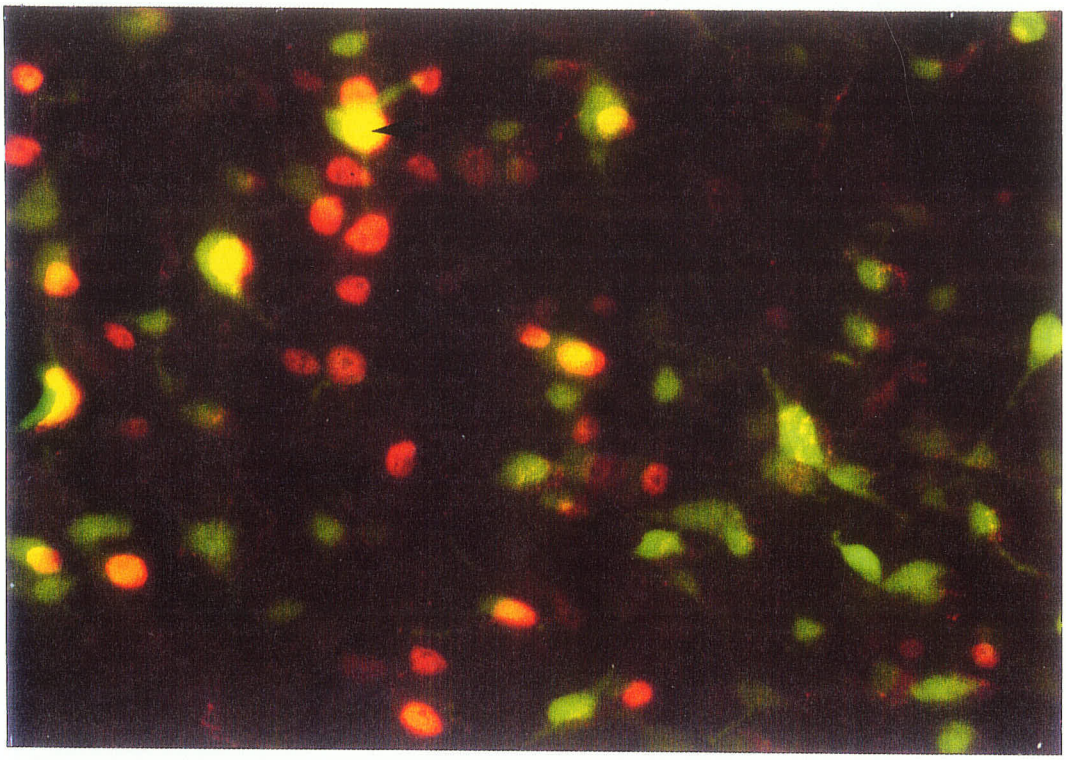

M
L
M
L
100 μm

FIGURE 11.

Photomicrograph of the MLR (level of the cuneiform nucleus) of a rat injected with FG+TMRD in the vMRF and exercised in a treadmill for 60 min. C-fos positive cells are red (Cy3) and FG cells are green. In this photo, 13% of the FG labeled cells were identified as also showing c-fos protein (one of the double labeled cells is pointed by arrowhead).

L: Lateral

M: Medial



M L

100 μm

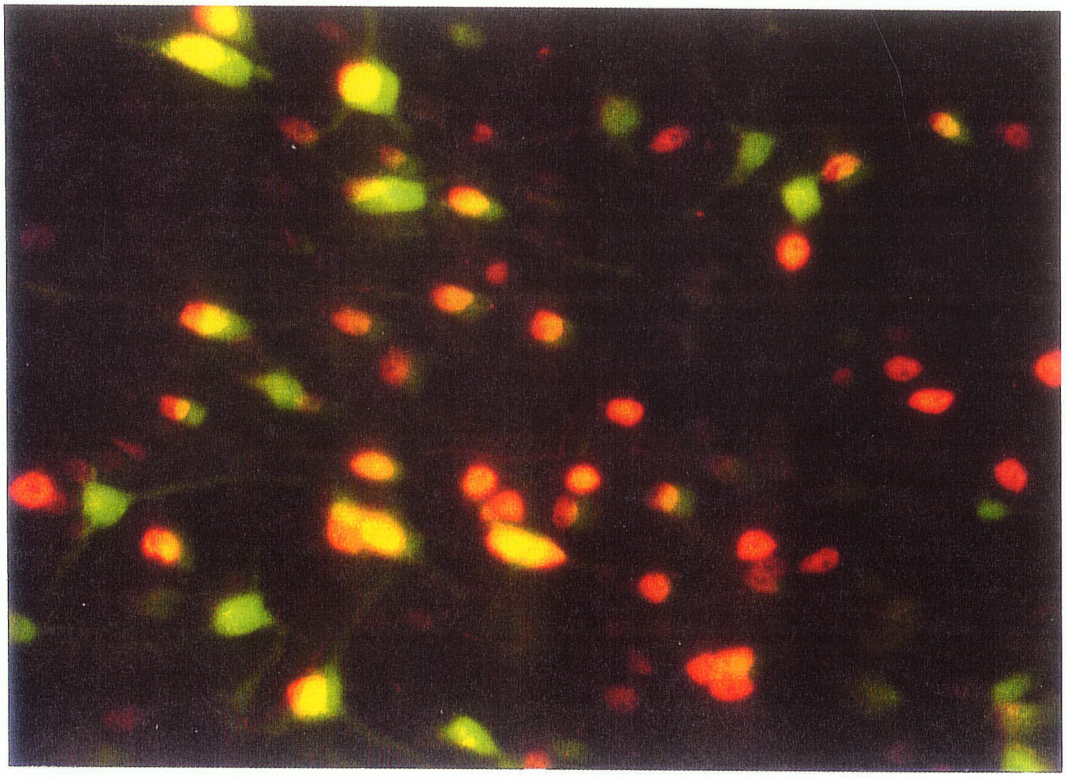
Bregma: -8.72 mm

FIGURE 12.

Photomicrograph of the more rostral MLR of a rat injected with FG+TMRD in the vMRF and exercised in a treadmill for 60 min. C-fos positive cells are red (Cy3) and FG cells are green. In this photo 24% of the FG labeled cells were identified as also showing c-fos protein.

L: Lateral

M: Medial



M L

100 μm

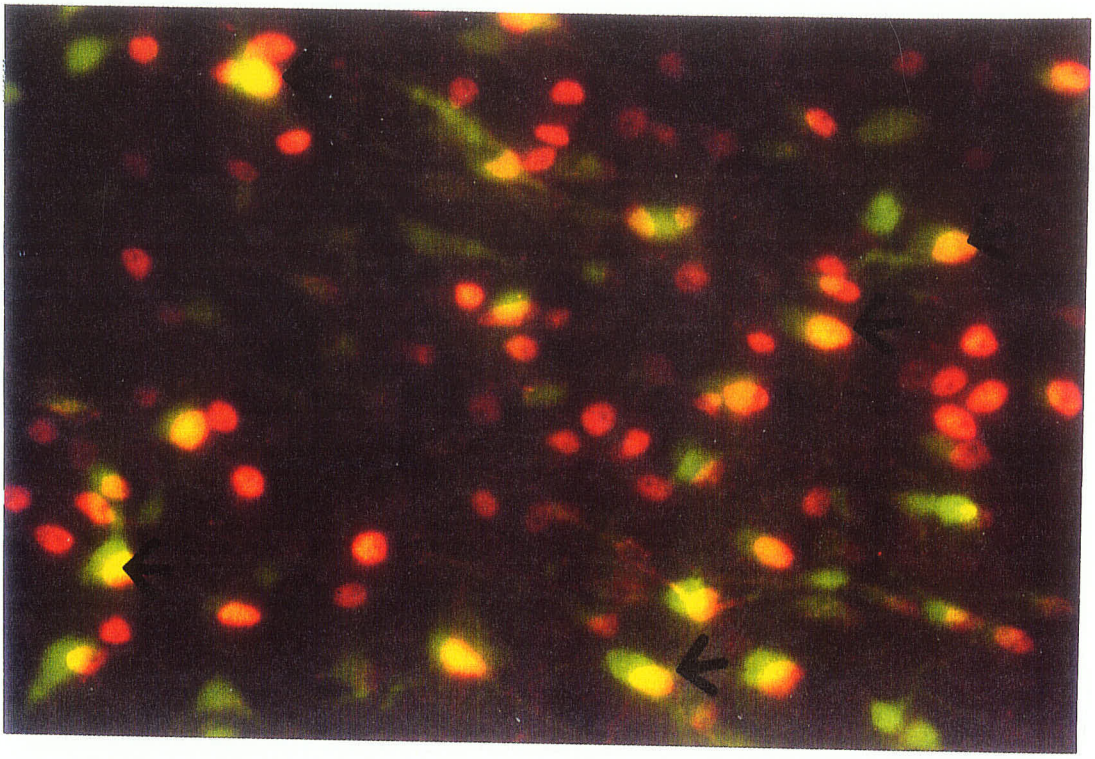
Bregma: -7.30mm

FIGURE 13.

Photomicrograph of the deep mesencephalic nucleus (most rostral extension of the MLR) of a rat injected with FG+TMRD in the vMRF and exercised in a treadmill for 60 min. C-fos positive cells are red (Cy3) and FG cells are green. In this photo 37% of the FG labeled cells were identified as also showing c-fos protein (some are pointed by arrows). This represents the region with the maximal number of double labeled cells.

L: Lateral

M: Medial



M L

100 μm

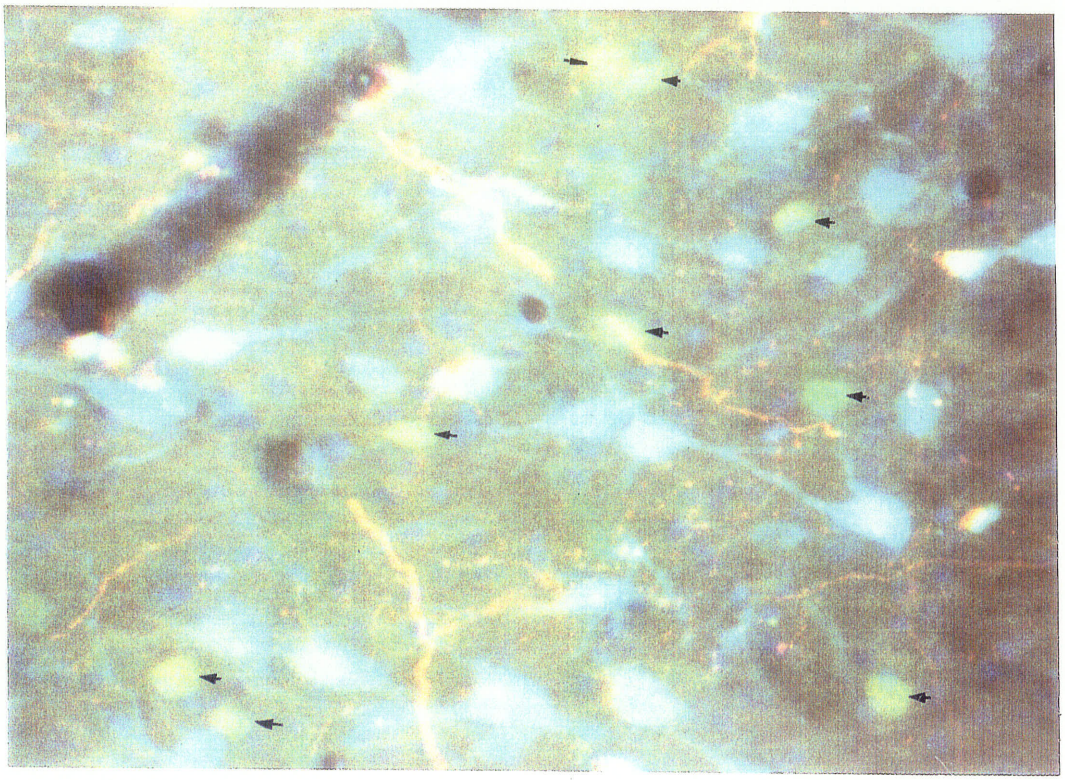
Bregma: -6.30 mm

FIGURE 14.

Photomicrograph of the cuneiform nucleus of a rat injected with TMRD +FG in the vMRF and exercised in a treadmill for 60 min. C-fos positive cells are green (indicated by arrows), retrogradely labeled (FG) cells are blue/white and anterogradely labeled (TMRD) terminal and fibers are red.

L: Lateral

M: Medial



M L

100 μm

Bregma: -8.72 mm

FIGURE 15.

Photomicrographs of the lumber cord (lamina I-X) showing c-fos positive cells following 60 min treadmill locomotion.

L: Lateral

M: Medial

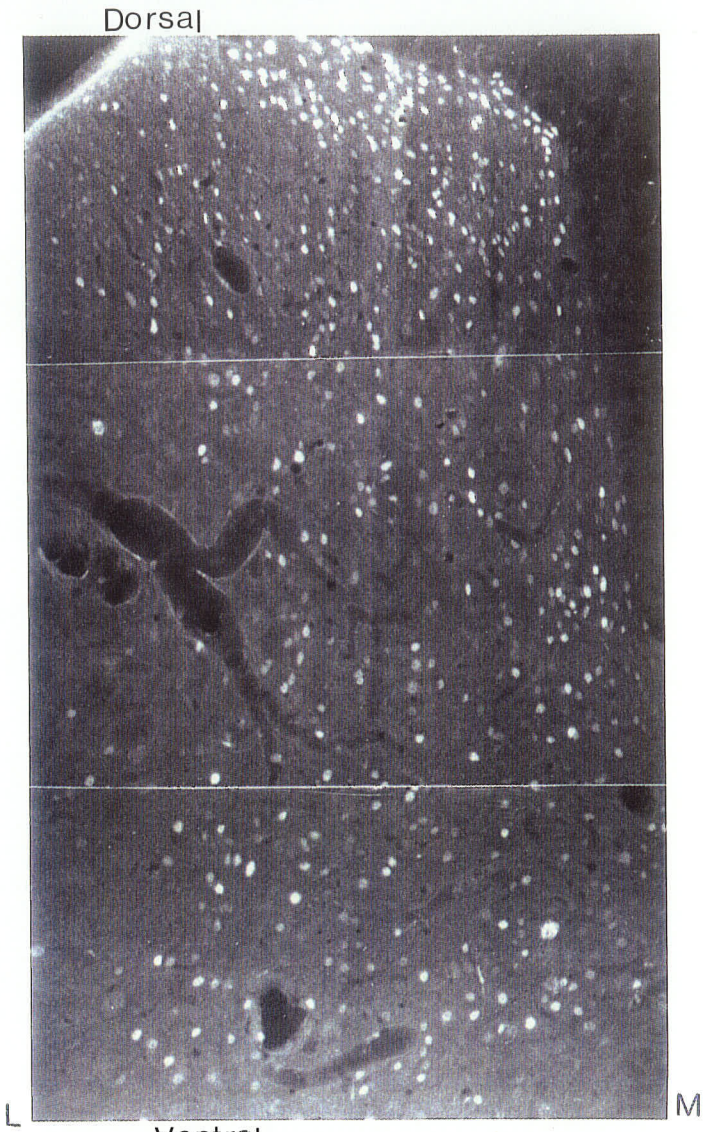

Dorsal
L
M
Ventral
0.5 mm

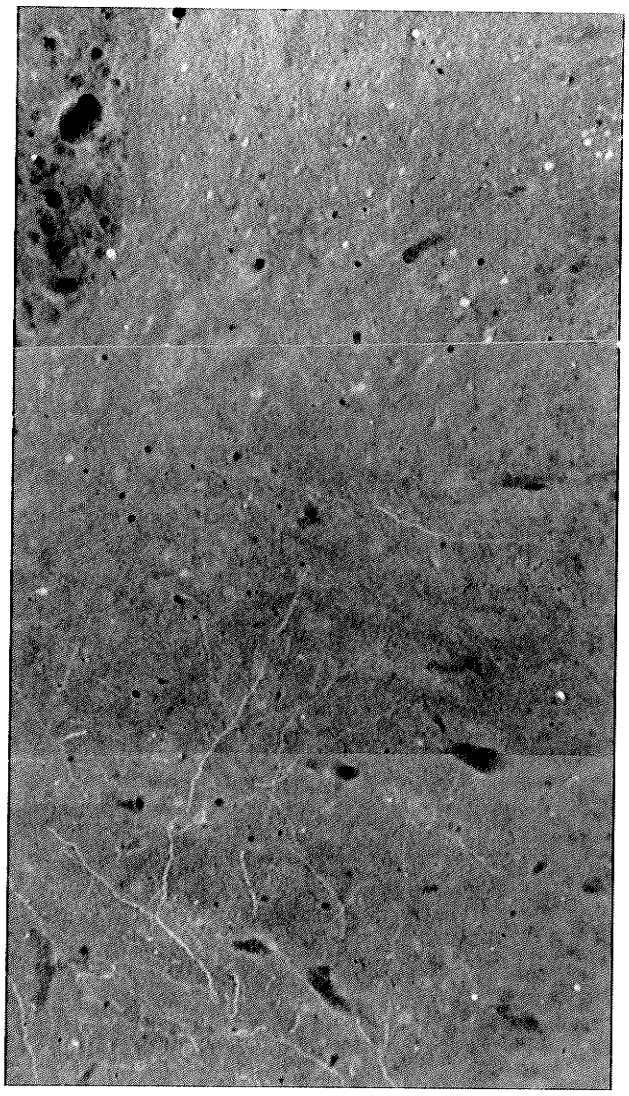
FIGURE 16.

Photomicrograph of the cervical cord (lamina I-X) of a non-exercised control rat illustrating the absence of c-fos positive cells and anterogradely labeled terminals. Absence observed at all levels of the spinal cord.

L: Lateral

M: Medial

Dorsal



M L

Ventral

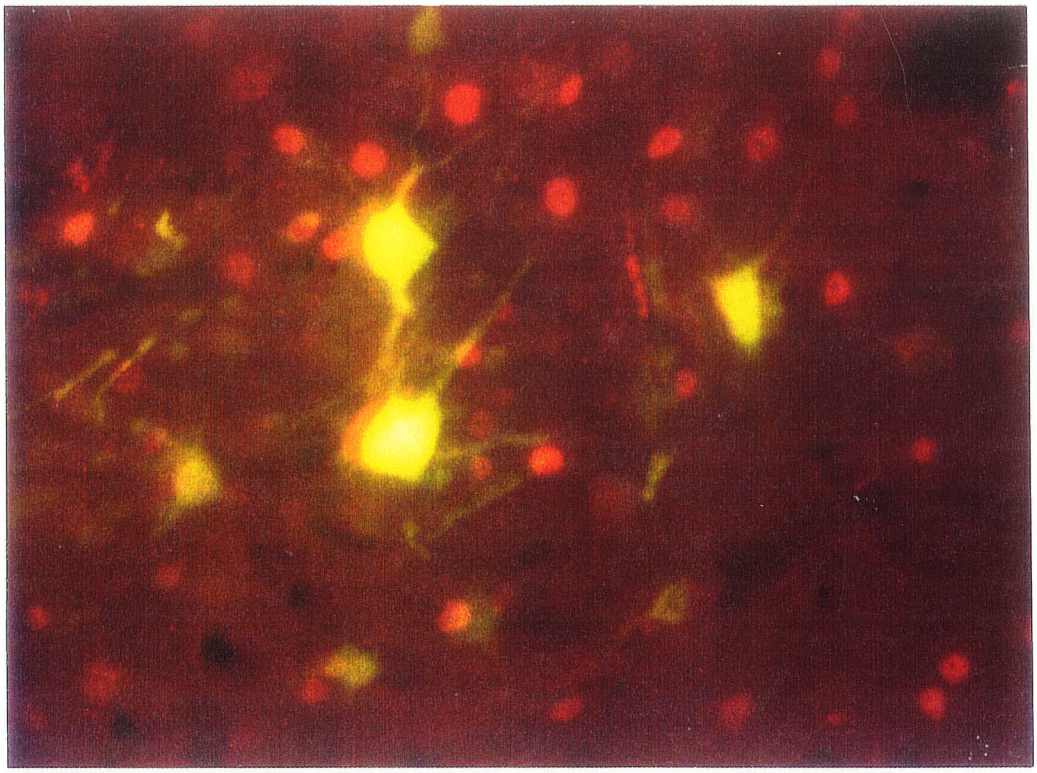
0.5 mm

FIGURE 17.

A double exposure photomicrograph of the lumbar cord (lamina VII and VIII, X) of a rat injected with FG+TMRD in the vMRF and exercised in a treadmill showing retrogradely labeled FG cells (Yellow/green; spinoreticular cells) and numerous c-fos positive cells (red).

L: Lateral

M: Medial



M

L

100 μm

L5

FIGURE 18.

Photomicrographs of the region adjacent to the central canal in horizontal sections of the lumbar spinal cord of a rat injected with FG+TMRD in the vMRF and exercised in a treadmill for 60 min. A. Dorsal to the central canal. B. Level at the central canal. C. Ventral to the central canal. FG labeled cells are green/white, c-fos positive cells are red.

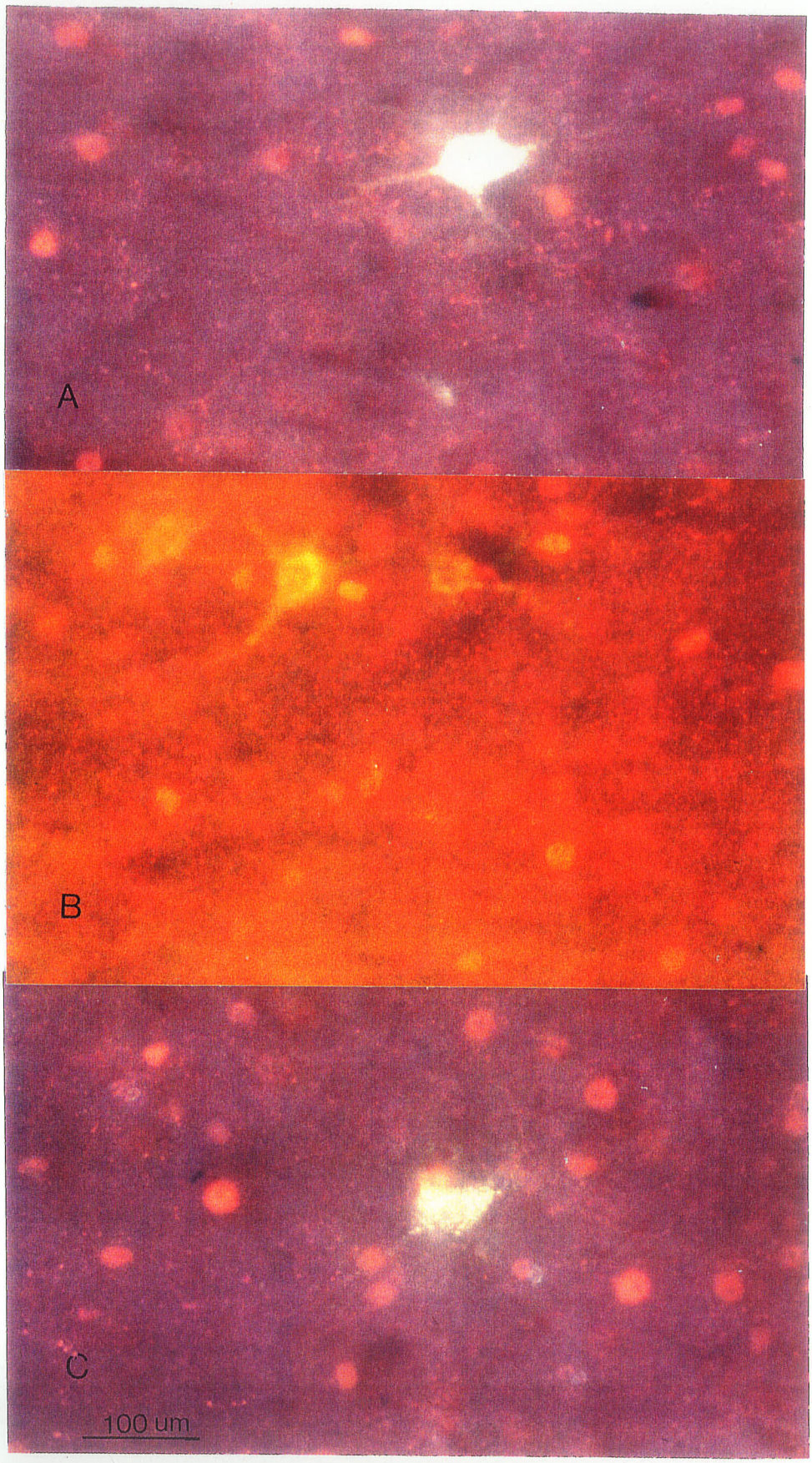
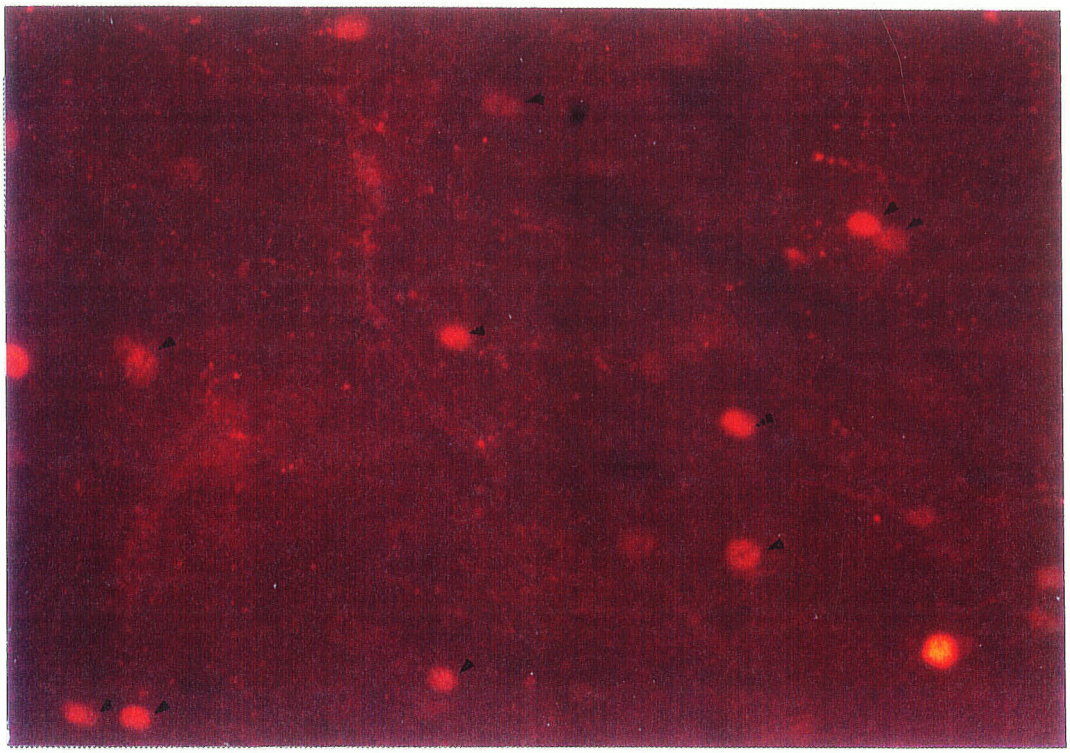

A
B
C
100 um

FIGURE 19

Photomicrograph of a horizontonal section at the region adjacent to the central canal in the lumbar cord of a rat injected with FG+TMRD in the vMRF and exercised in a treadmill for 60 min. TMRD labeled terminals and fibers are red fibers, c-fos positive cells are red (pointed by arrowheads).

L: Lateral

M: Medial



L M

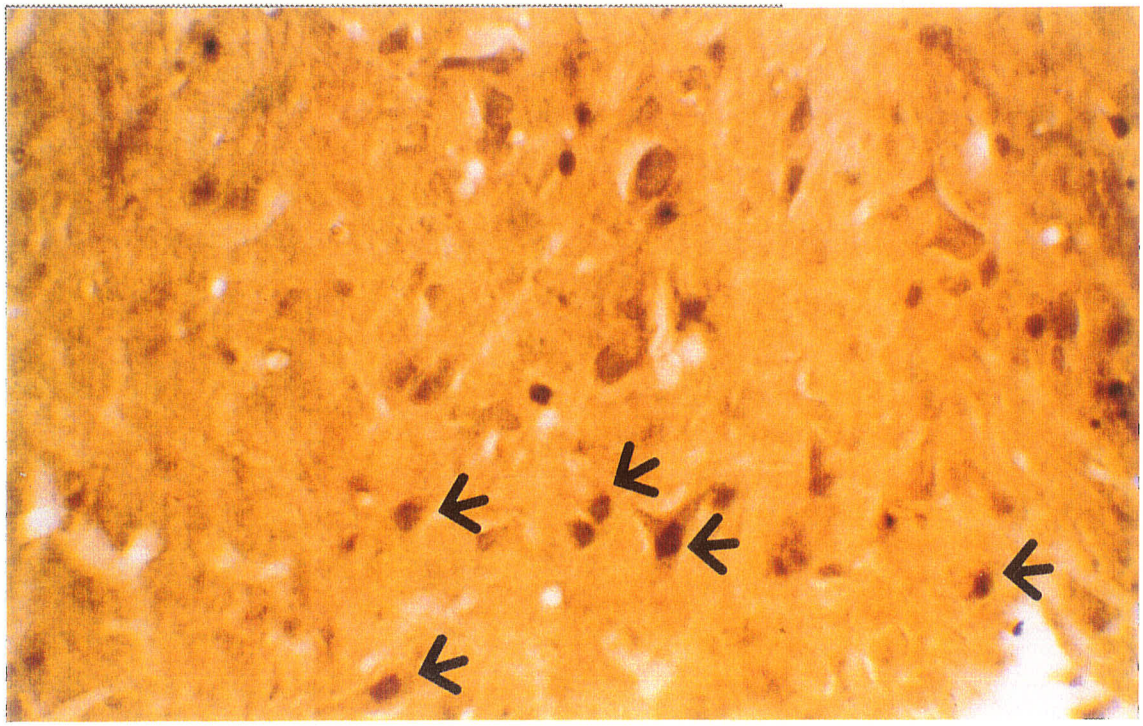
100 μm

FIGURE 20.

Photomicrograph of the cervical cord (lamina VII, VIII, X) of a rat exercised in a treadmill for 60 min and processed for c-fos and ChAT immunoreactivity. C-fos positive cells (nuclei) are black, whereas ChAT positive cells show brown cytoplasm. Double labeled neurons are pointed by arrows.

L: Lateral

M: Medial

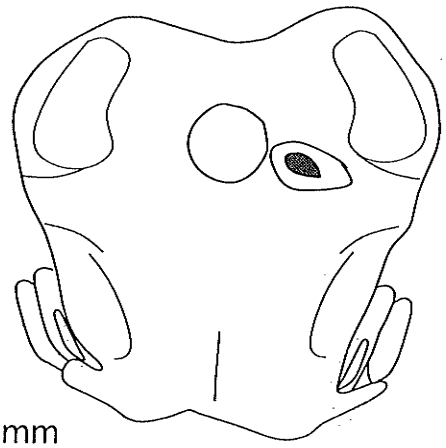


L M

100 μm

FIGURE 21.

Schematic illustration of the location of a FG+TMRD injection site in the MLR.



Interaural 1.50mm

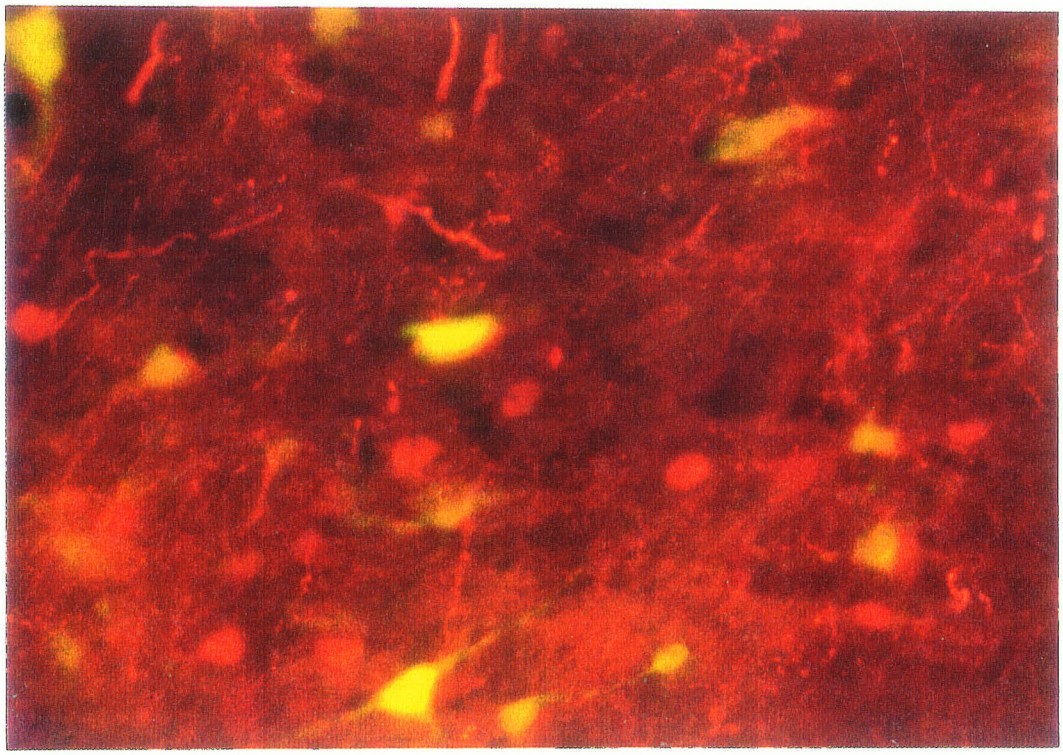
Scale: 1 mm

FIGURE 22.

Double exposure photomicrograph of the vMRF of rat injected with FG+TMRD in the MLR and exercised in a treadmill for 60 min. FG labeled cells are yellow/green, c-fos positive cells are red, and TMRD labeled fibers are red.

L: Lateral

M: Medial



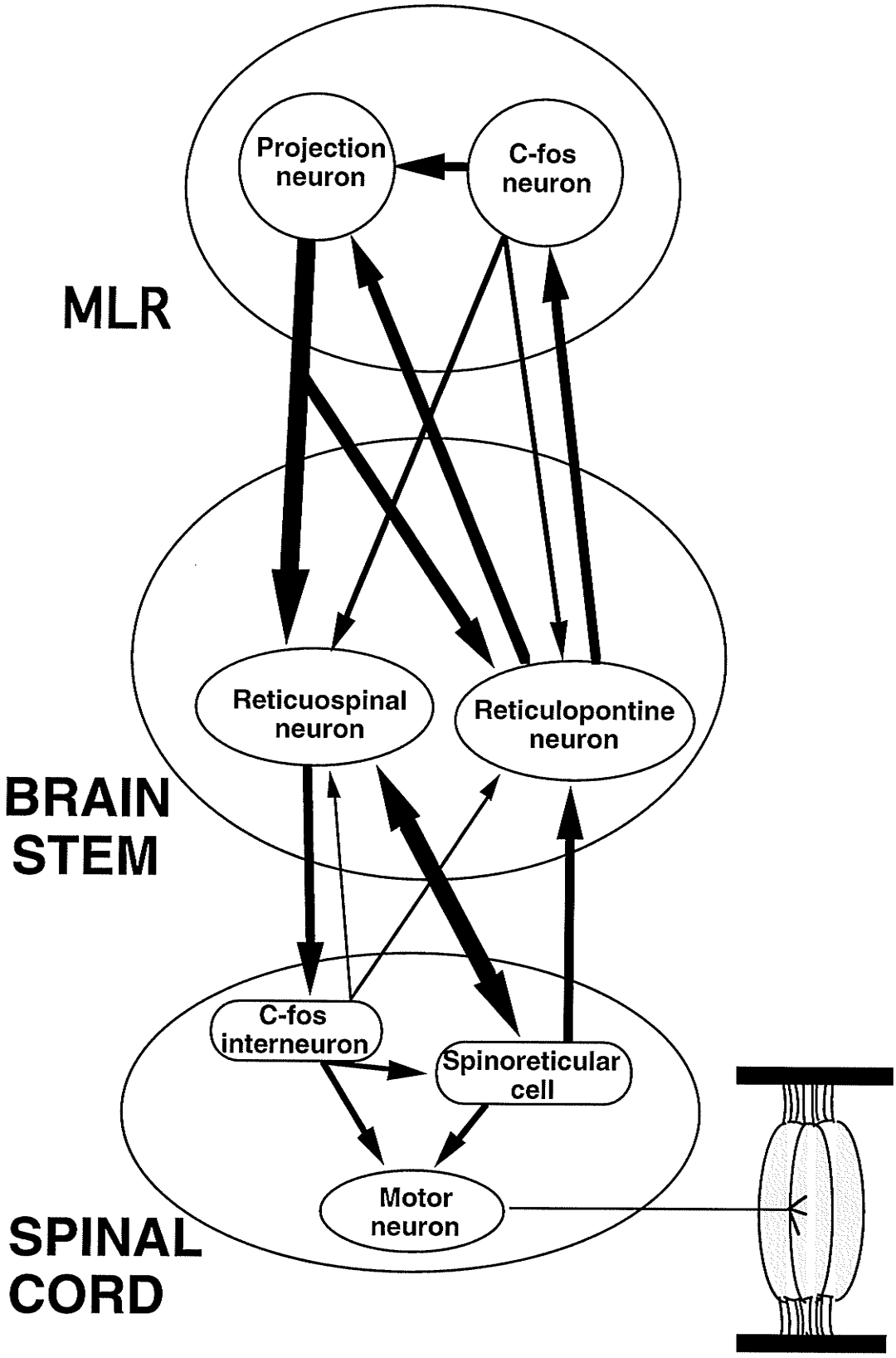
L M

100 μm

Bregma: -11.60mm

FIGURE 23.

Graphic representation of the interconnections between the MLR, VMRF and the spinal cord based on neuroanatomical tract tracing and activity dependent c-fos immunocytochemistry. The thickness of the arrows illustrates the relative extent of these identified connections.


Projection neuron
C-fos neuron
MLR
Reticuospinal neuron
Reticulopontine neuron
BRAIN STEM
C-fos interneuron
Spinoreticular cell
Motor neuron
SPINAL CORD

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ABBREVIATIONS

2-DG	2-deoxyglucose
MLR	mesencephalic locomotor region
MRF	pontomedullary reticular formation
vMRF	ventromedial reticuar formation
FG	fluorogold
TMRD	tetramethylrhodamine-dextrans
PPN	pedunculopontine nucleus
GABA	gamma aminobutyric acid
ChAT	choline acetyltransferase
NMDA	N-methyl-D-aspartate
CPGs	central pattern generators
FTG	gigantocelluar tegmental field
FTM	magnocellular tegmental field
VMM	ventromedial medulla
PLS	pontomedulllary locomotor strip
VLF	ventral lateral funiculi
LC	locus coeruleus
CA	catecholamine
L-DOPA	L-dihydroxyphenylalamine
IEGs	immediate early genes
PC12	pheochromocytoma cell line