LOCALIZATION OF A SUB-POPULATION OF COMMISSURAL CELLS ACTIVE IN TREADMILL LOCOMOTION IN THE ADULT RAT THORACOLUMBAR SPINAL CORD

BY

NALINI NAROTAM

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Physiology
University of Manitoba
Winnipeg, Manitoba
Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

LOCALIZATION OF A SUB-POPULATION OF COMMISSURAL CELLS ACTIVE IN TREADMILL LOCOMOTION IN THE ADULT RAT THORACOLUMBAR SPINAL CORD

BY

NALINI NAROTAM

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

MASTER OF SCIENCE

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

The author wishes to express her sincere gratitude to the following individuals for their assistance in the preparation of this thesis:

Dr L. Jordan, Head/Professor of Physiology, Director, Neuroscience Research Program for his expert guidance, encouragement, constructive criticism and supervision of the thesis.

Drs B. Schmidt, Associate Professor, Internal Medicine and **D. Nance**, Professor, Pathology, members of the thesis committee, for their guidance and valuable suggestions.

Dr B. Fedirchuk, Research Associate, for assistance and advice during the animal experiments.

Mr M. Sawchuk, Ms K. Madec and Ms S. Pylypas, Laboratory Technicians, for technical assistance.

Ms M. Setterbom, Laboratory Technician, for assistance in preparation of the graphics.

Fellow students at the **Spinal Cord Research Center** especially **Yue Dai, Stephen MacDonald, Cima Cina** and **Jason Maclean** for all their support and assistance.

The author wishes to make special mention of the unconditional love, encouragement, patience and support of her husband, two daughters and her parents during the course of her studies and during the preparation of this thesis.

This thesis is dedicated to my husband, Pradeep.

ABSTRACT

The aim of this study was to identify a selective population of commissural neurons in the adult rat spinal cord that were active during treadmill locomotion. Fluorogold, a retrograde neuroanatomical tracer was combined with the immunocytochemical localization of the activity dependant cellular marker c-fos protein in an in vivo animal.

We observed that the commissural neurons that were activated during locomotion are distributed from spinal segments T13 to L6. They are located primarily in Rexed's laminae VII & X of the intermediate zone and lamina VIII of the ventral horn. No c-fos and fluorogold double labeled cells were observed in the experimental animals that did not perform the treadmill locomotor task. This suggests that the identified neurons are specifically activated by the locomotor task. We also observed that ipsi-lateral propriospinal cells, known to have extensive synaptic connections within the spinal cord, were also active during treadmill locomotion.

These identified commissural cells are active during physiological locomotion and they may mediate left/right alternating rhythmic patterns and represent an important component of the spinal central pattern generator (CPG).

ABBREVIATIONS

2DG	2-deoxyglucose
5-HT	5-hydroxytryptamine
ACh	acetylcholine
aFGF	acidic fibroblast growth factor
APV	D-aminophosphonovalerate
cAMP	cyclic adenosine monophosphate
CAT	computerized axial tomography
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPG	central pattern generator
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanide perchlorate
E 13.5	embryonic age 13.5
E15	embryonic age 15
E19	embryonic age 19
EPSP's	excitatory post synaptic potentials
HCL	hydrochloric acid
HN	Hoffman Nuclei
HRP	horse radish peroxidase
IEG	immediate early gene
IM	intramuscular
IP	intraperitoneal
KAR	Ketamine Atropine Rompun
KR	Ketamine Rompun
L-DOPA	L-dihydroxyphenylalanine

MRI	magnetic resonance imaging
MLR	mesencephalic locomotor region
mRNA	messenger ribonucleic aid
NaCl	sodium chloride
$NaNO_2$	sodium nitrite
NaOH	sodium hydroxide
NMA	N-methyl-aspartate
NMDA	N-methyl-D-aspartate
NGF	nerve growth factor
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Triton
TRIS	Trizma base
trkA	tyrosine kinase receptor
UV	ultraviolet
VLF	ventrolateral funiculus
VMN	ventromedial nucleus
vMRF	ventromedial pontomedullary reticular formation
WGA-HRP	wheat germ agglutin-horseradish peroxidase

INTRODUCTION

Major advances in the clinical care of patients with acute spinal cord injury have occurred. These include efficient pre-hospital spinal immobilization, in field resuscitation and transport, special attention to secondary systemic factors i.e. hypotension and hypoxia, and more recently, the administration of high dose methylprednisolone. Technical advances in computerized axial tomography (CAT) and magnetic resonance (MRI) imaging have lead to better evaluation of bone and cord injury. Aggressive and radical surgical treatment in the form of early decompression of the neural elements followed by spinal column reconstruction, coupled with biomechanical advances, have predominantly benefited patients with incomplete spinal cord injuries. Unfortunately, patients with complete spinal cord injuries demonstrate minimal functional recovery and require extensive rehabilitation. Any hope of functional recovery for these patients lies in the endeavors of spinal cord researchers.

The new frontier in spinal cord injury research involves research into mechanisms of spinal cord repair and/or regeneration. A major breakthrough occurred recently when adult rats showed substantial spinal cord repair following a complete spinal cord transection. This was made possible by

reconnecting the spinal cord using peripheral nerve grafts and treated with fibroblast growth factor (aFGF) (Cheng et al., 1996).

In order to design mechanisms for regeneration of an injured spinal cord it is imperative to fully understand the basic patterns of locomotion and the neurocircuitary involved at the cellular level. There has been extensive progress in the past few years in the cellular analysis of central motor networks, thereby permitting the identification of some of the major classes of neurons that comprise this neural network.

Spinal interneurons active during locomotion

In the initial determination of the spinal mechanism of progression, Philippson (1905) emphasized the role of the exteroceptive reflexes in the production of locomotion. Sherington (1910) concluded after his investigations that the intrinsic stimuli for reflex stepping of the limb was clearly not as a result of stimulation of the skin of the limb but rather the stimulation of the sensory end-organs of the muscles of the limb. The control of locomotion was thus thought to be influenced largely by proprioceptive reflexes. One of the landmark achievements of the neuroscientists in the last century has been the repeated verification of the centralist theory of locomotion which describes motor output

patterns as being generated by central networks in contrast to chains of reflexes activated by sensory feedback references. Brown (1911) was the first neuroscientist to conclusively demonstrate this concept. He was able to evoke the act of progression in the hind limbs following rapid transection of the thoracic spinal cord of the decerebrate cat preparation after all proprioceptive reflexes were removed by deafferentation. He suggested that the central mechanism for the alternating rhythmic movements of spinal animals may be two antagonistic spinal centers paired with each other.

The cellular network of the spinal cord that is responsible for the generation of rhythmic locomotor movements is termed the central pattern generator (CPG) (Grillner,1975; Grillner and Wallen,1985). The CPG can be stimulated to produce rhythmic activity following primary afferent stimulation (Sherrington,1913, Grillner and Zangger, 1974); activity of the CPG interneurons induced by anesthesia (narcosis progression) (Brown,1913 ,1914); stimulation of specific brainstem nuclei in the mesenchephalic locomotor region (MLR) and ventromedial nucleus (VMN) of the thalamus (Garcia-Rill et al., 1985; Steeves and Jordan, 1984; Shik et al.,1966) or by the direct electrical and chemical manipulation of the presumptive CPG neurons (Barbeau and Rossignol,1991; Barbeau

et al 1993; Cazalets et al., 1990, 1992; Cowley and Schmidt, 1994; Kiehn et al.,1992; Sqalli-Houssaini et al., 1993; Rossignol and Dubuc, 1994). Grillner (1981) suggested that the spinal network producing rhythmic locomotor movements is made up of multiple "unit burst" generators which are distributed throughout the spinal cord in close proximity to the motorneuron pools they drive. These networks may be connected in alternative ways and the number of networks activated and their specific coupling determines the outcome of locomotor task. For example reciprocal inhibition between two generators may produce alternating activity such as walking, whereas mutual excitation of the generators may produce the synchronization of the limbs as in jumping or bounding. Several other studies suggest a similar distribution of unit generators in the locomotor system of mammalian and non-mammalian species, ie. dogfish (Grillner 1974); lamprey (Cohen and Wallen 1980); frog embryo (Khan and Roberts 1982); embryonic chick (Ho and O'Donovan 1993); cat (Deliagina et al. 1983) and turtle (Mortin and Stein 1989).

Stein et al., (1995) have postulated a different arrangement for the scratch CPG in the turtle. Their results indicated that the contralateral interneurons were members of the ipsilateral limb's rostral CPG and the organization was termed a "bilateral shared core". This concept differs from

the traditional view on locomotor phasing which proposes that the CPG of one limb is synaptically modulated by the CPG of the other limb to produce alternating rhythmic activity (Grillner, 1981). In the bilaterally shared core model, interlimb phasing is coordinated by appropriate interconnections between neurons of the core.

Numerous studies in mammalian preparations have revealed the presence of groups of spinal interneurons that may be involved in the process of locomotion. The first group of interneurons studied were the Ia reciprocal inhibitory interneurons and the Renshaw cells. The Ia inhibitory interneurons are located within a narrow zone in Rexed's lamina VII just dorsal to the motor nuclei. The Renshaw cells are situated in the medioventral border of the motor nuclei (Jankowska and Lindstrom 1972; 1971). The function of the Ia inhibitory interneuron is to prevent antagonistic muscle activation and the function of the Renshaw cell is to alter the motoneuron firing by a negative feedback mechanism. Electrophysiological studies show that the Ia inhibitory interneurons and the Renshaw cells display phasic activity which is closely coupled to their associated flexor or extensor motoneuron population during the fictive step cycle (Shik et al., 1966; McCrea et al., 1980; Pratt and Jordan, 1987).

In an attempt to localize the region of the locomotor neurons, Grillner and Zangger (1979) demonstrated that the spinal animal's ability to initiate locomotion was lost after spinal transections below caudal L5. This was consistent with the results of Deliagina et al., (1983) who showed that the L3-L5 segments were the most important segments in the production of rhythmic activity. These findings were also favored by Mortin and Stein (1989) and Ho and O' Donovan (1993) who indicated the presence of the rhythm generating elements in the rostral segments of the lumbosacral enlargements of the turtle and chick spinal cord, respectively. Noga et al., (1995) has also demonstrated using field potential recordings that the interneurons in L4-L6 were active following MLR induced fictive locomotion. Further studies revealed interneurons in Rexed's lamina VI, VII and the dorsal part of lamina VIII of the midlumbar segments which receive descending commands from the MLR via the vMRF (ventromedial pontomedullary reticular formation) (Edgley et al., 1988) and are also rhythmically active during fictive locomotion (Shefchyk et al., 1990). In addition, these interneurons receive multimodal peripheral inputs from afferents in muscle, skin and joint nerves which in turn project onto motoneurons (Edgley et al. 1988; Edgley and Jankowska, 1987a, 1987b).

By studying the regional distribution of the locomotor pattern-generating network in the neonatal rat spinal cord, Cowley and Schmidt, (1997) have concluded that: 1) the 5-HT network which is responsible for the descending rhythmic drive to the lumbar locomotor regions, is distributed throughout the supralumbar area of the spinal cord ; 2) NMA- and ACh- rhythmogenic elements are distributed throughout the spinal cord and; 3) a bilaterally intact system is essential for the activation of the ACh- network to produce alternating left and right rhythmic activity.

Last-order interneurons active during locomotion were detected via transneural labeling in the cat (Alstermark and Kummel, 1986; Jankowska, 1986; Jankowska and Skoog, 1986). These interneurons were located in laminae V-VII, ipsilateral to the filled motoneurons and in lamina VIII, contralaterally to the filled motoneurons. A similar distribution of last order interneurons participating in locomotion were located mainly in lamina VII of spinal segments L5-L7, via retrograde transneuronal labeling with wheat germ agglutinin-horseradish peroxidase (WGA-HRP) in the MLR induced fictive locomotion preparation (Noga et al., 1987). Hoover and Durkovic, (1992) retrogradely labeled last order interneurons following injections of fluorescent microspheres into hindlimb motoneurons (1992). The labeling pattern was consistent with

that of the above studies but in addition interneurons were labeled in laminae V and X. Transneural injections of psuedorabies virus in the rat medial gastrocnemius muscle resulted in the labeling of the last order interneurons in laminae I, II, IV-VIII and X (Rotto and Percelay et al., 1992).

Activity-dependant labeling has been employed in numerous studies to identify the neurons active in locomotion. The activity dependant metabolic marker, 2-deoxyglucose, was used to label locomotor neurons during L-DOPA induced fictive locomotion in low-spinal rabbits. The degree of the uptake of 2-deoxyglucose is closely correlated with the intensity of metabolic activity which is especially large at active synapses. The greatest uptake of this marker was in the region of the intermediate gray matter along L6-S1 (Viala et al., 1988).

C-fos immunocytochemistry has been used as a metabolic activity marker which allows for excellent cellular resolution of activated neurons. This technique has been used to label neurons in the decerebrate cat preparation undergoing fictive locomotion. Labeling occurred in lamina VII, especially the medial part, in lamina VIII and in lamina X (Dai et al 1990). Unilateral scratching also induces c-fos expression in the decerebrate cat preparation. Here, the

labeling pattern was focused in the dorsolateral part of the ventral horn, in the intermediate zone (lamina VII), medial part of lamina VII and in lamina VIII (Barajon et al., 1992).

Sulphorhodamine, a fluorescent dye known to be taken up endocytotically in synaptically active neurons has been used to translate this locomotor activity into neuronal labeling in the in-vitro neonatal rat preparation. Fictive locomotion was chemically induced by the application of NMDA and 5-HT. Labeled cells have been localized around the central canal, the intermediate zone, laminae VI-VII and X (Kjaerulff et al., 1994).

Jasmin et al., (1994) used a Rota-Rod walking task to induce c-fos expression in the in vivo rat preparation. Labeling had occurred in the dorsal horn and especially in laminae VII and X of the ventral horn in the cervical and lumbar cords. The labeling was dramatically reduced in both the dorsal and ventral horns following deafferentation but persisted in the area around the central canal. It is interesting to note that the neurons around the central canal of the in vitro neonatal rat spinal cord exhibit N-methyl-D-aspartate mediated bursting properties (Hochman et al., 1994). This property is consistent with the role of these neurons in the generation

of the locomotor rhythm as demonstrated in the studies above. Further labeling experiments by Carr et al., (1995) demonstrated c-fos immunoreactivity in cells in laminae VI, VII, VIII and X of the lumbosacral segments of the decerebrate cat spinal cord. There appears to be considerable overlap in the lamina distribution of the neurons displaying negative field potentials and other neurons active during locomotion. Electrophysiological and anatomical identification of these interneurons is the next step in unraveling the intricacies of the mammalian locomotor central pattern generator.

Origin and morphological development of commissural interneurons

Ramon y Cajal, (1911) was the first to describe broad groups of interneurons in lamina V and VI of the dorsal horn, the intermediate zone (lamina VII) and lamina VIII of the ventral horn. Commissural interneurons send axons across the ventral commissure to link different areas of the spinal cord (propriospinal or intersegmental). These axons also ascend to various locations in the brainstem, cerebellum and thalamus.

Attempts at the characterization of spinal commissural cells in different species

The classification of spinal neurons in non-mammalian species, has been accomplished by using crystals or focal injections of horseradish peroxidase (HRP). Eight classes of spinal neurons have been identified in the Xenopus laevis embryos. Six of these classes are interneurons with ipsi-, or contra-laterally projecting axons (Roberts and Clark, 1982). The spinal cord organization in the zebra fish embryos has been found to be almost identical to that of the Xenopus (Bernhardt et al.,1990). A similar study in the chick has identified five or six classes of neurons that interconnect spinal segments (Oppenheim et al.1988).

Initial mammalian studies using Golgi and intracellular techniques were not successful in showing the complete characteristic morphology of the commissural and association interneurons (Scheibel and Scheibel, 1966a; Brown, 1981). Only one class of intersegmental neuron and one class of neuron projecting to the cerebellum have been previously described in terms of both location and dendritic morphology (Bras et al.,1988, 1989; Edgley and Gallimore, 1988). Silos-Santiago and Snider, (1992) were the first to conduct a detailed developmental study of spinal interneurons in mammals. The study utilized the lipid soluble tracer, DiI to

reveal the morphological characterization of spinal neurons in rat embryos. It is no longer necessary to maintain a live embryonic preparation for the DiI to be transported since this tracer is able to diffuse in aldehyde-fixed tissues making mammalian tissue more accessible. The main findings are summarized below:

a) The migration of commissural neurons proceeded directly laterally from the midline.

b) Commissural cells were labeled in the dorsal horn, intermediate zone and the ventral horn at embryonic ages (E), E13.5, E15 and E19.

c) Seven groups of these cells were present at E13.5. and a final total of 18 different groups were present by E19.

d) The groups with similar morphology tended to form clusters.

e) Most commissural cells are located dorsally and medially in relation to the ventral motor pools.

f) The dendritic arbors of the commissural interneurons are restricted to the transverse plane in early development.

Comparison and function of spinal commissural interneurons in different species

Eighteen classes of commissural neurons in mammals have been located whereas studies in non mammalian species have identified only a small number of distinguishable neuronal

cell types. Only two interneuron types have been identified in the Xenopus (Roberts and Clark, 1982) and three in the zebra fish and lamprey (Bernhardt et al., 1990; Buchanan, 1982). These extend an axon across the commissure. Two of the commissural interneurons localized in the lamprey have axons projecting contralaterally, and caudally and appear to be inhibitory. The third type is excitatory. The inhibitory commissural interneurons have been shown to be glycinergic (Grillner and Matsushima) and play an important role in the alternation between the left and right sides of the spinal cord. This reciprocal inhibition allows for the contraction of the ipsilateral muscles with the concomitant relaxation of the contralateral muscles, resulting in the undulatory movements characteristic of the swimming pattern. In amphibians and fish (Roberts and Clark, 1982; Bernhardt et al., 1990) spinal commissural cells have axons that ascend to the midbrain and thalamus and other commissural cells have bifurcating ascending and descending axons that connect the different segments of the spinal cord (Fetcho, 1990). Commissural interneurons may form an important component of the spinal swimming network in Xenopus and lamprey (Buchanan, 1982; Grillner et al., 1987; Roberts and Sillar, 1990) and in the escape reflex in goldfish (Fetcho, 1990). It is not clear which classes of the mammalian commissural cells could correspond to the commissural cells in these lower species. Some of the commissural cell groups in the dorsal horn,

intermediate zone and ventral horn of the rat are similar in location to those found for the intersegmental interneurons in the chick embryos (Oppenheim et al., 1988). The commissural interneuron that stained intracellularly following electrophysiological identification in the cat, (Bras et al., 1989), may correspond to the the intermediolateral border commissural interneuron of the rat (Silos-Santiago and Snider, 1992).

Synaptic connections of commissural cells - interneurons in lamina VIII and X

It has been shown that interneurons with axons that cross the spinal cord in the commissural region are the recipients of monosynaptic excitatory connections descending in the ventral white matter of the cord. Their axons were found to extend as far rostrally as the thoracic cord and it is likely that these axons belong to a tract ascending to the brain. Thus, these interneurons may be excited monosynaptically by fibres of descending pathways which are known to relay in the ventral horn (Willis, 1969). Spinal motorneurons in the cat and rat have been shown to be contacted by ipsilateral interneurons of lamina V-VII and commissural interneurons of lamina VIII (Harrison et al., 1984). Several other studies also describe commissural interneurons that project to contralateral motorneurons in the cat, (Alstermark and

Kummel, 1990,1986; Harrison et al.,1986; Jankowska and Skoog,1986; Noga et al., 1987, rat (Hultborn, lab communication), and guinea pig (Chantanez and Skoog, in preparation). The columns of these interneurons extend over many segments, but the density of their distribution is not uniform. Lamina VIII commissural interneurons in the lumbar region of the cat spinal cord were found within segments L7, L6, caudal L5 and at the border of L4 and L3 (Harrison et al.,1986; Jankowska and Skoog, 1986). The caudal groups have been found to be excited mainly by high threshold muscle and skin afferents and by descending tract fibres with weak mono- or di-synaptic EPSPs from group 1 afferents (Harrison et al.,1986).

It is of interest to note that lamina VIII of the mid- and lower lumbar spinal segments of the cat has been a focus of electrical activity during locomotion (Noga et al., 1995). This electrical activity may arise from lamina VIII cells activated by the cuneiform nucleus stimulation (Jankowska and Noga,1990) In addition, those cells have been shown to be be interposed in cross reflex pathways in the cat (Harrison et al., 1986). The role of these interneurons in locomotion is further supported by evidence that some spinal interneurons in lamina VIII that are activated by mesencephalic locomotor region (MLR) stimulation. They exhibit rhythmic activity which is phase-locked to the locomotor step cycle of the

contralateral side during MLR-evoked fictive locomotion in the cat (Jordan and Noga 1991).

Commissural projections of the Hofmann Nuclei (HN) of the chicken embryo have been recently shown to send terminal collaterals to lamina VIII. This is important because lamina VIII commissural interneurons are known to be presynaptic to motorneurons (Harrison et al., 1986; Jankowska and Noga, 1990). The commissural Hofmann Nuclei are thus probably an important component of the spinal interneuronal network for locomotion.

Role of commissural cells in interlimb locomotion

Commissural cells play an important role in the neural control of locomotion because they are required for interlimb coordination (Kulagin and Shik 1970; Forssberg et. al., 1980). It has been demonstrated that powerful contralateral effects on motorneurons are produced by the stimulation of muscle afferents (Ayra et al 1991), and a fairly large number of group II interneurons are activated by contralateral group II afferents (Bajwa et al 1992). These are probably the same neurons rhythmically active during fictive locomotion (Shefchyk et al., 1990). One of the major conclusions drawn from past studies is that the step cycle of one limb is strongly influenced by the step cycle in the contralateral limb. Commissural interneurons have also been shown to be

involved in the resetting of the locomotor rhythm produced by group I and group II muscle afferent stimulation (Perreault et al 1994).

Bilateral field potential recordings in the lumbar enlargement of the rat spinal cord suggest that when the command signals from the MLR and medial reticular formation (MRF) descend to the spinal cord via the fibers of the ventrolateral funiculus (VLF), they cross over the rostral lumbar segments and terminate in the ventral and intermediate laminae (Magnusson and Trinder 1997). This clearly indicates that the fibers carrying important locomotor signals in the spinal cord cross over at the midline. Several lesion studies have also revealed the role of the commissural cells in the mediation of coordinated left/right alternating activity during locomotion. Harder and Schmidt (1992) showed that left-right coordination is possible with only a small number of commissural connection remaining, while Kjaerulff and Kiehn demonstrated that left/right alternating rhythms are contingent upon intact commissural fibers of the spinal cord (1996). After examining the organization of the transverse coupling system in the neonatal rat, Kremer and Lev-Tov, (1997) concluded that the T12-L4 segments have important cross connections which aid in the mediation of left and right alternating activity during locomotion.

Important characteristics of commissural cells

A sub-population of commissural cells in the turtle spinal cord have an important intrinsic cell property, ie. calcium mediated plateau potentials. These bi-stable potentials would be valuable in enhancing the cells' ability to generate alternating rhythmic activity (Hounsgaard 1992). A study in the cat revealed that some of the cells active during MLR induced fictive locomotion were also cholinergic (Carr et al., 1994). Upon reconstruction, these cells possessed contralaterally projecting axons. A sub-set of cholinergic commissural cells has also been described in previous studies (Houser et al., 1983; Barber et al., 1984; Phelps et al., 1984; Borges and Iverson, 1986; Phelps et al 1990). Acetylcholine has been shown to produce coactivation of flexors and extensors on one side of the spinal cord and alternation between the left and right sides of the cord during fictive locomotor studies (Cowley and Schmidt 1994).

Expression of c-fos as a marker of neuronal activity

Synaptic activity alters the post synaptic gene expression in two ways. An early response is the rapid induction of immediate early genes (IEG's) in response to neuronal stimuli (Greenberg et al., 1985; Morgan and Curran, 1986; Bartel et al., 1989; Barzilai et al., 1989), and a delayed expression of late-onset genes (Merlie et al., 1984; Castellucci et al., 1988; Goldman et al., 1988; Barzilai et al., 1989; Offord and

Catterall, 1989; Klarsfeld et al., 1989). Together, these genes encode differentiated neuronal products eg. neuropeptides and neurotransmitter biosynthetic enzymes.

Regulation of immediate early genes

Immediate early genes were first detected in growth factor stimulated fibroblasts and subsequently, in neuronal cell lines (Curran and Morgan, 1987; Lau and Nathans 1987). They have several distinguishing features. Their expression is low in non-activated cells, but are rapidly induced ie. within minutes of extracellular stimulation (30-60 minutes), at the transcriptional level. This induction is transient and independant of new protein synthesis. The mRNA's that are transcribed from these genes typically have a short half-life, eg. c-fos mRNA has a half-life of 10-15 minutes (Sheng and Greenberg, 1990).

One of the best characterized IEG to date is the c-fos gene (Curran, 1988). It was initially characterized as a viral oncogene in feline osteosarcoma cells. The cellular proto-oncogene counterpart, c-fos, probably has a regulatory function in encoding transcription factors. C-fos combines with another IEG, c-jun, to form a Fos/Jun hetero-dimer which activates the transcription of target genes (Halazonetis et al., 1988; Kouzarides and Ziff, 1988; Nakabeppu et al., 1988;

Rausher et al., 1988a). C-fos expression is rapidly activated in response to neuronal stimuli and is turned off within an hour (Armstrong and Montiminy, 1993). The c-fos gene can be induced by a wide variety of stimulants which utilize different second messenger pathways. In PC12 cell cultures, the expression of c-fos mRNA and protein have been induced by the following: depolarization (Morgan and Curran, 1986); agents that activate voltage dependant calcium channels (Morgan and Curran, 1986); neuro-transmitters (Greenberg et al., 1986) and neurotrophic factors (Curran and Morgan, 1985). These various agents employ many second messenger systems that include the modulation of the intracellular calcium levels, cyclic AMP and protein kinase C activity (Morgan and Curran, 1989).

Several studies have also successfully demonstrated the induction of c-fos in the intact nervous system, further supporting the idea that c-fos is an important regulator of nerve cell responses in vivo. Stimulation of the mammalian nervous system by pharmacological agents (Morgan et al., 1987), electrical stimuli (Dragunow and Robertson, 1987; Sagar et al., 1988), surgical trauma (White and Gall, 1987) and physiological challenges (Hunt et al., 1987; Sagar et al., 1988; Bullit, 1989) resulted in increased c-fos expression. Numerous investigators have also used c-fos

expression as a means of identifying the cells active during locomotion (Dai et al., 1990; Jasmin et al., 1994; Carr et al., 1995).

C-fos immunoreactivity

The c-fos mRNA encodes a protein product, Fos protein. This protein is rapidly synthesized and translocated to the nucleus. An antibody directed at the N-terminal of Fos (Franza et al., 1987) is utilized in the immunocytochemical studies to selectively observe c-fos immunoreactivity. The nuclear localization of the antigen allows for excellent cellular resolution of activated cells and is ideal for double-labeling immunocytochemical techniques where the second antigen is located elsewhere in the cell, e.g. the cytoplasm. These cells may be characterized by location, type of neurotransmitter, cytologic appearance and other morphological features. The mapping of c-fos induction is therefore a powerful tool in identifying the cellular and neuroanatomical targets of pharmacological stimuli or the intricate pathways involved in neurophysiological responses.

Fluorogold as a retrograde tracer

Fluorogold is a fluorescent *stilbene* derivative and is made up of two benzene rings connected by a vinyl link. This alternating single and double bond configuration of the whole molecule has electrons lying within the outer p-orbital

clouds. Ultraviolet light (UV) excites these electrons to a higher energy level and upon return to their original energy level they emit an intense blue light. This valuable fluorescent compound, fluorogold, has found several applications in neuroanatomical studies, especially as a retrograde axonal tracer, because of its unique set of properties gives it a distinct advantage over the other fluorescent tracers ie. an intense white/blue fluorescence in an an acidic medium, its capability for extensive dendritic filling, its resistance to fading easily, its demonstrated compatibility with other neuroanatomical and immunohistochemical techniques, its confinement within the cell without any detectable leakage, it allows for various survival times, and it does not appear to be taken up from undamaged fibres (Schmued, 1990). Fluorogold, known to be taken up endocytotically from the ends of cut axons, is transported only in fresh tissue. Since it is not toxic to the tissue, the animal is able to survive as long as necessary to ensure adequate labeling of the desired structures. The average length of survival time for in vivo preparations is about one week. It is also important to note that there is minimal necrosis observed at the site of the fluorogold injection (Schmued and Fallon, 1986). The tissue does not require post injection processing and the retrogradely labeled cells may be visualized immediately upon slicing.

C-fos immunofluorescence is excited by a longer wavelength than that of the fluorogold fluorescence (UV), thereby allowing the detection of both the tracers within the same cell by utilizing separate excitation filters for separate photomicrographs. This greatly enhances the visualization and mapping of double labeled cells in neuroanatomical studies.

Aims of Study

Although there is substantial evidence for the role of spinal commissural cells in coordinating phasing of the left and right sides of the body during various rhythmic activities e.g. walking, swimming and scratching, there are several important distinguishing features of the mammalian locomotor commissural cells which have not yet been established:

1. their location in the spinal cord
2. their mechanism of excitation or inhibition
3. which neurotransmitters are employed
4. and what of their synaptic connections?

This study therefore attempts to localize anatomically the population of commissural cells active during treadmill locomotion using fluorogold retrograde fluorescent tracing and c-fos immunocytochemistry in a double labeling experiment using an in vivo adult rat model of locomotion.

MATERIAL AND METHODS

Experimental animals with locomotion

Three adult male rats (220g-270g) each underwent a laminectomy and fluorogold injection followed by a 60 min treadmill task one week later, to induce c-fos protein expression in the spinal cord. The animals were perfused immediately thereafter and the spinal cords removed. The tissue was processed for c-fos immunocytochemistry.

Experimental animals without locomotion

This group consisted of three adult, male rats (220g-270g). Each rat underwent similar surgical procedures as the experimental animals followed by fluorogold injection. Importantly, no locomotor task was performed. After the one week recovery period, these rats were all allowed to sit in the treadmill for 60 min without locomotion and perfused immediately at the end of the session and the spinal tissue processed for c-fos immunocytochemistry.

Positive control animals

Two adult, male rats (220g-270g) performed the 60 min treadmill locomotor task only and did not undergo a laminectomy nor were they injected with fluorogold. The purpose of this phase of the experiment was to establish the pattern of c-fos expression induced by treadmill locomotion and to ensure that the c-fos antibody and the

immunohistochemical procedures were indeed reliable in detecting activity induced c-fos immuno-reactive cells. The tissue from these animals was processed simultaneously with those of the experimental and control animals.

LABORATORY TECHNIQUES

a. Fluorogold injection

Each rat was weighed and anesthetized via an IP injection of the appropriate dosage of a Rompun, Ketalean and Atropine mixture (KAR). K.A.R. surgical dose: Ketamine 100 mg/kg (10 mg/100g rat); Atropine 0.05mg/kg (0.005mg/100g rat); Rompun 10 mg/kg (1mg/100g rat); saline 0.1 ml/100g rat. Anesthesia was facilitated by placing the animal in a closed chamber containing halothane and nitrous oxide, immediately prior to KAR injection. When the animal became light as determined by the toe-pinch reflex, halothane (0.8-1.5%) was used to maintain a surgical depth of anesthesia. The animal was then injected with an antibiotic, Derapan, 0.01ml/100g. The eye blink reflex and the toe pinch reflex were monitored to determine how deeply the rat was anesthetized. Once the animal was anesthetized, gentocin opthalmic ointment was applied to the eyes, the dorsal surface shaved and the area wiped down with betadine. The animal was placed in a stereotaxic apparatus. The rostral end of the animal was stabilized by clamping the head in the stereotaxic frame and

the spinal column was stabilized by clamping the L2 vertebra. The laminectomy was performed at T13 vertebral level, to expose a small area of the spinal cord of the corresponding lumbar segment. Stereotaxic measurements were set to pressure inject 0.1ul of 1%fluorogold, 0.75mm lateral to the central canal on the right hand side at an angle of 20^0 ventrolateral to the spinal cord and to a depth of 1.6 mm. A few drops of xylocaine were applied onto the cord and after a few minutes fluorogold was gradually injected over a 10min. period. The needle was allowed to remain in position for 5min. and then slowly removed. A small strip of gel foam was gently placed into the incision area and the skin closed with stainless steel clips. Betadine and xylocaine paste were applied over the incision area. A 0.15 ml IM injection of an analgesic, Banamine, was administered for pain control immediately after surgery and as required thereafter. The animal was allowed to recover for 1 week before exposure to the treadmill.

b. Treadmill procedure

Day 1

A motorized 4 lane treadmill (Columbus Instrument International Co. Columbus, OH) was used. The power switch was turned on , the shock grid turned off and the belt speed set to zero. The rat was placed in a lane and allowed to adapt for 15 min. It was then carefully removed, given a

peanut butter treat and returned to the animal room.

Day 2

The power was switched on only. The rat was placed in the center lane and then the shock grid was switched on. The intensity and repetition rate were each set at 5. These settings were maintained or gradually increased until an adequate stimulus was attained to prevent the rat from approaching the grid. The belt speed was slowly increased over a 5 min. period up to 0.14m/s. The rat was maintained at this walking speed for about 5 min. It was then carefully removed once all switches were tuned off, given a peanut butter treat and returned to the animal room.

Day 3

The initial steps as in "Day 2" were repeated and the rat was allowed to walk comfortably at 0.14m/s for a total time of 60 min. It was removed carefully and overdosed with an mixture of Ketalean and Rompun (KR). The eye blink reflex and the toe pinch reflex were carefully monitored, and the perfusion procedure was performed only once an appropriate level of anesthesia was achieved.

c. Perfusion procedure

Perfusion solutions were made up fresh on the morning of the perfusion and stored at 4 ^{0}C.

i. Perfusion solutions per animal: 100ml Pre - fixative

0.1g $NaNO_2$

0.1ml heparin

10ml 0.5 M phosphate buffer

10ml 9% NaCl stock soln.

topped up to 100ml with distilled water

ii. 500ml 4% Paraformaldehyde (100ml/100g weight of animal)

20g paraformaldehyde was weighed out, 200ml distilled water added and the mixture heated and stirred to 60 ^{0}C. A few drops of NaOH was added to clear the solution. Upon clarification, the mixture was removed from the stirrer and cooled to room temperature. 100ml 0.5M phosphate buffer was added and then filtered through no. 1 filter paper. The pH was adjusted to 7.4 with NaOH and HCl and the mixture topped up to 500ml using distilled water.

Perfusion Pump Calibration

The tubing was first washed through with distilled water. Thereafter it was primed prior to perfusion with cold pre - fixative solution, and the flow rate was adjusted to 14ml/min.

Anesthetic overdose procedure

The rat was removed from the treadmill once it had completed 60 minutes of locomotion. It was overdosed with a Ketalean and Rompun mixture via an IP injection. K.R. euthanizing dose: Ketamine 400 mg/kg (40 mg/100g rat); Rompun 10 mg/kg (1mg/100g rat); saline 0.1 ml/100g rat. The toe pinch reflex and the blink reflex were monitored to determine how deeply the animal was anesthetized.

Perfusion procedure

Once it was established that no reflexes were present, the heart was exposed. The cannula attached to the perfusion pump was inserted into the apex of the left ventricle, the pump was switched on and the pre- fix perfusion was commenced. A small incision in the right atrium was made to allow the blood to drain out. Thereafter, the animal was perfused with the 4% paraformaldehyde solution. A laminectomy was performed to expose the spinal cord and the dura mater was removed. The spinal cord was isolated using a pair of fine scissors and then placed in 4% paraformaldehyde solution for two hours. Thereafter the cord was transferred to a 25% sucrose solution in 0.1M phosphate buffer and 10% glycerol for a minimum of three days for cryo-protection. The sucrose solution was changed at least once before slicing.

Cryo - Protectant Solution (25% sucrose in 0.1M phosphate buffer and 10% glycerol)

125g sucrose

300ml distilled water

50ml glycerol

50ml 0.5M phosphate buffer, pH 7.4 top up to 500ml with distilled water store at 4 ^{0}C

d. C-fos immunocytochemistry

Solutions for Immunocytochemistry:

0.1M Phosphate buffered 0.9% saline (PBS)

70ml distilled water

20ml 0.5M phosphate buffer, pH 7.4

10ml 9% NaCl stock soln.

0.1M Phosphate buffered 0.9% saline and 0.3% Triton-X (PBST)

200ml 0.5M phosphate buffer, pH 7.4

100ml 9% NaCl stock soln.

3ml Triton - X

The solution was topped up to 1000ml using distilled water and stirred until homogenized.

50mM TRIS (Trizma Base)

5ml 1M TRIS soln.

95ml distilled water

Immunocytochemistry

The cord was sectioned at 16 um on the sliding microtome and serial sections were collected in individual wells in PBS. Every third section was washed 3x10 min. in PBST solution and then left in the fourth PBST solution overnight in the cold room at 4 ^{0}C. They were then mounted in 50 mM TRIS solution onto subbed slides and allowed to dry for at least 4 hrs. at room temperature. The sections were rehydrated for 30 min. in PBST. The back of the slides and around the sections were dried and a pap pen used to make a well around the sections. Well markings were allowed to dry for a few seconds and then the primary antibody , Polyclonal rabbit anti - c - fos (Santa Cruz Biotechnology), diluted 1:5000 in 0.1M PBS, 0.9% sodium chloride, 0.3% Triton X100 and 1% horse serum, was inserted. The slides were stored in the cold room for 3 days and the antibody replenished as required. On the fourth day, the sections were washed 3x30 min. in PBST solution and the secondary antibody, Donkey anti-rabbit Cy3 (Jackson Immuno Research Laboratory Inc), diluted 1:250 in 0.1M PBS, 0.9% sodium chloride, 0.3% Triton X100 and 1% horse serum, inserted and the sections incubated for 2.5 hrs. at room temperature. Thereafter they were washed 1x20 min. in PBST solution and 2x20 min. in 50 mM TRIS solution and air dried overnight. The slides were cover slipped using anti - fade commercial vector medium and stored in the freezer.

e. Neurolucida image analysis

Spinal cord slices extending from the T13-L6 segments were examined under epifluorescence microscopy or light microscopy and the gray and white matter outlines as well as the location of labeled cells were drawn using an image analysis program, Neurolucida™. The specific location of the sections in the spinal cord was determined by the shape of the gray matter. Both fluorescent tracers, c-fos and fluorogold, were detected in either the same cell or separate cells by the alternate utilization of two different excitation filters, Rhodamine and UV, respectively.

RESULTS

Several retrogradely labeled spinal locomotor neurons were identified in this study. They were localized primarily in laminae VII and X of the intermediate zone and lamina VIII of the ventral horn (Fig.1 & Fig.2).

Pattern of c-fos labeling:

Animals with no locomotion

C-fos expression in the spinal cord of the animals with no locomotion was minimal and the neurons were restricted to the dorsal horns bilaterally. No c-fos positive cells were detected in laminae VII, VIII, IX or X, bilaterally (Fig.4).

Animals with locomotion

In all 3 experimental animals that walked on the treadmill for 1 hr, numerous c-fos positive neurons were distributed in the spinal cord from the T13 to the L6 segments. These neurons were located bilaterally in laminae I to lamina VI of the dorsal horn, laminae VII and X of the intermediate zone and laminae VIII and IX of the ventral horn (Fig.5-Fig.11).

Pattern of fluorogold labeling:

i. Ipsi-lateral to the injection site

a. Experimental animal 1- with locomotion

There was extensive and intense fluorogold labeling at the injection site (~0.5mm in diameter), which was localized to the spinal cord segment L5. The injection site extended from the dorsal horn to the ventral horn. Few fluorogold positive cells extended rostrally and caudally from the injection site from the T13 to the L6 segments. The distribution of these cells was restricted to laminae II to VI of the dorsal horn, laminae VII and X of the intermediate zone and lamina VIII of the ventral horn (Fig.12-Fig.14).

b. Experimental animal 2- with locomotion

Fluorogold cells were well distributed in both the dorsal and ventral horns at the injection site (~0.6mm in diameter) in the region of the L3/L4 segment. Many fluorogold positive cells extended rostrally and caudally from the injection site to involve the T13 to the L6 segments. The laminar distribution was as follows: laminae I-VI of the dorsal horn, laminae VII and X of the intermediate zone and lamina VIII and IX of the ventral horn (Fig.15 & Fig.16).

c. Experimental animal 3- with locomotion

There was extensive fluorogold labeling in both the dorsal

and ventral horns at the injection site (~0.5mm in diameter) of the L6 segment. Several fluorogold cells were also distributed rostrally and caudally from the injection site extending from segments T13 to the L6. The involved laminae were: laminae II-VI of the dorsal horn, laminae VII and X of the intermediate zone and lamina VIII of the ventral horn (Fig.17 & Fig.18).

d. Experimental animals with no locomotion

There was extensive and intense fluorogold labeling at the injection sites located at L3-L6 in the lumbar enlargement. Similar to the rostral-caudal distribution in the experimental animals with locomotion, the fluorogold labeled neurons extended from the T13 to the L6 segments. The laminar distribution was also similar to that of the animals with locomotion.

ii. Labeling (FG) contralateral to the injection site:

The rostral-caudal distribution of fluorogold labeling on the side contralateral to the injection site was identical in the experimental animals with and without locomotion and extended from T13 to L6 in all animals. There were slight differences in the lamina distribution of fluorogold staining neurons in the animals with locomotion. At the level of injection in animal 1, a few fluorogold positive cells were distributed throughout laminae I-VI of the dorsal horn contralateral to

the site of injection. In the second animal laminae II-VI were involved while in the third animal fluorogold cells were found in lamina III-IV. However, in all the animals, laminae VIII of the ventral horn and laminae VII and X of the intermediate zone were uniformly involved, which was the location of the majority of the retrogradely labeled neurons (Fig.12 - Fig.18).

C-fos and fluorogold double labeling in animals with locomotion:

The double labeled neurons in all experimental animals with locomotion, were localized bilaterally primarily in the region of the injection site (Figs.21, 24 & 27; tables 1, 2,& 3). However, the number of c-fos and fluorogold double labeled neurons in animal 1, was less than that observed in both animals 2 and 3. This may be attributed the intensity of fluorogold labeling which was much reduced in animal 1.

1. Ipsi-lateral double labeling

a. <u>Experimental animal 1-with locomotion</u>

The rostral-caudal distribution was from segments L2-L6 in laminae I-VI of the dorsal horn, laminae VI-X of the intermediate zone and lamina VIII of the ventral horn (Fig.19-Fig.20).

b. Experimental animal 2-with locomotion

Double labeled cells were well represented from segments T13-L6, in laminae I-VI of the dorsal horn, laminae VII and X of the intermediate zone and laminae VIII-IX of the ventral horn (Fig.22-Fig.23).

c. Experimental animal 3- with locomotion

The rostral-caudal distribution of the retrogradely labeled cells extended from segments L3-L6 in laminae I-VI of the dorsal horn, laminae VII and X of the intermediate zone and laminae VIII and IX of the ventral horn (Fig.25-Fig.26).

2. Double Labeling contralateral to the injection site

a. Experimental animal 1- with locomotion

The rostral-caudal distribution of double labeled cells was from segments L4-L6 in laminae III-VI of the dorsal horn, laminae VII and X of the intermediate zone and lamina VIII of the ventral horn (Fig.19-Fig.20).

b. Experimental animal 2- with locomotion

Several double labeled cells were found in segments T13-L6, in laminae II-VI of the dorsal horn, laminae VII and X of the intermediate zone and lamina VIII of the ventral horn (Fig.22- Fig.23).

c. Experimental animal 3- with locomotion

The rostral-caudal distribution extended from segments L3-L6 in laminae III-VI of the dorsal horn, laminae VII and X of the intermediate zone and lamina VIII of the ventral horn (Fig.25-Fig.26).

C-fos and fluorogold double labeling in animals without locomotion:

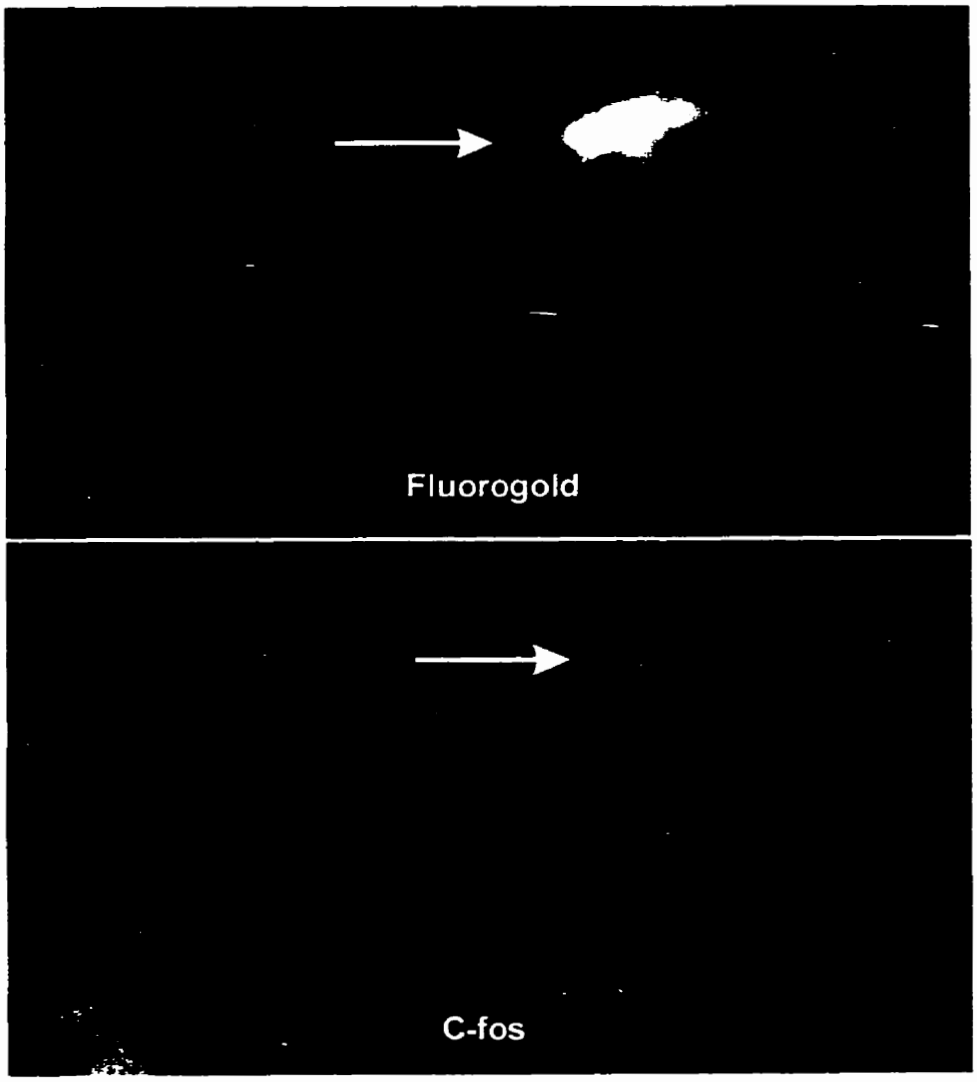
No double labeled cells were observed in these animals. Although the fluorogold labeling was substantial and extended from the T13 to the L6 segments, the absence of double labeled cells is a reflection of the minimal c-fos expression in the spinal cord. The c-fos labeled neurons were restricted to both dorsal horns. No c-fos immunoreactivity was detected in either laminae VII and X of the intermediate zone or lamina VIII of the ventral horn, bilaterally(Fig. 4).

Figure 1

Fluorescent photomicrographs (20x) to show a single, double labeled, spinal cell in an experimental animal with treadmill locomotion. This cell is labeled with both fluorogold and c-fos and is located in lamina VIII of the lumbar spinal cord (L6), contralateral to the site of the fluorogold injection.

Figure 1

Fluorogold and C-fos double-labeled cell in an experimental animal with locomotion.



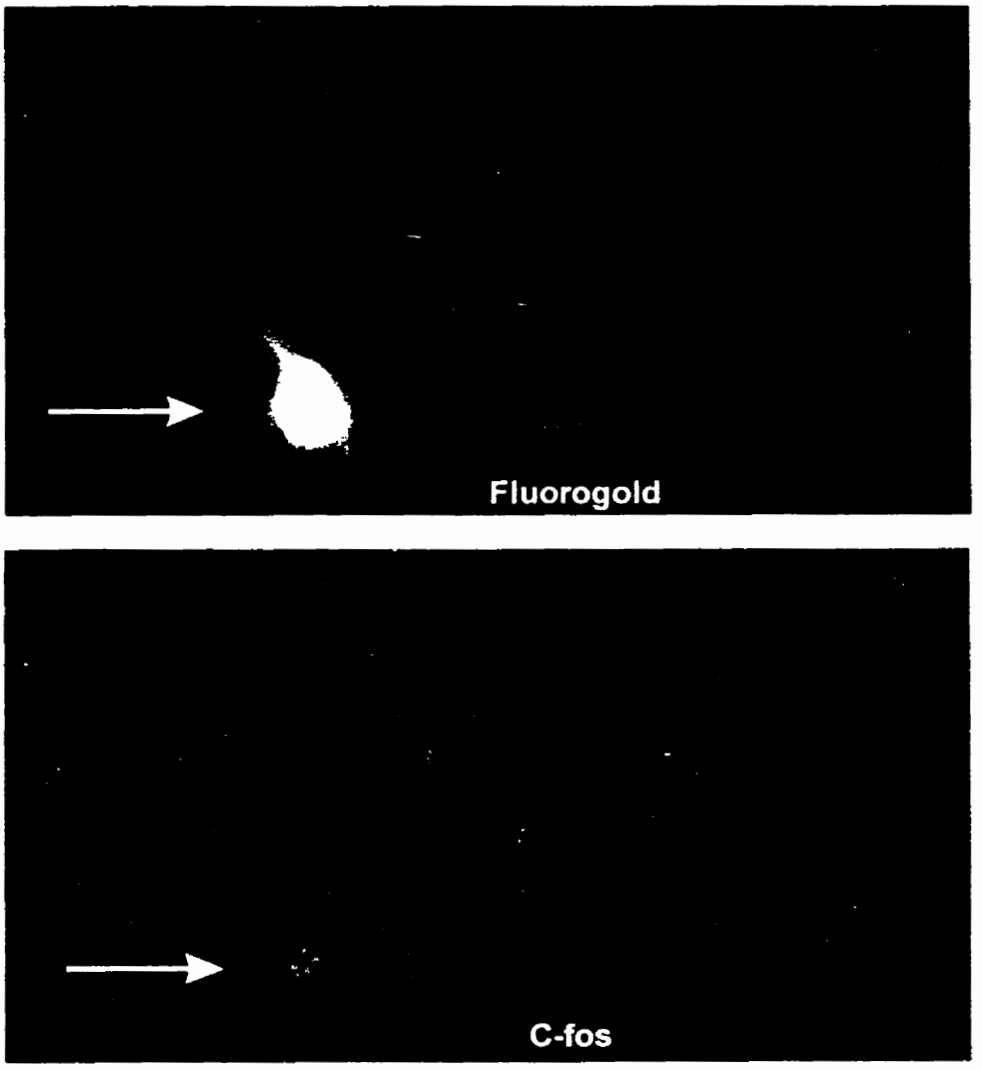
Fluorescent micrographs indicating a fluorogold and c-fos double labeled cell and its location in the spinal cord.

Figure 2

Fluorescent photomicrographs (20x) to show a single, double labeled, spinal cell in an experimental animal with treadmill locomotion. This cell is labeled with both fluorogold and c-fos and is located in lamina VII of the lumbar spinal cord (L5), contralateral to the site of the fluorogold injection.

Figure 2

Fluorogold and C-fos double-labeled cell in an experimental animal with locomotion



Fluorescent micrographs indicating a fluorogold and c-fos double labeled cell and its location in the spinal cord.

Figure 3

Fluorescent photomicrographs (10x) to show c-fos and fluorogold labeling following one hour treadmill locomotion in the rat lumbar spinal cord, contralateral to the fluorogold injection site.

C-fos and Fluorogold labeling in an experimental animal with locomotion

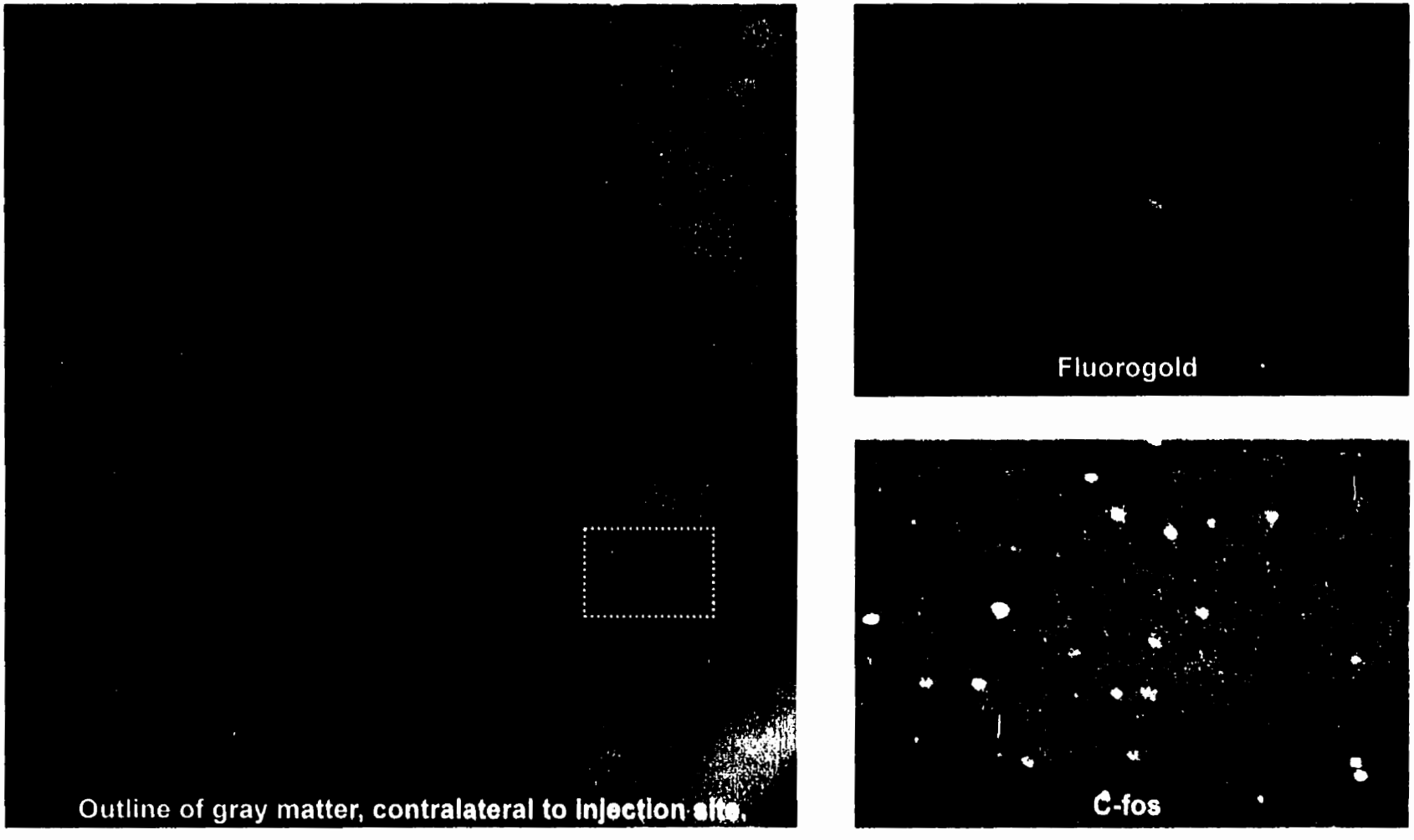


Figure 3 Fluorescent micrographs indicating c-fos and fluorogold labeling in a rat with locomotion

Figure 4

Fluorescent photomicrographs (10x) of an experimental animal with no locomotion to show c-fos and fluorogold labeling in the lumbar spinal cord, contralateral to the fluorogold injection site.

C-fos and Fluorogold labeling in an experimental animal with no locomotion

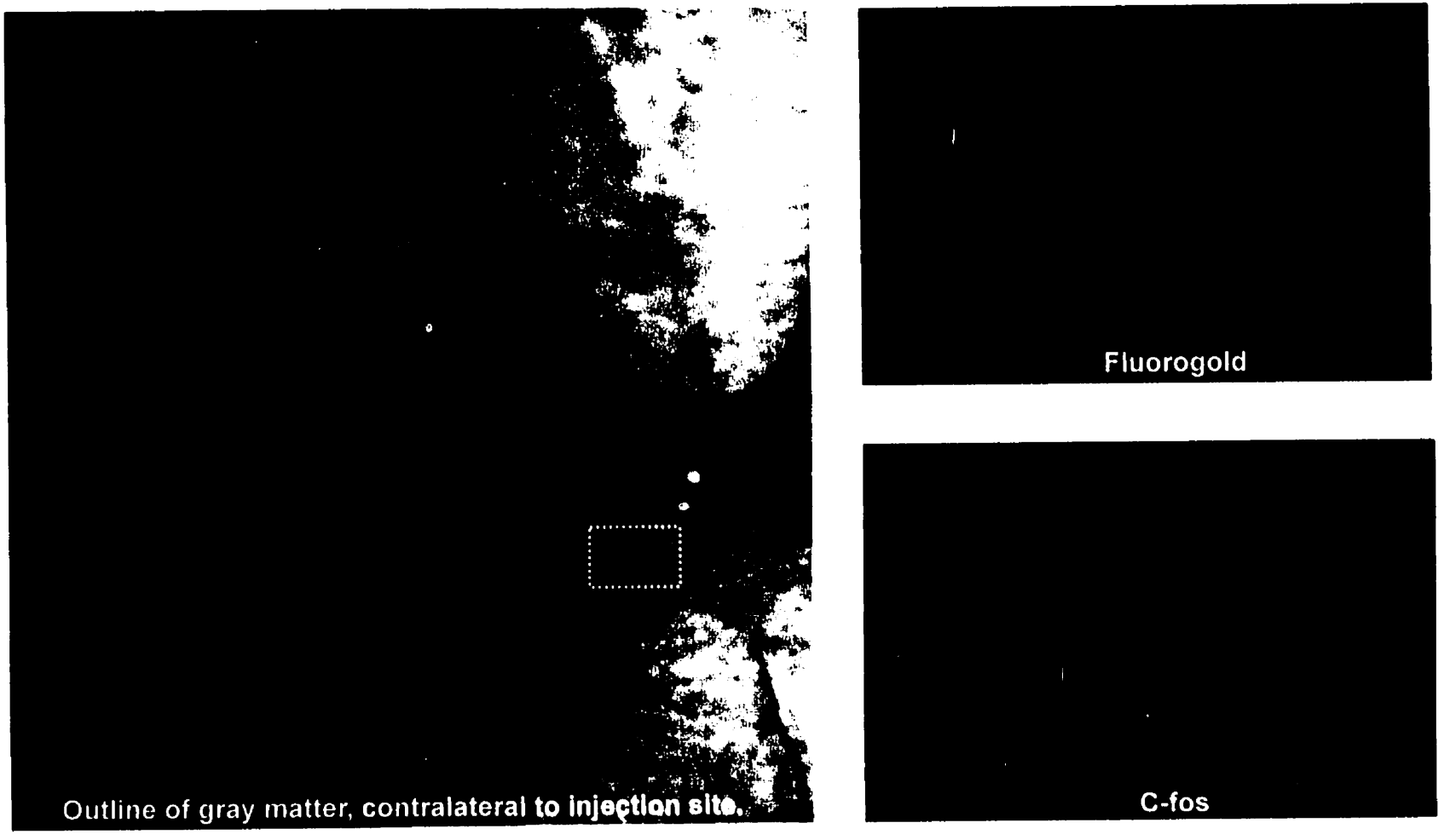


Figure 4 Fluorescent micrographs indicating c-fos and fluorogold labeling in a rat with no locomotion

Figure 5-11

Camera lucida drawings to show the rostro-caudal distribution of c-fos positive cells from spinal segments T13-L6, in all the experimental animals following one hour of treadmill locomotion. The open triangles represents c-fos positive neurons.

Distribution of c-fos labeled cells in transverse spinal sections T13-L1

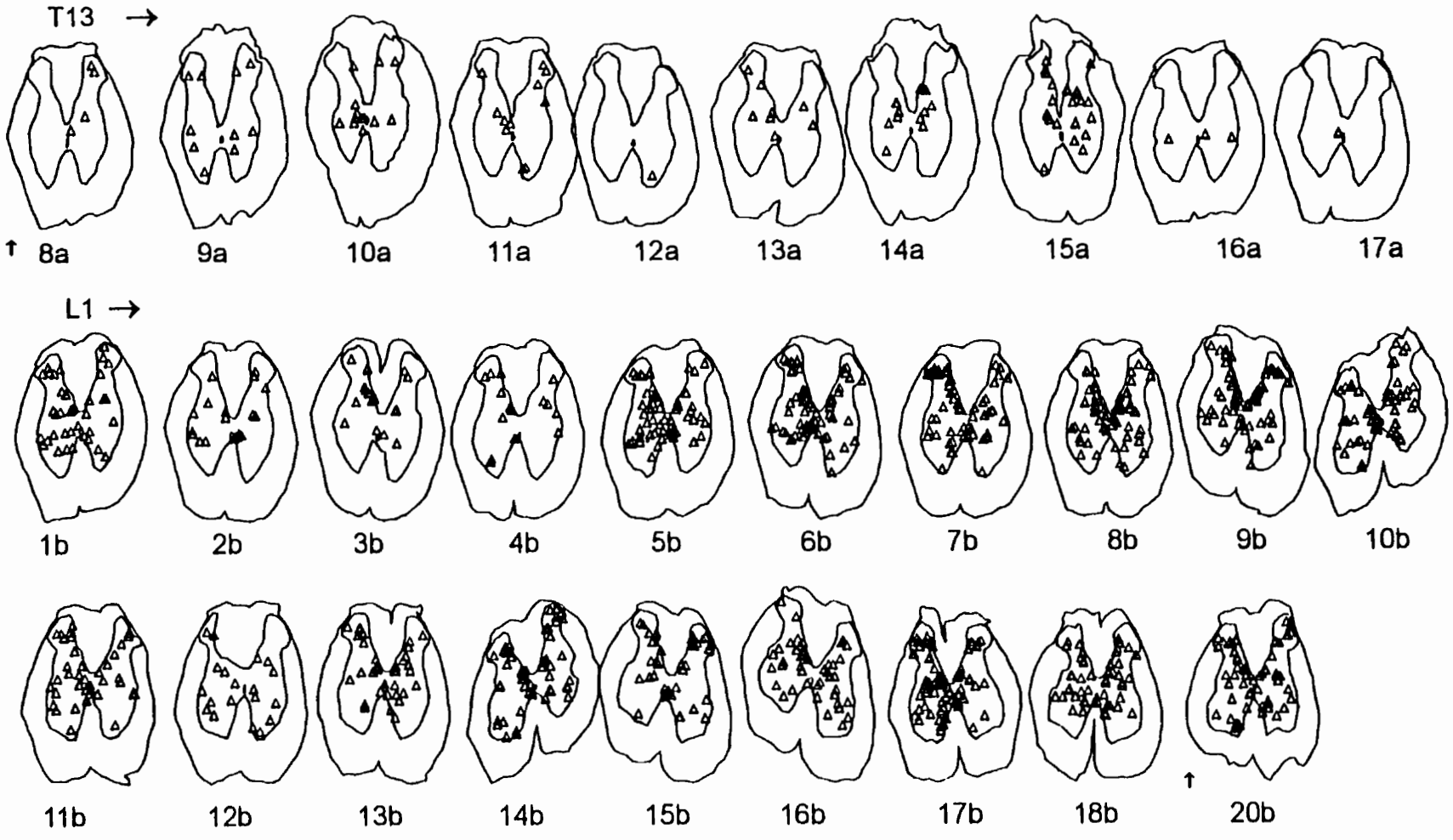


Figure 5 Experimental animal 1, after locomotion.

Distribution of c-fos labeled cells in transverse spinal sections L2-L5

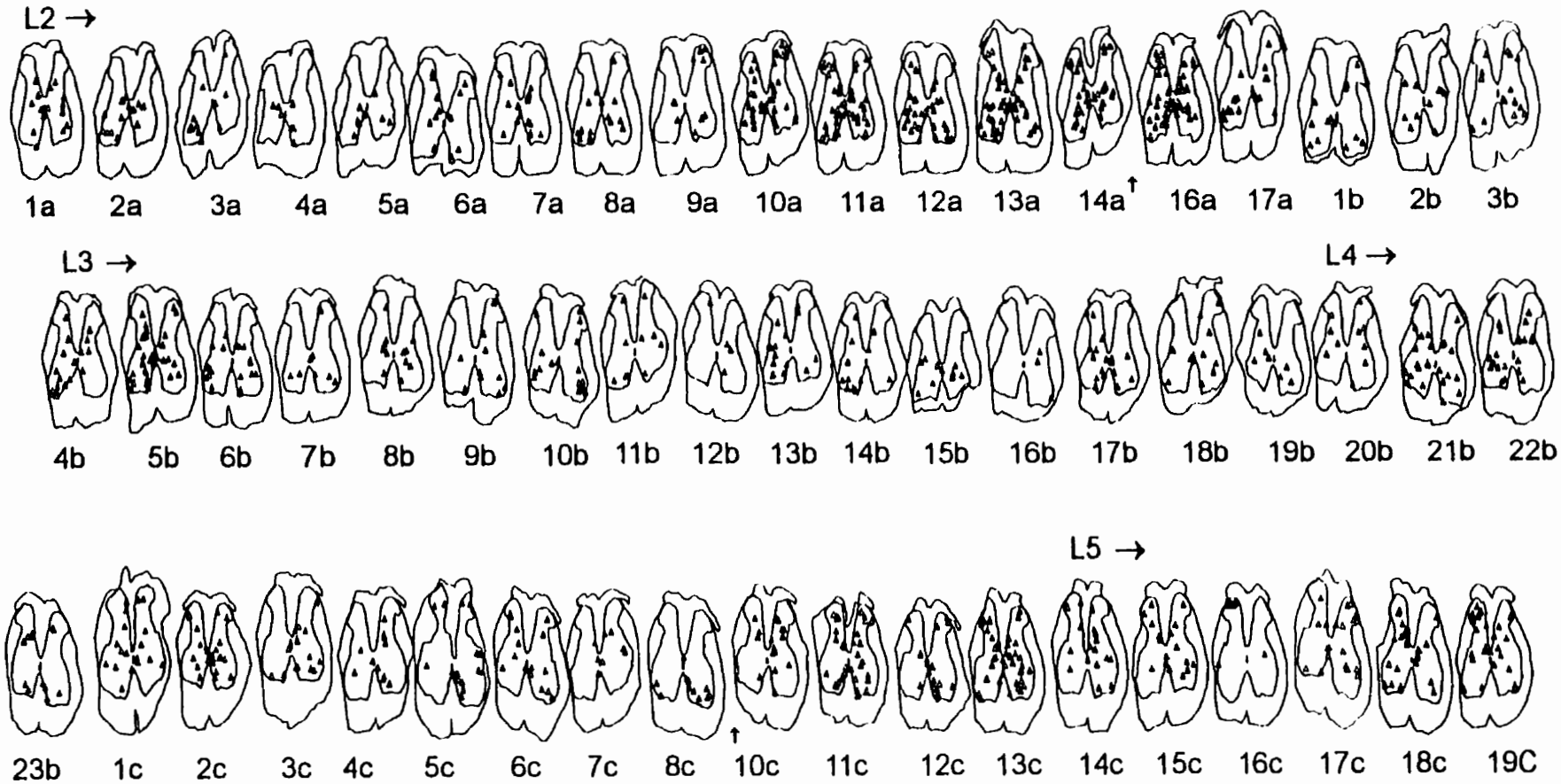


Figure 6

Experimental animal 1, after locomotion

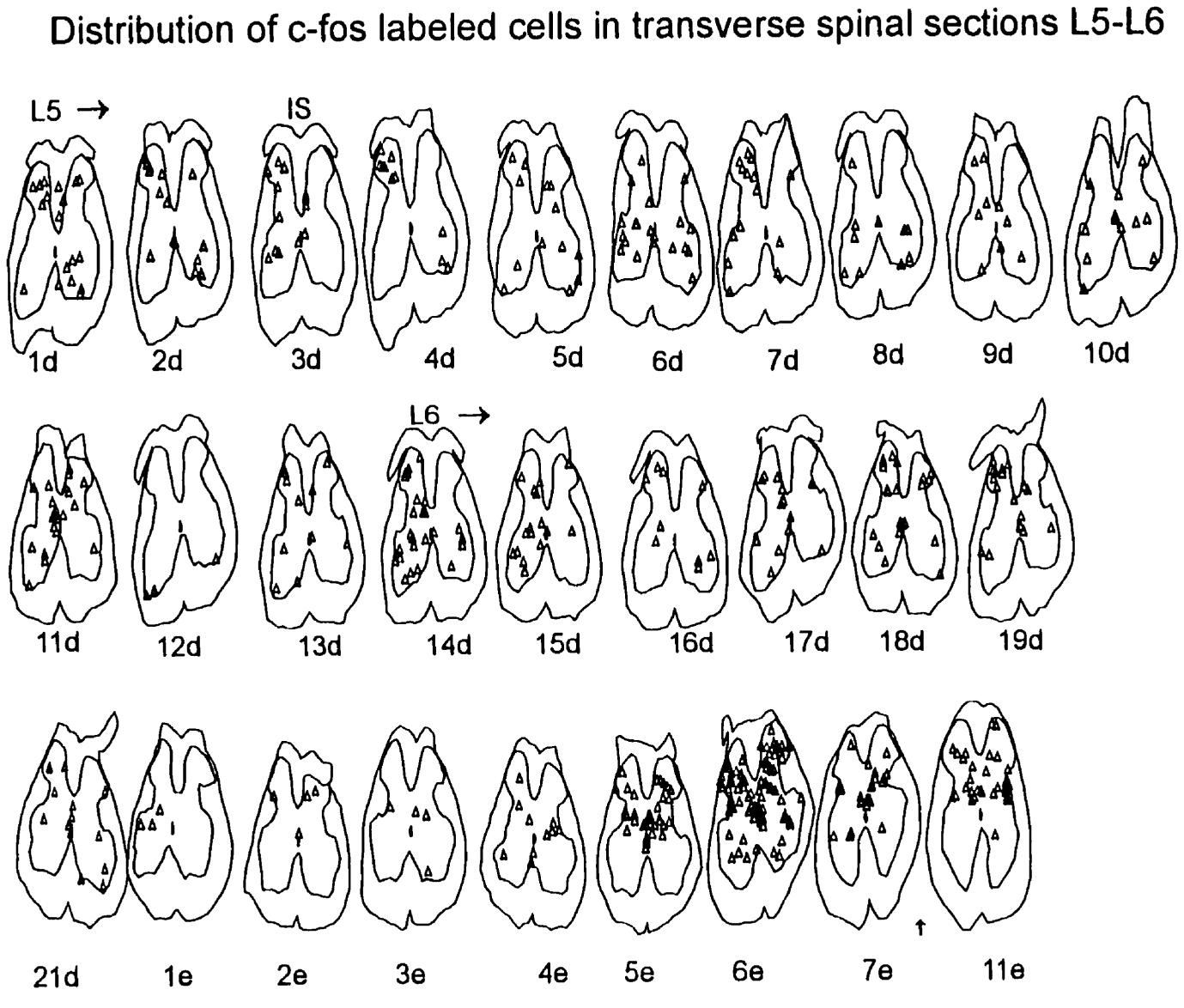


Figure 7 Experimental animal 1, after locomotion. Sections L5-L6, including injection site.

Distribution of c-fos labeled cells in transverse spinal sections T13-L1

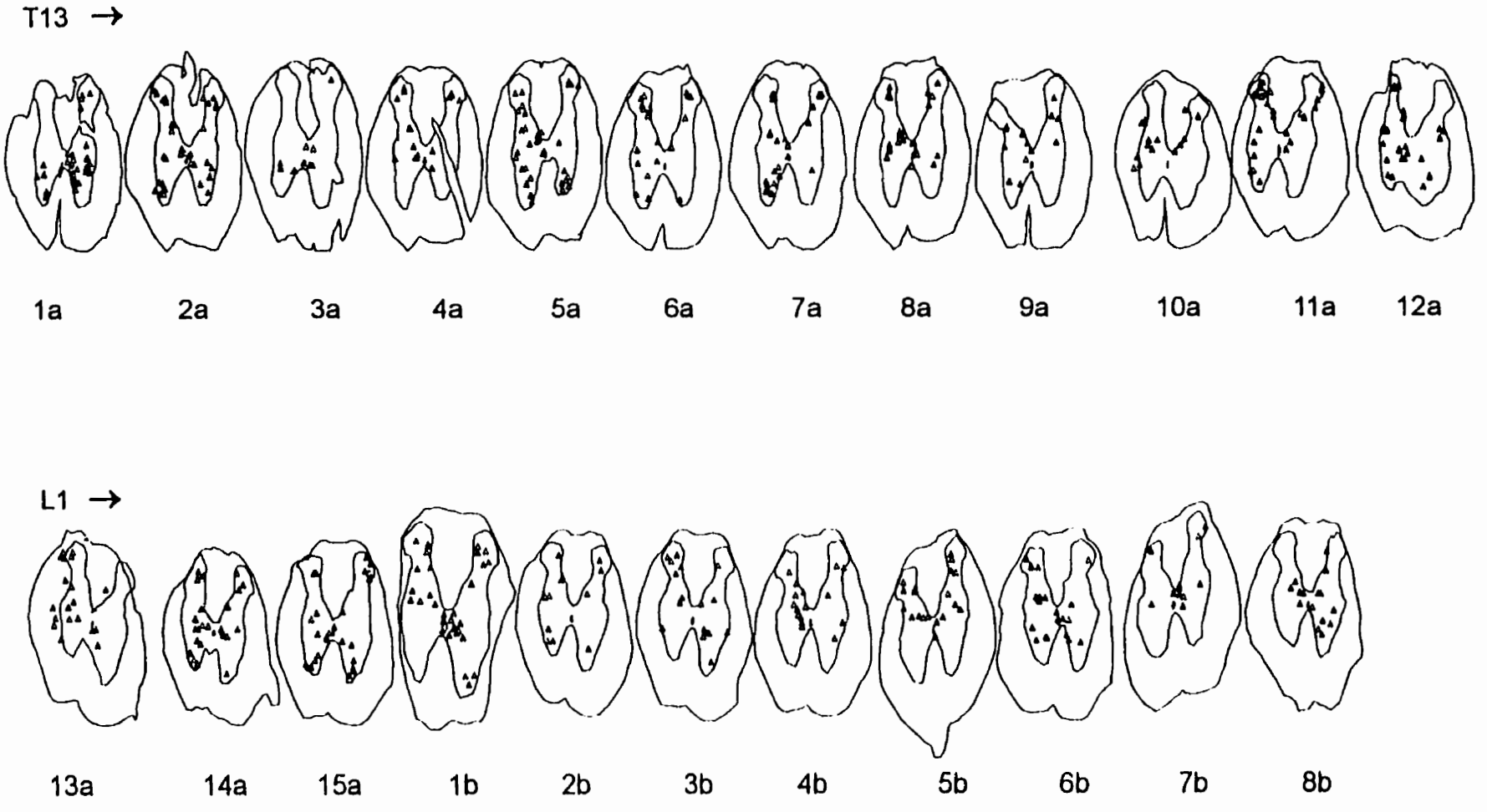


Figure 8 Experimental animal 2, after locomotion.

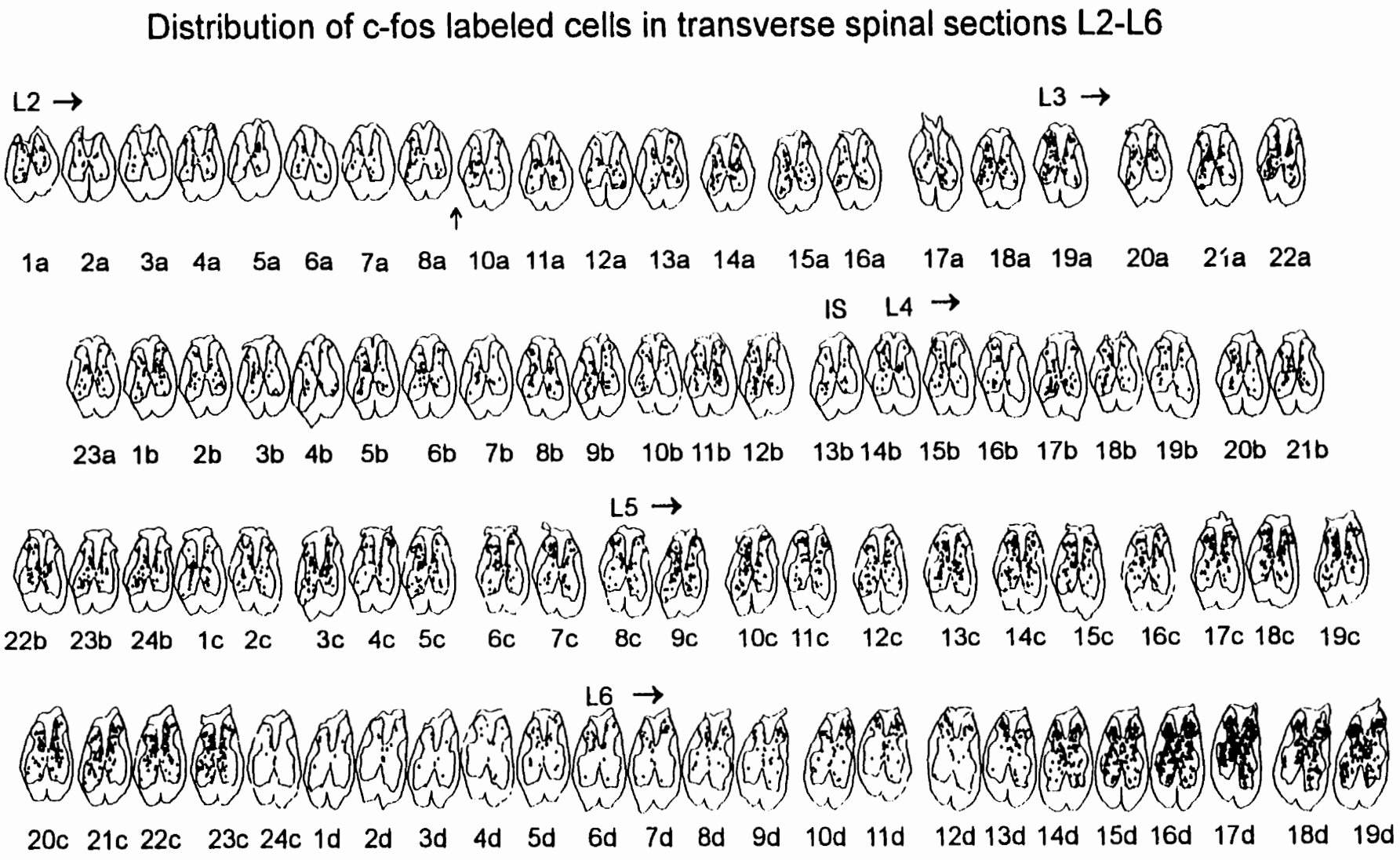


Figure 9 Experimental animal 2, after locomotion. Sections L2-L6, including injection site.

Distribution of c-fos labeled cells in transverse spinal sections T13-L3

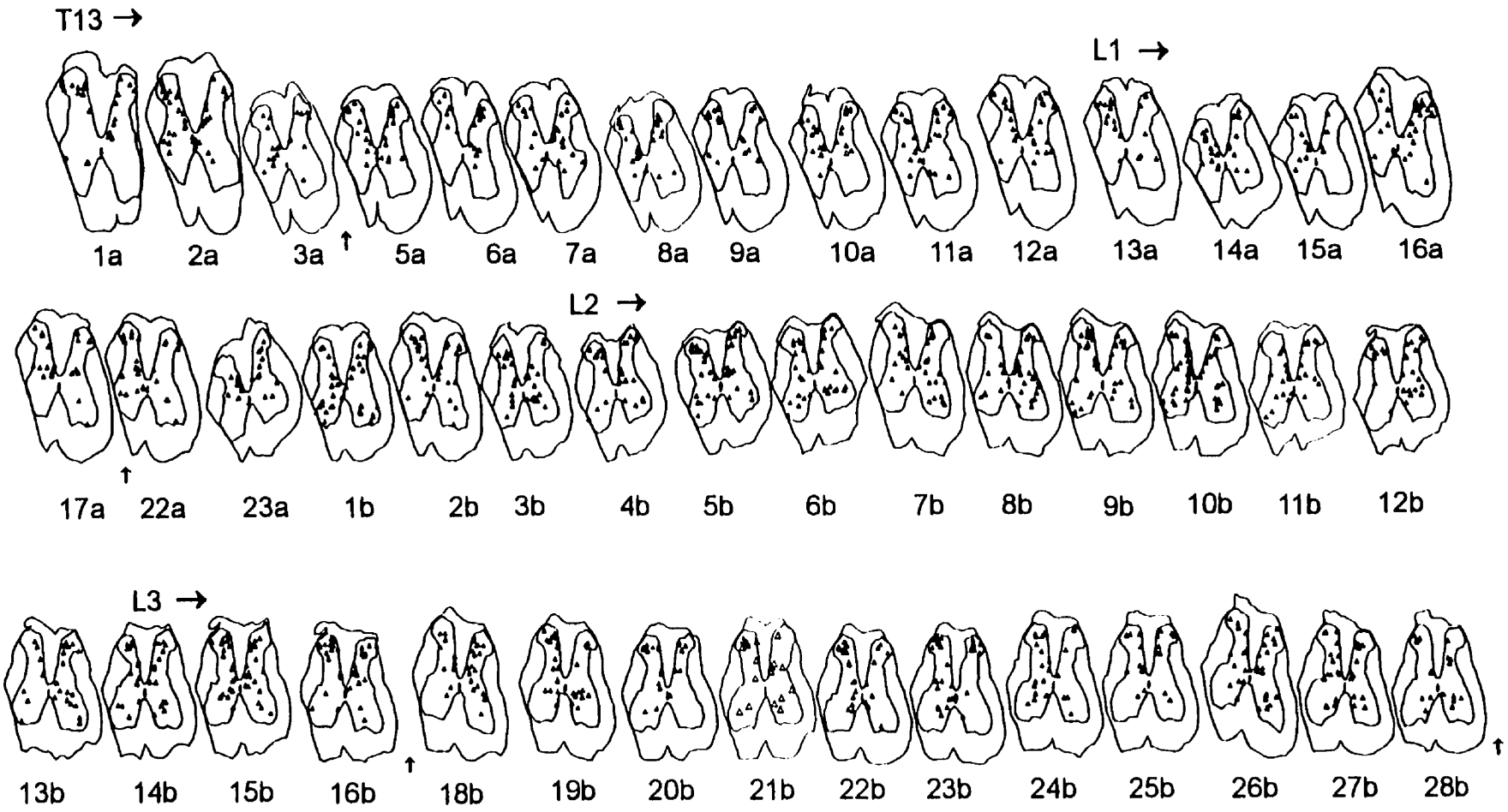


Figure 10

Experimental animal 3, after locomotion.

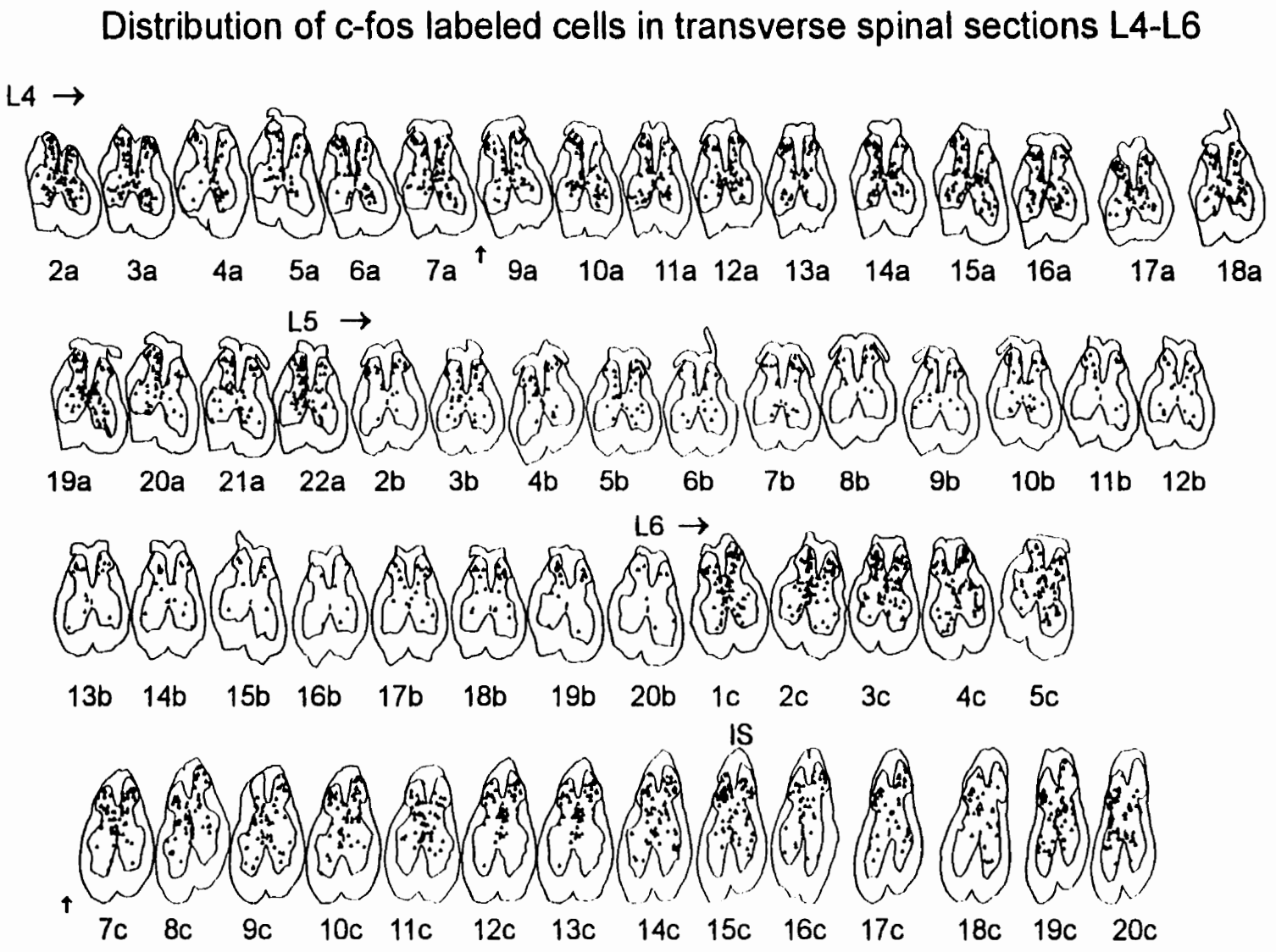


Figure 11 Experimental animal 3, after locomotion. Including injection site.

Figure 12-18

Camera lucida drawings to show the rostro-caudal distribution of

fluorogold labeled cells from spinal segments T13-L6, in all the experimental animals with locomotion. The maps indicate both ipsi-laterally (right side of spinal cord) and contralaterally (left side of spinal cord) labeled cells in serial sections, including the injection site (IS). The open crosses indicates individual fluorogold labeled neurons.

Distribution of fluorogold labeled cells in transverse spinal sections T13-L1

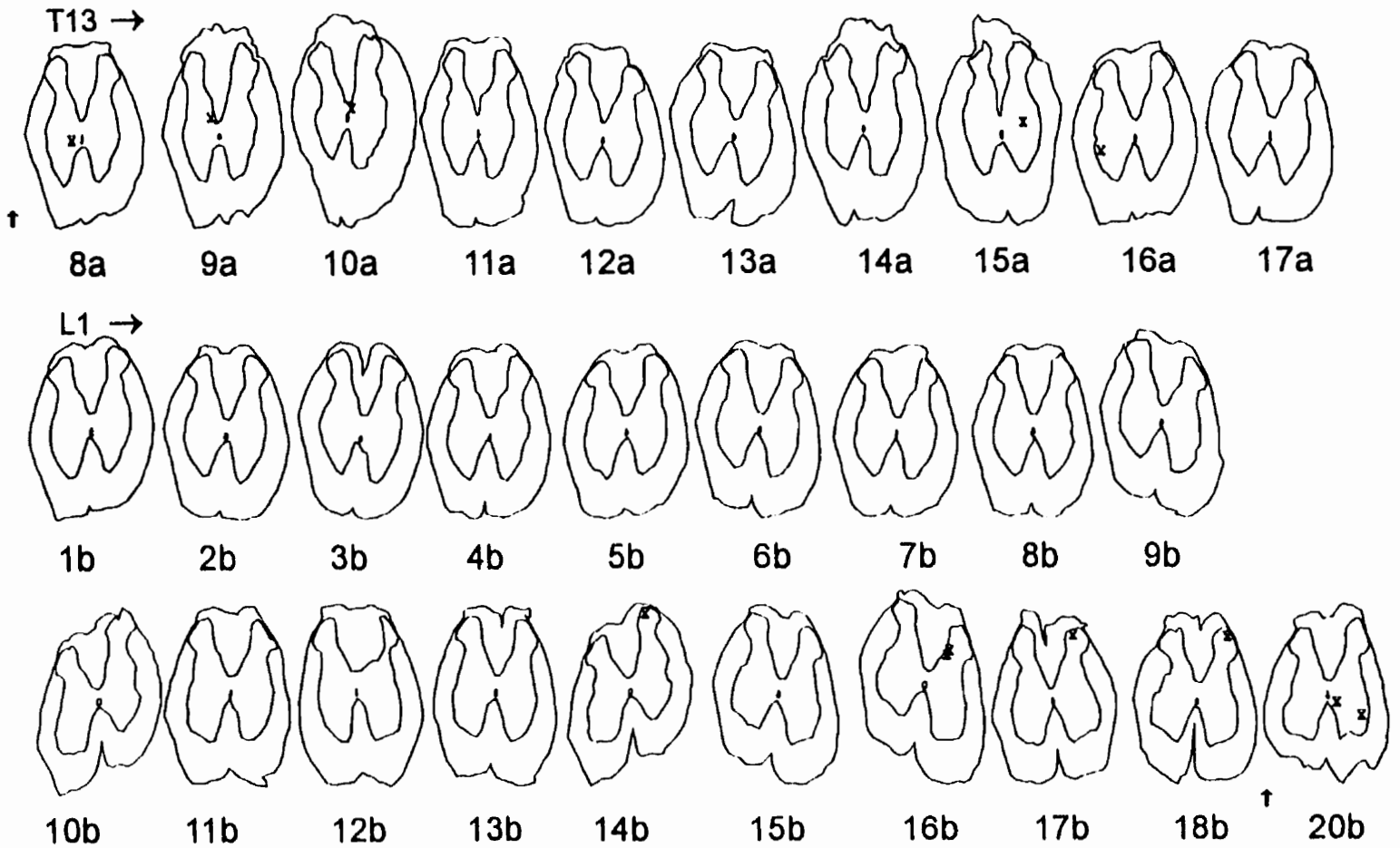


Figure 12 Experimental animal 1, after locomotion.

Distribution of fluorogold labeled cells in transverse spinal sections L2-L5

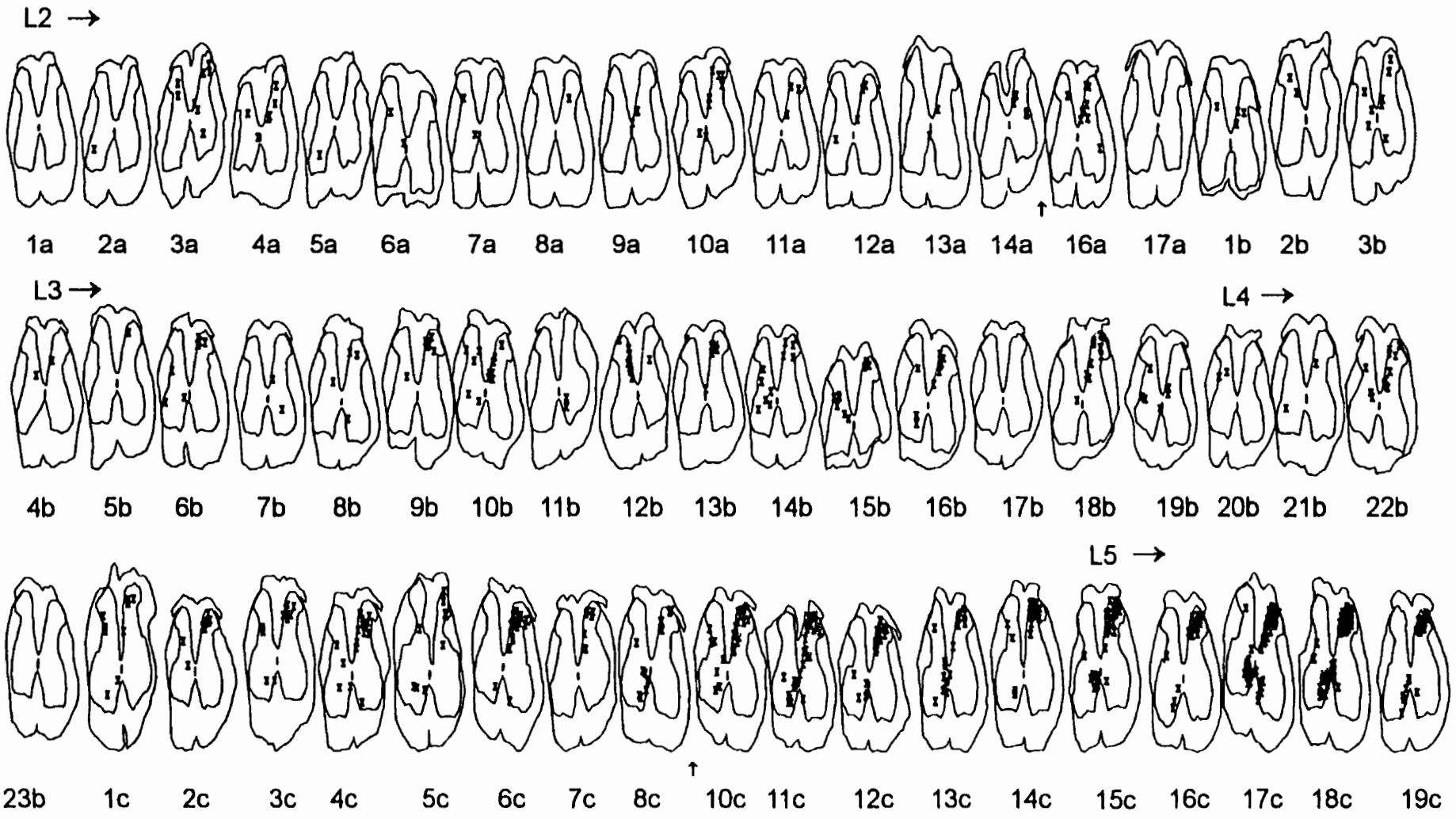


Figure 13

Experimental animal 1, after locomotion.

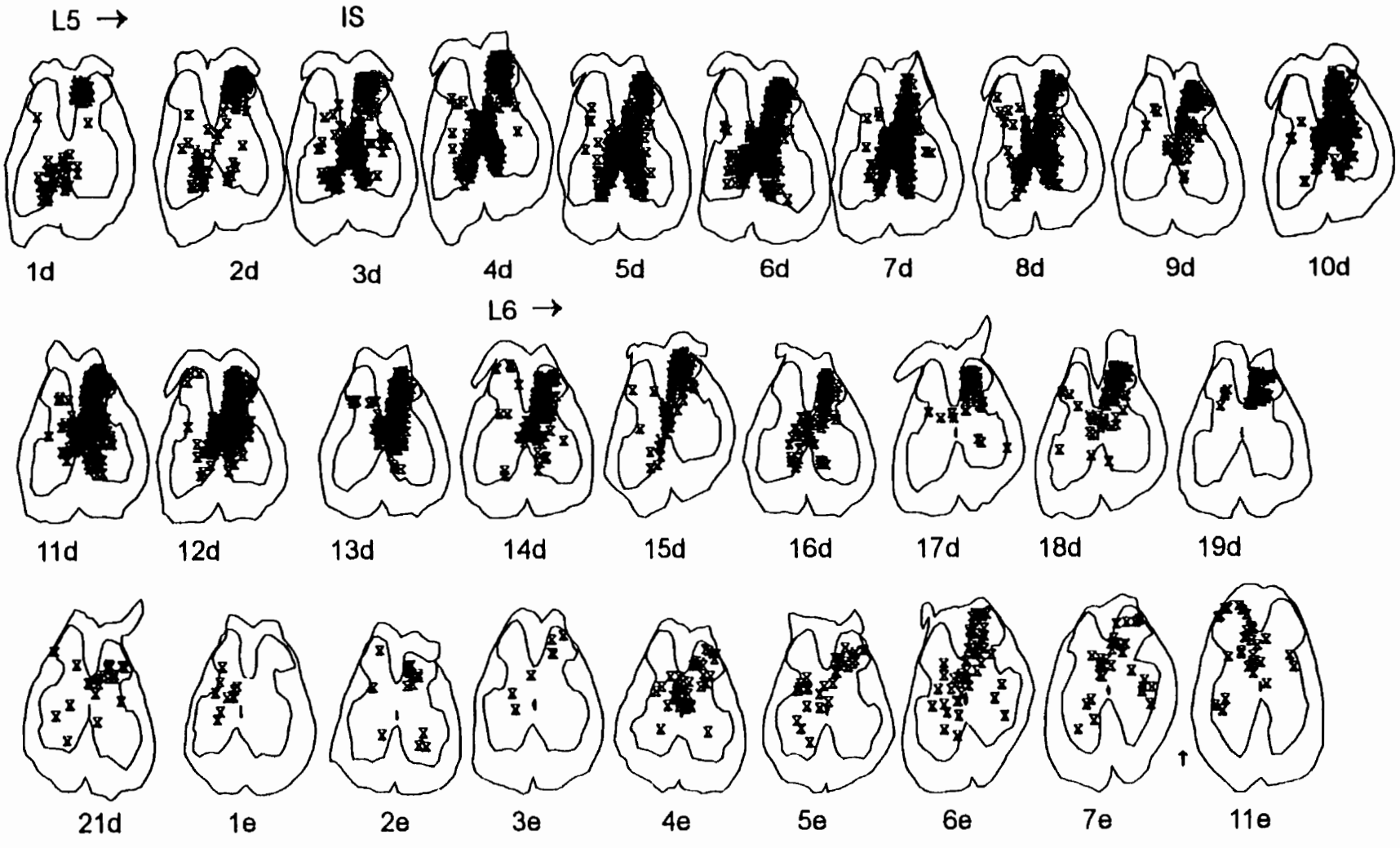


Figure 14 Experimental animal 1, after locomotion. Including injection site.

Distribution of fluorogold labeled cells in transverse spinal sections T13-L1

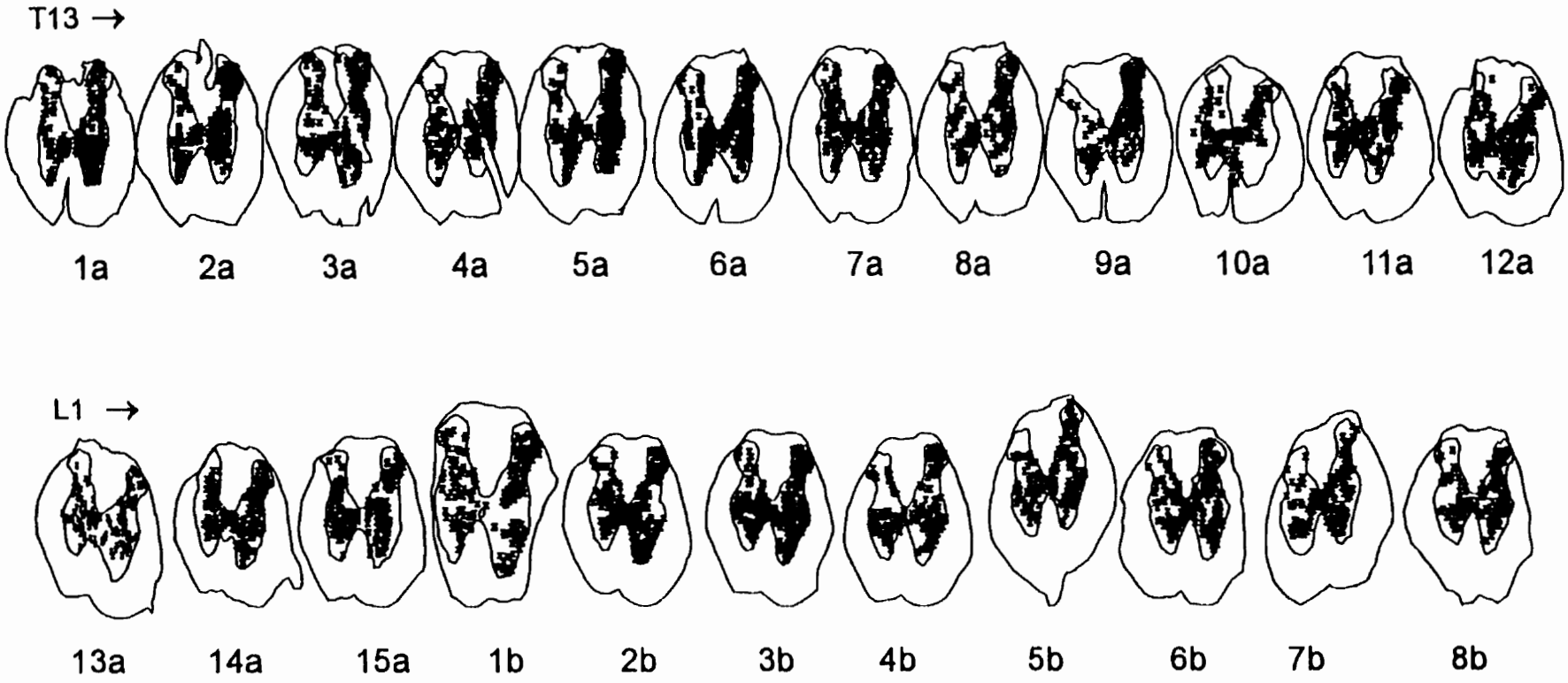


Figure 15

Experimental animal 2, after locomotion.

Distribution of fluorogold labeled cells in transverse spinal sections L2-L6

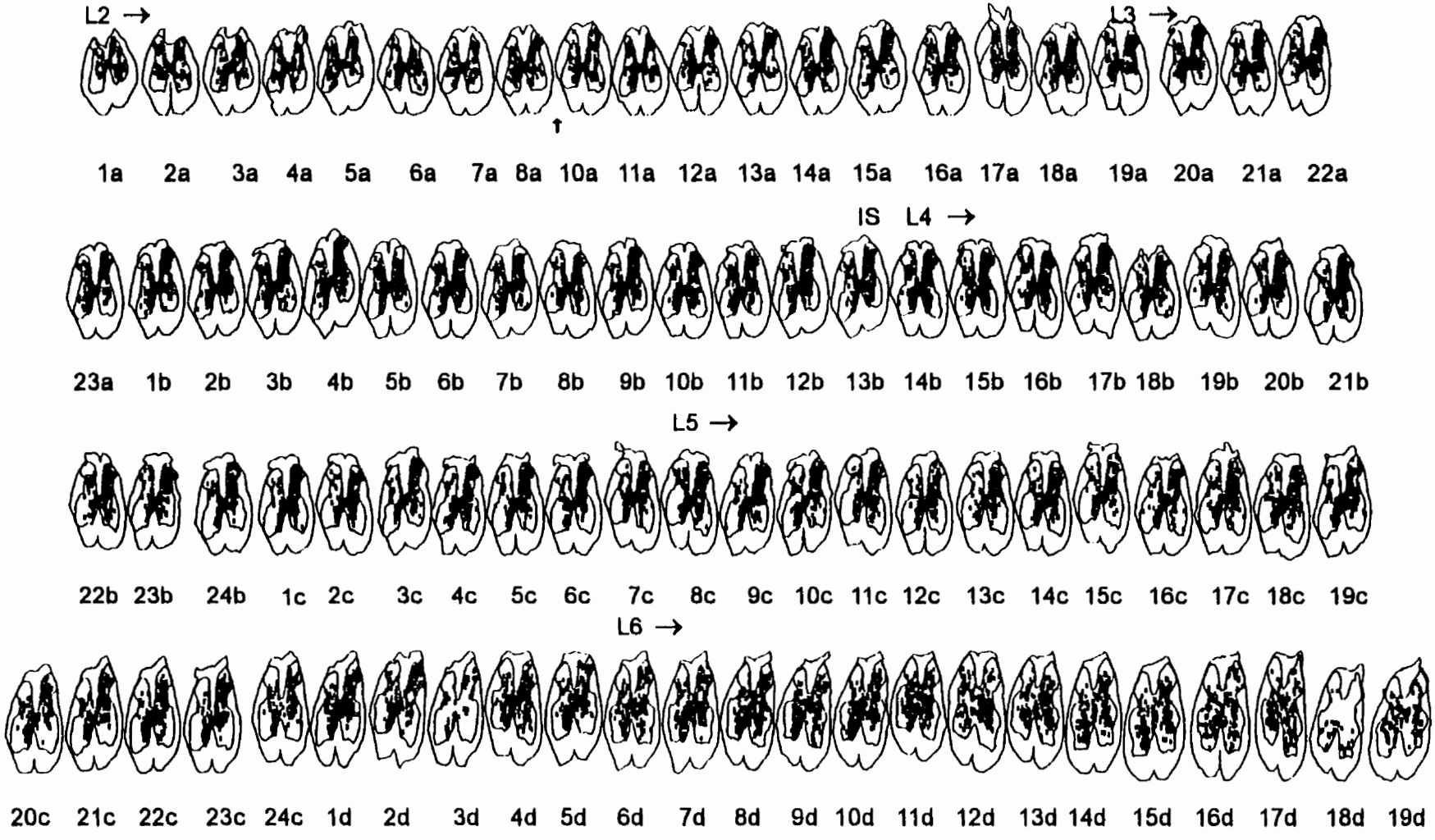


Figure 16 Experimental animal 2, after locomotion. Including injection site.

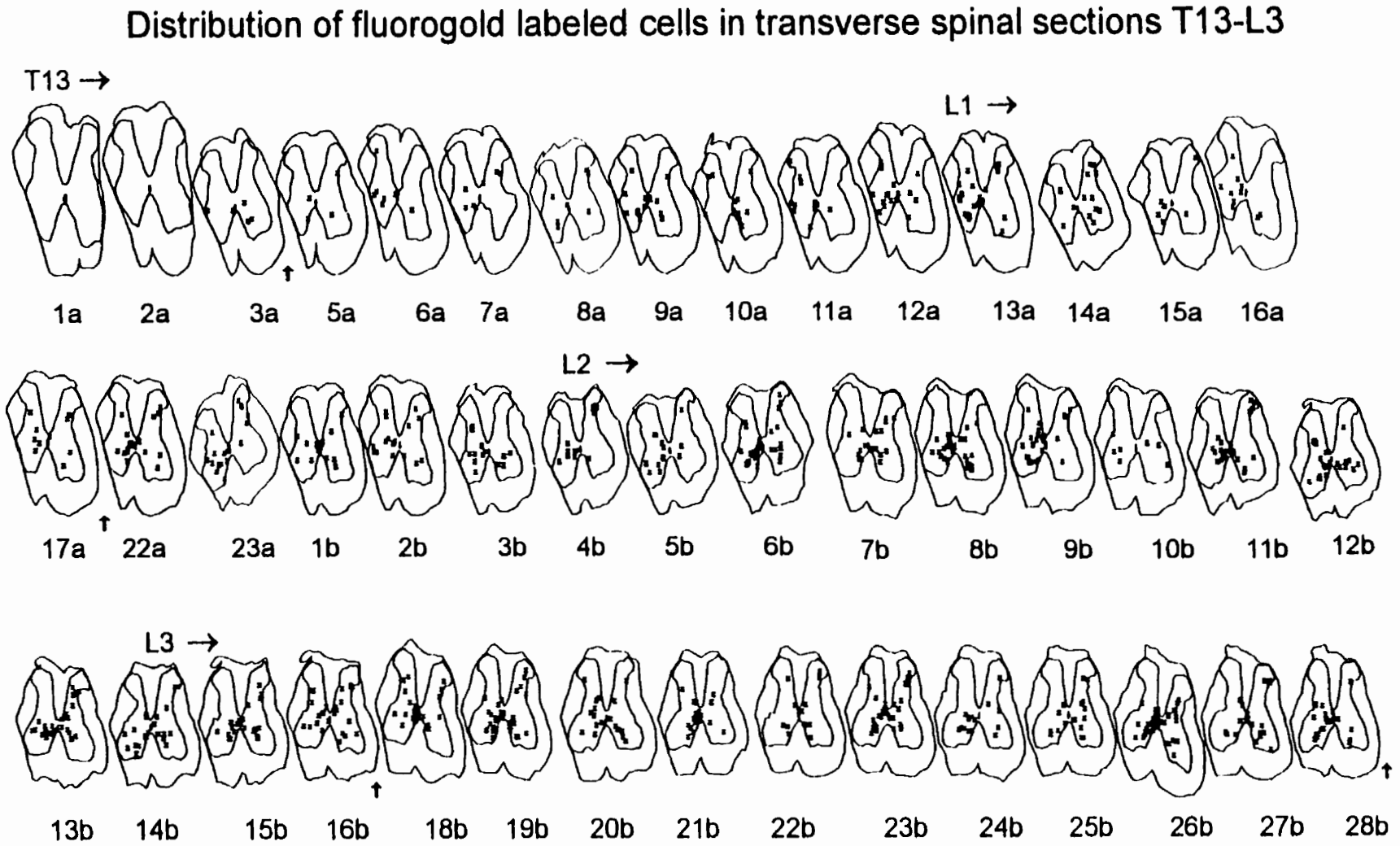


Figure 17

Experimental animal 3, after locomotion.

Distribution of fluorogold labeled cells in transverse spinal sections L4-L6

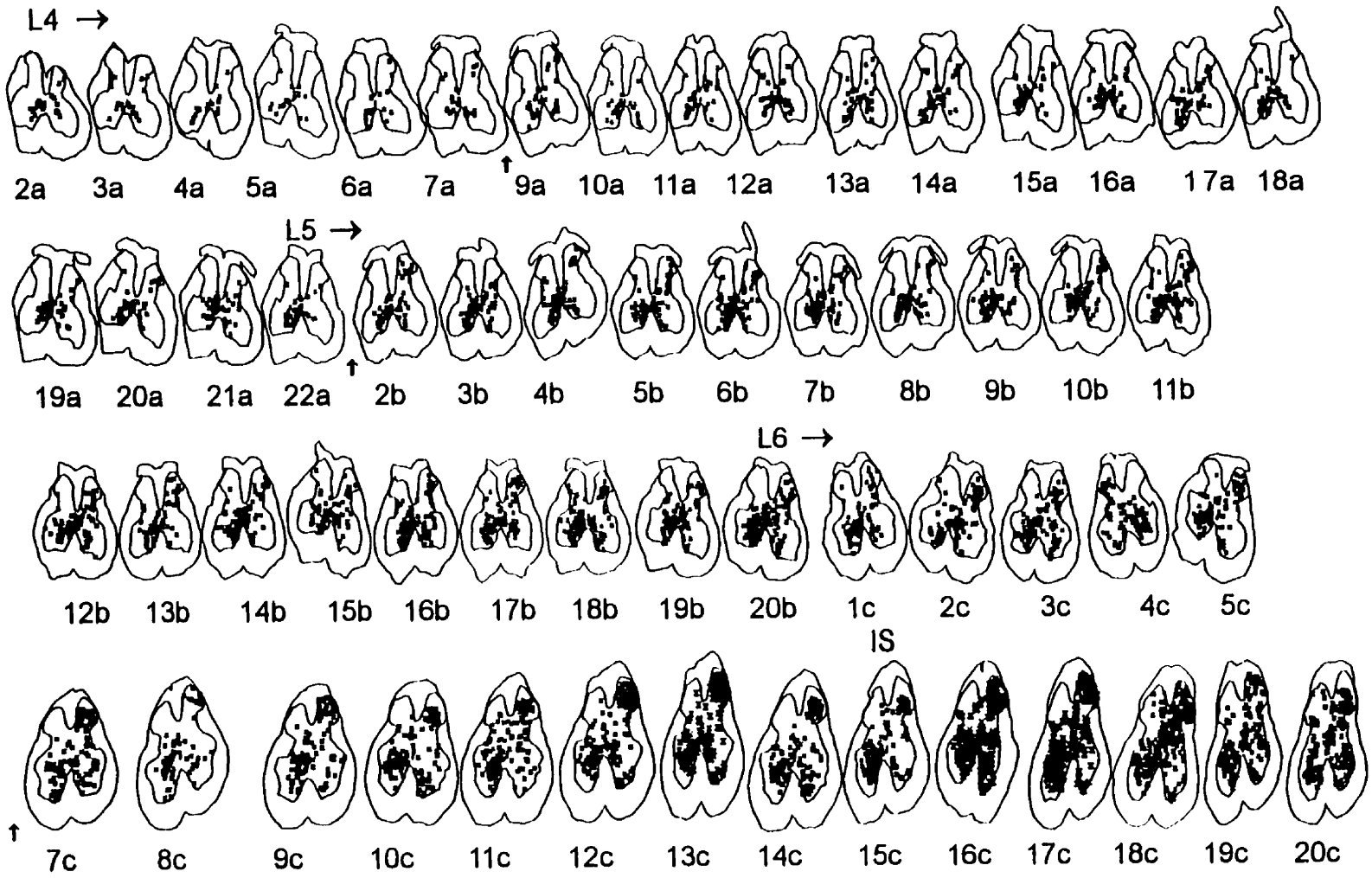


Figure 18 Experimental animal 3, after locomotion. Including injection site.

Figure 19-20

Camera lucida drawings to show the rostro-caudal distribution of fluorogold and c-fos double labeled cells from spinal segments T13-L6 in experimental animal 1, with locomotion. The maps indicate both ipsi-laterally (left side of spinal cord) and contralateral (right side of spinal cord), retrogradely labeled cells. The injection site (IS) is in L5. The filled crosses indicate all c-fos and fluorogold double labeled neurons.

Distribution of c-fos and fluorogold double labeled cells in transverse spinal sections T13-L1

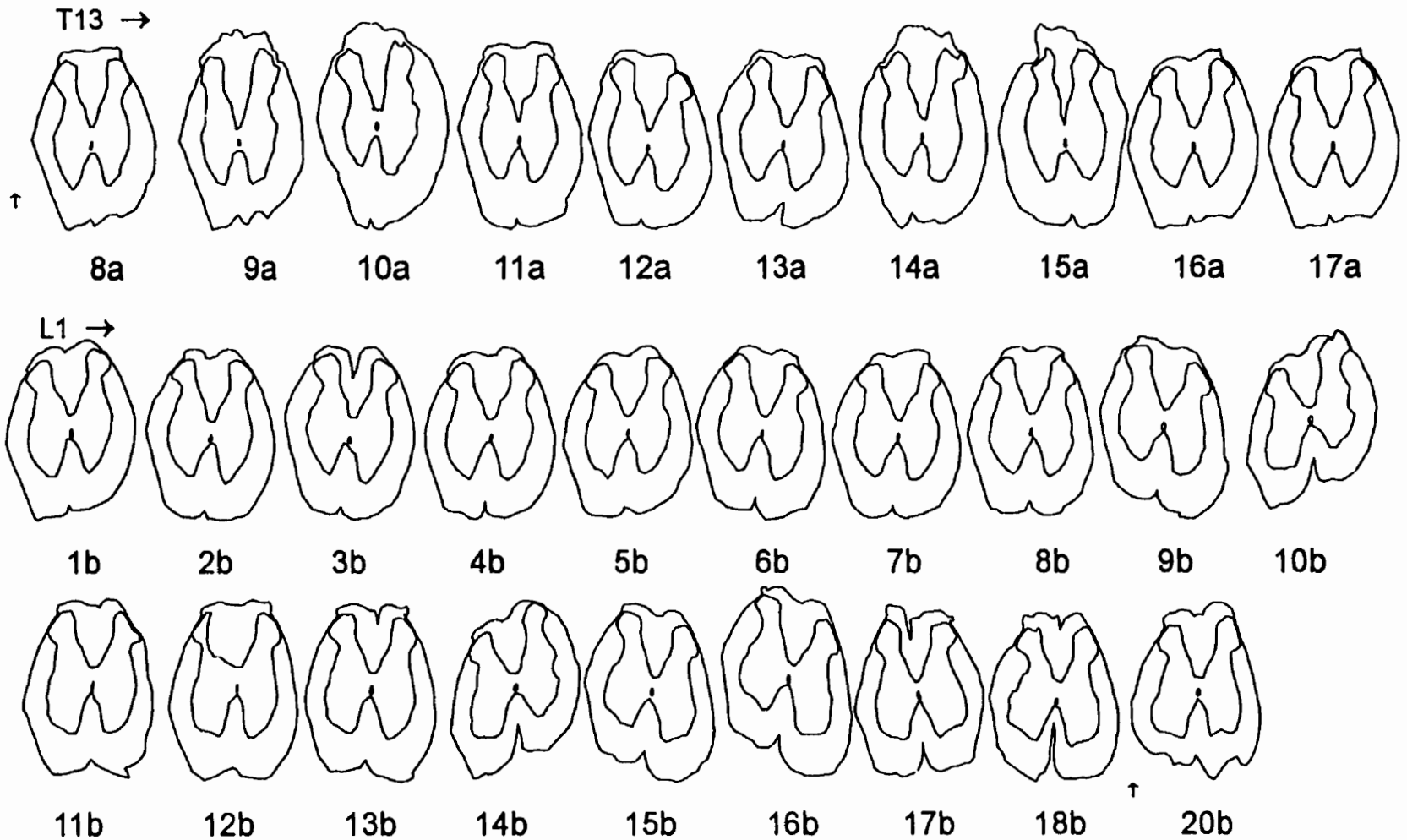


Figure 19

Experimental animal 1, after locomotion.

Distribution of c-fos and fluorogold double labeled cells in transverse spinal sections L2-L6

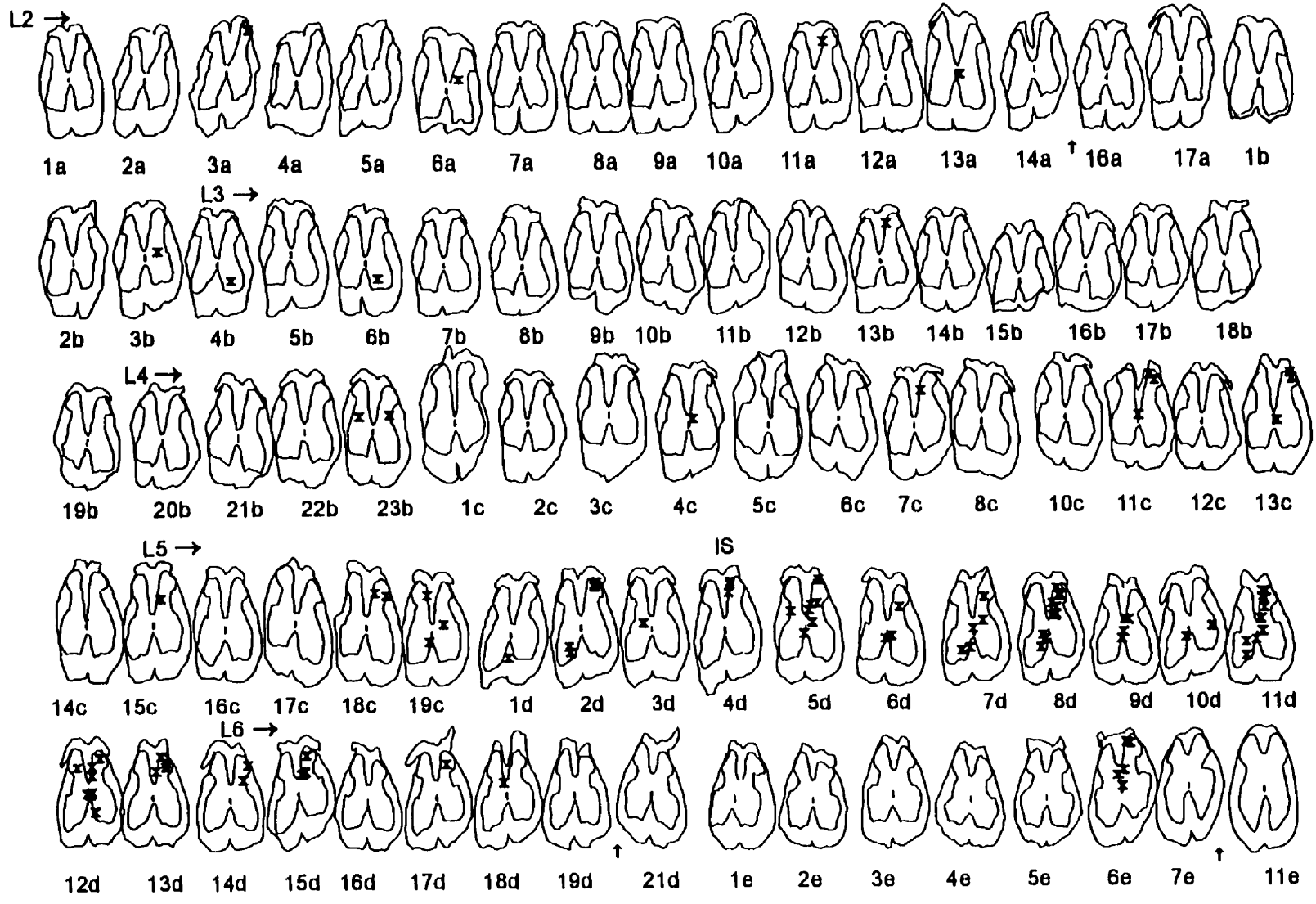


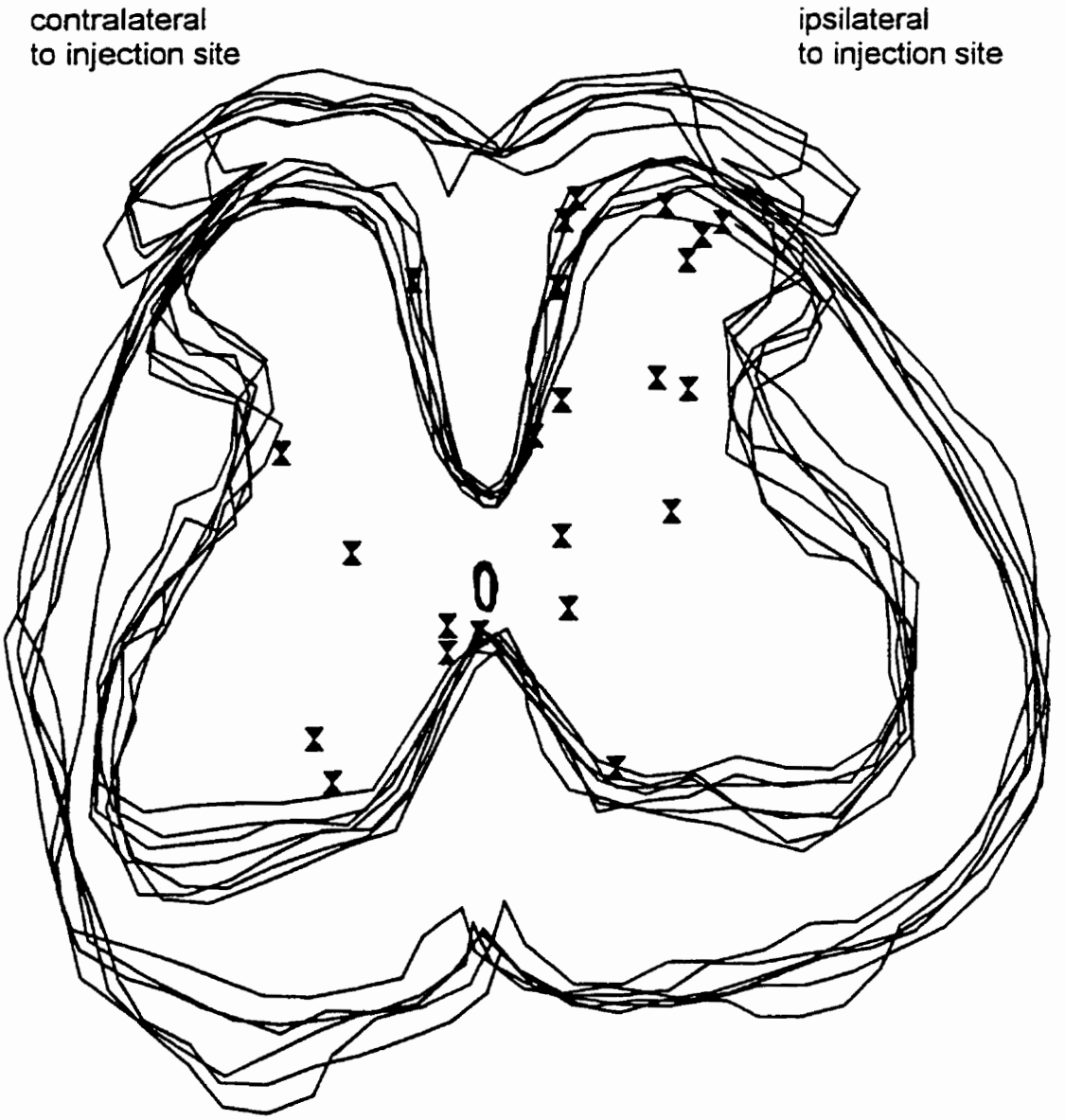
Figure 20 Experimental animal1, after locomotion. Including injection site.

Figure 21

Composite of 7 camera lucida drawings to show the distribution of fluorogold and c-fos double labeled cells at the injection site (L5) in experimental animal 1, with locomotion. The maps indicate both ipsi-laterally (left side of spinal cord) and contralaterally (right side of spinal cord) double labeled cells.

Figure 21

Distribution of c-fos and fluorogold double labeled cells in 7 sections at injection site (L5)

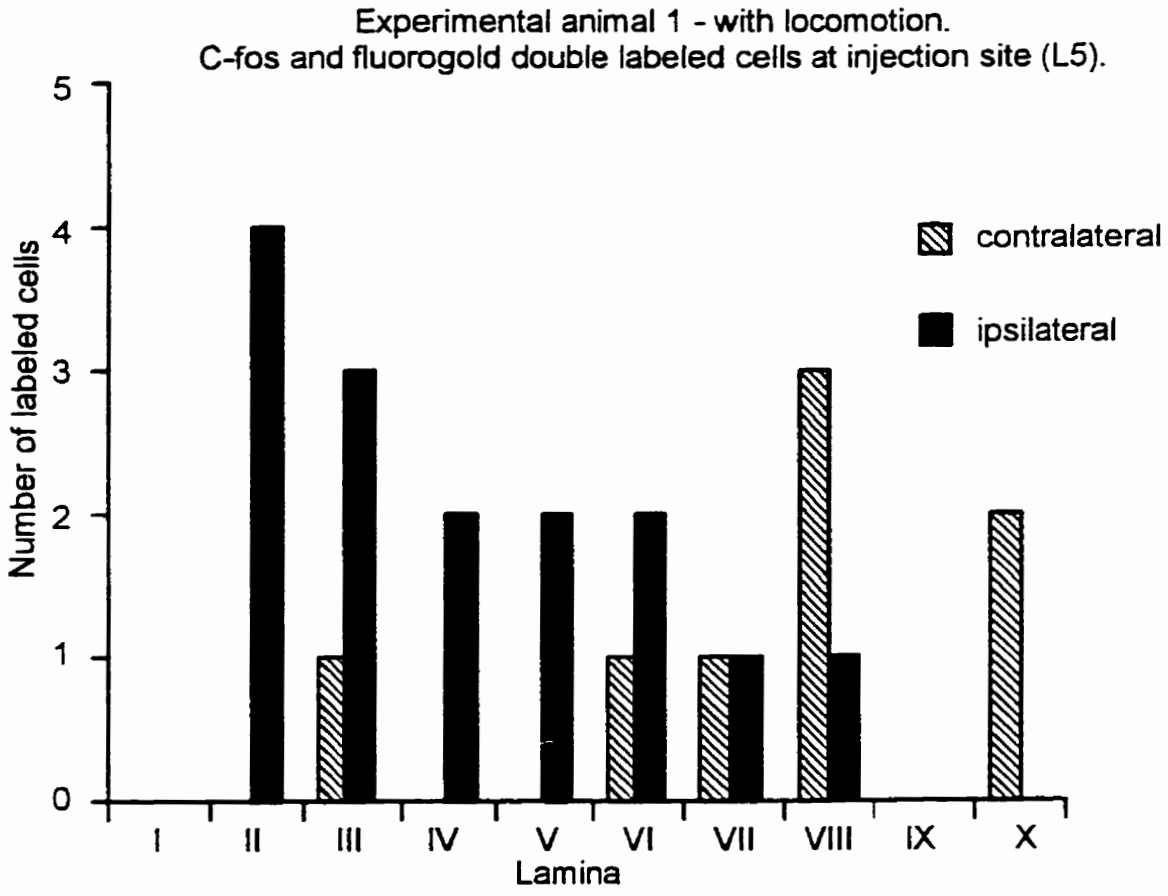


Experimental animal 1,after locomotion

Table 1

Histogram indicating the number of bilateral, c-fos and fluorogold double labeled cells (mapped in fig. 21), in each lamina.

Table 1



Histogram indicating the number of c-fos and fluorogold double labeled cells in 7 sections at injection site (L5)

Figure 22-23

Camera lucida drawings to show the rostro-caudal distribution of fluorogold and c-fos double labeled cells from spinal segments T13-L6 in experimental animal 2, with locomotion. The maps indicate both ipsi-laterally (left side of spinal cord) and contralateral (right side of spinal cord), retrogradely labeled cells. The injection site (IS) is at the border of L3/L4. The filled crosses indicate all c-fos and fluorogold double labeled neurons.

Distribution of c-fos and fluorogold double labeled cells in transverse spinal sections T13-L1

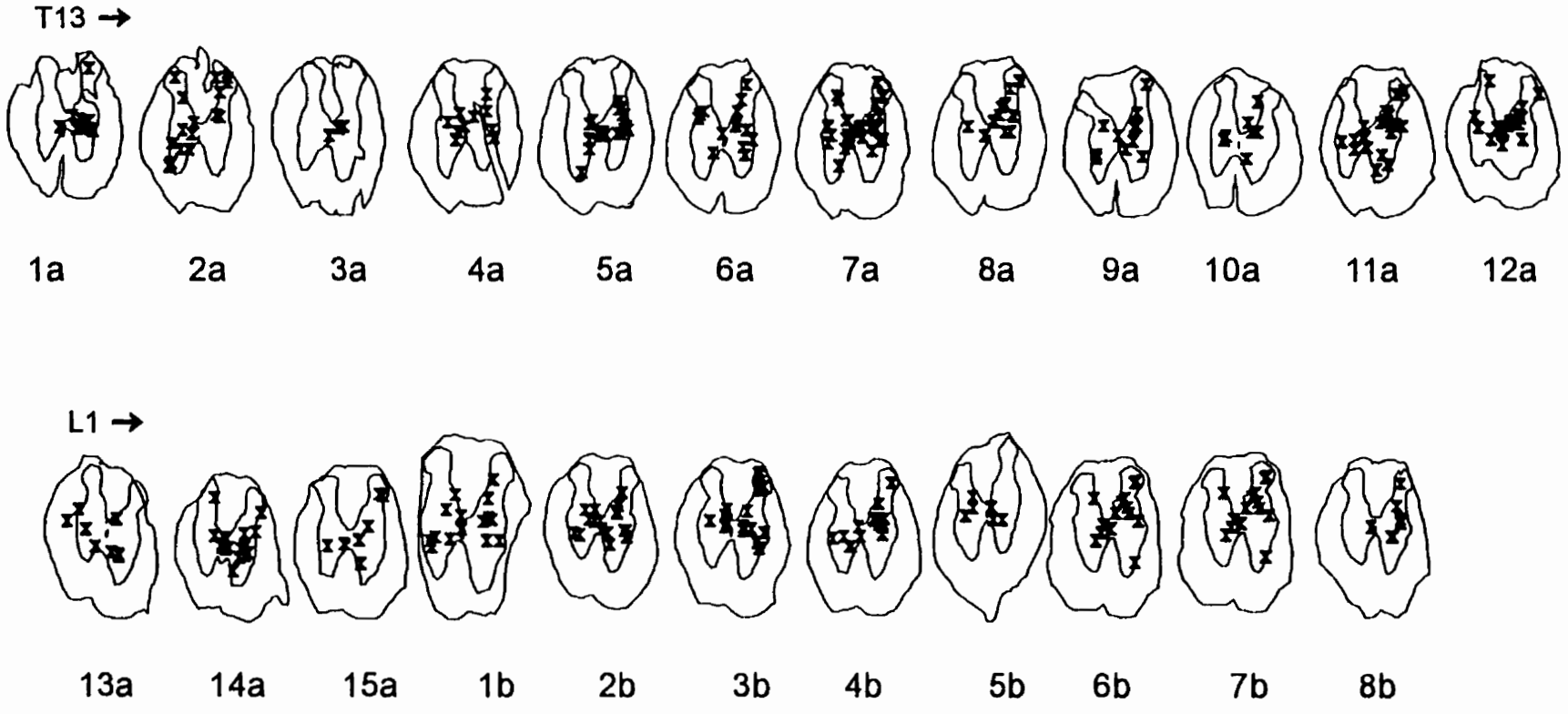


Figure 22

Experimental animal 2, after locomotion.

Distribution of c-fos and fluorogold double labeled cells in transverse spinal sections L2-L6

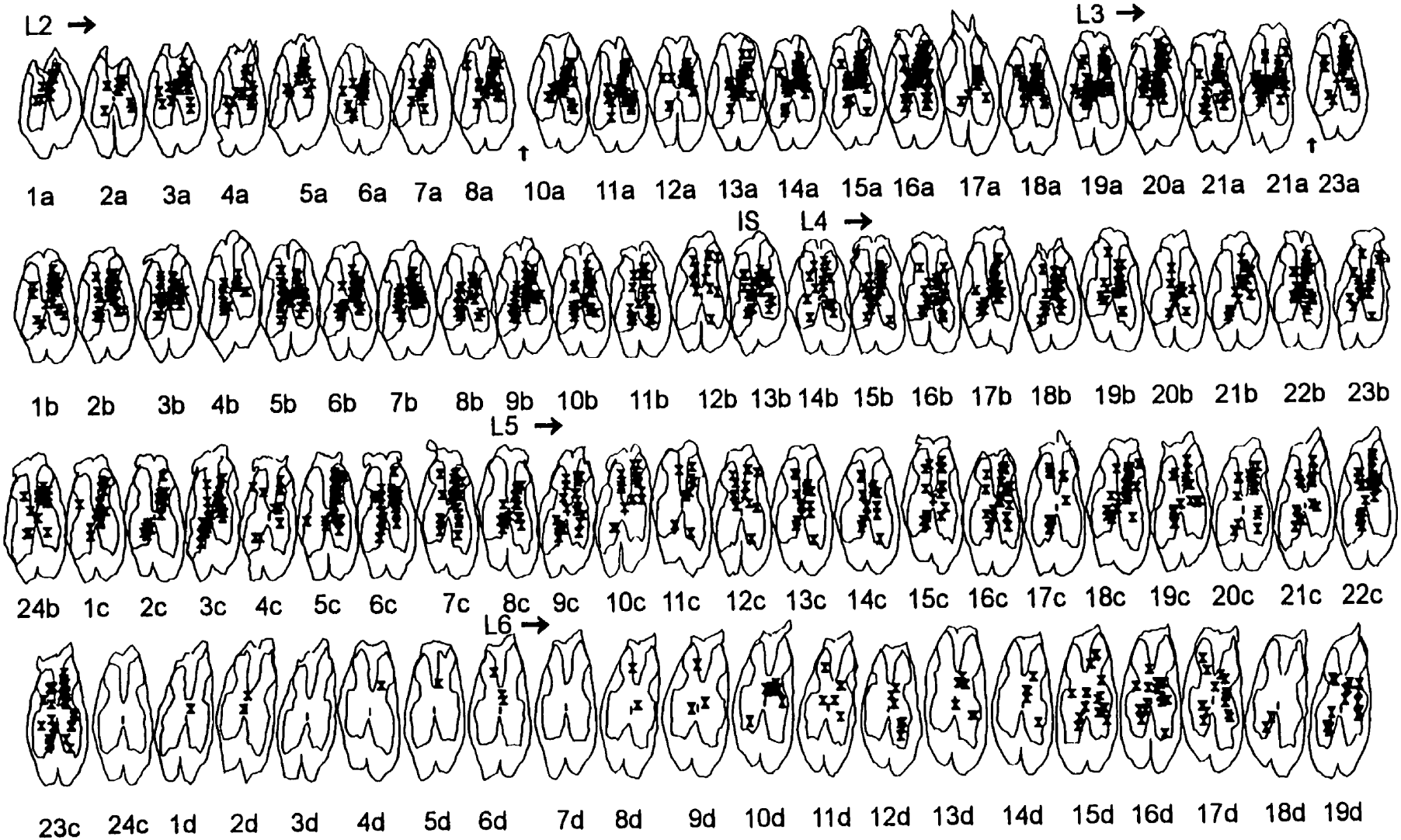


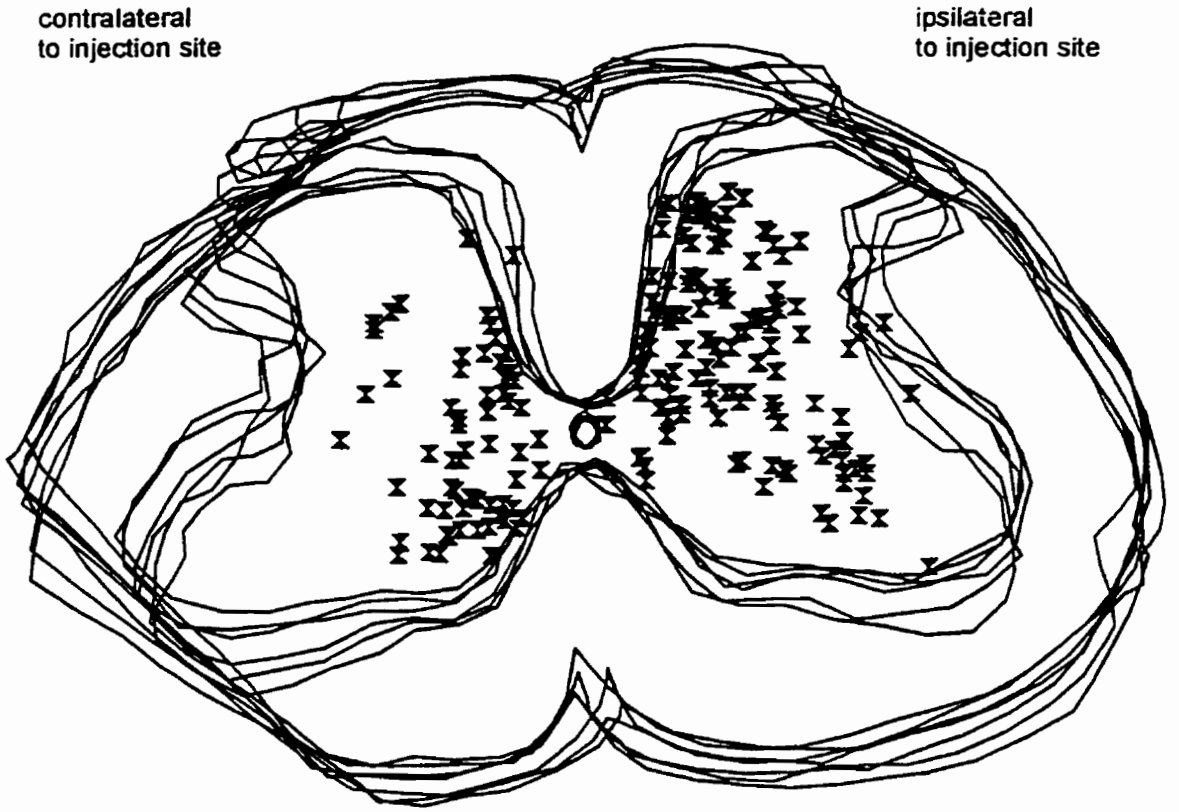
Figure 23 Experimental animal 2, after locomotion. Including injection site.

Figure 24

Composite of 7 camera lucida drawings to show the distribution of fluorogold and c-fos double labeled cells at the injection site (border of L3/L4) in experimental animal 2, with locomotion. The maps indicate both ipsi-laterally (left side of spinal cord) and contralaterally (right side of spinal cord) double labeled cells.

Figure 24

Distribution of c-fos and fluorogold double labeled cells in 7 sections at injection site (border L3/L4)



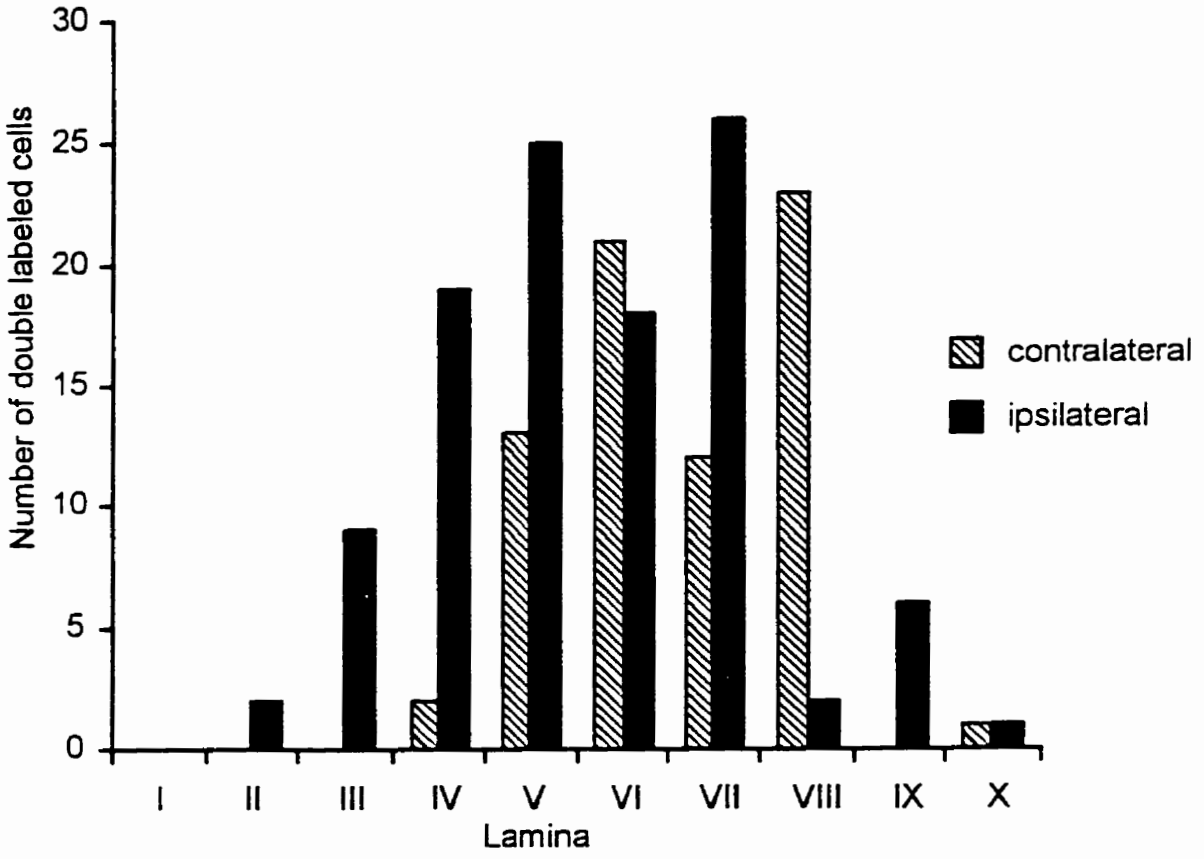
Experimental animal 2, after locomotion

Table 2

Histogram indicating the number of bilateral, c-fos and fluorogold double labeled cells (mapped in fig. 24), in each lamina.

Table 2

Experimental animal 2 - with locomotion.
C-fos and fluorogold double labeled cells at injection site (border L3/L4).



Histogram indicating the number of c-fos and fluorogold double labeled cells in 7 sections at injection site (border L3/L4)

Figure 25-26

Camera lucida drawings to show the rostro-caudal distribution of fluorogold and c-fos double labeled cells from spinal segments T13-L6 in experimental animal 3, with locomotion. The maps indicate both ipsi-laterally (left side of spinal cord) and contralateral (right side of spinal cord), retrogradely labeled cells. The injection site (IS) is in L6. The filled crosses indicate all c-fos and fluorogold double labeled neurons.

Distribution of c-fos and fluorogold double labeled cells in transverse spinal sections T13-L3

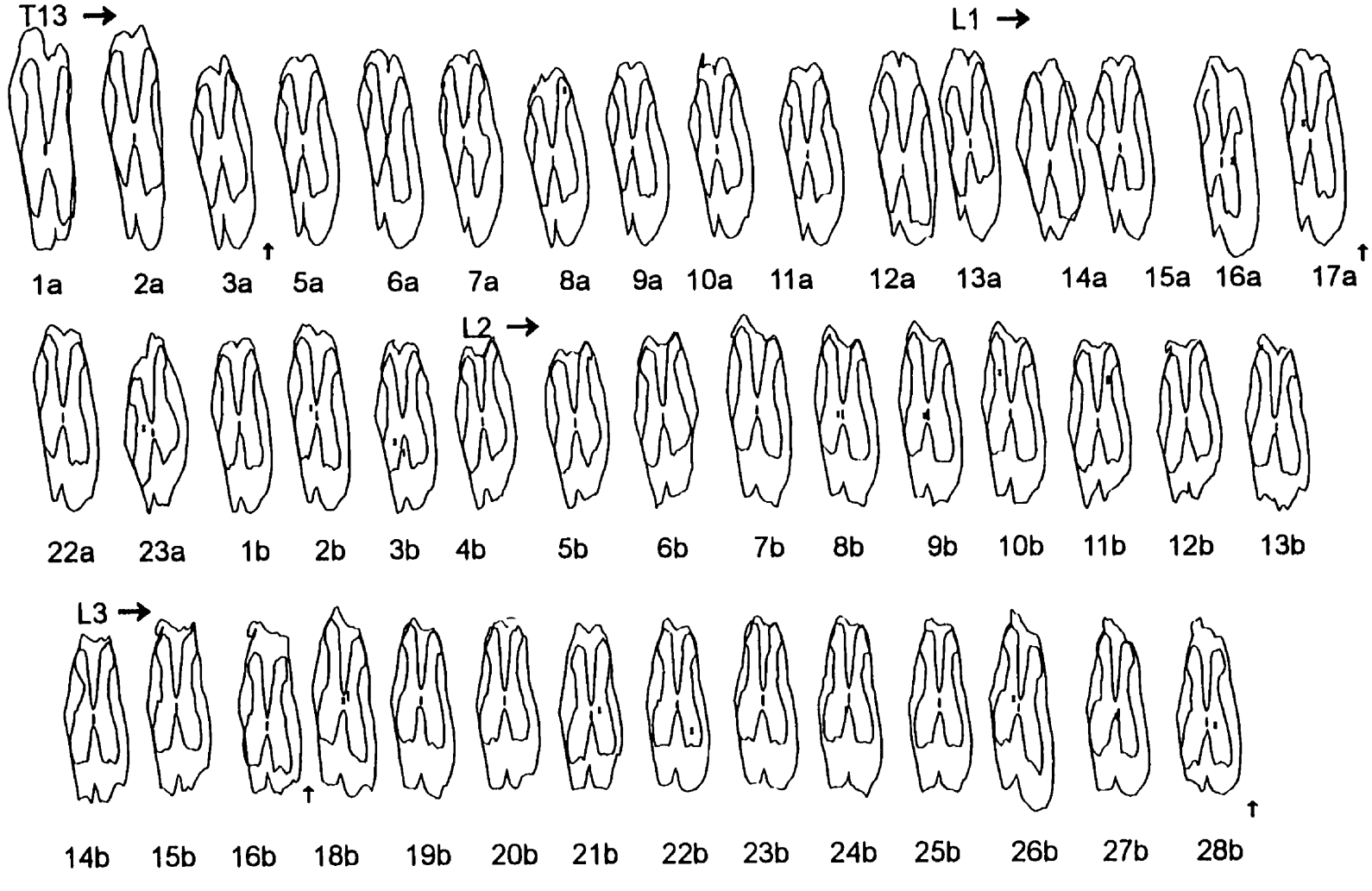


Figure 25 Experimental animal 3, after locomotion.

Distribution of c-fos and fluororgold double labeled cells in transverse spinal sections L4-L6

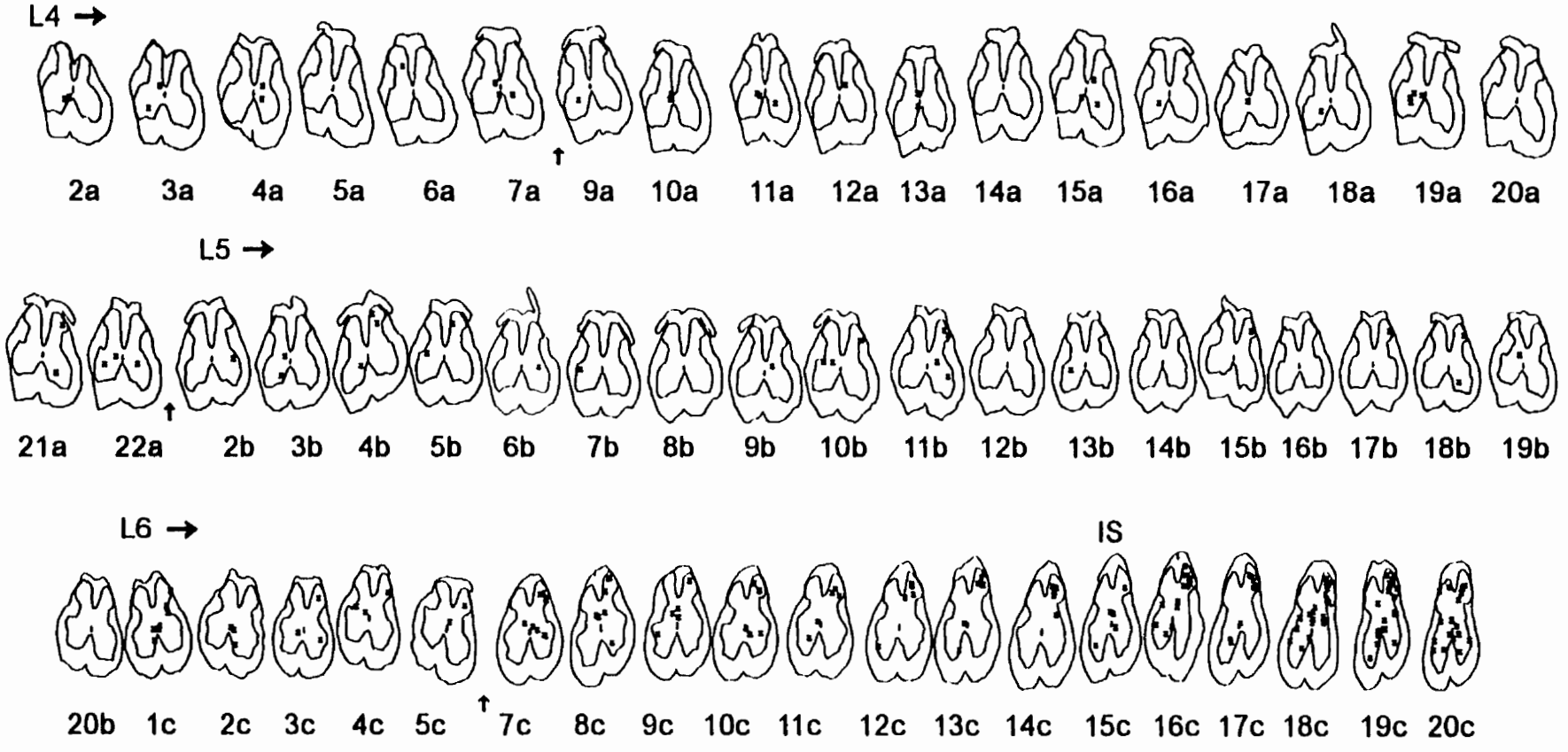


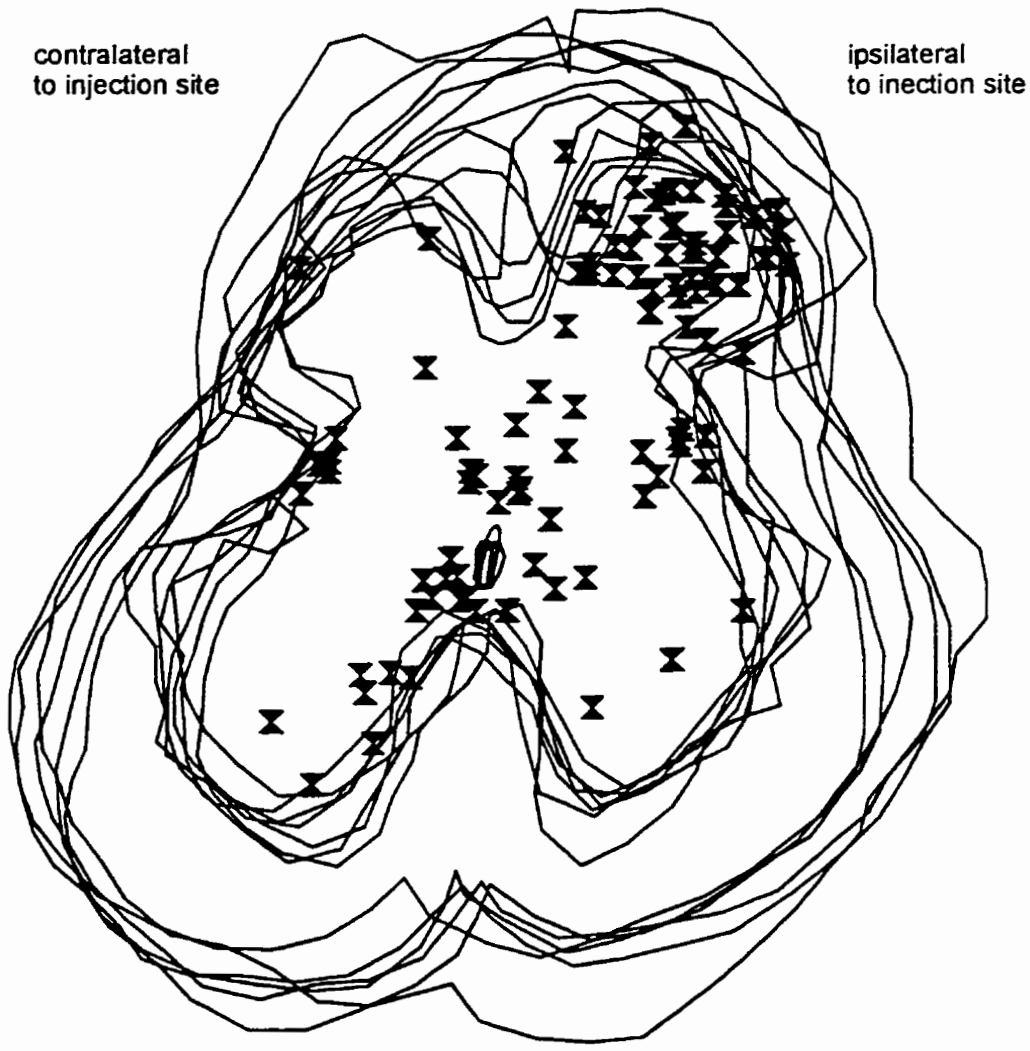
Figure 26 Experimental animal 3, after locomotion. Including injection site.

Figure 27

Composite of 7 camera lucida drawings to show the distribution of fluorogold and c-fos double labeled cells at the injection site (L6) in experimental animal 3, with locomotion. The maps indicate both ipsi-laterally (left side of spinal cord) and contralaterally (right side of spinal cord) double labeled cells.

Figure 27

Distribution of c-fos and fluorogold double labeled cells in 7 sections at injection site (L6)

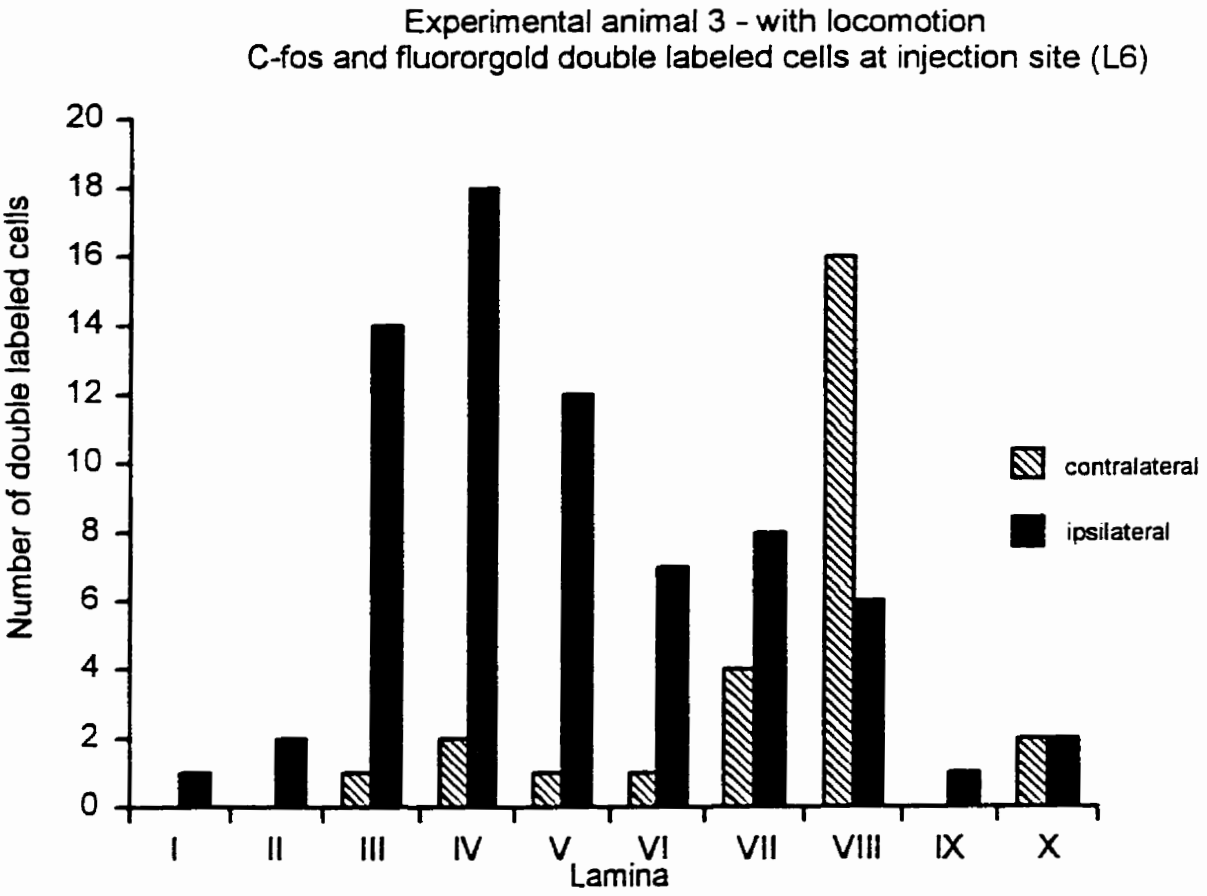


Experimental animal 3, after locomotion

Table 3

Histogram indicating the number of bilateral, c-fos and fluorogold double labeled cells (mapped in fig. 24), in each lamina.

Table 3



Histogram indicating the number of c-fos and fluorogold double labeled cells in 7 sections at injection site (L6).

DISCUSSION

The spinal neurons necessary for the mediation of alternating rhythmic activity during locomotion have not yet been identified. The objective of the present study was to determine the location of candidate cells in the lumbar spinal cord. The study of the nervous system of the adult rat has distinct a advantage over that of the neonate. The neonatal nervous system is immature and still subject to a variety of developmental changes, while that of the adult is fully formed and established. The muscles of the adult animal are also much stronger to perform appropriate, physiological locomotor tasks viz. walking on the treadmill.

The fluorescent markers used in this double-labeling study have been carefully chosen in order to meet specific criteria. The use of fluorogold provides significant advantages over other retrograde tracers e.g. horseradish peroxidase (HRP) or its conjugates. These advantages include: minimal tissue processing; several fluorescent tracers may be combined to demonstrate collateralization of efferents; and tissue labeled with fluorogold may be processed for immunocytochemistry to further characterize the labeled cells, e.g. the presence of putative neuro-transmitters (Pieribone and Aston-Jones, 1988).

The c-fos immunocytochemical method has some important advantages over other tracing techniques such as 2-deoxyglucose (DG), horseradish peroxidase conjugated with wheat germ agglutin (WGA-HRP) and sulphorhodamine. The population of cells demonstrating 2-DG uptake and c-fos immunoreactivity indicates an overlap in distribution. This distribution is however not always identical and may be attributed to the fact that fos expression requires the integration of various stimuli and intracellular pathways. These pathways in turn may or may not be associated with an increase in metabolic activity (Morgan and Curran, 1989). Cellular resolution is always obtained with c-fos immunocytochemistry while the 2-DG method of labeling has not been successful in localizing single neuronal perikarya active during various types of stimulation (Sharp et al., 1993). Retrograde WGA-HRP activity dependant labeling allows for the identification of only the last order interneurons whereas several studies using c-fos expression are able to identify multiple interconnected neurons.

Sulphorhodamine, a fluorescent dye, which labels synaptically active cells, has one major disadvantage in that it fades rapidly and does not permit additional labeling procedures. This problem is overcome by using c-fos immunocytochemistry, which labels the cells more permanently and allows for further characterization of the cells using different

labeling techniques. C-fos immunocytochemistry thus provides unique advantages over other neuro-anatomical tracers in neuronal physiology. In this study, Fluorogold, the retrograde tracer, was compatible with the c-fos immunocytochemistry; and since the c-fos immunofluorescence and fluorogold fluorescence are detected at different wavelengths, the visualization of both the tracers in an individual cell was greatly enhanced by the simple manipulation of the fluorescent cubes.

Location of spinal interneurons following treadmill locomotion

C-fos positive cells

Few c-fos positive cells were observed in the dorsal horn in the more superficial laminae, I and II, similar to that of the control animals. These laminae contain neurons that react primarily to noxious stimuli (Besson and Chaouch, 1987; Presley et. al., 1990). More c-fos positive cells were observed in laminae III and IV, areas known to contain neurons of the post synaptic dorsal column and spino-cervical tract (Bennet et al 1983; Brown, 1981). These neurons are activated either by cutaneous mechano-receptors or nociceptors (Cervero, 1986; Brown, 1981). The dorsal horn also contains interneurons which have inputs from both Ia and Ib afferents. (Hongo et. al., 1966; Jankowska et al, 1981). These cells are located in laminae V and VI. Lamina VI has

previously been shown to contain cells involved in locomotion. (Viala et. al., 1988).

The C-fos positive cells in this study were localized especially in the medial aspect of laminae V and VI and might have been labeled due to activation from muscle afferents of the moving limbs during the treadmill-walking task. The few C-fos positive cells observed in the control animals were located in laminae III and IV.

The majority of the c-fos labeled cells were localized bilaterally in the intermediate zone (laminae VII, X), and in the ventral horn (lamina VIII). Fewer labeled cells were observed in lamina IX. Lamina VII contains several types of interneurons: those that mediate reflex effects on motor nuclei (Rexed, 1954); Ia reciprocal interneurons and Renshaw cells (Jankowska and Lindstrom, 1972, 1971); and a sub-population that receive inputs from group II muscle afferents and from other muscle, joint and skin afferents that in turn project to motor nuclei (Edgley and Jankowska 1987a, 1987b, Edgley et al 1988). All three types of interneurons have been shown to be active in MLR-induced fictive locomotion (McCrea et al 1980, Pratt and Jordan 1987, Noga et al 1987, Shefchyk et al 1990).

Some c-fos positive cells in lamina X have also been localized in previous locomotor investigations (Dai et. al., 1995, Jasmin et. al., 1994). In a separate study, several cells from this region have also been found to exhibit N-methyl-D-aspartate mediated bursting properties (Hochman et. al., 1994). This property could be useful in a network engaged in alternating rhythms.

Commissural cells are known to be found in lamina VIII. Several studies indicate that a sub population of these commissural cells project to the contralateral motor neurons (Scheibel and Scheibel, 1966a; Harrison et al 1986). These cells are probably involved in either crossed extension reflexes (Grillner and Hongo, 1972) or in the excitation or inhibition of contralateral motor neurons evoked by the vestibulo- and reticulo-spinal tracts. Lamina VIII cells has also been shown to be active during locomotion (Noga et. al., 1987).

Lamina IX contains the alpha-motor neurons. These cells are significantly larger than the interneurons and are easily distinguishable. Several of the motor neurons were labeled with c-fos following the treadmill task (fig.1-fig.7). This finding is consistent with that of Jasmin et. al. , 1994. No c-fos positive cells were observed in laminae VII, X, VIII or IX in the absence of locomotion.

In general, the present results are consistent with that of previous labeling studies. That is c-fos positive cells were found in laminae III, IV, VII, VIII and X of the lumbar cord in the cat following treadmill locomotion (Dai, et. al., 1995), and more recently in the neonatal rat chemically induced fictive locomotion resulted in cells being labeled with sulphorhodamine in laminae V, VI, VII, VIII and X. The labeling extended from lumbar segments, L1-L6 (Cina, Thesis, 1997).

Fluorogold labeling and injection sites

The fluorogold injection sites corresponded to the T13 vertebral level in all three experimental animals and involved several levels of the lumbar region known to contain the motorneurons of important locomotor muscles viz. the anterior biceps and the semi-membranosous muscles of the rat (Nicolopoulos and Iles, 1983). The commissural cells closely associated with these motorneurons are likely to be located nearby. Electrophysiological studies in this laboratory using the in vivo cat model have shown that commissural cells often cross over the spinal cord within a segment. The injection sites, however, were at a different locations in each of the experimental animals, and may be attributed to anatomical variations within the animals. Thus differences in the rostral to caudal pattern of labeling was observed. However, the labeling at each injection site was

nevertheless comparable among the animals.

C-fos and fluorogold double labeled cells

In all the experimental animals with locomotion, the double-labeled cells were found in a similar laminar distribution, but the rostro-caudal pattern differed and was dependant on the intensity of the fluorogold labeling and the injection site. In animal 2 the injection site was more rostral (border of L3/L4) compared to that of both animals 1 (L5) and 3 (L6). The more rostral injection site may explain the greater number of commissural cells labeled in experimental animal 2, while the more caudal injection sites in experimental animals 1 and 3, probably resulted in a larger number of sensory cells being labeled. The sensory cells may represent a group of intermediolateral cells which may be located in L6. However, definite conclusions cannot be made because of the limited number of animals used in this study. The cells labeled on the side ipsi-lateral to the injection may represent a group of propriospinal cells involved in locomotion. It has recently been observed that descending propriospinal axons located below the lesion of spinal cord transection in the lamprey are responsible for the functional recovery of caudal locomotor networks and the restoration of normal locomotor patterns. (Rouse, D.T., et. al., 1997). Propriospinal cells traveling in the ventrolateral tracts in the chick have also been implicated in rostro-caudal

coordination during locomotion (Ho and O'Donovan, 1993). In the cat, long descending propriospinal neurons in the cervical enlargement have been implicated in synchronizing the walking of forelimbs and hind limbs (Remmel, R.S. and Skinner R.D., 1980).

In this study, the double labeled commissural cells on the contralateral side were observed primarily in laminae VII, VIII and X. This substantiates previous studies which identified neurons with crossed axons i.e. commissural cells concentrated in the medial part of laminae VII and VIII (Matsushita et. al., 1979; Mentetry et. al., 1985; Skinner et. al., 1979). It is therefore likely that these double labeled commissural cells in laminae VII, X of the intermediate zone and lamina VIII of the ventral horn are indeed active during treadmill locomotion because, in the animals without the treadmill locomotion no double labeling was observed. These commissural cells are most likely involved in coordinating left/right alternating locomotor rhythms. However, they may not be exclusively essential in this function. In a recent study, Cowley & Schmidt, 1997 demonstrated that reciprocal connections in the lumbar cord are not essential for interlimb coordination, provided that bilateral supralumbar connection are intact. The few double labeled cells in the contralateral dorsal horn could contribute to crossed ascending pathways, viz., spino-

thalamic or spino-cerebellar pathways, cells projecting to the brainstem and limbic system or even to the red nucleus.

Pathways involved in alternating rhythms

Numerous studies in a variety of species have used strychnine, an inhibitory amino acid inhibitor, to reveal the inherent crossed excitatory connections in the spinal cord. These studies demonstrate that reciprocal inhibitory synaptic connections mediated by glycine modulate the bilateral synchronous activity produced by excitatory inputs to achieve left/right alternating rhythmic activity in lamprey (Hagevik, 1994); turtle (Currie, 1996) and neonatal rat (Cowley and Schmidt, 1995; Kremer and Lev-tov, 1997; Kjaerulff and Kiehn, 1997). Kudo and Yamada, (1991) showed that the crossed mutually excitatory pathways responsible for bilateral synchronous rhythms are present as early as embryonic age E15.5. The bilateral synchrony is then changed into left/right alternating rhythms with the onset of the development of the crossed inhibitory connections at E18.5. This suggests that independent bilateral oscillators of the rat spinal cord, which regulate alternating rhythmic activity, may be coupled via strong inhibitory and weak excitatory connections. However in a recent study by Kjaerulff and Kiehn, (1997) the crossed excitatory connections in the spinal locomotor network of the neonatal rat have not conclusively been shown to be glutaminergic.

Although CNQX and APV, NMDA and non NMDA antagonists, did block the excitation of the inhibitory pre-motoneurons and in turn the inhibition of the motoneurons, the source of the glutaminergic excitation was not clearly demonstrated as originating from the contralateral side of the spinal cord. This excitation may have originated from ipsi-lateral glutaminergic interneurons. The crossed excitatory connections may thus be mediated by a different excitatory neurotransmitter.

One possibility is acetylcholine. This is supported by the presence of cholinergic commissural interneurons in the different developmental stages of the rat (Houser et al., 1983; Barber et al., 1984; Phelps et al., 1984; Borges and Iverson, 1986; Phelps et al 1990). The possible role of acetylcholine in locomotion has also previously been observed in the rat (Cowley and Schmidt 1994). Recently, a group of central canal and partition propriospinal cells expressing tyrosine kinase receptor (trkA), a high affinity receptor for nerve growth factor (NGF) was found to be cholinergic (Michael, et al., 1997). In addition to the importance of acetylcholine in the phasing of the left and right rhythms during locomotion, the trkA in these cells has significant implications in the design of effective mechanisms for spinal cord repair and regeneration because these cells might be expected to respond well to an exogenous source of NGF. A

whole-cell patch-clamp study has also revealed nicotinic acetylcholine receptors in a sub population of lamina X neurons located dorsally to the central canal (Bordey, A., et. al., 1996). These receptors may be involved in the modulation of important neural locomotor transmission. Presently cholinergic interneurons in the cat that are active during fictive locomotion are being reconstructed to reveal crossed axonal projections, which are subject to further study.

CONCLUSIONS

This study was conducted in adult rats using treadmill locomotion and the principal findings are summarized below:

1. The commissural cells involved in locomotion were identified in spinal segments T13 to L6.

2. These neurons were localized primarily in laminae VII & X of the intermediate zone and lamina VIII of the ventral horn.

3. These neurons are active during physiological locomotion.

4. These neurons may mediate left/right alternating rhythmic patterns during locomotion in conjunction with supra lumbar inputs.

5. These identified commissural cells may form an important component of the spinal CPG.

6. Propriospinal cells, known to have extensive synaptic connections in the spinal cord, are also active in locomotion.

7. Further studies should examine the excitatory/inhibitory properties of these commissural cells.

REFERENCE LIST

Alstermark, B., and Kummel, H. (1986). Transneuronal labeling of neurons projecting to forelimb motorneurons in cat performing different movements. Brain Research 376, 387-391.

Alstermark, B., and Kummel, H. (1990). Transneuronal transport of wheatgerm agglutinin conjugated horseradish peroxidase into last orders spinal interneurons projecting into Acromio-Spinodeltoideus motorneurons in the cat. 1 and 2, Exp. Brain Res. 80, 83-103.

Armstrong, R. C., and Montminy, M. R. (1993). Transsynaptic control of gene expression. Annu. Rev. Neurosci. 16, 17-29.

Arya, T., Bajwa, S., and Edgley S. A. (1991). Crossed reflex actions from group II muscle afferents in the lumbar spinal cord of the anesthetized cat. J. Physiol. (Lond.) 444, 117-131.

Bajwa, S.,Edgley, S. A., Harrison, P. J., (1992). Crossed actions on group II activated interneurons in the midlumbar segments of the cat spinal cord. J. Physiol. 455, 205-217.

Barbeau, H., and Rossignol, S. (1991). Initiation and modulation of the locomotor pattern in the adult chronic spinal cat by noradrenergic, serotonergic and dopaminergic drugs. Brain Res. 546, 250-260.

Barbeau, H., Chau, C., and Rossignol, S. (1993). Noradrenergic agonists and locomotor training affect locomotor recovery after cord transection in adult cats. Brain Res. Bull. 30, 387-393.

Barber, R. P., Phelps, P. E., Houser, C. R., Crawford, G. D., Salveterra, P.M. and Vaughn, J. E. (1984). The morphology and distribution of neurons containing choline acetyltransferase in the adult rat spinal cord: an immunocytochemical study. J. Comp. Neurobiol. 229, 329-346.

Bartel, D. P., Sheng, M., Lau, L. F., and Greenberg, M. E. (1989). Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of fos and jun induction. Genes Dev.3, 304-313.

Barzilai, A., Kennedy, T. E., Sweatt, J. D., and Kandel, E. R. (1989). 5-HT modulates protein synthesis and the expression of specific proteins during long-term facilitation in Aplysia sensory neurons. Neuron 2, 1577-1586.

Bennet,G.J.,Seltzer,Z., Lu, G.W., Nishikawa,N., and Bubner,R. (1983). The cells of origin of the dorsal column projection in the lumbo-sacral enlargements of cats and monkeys. Somatosens Res 1:131-149.

Bernhardt, R. R., Chitnis, A. B., Lindamer, L., and Kuwada, J. Y. (1990).Identification of spinal neurons in the embryonic and larval zebra fish.J. Comp. Neurol. 302, 603-616.

Besson,J.M. and Chaouch,A. (1987). Peripheral and spinal mechanisms of nociception. Physiol Rev. 67, 67-185.

Borges, L., and Iversen, S. (1986). Topography of choline acetyltransferase immunoreactive neurons and fibres in the rat spinal cord. Brain Res. 362, 140-148.

Bordey, A., Feltz, P., and Trouslard, J. (1996). Patch-clamp characterization of nicotinic receptors in a subpopulation of lamina X neurones in rat spinal cord slices. J. Physiol. (Lond). 490:3, 673-678.

Brown, T. G. (1911). The intrinsic factors in the act of progression in the mammal. Proc. Roy. Soc. B 84, 308-319.

Brown, T. G. (1913). The phenomenon of "narcosis progression" in mammals. Proc. Roy. Soc. B 86, 140-164.

Brown, T. G. (1914). Only the nature of fundamental activity of the nervous centers: Together with an analysis of the conditioning of rhythmic activity in progression, and a theory of the evolution of function in the nervous system. J. Physiol. 48, 18-46.

Brown, A. G. (1981). Organization of the dorsal horn. In A. G. Brown (ed).

Brown,A.G. (1981). Organization in the spinal cord, Springer Verlag, Heidelberg.

Organization of the Spinal Cord. the Anatomy and Physiology of Identified Neurons. Berlin: Springer-Verlag, 136-153.

Bras, H., Cavallari, P., and Jankowska, E. (1988). Demonstration of initial axon collaterals of cells of origin of the ventral spinocerebellar tract in the cat. J. Comp. Neurol. 274, 584-592.

Bras, H., Cavallari, P., and Jankowska, E., and Kubin, L. (1989). Morphology of midlumbar interneurons relaying information from Group II muscle afferents in the cat spinal cord. J. Comp. Neurol. 290, 1-15.

Buchanan, J. T. (1982). Identification of interneurons with contralateral, caudal axons in the the lamprey spinal cord: Synaptic interactions and morphology. J. Neurophysiol. 47, 961-975.

Bullit, E. (1989). Induction of c-fos-like protein within the lumbar spinal cord and thalamus of the rat following peripheral stimulation. Brain Res. 493, 391-397.

Carr, P. A., Noga, B. R., Nance, D. M., and Jordan, L. M. (1994). Intracellular labeling of cat spinal neurons using tetramethylrhodamine-dextran amine conjugate. Brain Res. Bull. 34, 447-451.

Carr, P. Huang, A., Noga, B., Dai, X., and Jordan, L. M. (1995). Cytochemical characteristics of cat spinal neurons activated during fictive locomotion. Brain Res. Bull. 37:2, 213-218.

Castellucci, V. F., Kennedy, T. E., Kandel, E. R., and Goelet, P. (1988). A quantitative analysis of 2-D gels identifies proteins in which labeling is increased following long-term sensitization in Aplysia. Neuron 1, 321-328.

Cazalets, J. R., Grillner, S., Menardi. I., Creieux, J., and Clarac, F. (1990). Two types of motor rhythm induced by NMDA and amines in an in vitro spinal cord preparation of the neonatal rat. Neurosci. Lett. 111, 116-121.

Cazalets, J. R., Sqalli-Houssaini, Y., and Clarac, F. (1992). Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. J. Physiol. (Lond) 474, 173-181.

Cervero,F. (1986). Dorsal horn neurons and their sensory inputs. In:Spinal afferent processing, Yuksh,T.L. (Ed),197-215, Plenum Press, New York

Cheng, H., Jihai, C., and Olsen, L. (1996). Spinal cord repair in adult paraplegic rats: Partial restoration of hindlimb function. Science 273, 510-513. Cina,C. Msc Thesis (1997).

Cohen, A. H., and Wallen, P. (1980). The neuronal correlates of locomotion in fish: "fictive swimming" induced in an in vitro preparation of the lamprey spinal cord. Exp. Brain Res. 41, 11-18.

Khan, J. A., and Roberts, A. (1982). The central nervous system origin of the swimming motor pattern in embryos of Xenopus laevis J. Exp. Biol. 99, 185-196.

Cowley, K. C., and Schmidt, B. J. (1994). A comparison of motor patterns induced by N-methyl-D-aspartate, acetylcholine and serotonin in the in vitro neonatal rat spinal cord. Neurosci. Lett. 171. 147-150.

Cowley, K. C., and Schmidt, B. J. (1995). Effects of inhibitory amino acid antagonists on reciprocal inhibitory interactions during rhythmic motor activity in the in vitro neonatal rat spinal cord. J. Neurophysiol. 74:3, 1109-1117.

Cowley, K. C., and Schmidt, B. J. (1997). Regional distribution of the locomotor pattern-generating network in the neonatal rat spinal cord. J. Neurophysiol. 77:1, 247-259.

Curran, T., and Morgan, J.I. (1985). Super induction of c-fos by nerve growth factor in the presence of peripherally active benzodiazepines. Science 229, 1265-1268.

Curran, T., and Morgan, J. I. (1987). Memories of fos . Bioessays 7, 255-258.

Curran, T. (1988). The fos oncogene. The Oncogene Handbook, ed. E. P. Reddy, A. M. Skalka, 307-325. Amsterdam: Elsevier.

Currie, S. N., and Lee, S. (1996). Glycinergic inhibition in the turtle spinal cord regulates the intensity and pattern of fictive flexion reflex motor output. Neurosci. Lett. 205, 75-78.

Dai, X., Douglas, J. R., Nagy, J. I., Noga, B. R., and Jordan, L. M. (1990). Localization of spinal neurons activated during treadmill locomotion using c-fos immunohistochemical method. Soc. Neurosci. Abstr. 16, 889.

Deliagina, T. G., Orlovsky, G. N., and Pavlova, G. A. (1983). The capacity for generation of rhythmic oscillations is distributed in the lumbosacral spinal cord of the cat. Exp. Brain Res. 53, 81-90.

Dragunow, M., and Robertson, H. A. (1987). Kindling stimulation induces c-fos protein(s) in granule cells of the rat dentate gyrus. Nature 329, 441-442.

Edgley, S. A., Jankowska, E. (1987a). Field potentials generated by group II afferents in the middle lumbar segments of the spinal cord. J. Physiol. (Lond). 385, 393-413.

Edgley, S. A., Jankowska, E. (1987b). An interneuronal relay for group I and group II muscle afferents in middle lumbar segments of the cat. J. Physiol. (Lond). 389, 647-674.

Edgley, S. A., Jankowska, E., and Shefchyk, S. J. (1988). Evidence that mid-lumbar neurons in reflex pathways from group II afferents are involved in locomotion in the cat. J. Physiol. (Lond). 403, 57-71.

Eide, A. L. (1996). The axonal projections of the Hofmann nuclei in the spinal cord of the late stage chicken embryo. Anat. Embryol. (Berl). 193,4543-557.

Fetcho, J. R. (1990). Morphological variability, segmental relationships, and functional role of a class of commissural interneurons in the spinal cord of goldfish. J. Comp. Neurol. 299, 283-298.

Forssberg, H., Grillner, S., Halbertsma, J., and Rossignol, S. (1980). The locomotion of the low spinal cat. II. Interlimb coordination. Acta Physiol. Scand. 108, 283-295.

Franza, B. R., Sambucetti, L.C., Cohen, D. R., and Curran, T. (1987). Analysis of Fos protein complexes and Fos-related antigens by high resolution two-dimensional gel electrophoresis. Oncogene 1, 213-221.

Garcia-Rill, E., Skinner, R. D., and Fitzgerald, J. A. (1985). Chemical activation of the mesenchephalic locomotor region. Brain Res. 330, 43-54.

Goldman, D., Breener, H. R., Heinemann, S. (1988). Acetylcholine receptor a, b, g and d subunit mRNA levels are regulated by muscle activity. Neuron 1, 329-333.

Greenberg, M. E., Greene, L. A., and Ziff,E. B. (1985). Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. J. Biol. Chem. 260, 14101-14110.

Greenberg, M. E., Ziff, E. G., and Greene, L. A. (1986). Stimulation of neuronal acetylcholine receptors induces rapid gene transcription./ Science 234, 80-83.

Grillner,S., and Hongo,T. (1972). Vestibulospinal effects on motorneurons and interneurons in the lumbosacral cord. Progress in Brain Res. 37, 243-262.

Grillner, S. (1974). On the generation of locomotion in the spinal dogfish. Exp. Brain Res. 20, 459-470.

Grillner, S., and Zangger, P. (1974). Locomotor movements generated by the deafferented spinal cord. Acta. Physiol. Scand. 91, 38A-39A.

Grillner, S. (1975). Locomotion in vertebrates: central mechanisms and reflex interaction. Physiology Reviews 55:2, 247-304.

Grillner, S., and Zangger, P. (1979). On the central generation of locomotion in the low spinal cat. Exp. Brain Res. 34, 241-261.

Grillner, S. (1981). Control of locomotion in bipeds, tetrapods, and fish. In: Handbook of Physiology, The Nervous System, vol, 2 chapter 26, 1179-1236.

Grillner, S., and Wallen, P. (1985). Central pattern generators for locomotion, with special reference to vertebrates. Ann. Rev. Neurosci. 8, 320-333.

Grillner, S., Walle'n, P., Dale, N., Brodin, L., Buchanan, J., and Hill, R. (1987). Transmitters, membrane properties and network circuitry in the control of locomotion in lamprey. Trends Neurosci.10, 34-41.

Grillner, S., and Matsushima, T. (1991). The neural network underlying locomotion in lamprey-synaptic and cellular mechanisms. Neuron. 7:1,1-15.

Hagevik, A., and McClellan, A. D. (1994). Coupling of spinal locomotor networks in larval lamprey revealed by receptor blockers for inhibitory amino acids: Neurophysiology and computer modeling. J. Neurophysiol. 72:4, 1810-1829.

Halazonetis, T. D.., Georgopoulos, K., Greenberg, M. E., and Leder, P. (1988). c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. Cell 55, 917-924.

Harder, K. C., and Schmidt, B. J. (1992). Effects of inhibitory amino acid agonists on locomotor rhythmicity in the rat spinal cord in vitro. Soc. Neurosci. Abstr. 139, 17.

Harrison, P. J., Hultborn, H., Jankowska, E., Katz, R., Storai, B., and Zytnicki, D. (1984). Labeling of interneurons by retrograde Transsynaptic transport of horseradish peroxidase from motorneurons in rats and cats. Neurosci. Lett. 45, 15-19.

Harrison, P. J., Jankowska, E., and Zytnicki, D. (1986). Lamina VIII interneurons interposed in crossed reflex pathways in the cat. J. Physiol. 37, 147-166.

Ho, S., and O'Donovan, M. J. (1993). Regionalization and intersegmental coordination of rhythm generating networks in the spinal cord of the chick embryo. J. Neurosci. 13, 1354-1371.

Hochman, S., Jordan, L. M., MacDonald, J. F. (1994). N-Methyl-D-aspartate receptor-mediated voltage oscillations in neurons surrounding the central canal in slices of rat spinal cord. J. Neurophysiol. 72, 565-577.

Hongo, T., Jankowska, E., and Lundberg, A. (1966). Convergence of excitatory and inhibitory action on interneurons in the lumbosacral cord. Expl Brain Res. 1, 338-358.

Hoover, J. E, and Durkovic, R. G. (1992). Retrograde labeling of lumbosacral interneurons following injections of red and green fluorescent microspheres into hindlimb motor nuclei of the cat. Somatosens. Motor. Res. 9, 211-226.

Hounsgaard, J., and Kjaerulff, O. (1992). Ca2+-Mediated plateau potentials in a subpopulation of interneurons in the ventral horn of the turtle spinal cord. Eur. J. Neurosci. 4, 183-188.

Houser, C. R., Crawford, G. D., Barker, R. P., Salvaterra, P. M., and Vaughn, J. E. (1983). Organization and morphological characterization of cholinergic neurons: an immunohistochemical study with a monoclonal antibody to choline acetyltransferase.

Hunt,S. P., Pini, A., and Evan, G. (1987). Introduction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature 328, 632-634.

Jankowska, E., and Lindstrom, S. (1971). Morphological identification of Renshaw cells. Acta Physiol. Scand. 81, 428-430.

Jankowska, E., and Lindstrom, S. (1972). Morphology of interneurons mediating reciprocal inhibition of motorneurons in the spinal cord of the cat. J. Physiol. 226, 805-823.

Jankowsksa,E., Johannisson,T., and Lipski,J. (1981). Common interneurons in reflex pathways from group 1a and 1b afferents of ankle extensors in the cat. J Physiol 310, 381-402.

Jankowska, E. (1986). Further indications for enhancement of retrograde transneuronal transport of WGA-HRP by synaptic activity. Brain Re. 341, 403-408.

Jankowska, E., and Skoog, B. (1986). Labeling of midlumbar neurones projecting to cat hindlimb motorneurones by transneuronal transport of horse radish peroxidase conjugate. Neurosci. Lett. 71, 163-168.

Jankowska, E., and Noga, B. R. (1990). Contralaterally projecting lamina VIII interneurons in middle lumbar segments in cat. Brain Res. 341, 403-408.

Jasmin, L., Gogas,K. R., Ahlgren, S. C., Levine, J. D., and Basbaum, A. I. (1994). Walking evokes a distinctive pattern of fos-like immunoreactivity in the caudal brainstem and spinal cord of the rat. Neuroscience 58:2, 275-286.

Jordan, L. M., and Noga, B. R. (1991). Spinal interneurones synaptically activated by stimuli applied to the mesencephalic locomotor region. Soc. Neurosc. Abstr. 17,1223.

Kiehn, O., Hultborn, H., and Conway, B. A. (1992). Spinal cord locomotor activity in acutely spinalized cats induced by intrathecal application of noradrenalin. Neurosci. Lett. 143, 243-246.

Kjaerulff, O., Barajon, I., and Kiehn, O. (1994). Sulphorhodamine-labelled cells in the neonatal rat spinal cord following chemically induced locomotor activity in vitro. J. Physiol. (Lond). 478, 265-273.

Kjaerulff, O., and Kiehn, O. (1996). Distribution of networks generating and coordinating locomotor activity in the neonatal rat spinal cord in vitro: A lesion study. J. Neurosci. 16:18, 5777-5794.

Kjaerulff, O., and Kiehn, O. (1997). Crossed rhythmic synaptic input to motorneurones during selective activation of the contralateral spinal locomotor network. J. Neurosci. 17:24, 9433-9447.

Klarsfeld, A., Laufer, R., Fontaine, B., Devilliers-Thie'ry, A., Dubreuil, C., and Changeux, J. P. (1989). Regulation of the muscle AChR a subunit gene expression by electrical activity: involvement of protein kinase C and Ca2+. Neuron 2, 1229-1236.

Kouzarides, T., and Ziff, E. (1988). The role of the leucine zipper in the fos-jun interaction. Nature 336, 646-651.

Kremer, E., and Lev-Tov, A. (1997). Localization of the spinal network associated with generation of hindlimb locomotion in the neonatal rat and organization of its transverse coupling system. J. Neurophysiol. 77, 1155-1170.

Kudo, N., Ozaki, S., and Yamada, Y. (1991). Ontogeny of rhythmic activity in the spinal cord of the rat. In : Neurobiological Basis of Human Locomotion. Shimamura, M., Grillner, S., and Edgerton, V.R. (eds.) Tokyo: Japan Scientific Societies Press. 127-136.

Kulagin, A. S., and Shik, M. L., (1970). Interaction of symmetrical limbs during controlled locomotion. Biofizika 15, 164-170.

Lau, L. F., and Nathans, D. (1987). Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc. Proc. Natl. Acad. Sci. USA 84, 1182-1186.

Magnusson, D. S. K., and Trinder, T. C. (1997). Locomotor rhythm evoked by ventrolateral funiculus stimulation in neonatal rat spinal cord. Amer. Physiol. Soc. 200-206.

Matsushita,M., Ikeda,M., and Hosoya,Y. (1979). The location of spinal neurons with long descending axons (long descending propriospinal tract neurons) in the cat: a study with the horseradish peroxidase technique. J Comp Neurol. 184, 53-80.

McCrea, D. A., Pratt, C. A., and Jordan, L. M. (1980). Renshaw cell activity and recurrent effects on motorneurons during fictive locomotion. J. Neurophysiol. 44, 474-488.

Menetry,D., DePommery,J., and Roudier,F. (1985). Propriospinal fibres reaching the lumbar enlargement in the rat. Neurosc Lett. 58, 257-261.

Merlie, J. P., Isenberg, K. E., Russel, S. D., and Sanes, J. R. (1984). Denervation supersensitivity in skeletal muscle: analysis with a cloned cDNA probe. J. Cell Biol. 99, 332-335.

Michael, G. J., Kaya, E., Averill, S., Rattray, M., Clary, D. O., and Priestley, J. V. (1997). Trka immunoreactive neurones in the rat spinal cord. J. Comp. Neuro. 385, 441-455.

Morgan, J. I.,and Curran, T. (1986). Role of ion influx in the control of c-fos expression. Nature 322, 552-555.

Morgan, J. I., Cohen, D. R., Hempstead, J. L., and Curran, T. (1987). Mapping patterns in the central nervous system after seizure. Science 237, 192-197.

Morgan, J. I., and Curran, T. (1989). fos and the immediate-early response in the central nervous system. In: N. H. Colburn (Ed.), Genes and Signal Transduction in Multistage Carcinogenesis, Marcel Dekker, New York and Basel 377-390.

Mortin, L. I., and Stein, P. S. G. (1989). Spinal cord segments containing key elements of the central pattern generators for three forms of scratch reflex in the turtle. J. Neurosci. 9, 2285-2296.

Nakabeppu, Y., Ryder, K., and Nathans, D. (1988). DNA binding activities of three murine Jun proteins: stimulation by Fos. Cell 55, 907-915.

Nicolopoulos-Stournaras, S., and Iles, J. F. (1983). Motor neuron columns in the lumbar spinal cord of the rat. J. Comp. Neurol. 217, 75-85.

Noga, B. R., Shefchyk, S. J., and Jordan., L. M. (1987). Localization of last order spinal interneurones participating in the production of locomotion in the cat. Soc. Neurosci. Abstr. 13, 826.

Noga, B. R., Shefchyk, S. J., and Jordan, L. M.(1987b). Localization of last-order interneurons participating in the production of locomotion in the cat. Soc. Neurosci. Abstr. 13, 826.

Noga, B. R., Fortier, P. A., Kriellaars., D. J., Dai, X., Detilliex, G. R., and Jordan, L. M. (1995). Field potential mapping of neurones in the lumbar spinal cord activated following stimulation of the mesencephalic locomotor region. J. Neurosci. 15:3, 2203-2217.

Offord, J., and Catterall,W. A. (1989). Electrical activity, cAMP, and cytosolic calcium regulate mRNA encoding sodium channel a subunits in rat muscle cells. Neuron 2, 1447-1452.

Oppenheim, R. W., Schneiderman, A., Shimizu, I., and Yaginuma, H. (1988). Onset and development of intersegmental projections in the chick embryo spinal cord. J. Comp. Neurol. 275, 159-180.

Perreault, M. C., Angel, M. J., Guertin, P., and McCrea, D. A. (1995). Effects of stimulation of hindlimb flexor group II afferents during fictive locomotion in the cat. J. Physiol. 487:1, 211-220.

Phelps, P.E., Barber, R. P., Houser, C. R., Crawford, G. D., Salvaterra, P. M., and Vaugn, J. E. (1984). Postnatal development of neurons containing choline acetyltransferase in rat spinal cord: an immunocytochemical study. J. Comp. Neurol. 229, 347-361.

Phelps, P. E., Barber, R. P. Brennan, L. A., Maines, V. M., Salvaterra, P. M. and Vaughn, J. E. (1990). Embryonic development of four different subsets of cholinergic neurons in rat cervical spinal cord. J. Comp. Neurol. 291, 9-26.

Philippson, M. (1905). L'autonomie et la centralisation dans le syst'eme nerveux des animaux, Trav. Lab. Physiol.. Inst. Solvay Bruxelles 7,1-208.

Pieribone, V. A., and Aston- Jones, G. (1988). The ionotophoretic application of Fluoro-Gold for the study of afferents to deep brain nuclei. Brain Res. 475, 259-271.

Pratt, C. A., and Jordan, L. M. (1987). Ia inhibitory interneurons and Renshaw cells as contributors to the spinal mechanism of fictive locomotion. J. Neurophysiol. 57, 56-71.

Presley,R.W., Menetry,D., Levine,J.D., and Basbaum,A. (1990). Systemic morphine suppresses noxious stimulation-evoked fos protein like immunoreactivity in the rat spinal cord. J Neurosci. 10, 323-335.

Roberts, A., and Clark, J. D. (1982). The neuroanatomy of an amphibian embryo spinal cord. Phil. Trans. R. Soc. Lond. 296, 195-212.

Roberts, A., and Sillar, K. T. (1990). Characterization and function of spinal excitatory interneurons with commissural projections in xenopus laevis embryos. Eur. J. Neurol. Sci. 2, 1051-1062.

Ramo'n y Cajal, S. (1911). Histologiedu Syste'me Nerveux de l'Hommet et des Verte'bre's. Vol. 1. Paris: A. Maloine.

Rauscher, F. J., Voulalas, P. J., Franza, B. R., Jr., and Curran, T. (1988a). Fos and Jun bind cooperatively to the AP-1 site: reconstruction in vitro. Genes Dev. 2, 1687-1699.

Remmel, R. S., and Skinner R. D. (1980) "Walking" rhythms in long descending propriospinal neurons in cats. Soc. Neurosci. Abstr. 6:24.

Rexed,B. (1954). A cytoarchitectonic atlas of the spinal cord in the cat. J Comp Neurol 100, 297-379.

Rossignol, S. A., and Dubuc, R. (1994). Spinal pattern generation. Curr. Opinion. Neurobiol. 4, 894-902.

Rotto-Percelay, D. M., Wheeler, J. G., Orsorio, F. A., Platt, K. B., and Loewy, A. D. (1992). Transneuronal labeling of spinal interneurons and sympathetic preganglionic neurons after pseudorabies virus injections in the rat medial gastrocnemius muscle. Brain. Res. 574, 291-306.

Rouse, D. T., and McClellan, A. D. (1997). Descending propriospinal neurons in normal and spinal cord-transected lamprey. Experimental Neuro. 146, 113-124.

Sagar, S.M., Sharp, F. R., and Curran, T. (1988). Expression of c-fos protein in brain: a novel method of neuroanatomic metabolic mapping at a cellular level. Science 240, 1328-1331.

Scheibel, M. E., and Scheibel, A. B. (1966a). A structural analysis of spinal interneurons and Renshaw cells. Arch. Ital. Biol. 104, 328-353.

Schmued, L. C., and Fallon, J. H. (1986). Fluoro-Gold: a new fluorescent retrograde axonal tracer with numerous unique properties. Brain Res. 377, 147-154.

Schmued, L. (1990). Fluoro-Gold and 4-Acetomido-4'-isothiocyanostilbene-2,2'-disulphonic Acid: Use of substituted stilbenes in neuroanatomical studies. Meth. in Neurosci. 3, 317-330.

Sharp, F. R., Sagar, S. M., and Swanson, R. A. (1993). Metabolic mapping with cellular resolution: c-fos vs. 2-Deoxyglucose. Critical Reviews in Neurobiol. 7:3/4, 205-228.

Shefchyk, S., McCrea, D. A., Kriellaars, D. J., Fortier., P. A., and Jordan, L. M. (1990). Activity of interneurons within the L4 spinal segments of the cat during brainstem-evoked fictive locomotion. Exp. Brain Res. 80, 290-295.

Sheng, M., and Greenberg, M. E. (1990). The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron 4, 477-485.

Sherrington, C. S. (1910). Flexion-reflex of the limb, crossed extension-reflex, and reflex stepping and standing. J. Physiol. 40, 28-121.

Sherrington, C. S. (1913). Nervous rhythm arising from rivalry of antagonistic reflexes: Reflex stepping as outcome of double reciprocal innervation. Proc. Roy. Soc. B 86, 233-261.

Shik, M. L., Severin, F. V., and Orlovsky, G. N. (1966). Control of walking and running by means of electrical stimulation of the mid-brain. Biofizika (engl transl) 11, 659-666.

Shik, M. L., Orlovsky, G. N., and Severin, F. V., (1966). Organization of locomotor synergism. Biofizika 11, 1011-1019.

Silos-Santiago, I., and Snider, W. D. (1992). Development of commissural neurons in the embryonic rat spinal cord. J. Comp. Neurol. 325, 514-526.

Skinner, R.D., Coulter, J.D., Adams, R.J. and Remmel, R.S. (1979). Cells of origin of long descending propriospinal fibres connecting the spinal enlargements in cat and monkey determined by horseradish peroxidase and electrophysiological techniques. J Comp Neurol 188, 443-454.

Sqalli-Houssaini, Y., Cazalets, J. R., and Clarac, F. (1993). Oscillatory properties of the central pattern generator for locomotion in neonatal rats. J. Neurophysiol. 70, 803-813.

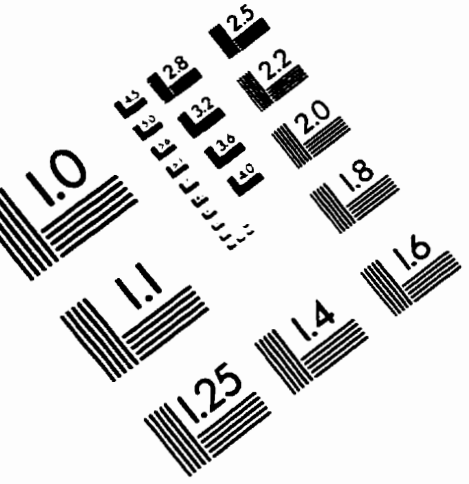
Steeves, J. D., and Jordan, L. M. (1984). Autoradiographic demonstration of the projections from the Mesencephalic Locomotor Region. Brain Res. 307, 263-276.

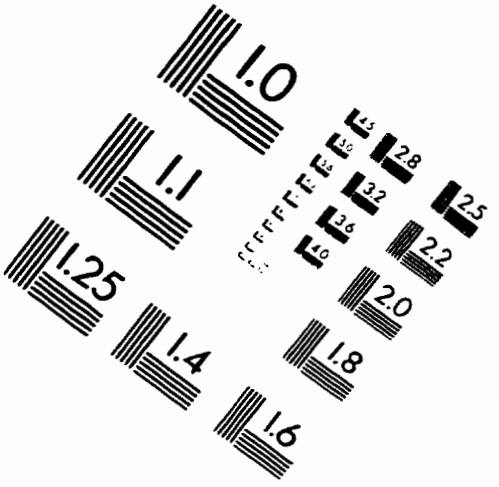
Viala, D., Buisseret-Delmas, C., Portal, J. J. (1988). An attempt to localize the lumbar locomotor generator in the rabbit using 2-deoxy-[14 C] glucose autoradiography. Neurosci. Lett. 86, 139-143.

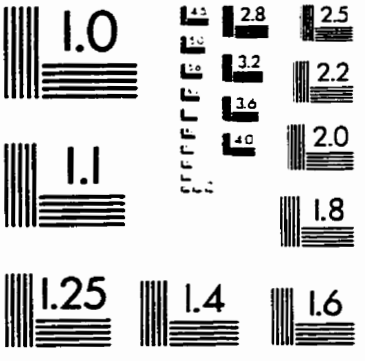
White, J. D., and Gall, C. M. (1987). Differential regulation of neuropeptide and proto-oncogene mRNA content in the hippocampus following recurrent seizures. Mol. Brain Res. 3, 21-29.

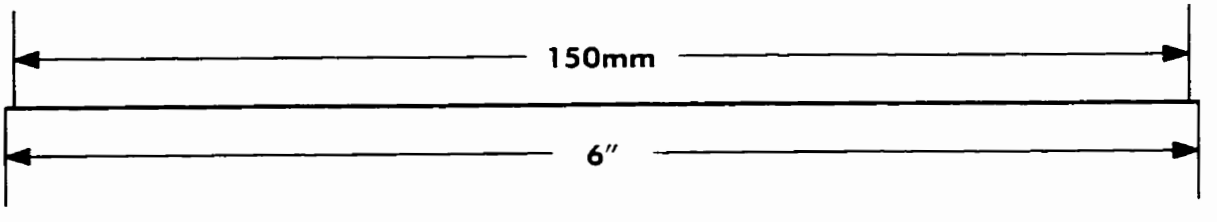
Willis, W. D. Jr. (1969). The localization of functional groups of interneurons. UCLA Forum Med. Sci. 11, 267-287.

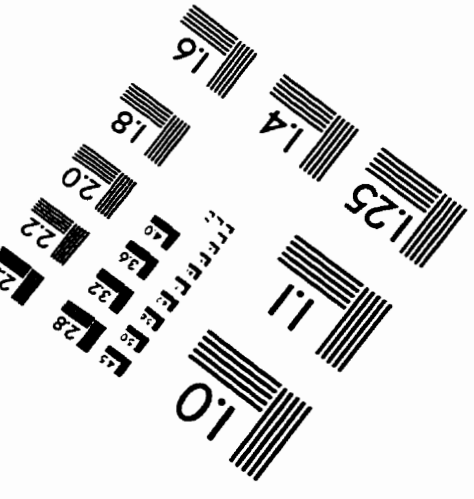
IMAGE EVALUATION
TEST TARGET (QA-3)

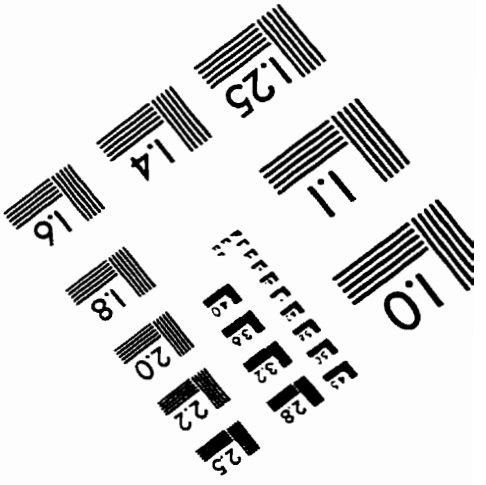












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989