LOCALIZATION OF SPINAL CHOLINERGIC NEURONS ACTIVATED DURING LOCOMOTION IN CATS USING C-FOS AND CHOLINE ACETYLTRANSFERASE IMMUNOHISTOCHEMISTRY

BY
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ABSTRACT

The objective of the present study was to determine the location of the cholinergic neurons activated in the spinal cord of decerebrate cats during fictive locomotion.Locomotion was induced by stimulation of the mesencephalic locomotor region (MLR). After bouts of locomotion during a 7-9 hour period, the animals were perfused and the L_3-S_1 spinal cord segments removed. Cats in the control group were subjected to the same surgical procedures but no locomotor task. The tissues were sectioned and then stained by immunohistochemical methods for detection of the c-fos and choline acetyltransferase (ChAT) .

The resultant c-fos labelling in the lumbar spinal cord was similar to that previously described in the fictive locomoting cat . ChAT-positive cells were also clearly active during locomotion. Double labelling of c-fos with the ChAT marker displayed a scattered distribution within ventral lamina VII, VIII and possibly IX. Most of them were concentrated in the medial portion of lamina VII close to lamina X, in which partition and central canal cells were found by Barber and her collaborators (1984). The number of ChAT and c-fos labelled neurons following fictive locomotion was increased and was greatest in the intermediate grey, relative to dorsal and ventral regions. The results suggest that cholinergic interneurons in lumbar spinal cord are involved in the production of fictive locomotion.

ABBREVIATIONS

2-DG	2-deoxyglucose
AB	anterior biceps
ACh	acetylcholine
AChE	acetylcholinesterase
ChAT	choline acetyltransferase
CPG	central pattern generator
EDRO	edrophonium
ENG	electroneurogram
FC-cat	fictive control cat
FL-cat	fictive locomotion cat
MEC	mecamylamine
MLR	mesencephalic locomotor region
NMDA	N-Methyl-D-aspartate
PB	posterior biceps
SA	sartorius
SM	semimembranosus
ST	semitendinosus
THA	9-amino-1,2,3,4-tetrahydroacridine
WGA-HRP	wheat germ agglutinin-horse radish peroxidase

INTRODUCTION

This study was designed to determine the location of cholinergic interneurons that are involved in the control of locomotion at the spinal level using the C-fos and choline acetyltransferase (ChAT) immunohistochemical methods. The following survey of acetylcholine (ACh) literature will focus primarily on the spinal cord, and is intended to establish the foundation upon which the experiments presented in this thesis are based. The distribution of ChAT-positive neurons and characteristics of cholinergic propriospinal connections will be described first, followed by a discussion of ACh function in the central pattern generator for locomotion in the spinal cord. This will lead to a search of what is known about the roles played by ACh in spinally-organized rhythmic activity.

Cholinergic structures within the spinal cord

There is long-standing evidence that ACh is released by the motoneuron axon terminus and its axon collateral (Eccles 1954). As early as 1936, Dale et al observed that stimulation of spinal motor nerves caused a release of ACh upon somatic muscles and a concomitant muscular contraction. Shortly thereafter, Macintosh (1941) and Feldberg (1948) demonstrated the presence and synthesis of ACh in the spinal cord in animal experiments. The first application of acetylcholinesterase (AChE) histochemistry also depicted intense staining of the motor neurons (Giacobini and Holmstedt 1958).

Since these achievements were reported, there has been considerable interest in identifying different cholinergic neurons, including motoneurons and other

neurons, fibers and synapses where ACh or its synthesizing enzyme may be present within the spinal cord.

Several histochemical studies on the spinal cord of cat (Koelle 1950, Koessmann and Friede 1967, Silver and Wolstencroft 1971), rat (Toth et al 1972, Kasa 1978) and monkey (Odutola 1972) have shown that AChE-positive structures are widely concentrated or dispersed not only in the motor cell columns but also in other areas. Biochemical assay has revealed a high activity of ChAT in all of Rexed's laminae (Kasa 1978). Further, following selective lesions of the interneurons (using an ischemic spinal cord), both ChAT and AChE activities decreased in parallel with the disappearance of interneurons from laminae VII (Kasa 1978). These results indicated that, besides the motoneurons, cholinergic interneurons were present in the spinal cord.

The most significant advances were obtained with the recently-developed immunohistochemical technique. The use of AChE to visualize cholinergic neurons has been hampered due to its presence not only in cholinergic neurons, but also in several other noncholinergic and noncholinoceptive tissue cells (Carvalheira and Pearse 1967). Choline acetyltransferase, the enzyme that synthesizes ACh, is now generally accepted as a definitive marker for cholinergic neurons (Kuhar 1976, McCaman 1976, Storm - Mathisen 1977) since its distribution is more closely correlated with the distribution of ACh itself (Hebb 1956). Purified fractions of ChAT have been used to develop polyclonal (Kimura 1980,1981) and monoclonal (Crawford 1982, Levery 1983) antibodies that can identify cholinergic neurons with immunohistochemical techniques.

Immunocytochemical studies showed that ChAT cells are present throughout spinal cord from cervical levels to the coccyx. At least five different types of ChAT-

positive neurons have been identified in the spinal cord (Barber et al 1984, Berges and Iversen 1986, Houser et al 1983, Phelps et al 1984, Ribeiro-da-silva and Cuello 1990) and these comprise (1) motoneurons (2) preganglionic neurons in the thoraco-Lumbar T_{1-3} and L_{1-3} (sympathetic) and lumbosacral L_6-S_1 (Parasympathetic) levels of the spinal cord (3) partition cells (lamina VII) (4) central canal cluster cells (lamina X), and (5) small dorsal horn cells scattered in lamina III-V extending dendrites dorsally into lamina III.

Partition cells and central canal cells were identified recently. Partition cells formed a ChAT-positive network across the intermediate spinal region of non-autonomic levels extending from the central canal to the lateral edge of the grey matter, thereby delineating the dorsal from the ventral horn (Barber et al 1984, Borges and Iversen 1986). In horizontal sections, the dendrites of medial partition neurons coursed rostrocandally forming a loose, longitudinal plexus that was joined by dendrites from central canal cells. In contrast, intermediate partition cells had dendrites that radiated in all directions within the horizontal plane and interdigitated with those of other partition neurons and lateral motoneurons. Lateral partition neurons often were embedded in the dorsally oriented dendrites of lateral motoneurons (Barber et al 1984, Phelps et al 1984). These characteristics of the partition cells suggest that they may make propriospinal connections since Golgi studies (Scheibel and Scheibel 1966) reported large propriospinal neurons with axons coursing in a ventromedial direction and projecting contralaterally in a pathway similar to that of the partition cell fibers which traverse the ventral commissure. Also, Skinner et al (1979) described some long descending propriospinal neurons within the cervical enlargement as among the largest neurons within the medial and intermedial portions of lamina VII.

Central canal cells are small ChAT-positive neurons. They are located throughout the spinal cord and grouped together in the central grey matter encircling the central canal within lamina X (Barber et al 1984, Phelps et al 1984, Borges and Iversen 1986). Some of them project to dorsal, intermediate and ventral gray matter. Some extend to the contralateral side of the spinal cord. Some contribute to small, longitudinal fascicles of ChAT-positive observed in horizontal sections. Besides the somata and processes of these cells, area X also contains ChAT-positive dendrites of both medial motor neurons and medial partition cells, as well as ChAT-positive punctate structures that may represent terminals from local cholinergic neurons (Barber et al 1984, Phelps et al 1984). These interconnections within lamina X may provide a cholinergic relay between the dorsal, intermediate and ventral grey matter of the spinal cord, and could be involved in integration between the sensory, motor and autonomic segments of the spinal cord (Borges and Iversen 1986).

Although partition and central canal cells make up only a portion of the total population of neurons in the spinal cord, they may provide several overlapping levels of regulation of spinal cord neurons and play an important role in the cholinergic innervation of the spinal cord (Sherriff 1994).

So far, the cholinergic innervation of the spinal cord is unclear. To determine whether there are ascending and / or descending cholinergic fibers in the spinal cord the most commonly used technique has been to perform lesions (hemisections) above the segment under study. The observation that ChAT activity in the ventral horn is reduced ipsilateral to and below a spinal cord lesion suggests that cholinergic fibers may arise from higher levels (Gillberg and Wiksten 1986). There is no chang in cholinergic receptors. This is not in agreement with an earlier ChAT study (Kanazawa et al 1979), but is in accordance with results of Gwyn et al (1966, 1972),

who found evidence of ascending and descending cholinergic fibers in the spinal cord white matter. Borges and Iversen (1986) using immunohistochemical techniques with a monoclonal antibody to ChAT could not observe any evidence of cholinergic fibers within the dorsolateral or dorsal funiculus that would support a descending cholinergic projection to the dorsal horn. However, they observed fine ChAT immunoreactive fibers entering the midlateral funiculus from the ventral grey matter. These fibers appeared to represent cholinergic propriospinal neurons, perhaps from recurrent axon collaterals of motor neurons.

Besides this evidence both in favour and against the existence of descending and ascending cholinergic tracts in the spinal cord, intrinsic cholinergic innervation of the spinal cord is obvious. With a combination of retrograde axonal tract tracing and choline acetyltransferase immunocytochemistry, Sherriff (1994) observed the presence of long- and short-range propriospinal connections in the rat spinal cord, and showed that the intraspinal cholinergic circuitry involved short-range propriospinal projections. Barber (1984) proposed that the cells contributing to these intersegmental cholinergic projections are central canal and partition cells which probably project diffusely to all laminae of the spinal cord, but not preganglionic or dorsal horn cholinergic neurons or motoneurons . These results were in line with classic studies indicating the presence in the spinal cord of propriospinal fiber systems that descend and ascend for short distances in the spinal white matter (Sheibel and scheibel 1966) and with other, more indirect lesioning and immunocytochemical studies which have predicted the presence of short-range cholinergic projections (Barber et al 1984, Kanazawa 1979).

Spinal motoneurons and dorsal horn neurons may involve in intrasegmental cholinergic innervation (Culheim et al 1977, Lipski and Martin-Body 1987, Ribeiro

and Cuello 1990). Ultrastructural studies have suggested that the dorsal horn cholinergic cells provide a local innervation of the dorsal horn upper layers (Ribeiro and Cuello 1990). Motoneurons have recurrent collaterals which terminate on the soma and proximal dendrites of neighbouring motoneurons and in the adjacent Renshaw cell (Culheim et al 1977, Lipski and Martin-Body 1987).

This pattern of widespread interconnectivity of spinal cholinergic neurons, fibers and terminals could mediate coordinated firing patterns within local pockets of contiguous cholinergic neurons. This is potentially important in locomotor movements since motor programs are produced by connections made between motor neurons and local circuit cells (Woolf 1991). Schoenen (1982) proposed that cholinergic longitudinal bundles link together motor neurons innervating the same or agonist muscles and mediate synchronous and synergistic activity. Conversely, dendritic bundles extending in the transverse plane mediate central motor programs of locomotor functions such as walking, based upon the various functional types of motor neurons that are interlimbed (Schoenan 1982).

Based upon the observations reported above, I suggest there are at least 2 cholinergic 'systems ' within the spinal cord. One is confined to single segments and neighbouring lamina and includs the small dorsal horn cells and the motoneurons of the ventral horn. The former appears to be a local circuits of dorsal horn. The latter project to muscles to influence contraction directly and neighbouring motoneurons via recurrent axon collaterals. The second system, with longer-range effects and a more diffuse distribution , includes the central canal and partition cells. The system is likely to have an intersegmental sphere of influence and a possible coordinating function.

Spinal cholinergic effects

Since the fundamental work of Eccles et al (1954) on the role of acetylcholine in the recurrent inhibitory pathway of spinal motoneurons, there have been many investigations of ACh as a transmitter candidate and presence of both nicotinic and muscarinic receptor systems in mammalian spinal cord (Kava et al 1970, Myslinski and Randic 1977, Odutola 1972, Silver and Wolstencroft 1970, 1971). In general, cholinomimetics predominantly excite CNS neurons (Evans 1977, Myslinski and Randic 1977, Weight and Salmoiraghi 1966), although biphasic responses exhibiting an initial hyperpolarizing phase followed by depolarizating have been revealed by intracellular studies (Benardo and prince 1981, Segal 1982). Excitation seems mediated by activation of both muscarinic and nicotinic receptors, and muscarinic receptors may also mediate inhibitory responses in spinal neurons (Zieglgansberger and Reiter 1974).

In vitro receptor autoradiography studies showed that both muscarinic and nicotinic receptors are distributed extensively in the spinal cord (Scatton et al 1984, Gillberg and Aquilonius 1985, Gillberg and wiksten 1986, Gillberg and Askmark 1989). Renshaw cells have both nicotinic and muscarinic receptors on the perikarya (Curtis and Ryall 1966, King and Ryall 1981) and are inhibitory to the motoneuron (Eccles et al 1954, Wilson et al 1960). Intracellular studies provide support for nicotinic and muscarinic receptor modulation of certain potassium channels. Tokimasa and North (1984) indicated that nicotinic receptor stimulation enhances inward Ca current which in turn triggers Ca-dependent K conductances in rat. Nowak and MacDonald (1983a) reported that muscarine increased action potential firing and evoked a slow, sustained depolarization accompanied by decreasing membrane conductance of mouse spinal cord neurons. Action potentials but not slow membrane

depolarizations were eliminated by the sodium channel blocker tetrodotoxin. Muscarine also decreased a time- and voltage- dependent potassium current (Brown and Adams 1980, Nowak and Macdonald 1983b). In chicken spinal cord neurons in dissociated cell cultures, excitatory synaptic interactions between spinal cord neurons were augmented by ACh or muscarine, while inhibitory synaptic interactions were diminished (Wang et al 1990). Thus, both nicotinic and muscarinic effects have different characteristics. The rapid, large, high conductance nicotinic mechanisms in motor and preganglion neurons made the unambiguous positive effect, such as contraction of skeletal muscle (Wang et al 1990). According to one study (Gillberg et al 1989), spinal cholinergic nicotinic receptors have no significant effect on total activity, locomotion and rearing. Another nicotinic agonist, cytisine, only activated the nicotinic receptors on Renshaw cells, thus causing inhibition of the motor neuron and therefore a reduced spontaneous motor activity (Gillberg et al 1990). The muscarinic cholinergic slower synaptic phenomena seems more involved in mediating movement. Microiontophoretically applied ACh onto the motor neuron gave a depolarization of the cells, whereas Renshaw cells respond with both a rapid depolarization and an increase of firing rate (Zieglgansberger and Reiter 1974). Gillberg (1990) suggested that the decrease of spontaneous activity of the motor neuron caused by the AChE inhibitors, physostigmine and 9-amino-1,2,3,4-tetrahydroacridine (THA) might be explained by both a direct depolarization of the motor neuron and an inhibition mediated via Renshaw cells.

Our current understanding of the role of ACh in spinally-organized rhythmic activity owes a great deal to the innovative vertebrate preparations. In some lower vertebrate, investigations of the central pattern generators for locomotion have yielded considerable information. In vitro preparations of the lamprey spinal cord

(Cohen and Wallen 1980) and the paralysed Xenopus frog embryo (Roberts et al 1981) were shown to exhibit locomotor rhythms and were established as legitimate models for detailed studies of spinal locomotor circuitry. The isolated nature of the preparations reduced the number of variables with which investigators had to contend and permitted detailed cellular studies previously possible only with invertebrates (McClellan 1987). Florey (1973) opened the door to ACh with his established spinal locomotor model in isolated crayfish abdominal nerve cords. He found that direct perfusion of ACh or eserine, an AChE inhibitor, through the crab sternal artery induced movement of the locomotory appendages. Extracellular and intracellular recordings had shown that the muscarinic agonist oxotremorine increases the frequency of the swimmeret rhythm in a dose-dependent manner and induces slow rhythmic activity in the abdominal positioning system consisting of opposite firing between the flexor and extensor motor neurons (Chrachri and Neil 1993). Since both forms of coordination between the swimmeret and the abdominal positioning systems can be induced by depolarization of certain abdominal interneurons, the author proposed that abdominal interneurons may play the major role in determining this oxotremorine-induced rhythmic activity. Using a different muscarinic agonist, pilocarpine, Braun and Mulloney (1993) confirmed that cholinergic agonists can activate the swimmeret motor pattern and modulate the burst frequency in a dose-dependent manner. Nicotine did not elicit rhythmic activity in resting isolated preparations but increased the burst frequency in active preparations. The ACh-esterase inhibitor eserine also increased the burst frequency in active preparations. Because there was no sensory feedback present in isolated nerve cords and the terminals of sensory afferents were not active, this suggested that the observed effects of cholinergic drugs reflect the activity of central cholinergic interneurons. Since

eserine had no effect on resting preparations, its action on active preparations implicate a role for cholinergic neurons in pattern-generation or coordinating circuits (Mulloney 1993, Murchison 1993). Even in the isolated crayfish thoracic ganglia, oxotremorine induced expression of the swimmeret motor pattern (Chrachri 1990). Taken together, these results provided strong evidence that acetylcholine acting on muscarinic receptors involved in the spinal rhythm generating system.

ACh occurs as a neurotransmitter in arthropods, including crustaceans (Barker et al 1972, Florey 1973), and receptors similar to vertebrate nicotinic and muscarinic receptors have been described pharmacologically (Barker et al 1986, Freschi 1991, Pfeiffer-Lynn and Glantz 1990). Thus the discovery that cholinergic interneurons involved in rhythmic motor activity in crayfish suggest the possible contribution of ACh to locomotion in other vertebrates.

Roberts and his colleagues (1991) observed ACh involvement in rhythmic motor activity from a central pattern generator perspective. They studied the effects of ACh in Xenopus embryos, which displays a developmental progression from undulatory swimming to pedal locomotion, by recording either extracelluarly with suction electrodes on the ventral roots or intracellularly with microelectrodes from spinal neurons. Bath application of ACh evoked a burst of activity, followed by an increased frequency of spontaneous swimming episodes. The muscarinic antagonist atropine reversibly blocked the effects of ACh. Atropine also considerably shortened both spontaneous and dimming-evoked bursts of fictive swimming, while nicotinic antagonists appear to have little effect. In order to eliminate an action on neurons in the brain which probably mediated the effect of ACh on frequency of occurrence of spontaneous episodes of activity, the same ACh experiments were performed in spinalised embryos where no spontaneous swimming was seen. They found that the

increased duration of swimming episodes was also present in spinal animals, suggesting that ACh was acting on the spinal central pattern generator. The observation that atropine decreased the duration of spontaneous episodes suggested that ACh was being released during fictive locomotion. Thus, they concluded that ACh has a strong excitatory effect on the central pattern generator and that this effect is blocked by the muscarinic antagonist atropine.

Meanwhile, Hounsgaard's group has attempted to characterize the intrinsic response properties of spinal motoneurons to transmitter modulation. They (Hounsgaard and Mintz 1994) recorded intracellularly from motoneurons in transverse sections of turtle spinal cord and found that activation of muscarinic receptors may facilitate phasic motor activity by inducing voltage regulated oscillatory properties in motoneurons. Muscarine-induced oscillations were blocked by atropine. They were also blocked by nifedipine and other Ca-channel blockers, while moderate changes in the extracellular concentration of Mg^{2+} had minimal effect. The phasic state induced by muscarine seemed to involve a basic transient depolarization in which the frequency of occurrence of depolarization rather than the duration of the transient depended on the holding current. This suggested that Ca^{2+} current through the nifedipine sensitive Ca-channels acts as a trigger signal for the depolarizing. In previous experiments, however, activation of the Ca^{2+} plateau alone by block of K^{+} channels or by application of L-channel agonists never led to oscillations (Hounsgaard and Mintz 1988). In fact, sometimes on-going muscarine-induced oscillations were obliterated by activating the plateau and promptly returned when the plateau was ended by a current pulse. Probably muscarine induced oscillations in motoneurons, in addition to membrane properties, produce a change in intracellular Ca^{2+} homeostasis, since muscarine-induced oscillations were blocked by

ryanodine and by caffeine which interfere with Ca^{2+} sequestering. Although neither of these agents are selective, the fact that they act by different mechanisms strongly suggests that muscarine induced oscillations depend on intact Ca^{2+} sequestering. These agents did not prevent NMDA induced oscillations. It is important to note that the oscillatory properties induced by muscarine differs substantially from the better known NMDA induced oscillations. It is therefore possible for vertebrate motoneurons to be optimized for different motor behaviours by regulation of their intrinsic response properties.

The marked similarities which have come to light concerning the generation of locomotion in such different animals as the crayfish and the frog embryo suggest that some mechanisms might also be conserved in higher animals (Kudo et al 1987, 1991). Smith and Feldman (1985) introduced an in vitro brainstem-spinal cord preparation of the neonatal rat, which made it possible to test the findings observed in lower vertebrates in a mammal since this novel experimental model retains functional circuitry for locomotor pattern generation and sensorimotor integration (Smith and Feldman 1985, Smith et al 1986, Smith and Feldman 1987a, 1987b). It quickly became apparent that bath application of cholinergic drugs, such as ACh and edrophonium, to the spinal cord partition could elicit stepping-like movements (Smith et al 1986) with interlimb coordination (Smith and Feldman 1987b) when the limbs were left attached. ACh-induced rhythmic activity in the spinal cord was completely suppressed by bath application of the muscarinic receptor antagonist atropine sulfate. The locomotor cycle period decreased with increasing ACh or cholinergic agonist concentration (Smith et al 1988). Nonselective neuronal excitation was unlikely, because generalized depolarization of spinal neurons produced by elevating extracellular K^+ concentration, sufficient to evoke motoneuronal discharge, did not

induce locomotor activity (Wallen et al 1984). With this groundwork established, Smith et al (1988) went on to probe the system with neurochemical uptake or breakdown inhibitors to test for involvement of related endogenous neurochemicals. They found edrophonium (EDRO), an anticholinesterase, applied to the spinal cord alone elicited motor patterns for walking in a dose-dependent manner, indicating ongoing endogenous release of ACh whose synaptic concentrations can be brought to threshold for activation of the locomotor circuitry.

Atsuta et al (1991) looked at ACh involvement in rhythmic motor activity from electromyographic recordings in the hindlimb-attached in vitro brainstem-spinal cord preparation. They reported that application of ACh to the spinal cord bath produced long-lasting co-contractions of both flexor and extensor groups. Even at the lowest concentration that was effective in producing movement, either a single muscle burst or coactivation of both limbs was observed. It was unlike application of a cholinergic agonist to the brainstem bath, which induced airstepping. The reason that bath applied cholinergic agonists failed to elicit rhythmic alternation is unclear, but probably involves in drug concentration. Cowley and Schmidt (1994) observed only a transient increase in tonic flexor and extensor activity during application of low concentration ACh and EDRO. They systematically monitored bilateral flexor and extensor ENG activity and confirmed that cholinergic receptor activation regularly induced rhythmic activity in this preparation, with side-to-side alternation of co-activated intralimb flexor-extensor pairs. It was not compatible with an overground locomotor pattern in the adult intact rat (Gruner et al 1980). Althought exogenously applied neurochemicals induced inconsistent patterns of motor activity, these differences presumably reflect differences in the number of active neuronal elements and site of action of the neurochemicals in the locomotor circuitry. The

possibility also exists that this variability reflected different types of locomotor gaits.

In studies designed to investigate locomotion more specifically, decerebrate animals have been used, largely because they offer the experimenter a greater degree of control over the animal behaviour. Studies on the mechanism of central pattern generator for locomotion have demonstrated that Renshaw cells are rhythmically active during treadmill and fictive locomotion in decerebrate cats (McCrea et al 1980, Pratt and Jordan 1980). Noga et al (1987) postulated that if Renshaw cells comprised a vital part of the locomotor central pattern generator for locomotion (Miller and Scott 1977, 1980), the removal of nicotinic mediated activation of Renshaw cells during fictive locomotion would affect the induction of movement. They found that after the disruption of Renshaw cell activity with the nicotinic antagonist mecamylamine (MEC), locomotor drive potentials in motoneurons during the fictive step cycle were only slightly modified. Increased frequency and numbers of spikes per burst were also observed in motoneurons and Ia inhibitory interneurons. They concluded that the Renshaw cells are not an integral part of the spinal central pattern generator for locomotion, not do they control the timing of the motor neuron or Ia interneuron bursts of firing during fictive locomotion. The Renshaw cells instead limit the firing rates of motor neurons and Ia interneurons during each burst. Using intracellular dye injection in physiologically identified neurons coupled with immunohistochemical markers, Carr et al (1994) reported that a subpopulation locomotor-related commissural interneurons may be cholinergic. Some of these cells were located within the territory of partition cells and were observed to terminate on contralateral motoneurons. To date, there have been no studies which have provided a clear, complete picture of the locations of cholinergic interneurons activated during locomotion. Also, few investigations have been done to examine in

vivo the role of ACh in the generation of locomotion at the spinal level.

Application of c-fos immunohistochemical method

C-fos is the cellular homologue of the viral oncogene, v-fos and exists in normal cells in vertebrates. Expression of the gene is rapid. Under an appropriate stimulus, the mRNA product, Fos protein, is rapidly synthesized and translocated to the nucleus in which it combines with Jun protein to form a heterodimeric protein complex, then the complex binds to the DNA regulatory element known as the AP-1 binding site that regulate the expression of other genes. Thus, Fos is a marker of functional activity, with resolution at the level of the single cell nucleus (Greenberg and Ziff 1984, Kruiger et al 1984, Greenberg et al 1985, 1986, Curran and Morgan 1986, Curran and Franza 1988, Ran et al 1986). Recently, it was found that the c-fos proto-oncogene is rapidly and transiently induced by growth factors (Cochran et al 1984, Muller et al 1984, Bravo et al 1985, Muller et al 1985), neurotransmitters (Greenberg et al 1986, Szekely et al 1987), depolarizing conditions (Morgan and Curran 1986) and agents that provoke an influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (Morgan and Curran 1986) in cultured cells, as well as by pharmacological (Morgan et al 1987), electrical (Dragunow and Robertson 1987), surgical (White and Gall 1987) and physiological stimuli (Hunt et al 1987, Sagar et al 1988) in neurons of intact animals. The fact that c-fos can be induced by various stimuli in a broad range of cells has suggested that c-fos plays an important role in coupling extracellular signals to alternation in gene expression (Morgan and Curran 1986). This activity-driven expression of c-fos protein has been used as a monitor of functional activation of second- and higher-order neurons in several systems. It has been shown that the c-fos immunohistochemical method is effective for revealing

spinal cord neurons which are activated by a peripheral stimulus (Hunt et al 1987) or for revealing neurons activated by a selected stimulus in other parts of the central nervous system (Sagar 1988). It was also demonstrated that neurons in the nucleus proprius, medial Rexed's lamina V and VI and ventral horn were labelled with c-fos in rats after walking on a rotarod treadmill (Gogas et al 1990, Jasmin et al 1994). For MLR stimulation induced locomotion in cats, this activity-dependent labelling has been applied successfully to locate the neurons in the lumbar spinal cord and the brainstem which are activated (Dai et al 1994).

The c-fos immunohistochemical method has some advantages over other tracing techniques such as 2-deoxyglucose autoradiography (2-DG) and horseradish peroxidase conjugated with wheat germ agglutinin (WGA-HRP). It gives single cell resolution and it potentially localizes all interneurons activated during certain manipulations rather than only the last order interneurons, as in the case with WGA-HRP activity-dependent labelling. Comparisons of patterns of 2-DG uptake with Fos-like immunoreactivity have shown overlap, but there is not always an identical distribution (Sagar et al 1988). One of the contributing factors may be that autoradiographs prepared from tissue treated with 2-DG provide a measure of glucose utilization throughout the cell and may be more sensitive to alternations in functional activity in neuropil than in cell bodies (Sagar et al 1988). Another possibility suggested by Morgan and Curran (1989) is that induction of Fos expression requires the integration of various stimuli and intracellular signalling pathways, which may or may not be associated with increased metabolic activity. Therefore, Fos expression provides a unique window on neuronal physiology.

It is known that cell networks which produce rhythmic locomotor movements are located within the spinal cord. In almost all species, these movements can be

generated in the absence of afferent input (Delcomyn et al 1980), and the neuronal networks have been termed central pattern generators (CPGs). This problem has been studied in a number of different systems, with different levels of analysis. An alternative approach is to screen for endogenous chemical substances that modulate the CPG when applied in a bath or iontophoresed. Furthermore, the morphology and ultrastructure of the neurons that use these compounds to affect the CPG can be studied with immunocytochemical and histochemical procedures. It has been shown that some of them may normally act as triggers to turn on CPG activity; whereas others appear to have a more general role as gain-setters or enablers to prepare the CPG for activation by other neuronal inputs (Harris-Warrick RM 1988). Thus, determining the locations and transmitters released by spinal neurons involved in CPGs is a necessary step toward understanding the mechanism of locomotion and for development of pharmacological strategies for restoration of locomotion after spinal injury. So far, considerable detail about the effects of excitatory amino acids on cellular and network mechanisms in generating motor programs has been generated in invertebrates and in some simple vertebrates (Roberts et al 1986, Harris-warrick RM 1988, Grillner and Matsushima 1991). Howerver, much less is known about the effects of other transmitters on motor CPGs, especially in mammals. As introduced earlier, a system of cholinergic neurons active during locomotion may be involved in interlimb coordination and play an important role in the control of locomotion. Also we know immunohistochemical procedure for choline acetyltransferase, the most reliable way of localizing cholinergic systems, has been combined with c-fos activity-dependent labelling in order to study a sub-population of cholinergic interneurons which are active during MLR-induced locomotion in cats was the focus of the present study.

The experiments described in this thesis were designed to provide a detailed map of the locations of cholinergic cells in the spinal cord which are active during locomotion, and will form the basis for future electrophysiological intracellular experiments to functionally identify these cells. To this end we used precollicular postmammillary decerebrate cats. With this preparation the experimenter can turn locomotion on and off at will in a reasonably reproducible fashion by stimulating the MLR in the midbrain, and can be assured that spontaneous locomotion will not occur.A double labelling method was used to identify spinal interneurons which were labelled with the activity-dependent marker c-fos as a result of a locomotion task (fictive locomotion) and were also positive for choline-acetyltransferase. These cells and their targets are likely to represent the cholinergic substrate for the control of locomotion. Future electrophysiological intracellular recording coupled with injections of a fluorescent dextran-amine will be carried out to impale and trace cholinergic cells localized in these experiments to determine their targets and receptors.

METHODS

The experiments were carried out on 6 adult cats weighing 1.8 to 2.6 kg. The data was obtained from precollicular postmamillary decerebrate cats, similar to a preparation described previously (Jordan et al 1979). The animals were subjected to intubation, hindlimb nerve dissections, decerebration, and either fictive locomotion (locomotion experiments) or no brainstem stimulue (control experiments). All animals were perfused at the end of experiment. The detailed procedures are described below.

Surgery preparations and production of locomotion

Animals were initially anaesthetized with a mixture of nitrous oxide and halothane through a face mask. The trachea was intubated for direct administration of the anaesthetic and later for connection with a respiratory pump after muscle paralysis. The left common carotid artery was cannulated and connected to a pressure transducer for blood pressure monitoring. The right common carotid artery was dissected free from the surrounding tissue and a ligature was looped around it in order to permit temporary occlusion of the artery during decerebration. The right jugular vein was cannulated for all drugs and fluid administration. A 5% glucose-sodium bicarbonate buffer solution was infused to replace fluid loss and help maintain a normal pH balance in the animals through the experiments. Each animal was given 2 mg dexamethasone (Hexadrol phosphate, organon) intravenously to reduce tissue swelling. Animal body temperature was monitored via an esophageal temperature probe and maintained at 36-38°C by a heating pad underneath the surgery table and a heating lamp over the animal during the surgery.

In order to monitor fictive locomotion in paralysed cats (3 experiments), various muscle nerves were dissected to monitor motor activity. The nerve to the sartorius (SA) muscle in the left and right hindlimbs were inserted into recording nerve cuffs. The sciatic nerves were placed in custom-made boats filled with mineral oil. The anterior biceps (AB), semimembranosus(SM), posterior biceps (PB), semitendinosus (ST) branches of the sciatic nerve in both hindlimbs were placed on bipolar electrodes.

Animals were transferred from the surgical table to a spinal frame. They were placed in the frame with legs pendant and were supported by two vertebral clamps and pins attached to the iliac crests. The head of each animal was fixed in a stereotaxic headholder. A precollicular, postmammillary decerebration was performed in all of cats. The anaesthesia was subsequently terminated. Up to 5ml of oxypherol (Alpha Therapeutic Corporation), an agent capable of carrying oxygen, was given to compensate for blood lost during decerebration. Dextran 70 (Travenol) was injected after the decerebration if blood pressure did not return to at least 80 mmHg.

After a 1 hour recovery period following the decerebration, animals were paralysed with 1ml (8mg/ml) Flaxedil (Gallamine triethiodide, Rhone-Poulenc) given intravenously. Periodic injections of Flaxedil were used throughout the experiment to keep the animals in a state of flaccid paralysis. Artificial ventilation was adjusted to maintain end tidal CO_2 between 3.5% and 4.5%.

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

Locomotion was induced by electrical stimulation (1.0 ms pulses, 15Hz, 50-160 uA) of the mesencephalic locomotor region and monitored by electroneurograms

(ENGs). The ENGs were amplified, band-pass filtered (30-3000Hz), rectified and integrated, then digitized on a Masscomp computer for analysis.

Because C-fos expression is activated by various stimuli (Morgan and Curran 1989), the surgery itself may induce c-fos expression in the spinal cord. To eliminate fos expression induced by surgery from the analysis, a constant interval of 8-9 hours between decerebration (final surgery) and perfusion was established for all locomotion and control experiments. Within the time length, locomotion was tried to be obtained about 3 hours.

All control animals were subjected to the same amount of surgery as the locomotion test animals, and they were all decerebrated at the precollicular-postmammillary level. These animals were not subject to the locomotor task.

Tissue perfusion and procession

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

Pre-fixative solution (10% saline with $NaNO_2$ and heparin) : Total volume 100ml, 90ml dH_2O, 10ml 9% saline, 0.1g $NaNO_2$, and 10ul heparin. It was used at 0.3ml/g of animal weight. The solution was kept at 4°C. Fixative solution (4% paraformaldehyde-0.2% picric acid) : Total volume 400ml, 16g paraformaldehyde, 80 ml 0.5M phosphate buffer (PH 7.4), 60ml saturated picric acid, and dH_2O. It was used at 1ml/g of animal weight. The solution was kept at 4°C. Sucrose solution (25% sucrose) : Total volume 500ml, 125g sucrose, 50ml glycerol, 50 ml 0.5M phosphate buffer (PH 7.4), and dH_2O. It was kept at 4°C.

The animal was deeply anaesthetized with sodium pentobarbital (30 mg/kg) before perfusion and quickly transferred from the frame to a tray. The thoracic cavity was opened. A needle connected with the perfusion apparatus was inserted into the left ventricle and secured with a haemostat. The inferior vena cava was cut to allow blood and perfusion fluid out. The perfusion apparatus was then turned on carrying pre-fixative solution, followed by fixative solution. Perfusion pressure was kept at 80mmHg.

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

A sliding microtome was used for tissue cutting. The spinal cord segments were sectioned coronally. Tissue sections of 20-30 um thickness were collected in 0.1 phosphate buffer solution (PBS). There were 25 sections collected from each spinal segment. These sections were collected in even distribution from the rostral to the caudal end of each spinal segment.

Solutions and antibodies used:

Solution A: 0.2M PBS-0.3% TritonX (t-octylphenoxypoly-ethoxyethanol)-2% BSA (Albumin, Bovine). This solution was used as a base for incubation of primary antibody.

Solution B: 0.1M PBS - 0.3% TritonX - 2% BSA. This solution was used as a base for incubations of secondary and third antibodies.

Solution C: 0.1M PBS - 0.3% TritonX. This solution was for a rinse, in which non-specific binding would be washed out.

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

Antibody 1: Anti-c-fos antibody (Cambridge research biochemicals inc., sheep polyclonal)

Antibody 2: Anti-choline acetyltransferase antibody (Chemicon' International Inc.,rabbit polyclonal)

Antibody 3: Anti sheep/goat Ig, biotinylated antibody (Amersham international plc)

Antibody 4: Streptavidan Cy3 (Sigma)

Antibody 5: Goat anti-rabbit IgG (Sigma)

Antibody 6: Rabbit PAP complex (Sigma)

For immunohistochemical double staining, c-fos immunoreactivity was demonstrated first by an indirect fluorescence method. Free-floating sections were transferred sequentially by the following procedures:

Day 1: Tissue sections were put in solution C overnight.

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

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

Secondary antibody incubation. Concentration was 1:100=antibody 3:solution B. Incubation lasted 1.5 hours on a shaker at room temperature. Then, the tissue was washed with solution C twice, 30 minutes each time on a shaker. Third antibody incubation. Concentration was 1:100=antibody 4: solution B. Incubation

lasted 1.5 hours on a shaker at room temperature. Following, the tissue was washed with solution C, 30 minutes on a shaker and with solution D, 30 minutes on a shaker.

All tissue was mounted onto gelatinized slides from 50mM Tris-Hcl buffer, dried 15 minutes at room temperature and coverslipped with a glycerol-based anti-fade medium (ref). Sections were viewed with a Nikon optiphot, labophot combination light/epifluorescence microscope equipped with an X/Y movement sensitive stage and CCD video camera attached to an IBM PC computer. An Neurolucida image analysis system (MicroBrightField Inc USA) made it possible to draw outlines of sections at low magnification and to plot c-fos +neurons within these drawings at high magnification.

Subsequently, ChAT immunoreactivity was visualized in the same sections using the peroxidase-autiperoxidase (PAP) method. The coverslips were carefully removed by dropping solution D onto the slides. The slides were then washed twice in solution C, then incubated as follows:

a) rabbit anti-ChAT antibody incubation (1:1000 in solution A, 3 days in 4°C room).

b) Goat anti-rabbit IgG (1:100 in solution B, 1.5 hours)

c) Rabbit PAP complex (1:100 in solution B, 1.5 hours)

After each incubation step, the tissue sections were washed twice in solution C. Last step, the tissue was immersed into the solution for staining with DAB (3,3-diaminobenyidine tetrahydrochloride):

100ml solution D

20mg DAB

15ml H_2O_2

H_2O_2 was mixed with other two ingredients just before the tissue sections were immersed. Staining was closely monitored under the microscope. When the staining was done, the tissue sections were transferred to solution D to stop further staining. After DAB, tissue slides were dried completely (overnight),and cover-slipped with a glyceral-based anti-fade medium .

The ChAT-positive cells were analyzed using the Neurolucida program and photographed again with bright-field illumination.

The intensity of both c-fos and ChAT cell labelling was variable, with brightly and faintly stained cells intermingled. We only plotted intensively stained cells. This method is likely to underestimate the number of active cells. Distribution of double labelled cells was determined by adding all ChAT-/C-fos positive cells in 18 sections (FL-cat1), 21 sections (FL-cat2) and 19 sections (FL-cat3).

Statistics: statistical significance was calculated by student's t test on data about difference of labelled cells between locomotor and control tissue.

RESULTS

Slides were examined and photographed with a Nikon optiphot, labophot combination light/epifluorescence microscope. Double labelling of c-fos and ChAT-immunoreactivity in the same cell was determined by alternate observation of the same area of the sections using the appropriate filter cube for visualization of CY3 fluorophore and the bright-field illumination for PAP. A Neurolucida image analysis system was employed to plot both c-fos and ChAT-positive cells. Only those neurons which obviously contained reaction product in the nucleus were considered to be labelled for c-fos, while ChAT-immunoreactivity appears in the cytoplasm.

The quality of the labelling is depicted in Fig.1A,B,C and D. They are high-power (X400) photomicrographs. Every pair shows the same field in sections double-labelled by immunofluorescence for c-fos (A, C) and by immunoperoxidase for ChAT (B, D). They also illustrate intensity difference between labelled and unlabelled cells. Double-labelled cells in A,B were located in ventral lamina VII or lamina IX; those in C,D were located in lamina VII. Another photomicrograph group shows the difference of c-fos and ChAT labelled cells between locomotor and control cats (Fig.2).

Distribution of labelled cells in the spinal cord

Bouts of fictive locomotion were evoked by MLR stimulation. Good locomotion was recorded in three fictive locomotion cats 1 (FL-cat1), 2 (FL-cat2) and 3 (FL-cat3). Total time was recorded was 2 hours 55 minutes in FL-cat1, 3 hours 05 minutes in FL-cat2 and 3 hours 12 minutes in FL-cat3 . as illustrated in Fig.3.

As previously reported, extended periods of fictive locomotion resulted in a characteristic distribution of c-fos-immunoreactive neurons among spinal lamina in

the cat lumber cord. Immunoreactive cells were most heavily concentrated in medial lamina VI, VII and in lamina VIII and X, while occasional c-fos-positive neurons were observed in lamina I to V of the dorsal horn and in the reticulate grey matter of lateral lamina V and VI.

The appearance and distribution of ChAT-positive neurons in transverse sections of lumbar spinal cord (Fig 1B,D) were very similar to those described in detail by Barber et al (1984). Stained neurons were present throughout the spinal cord and were particularly concentrated in the ventral horn, central grey matter and intermediate grey matter. Some of them were scattered in the dorsal horn (lamina III-V). The density of neuronal staining in different regions was not uniform: many cells in lamina VIII, IX, in medial part (lamina VII) and around the central canal were intensely stained, while cells in the dorsal horn were usually more lightly stained. When these sections from the lumbar spinal cord were incubated with both c-fos and ChAT antibodies, some c-fos immunoreactive neurons were found to be ChAT-positive. In FL-cat1, 24.35% of c-fos positive neurons were ChAT positive; in FL-cat2, 15.86% of c-fos positive neurons were ChAT positive; in FL-cat3, 18.89% of c-fos positive neurons were ChAT positive (Fig.4). The distributions of the double labelled cells in each preparation is illustrated in Fig.5,6.7. Maps of labelled cells were derived from Neurolucida drawings, as described in the Method section. Most of double labelled neurons were distributed among the deeper lamina with the majority of c-fos / ChAT-positive cells situated in lamina VII and VIII. There was also a considerable amount of double labelled cells in the central part of the intermediate grey matter around the central canal.(Fig.8). Some labelling was observed in large neurons in the ventral horn, suggesting that motoneurons may be labelled using our staining protocol. Differentiation between putative motoneurons

and other spinal neurons was based on soma size and lamina location alone and was not considered unequivocal. In general, very few double labelled cells were found in lamina I-III. The locations of double labelled cells were similar in the three locomotor cats.

Difference of labelled cells between locomotor and control cats

Three animals were used as controls for fictive locomotion. They were subjected to the same surgical procedures as the fictive locomotion animals, including the nerve dissection and paralysis with flaxedil, and artificially ventilated following the decerebration. Figure 2 shows an example of photomicrographes taken from the spinal cords of control (C,D) and locomotor task (A,B) animals. Both pairs of sections processed for c-fos (A,C) by immunofluorescence and for ChAT (B,D) by immunoperoxidase. These low-power (X200) micrographs illustrate that the locomotor task produces clear c-fos nuclear labelling which is not evident in the control spinal cord. The number of ChAT-positive cells in cats subjected to the locomotor task is greater than that in control animals. In the three locomotor preparations, the total number of cells (c-fos, ChAT, double labelling) was increased compared with the total cell count in the three control preparations (Table II). The number of c-fos and double labelled cells in spinal segments (L_3-S_1) in locomotor cats is much higher than that in controls (Table I and Fig.9,10,11). The difference is statistically significant (student's test, $p < 0.01$). The proportion of double or c-fos labelled cells was much greater in locomotor cats compared to controls (Table 1). The difference of double labelled cells was ranged between 48 and 92% ; the difference of c-fos labelled cells was ranged between 39 and 78% (Table 1). These results confirm the previous report showing the location of interneurons in the

lumbar spinal cord involved in the production of locomotion (Dai et al 1994). The increase in the number of double labelled cells is most likely due to both increases of c-fos and ChAT cells in sections from lumbar segments of animals subjected to locomotor trials.

The proportion of ChAT-labelled cells was also greater in locomotor cats compared to controls (Table 1). The difference was ranged from 3 to 48% (Table 1). segments the number of ChAT labelled cells as a whole was increased compared with controls, but in two segments (S_1 in FL-cat2, L_5 in FL-cat3) ChAT-labelled cells decreased 5% and 9%. In two thirds of the segments, the difference is significant ($p < 0.01$, $p < 0.05$). These changes between locomotor and control preparations can also be seen in Table I and Fig.9,10,11. The increase was marked in the intermediate grey matter (lamina VII, VIII) (Fig.12). These regions are almost consistent with the reported territory of partition cells (Barber et al 1984).

It appears from these experiments that fictive locomotion resulted in a characteristic distribution of c-fos positive neurons among spinal lamina in the cat lumbar cord. ChAT positive cells are also clearly active during locomotion. Approximately a fourth to a third of the cells which express the activity-dependent marker c-fos as a result of fictive locomotion are also positive for ChAT. An obvious change of the number of ChAT cells in spinal lumbar cord between locomotor and control preparations was observed.

DISCUSSION

These experiments provide a first attempt to localize cholinergic interneurons which may be engaged in spinal locomotor generation in the adult decerebrate cat. The most striking information that we have obtained relating to locomotor generation is a distribution of ChAT/c-fos-positive neurons in the spinal cord. We have also shown an increase in the number of ChAT-positive neurons following fictive locomotion. These results will first be discussed individually, and then some general observations and speculations will be made in light of previous findings by other investigators.

As previously reported, extended periods of fictive locomotion in the decerebrate cat preparation result in a characteristic distribution of c-fos-positive neurons in the lumbar spinal cord (Dai et al 1994). Although this preliminary report does not purport to be a conclusive quantitative study of the cytochemistry of a group of physiologically-identified spinal neurons, it describes certain aspects of the neurochemical heterogeneity of the c-fos labelled locomotor-related neurons. ChAT-positive cells are also clearly active during locomotion. Subpopulations of c-fos-positive neurons were seen to contain ChAT-positive reaction product. These double labelled cells displayed a scattered distribution extending to ventral lamina VII, VIII and possibly IX. Most of those are located in the medial portion of lamina VII close to lamina X and seemed to be more numerous than in the lateral portion. We suggest that these cells are involved in the generation of locomotion. As mentioned in the results the putative identification of motoneurons was based solely on laminar location and somal size. Using these limited identification criteria it appears that only a small subpopulation of large, ventrally located neurons were c-fos positive.

It is well known that many interneurons which mediate reflex effects on the motor nuclei are located in lamina VII. There are three types of identified interneuron which are found in the ventral part of lamina VII adjacent to the motor nuclei which includes Ia reciprocal interneurons, Renshaw cells and a population of interneurons of lumbar segments which receive inputs from group II muscle afferents and from other muscle, skin and joint afferents, and project to motor nuclei (Jankowsk and Lindstrom 1971, 1972, Edgley and Jankowska 1987a, 1987b, Edgley et al 1988). All of them have been shown to be rhythmically active during MLR-induced locomotion (McCrea et al 1980, Pratt and Jordan 1987, Noga et al 1987, Shefchyk et al 1990) and display phasic activity during the step cycle which is tightly coupled to the extensor or flexor neurogram.

Characteristically, there is a significant proportion of commissural interneurons in lamina VIII that have been shown to project to contralateral motor nuclei by means of WGA-HRP injections into a muscle nerve (Harrison et al 1986). These cells may be involved in crossed extension reflexes (Grillner and Hongo 1972) or else mediate excitation or inhibition of contralateral motoneurons evoked by the vestibulo- and reticulospinal tracts (Sasaki et al 1962, Hongo et al 1975). These lamina VIII neurons receive a strong input from the descending locomotor command pathway. Their axons cross the midline and provide collateral branches that terminate in the contralateral grey matter (Noga et al 1987, Jankowska and Noga 1990). Also, they appear to be involved in the first rhythmic activity which appears during development of the fetal rat (Kudo et al 1991).

Investigations using various activity markers confirm these areas to be important for generating spinal locomotion. In the rabbit the uptake of 2-deoxyglucose during L-B-3,4-dihydroxyphenylalanine (L-DOPA)-induced fictive

locomotion was found to be localized in cells in the intermediate grey (Viala et al 1988), while in locomoting deafferented cats c-fos was detected in cells in the intermediate grey and area X (Dai et al 1994). Isopotential field studies in the cat have also identified these areas as potential network areas (Jordan 1991, Noga et al 1994). In addition, studies using extracellular recordings have revealed rhythmically active cells in the intermediate grey during fictive locomotion (Orlovsky and Feldman 1972, Jordan 1991). Recent experiments using optical imaging and ablation have also pointed toward ventral and intermediate regions as potential locomotor generators in the chick embryo spinal cord (O'Donovan et al 1992). In agreement with previous reports, motoneurones have been proved difficult to label both with the c-fos and 2-deoxyglucose methods (Viala et al 1988, Barajon et al 1992, Dai et al 1994).

Interestingly, previous studies regarding cholinergic elements in the spinal cord have identified partition and central canal cluster cells which are located in the intermediate grey and the area around the central canal (Barber et al 1984, Borges and Iversen 1986). Partition neurons form a ChAT-positive network across the intermediate spinal region and may make propriospinal connections. Central canal cells in lamina X project to dorsal, intermediate and ventral grey matter and have been proposed to provide integration between the sensory, motor and autonomic segments of the spinal cord (Borges and Iversen 1986). Both partition and central canal neurons contribute to the intraspinal cholinergic circuitry (Barber et al 1984, Borges and Iversen 1986) which forms the main cholinergic innervation of the spinal cord and modulates the transmission of information in the spinal cord (Borges and Iversen 1986). It has been proposed that these widespread intersegmental connections may mediate central motor programs for locomotor functions such as walking (Woolf 1991).

The results obtained in this study confirm and extend the previous finding that lamina VII and lamina VIII cells are involved in locomotion. It is noted that many of c-fos / ChAT labelled cells are concentrated within the area of partition cells (Fig.5,6,7). In this area, the number of ChAT-positive cells showed the largest relative increase, as compared to dorsal horn and ventral horn cells, during fictive locomotion. Using the intracellular staining technique with tetrametlylrhodamine-dextran as a tracer in combination with ChAT immunocytochemistry for identifying locomotor-related spinal neurons, suggests that many of these cells are cholinergic and some of these cells terminate on contralateral motoneurons (Carr et al 1994). Retrograde transport of fluorescent microphores injected into selected motor nuclei has revealed that commissural cells which project to motoneurons overlap the area where partition cells and cluster cells are located (Hoover and Durkovic 1992). Partition cells send axons into the motoneuron pools (Phelps et al 1984). Immunohistochemical and electron microscopic methods have shown the terminals of cholinergic fibers onto motoneurons (Nagy et al 1993, Li et al 1994). Thus, the double labelled cells in the present study may correspond to these cholinergic "partition cells" and central canal "cluster cells". Likewise, some of them may represent cholinergic commissural neurons.

Spinal cholinergic neurons are of considerable interest in the investigation of locomotion. Initial studies of locomotion using in vitro rat preparations suggested a possible role for cholinergic mechanisms in the induction of rhythmic spinal cord activity (Smith and Feldman 1987, Smith et al 1988). A recent study of the Xenopus spinal cord activity also implicated cholinergic mechanisms in the operation of the central pattern generator (Panchin et al 1991). Using pharmacological methods, stimulation of cholinergic receptors in the neonatal rat cord was shown to produce

ipsilateral synchronous flexor and extensor bursts with alternation between the two hind limbs (Cowley and Schmidt 1994). This indicates that cholinergic neurons may be involved in interlimb coordination. We speculate that a system of cholinergic commissural and propriospinal cells plays a prominent role in the generation of rhythmic activity in the spinal cord.

From the experiments performed in this study, at least one thing seems clear. In the adult decerebrate cat, cholinergic interneurons in lumbar spinal cord are involved in the production of fictive locomotion. Since our window on spinal cholinergic cell activity is ChAT/c-fos-immunoreactive, it is difficult to draw more specific conclusions than this. It is impossible to know which cells are labelled due to activation by directly descending inputs from the brain stem or by interactions with other activated interneurons. Likewise,we cannot determined which type of cholinergic receptor is activated during these experiments nor the effect of cholinergic activity, is on the functions of the central pattern generator for locomotion.

Another possible explanation of the increase in the number of ChAT cells during fictive locomotion is the effects of neuronal depolarization. It is noted that neuronal depolarization can influence ChAT activity levels and therefore ACh production. The neurons treated with depolarizing agents or stimulated directly by electric current tended to display an adrenergic character, which causes an increase in ChAT enzyme activity (Walicke et al 1977, Ishida and Deguchi 1983). Nishi and Berg (1981) reported that a high KCl concentration enhanced ChAT activity, protein synthesis, and other activities in neurons cultured from chick ciliary ganglion, from mouse spinal cord. The increase of ChAT activity was blocked by Ca^{2+} antagonists (Ishida and Deguchi 1983). Depolarization by high concentration of KCl increased

the number of surviving neurons and stimulated the differentiation of cerebellar neurons (Lasber and Zagon 1972), dorsal root ganglion neurons (Scatt 1977, Chalazonitis and Fischbach 1980), sympathetic neurons (Philipson and Sandler 1975), and ciliary ganglion in cultures (Bennett and White 1979, Nishi and Berg 1981). The effect of depolarization on ChAT activity could be due to a selective survival of cholinergic neurons or a general stimulation of protein synthesis in ChAT-producing cells. In addition to typical rhythmic oscillations in motoneurons during locomotion, all of the interneurons which are rhythmic active undergo depolarizing and hyperpolarzing membrane potential oscillations similar to the locomotor drive potentials described in alpha motoneurons. They fire action potentials during the time of activity in the ipsilateral hindlimb flexor neurogram (Shefchyk et al 1990). With extracellular recording, it has been observed that the Ia inhibitory interneurons and the Renshaw cells displayed phasic activity during fictive step cycle which is tightly coupled to the particular extensor or flexor motoneuron population with which they were associated (McCrea et al 1980, Pratt and Jordan 1987). In investigations of the effect of excitatory amino acid on locomotion, interneurons making EAA-dependant synapse on motoneurons have been shown to have rhythmic "motoneuron-like" activity themselves, and appear to receive similar EAA-mediated synaptic inputs to those received by motoneurons (Dale and Roberts 1985). The ventrally located interneurons seem to be excited at short latencies from the MLR and to project to motor nuclei. However, it is less easy to explain the degree of correspondence between the ChAT/c-fos population and the ChAT-positive population in the non-locomoting cat due to the increase in the number of ChAT-positive neurons following fictive locomotion.

Recently, Bausero et al (1993) proposed a possible functional relationship

between c-fos and ChAT by studying the sequence of human ChAT gene promoter. They demonstrated a 4-13 X increase in ChAT activity in cos-1 cells and NE-1-115 neuroblastoma cells following co-transfection with c-fos / c-jun expression vectors and suggested that the ChAT promoter region may be inducible by c-fos /c-jun and thus may be a target for c-fos / c-jun proto-oncogene. This indicates that at least in the system examined, c-fos / c-jun alter ChAT expression/activity. In light of this, the degree of colocalization of c-fos and ChAT in cat spinal cord following locomotor trials may be either 1) a function of the involvement of cholinergic neurons constitutively expressing ChAT that are induced to express c-fos; or 2) a result of the induction of ChAT in cells expressing c-fos. Two possibilities are yet to be determined.

This discussion has raised a number of possible explanations for the results of the experiments in this study. In order to narrow down the list of possibilities, further electrophysiological experiments combined the intracellular injection with a fluorescent dextran-amine would be necessary to functionally identify these ChAT / c-fos-positive cells and define their role in locomotion.

Figure 1

Pairs of photomicrographs showing the same field in sections double-labelled by immunofluorescence for C-Fos (A,C) and by immunoperoxidase for Choline acetyltransferase (B,D). Labelled neurons are pointed by an arrow and unlabelled neurons are pointed by an arrowhead. (X400)

A,B: Cells located in ventral lamina VII or lamina IX.

C,D: Cells located in medial lamina VII.

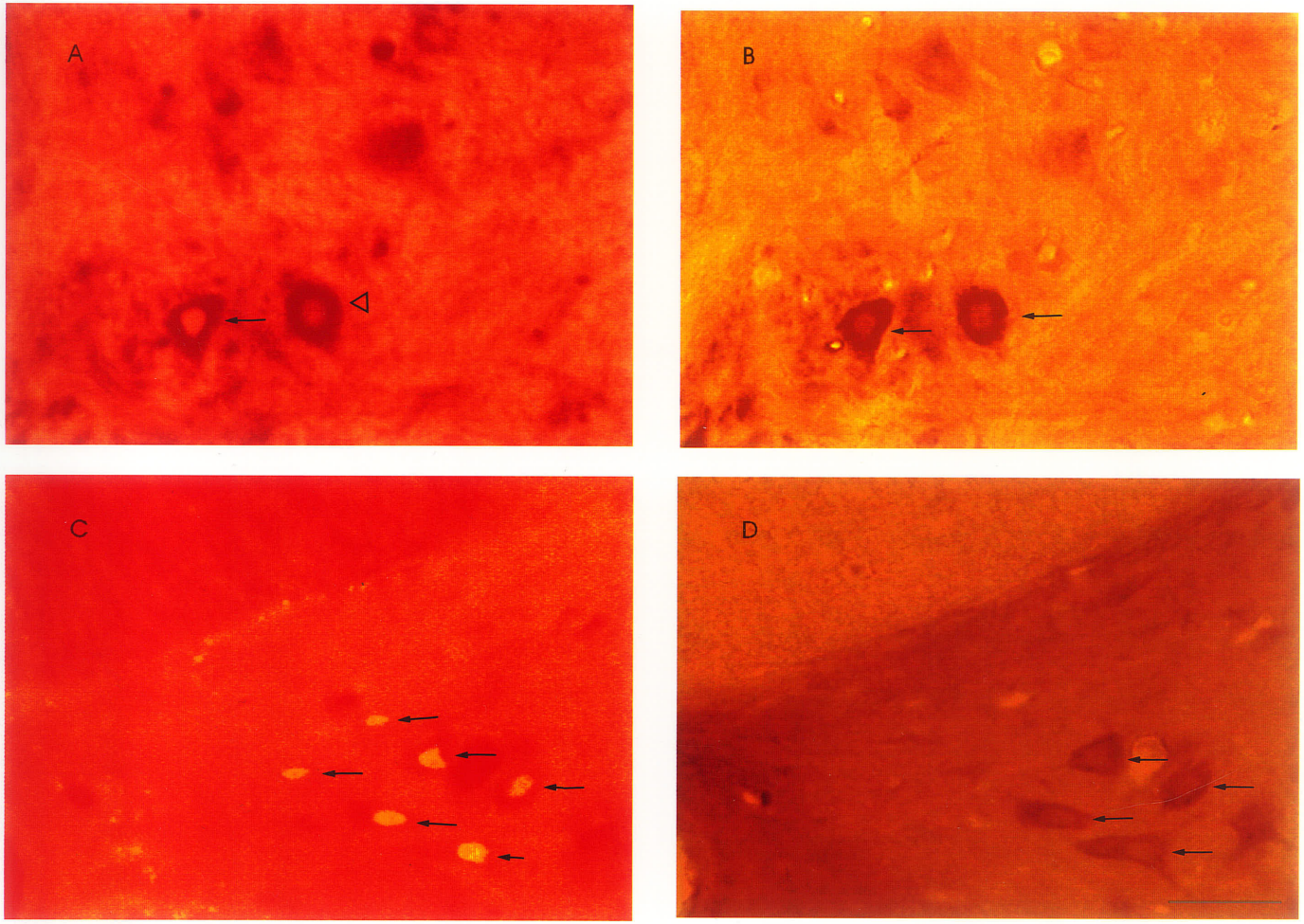

Fig.1

Figure 2

Photomicrographs showing the difference of labelled neurons between Locomotor and Control sections. A,B: The same field of spinal neurons in the Locomotor cat. C,D: The same field of spinal neurons in the Control cat. A,C: Immunofluorecence for C-Fos (arrows in left set of micrographs). B,D: Immunoperoxidase for ChAT (arrows in right set of micrographs). (X200)

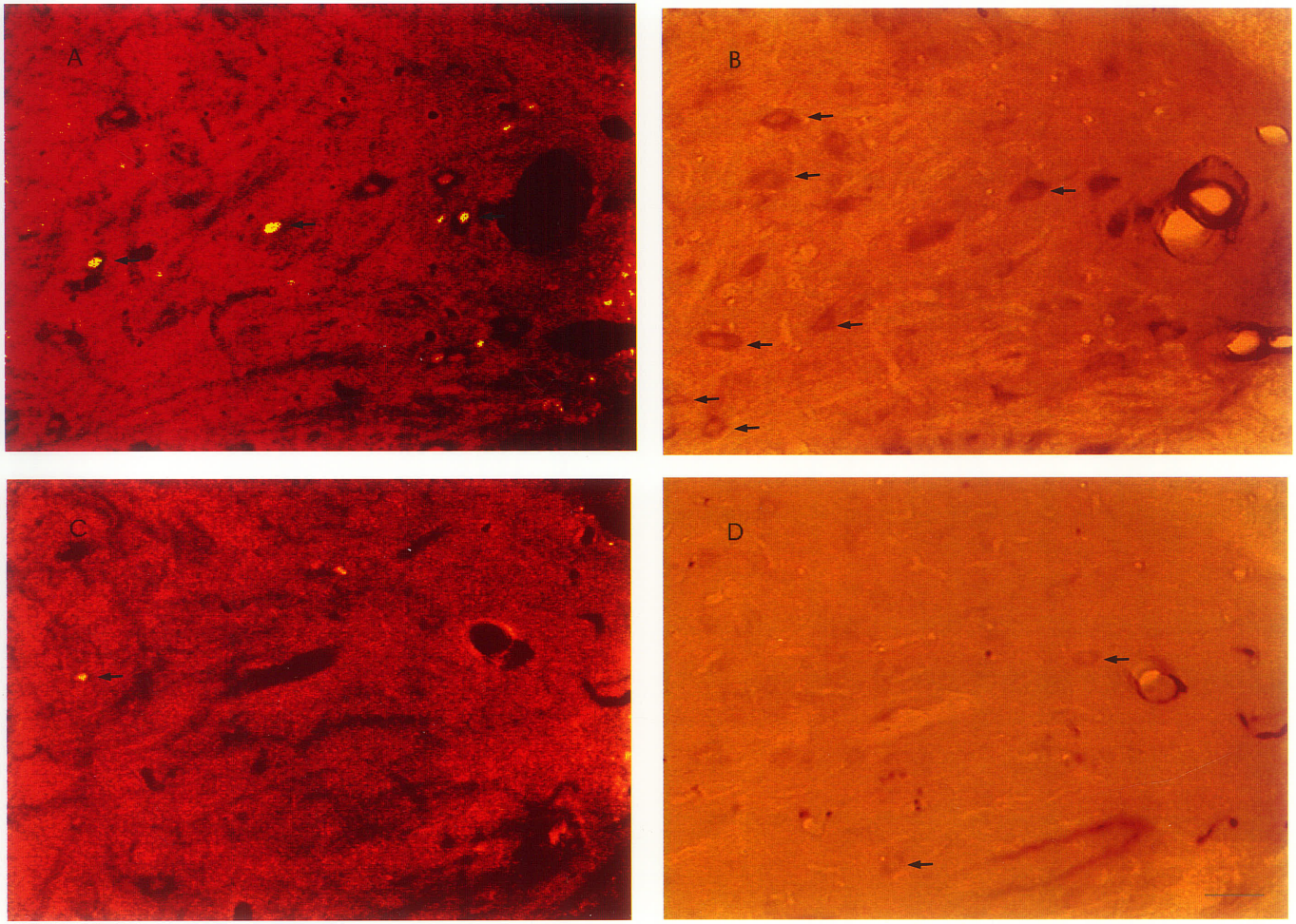

Fig.2

Figure 3

In locomotor cats, ENG bursts in both hindlimbs were elicited with 120uA MLR stimulation. SA-sartorius; SMAB-semimembranosus and anterior biceps; PBST-posterior biceps and semitendinosus.

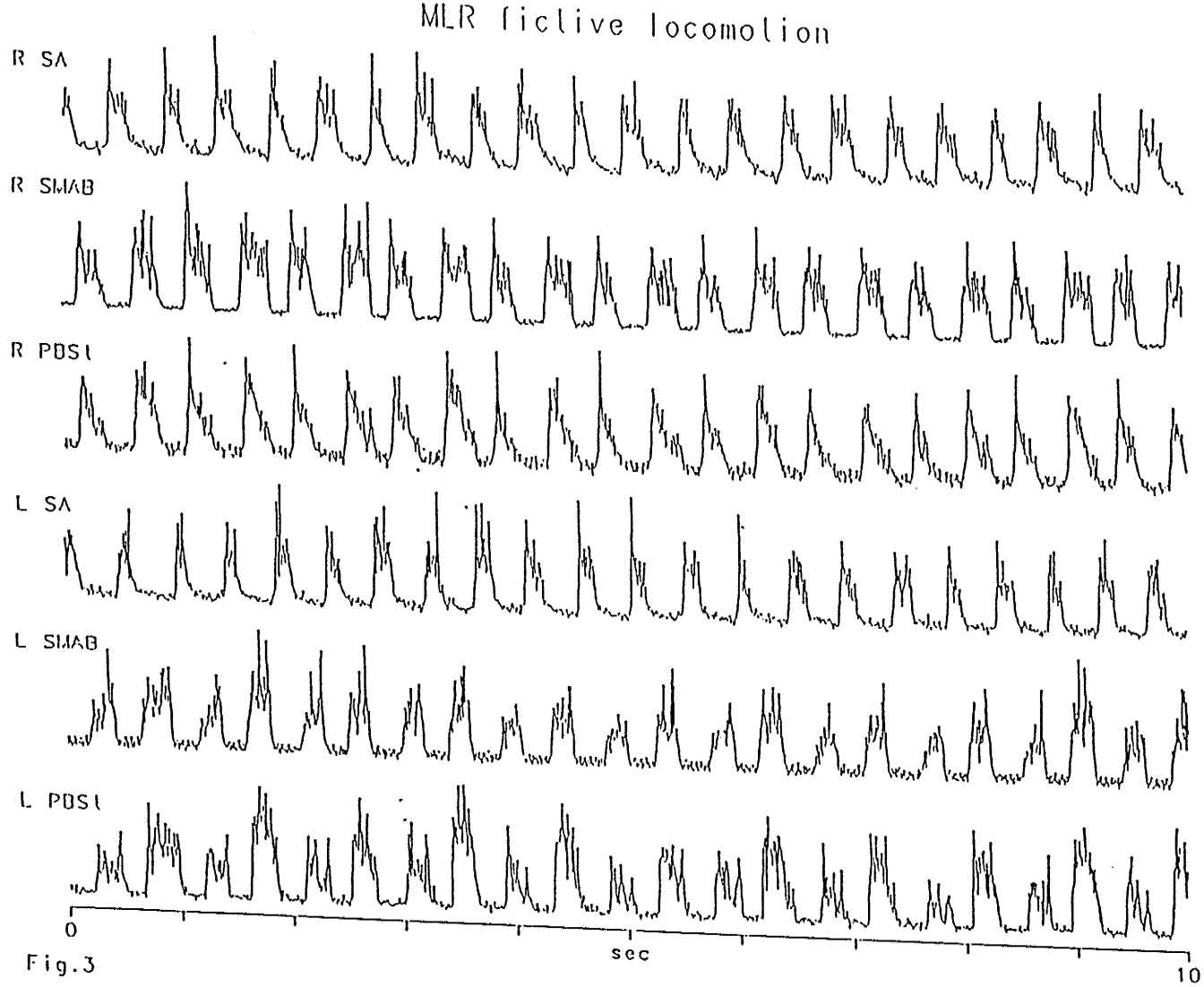
MLR fictive locomotion
R SA
R SMAB
R PDST
L SA
L SMAB
L PDST
0
sec
10

Fig.3

Figure 4

A. Percentage of double labelled cells to C-fos Labelled cells in each Locomotor cat. In FL-cat1, 24.35% of c-fos positive neurons were ChAT positive; In FL-cat2, 15.86% of c-fos positive neurons were ChAT positive; In FL-cat3, 18.89% of c-fos positive neurons were ChAT positive.

B. Percentage of double labelled cells to ChAT labelled cells in each Locomotor cat. In FL-cat1, 1.95% of ChAT positive neurons were c-fos positive; In FL-cat2, 2.13% of ChAT positive neurons were c-fos positive; In FL-cat3, 1.20% of ChAT positive neurons were c-fos positive.

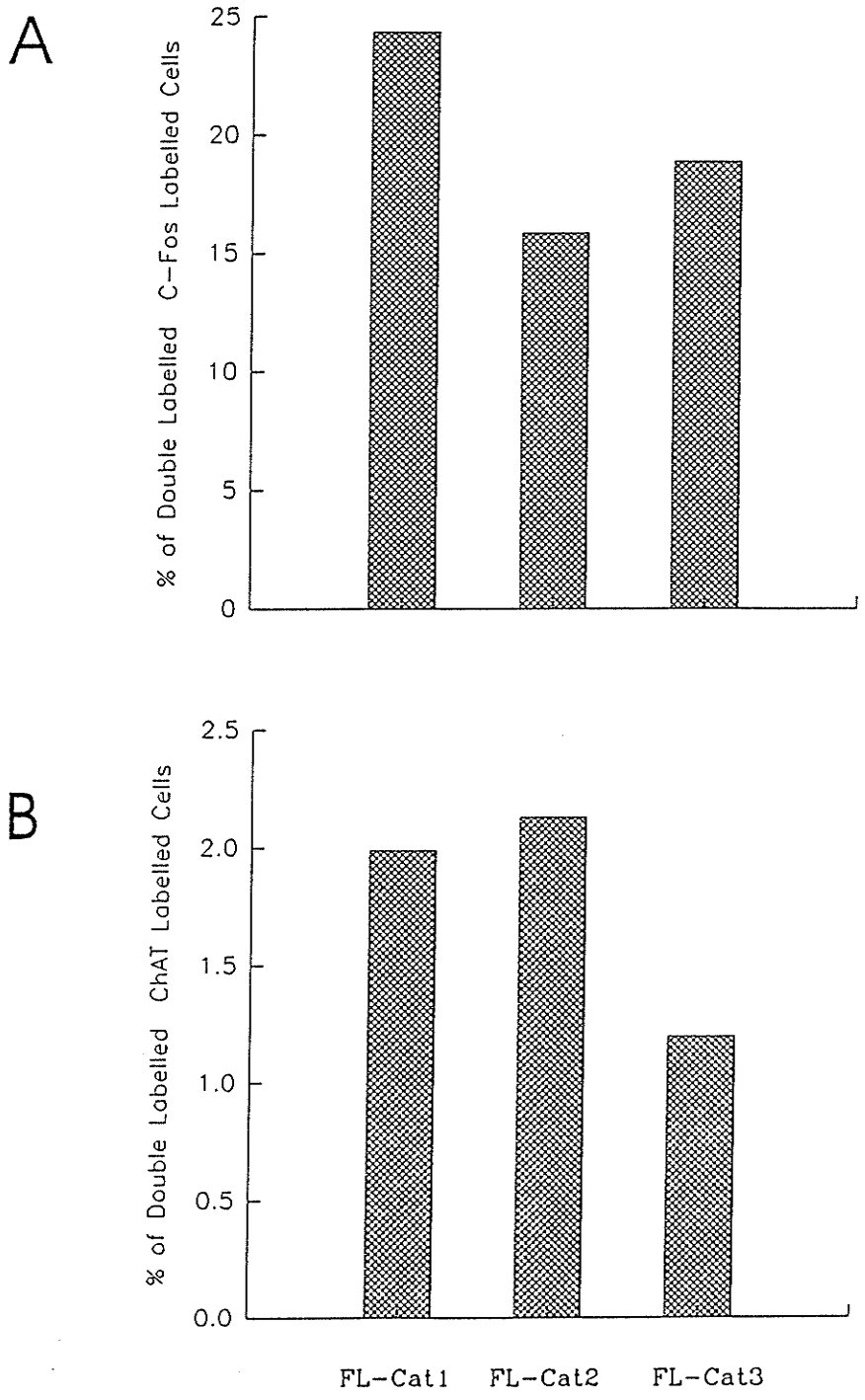
A
25
20
15
10
5
0
% of Double Labelled C-Fos Labelled Cells
B
2.5
2.0
1.5
1.0
0.5
0.0
% of Double Labelled ChAT Labelled Cells
FL-Cat1
FL-Cat2
FL-Cat3

Fig.4

Figure 5

Neurolucida drawings showing the distribution of double labelled neurons (C-Fos and ChAT) in the spinal segments L_3-S_1 in FL-Cat1 (upper panel) and FC-Cat1 (lower panel). Each diagram includes all double labelled cells (excluding ventral motoneurons) in 18 sections of that segment. Each dot represents one double labelled cell.

Location of Double Labelled Cells (C-Fos and ChAT)

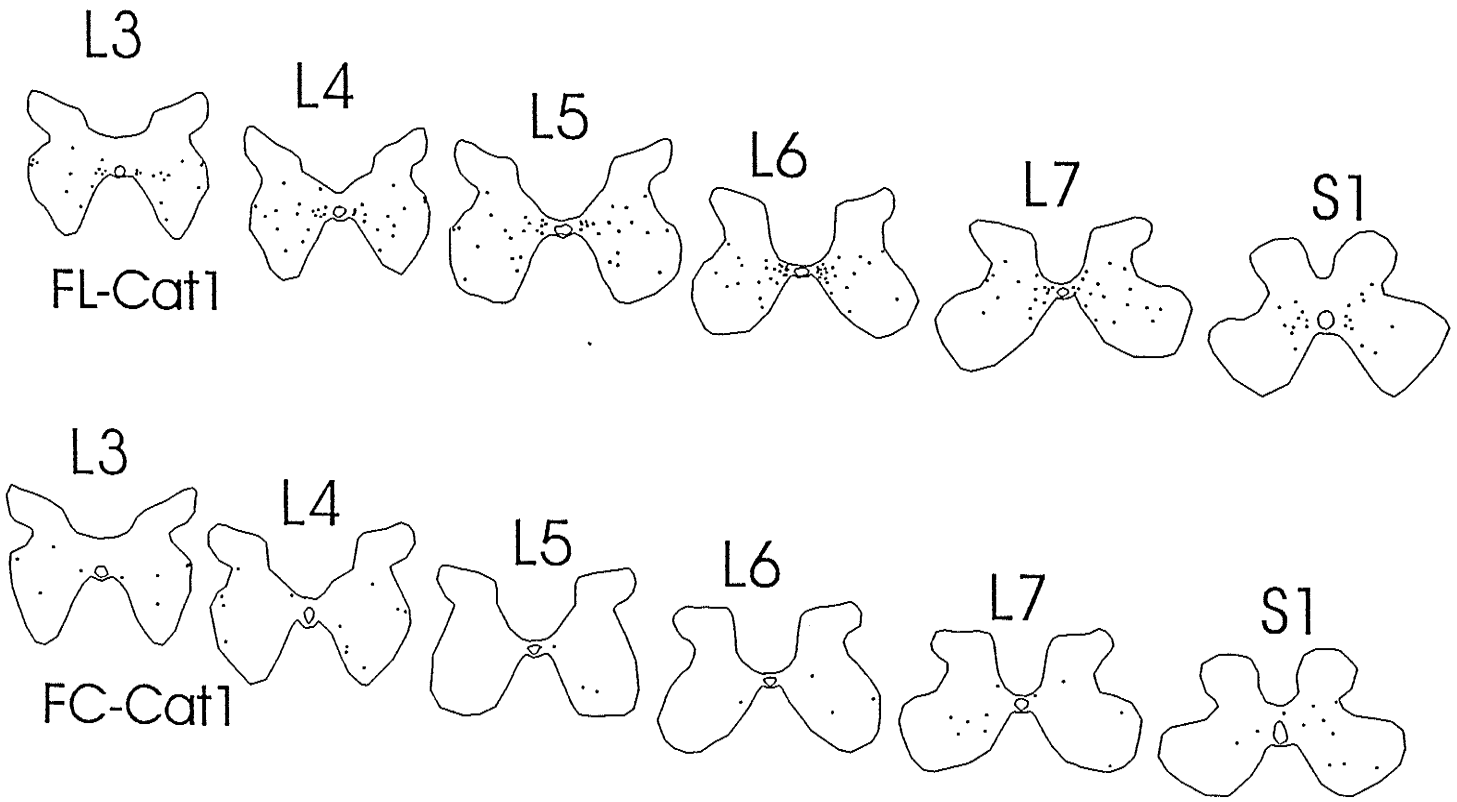

Fig.5

Figure 6

Neurolucida drawings showing the distribution of double labelled neurons (C-Fos and ChAT) in the spinal segments L_3-S_1 in FL-Cat2 (upper panel) and FC-Cat2 (lower panel). Each diagram includes all double labelled cells (excluding ventral motoneurons) in 21 sections of that segment. Each dot represents one double labelled cell.

Location of Double Labelled Cells (C-Fos and ChAT)

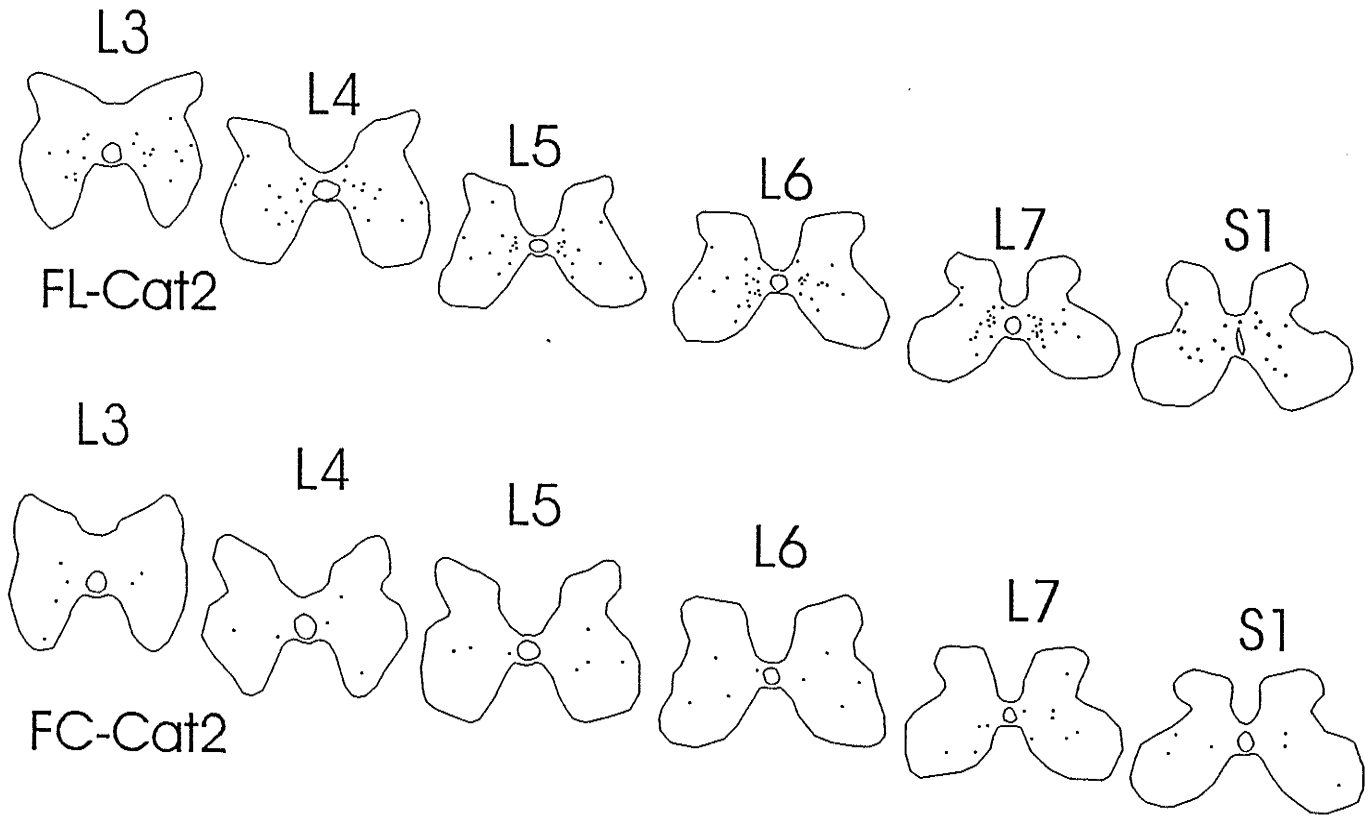

Fig.6

Figure 7

Neurolucida drawings showing the distribution of double labelled neurons (C-Fos and ChAT) in the spinal segments L_3-S_1 in FL-Cat3 (upper panel) and FC-Cat3 (lower panel). Each diagram includes all double labelled cells (excluding ventral motoneurons) in 19 sections of that segment. Each dot represents one double labelled cell.

Location of Double Labelled Cells (C-Fos and ChAT)

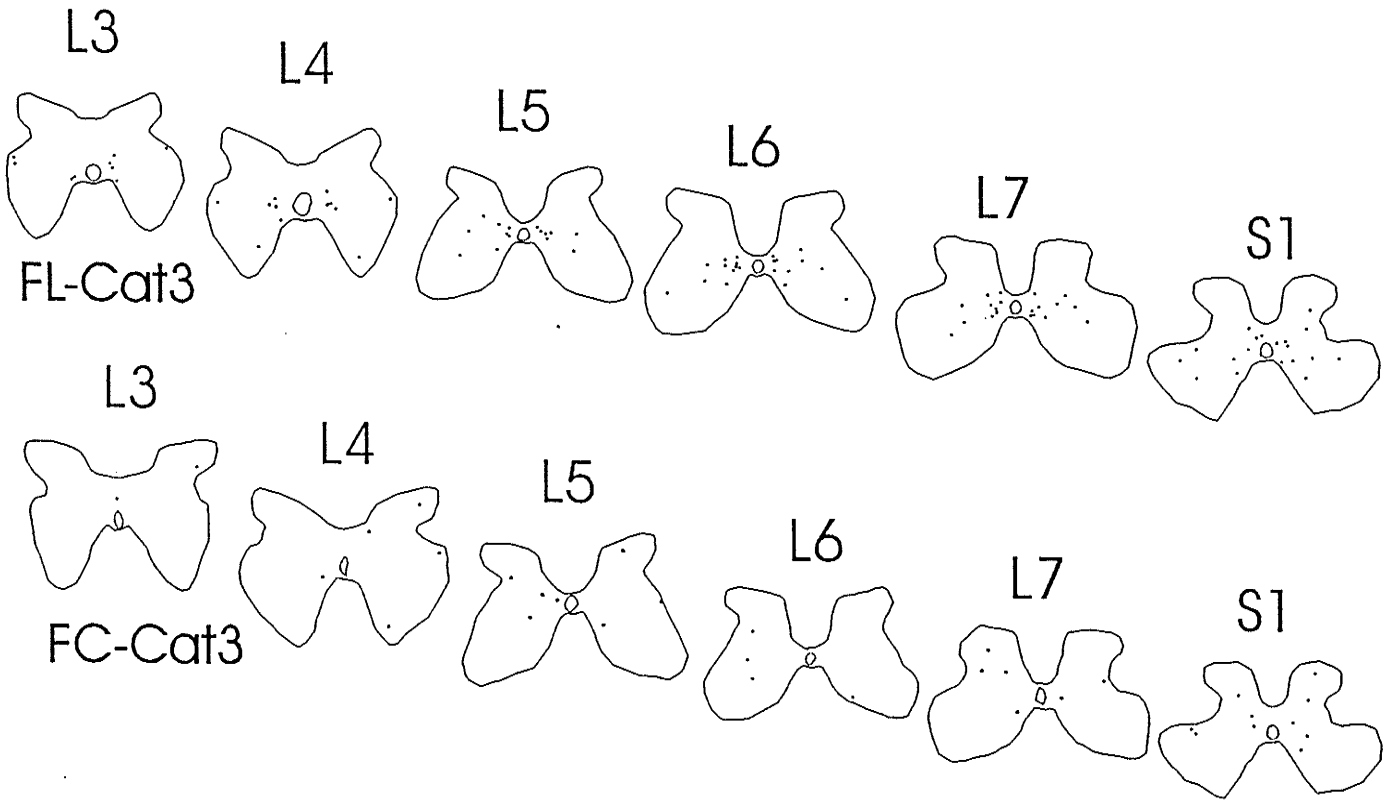

Fig.7

Figure 8

Laminar distribution of double labelled cells in each Locomotor cat. A. FL-Cat1 . B. FL-Cat2 . C. FL-Cat3 .

The calculation was done separately in each cat. The percentage of double labelled cells (bar height) in each lamina was obtained by total numbar of double labelled cells in that lamina from L_3 to S_1 divided by the total number of double labelled cells in the cat.

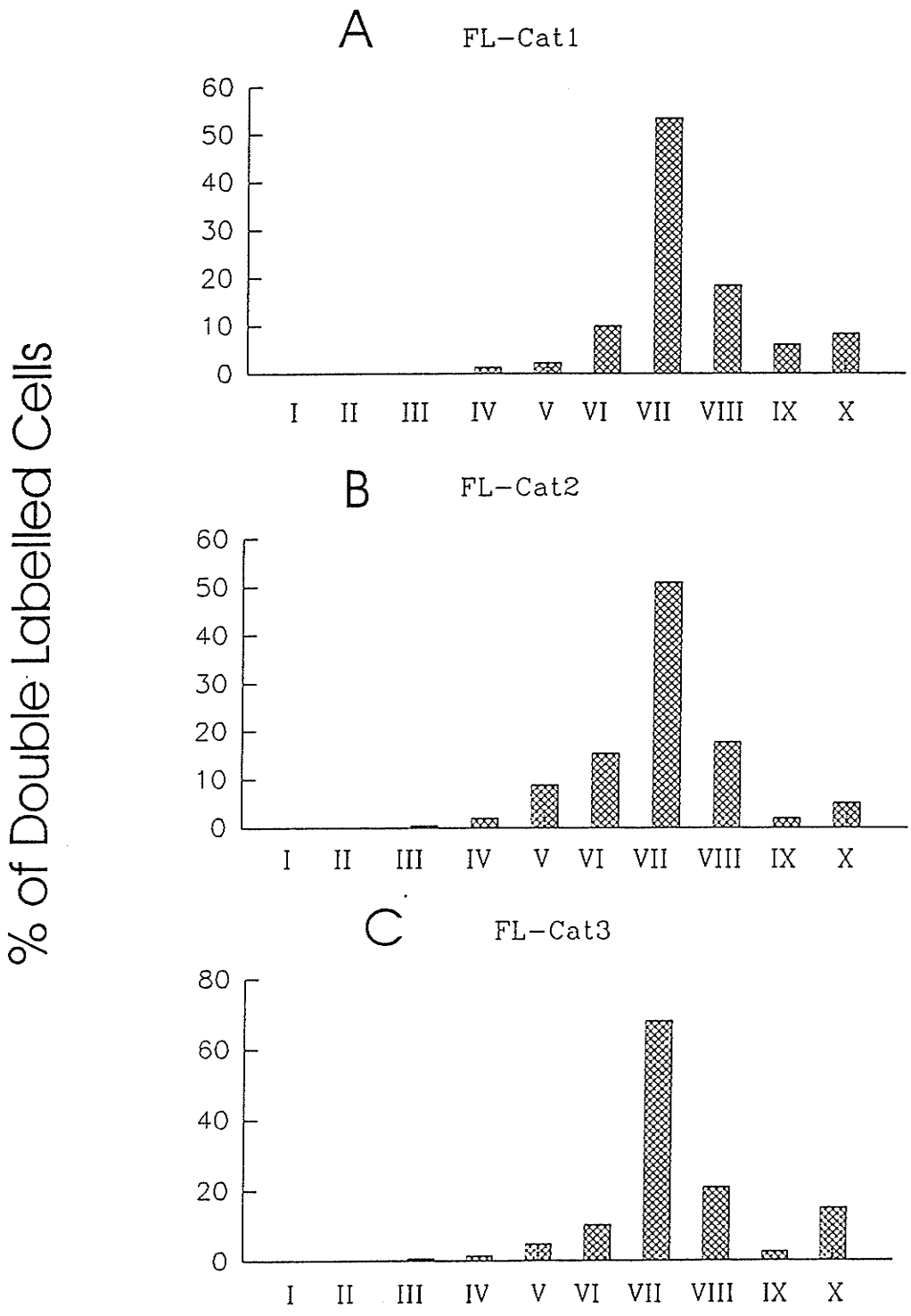
A
FL-Cat1
B
FL-Cat2
C
FL-Cat3
% of Double Labelled Cells
0
10
20
30
40
50
60
80
I
II
III
IV
V
VI
VII
VIII
IX
X
Spinal Laminae

Fig.8

Figure 9

Segmental distribution of labelled cells in FL-Cat1 and FC-Cat1. A. Double labelled cells . B. C-Fos labelled cells . C. ChAT labelled cells .

The calculation was done separately in the cats. Averaged number of labelled cells per section can correct the bias which is induced by uneven number of tissue sections collected during tissue processing.

Note: Averaged number of double labelled cells and C-Fos labelled cells in Locomotor cat is much higher than those in the Control cat. Averaged number of ChAT labelled cells in Locomotor cat is also higher than those in the Control cat.

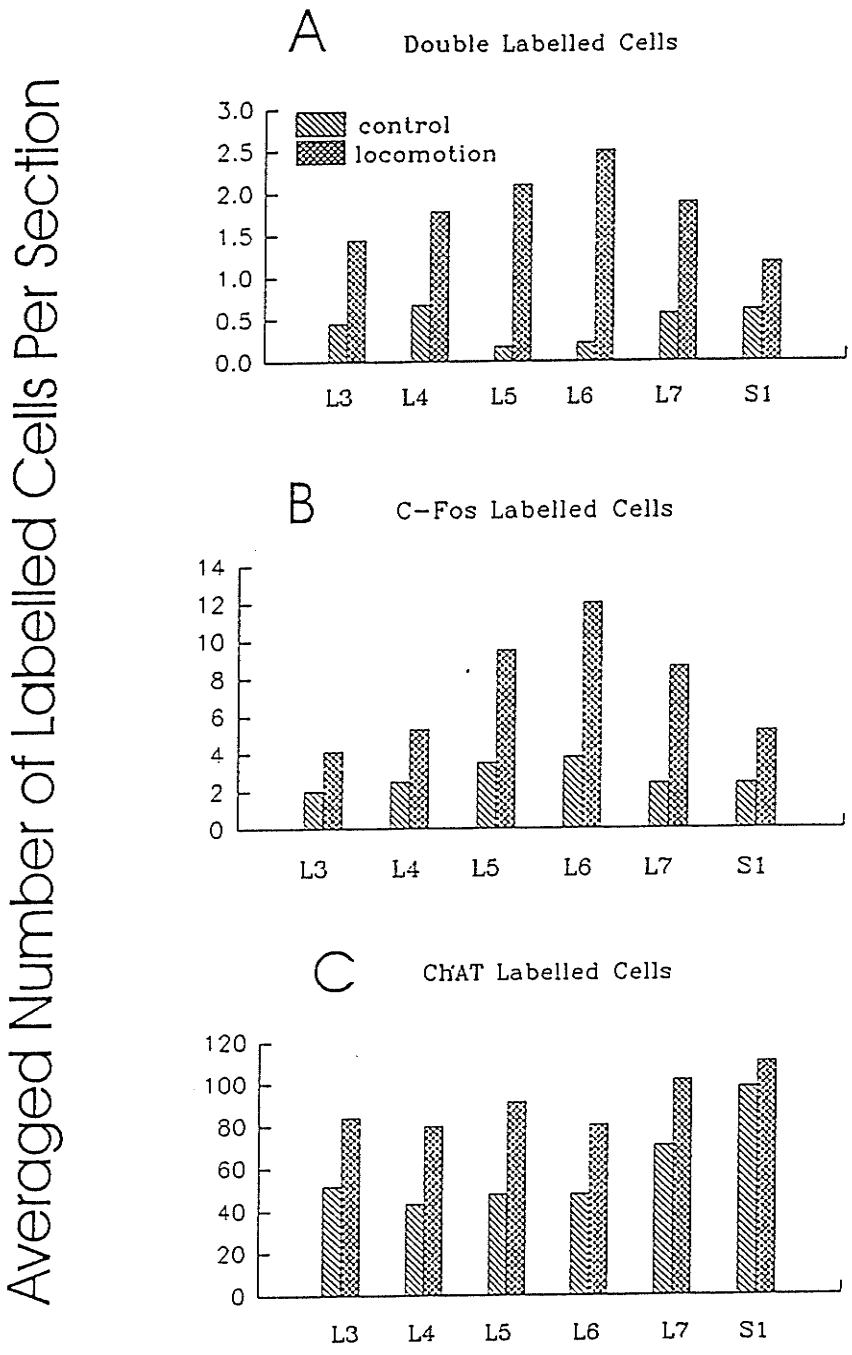
A
Double Labelled Cells
control
locomotion
3.0
2.5
2.0
1.5
1.0
0.5
0.0
L3
L4
L5
L6
L7
S1
B
C-Fos Labelled Cells
14
12
10
8
6
4
2
0
L3
L4
L5
L6
L7
S1
C
ChAT Labelled Cells
120
100
80
60
40
20
0
L3
L4
L5
L6
L7
S1
Averaged Number of Labelled Cells Per Section
Spinal Segments

Fig.9

Figure 10

Segmental distribution of labelled cells in FL-Cat2 and FC-Cat2. A. Double labelled cells . B. C-Fos labelled cells . C. ChAT labelled cells .

The calculation was done separately in the cats. Averaged number of labelled cells per section can correct the bias which is induced by uneven number of tissue sections collected during tissue processing.

Note: Averaged number of double labelled cells and C-Fos labelled cells in the Locomotor cat is much higher than those in the Control cat. Averaged number of ChAT labelled cells in most segments in the Locomotor cat is also higher than those in the Control cat.

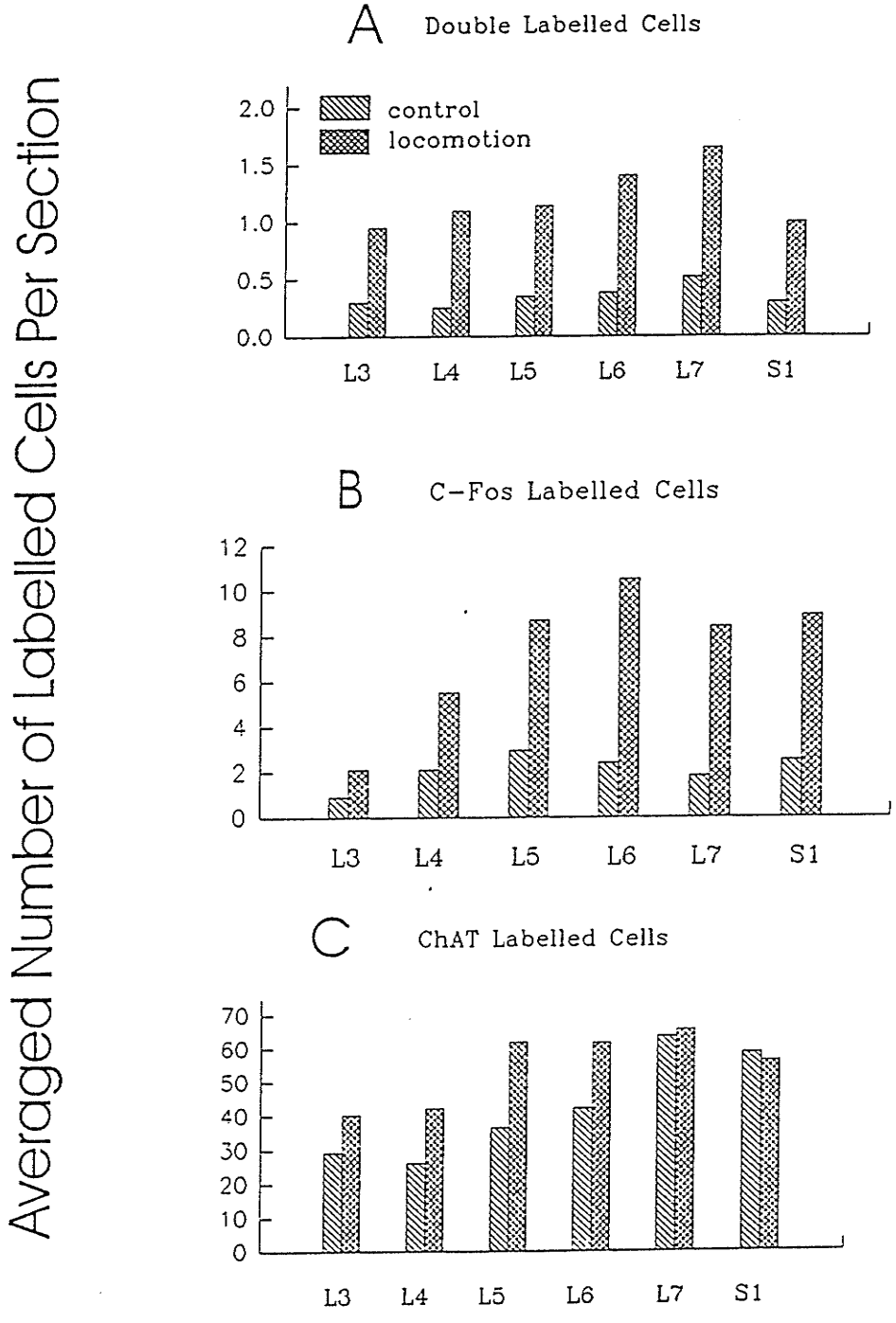
A
Double Labelled Cells
control
locomotion
2.0
1.5
1.0
0.5
0.0
L3
L4
L5
L6
L7
S1
B
C-Fos Labelled Cells
12
10
8
6
4
2
0
L3
L4
L5
L6
L7
S1
C
ChAT Labelled Cells
70
60
50
40
30
20
10
0
L3
L4
L5
L6
L7
S1
Averaged Number of Labelled Cells Per Section
Spinal Segments

Fig.10

Figure 11

Segmental distribution of labelled cells in FL-Cat3 and FC-Cat3. A. Double labelled cells . B. C-Fos labelled cells . C. ChAT labelled cells .

The calculation was done separately in the cats. Averaged number of labelled cells per section can correct the bias which is induced by uneven number of tissue sections collected during tissue processing.

Note: Averaged number of double labelled cells and C-Fos labelled cells in the Locomotor cat is much higher than those in the Control cat. Averaged number of ChAT labelled cells in most segments in the Locomotor cat is also higher than those in the Control cat.

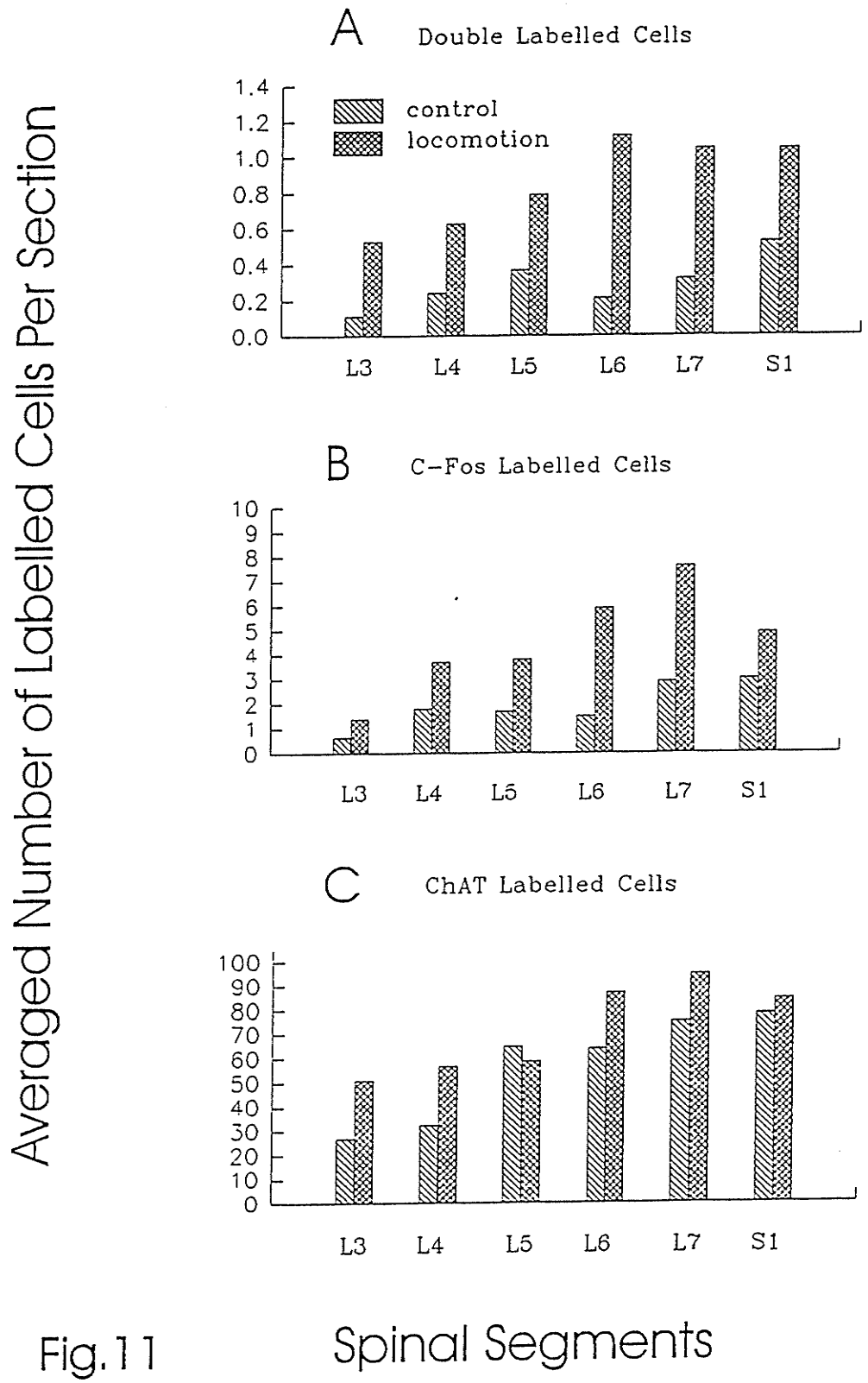
A
Double Labelled Cells
control
locomotion
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
L3
L4
L5
L6
L7
S1
B
C-Fos Labelled Cells
10
9
8
7
6
5
4
3
2
1
0
L3
L4
L5
L6
L7
S1
C
ChAT Labelled Cells
100
90
80
70
60
50
40
30
20
10
0
L3
L4
L5
L6
L7
S1
Averaged Number of Labelled Cells Per Section
Spinal Segments

Fig.11

Figure 12

Percent increase of ChAT-labelled cells in spinal dorsal horn, intermediate grey and ventral horn in locomotor cats compared to control cats.

A. FL-cat1

B. FL-cat2

C. FL-cat3

The calculation was done separately in the cats. The percent increase of ChAT-labelled cells (bar height) in each place was obtained by the total number of ChAT-labelled cells in that place from L_3 to S_1 in locomotor cat minus the total number of ChAT-labelled cells in same place in contral cat divided by the former.

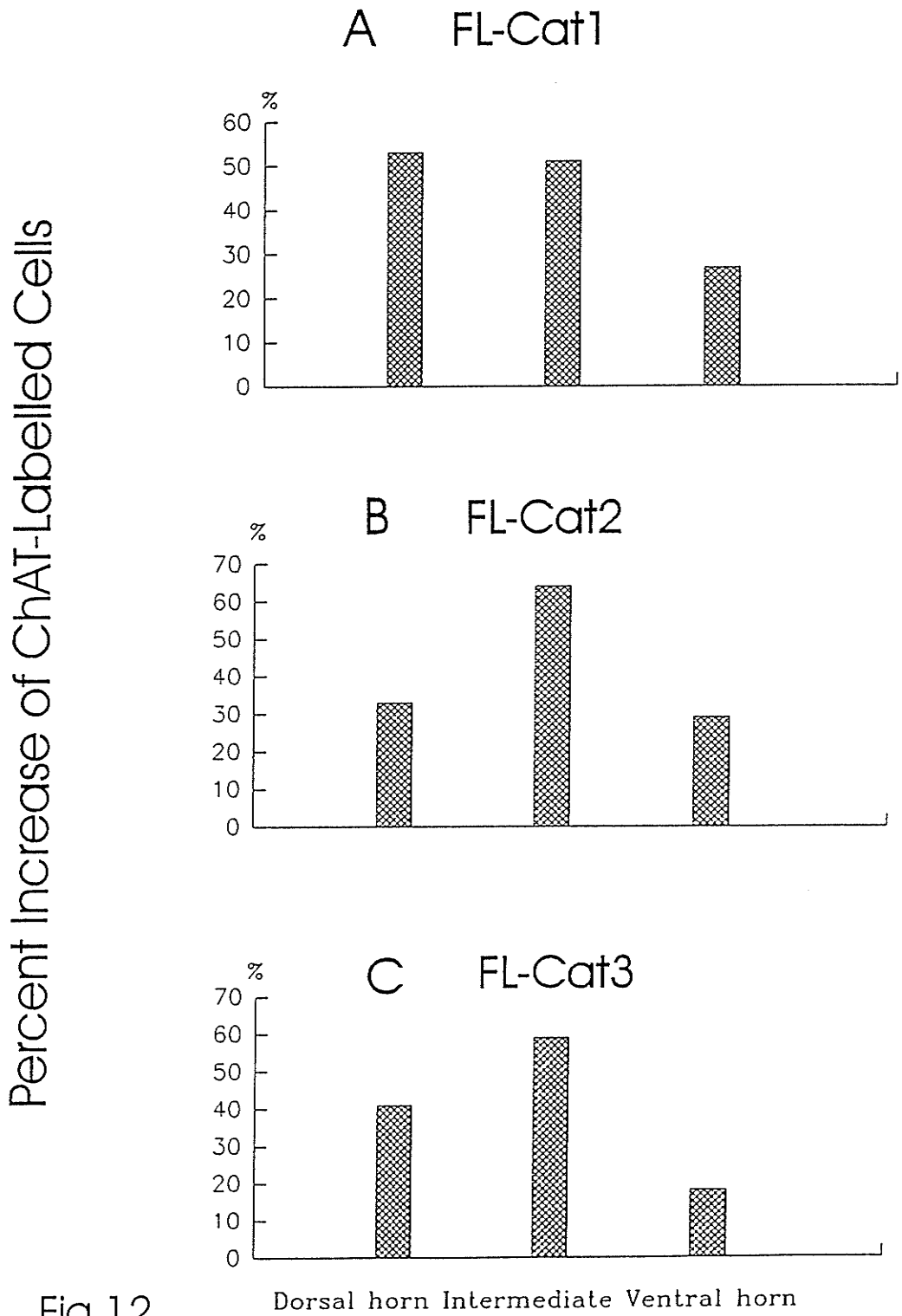
A FL-Cat1
%
60
50
40
30
20
10
0
B FL-Cat2
%
70
60
50
40
30
20
10
0
C FL-Cat3
%
70
60
50
40
30
20
10
0
Percent Increase of ChAT-Labelled Cells
Dorsal horn Intermediate Ventral horn

Fig.12

Table I

Comparison of labelled cells in different spinal segments for Locomotor cats and Control cats.

Values are means ± SD for the number of positive labelled cells indicated in parentheses.

$P < 0.05$ and $P < 0.01$ significantly different from Control.

Table I Mean labelled cells in different spinal segments in Locomotor and Control cats

Name of Labelled Cells	Labelled Cells (mean ± SD, n=18)		% difference	P	Labelled Cells (mean ± SD, n=21)		% difference	P	Labelled Cells ((mean ± SD, n=19)		% difference	P
	Locomotion 1	Control 1			Lomotion 2	Control 2			Locomotion 3	Control 3		
Double												
L3	1.44±1.10	0.45±0.30	69	<0.01	0.95±1.06	0.30±0.35	68	<0.01	0.53±0.42	0.11±0.12	79	<0.01
L4	1.78±1.05	0.67±0.51	62	<0.01	1.10±1.11	0.25±0.20	77	<0.01	0.63±0.46	0.24±0.22	62	<0.01
L5	2.10±1.12	0.17±0.11	92	<0.01	1.14±0.98	0.36±0.30	69	<0.01	0.79±0.64	0.37±0.25	53	<0.01
L6	2.50±0.95	0.22±0.20	91	<0.01	1.40±0.75	0.38±0.22	73	<0.01	1.12±0.95	0.21±0.14	81	<0.01
L7	1.89±1.21	0.56±0.35	70	<0.01	1.65±1.22	0.52±0.61	68	<0.01	1.05±1.10	0.32±0.40	70	<0.01
S1	1.17±1.52	0.61±0.37	48	<0.01	1.00±1.02	0.30±0.30	70	<0.01	1.05±0.84	0.53±0.27	50	<0.01
C-Fos												
L3	4.10±3.03	2.00±1.48	51	<0.01	2.10±1.12	0.90±0.64	57	<0.01	1.40±1.75	0.65±0.52	54	<0.01
L4	5.30±3.56	2.50±1.45	53	<0.01	5.50±2.25	2.10±1.21	62	<0.01	3.70±1.95	1.80±1.44	51	<0.01
L5	9.50±6.07	3.50±1.64	63	<0.01	8.70±3.39	2.95±1.59	65	<0.01	3.80±3.65	1.70±1.53	55	<0.01
L6	12.00±5.02	3.80±2.10	68	<0.01	10.50±5.17	2.40±1.42	77	<0.01	5.90±4.91	1.50±0.86	74	<0.01
L7	8.60±5.74	2.40±1.43	72	<0.01	8.40±3.33	1.80±0.94	78	<0.01	7.60±2.87	2.90±1.62	62	<0.01
S1	5.20±3.77	2.42±1.58	54	<0.01	8.90±1.95	2.50±1.04	72	<0.01	4.90±4.52	3.00±1.85	39	<0.05
ChAT												
L3	84.00±23.51	51.64±17.42	39	<0.05	40.34±10.38	29.22±11.12	28	<0.05	50.99±16.73	26.99±12.47	47	<0.01
L4	79.82±21.90	43.02±17.62	46	<0.01	42.21±8.34	26.21±8.17	38	<0.05	56.38±16.35	32.21±16.71	43	<0.01
L5	91.02±13.49	47.69±14.09	48	<0.01	61.83±16.00	36.57±8.00	41	<0.01	59.02±33.59	64.88±27.87	9	>0.05
L6	80.27±12.45	47.51±10.17	41	<0.05	61.75±12.38	42.29±9.33	32	<0.05	87.16±30.72	63.96±15.65	27	<0.05
L7	101.60±9.29	70.40±14.38	31	<0.05	65.56±13.37	63.62±14.72	3	>0.05	94.82±7.40	75.41±10.74	20	>0.05
S1	110.40±22.40	98.20±17.61	11	>0.05	56.27±8.29	58.94±8.88	5	>0.05	84.66±18.43	78.51±20.48	7	>0.05

Table II

Total number of labelled cells in each animal.

The total number of labelled neurons in the MLR-induced Locomotor cats is higher than that in the controls.

TABLE II

Total Number of Labelled cells

Cat Names	Labelled Cells			No.
	Double	C-Fos	ChAT	
FL-Cat1	196	805	9848	18
FC-Cat1	49	192	6398	18
FL-Cat2	147	927	6887	21
FC-Cat2	43	134	5394	21
FL-Cat3	98	518	8170	19
FC-Cat3	34	141	6177	19

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