DISTRIBUTION OF LOCOMOTOR-LABELLED NEURONS IN THE NEONATAL RAT THORACOLUMBAR SPINAL CORD

BY

CIMA CINA

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

MASTER OF SCIENCE

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ABSTRACT

The present study was undertaken to identify thoracolumbar spinal neurons (T10-L6) active during 5-HT - induced hindlimb locomotor-like activity in the isolated intact neonatal rat spinal cord preparation *in vitro*. The synaptic transmission-dependent endocytosis and consequent retrograde transport of the fluorescent dye sulforhodamine 101 was used to identify activated cells. Immunohistochemical labeling of spinal cells into neurons and astrocytes suggested that cells labeled during locomotor-like activity were most likely to be neurons. We show that serious methodological errors in a similar previous study (Kjærulff et al 1994) necessitated the present re-examination and identification of neurons active during the locomotor rhythm. In particular, we demonstrate that bath application of the exogenous neuroexcitant NMDA (5 μM), used by the Kjærulff et al (1994) study to help induce and maintain locomotion, results in pronounced activity-dependent labeling of cells even in the absence of locomotor activity. Further, in the presence of bath applied NMDA, the distribution and number of labeled neurons varied with incubation time (comparison at 45, 90 and 180 minutes). We conclude that any studies which include NMDA to induce locomotion may recruit additional populations of neurons unrelated to the spinal locomotor network. In contrast to NMDA, we show that bath application of the endogenous neuromodulator 5-HT (10 - 100 μM) will only induce significant sulforhodamine cellular labeling when the locomotor network is active. We conclude that 5-HT, when applied alone, is a reliable agent for the selective labeling of neurons recruited during locomotor-like activity. We observed that the topographical distribution of labeled neurons was diffusely distributed within the spinal cord. However, predominant labeling was observed in lamina VII and motor nuclei (lamina IX), whereas superficial laminae (I-II) were typically devoid of labeled cells. The rostrocaudal incidence and distribution of labeled neurons was uniform in spinal segments T10-L5 with reduced number observed in the L6 spinal segment. Total cell labeling was less than 1200 per lumbar segment suggesting that the population of neurons positively labeled by the activity-dependent labeling procedure during locomotion represent a very small fraction of the neurons contained within the spinal cord.

ABBREVIATIONS

2-DG	2-deoxyglucose
5-HT	5-hydroxytryptamine
ACSF	artificial cerebrospinal fluid
APV	D-aminophosphonovalerate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPG	central pattern generator
DA	dopamine
DOPA	dihydroxyphenylalanine
EMG	electromyogram
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
MAP-2	microtubule associated protein 2
MLF	medial longitudinal fasciculus
MLR	mesencephalic locomotor region
NMDA	N-methyl-D-aspartate
NMDLA	N-methyl-D,L-aspartate
SP	Substance P
SR-101	sulforhodamine 101
TTX	tetrodotoxin
5-HTP	5-hydroxytryptophan

DAGO	(D-Ala^2,N-Me-Phe^4,Gly^5-ol)-enkephalin
DSLET	D-Ser^2-Lev-enkephalin-Thr^6
FRA	flexor reflex afferent
6-OHDA	6 hydroxy-dopamine
5,6-DHT	5,6-dehydroxytriptamine
4-DI-2ASP	4-(4-Diethyllaminostyryl)-N-methylpyridiniu iodide

INTRODUCTION

I. The central pattern generator for locomotion

I.1. History

As early as 1906, it was known that locomotor activity can be induced in the absence of brainstem input. Sherrington (1906) observed that some locomotor movements could be organized without descending input from the brain. Then in order to find hindlimb function without sensory inputs, Graham Brown (1911), executed the transection of the spinal cord and there were alternating contractions in antagonist muscles. As a conclusion he said that these muscle contractions were produced within the spinal cord. Brown (1914) hypothesized that there are two half-centers, one controlling extensor motoneurons and one controlling flexor motoneurons which are connected by inhibitory interneurons and these half-centers, produce the alternation between antagonist motoneurons. The interesting part is that when one half-center is active, it silences the other half-center by inhibitory interneurons. In 1967, Jankowska et al, by recording extracellularly from interneurons in the lumbar spinal cord, revealed that after intravenous injection of DOPA, which evokes rhythmic activity in interneurons, reciprocal innervation is produced at this level and this produces a discharge of flexors or extensors motoneurons. Therefore, this finding reports the concept of interneurons having inhibitory and excitatory connections responsible for generating rhythmic activity.

Grillner, (1981) defined the central pattern generator (CPG) as a group of neurons that are capable of generating motor pattern activities. These neurons function without input from the brain or sensory afferents. There are CPGs for many behaviors such as locomotion, respiration and mastication and it is possible that some interneurons which are part of CPG for locomotion act as CPG neurons for other behaviors (Grillner 1981). Each CPG can generate rhythmic activity by itself. The CPG may also act to change the phase relationship between different muscle groups.

I.1.a. Possible locomotor rhythm-generating mechanisms

Brown's half center hypothesis (1914): In this model there is reciprocal inhibition between interneurons that excite flexors or extensors motoneurons to drive an alternating pattern. When one interneuronal pool is active, (e.g. the flexor half center) it inhibits the antagonist pool (e.g. the extensor half center), and in so doing, also decreases the inhibition of the dominating pool. Hence an alternation will occur (Grillner, 1981).

Miller and Scott model (1977): This model suggests that the CPG consists of interactions between antagonist Ia inhibitory interneurons (IaINs) and Renshaw cells (RCs). IaINs receive input from ipsilateral group Ia spindle afferents and inhibit the motoneurons of antagonists. The model generates periods of alternating inhibition in flexor and extensor motoneurons. If the motoneuron excitability level is sufficiently high, the motoneurons burst in an alternating fashion. The alternation between flexor and extensor activity was determined by phasic IaIN inhibition of motoneurons. The activity of IaIN was

determined by the inhibitory action of RCs. RCs are activated by axon collaterals from motoneurons and mediate recurrent inhibition, therefore allow an alternation between flexors and extensors.

1.2. The locomotor CPG in lower vertebrates

The interaction of neurons comprising the CPG for locomotion have been studied in lower vertebrates. Oscillatory activity within motor pattern-generating networks results from the combination of intrinsic electrical properties of the component neurons and their synaptic interactions (Calabrese 1995). Calabrese emphasized the importance of reciprocal inhibition to produce oscillatory activity.

Xenopus: In *Xenopus*, reciprocal inhibition, plateau potentials, and post inhibitory rebound are properties within the CPG network of neurons which control rhythmic activity in each spinal segment. Motoneurons that innervate muscles on each side of the body produce alternating spike-like activity and in each hemi-segment, inhibitory interneurons form reciprocal inhibitory connections responsible for the production of these alternating spike-like potentials. These interneurons, when hyperpolarized, show strong post-inhibitory rebound which is responsible for a robust rhythm based on reciprocal inhibition and cycle period in synaptic potential activity. Swimming in this animal can be produced in response to cutaneous stimulation (Sillar and Roberts, 1993). There are four types of neurons in the *Xenopus* spinal cord which are active during locomotion. They are: (i) one type of dorsal sensory interneuron which is located in the initiation pathway, (ii) two types of premotor interneurons which are active during

swimming (Roberts, 1990), (iii) descending interneurons (Roberts and Clarke, 1982) which excite ipsilateral motoneurons via the activation of excitatory amino acid receptors (Dale and Roberts, 1985), and (iv) commissural interneurons (Roberts and Clarke, 1982) which inhibit motoneurons on the opposite side at strychnine-sensitive, presumably glycinergic synapses (Dale, 1985). Alternation between the two sides depends on excitation and inhibition produced by the commissural interneurons and occurs during locomotion.

Lamprey: In the lamprey, during swimming, laterally directed undulations going along the body in a caudal direction act as the propulsive force. The circuitry for locomotion includes: the pattern responsible for segmental motor activity, sensory input into the segmental pattern and the descending reticulospinal system. The segmental pattern generator includes; motoneurons, one group of excitatory interneurons, two groups of inhibitory interneurons, lateral and contralateral interneurons. Excitatory interneurons excite interneurons on the same side and motoneurons while contralateral interneurons inhibit all interneurons and motoneurons in the opposite side (Grillner et al, 1991). However the pattern for locomotor activity is not completely understood.

1.3. Localization of the locomotor CPG in mammals

The exact location of the CPG itself is still unknown in mammals because of the complexity of the spinal cord, and to date it has not been possible to tell whether the basic oscillator occupies a specific location in the spinal cord or whether it presents a multisegmental distribution in the lower spinal cord (Cazalets et al, 1995; Cowley and

Schmidt, 1994; Kjaerulff et al, 1994)

In 1967 Lundberg and colleagues, after induction of locomotor-like activity following intravenous injection of DOPA, showed that there are two groups of interneurons that are located reciprocally in the spinal cord, which have long- lasting reflex effects from flexor reflex afferents (FRA). These interneurons are located in the dorsolateral part of Rexed's lamina VII (Rexed, 1954). Type A get excited by ipsilateral high-threshold afferents and get inhibited by high threshold afferents from the contralateral side. Type B are activated by contralateral high threshold afferents and are inhibited by ipsilateral afferents. Further study of neurons rhythmically active during locomotion was undertaken by Orlovsky and Feldman in 1972. They recorded extracellularly from interneurons in the spinal cord which were rhythmically active during mesencephalic locomotor region (MLR) stimulation evoked locomotion in the cat and they reported that the most of the active neurons are in the ventral intermediate gray matter and the motor nucleus.

The spinal cord interneurons which influence the action of alpha-motoneurons and are active during locomotion have been recognized on the basis of known synaptic connections, they are Renshaw cells (RCs) and Ia inhibitory interneurons (IaINs) (Jordan, 1983). Renshaw cells are rhythmically active during fictive locomotion. The inhibitory actions of Renshaw cells on their target cells maintains active during fictive locomotion (McCrea, et al, 1980), and it was shown that rhythmically active segmental afferents do not supress the recurrent inhibitory pathway to alpha-motoneurons during treadmill walking (Pratt and Jordan, 1980).

Also the action group II interneurons during locomotion has been determined. In 1990, rhythmic activity during fictive locomotion in a specific set of interneurons in the L4 segment was recorded by Shefchyk and colleagues. These L4 interneurons received input from group II afferents, which are located in laminae VII and VIII, and projected caudally to motoneurons in lower lumbar segments where they produced monosynaptic excitation or disynaptic inhibition of motoneurons.

Disynaptic group I excitation during extension is widely distributed to hip, knee, and ankle extensor motoneurons (Angel et al, 1996). Recent work shows that the interneurons producing disynaptic group I excitation of extensors are excited from the midbrain locomotor region and these interneurons are spontaneously active during fictive locomotion (M.J. Angel, E. Jankowska, and D.A. McCrea, unpublished).

The reciprocal inhibition of IaINs by Renshaw cells and the role of IaINs during MLR-evoked locomotion, was examined by Pratt and Jordan (1987), using intravenous administration of strychinine. They found that strychinine removed the hyperplorized phase of the locomotor drive potential (LDP, rhythmic alternation in mean voltage) in the motoneurons, but the rhythm and depolarized phase of the LDP were conserved (Jordan 1983).

In 1995 Cazalets and colleagues used a brainstem-spinal cord neonatal rat preparation in an attempt to localize the neurons that are part of the CPG and active during locomotion. They applied a mixture of neuroactive substances (NMDA and 5-HT) to the spinal cord

and, at the same time, recorded from ventral roots (L2-L5) which represent activity in flexor and extensor motoneurons respectively (Cazalets et al, 1995; however see Cowley and Schmidt 1994). In their experiments they also separated the spinal cord with a petroleum jelly wall into a rostral part (L1-L2) and a caudal part (L3-L4-L5). During the application of transmitters to the rostral part there was rhythmic activity in whole lumbar cord but when transmitters were applied only to the caudal part no rhythmic ventral root activity was seen in L1 and L2. Therefore they concluded that the circuitry that generates locomotor activity in a mammal is only in the rostral part of the lumbar spinal cord. Also, following experiments which employed hemisection of the cord from the caudal end forward to L2, Cazalets et al, (1995) suggested that right and left alternating pattern is also organized in L1 and L2 and from this region there is monosynaptic drive to motoneurons. They also added that the caudal part of the lumbar spinal cord has no effect in generating locomotor activity.

In 1996, Kjaerulff and Kiehn investigated the localization of rhythm-generating networks along the dorsoventral axis by horizontal and sagittal sections in the spinal cord neonatal rat. In horizontal sections, they divided the spinal cord into dorsal half and ventral half and recorded from ventral roots following application of 5-HT and NMDA. They concluded that the ventral third of the spinal cord is sufficient to generate coordinated rhythmic activity. Also from sagittal sections, they concluded that the locomotor neurons are most likely in medial gray matter. They also investigated the left/right coordination in T_{12}-L_6 spinal cord preparations using caudal and rostral midsagittal sections following application of 5-HT and NMDA. they recorded the activity of ventral roots. It was

concluded that left/right alternation is distributed along the entire rostrocaudal axis in the caudal thoracic-lumbar spinal cord and it is not restricted only in L1-L2 segments as suggested by Cazalets et al, (1995).

In 1997, Cowley and Schmidt investigated the rostral-caudal distribution of the locomotor-like network elicited by 5-HT. In their experiments the spinal cord was transected at several levels starting rostrally and proceeding caudally. They found that the network which was activated by 5-HT and produced a locomotor-like activity in flexors and extensors is distributed along the supralumbar region of the spinal cord. This region is not only responsible for generating motor activity, but also for coordinating left/right interactions for more caudal regions.

In 1997, Magnuson and Trinder, investigated the activity in lumbar ventral roots to stimulate a specific descending pathway in VLF of the isolated rat spinal cord using midsagittal lesions. Rhythmic activity was elicited after midsagittal section of the entire cervical cord and the thoracic cord to level of T9 or T10 Also the activity was elicited after the midsagittal section of the lumbar part caudal to L3. However, any midsagittal lesion of spinal cord segments T10-L3 attenuated the VLF-induced rhythmicity. Hence, one can conclude that lower thoracic and upper lumbar segments are important for rhythmicity.

I.4. Afferent input to the CPG

Even though the isolated spinal cord is capable of generating locomotion, rhythmic motor activity normally recruits sensory feedback from the limbs. There are three different types of sensory input that have been shown to influence activity of the CPG in studies of cat hindlimb locomotor activity. 1) Load sensitivity from extensor muscles: if the load on extensor muscle remains high, it prevents flexion (Pearson et al, 1976). 2) Effect of hip position: Sherrington (1910) suggested that the step cycle can be induced from extension of the limb by gravity followed by flexion (Grillner, et al, 1978, Conway et al, 1987, Guertin et al, 1995). 3) Sensitivity in hip movement; when all muscles controlling the knee joint and lower limb were dennervated and only the afferents from hip muscles and joint were intact, the spontaneous locomotor activity becomes modified by which flexor activity occurred during hip flexion and extensor activity occurred during hip extension (Grillner and Rossignol 1978; Kriellars, 1994).

Pearson (1993) suggested that sensory feedback from a limb during locomotion consists of; 1) input from receptors in muscle (proprioceptors) - this feedback is responsible for regulation of muscle activation between the phases of the step cycle, 2) input from joint receptors - responsible for locomotor entrainment during fictive locomotion; and 3) input from skin receptors - contact of the foot by an obstacle during the swing phase of walking induces an increase flexion in limb.

I.5. The descending input into the CPG

One of the regions in brainstem that can activate the pattern generator for locomotion in the spinal cord is the mesencephalic locomotor region (MLR). The MLR has projections to the reticular formation which contains large number of reticulospinal neurons that descend via the ventral funiculus of the spinal cord to form synapses in the gray matter at all segmental levels of the cord. This projection can act on spinal neuronal circuits by recruiting the CPG (Armstrong, 1988). Orlovsky et al (1972) performed experiments in which brief pulse stimulation of the medial reticular formation during decerebrate stepping could decrease or increase the locomotor activities in flexor and extensor muscles.

The MLR which projects to reticulospinal neurons, forms the major locomotor pathway to the spinal cord (Jordan, in press). This area has two major nuclei: the nucleus cuneiformis (CNF) and the pedunculopontine nucleus (PPN). Electrical stimulation of CNF by implanting an electrode in the MLR produces a large increase in the velocity of locomotor activity (Sterman and Fairchild, 1966). The CNF appears to be related to the medial hypothalamus and has a role in escape and flight. The PPN is related to lateral hypothalamic activity (exploratory locomotion).

In the reticulospinal neurons, which are the descending pathway for the initiation of locomotion, it is thought that glutamatergic cells are critical, since the initiation of locomotor activity from the brainstem can be prevented by antagonists of excitatory

amino acids (Smith et al 1988; Douglas et al 1993; McClellan et al 1994). Other nuclei in brainstem are also implicated in the central activation of motor activity. For example, the raphe nuclei contain serotonergic cells which release 5-HT and promote the development of plateau potentials in spinal cord neurons (Kiehn, 1991)

I.6. The *in vitro* neonatal rat preparation

There are different experimental preparations and animal models that have different advantages and disadvantages for the study of locomotor activities. *In vitro* preparations of CNS tissue offer a number of well-publicized advantages over *in vivo* approaches for neuronal behavior (Smith and Feldman, 1987). For example, due to its small size and small amount of myelination, the neonatal rat spinal cord can survive for many hours with the passive diffusion of metabolites and gas. Further, the concentration of applied neuropharmacological agents can be tightly controlled (Otsuka and Konishi 1974). With these advantages Smith and Feldman (1987) demonstrated that the isolated spinal cord from neonatal rat up to 7 days old can generate locomotor activity *in vitro*. Also, the tolerance of mammalian neonatal nervous tissue to hypoxia (anoxia) permits neuronal energy metabolism to be maintained under the conditions of reduced tissue oxygenation *in vitro*. Hence the isolated neonatal rat preparation is well suited to study mammalian locomotor activity. Motor output activities can be generated by the isolated nervous system *in vitro*, even in the absence of movement-related and other sensory inputs. Air stepping can be induced both pharmacologically (e.g. Kudo and Yamada 1987) and electrically (Atsuta et al 1990) in the neonatal rat. Kudo and Yamada (1987) demonstrated that alternating activity between antagonists tibialis and gastrocnemius was

produced when NMDA was applied to the bath. Currently several laboratories are using the isolated neonatal rat spinal cord preparation to study locomotor behavior (e.g. Cazalets et al 1990, 1992, 1995; Cowley and Schmidt 1994, 1997; Kjærulff and Kiehn 1996; Kiehn et al 1996; Raastad et al 1996; Magnuson and Trinder 1997).

II. The induction of locomotion

II.1. NMDA receptors.

NMDA receptors are a subtype of ionotropic excitatory amino acid (glutamatergic) receptor whose unique properties have been shown to support cellular oscillatory behavior (for review see Daw et al 1993). NMDA is a selective exogenous receptor agonist of the NMDA receptor. In 1985 Grillner et al. showed pacemaker-like activity elicited by NMDA receptor activation in interneurons and motoneurons in lamprey spinal cord during locomotion which was maintained after blockade of action potential-dependent synaptic transmission with tetrodotoxin (TTX). Also bath application of NMDA initiates locomotion in the *Xenopus* embryo (Dale and Roberts 1985). In 1987, Smith and Feldman used an *in vitro* preparation of the brain stem-spinal cord from neonatal rat to investigate the motor pattern for locomotion. They recorded from ventral roots (L2-L6) innervating the hind limbs to see the patterns of inter limb coordination. After application of excitatory amino acid NMDA to the spinal cord, locomotor output patterns were induced.

II.1.a. The voltage dependent properties of the NMDA receptor and cellular pacemaker properties.

NMDA acts on spinal neurons through activation of NMDA receptors which possess a characteristic voltage sensitivity. When the membrane potential is at a resting level, the NMDA voltage sensitive receptor is blocked by extracellular Mg^{2+}. Glutamatergic synapses normally contain NMDA and non-NMDA receptors postsynaptically, and during repetitive synaptic activation, sufficient depolarization of the postsynaptic membrane, largely via Na^+ entry through non-NMDA receptor-coupled channels, results in removal of Mg^{2+} from the NMDA ion channel allowing ion entry (Na^+ and Ca^{2+}). Entrance of Ca^{2+} through NMDA channels causes activation of outward currents generated via Ca^{2+} dependent K^+ channels causing membrane hyperpolarization. Mg^{2+} block of the NMDA channel is then reinstated, Ca^{2+} entry is blocked, and the cycle begins again.

II.2. Other transmitter systems implicated in locomotion (in rats)

Locomotor activity can be initiated by; 1) induction of locomotor activity at the spinal level or activation of descending spinal inputs from brainstem, and, 2) stimulation of sensory pathways (e.g. pinching the tail in the neonatal rat brainstem-spinal cord preparation can induce alternating locomotor activity, Smith et al 1988). As early as birth there are a number of neurotransmitters which are capable of activating the locomotor pattern. NMDA and dopamine (DA) are effective in inducing air stepping (Kudo and Yamada, 1987; Dales and Roberts, 1985; Grillner, 1985; Atsuta, 1991), but GABA has

the opposite action. Also combined application of 5-HT and SP on brainstem produces faster and longer alternation between flexors and extensors (Atsuta 1991). Ozaki et al (1996) investigated the effect of NMDA on the activity of ventral roots in neonatal rat spinal cord in which application of 7.5 μM NMDA evoked alternating rhythm on both sides of the ventral roots. The mammalian spinal cord contains descending monoaminergic pathways that are either serotoninergic (5-HT), adrenergic or dopaminergic (Grillner, 1975). Cazalets et al (1990), demonstrated that bath application of 5-HT in the spinal cord neonatal rat is capable of producing locomotor activity. Cowley and Schmidt (1994) showed in *in vitro* neonatal rat preparation, the rhythmic activity which produced by 5-HT is more regular than with NMDA alone. Simultaneous application of 5-HT and NMDA evoked a more stable and reliable locomotor pattern (Squalli-Houssaini et al, 1993). In 1991 Cazalets et al, suggested that the locomotor-inducing action of 5-HT is mediated through 5-HT_1 and 5-HT_2 receptors subtypes and 5-HT antagonists like propanolol blocks the rhythmic activity. Glutamate receptor agonists like aspartate act to increase locomotor activity and their action is mediated through both NMDA and non-NMDA receptors. In adult rats the serotonergic terminals that innervate the spinal cord, mostly originate in raphe nucleus in the brainstem (Steinbusch, 1984). At birth the innervation of serotonergic neurons is less than adults. 5-HT acts directly on the component of the CPG and induces an alternating pattern between flexors and extensors and dose dependent increases in the ventral root activity (Cazalets et al 1992). Stimulation of serotonergic neurons enhances the locomotor rhythm and motoneuron discharges. Therefore serotonergic neurons are most likely capable of directly affecting

the CPG. In studying the CPG in vertebrates, it was shown that command pathways as well as agonists of command pathway release transmitter induces rhythmic activity in interneurons (Hochman et al, 1994).

II.3. Other transmitter systems implicated in locomotion (in cats)

In the mid-60s, Lundberg and colleagues observed an interneuronal organization of inhibitory and excitatory connections to produce activity which could account for the production of a locomotor rhythm. In acute spinal cats given DOPA (dihydroxyphenylalanine), ipsilateral high threshold cutaneous, joints, and contralateral high threshold afferents were stimulated within 2 ms of each other. The first stimulation resulted in reflex discharge in flexor motoneurons. The second stimulation resulted in reflex excitation of ipsilateral extensor motoneurons. These two pathways showed reciprocal inhibition. Hence, reciprocal connections between flexors and extensors were mediated by inhibitory interneurons.

In 1995, E.D. Schomburg et al, investigated the effect of opioids(DAGO and DSLET) and naloxone on rhythmic motor activity in spinal cats. The DAGO and DSLET which were injected intravenously or applied to the lumbar spinal cord, inhibited the rhythmic motor activity. In contrast, naloxone removed the rhythm-depressing action of opioids. The application of naloxone after injection of opioids was more regular and more pronounced. The effect of opioids was partly due to the suppression of excitatory FRA pathway and facilitation of inhibitory ones.

In 1991, Barbeau and Rossignol showed that in chronic spinal cats the application of 5-HTP alone increases the tonic activity in motoneurons but does not elicit locomotion. However, coapplication of 5-HTP and clonidine (a noradrenergic agonist), produces a locomotor pattern.

In 1980, Steeves et al. showed that the production of locomotor activity is mediated partly by the descending monoaminergic pathways. However, a partial depletion of spinal cord NA and 5-HT contents by injection of 6hydroxy-dopamine(6-OHDA) and 5,6-DHT did not abolish MLR evoked locomotion.

III. Activity dependent labeling

Several low-mass, highly-charged, sulfonated fluorescent molecules are taken up endocytotically from presynaptic nerve terminals in an activity-dependent manner (Lichtman et al 1985) and are transported retrogradely to the soma, probably within lysosomal organelles. They include sulforhodamine 101 and Lucifer yellow. The unique strength of these uptake-dependent dyes is their ability to directly translate activity into neuronal labeling. The labeling of living nerve terminals with fluorescent dyes taken up in an activity-dependent manner was first described at the snake neuromuscular junction (Litchman et al., 1985). Since this landmark application, several investigators have used uptake-dependent dyes to study the mechanisms and kinetics of synaptic vesicle recycling at presynaptic terminals.

III.1. The biological properties of uptake-dependent dyes

These fluorescent dyes can be visualized at relatively low concentrations, showing stained structures in detail (Lichtman, 1987). These dyes (N-methylpyridinum iodide; Safranine O; Rhodamine.123; 2-(4-Dimethylaminostyryl)-N-ethylpyridinium iodide; 4-Di-2-ASP) appear to be taken up via endocytosis at the synaptic terminals of stimulated axons. Lichtman in 1987 used 4-DI-2-ASP to label motor nerve terminals. This dye is non toxic and is used for neuromuscular junction. It stains the presynaptic nerve terminals in animals such as frog, snake, fish and lamprey. Lichtman suggested that the dye does not produce any lasting changes in synaptic mechanisms. High concentration of the dye abolishes spontaneous miniature endplate potential activity in fibers. In 1992, Keifer and Houk used the fluorescent dye sulforhodamine 101 to study the pattern of neural activity in the *in vitro* turtle brainstem-cerebellum preparation. Sulforhodamine, which is also taken up endocytotically at the presynaptic nerve terminal, labeled active neurons during bursting activity in the red nucleus. Keifer and colleagues applied electrical stimulation to the dorsolateral funiculus on one side of the spinal cord and it induced burst activity in 50% of red nucleus neurons on the other side of stimulation. They also bathed the preparation in sulforhodamine and observed that the labeling was mostly seen in cell bodies. The labeled cells were only on one side of the brain. They also found staining in the ipsilateral nucleus of the MLF and in cerebellar purkinje cells bilaterally and several zones of reticular formation. It was suggested that dye uptake is due to the production of action potentials in red nucleus, since, when they blocked the burst discharges in red nucleus, only a few cells were labeled. In comparison with C-fos,

a nuclear regulatory protein that only labels the nucleus, sulforhodamine stains dendrites, terminals and somas. Sulforhodamine also has an advantage when compared to 2-deoxy-glucose (2-DG). With 2-DG there is a long processing time for autoradiography and it is difficult to differentiate between synaptic excitation and inhibition (see discussion in Keifer, 1992). Further, this technique does not allow for visualization of individually activated cells.

III.2. Activity dependent labeling of neurons active during locomotion

Viala et al (1987) attempted to find the anatomical localization of the lumbar locomotion generators using 2-DG radioactive uptake in acute low spinal preparation of rabbits. The animals were injected with DOPA and nialamide to produce locomotion. Labeled cells were found in Rexed's laminae VI and the dorsolateral portion of lamina VII in segments L6-S1.

In 1990 Jordan and colleagues attempted to localize the interneurons in the spinal cord that were activated during locomotion in decerebrate cats using immunohistochemical detection for expression of the immediate early gene c-fos. In order to produce treadmill locomotion the mesencephalic locomotor region (MLR) was stimulated. Spinal segments L3-S1 were removed and the tissues were sectioned. Then it was processed for fos labeling. The results suggest that the labeled cells in laminae VII, VIII and X are involved in locomotor activity.

In 1994, Kiehn and colleagues, in order to explore the localization of the CPG during locomotion in neonatal rat, used sulforhodamine 101 to label the cells that are synaptically active during locomotion. These experiments were undertaken in the isolated spinal cord usually with hindlimbs attached. Bath application of serotonin and NMDA were used to evoke locomotor-like activity. The dye was then applied to the bath for 4 hours, while activity was monitored with EMG electrodes. They observed that locomotor-labeled cells were located in the medial intermediate gray (lamina VII) and around central canal. In preparations with the hind limb attached, stained cells were also found in the dorsal horn. Kiehn suggested that cells located in these areas are involved in generating, spinal locomotor activity. One disadvantages of this dye is that it can have direct actions on neurons at higher concentrations (Kjærulff et al 1994). For example, in some experiments, even at a concentration of 0.0001-0.0005%, SR may alter the locomotor pattern from alternating to a synchronized motor activity (Kjaerulff, et al, 1994). Hence, caution must be used when using activity-dependent dyes.

IV. Limitations of previous studies

In 1994, Kiehn's group used the *in vitro* neonatal rat spinal cord preparation in order to investigate the role and location of the spinal CPG engaged in locomotion. They combined chemically induced locomotion with SR 101 to label the cells that are rhythmically active during locomotion. After EMG recording from hind limb muscles following bath application of NMDA and 5-HT, they suggested that, in the hindlimb

attached preparation, with stained sections from L1-L6, the neurons responsible for locomotion is located in lamina I-II and the lateral part of lamina III and IV in the dorsal horn, in the intermediate gray (VI-VIII) and in lamina X (around the central canal). In the isolated spinal cord, labeled cells were around the central canal and intermediate gray matter (VI-VII). Unfortunately this study was not properly controlled because high concentrations of NMDA (5.0-7.5 μM) and extracellular K^+ (6 mM) were used to induce locomotion. High concentrations of NMDA would be predicted to activate many cells even those not related to locomotion. This is particularly true in the neonate because NMDA receptors are densely distributed during this developmental period (Kalb et al, 1992). Our results will show that high concentrations of NMDA can induce the same pattern of labeling observed by Kiehn (1994) even in the absence of locomotion. Also we have observed with the use of NMDA that there is a time dependent recruitment of neuronal populations which was not taken into account by Kiehn's group. Also in those experiments they used high extracellular K^+ (6 mM) which would also be predicted to depolarize and activate cells not related to locomotion. Therefore in the present study serotonin alone was used to activate locomotion to identify neurons active during locomotor activity. 5-HT, is a strong neuromodulatory drug in the CNS, which is capable of activating of locomotor pattern that can last for several hours (Cazalets, 1992). We show that 5-HT, unlike NMDA, does not evoke significant spinal neural labeling in the absence of locomotion. Our studies were undertaken in order to shed light on the organization of the locomotor network.

V. Aims of the study

Since 1967, it has been known that there is a group of interneurons in the spinal cord that are organized with the appropriate excitatory and inhibitory connections to generate locomotor activity. However, little is known about locomotor CPG in mammals (Gossard and Hultborn, 1991). Normally the locomotor pattern is activated by the projection of neurons from brainstem to spinal cord. It is important to be able to identify and characterize CPG neurons, in order to understand the manner in which rhythm generation occurs. One important factor is localization of the network. If we know the exact location of locomotor-generating neurons, we could focus our efforts into a spatially discrete region and perhaps find methods of targeting this region for treatment following spinal cord injury.

In the present study, the activity-dependent labeling method is used in order to localize and identify the spinal cord interneurons responsible for locomotor activity. In order to reach this goal we must first; (i) show that labeled cells are neurons, and (ii) control for false-positive labeling. As is indicated in Results section, in order to investigate whether the sulforhodamine labeled cells were neurons, immunohistochemical procedures were employed following activity labeling to identify neurons and astrocytes. In order to reduce false-positive labeling, normal $[K^+]_o$-containing ACSF and no NMDA were used in our experiments.

The present study provides the first realistic topographical identification of neurons recruited during locomotion in the neonatal rat. Identification of locomotor-labeled neurons further allows for cell targeting with single cell electrophysiological approaches in a slice preparation. This is important. For example Hochman et al (1994) identified a population of interneurons in lamina X that displayed conditional bursting properties in the presence of NMDA in the neonatal rat spinal cord slice. Since conditional bursting properties may be critical to CPG function, it would be important to identify conditional bursting in neurons previously identified as having participated in the generation of locomotion. Recordings from locomotor-labeled neurons would provide such information.

METHODS

I. Dissection

Sprague-Dawley rats (aged 0-4 days) were decapitated and eviscerated prior to being transferred to a dissection chamber filled with cold (4°C), oxygenated (95% 0_2, 5% CO_2) artificial cerebrospinal fluid (ACSF). The ACSF contained in mM; NaCl 125, KCl 2.5, NaH_2PO_4 1.25, D-Glucose 25.0 $MgCl_2$ 1.0, $CaCl_2$ 2, at a pH of 7.4. Two kinds of preparations were used: the isolated spinal cord (with attached nerve roots) and the isolated spinal cord with hindlimbs attached. The spinal cord was transected at the C_2 cervical level. The remaining spinal cord (thoracic, lumbar and sacral segments) was exposed by a ventral laminectomy. After exposing the spinal cord, the temperature of the chamber was allowed to warm to room temperature (22°C) where it was maintained. During the dissection, the solution bathing the spinal cord was replaced with fresh ACSF several times. All preparations were pinned down ventral side up in a chamber having a Sylgard bottom. For isolated preparations, 0.1 mm insect pins were used to stabilize the spinal cord along its entire length and at the end of the nerve roots.

II. Experimental configuration

Following warming the bath to room temperature, locomotor activity was induced by 5-HT (10-100 μM) and in 10% of experiments naloxone (10-30 μM) was included (Sigma) to facilitate the locomotor rhythm (Pearson, et al, 1992; Schomburg, et al, 1995). The neurochemicals to be applied were initially dissolved in distilled water and stored at -

20°C as 10 mM stock solutions. Stock concentrations of drugs were added to the static bath in order to achieve a final concentration of the agent in the chamber.

In order to record motoneurone activity from ventral roots, suction electrodes were constructed from glass capillaries (outside diameter 1.2 mm and inside diameter 0.9 mm) which were heated and pulled in to fine diameter tips using a micropipette puller (Sutter Instrument Co.). The desired diameter of the pipette tip was achieved using a sharpening stone, then rinsed of debris with distilled water and polished over a Bunsen burner. This procedure was repeated until an inside diameter of 80 - 160 μM was achieved. Two suction electrodes were placed on ventral roots, either L2 or L5 ipsilaterally and contralaterally, or L2 and L5 ipsilaterally to monitor motor activity. During locomotion L2 ventral roots predominantly signal activity in flexors while L5 signals activity in extensors (Cazalets et al 1995). Ventral root activity was monitored on-line using Axotape (version 2.0.2., Axon Instruments). Locomotor-like activity observed from ventral root recordings was elicited with 5-HT at low concentration.

III. Experimentation

The spinal cord preparation was considered to be undergoing locomotor-like activity, if the activity of two recorded ventral roots were approximately 180° out of phase. After stable induction of locomotor activity, sulforhodamine (Sigma, or Molecular Probes) was applied to the bath at a concentration of 0.0005% in order to label the neurons which were active during locomotion. Sulforhodamine was left in the bath chamber for three

hours during the neurochemically-induced locomotor activity. Then the solution of the bath was exchanged for fresh ACSF several times to wash out any remaining sulforhodamine. This 'wash-out' procedure was required in order to avoid having a dense background of non-specific fluorescence. The cord was then immersed in fixative for 30 minutes after solution exchange. (see Tissue Processing below)

In another series of experiments, the hindlimbs attached preparation was used in order to duplicate aspects of the experiments of Kjærulff et al (1994), which included their ACSF solution and neurochemical concentrations. Hence, regular ACSF was changed to high K^+-containing ACSF (6 mM). Three types of control experiments were undertaken in order to identify potential sources of false-positive labeling that may have occurred in the work of Kjærulff et al (1994) due to their use of high concentration of NMDA and K^+. First, the hindlimbs-attached preparations were perfused with high K^+-containing ACSF solution and 5 μM NMDA. Air stepping was induced, typically for approximately 30 minutes, which confirmed that the cord had viable rhythmogenic circuitry. After locomotion spontaneously stopped, sulforhodamine (0.0005%) was then applied to the bath for 3 hours to label active neurons not related to locomotor output. The solution was then changed with new ACSF several times and left for 30 minutes prior to fixation. In other control experiments, instead of NMDA, 10 μM 5-HT was applied and the same procedure was employed as above. In the third control experiments, the effect of high K^+ -containing ACSF on neuronal labeling was examined.

Recordings were monitored continuously and captured on computer once the neurochemical was applied to the bath and until a stable rhythmic motor activity was achieved. Epochs of data were captured every 10 minutes thereafter. Data capture was achieved using the TL-1 data translation board (Axon) and Axotape software. The sampling rate per channel was 1 KHz. Electrical activity in ventral roots was amplified using amplifiers built in house at a gain of 10,000 and band-pass filtered at 30-3000 Hz. Electrophysiological data analysis was performed on a PC computer and wave forms were analyzed with specialty software written in-house and run using a Linux operating system.

IV. Tissue processing

In order to fix the lumbar spinal cord for histological processing, the tissue was cut from segments T11-L3 or L1-L6 with fine scissors and the roots were separated. The tissue was fixed in fresh 4% paraformaldehyde, left overnight at 4°C, then the next day it was placed in 30% sucrose for one hour on a shaker and then sectioned (frozen) using a cryostat (Leitz 1720). In all experiments the spinal cord was sectioned at 40 μm thickness and cut rostrocaudally. Between 8-12 sections were collected from each spinal segment (every third section). The sections were mounted on slides and then placed in a desiccator for 48 hours to dehydrate. The sections were then cleared in xylene for 15 minutes, mounted using Fluormount (BDH) and covered with a coverslip. Mounted sections were stored at room temperature.

Reconstruction of the distribution of fluorescently labeled cells in spinal segments were made using a Nikon Optiphot combination light/epifluorscent microscope. The microscope was equipped with an X/Y movement sensitive stage and CCD video camera attached to a PC computer. A Neurolucida image analysis system (Micro Bright Field Inc USA) made it possible to reconstruct the sections. The Neurolucida was used to draw outlines of sections at (100x) and to plot sulforhodamine-labeled neurons within these drawing at higher magnification (200x). Sulforhodamine-labeled cells were visualized under epifluorescent illumination with a standard Nikon rhodamine filter cube (G2-A).

V. Immunohistochemistry

In order to determine whether activity labeled cells were neurons, immunohistochemical procedures were performed to identify neurons (Pan-axonal and MAP-2) and glia GFAP). The processing was performed as follows:

1. Lumbar spinal cord slices were fixed in 4% paraformaldehyde overnight.
2. All subsequent washes and incubations were performed in 0.1 M phosphate buffered saline (PBS) containing 0.3% Triton X-100 (PBS-T).
3. Tissue was incubated in anti-Panaxonal and anti-MAP_2 (Sternberger Monoclonals) in order to identify neurons, and anti-GFAP (Chemicon) to identify astrocytes. All antibodies were incubated at a dilution of 1:1000 for four hours at 4°C.
4. Tissue was washed 3 x 10 minutes at room temperature, then incubated for 1-5 hours at room temperature in Cy3 conjugated sheep anti-mouse (Sigma) and FITC conjugated hours anti rabbit (Sigma), both diluted at 1:1000.

Tissue was washed for 20 minutes in PBS-T then 20 minutes in 50 mM Tris·HCl pH 7.4, dried and coverslipped using Aquamount (BDH).

RESULTS

I. Labeled cells are neurons

In the present study we first tested whether the cells which were labeled during activity-dependent labeling were neurons. Figure 1 shows a sample of GFAP immunostaining of one section of spinal cord. This section was previously reconstructed for sulforhodamine labeling and was realigned to compare the location of GFAP-positive astrocytes with locomotor-labeled cells. As shown in the Figure there are no GFAP-positive cells that previously stained positive with sulforhodamine. Panaxonal and MAP-2 immunostaining procedures were also used to identify neurons and compare their location with previously identified sulforhodamine-labeled cells. While we observed consistent overlap between immunostaining for neurons and the location of previously activity-labeled neurons, the immunostaining for neurons was intense and widespread and it was thus easier to positively identify locomotor-labeled cells as not being astrocytes than it was to identify them as neurons. We thus suggest that the locomotor labeled cells are most likely to be neurons. The likelihood of activity-labeled cells being neurons is further supported by several patch clamp recordings from these cells in slice and dissociated cell preparations (Hochman, personal communication).

II. NMDA but not 5-HT contributes significantly to false-positive labeling

Recently, Kjærulff et al (1994) undertook a similar study using sulforhodamine as an activity label during locomotion in the neonatal rat. Locomotion was induced

neurochemically with 5-HT but also included NMDA, a potent neuroexcitant. We assert that NMDA is a general neuroexcitant that unlike 5-HT will label neurons unrelated to locomotor activity. Hence, we examined the effects of NMDA on activity-labeling neurons not related to the locomotor event. We chose the lowest concentration of NMDA (5 μM) used by Kjærulff et al (1994) in their studies as well as their concentration of extracellular K^+ (6 mM).

In order to examine the effects of NMDA (in high $[K^+]_o$ ACSF) on activity-dependent labeling, two hindlimb-attached preparations were used. After application of 5 μM NMDA, air-stepping consistent with locomotor movements (alternating pattern between extensors and flexors) started and usually continued for ~30 minutes. When locomotion spontaneously stopped, sulforhodamine was applied (0.0005% concentration) to the bathing chamber in order to label active neurons in the absence of locomotor activity. Also, simultaneous extracellular recording from ventral roots L2 and/or L5 ipsilaterally or contralaterally, confirmed that no rhythmic motor activity was elicited while sulforhodamine was in the bath. Figure 2 shows the experimental setup for extracellular recording from ventral roots. Following experimentation, these spinal cords were fixed and sectioned in order to reconstruct the distribution of sulforhodamine-labeled cells in the lumbar spinal cord. Figure 3 presents the distribution of NMDA-induced sulforhodamine-labeled cells in one of the hindlimb-attached preparations with intact dorsal roots. Note that there was no rhythmic motor activity in the recorded ventral roots and background motor activity was low (Fig. 3B). Maps of labeled cells were derived

from superimposed drawings, as described in Methods. This figure demonstrates that in the presence of NMDA there are numerous activity labeled cells in all spinal segments which were not directly related to locomotor activity (to compare the number of labeled cells see Fig. 9). In two other preparations, instead of NMDA, 5-HT (10 μM) was applied to the bathing chamber in order to evoke locomotion (in high K^+-containing ACSF). Approximately 10 minutes after application of 5-HT air-stepping was observed and usually continued for at least 90 minutes. When locomotion eventually stopped, sulforhodamine was applied to the bathing chamber for three hours. The reconstructed distribution of labeling for one animal is shown in Figure 4A. Note that, in comparison to NMDA bath application, very few cells are labeled. (also see Fig. 9).

Kjærulff et al (1994) used a comparatively high concentration of K^+ (6 mM) in their bathing media that would depolarize neurons and possibly contribute to false-positive labeling. Thus the effect of high extracellular K^+ in ACSF solution on activity labeling was also investigated in two preparations. The procedure was the same as in the above experiments except no air stepping was observed. Figure 5 illustrates the distribution of activity-labeled cells observed in the presence of high $[K^+]_o$ and in the absence of locomotion. Labeled cells were observed in laminae III-V, VI, VII, and X.

The effect of low (regular) K^+-containing ACSF (2.5mM) on activity labeling was also investigated in two other preparations. The procedure was the same as figure 5. Figure 6 illustrates the distribution of activity-labeled cells observed in the presence of low K^+-containing ACSF and in the absence of locomotion. Note that the number of labeled

neurons are decreased compare to figure 5.

Because both NMDA and high $[K^+]_o$ contribute significantly to the false-positive labeling of neurons unrelated to the locomotor event, we can conclude that the distribution and/or number of neurons presumably active during locomotion reported by Kjærulff et al (1994) may have been significantly erroneous. We therefore felt it was necessary to re-evaluate the distribution of locomotor labeled neurons in the neonatal rat preparation. In our primary series of experiments (Section IV below) we chose to use a more typical value of [K+] in our ACSF (2.5 mM) and use only the endogenous neuromodulator 5-HT as the locomotor-inducing neurochemical agent. In order to investigate whether 5-HT alone, in the absence of locomotor activity contributes to false-positive labeling four experiments were performed using low (regular) K^+-containing ACSF. Air-stepping movement was evoked with 10 μM 5-HT in hindlimb attached preparations and, after locomotor activity ceased, sulforhodamine was applied in the same manner as above. As illustrated in Figure 7 there were very few neurons labeled due to 5-HT in the absence of locomotion. Hence, we conclude 5-HT alone does not contribute significantly to neuronal labeling in the absence of locomotor activity. We thus chose 5-HT as the locomotor-inducing agent in which to study the distribution of neurons labeled due to the recruitment of the locomotor circuitry.

We also compared the effect of normal 2.5 mM K^+-containing ACSF (Fig. 8A) and high K^+-containing ACSF (Fig. 8B) on ventral root activity. As shown in this figure there is

an increase in background spontaneous ventral root activity confirming that more neurons are recruited more depolarized by high K^+.

Figure 9, presents the total number of labeled cells observed in the absence of locomotor activity. Overall, we observed that the great majority of false-positive labeling is due to the presence of NMDA in the bath while low $[K^+]_o$ and 5-HT contributed minimally to false-positive neuronal labeling.

III. Time- dependence of activity labeling

The hindlimb attached preparation was used to study the time dependence of activity-dependent labeling in the presence of. NMDA (5 μM) was applied to induce activity-dependent labeling at three different time periods. The procedure employed was the same as Kjærulff et al., (1994) in order to compare our observations with their results. Figure 10A shows the scanned images of L3-L4 segments from Kjærulff's experiments which indicates the distribution of sulforhodamine-labeled cells after a 4 hour incubation in NMDA and 5-HT in the presence of locomotor activity. The labeled cells are mostly seen in dorsal horn, around central canal and intermediate gray matter areas. The results of our experiments are shown in Figure 10B. In the absence of locomotor activity and after 45 minutes labeling with sulforhodamine, labeled neurons were seen predominantly in the dorsal horn (lamina I-II) with a few neurons in lamina V. Then after 90 minutes more labeled neurons were pronounced in laminae V-VI. Finally after 180 minutes of incubation there was very intense staining of neurons in dorsal horn, intermediate gray

matter and around central canal. We conclude that the distribution of neurons labeled in the presence of NMDA is time-dependent. Further we observed that the pattern of labeling observed after 180 minutes incubation (in the absence of observable air-stepping) is nearly identical to that seen by Kjærulff et al (1994) (compare Figures 10A and 10B).

In contrast to NMDA, the effect of 5-HT on the distribution or number of neurons labeled does not appear to be time-dependent within the time range we examined. This is shown in Figure 11 where we plot the number of neurons labeled in the L2-L3 spinal segments versus different time periods allowed for 5-HT evoked locomotor activity-dependent labeling in 8 animals. Note that with different time periods the number of labeled neurons are remarkably similar.

IV. Topographical distribution of labeled cells resulting from 5-HT evoked locomotor-like activity

The isolated spinal cord preparation was used in twenty neonatal rats aged from 0-4 days old in order to investigate the distribution of the locomotor neurons evoked by 5-HT (10 - 100 μM) in regular K^+-containing ACSF (2.5 mM). In twelve of those preparations there was a consistent rhythmic motor activity with frequencies that varied from animal to animal between 0.02 and 0.18 Hz, which we considered as low frequencies. Further as reported by Cowley and Schmidt 1997 Fig. 1, there was a gradual tendency for the frequency of rhythmic motor activity to decreased with time in a given animal (figure 12

shows frequency versus time in 3 different animals). In most experiments, locomotor activity was maintained for over two hours. Figure 13 presents a representative example of the distribution of labeled neurons evoked by 5-HT during locomotor-like activity as verified with alternating activity observed between L2 ventral roots (frequency = 0.1 Hz). In contrast to the observations of Kjærulff et al (1994), which indicated that locomotor labeled cells are located around central canal and medial lamina VII (cp. their Figure 5), our results suggest that distribution of interneurons labeled by activity dependent labeling evoked by 5-HT, is relatively diffusely scattered.

Because the lower thoracic and upper lumbar spinal segments appear to be primarily involved in the central pattern generation of hindlimb locomotion (Cazalets et al , 1995; Kjærulff and Kiehn 1996; Cowley and Schmidt 1997) we recently extended our analysis of locomotor-labeled neurons to lower thoracic segments. In order to show the distribution of locomotor-labeled cells in lower thoracic spinal cord, three animals were used. The locomotor neurons were evoked by 30 μM 5-HT then the lower thoracic and upper lumbar (T11-L3) segments were reconstructed. An example is shown in Figure 14. As observed in the lumbar spinal cord, locomotor-labeled neurons are observed to be diffusely distributed along these segments.

In comparison to the results of Kjærulff et al (1994) we conclude that the distribution of labeled neurons evoked by NMDA alone, in the absence of locomotion, is the same as distribution of neurons during locomotion evoked by combination of NMDA and 5-HT.

Hence, studies which include NMDA in the bath to induce locomotion are recruiting additional populations of neurons unrelated to the spinal locomotor network. Thus the present distribution and number of activity labeled cells evoked during 5-HT induced locomotion is more likely to represent neurons that participate directly in the generation of locomotion.

V. Laminar distribution of locomotor-labeled neurons resulting from 5-HT evoked locomotor-like activity

In order to examine the laminar distribution of labeled neurons, the distribution of labeled neurons were superimposed on an outline of spinal cord divided into Rexed's laminae (modified from Kjærulff et al 1994) along the lumbar spinal cord. The average number of labeled cells active during locomotion were counted in 5 different animals. As shown in the example reconstruction in Figure 15 (right hand side), the locomotor neurons are most abundant in lamina VII (medial and lateral) but are also seen scattered in other laminae. The average distribution is also presented graphically in Figure 15 (left hand side). Motor nuclei (lamina IX) and superficial laminae (I-II) were typically devoid of labeled neurons.

VI. Relative segmental distribution of locomotor-labeled neurons

Figure 16 presents the relative percent distribution of labeled neurons in the lumbar spinal segments (average of 5 animals). Note that in spinal segments L1 though L5 there is an almost identical percent distribution while there are relatively fewer labeled neurons in

L6 (This reduction can be due to the fact that L6 is the segment where there is an abrupt reduction in size). Total cell labeling was less than 1200 per lumbar segment. If the number of labeled cells accurately reflects the interneuronal population recruited during locomotion, it suggests that only a very small fraction of the neurons contained within the spinal cord comprise the pre-motoneuronal locomotor circuitry.

VII. Frequency dependence of locomotor labeling

In *Xenopus*, Roberts et al (1993) showed that the rhythmic activity during swimming starts with high frequency, then gradually declines to a lower level and finally swimming ceases. They observed that the firing frequency of premotor interneurons increased as locomotor frequency increased. Therefore, they proposed that a portion of premotor interneurons pool may be silent at low frequencies but could be recruited in response to increasing the frequency and contribute to more intense behavior. Similarly, in the marine pteropod mollusk *Clione*, increases in the frequency of swimming are associated with the recruitment of interneurons and they are otherwise subthreshold at low frequencies (Arshavsky et al 1985). Based on these results, we investigated whether in mammalian preparations, recruitment of interneurons active during locomotion increases with higher frequencies. To achieve this, 12 isolated spinal cords were used and locomotor activity was evoked by application of 5-HT. In order to increase locomotor frequency, 1-3 mM KCl was applied to our regular ACSF (which contained 2.5 mM KCl). We chose to control locomotor frequency with $[K^+]_o$ because; (i) Squali Houssaini et al (1993) demonstrated in neonatal rat that increasing $[K^+]_o$ was associated with increased

locomotor frequencies, and (ii) we have already shown (Fig. 5) that increasing $[K^+]_o$ does not contribute strongly to increased activity labeling in the concentration range used. In seven preparations increasing $[K^+]_o$ increased locomotor frequency to between 0.2-0.4 Hz. Although we have already shown the duration of SR incubation is not related to the number of neurons labeled in the time range we examined, we still chose to incubate spinal cord's which underwent faster frequencies for half as much time. Figure 17A shows two examples of the distribution of recruited locomotor neurons evoked by 5-HT (40 μM) during low and high frequency. Note that in this comparison, more neurons were recruited at the higher locomotor frequency. Figure 17B shows the relationship between the intensity of labeled neurons and frequency during low and high speed. We observed that increased locomotor frequencies were weakly correlated with increased number of sulforhodamine labeled cells (# labeled cells = 447 + (311 * frequency; R = 0.50 and P= 0.07). The weak positive correlation between locomotor frequency and number of neurons labeled could be accounted for by nonspecific recruitment related to adding K^+ and suggests that locomotor frequency may be predominately regulated by factors other than increased neuronal recruitment (e.g. increased firing frequency of recruited neurons).

DISCUSSION

In order to begin to understand the CPG, a classification of neurons active during locomotion in the *in vitro* neonatal rat is necessary. The present study is an attempt to localize and identify neurons which may be engaged in spinal locomotor generation in the neonatal rat using activity-dependent labeling and extracellular recordings during locomotion.

I. Comparison of sulforhodamine with other activity markers

The fluorescent dye sulforhodamine 101, seemed promising since, it has been used to label activated motor nerve terminals in snake (Lichtman et al, 1985) and epileptiform activity in turtle visual cortex (Kriegstein et al, 1988). These highly charged fluorescent molecules do not directly permeate cellular membranes but instead, enter the cell via pinocytotic vesicles. Molecules such as sulforhodamine and Lucifer yellow accumulate in the lysosomes over a time course of almost an hour (Swanson et al, 1985; Wang and Goren, 1987). Sulforhodamine has a good spatial resolution and fast processing step compare to two other activity markers, namely ^{14}C-2-deoxyglucose (2-DG) and the expression of the C-fos gene. The degree of 2-DG labeling contributes to the intensity of metabolic activity, which is particularly large and at active synapses. The main disadvantages are its limited resolution, the long processing time required for autoradiography, and difficulty in distinguishing between synaptic excitation and inhibition. With C-fos labeling active neurons can be stained using immunohistochemistry for the fos protein. However, the label is contained exclusively in the cell nucleus since, fos is a nuclear regulatory protein. Also, because there are many

unknown steps between synaptic or electrical activity and fos expression makes it difficult to relate C-fos labeling directly to neural activity (Keifer et al, 1992).

II. Relation of labeling to the intensity of synaptic input

The distribution of labeled cells evoked by 5-HT during locomotor-like activity supports the hypothesis that sulforhodamine uptake is activity dependent. During locomotion the number of diffusely distributed locomotor neurons in Rexed's laminae which are strongly active and take up the label, are more pronounced compare to labeling distribution in the absence of locomotion (compare figure 4 to figure 13). Clearly, not all neurons which receive synaptic input during locomotion are labeled. (For example neurons which receive subthreshold EPSPs during locomotion but do not fire action potentials would not release transmitter at their presynaptic terminals in order to take up SR-101.

In previous physiological studies of bursting in the red nucleus (Keifer and Houk, 1989, 1991a), it was estimated that 20-50% of red nucleus neurons produce burst discharges in response to spinal stimulus whereas the other do not. Therefore, many neurons do not receive intense synaptic input, and may contribute to unlabeled neurons observed in that study. Hence, they believe that only bursting neurons take up the label. In the present study we support this hypothesis in which labeling is correlated with the intensity of synaptic input which neurons receive during locomotion and since, only a small population of neurons are labeled during 5-HT evoked locomotion (Fig 15), we could argue that not all neurons which receive synaptic input are labeled but only neurons

which fire action potentials.

III. Labeling pattern

We have observed the distribution of activity-dependent labeled cells evoked by chemical stimulation under different experimental conditions are located throughout the isolated spinal cord. After removal of dorsal roots the number of locomotor-labeled neurons in the dorsal horn (laminae I-IV) was reduced compared to those experiments with dorsal roots intact, likely due to a reduction of afferent input into the dorsal horn. However, the diffuse distribution of labeled neurons in medial and lateral intermediate gray matter (laminae VI-VII) and above central canal (lamina V) and laminae VIII and X remained the same following isolation of the spinal cord. We suggest that the labeled cells diffusely located in these areas are engaged in the central locomotor rhythm generation in the neonatal rat spinal cord.

Keifer et al (1992) showed that sulforhodamine uptake by neurons depends on synaptic activity. They investigated this finding by blocking the synaptic transmission with low-calcium, high-magnesium solution or the excitatory amino acid receptor blockers 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D-aminophosphonovalerate (APV). This investigation was compatible with previous reports which suggested that sulforhodamine was taken up selectively in electrically-activated motor terminals through endocytosis (Lichtman et al 1985). Kjærulff et al (1994) indicated that sulforhodamine is taken up in an activity- dependent manner during drug-induced locomotor-like activity and found that less intensive or no labeling was obtained when the staining period was

decreased in locomoting animals, and a lack of staining was observed in non-locomoting animals (Kjærulff et al 1994).

Previous studies in the rabbit and cat using activity markers have indicated mostly intermediate gray matter and around the central canal as areas important for generating spinal locomotion. Viala et al (1988) visualized 2-DG radioactive uptake during L-DOPA-induced fictive locomotion in the rabbit. The labeled cells were found in the intermediate gray matter. Also in locomoting cats, activity induced c-fos labeling was found in cells in the intermediate gray and lamina X (Dai et al 1990). C-fos is marker regulatory protein that labels the nucleus.

Our results reveal that the locomotor-labeled cells are diffusely distributed throughout the cord. Our results are in part in contrast to Kjærulff et al (1994) in which they observed a dense distribution of labeled cells in lamina VI, medial intermediate gray matter (lamina VII) and around central canal (lamina X). We have performed experiments which provides evidence that this difference can be explained by their use of NMDA (5.0-7.5 μM). Further, their use of high external K^+ also would cause general cell depolarization and may cause some neurons to produce action potentials and consequently take up label non-specifically.

In our experiments, we have observed that the labeling pattern was similar throughout the Rexed's laminae except lamina IX and laminae I-II. This is in partial contrast to the study

of Kjærulff et al (1994). Also we suggest that the spinal locomotor neurons are diffusely distributed along all spinal segments examined (T11-L6) (see Results). By duplicating the Kjærulff et al (1994) experiments we have also shown that, even in the absence of locomotion, NMDA has a direct excitatory action on a significant fraction of neurons in the spinal cord.

In our experiments, there was a faint staining of motoneurons or no staining at all. The absence of motoneuronal labeling may be due to the fact that their synaptic terminals are predominantly located at the neuromuscular end plate, a distance too great to allow retrograde transport following uptake (Kjærulff et al 1994). Occasionally however, motoneurons are labeled. This may be a result of axon collaterals which synapse on Renshaw cells or due to retrograde transport from cut axons in experiments where ventral root were cut.

IV. The advantages of 5-HT over NMDA

In contrast to NMDA, 5-HT can activate and maintain a functionally relevant locomotor pattern. 5-HT is the best single agent for the induction of a locomotor-like pattern (Cowley and Schmidt 1994). This paper suggests that 5-HT plays an important role in the endogenous activation or operation of mammalian locomotor network. In the present study we have shown that 5-HT is a reliable agent which specifically recruits locomotion. The present study provides the first realistic appreciation of topography of neurons

recruited during locomotion (induced by 5-HT). The number of locomotor-labeled neurons evoked by 5-HT alone in the isolated spinal cord are relatively low in each segment (compared to the numbered induced with NMDA, see Results) and are scattered throughout the spinal gray matter except in Rexed's laminae I-II and IX. Hence, we conclude that the location of the neurons responsible for locomotor activity is not strongly restricted to specific areas of the spinal cord.

Another advantage of 5-HT over NMDA is that labeling produced by 5-HT unlike NMDA is not time-dependent. In the results section we have shown that with different labeling time, the number of labeled neurons are in the same range and very close together. This finding further supports the notion that the locomotor-labeling pattern observed with 5-HT is reliable. The NMDA receptor has a particularly widespread receptor spinal distribution in the neonatal rat (Kalb et al 1992) and, since NMDA causes more cells to fire, it is not surprising, then that many spinal cord neurons are labeled following bath application of NMDA and there is a time dependent recruitment of different population of neurons. In contrast to NMDA, bath application of 5-HT evokes only a small fraction of neurons during locomotor activity.

V. Localization of rhythm-generating network

Kjærulff et al (1996) investigated the localization of rhythm-generating networks as revealed by horizontal sections in which they separated the ventral half from dorsal half

in the spinal cord. They isolated the ventral half of the spinal cord and recorded from ventral roots, and they observed that left/right alternation and rostrocaudal alternations were still present. Hence they concluded that the rhythm-generating network exists in ventral horn. However, our results suggest that locomotor-labeled neurons evoked by 5-HT are diffusely distributed throughout the spinal cord. This in- compatibility perhaps could be explained due to the fact that in Kjærulff study it was not possible to determine whether rhythmic activity was generated in the isolated dorsal half during locomotion. However, because 5-HT receptors are distributed throughout the spinal cord, it is reasonable to assume that 5-HT evoked locomotion may excite neurons both above and below the central canal (Marlier et al 1991). Marlier et al (1991) investigated the pattern of 5-HT receptors including 5-HT_1, 5-HT_{1A}, 5-HT_{1B} and 5-HT_2 in the rat spinal cord using [H^3]5-HT for 5-HT_1 and [H^3]-ketanserin for 5-HT_2. It has been shown that 5-HT_{1B} receptors and 5-HT_2 receptors are present in both superficial and deep laminae of the dorsal horn consistent with the observation that serotonergic axons from raphe nuclei also project to the deep dorsal horn area. Marlier et al (1991) indicated both 5-HT_{1B} and 5-HT_2 receptors are present in intermediolateral laminae as well as central canal (lamina X) mostly in thoracic level and 5-HT_{1A} receptors are present in lamina V and in the ventral horn.

Another explanation for the ability to generate locomotor activity without the dorsal half of the spinal cord would be to imagine redundant interconnections throughout the dorsal and ventral spinal cord. Such a overlap in connectivity may help reinforce activity in the network as well as be responsible for subtle alterations motor coordination. Thus while

the Kjærulff and Kiehn (1996) would suggest that neurons in ventral half are more responsible and more important for rhythm generation, we forward the caveat that more dorsally located neurons may be equally as important in rhythm generation but lesioning studies are incapable of examining the effect of only intact dorsal horn connections to motor nuclei.

VI. The importance of lower thoracic segments in rhythm-generating

Cowley and Schmidt (1997), showed that the central pattern generator for locomotion is not restricted only to the upper lumbar segments (Cazalets et al, 1995), since the application of neurochemicals to supralumbar portions of the spinal cord was used to investigate the effect of supralumbar circuits on the lumbar cord. Rhythmic activity was induced in the cervical and thoracic spinal cord after application of 5-HT to the isolated cervicothoracic region. 5-HT application to the lumbosacral cord (which included the rostrolumbar segments (L1-L2) failed to elicit lumbar rhythmicity (Cowley and Schmidt, 1997; Magnuson and Trinder, 1997). There was only tonic excitation that is required to bring lumbar circuitry above threshold for responding to the descending rhythmic activity form the supralumbar oscillatory network (Cowley and Schmidt, 1997). Also L2 ventral root rhythmic activity was abolished by transection at the segments from T12 to L1 junction although the spinal cord was exposed to 5-HT/NMDA below the lesion. The present study shows that the distribution of the locomotor labeled neurons during rhythmic activity evoked by 5-HT was diffusely distributed in Rexed's laminae throughout the lower thoracic (T11-T13) of the neonatal rat spinal cord using activity

dependent labeling (Fig. 14). In the present study, the application of 5-HT to the entire cord including the thoracic part also elicited locomotor-like activity which was recorded from the lumbar ventral roots. Therefore, the network of neurons activated in lumbar cord may be due to a synaptic drive from supralumbar regions. The activation of rostral lumbar cord segments by neurochemicals is not critical for rhythm generation (Cowley and Schmidt, 1997). Also MacLean et al 1995, showed that the lumbar midsagittally sectioned neonatal rat spinal cord *in vitro*, with one lumbar hemicord removed but with thoracic segments intact, is capable of producing rhythmic activity in the presence of 5-HT and NMDA.

Magnuson and Trinder (1997) also showed the importance of lower thoracic segments to generate rhythmic activity. Responses to VLF stimulation was elicited after the midsagittal section of the entire cervical cord and the thoracic cord down to level T9-T10. Rhythmic activity was also elicited after the midsagittal section of the lumbar part caudal to L3. However, if the midsagittal thoracic lesion was extended caudally to include the lower thoracic segments (T12-T13) no rhythmic activity could be induced. On the other hand, Cowley and Schmidt clearly showed that midsagittal lesions restricted to the thoracolumbar region do not abolish locomotion.

VII. Frequency-labeling relation

Sillar and Roberts (1993) suggest that the frequency of swimming in *Xenopus* embryos is determined by the proportion of the premotor interneurons which are active and contributing to rhythm generation. In the majority of these interneurons, firing decreases

as a function of swimming frequency. As determined through direct recordings of spinal interneurons, the number of active neurons (descending excitatory interneurons) decreases as frequency declines although the cell continued to receive synaptic input. They proposed that in many other vertebrate motor systems a proportion of the premotor pool may lie silent at low frequency but can be recruited to increase the frequency and contribute to more rapid behavior (Sillar and Roberts, 1993). Therefore, given these results, we investigated the effect of higher frequency on the number locomotor-labeled cells by adding more K^+ (up to 3 mM) to the normal ACSF (2.5 mM K^+). We conclude that frequency is determined in part by the number of interneurons which are active during locomotion (correlation coefficient, $R = 0.50$; $P = 0.07$). As observed in *Xenopus* and *Clione*, it is possible that many neurons receive only subthreshold synaptic actions at low frequencies but are recruited at higher frequencies (e.g. due to an increase in synaptic activity). An alternate, not mutually exclusive possibility is that recruited neurons increase their frequency of firing to regulate locomotor frequency. Presumably the addition of 3 mM K^+ to increase locomotor frequency would be at least partly due to a generalized membrane depolarization and consequent increased firing frequencies. A final, but unlikely possibility is that different populations of neurons control different locomotor frequencies and that frequency is regulated by a switching between networks that are somehow 'tuned' to frequencies within narrow ranges.

VIII. Summary

In summary, we have explained the distribution of locomotor neurons by the use of activity-dependent labeling technique in the present study (diffusely distributed throughout the spinal cord). Further investigation should target these locomotor labeled neurons for single cell electrophysiology characterization in a slice preparation and record from the activity of individual neurons. Finally this study presents some of the principles and methodological approaches necessary to localize the locomotor neurons.

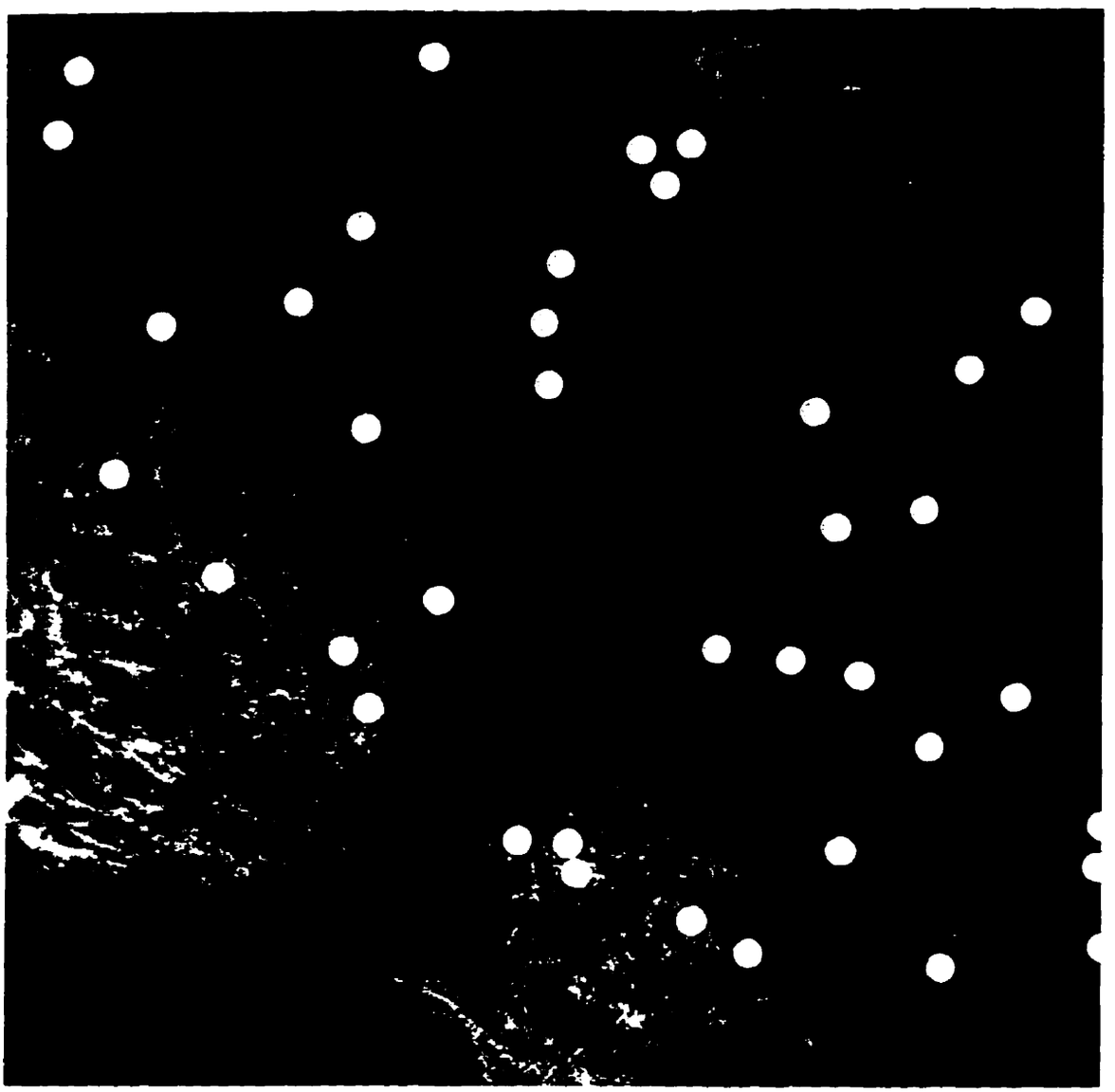

Figure 1. A sample of GFAP staining on one section of lumbar spinal cord to show that the location of immunohistochemically-identified astrocytes do not match with the locomotor labeled cells which labeled positively with sulforhodamine (represented by white dots).

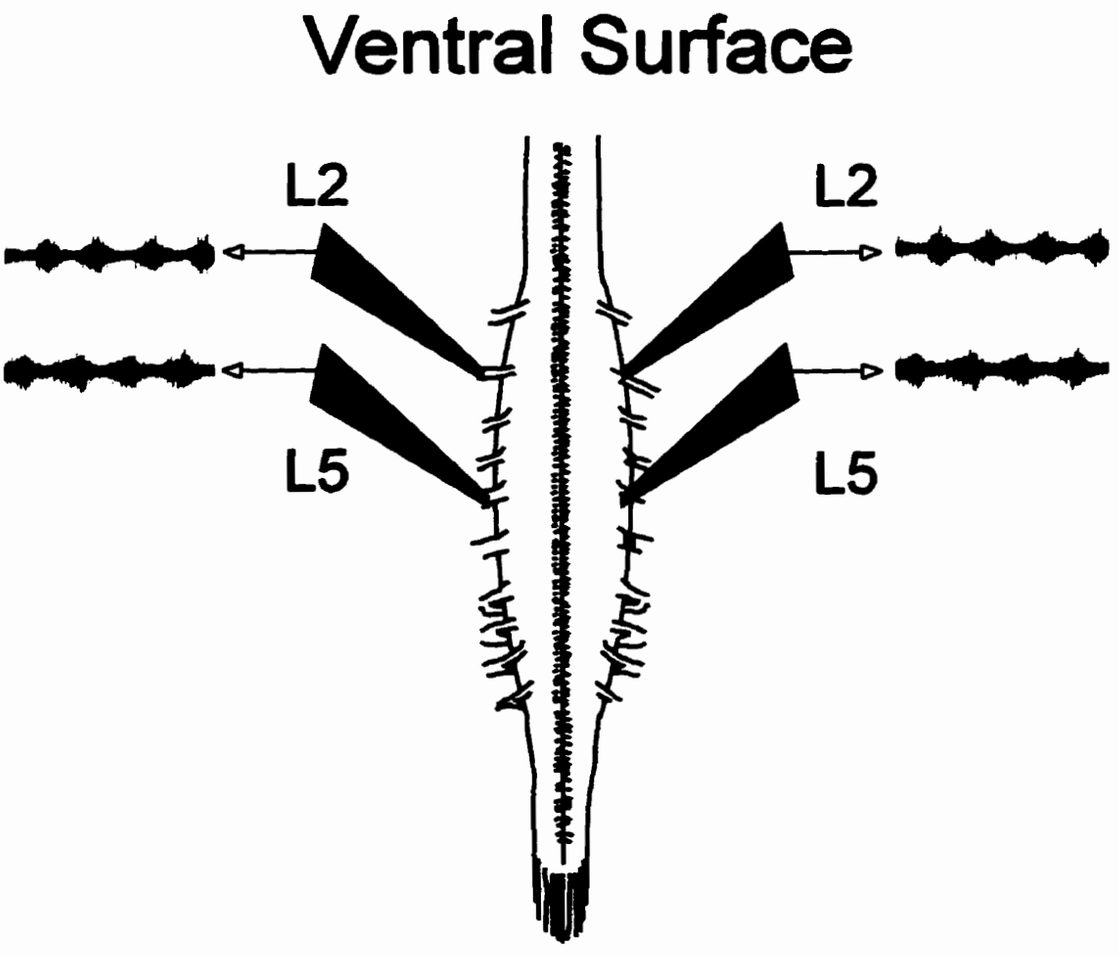

Figure 2. Experimental setup. Motor activity was monitored extracellularly with glass suction electrodes on ventral roots L2 and L5 in various combinations ipsilaterally and contralaterally.

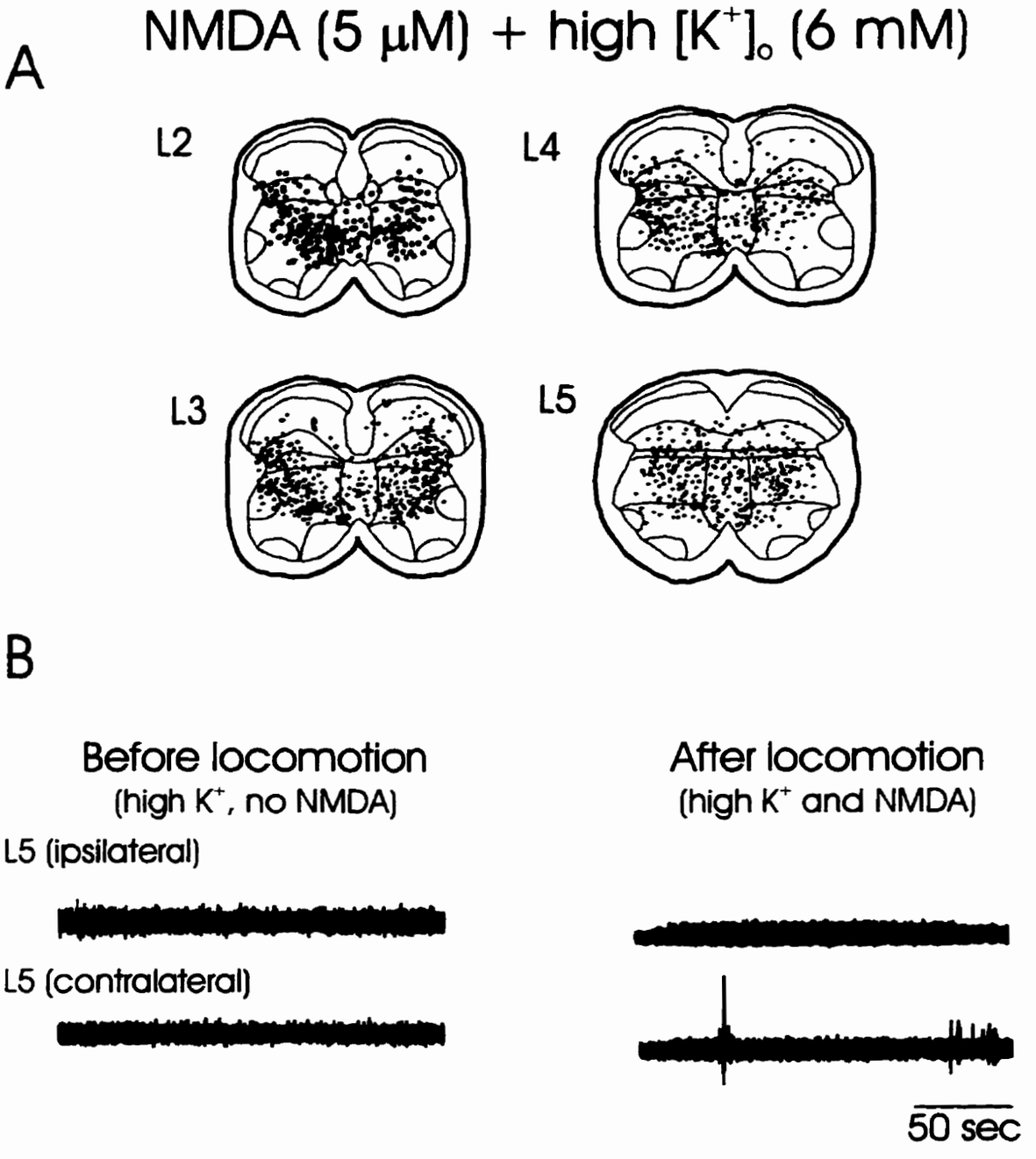

Figure 3. In this and other figures each spinal segment represents the summed reconstruction of every third 40 m section (8-12 sections reconstructed per lumbar segment). Unless otherwise specified all incubation periods were for 3 hours. **A.** Reconstructed distribution of SR activity labeled cells in L2-L5 lumbar segments following a 3 hour incubation in the presence of NMDA but in the absence of locomotor activity. **B.** Note that ventral root activity before NMDA application and in the presence of NMDA and SR (after locomotion has ceased) is virtually identical.

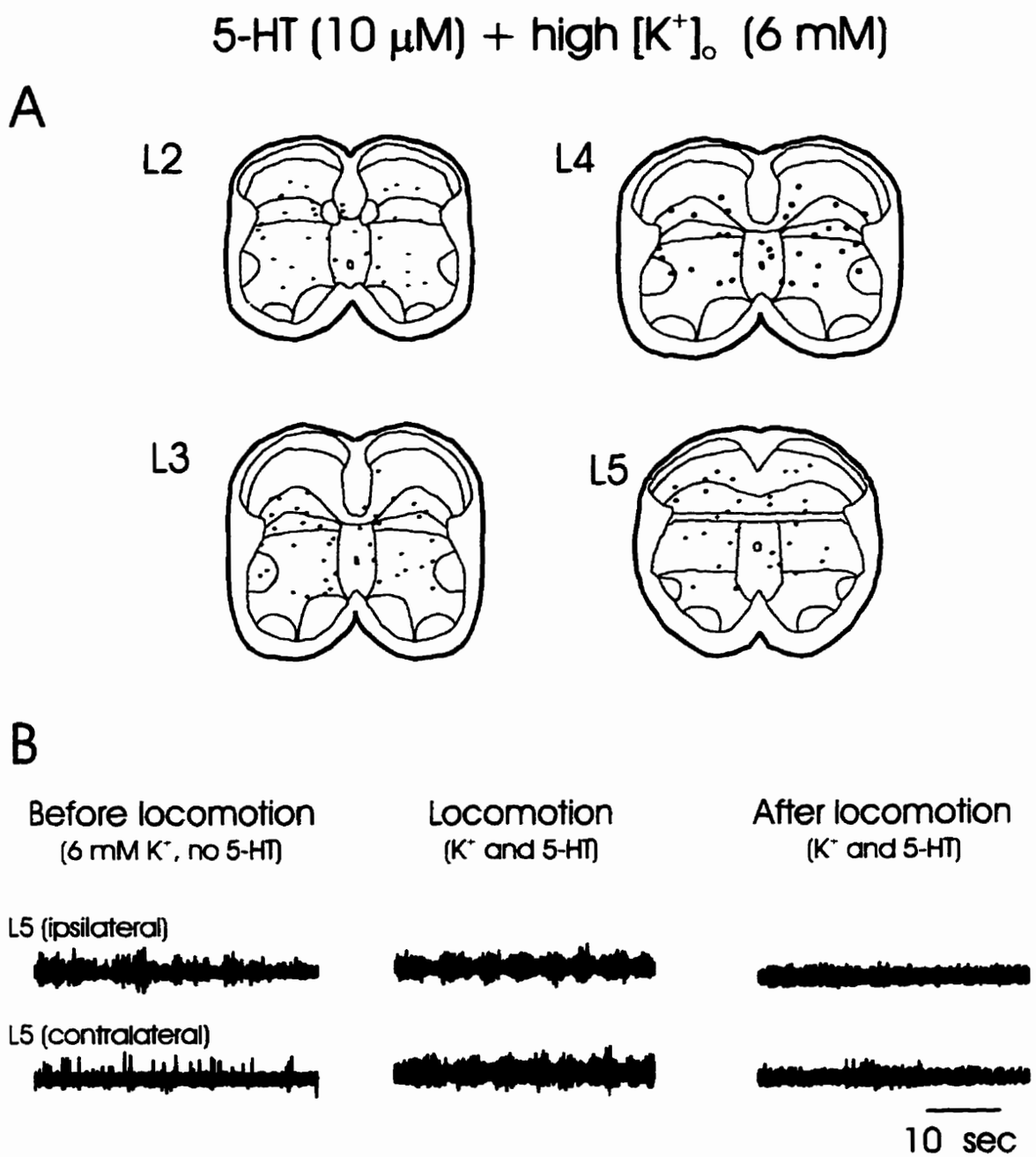

Figure 4. **A.** The effect of 5-HT and high $[K^+]_o$ on cellular activity-dependent labeling in the absence of locomotor-like activity. **B.** Sample epochs of ventral root activity before, during and after locomotion is provided.

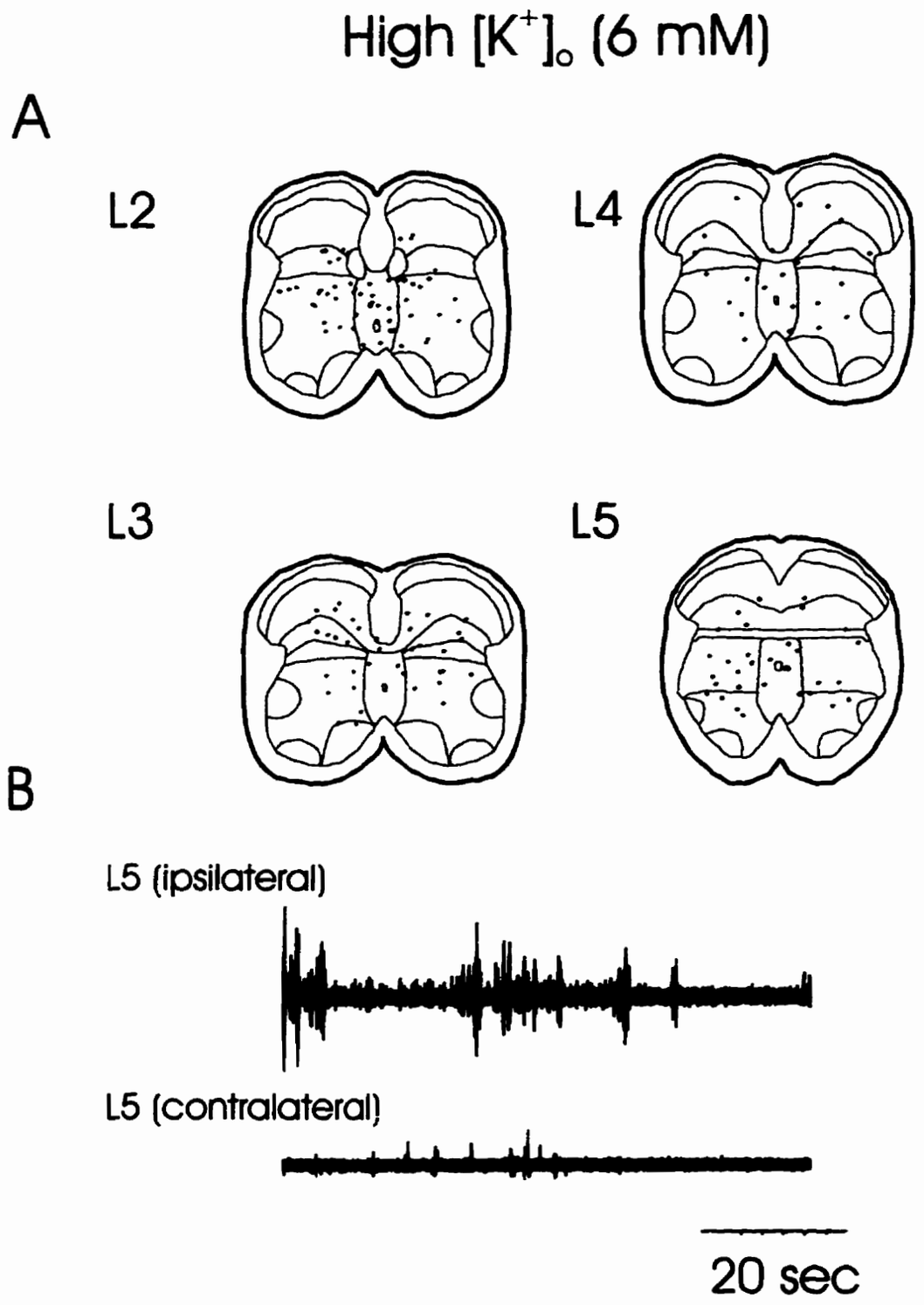

Figure 5. **A.** The effect of high K^+-containing ACSF on cellular activity-dependent labeling in the absence of locomotor-like activity. **B.** Sample epochs of ventral root activity is provided.

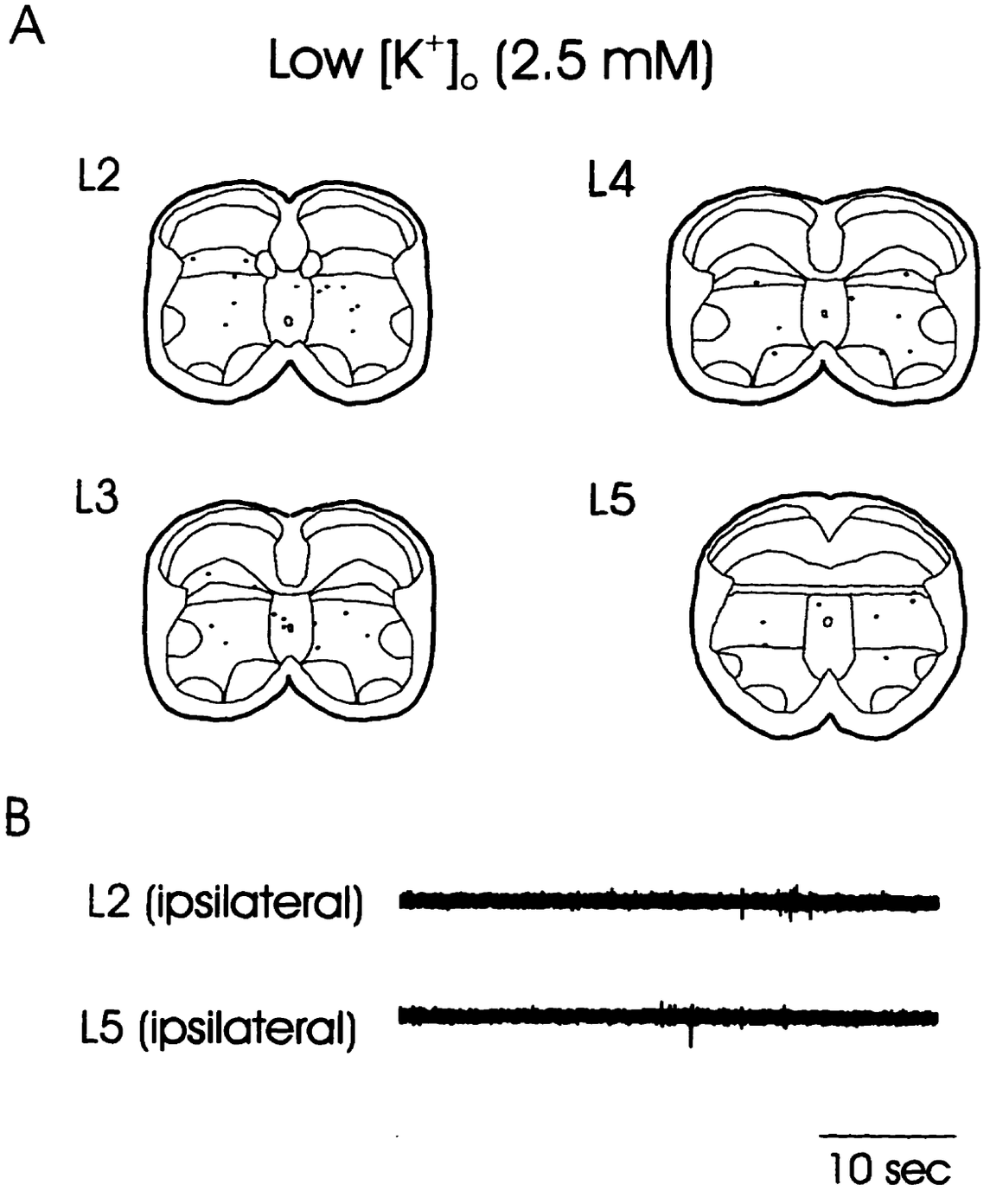

Figure 6. **A.** The effect of incubation of the spinal cord in normal ACSF (2.5 mM $[K^+]_o$) on sulforhodamine in the absence of locomotor-like activity. **B.** Sample epochs of ventral root activity are provided.

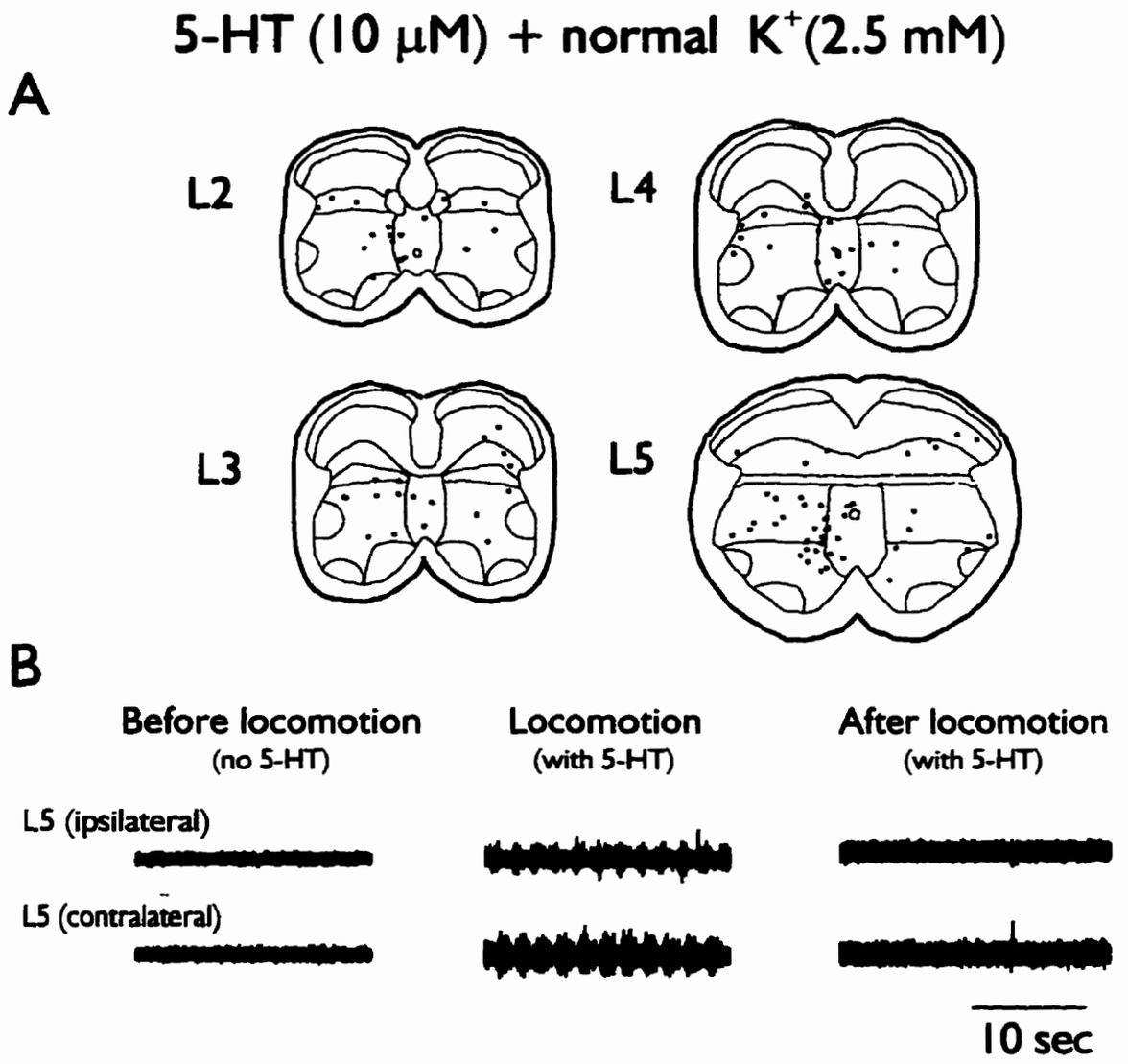

Figure 7. **A.** The effect of 5-HT on cellular activity-dependent labeling using normal ACSF (2.5 mM $[K^+]_o$) in the absence of locomotor-like activity. **B.** Sample epochs of ventral root activity before, during and after locomotion is provided.

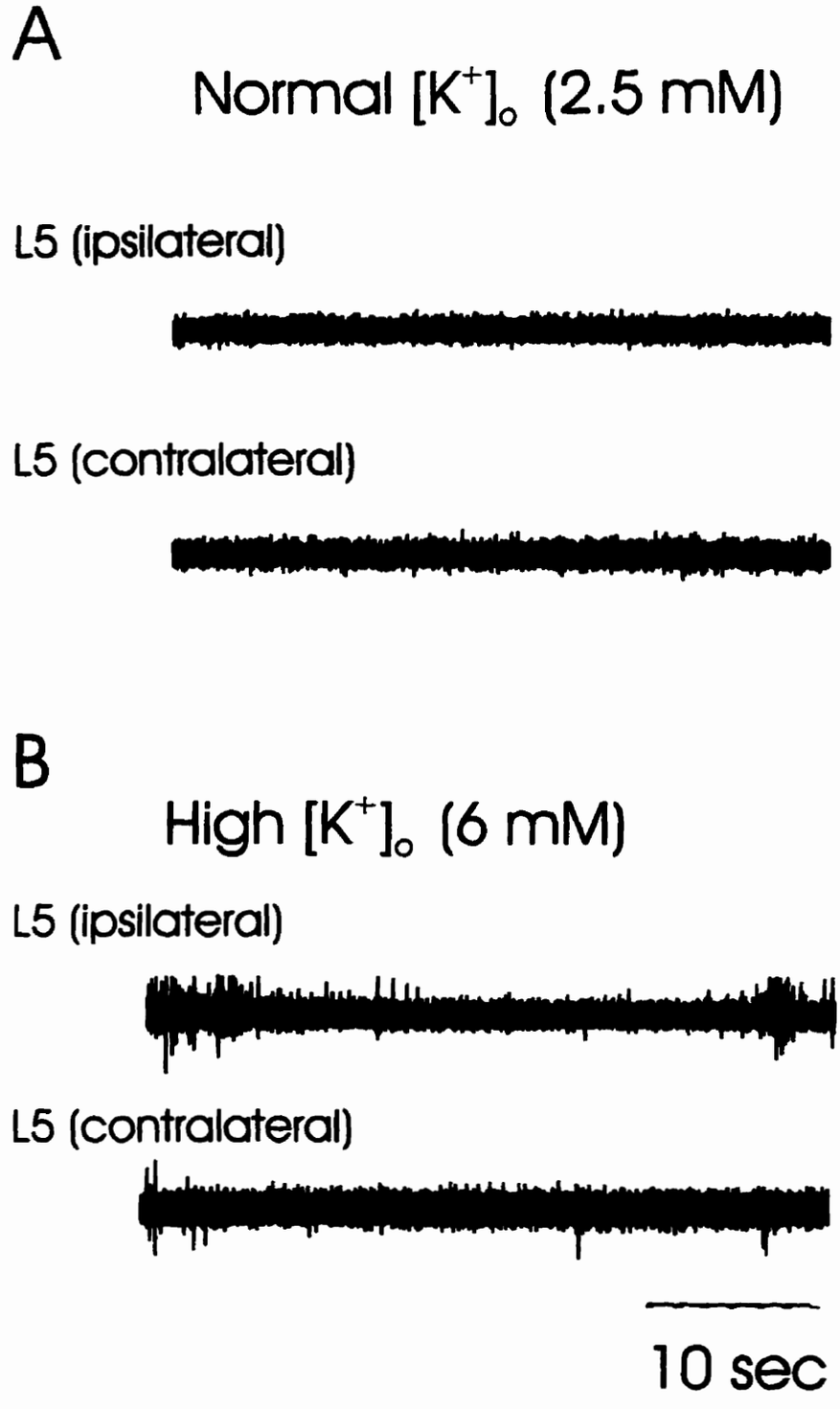

Figure 8. Comparison of the effect of low K^+-containing ACSF (**A**) and high K^+-containing ACSF (**B**) on recorded motor activity. Note that at higher $[K^+]_o$, spontaneous motor activity is observed.

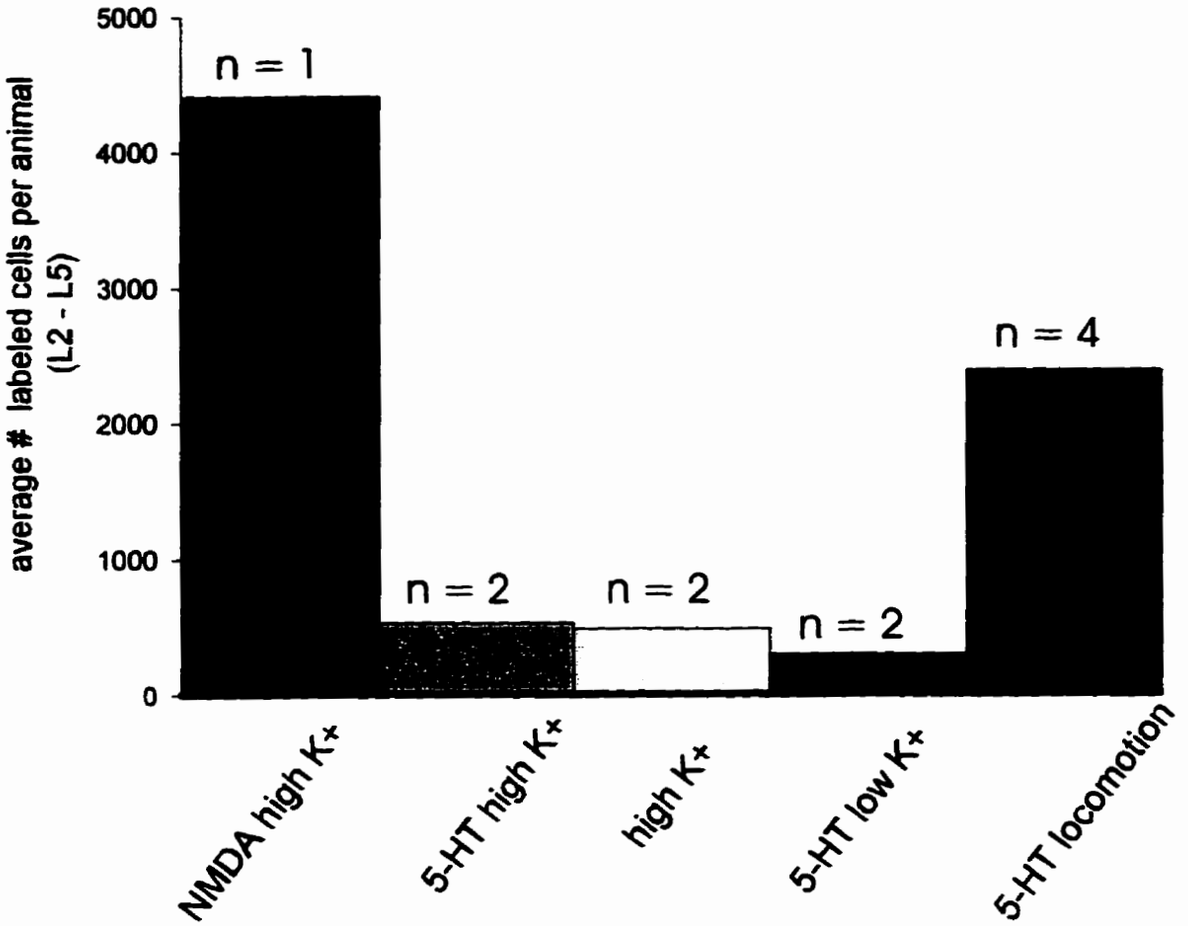

Figure 9. The average of total number of labeled cells per animal in the absence and presence of locomotor activity.

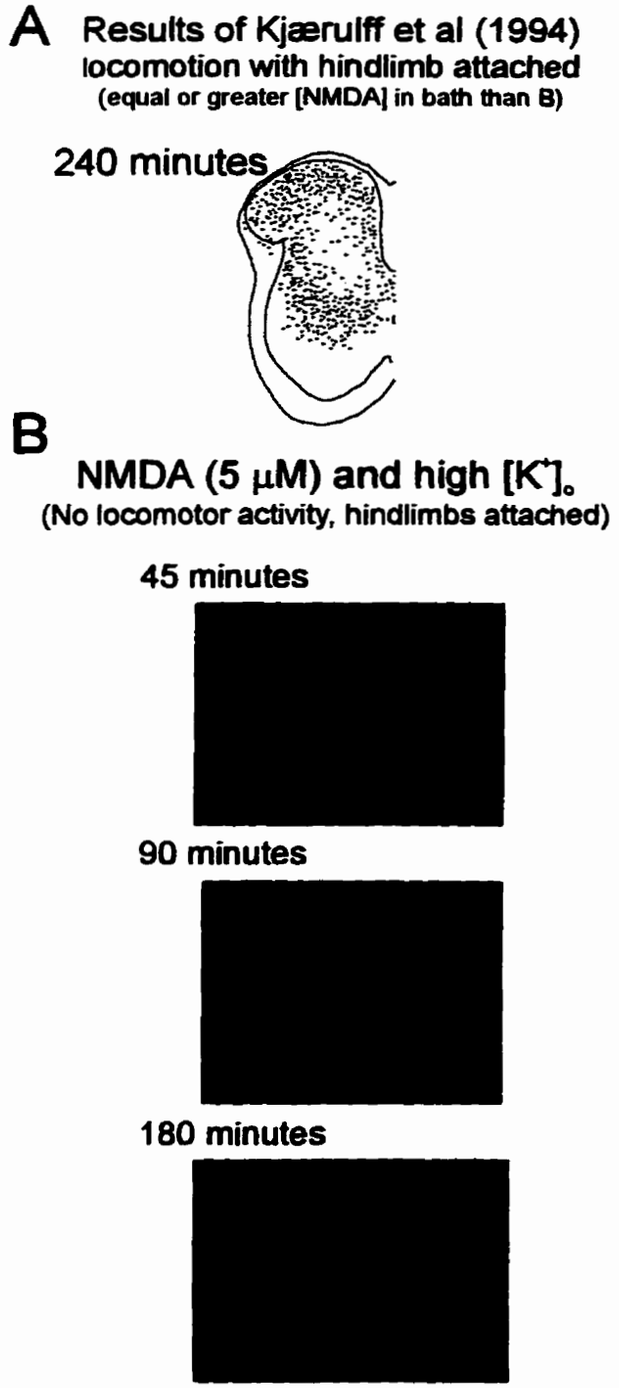

Figure 10. **A.** Scanned image of an L3-L4 spinal segment from Kjæ rulff et al (1994) showing distribution of sulforhodamine-labeled neurons after a 4 hour incubation in NMDA and 5-HT in the presence of locomotor-like activity. **B.** Distribution of NMDA-induced activity-dependent sulforhodamine labeling in 3 different animals at 3 different time periods in age- and littermate-matched spinal cords. Each photograph is from a 200 μm thick section from the L3 spinal segment taken in ACSF immediately following experimentation. Note that after 180 minutes, the labeling pattern observed is very similar to the presumed locomotor labeling of Kjærulff et al (1994).

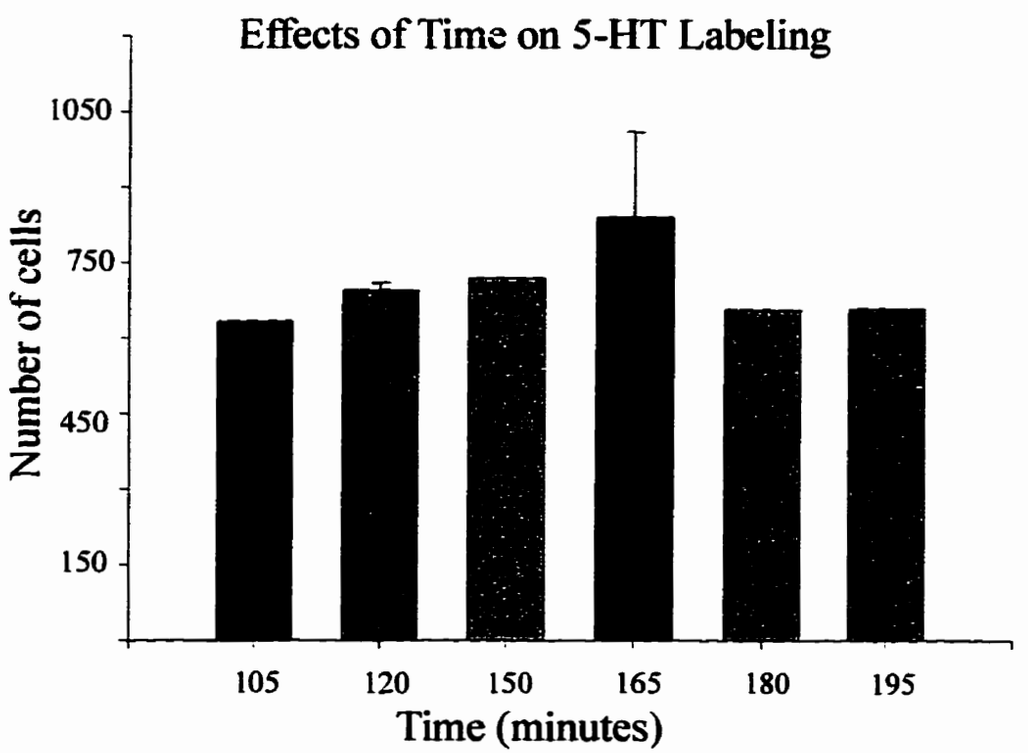

Figure 11. For 5-HT induced locomotion, between SR incubation periods of 105 to 195 minutes (in 8 animals), there is no apparent time-dependence in the number of labeled cells.

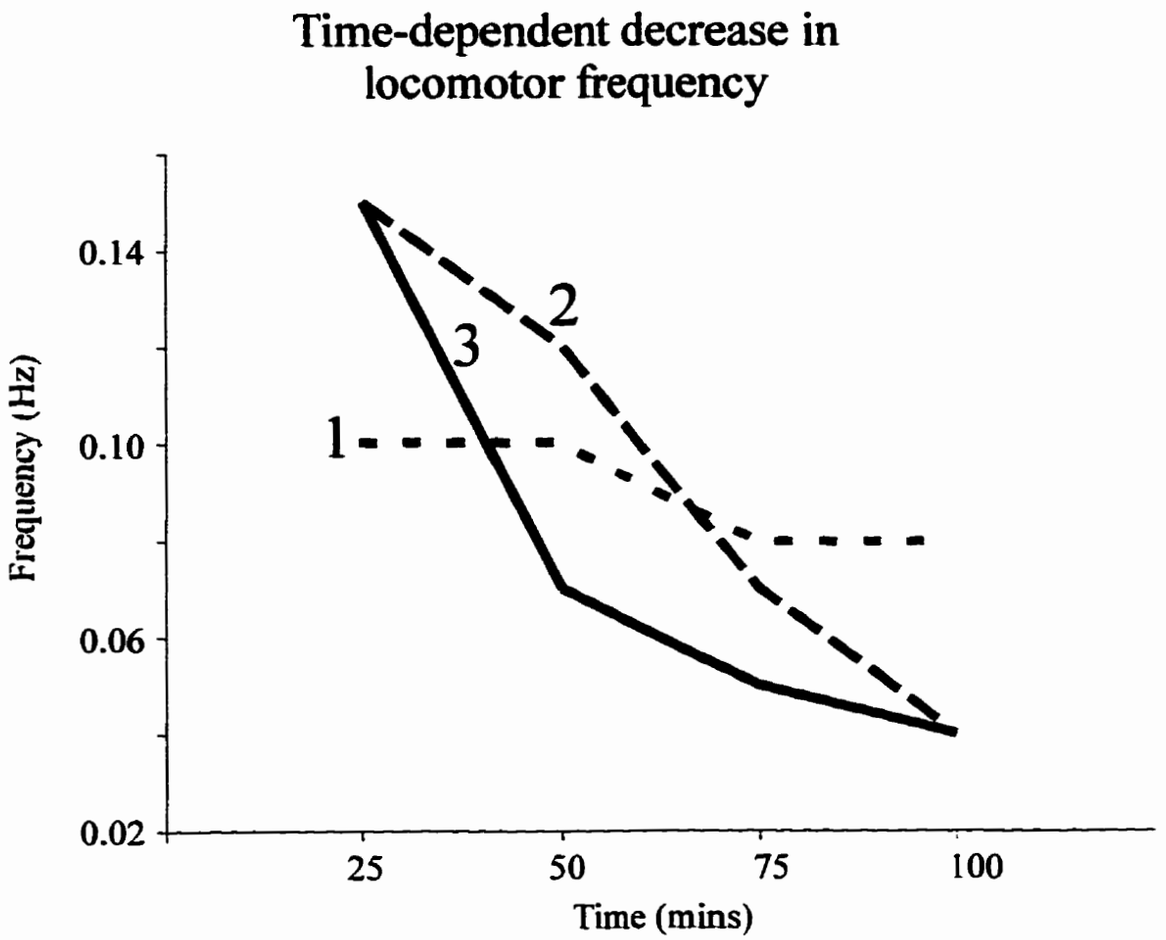

Figure 12. Time-dependence of locomotor frequency. Following the induction of the locomotor rhytyhm with bath application of 5-HT, frequency was observed to decrease progessively with time. Examples of the reduction in frequency with time is shown for 3 animals.

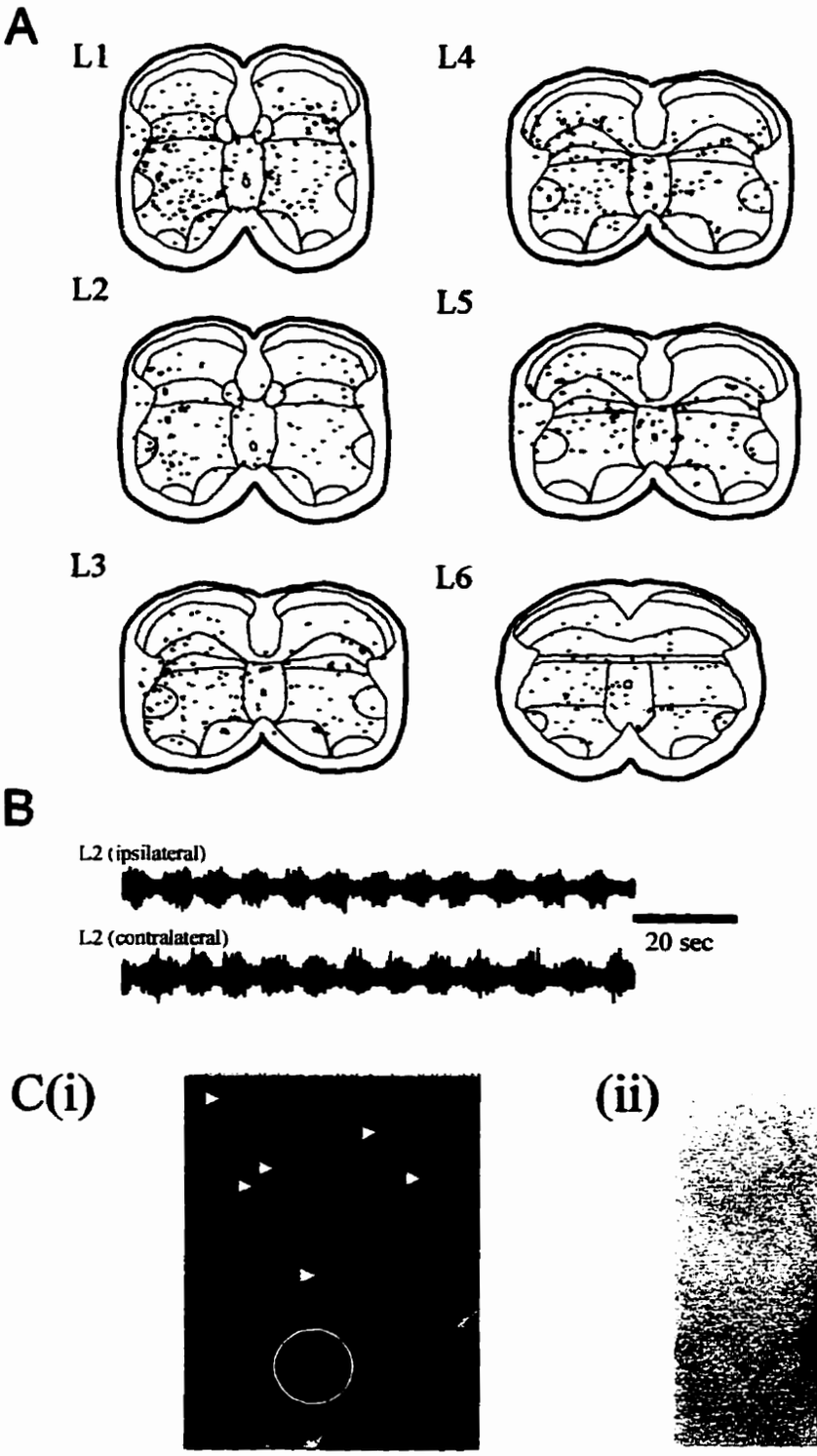

Figure 13. **A.** Sample distribution of labeled cells in lumbar segments of spinal cord resulting from 5-HT evoked locomotor activity (10 μM). Because only every third section was reconstructed, the total number of labeled cells represent approximately 1/3 of total labeling in each spinal segment. **B.** Extracellular recordings from ventral roots monitor locomotor activity. **C(i).** Sample 40 μm section illustrating sulforhodamine-labeled cells after fixation (white arrowheads). Motoneurons are also faintly labeled (white circle). **C(ii).** Sample neuron from a 40 μm fresh spinal slice to show puncta-like distribution of labeling in neuritic processes (typically lost following fixation).

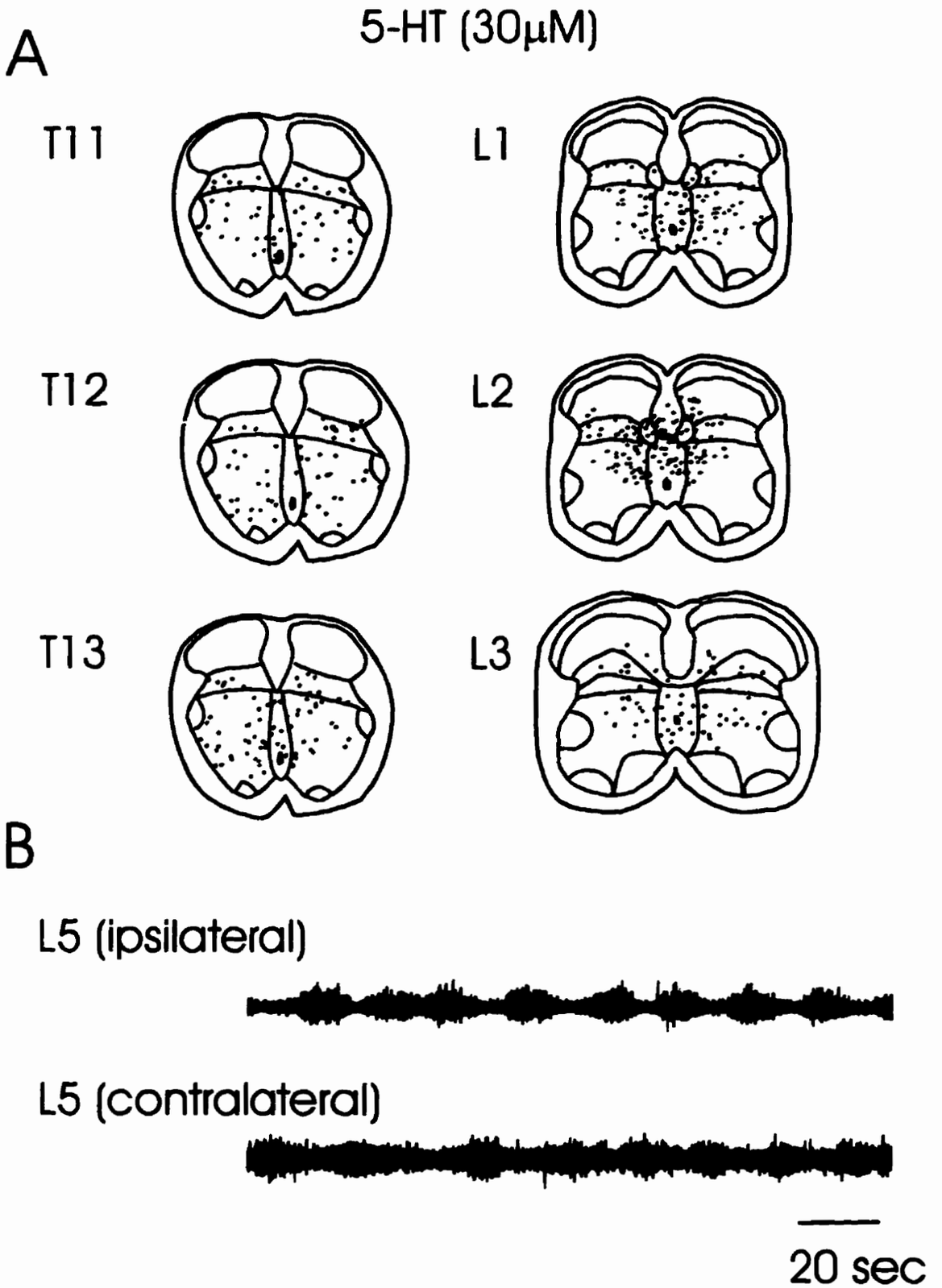

Figure 14. **A.** Example of labeled cells in lower thoracic and upper lumbar segments of spinal cord resulting from 5-HT evoked locomotor activity (30 M). Total number of labeled cells represent approximately 1/3 of total labeling in each spinal segment. **B.** Extracellular recordings from ventral roots monitor locomotor activity.

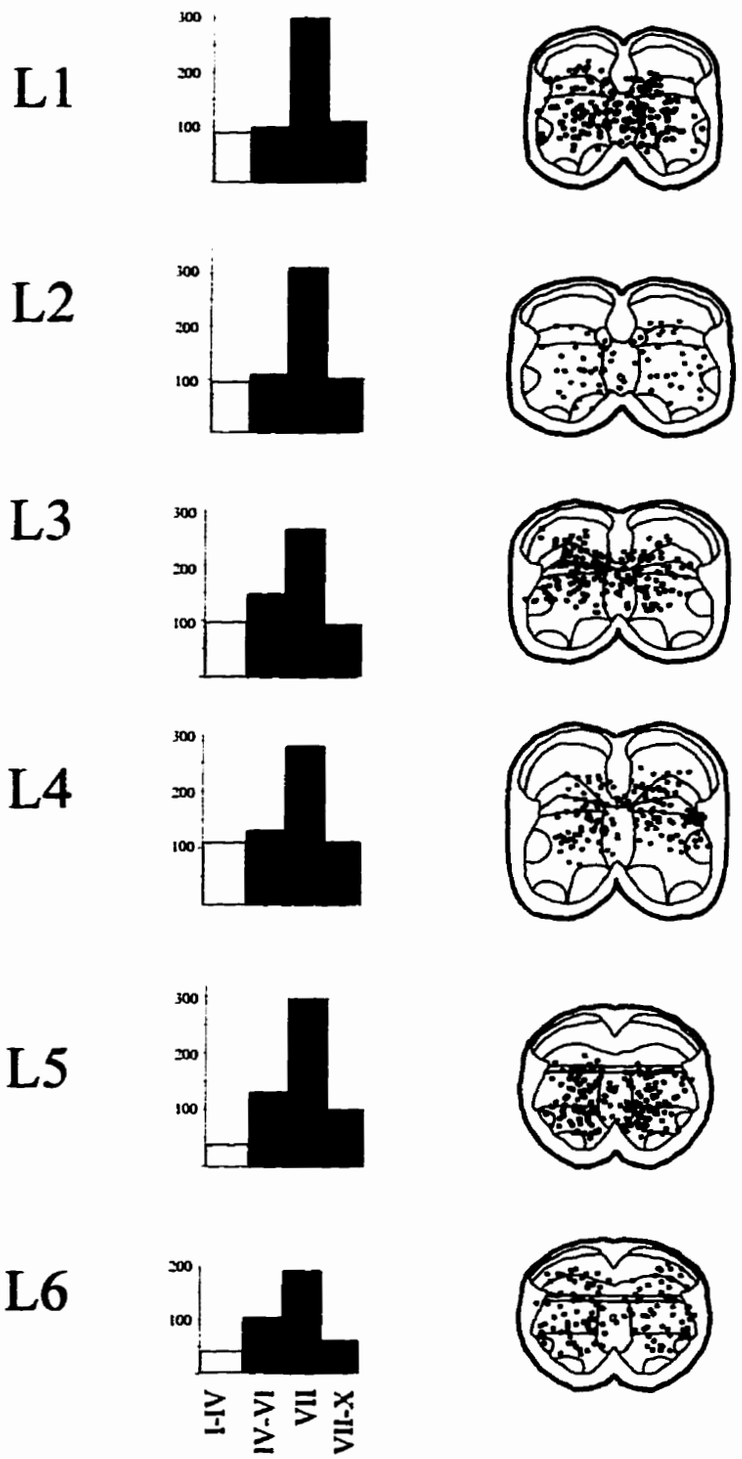

Figure 15. Left side: Average number of labeled cells per lumbar segment topographically-separated according to approximations of Rexed's laminae (n = 5). Quantitative estimate was obtained by multiplying average number of cells reconstructed in each animal by 3 (because reconstruction was from every third section). Right side: Representative distribution from one animal.

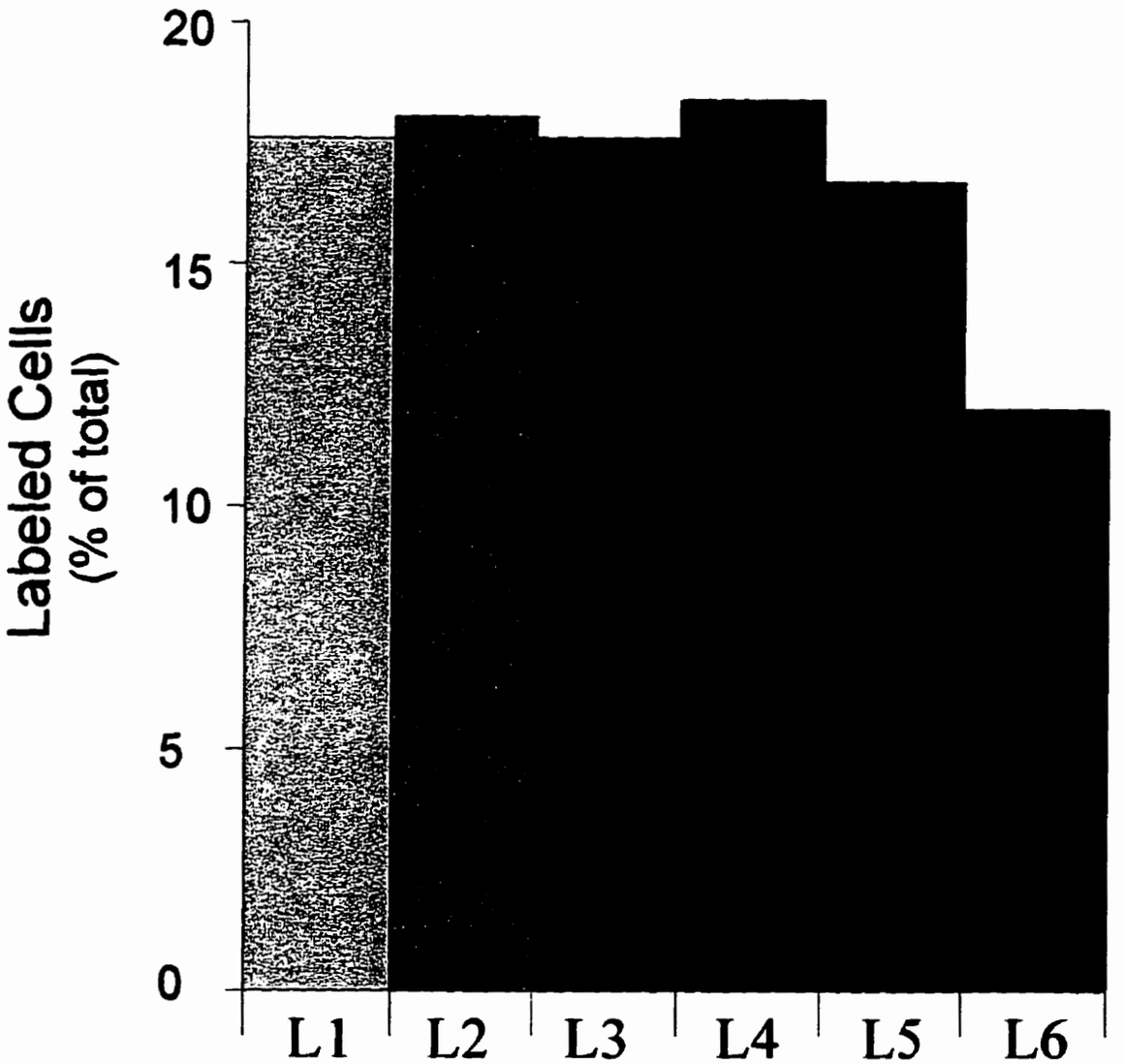

Figure 16. Percentage distribution of labeled cells per lumbar spinal segment. The segmental distribution is based on the average number of labeled cells from 5 animals.

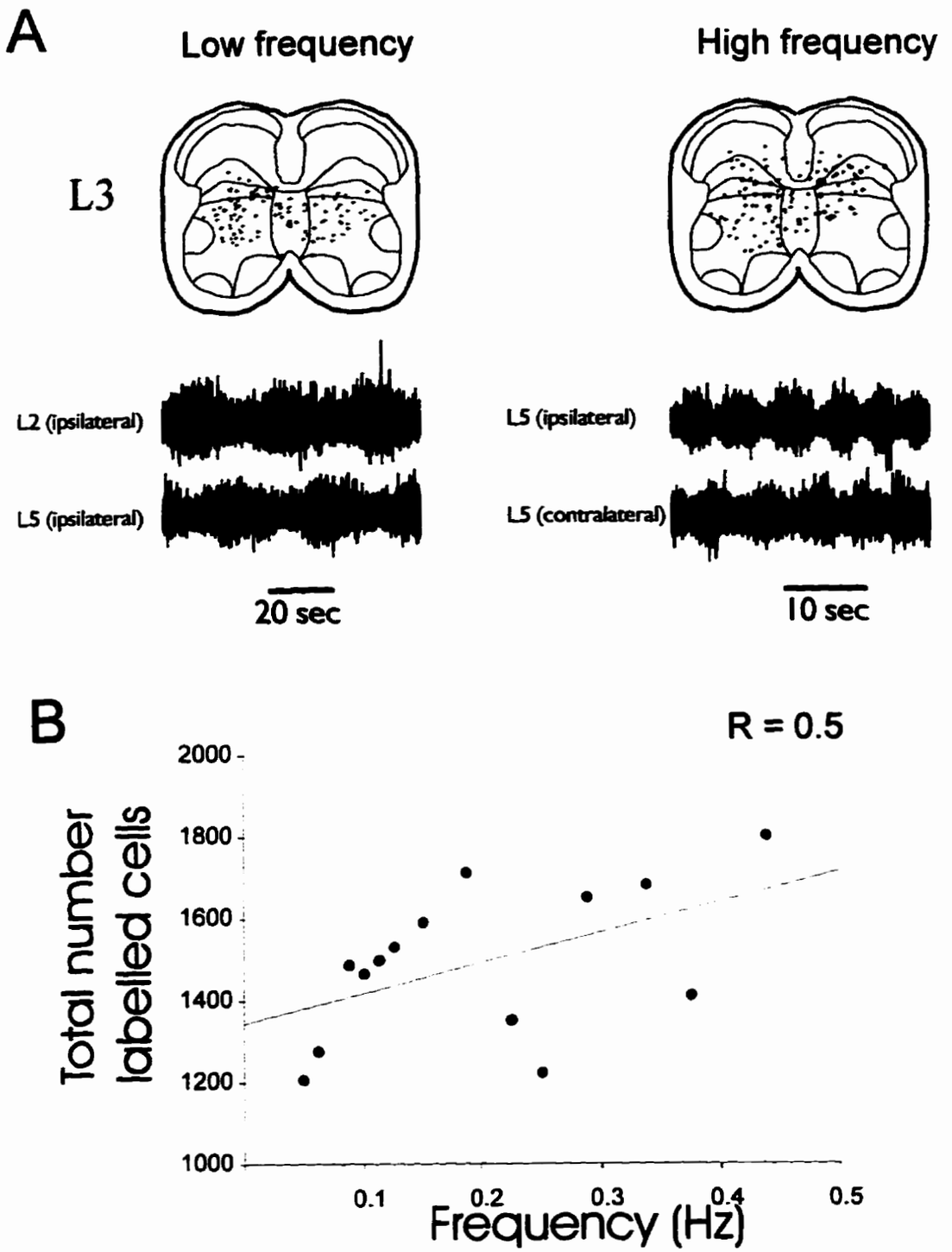

Figure 17. Frequency dependence of locomotor labeling. **A.** Distribution of locomotor-labeling at two frequencies (L3 spinal segment in both animals presented). In each animal locomotion was induced using 40 μM 5-HT. An additional 3 mM K^+ was added to spinal cord at right in order to increase locomotor frequency. **B.** Total number of labeled neurons in lumbar segments are compared to locomotor frequency. Animals with a mean locomotor frequency > 0.2 Hz were incubated in sulforhodamine for ½ normal period (i.e. 90 minutes) in order to help control for increased cycles of rhythmic activity at higher frequencies.

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