Do propriospinal neurons contribute to transmission of the locomotor command signal in adult mammals?

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A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Physiology and Pathophysiology

University of Manitoba

Winnipeg

ACKNOWLEDGEMENTS

First and foremost, I praise God, the almighty for providing me with this opportunity and strength to proceed successfully. I would like to offer my thanks to everyone due to which this thesis appears in its current form.

I would like to start by thanking the best supervisor and mentor, Dr. Brian J Schmidt. Your professionalism and hard –working nature has been an example for me that motivated me continuously to not only work hard but also in a meaningful and productive manner. I am extremely grateful for your continuous guidance throughout the two year long journey and I hope to continue benefiting from it in the future.

I would also like to cordially thank my co-supervisor Dr. Katinka Stecina who made the experiments possible and taught me nearly every technique that was required to successfully conduct an experiment. Your constructive feedback and persistent support has helped me understand the philosophy of the field of science.

I am grateful to my committee members, Drs. Kristine Cowley and Brian MacNeil, who guided me regularly, steered me in the right direction and extensively reviewed the thesis.

I would also like to acknowledge a long list of people that provided me with academic, technical and professional help. It includes and is not limited to Shannon, Maria, Sharon, Gail, Judy, Sarah, Zhicheng, Dr. Nagy, Bruce, Brett, Dr. Almiski, Ariff, Kannan, and all the course instructors and coordinators.

Finally, I must express my profound gratitude to my family, especially my parents Masroor and Rubina, Shahzeb, Faiza, Jahangir, Zain, Aimen & Amal and Areeba for constant support

throughout my years of study and through the process of research, thesis writing and USMLE exam. This accomplishment would not have been possible without them.

Thank you!

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List of Abbreviations

MLR – Mesencephalic locomotor region

PS – Propriospinal

NMDA – N-methyl-d-aspartate

5-HT – 5-Hydroxytryptamine

EGTA – ethylene glycol tetraacetic acid

Tib – Tibial nerve

CP – Common peroneal nerve

Abstract

Long projections from the brainstem to the lumbar cord activate locomotion. Using *in vitro* neonatal rats our laboratory showed that relay (propriospinal - PS) neurons also contribute to transmission of the locomotor signal. This thesis examines whether locomotor-related PS neurons exist in adult mammals, which has important clinical implications. The brainstem of adult decerebrate rats was stimulated to elicit stepping. The following manipulations were performed: 1) suppression of synaptic transmission to PS neurons, 2) lesioning of direct bulbospinal projections to lumbar segments, and 3) neurochemical excitation of PS neurons. In addition, in the absence of brainstem stimulation, the ability of neurochemically excited PS neurons to induce stepping was examined. Brainstem-evoked locomotion was suppressed by synaptic blockade, enhanced by PS neuron excitation, persists after lesioning of long-direct projections, and hindlimb stepping was elicited by PS neuron excitation alone. The findings support the existence of a locomotor-related PS system in adult mammals.

INTRODUCTION

Locomotion, which is simply defined as the ability of to move from one place to another, is a fundamental trait of animals, including humans. It enables them to carry out a variety of tasks ranging from a simple walk to a lifesaving run, or gallop, away from any harmful stimulus. The mode of locomotion for different animals, that is bipedal or quadrupedal, involves basic anatomical and physiological differences; however, the central nervous system mechanisms and circuitry generating rhythmic motor activity responsible for locomotion are largely conserved across a wide range of vertebrates.

Traumatic spinal cord injury (SCI), either partial or complete, is the major cause of the loss of locomotor function in humans. Non-traumatic causes such as tumors, infection and ischemia also contribute to SCI. The incidence of SCI in Canada is on the rise. In 2010 there were nearly 4300 new spinal cord injuries in Canada, 1800 of them due to trauma and 2,500 non- traumatic. The prevalence of SCI in Canada is approximately 86,000; nearly half of these individuals are paraplegic and half are tetraplegic. The health care costs are estimated to be $3.6 billion annually (Farry and Baxter 2010).

In this introduction I will first outline the origin and locomotor-specific role of supra-spinal descending motor control pathways. I will then describe neural circuitry in the spinal cord relevant to locomotor function. Finally, I will develop a rationale for investigating the potential role of a specific spinal cord pathway, the descending propriospinal system of neurons, in the transmission of the locomotor command signal in an adult mammalian preparation.

Cortical and Brainstem Centers

The major control center for all voluntary motor activities is located in the posterior part of the frontal lobe, the primary motor cortex (PMC). Anatomically, PMC lies in the precentral gyrus which is a part of Brodmann area 4. The body is represented in the PMC in a somatotopic organization, which is defined as "the point-for-point correspondence of an area of the body to a specific point on the central nervous system" (Mancall 2011). Just anterior to the PMC, lies the supplementary motor area (SMA) which is comprised of the supplementary area proper, pre-supplementary area, dorsal premotor cortex, pre-dorsal premotor cortex and ventral premotor cortex. Inputs to the fore-mentioned motor areas include primary somatosensory cortex, cerebellum, basal ganglia and various thalamic nuclei. These inputs provide the sensory information necessary to carry out appropriate motor actions.

The primary motor output pathway that originates from layer V of the cortex is the 'pyramidal tract', which descends and decussates in the medulla at the level of pyramids. The decussated fibers then descend as the corticospinal tract (CST) and most such fibers terminate on spinal interneurons; a few CST fibers (which originate from the PMC) terminate directly on motoneurons. Anatomical variations exist in the location of the descending CST fibers in human and rat. In humans the lateral and anterior corticospinal tracts lie in the lateral and anterior funiculi respectively, whereas in rats the crossed CST descends in the ventromedial part of the dorsal funiculus; ventral uncrossed tract travels in the ventral funiculus (Paxinos 2004; Watson, Charles, Paxinos, George, Kayalioglu 2008).

In the rostral midbrain, the magnocellular and parvicellular components of red nucleus give rise to the ‘rubrospinal tract’ (RST). In the rat, the axons of RST cross in the ventral tegmental decussation and continue to descend in the dorsolateral fasciculus of the cord. RST in association with CST, influence locomotion by facilitating contralateral flexor and extensor muscles and some skilled motor tasks including the excitation of flexor motoneurons during reaching and grasping of food (Paxinos 2004; Watson, Charles, Paxinos, George, Kayalioglu 2008; Baker 2011).

Descending projections from the vestibular nuclear complex in the brainstem comprise the lateral and the medial vestibulospinal tracts (VST). The lateral VST projects the entire length of the cord in the ventral part of the anterior funiculus; the medial VST primarily projects to the cervical cord in the medial margin of the anterior funiculus (Paxinos 2004).

The reticular formation comprises many groups of neurons that are responsible for a variety of functions. This includes the locomotor output relay from the mesencephalic locomotor region (see below), blood pressure regulation, control of the axial and respiratory musculature, and modulation of the) preganglionic sympathetic neurons. Of the many reticular nuclei, the gigantocellular nucleus contributes to the majority of the bilaterally descending reticulospinal fibers found in the lateral and ventral funiculi. Projections from the ventral medullary reticular nucleus reach the lower thoracic cord and axons from the mesencephalic reticular nuclei extend throughout the entire cord (Paxinos 2004).

The Mesencephalic Locomotor Region (MLR)

Supra-spinal control of locomotion has been intensively studied in animal models for several decades since the time of Sherrington. The discovery by Shik and Orlovskii, in 1966 that electrical stimulation of a specific region in midbrain elicits walking and running in the decerebrate cats provided another experimental paradigm that has expedited research in this field. The mode of locomotion depends on the intensity of electrical stimulation. Increasing frequency changes the pattern from walking to trotting and galloping and amplitude modifications can also change the locomotor output. The region from which locomotor activity could be evoked in decerebrate cats was named as the 'Mesencephalic Locomotor Region'
(MLR) (Shik et al. 1966).

The MLR is located in the brain stem, and receives inputs from both forebrain and brain stem structures. In the behavioral context, it can be divided into 'exploratory', 'defensive' and 'appetitive' systems which receive forebrain inputs from the basal ganglia, medial hypothalamus and lateral hypothalamus, respectively (Jordan 1998). The MLR mainly corresponds to the cuneiform, subcuneiform and pedunculopontine nuclei.

Figure 1.

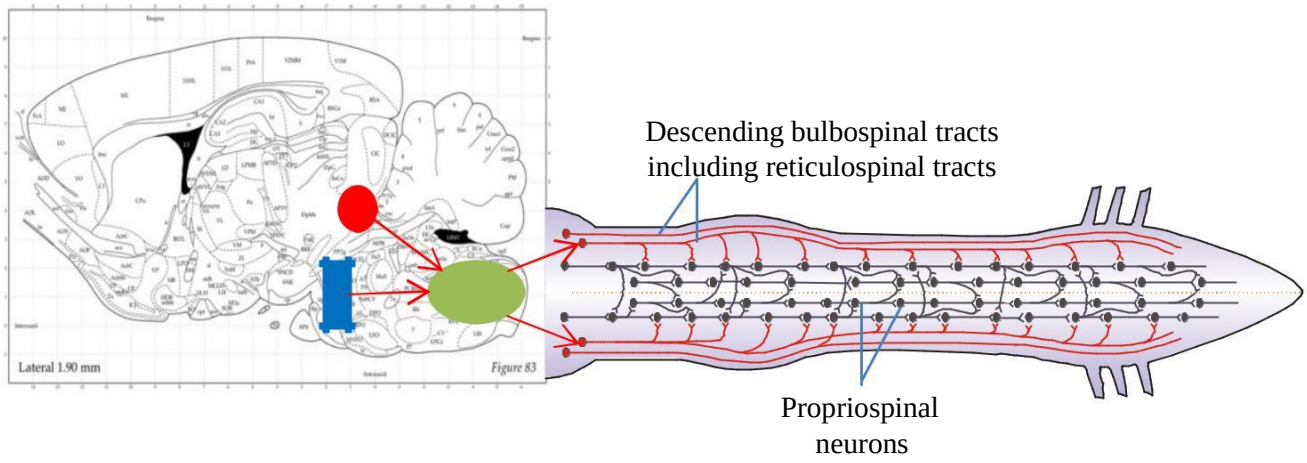


Fig 1. Schematic illustration of the MLR and its reticulospinal targets influencing the propriospinal neurons involved in locomotion.

On the left, sagittal section of brain is shown that is 1.90mm lateral to the midline *(Modified from Paxinos and Watson Rat Brain Atlas).* Approximately, the red cirle corresponds to area cuneiformis, blue rectangle corresponds to pedunculopontine area, and green oval represents the extent of the reticular formation that contains descending reticulospinal tracts cells that give off branches from their axons to the propriospinal neurons (small black circles on the right). The unidirectional red arrows are depicting the production and transmission of the locomotor command signal. On the right, a schematic transverse section of the spinal cord is illustrating the descending reticulospinal tracts (red) and propriospinal neurons in black.

Cuneiform and subcuneiform nuclei

Morphologically the cuneiform and subcuneiform nuclei are the components of the 'area cuneiformis'. In rats, the cuneiform extends from rostral pons to pretectal thalamus. Both nuclei are distinguished on the basis of their neuronal inter-connections (Canteras and Goto 1999).

The cuneiform nucleus, in addition to its role in locomotion, generates a response to stressful or threatening stimuli by appropriate cardiovascular responses (Korte et al. 1992). It also forms a part of the brain's defensive system. For instance, when a rat smells a cat, activity in the cuneiform nucleus is increased leading to neuronal firing in surrounding structures such as the amygdala, medial hypothalamus and periaqueductal grey (Dielenberg et al. 2001). Neuronal activity can be detected by immunohistochemical labeling of an activated early gene, c-fos (activity-dependent neuronal labelling). This method was used to establish the activation of neurons within the cuneiform nucleus during stress responses (Dielenberg et al. 2001). Physiological data also support a role for the area cuneiformis in locomotion and defensive behavior. In the rat, when glutamate is injected into the cuneiform area it produces freezing initially, followed by fast running (Mitchell et al. 1988). This reflects the usual behavior displayed when an animal encounters and then tries to escape from a predator.

A variety of neurotransmitters are expressed by these nuclei in varying concentrations including: glutamate (Richter and Behbehani 1991), serotonin (5-HT) (Beitz 1982a), acetylcholine (Ach) (Spann and Grofova 1992), gamma amino butyric acid (GABA) (Ford et al. 1995), and peptides

such as encephalin (Sar et al. 1978), neurotensin (Beitz 1982a), substance P (Beitz 1982b) and corticotropin-releasing factor (Sakanaka et al. 1987).

Afferent projections to these nuclei originate in various forebrain and midbrain structures. The cuneiform nucleus receives inputs from the amygdala, zona incerta, hypothalamus, periventricular grey matter, periaqueductal grey matter, substantia nigra pars lateralis, peripeduncular area, superior colliculus, lateral geniculate complex, prefrontal cortex, and the contralateral cuneiform nucleus. The subcuneiform nucleus receives inputs from the amygdala, zona incerta, and precommisural nucleus (Redgrave et al. 1988; Bernard et al. 1989; Sesack et al. 1989; Vaudano and Legg 1992; Cameron et al. 1995).

The cuneiform and subcuneiform nuclei project to several areas in the brainstem. In the rat, most cuneiform fibers descend ispsilaterally and terminate in the reticular formation (the gigantocellular, caudal pons, magnocellularis, reticularis parvocellularis and raphe magnus nuclei) (Bernard et al. 1989). Subcuneiform fibers descend bilaterally and terminate in the nucleus reticularis pontis oralis (in the pons).

Pedunculopontine Nucleus (PPN)

The PPN is an important area for postural control partly overlapping with the MLR. It is responsible for maintenance of posture and it was formerly thought to be responsible for

initiation of locomotion. Behaviorally, it can be considered as a part of an exploratory system (Jordan 1998).

Morphologically, cell density is used to divide the PPN into two parts. Densely packed cells are found in the 'sub nucleus compactus (pars compacta)'; low cell density is found in the 'subnucleus dissipatus (pars dissipata)'. The expression of neurotransmitters from these two subdivisions varies slightly. Pars dissipata contains 23% cholinergic, 37% glutamatergic and 40% GABAergic neurons, whereas pars compacta has 31% cholinergic, 50% glutamatergic and 13% GABAergic neurons (Wang and Morales 2009). It was recently reported that the glutamatergic neurons are responsible for the initiation and maintenance of stepping (Roseberry et al. 2016). These neurons present in the MLR were connected to basal ganglia by direct and indirect pathways that mediate stimulation and inhibition of MLR, respectively. In the same study GABAergic neurons were found to cause inhibition of locomotion, and cholinergic neurons primarily modulated and not initiated the locomotion.

Ascending outputs of the PPN reach the basal ganglia, thalamic nuclei, superior and inferior colliculi in tectum, areas of forebrain. Descending projections target the reticular formation and spinal cord. PPN inputs come from a wide range of areas including the cerebral cortex, thalamus, hypothalamus, cerebellum, basal ganglia, tectum, brainstem and spinal cord (Martinez-Gonzalez et al. 2011; Roseberry et al. 2016).

Previously it was thought that electrical stimulation of PPN and its neighboring area induced controlled locomotion on a treadmill. Histological analysis of c-fos labeling in active neurons

revealed the cholinergic dominance in and around PPN where locomotion was induced (Garcia-Rill et al. 1987). Recently, c-fos labeling of the MLR neurons responsible for locomotor function has found that it is actually the CnF and few neighboring areas, and not PPN that are involved in the induction of stepping like activity (Jordan 1998; Heise and Mitrofanis 2006).

Intrinsic neuronal systems related to locomotor function

The Central pattern generator (CPG)

A CPG is a neural circuit which when activated produces a stereotyped rhythmic motor output, even in the absence of any phasic afferent input. The CPG includes neurons that mediate and modulate excitatory and/or inhibitory inputs. These neuronal networks underlie a variety of rhythmic behaviors such as walking, breathing, swimming and feeding. Using an in vitro neonatal rat model our laboratory previously provided evidence that the hindlimb locomotor CPG, contrary to previous concepts, was not restricted to the lumbar enlargement. Instead, it is distributed along the entire length of the spinal cord (Cowley and Schmidt 1997).

Several models of CPG have been proposed over the past 100 years. In the early twentieth century Graham Brown suggested that a half-center organization was responsible for generating rhythmic motor activity (the 'half-center hypotheses') (Brown 1911). He proposed that the rhythmic alternation between flexor and extensor half-centers was due to inhibitory neuronal fatigue. More specifically, if the excitatory input is initially received by both the flexor and extensor half-centers, whichever half receives slightly stronger input would dominate and result in flexor or extensor motoneuron discharge while simultaneously exerting an inhibitory influence

on the antagonist half-center. As a result of neuronal fatigue activity would wane and the balance would shift to the opposite half-center. In this manner activity would repeatedly shift between both the halves resulting in the characteristic flexor-extensor alternation observed during stepping. In this single-level organization, each limb consists of a separate CPG which modulates two sets of excitatory half-centers, flexor and extensor. Although this model provides an explanation for simple behaviors, it fails to explain more complex sequences of muscle activation, such as co-activation of extensors at one joint with flexors at another joint, during stepping. It also fails to account for the role of sensory feedback on the motoneuron activity.

Grillner proposed the Unit Burst Generator (UBG) model of CPG that expanded the half-center idea into a more meaningful model (Paul S.G. Stein, Sten Grillner 1999). Accordingly, the CPG consists of a unilateral and modular set of neurons that regulates the activity of an agonist at a single joint and is able to produce rhythmic burst cycles even in the presence of a quiescent antagonist. The latter property enables this model to account for the phenomenon of 'deletions'. Deletion refers to the drop-out of an expected rhythmic burst (or bursts) during a stepping sequence, despite uninterrupted rhythmic activity in the antagonist (McCrea and Rybak 2008). This process is otherwise not unaccounted for by the half-center hypothesis. In addition, UBG model also explains the mixed synergistic effect of the CPG that occurs in movements involving the simultaneous activation of certain flexors and extensors at more than one joint.

The Rybak-McCrea CPG model depicts a more complex two-level CPG that addresses limitations of simpler single level CPG models (McCrea and Rybak 2008). Their model features a rhythm generator (RG) layer and pattern formation (PF) layer. It was designed to

accommodate observations made of fictive stepping in cats, such as the effect of specific afferent input and non-resetting deletions. Both RG and PF reciprocally inhibit their antagonist components by a set of specific interneurons. RG and PF receive descending excitatory input from the MLR which activates these neuronal circuits and initiates rhythmic locomotor activity. RG controls the PF by its direct projections, and PF in turn sends out its phasic excitatory outputs directly onto the motoneuronal pools. Inhibitory interneurons are also under the control of PF network; they inhibit their respective motoneuron targets. Renshaw cells regulate the activity of the circuit by inhibiting motoneurons, reciprocal inhibitory interneurons, and each other reciprocally. In addition, the circuit also receives afferent input from extensor muscle spindles (Ia afferents) and Golgi tendon organs (Ib afferents), which are important in the phase-dependent activity of the motoneurons.

Propriospinal neurons

The propriospinal system of fibers refers to the axons of neurons that originate, project and terminate within the boundaries of the spinal cord (Watson, Charles, Paxinos, George, Kayalioglu 2008). Not all spinal neurons terminating within its boundary are propriospinal neurons, instead some are also interneurons. As shown by the neuroanatomical studies done by Chung and colleagues, propriospinal neurons are the predominant neuron type in the spinal cord making up approximately 97% of the total; the remaining consisting of long descending axons and motoneurons (Chung et al. 1984). Propriospinal neurons that synapse onto other interneurons and motoneurons are responsible for various physiological functions. Aforementioned CPGs are an example of one of the many functions of PSP neurons.

Anatomically the propriospinal network is broadly classified into short and long projections. Short fibers project up to six spinal cord segments; long axons project beyond six segments (Watson, Charles, Paxinos, George, Kayalioglu 2008). There are both descending and ascending systems. These projections are involved in many important functions including the relay of supraspinal locomotor command signals (Zaporozhets et al. 2006), control of posture and axial musculature (Anderson 1963), and a group in the cervical region known as the C3-4 propriospinal system modulates voluntary target grasping and reaching tasks (Alstermark et al. 2007). Thoracic propriospinal neurons modulating respiratory muscles activity receive input from the respiratory centers in the medulla (Merrill and Lipski 1987). Long propriospinal projections traverse along many segments connect the cervical and lumbar enlargements and mediate coordinated fore and hind limb motor activity (Ballion et al. 2001).

In the early 1940s, Lloyd provided the evidence that reticulospinal projections relay signals to motoneurons via a propriospinal system. He showed that the short propriospinal networks relaying the reticulospinal signals in the lumbar cord strongly activated the hind limb motoneurons (Lloyd 1941).

In vitro work done in our lab has clearly shown that the propriospinal neurons relay brainstem locomotor signals in the spinal cord (Zaporozhets et al. 2006). In vitro neonatal rat spinal cord exposed to synaptic blockade using calcium-free/high magnesium concentration bath solutions, with or without AP5 and CNQX, inhibited locomotor signal transmission induced by brain stem electrical stimulation. Direct application of these agents to the cord blocks local synaptic

transmission in the propriospinal system but has no effect on long direct axons of passage originating in the brainstem. The effectiveness of propriospinal signal suppression was greater if a large number of the cord segments was exposed to synaptic blockade.

Commissural axons in the spinal cord are responsible for coordinating the right and left limbs during locomotion. The role of these projections in rhythm generation was determined using the *in vitro* spinal cord preparation (Cowley et al. 2009). The spinal cord was exposed to mid-sagittal lesions at different levels along its entire length. Locomotion was elicited by brainstem electrical stimulation and in some cases by the application of excitatory neurochemicals such as 5-HT and NMDA. In some cases it was found that in the presence of only two or three bilaterally intact adjoining cord segments, locomotor-like activity persisted. However, T13 or L1 segments needed to be intact, indicating the importance of these thoracolumbar segments. However, in some preparations limited mid-sagittal lesions which included both the T13 and L1 segments failed to abolish the coordinated locomotor-like activity provided that more rostral or caudal segments remained intact. Therefore the results indicate there are no essential locomotor-related commissural neurons rather the system is distributed and redundant.

After establishing the vital role of the propriospinal system in conducting the supraspinal locomotor command signal, it was important to determine whether this system alone is sufficient to conduct these signals. To investigate this, the in vitro neonatal rat cord was subjected to bilateral staggered hemisections, which theoretically interrupt all long direct descending fibers involved in the transmission of the supraspinal locomotor command signal (Cowley et al. 2008). Locomotor-like activity was successfully induced in approximately 30% of such preparations, by

electrical or chemical stimulation of the brainstem. The presence of activity was independent of the number of segments between the two hemisections. Even though propriospinal projections were also been damaged by these lesions, the results suggest that the residual intact propriospinal fibers alone were sufficient to conduct the descending command signal.

Subsequently, once the role of the locomotor-related propriospinal network was characterized, a similar set of *in vitro* experiments was performed to determine whether neurochemical excitation of propriospinal neurons facilitated transmission of the locomotor command signal in lesioned preparations where brainstem stimulation alone was otherwise unsuccessful (Zaporozhets et al. 2011). Exposure of thoracic propriospinal neurons to excitatory neurochemicals did facilitate brainstem-induced hind limb locomotor-like activity. Maximum facilitation was observed with serotonin, methoxamine, quipazine, NMDA, and a combination of dopamine and norepinephrine.

To examine the relevance of the results from the *in vitro* neonatal rat in the mature nervous system, our laboratory carried out a series of *in vivo* experiments using adult rats to examine the effect of the chronic bilateral staggered hemisections on spontaneous recovery of locomotor function. In that study the effect of increasing thoracic propriospinal neuron excitation by intrathecal infusion of excitatory drugs in chronic bilaterally hemisected adult rats was also examined (Cowley et al. 2015). Partial recovery of hindlimb stepping occurred, despite bilateral staggered hemisections being placed in the same operation. This is in contrast to work reported by Courtine and colleagues who suggested that no recovery occurred unless the second

hemisection was delayed by 10 weeks after the first hemisection (Courtine et al. 2008). Neurochemicals administered via intrathecal catheters improved hindlimb stepping, with quipazine inducing the most significant excitatory effect. However, coordination between forelimb and hindlimb stepping was not seen. The recovered stepping in these chronic bilaterally hemisected animals, and facilitation of stepping by neurochemical stimulation of thoracic segments, may have been due a propriospinal influence on the lumbar cord, completely independent of any role in transmitting the brainstem locomotor command signal. That is, the results may be analogous to the effect of chronic complete cord transection. Such animals regain the ability to walk on a treadmill due to the recovery of intrinsic pattern generator activity in the lumbar cord.

Summary of rationale for this thesis

There is convincing evidence that the propriospinal system contributes to transmission of the locomotor command signal and that neurochemical excitation of propriospinal neurons in this preparation enhances locomotor signal transmission in the in vitro neonatal rat spinal cord preparation. No study to date has clarified whether propriospinal neurons transmit the locomotor command signal in the adult mammal, in vivo. Nevertheless, this information is critically needed given the potential therapeutic implications for humans with spinal cord injury. This was one of the reasons for undertaking the experiments described in this thesis.

The central hypothesis addressed here was: **Propriospinal neurons contribute to transmission of the locomotor command signal in adult mammals, in vivo**.

In order to test this hypothesis, we performed four types of experiments using adult, acute decerebrate rats. **First**, the MLR was electrically stimulated while monitoring alternating hindlimb flexor and extensor nerve activity ('fictive' locomotion). We then examined the effect on hindlimb fictive stepping of synaptic blockade in the rostral thoracic region, using calcium-free / high magnesium concentration solution, with or without neurotransmitter antagonists.

Secondly, we examined the effect on MLR-induced hindlimb stepping of abolishing all long-direct bulbospinal projections, while preserving at least part of the propriospinal system, using staggered hemisections ($T_{4/5}$ and contralateral $T_{9/10}$). **Thirdly**, we examined whether neurochemical stimulation of propriospinal neurons in the rostral thoracic region, in the absence of MLR stimulation, was able to induce hind limb stepping. **Finally**, we examined whether neurochemical excitation of thoracic propriospinal neurons has a facilitatory effect on MLR-evoked stepping in animals, with or without bilateral cord hemisections.

Implicit in this experimental design is an additional hypothesis, contrary to certain recent literature (Courtine et al. 2008; van den Brand et al. 2012), as follows:

The propriospinal contribution to locomotor command signal transmission after spinal cord injury does not require time-dependent plasticity.

METHODS

Experimental protocols used in this work complied with the Canadian Council on Animal Care and University of Manitoba guidelines. The experiments involved 34 female Sprague-Dawley rats, weighing approximately 250-300 grams.

Anesthesia and pre-surgical preparation

Rats were transferred to a plastic anesthetic chamber in a fume-hood. Anesthesia was induced using isoflurane 4-5% for 2-3 minutes. The level of anesthesia was checked by gently tilting the chamber in different directions and deemed adequate if the rat did not resist the tilts and turns of the chamber i.e. the righting reflex was abolished. Rat was taken out of the chamber and then weighed on a digital scale. Then the rat transferred to the dissection table where it was immediately connected to the anesthetic by a nose-cone mask. The rat was placed on a heating pad and a rectal temperature probe (coated with petroleum gel) was inserted to provide automatic feedback control based on core temperature measurements A pulse oximeter probe was placed at the base of the tail or foot to monitor the oxygen saturation and heart rate. Maintenance anesthesia was achieved using isoflurane 2-3%. The exact concentration of the anesthetics was titrated, as needed, depending on the respiratory rate, blood pressure and heart rate monitored at regular intervals. The normal respiratory rate for rats ranged from 40 to 60 breaths per minute in the surgical state during these types of studies. Vitals were documented every 2 to 5 minutes during the first hour of the surgery. Thereafter, 10-15 minutes intervals were used. In addition, to check the depth of anesthesia, pedal reflexes were repeatedly checked, bilaterally, during the surgery. Artificial tears were applied to both eyes; this procedure was

repeated throughout the experiment as needed. Animals were shaved over the left and right hindlimb, and along the back and the scalp. To prevent excessive tracheal secretions, atropine 0.05 mg/kg in 0.2ml saline was administered subcutaneously.

Hind limb nerve dissection

Xylocaine gel was applied and the skin was incised along the dorsal aspect of the leg from the ankle to the buttocks. Muscles were bluntly dissected while using micro cautery to control bleeding, as needed. Connective tissue was removed until the sciatic nerve was exposed and its branches, the common peroneal and tibial nerves, were dissected from surrounding connective tissue. Nerves were protected with saline soaked cotton and incisions were temporarily closed with a clip.

Insertion of arterial line and tracheostomy tube

Rats were rotated to a supine position for further surgery. In order to establish an arterial line, the midline of the neck was shaved, followed by xylocaine application. Blunt dissection of muscles and connective tissue around the trachea was performed. The left carotid artery was isolated and a catheter was inserted for fluid and drug infusion, as well as blood pressure monitoring. Ideally, mean arterial blood pressure was targeted to stay in the range of 80-120 mmHg. A standard buffer solution was infused at a rate of 0.9 – 1.2 ml / hour during the course of the experiment. This solution contained 0.42 gm $NaHCO_3$ and 2.50 gm dextrose in 50 ml Nano pure water. The right carotid artery was isolated and a thread was loosely tied around it. This thread was tightened immediately before decerebration to occlude the carotid artery and reduce blood loss. A tracheostomy was performed by making an incision at about 1-3 cartilage

rings below the larynx. A tracheal tube (a shortened 20G Hamilton metal needle) was inserted for ventilation and the anesthesia was maintained at 1.8-2.5% isoflurane in oxygen.

Laminectomies

Rats were rotated back to a prone position to perform laminectomies. An incision was made along the midline, followed by blunt dissection of the paraspinal muscles and ligaments. Laminae and dorsal spinous processes were removed at T1-2 and at T7-8 levels in order to later perform contralateral spinal cord hemisections, and at T13-L1 to record cord MLR stimulus-evoked dorsum potentials. Before transfer to the experimental frame, dexamethasone 0.25 ml (0.5 mg/kg) was injected into the arterial line.

Decerebration and electrode set-up

Rats were transferred to an experimental frame. The head was stabilized in a stereotaxic head-holder. The spinal cord was stabilized by clamps holding the dorsal spinous processes at the T6-7 and L2-3 vertebral levels. The thread around the right carotid artery was tightened to occlude blood flow at this point. After applying xylocaine gel, a midsagittal incision of the scalp was made. The scalp flaps were reflected laterally and muscles were bluntly dissected until the bone was exposed. The occipital bone was drilled between the frontal and inter-parietal suture such that a small portion of the skull could be removed bilaterally. The dura was incised and brain was mechanically removed by suctioning tissue from the caudal end of the opening to the frontal end, followed by suctioning of remaining tissue. A pre-collicular transection of the remaining tissue was done. After decerebration, anesthesia was discontinued and pancuronium bromide (2 mg/ml stock solution) was given via the carotid arterial line to eliminate any movement after

decerebration. The first dose of pancuronium was 0.3-0.4 ml followed by 0.2 ml every 45 minutes. Hindlimb nerves were placed on bipolar hook electrodes and metal ball electrodes were placed on dorsal surface of spinal cord to record cord dorsum potentials.

Metal (Tungsten coated with parylene) electrodes were stereotaxically targeted in the midbrain locomotor region. The initially targeted region for stimulation was near the location of the Cunieform nucleus and it was determined by coordinates with respect to the surface of the exposed midbrain.. The electrode was either positioned at 30-40 degrees with the tip pointing caudal and entering 2 mm caudal to the exposed border between the superior colliculus and the midbrain and 1 – 2.5 mm lateral to the midline or it was positioned nearly perpendicular to the surface of the brain and entering at a point 8-9 mm caudal to Bregma and 1 – 2.5 mm lateral to the midline. The dorso-ventral positioning of the electrode was determined by the quality of the locomotor activity in response to the electrical stimulation, typically ranging from 2.5 – 5.5 mm ventral to Bregma. The electrical stimuli consisted of 0.2-0.5 ms single pulses at 20 Hz delivered continuously.

After successful induction of locomotion via MLR stimulation, spinal cord hemisections were performed using fine forceps while avoiding damage to major vessels. To administer pharmacological agents we used pipettes for topical administration and Hamilton needles (30-33G steel, 12 degree beveled tip) for drug injections.

Tissue analysis

At the end of each experiment, animals received bilateral pneumothorax. When the results required post-hoc verifications of spinal hemisection extent and sites of brain stem stimulation, brain and spinal cord tissue were collected and post-fixed in 4% Paraformaldehyde solution

overnight. The tissue was cryoprotected for 3- 4 days in 10% sucrose solution with 0.1 M phosphate buffer. Tissue sectioning was done on a LEICA CM3050 S cryostat, using 100 micron and 50-80 µm thick sections for brain stem and spinal cord, respectively. Sectioned tissue was stained in 2%Cresyl violet solution.

Data capture and analysis were performed using custom made software developed in the Spinal Cord Research Centre in the University of Manitoba running on a personal computer with a Pentium processor.

Figure 2.

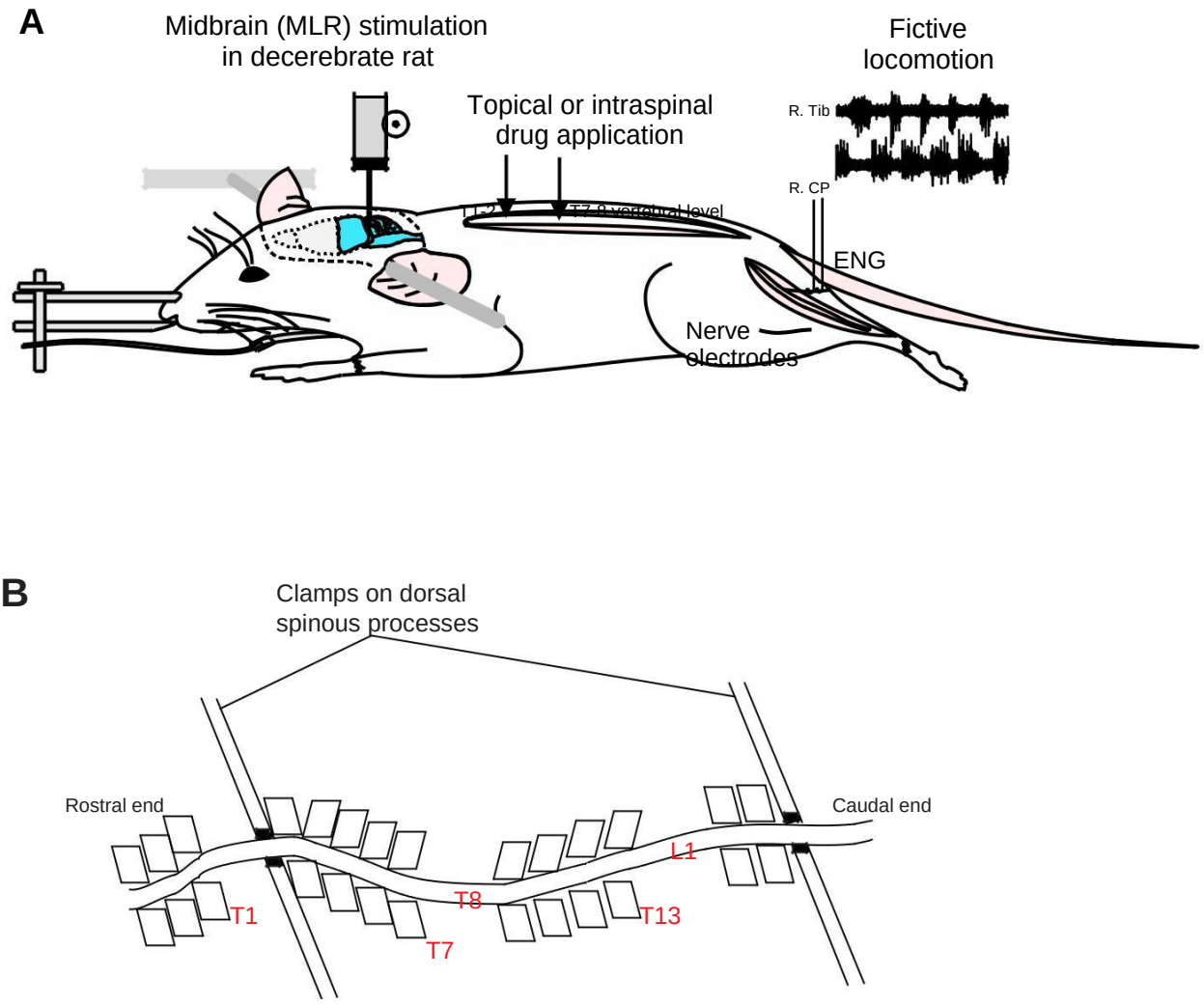


Fig 2. Schematic illustration of the rat in the experimental frame.

A. Rat placed in the frame with head stabilized by ear bars against the temporal bone. Dorsal opening over the thoracic region were used for administering drugs and performing hemisections. Hindlimb nerves were hooked onto the recording electrodes.

B. A simplified figure showing the curvatures formed along the surface of spinal cord while the vertebral column is clamped and animal is suspended. Rostral and caudal clamps were placed onto the T2 and L4/5 dorsal spinous processes respectively. Rectangular boxes are representing the vertebral bodies. Laminectomies were performed on C7, T1 and T7, T8 vertebral level to make openings for hemisections. T13, L1 laminectomy allowed an opening for cord dorsum potential recording.

RESULTS

Experiments were performed on a total of 34 animals. A brief summary of the number of animals used and the results obtained are shown in Figure 3. Experiments that failed to yield sufficient results included animals that died before the data could be collected due to bleeding, hypotension, hypocapnia, or unknown reasons (n=13).

Average duration of each experiment ranged from 10 to 12 hours. In the first 18 experiments majority of this entire duration was taken up by the surgical procedures that used to leave us with a very limited experimental data collection/recording time. Most of the animal deaths were also reported in this first half of experiments. Average duration of time taken by specific surgical procedures were

- Hindlimb nerve dissection: 1-2 hours
- Carotid arterial line and tracheal tube insertion: 1-2 hours
- Back dissection and laminectomies: 2-3 hours
- Decerebration: 1-1.5 hours

Another time consuming factor was the tracking of optimal MLR site to induce locomotion. This duration varied from 15 minutes to 2 hours and depended on a multiple other factors. Excessive sinus and/or arterial bleeding during decerebration probably lead to ischemia in the brainstem as that would result in more tracking time to find a functional or viable area, and/or due to recovery time needed after an ischemic shock. Visual appreciation of superior and inferior colliculi was an important landmark to target MLR but that required suctioning away the overlying cortex. In cases where the hemodynamic status was already declining, this suction was avoided as it caused more bleeding and further decline. In such an instance the MLR electrode was only guided

without the most crucial visual cues and resulted in more time to locate the most optimal position evoking locomotion. All of the aforementioned surgical durations substantially decreased by the end of this series of experiments.

Many other technical obstacles were also faced in the first half of experiments which were overcome considerably by the end of the second half. One of the major technical hurdles encountered initially was the blood loss during decerebration. Primary source of this bleeding were the superior sagittal and transverse sinuses. We tackled this issue by extensively cauterizing directly over and around the sutures overlying these sinuses, and to ensure its completeness we used to chip off the distal most portion of the bone along the course of each sinus to minimize potential bleeding.

Experiments generating results were broadly divided into those with or without MLR-induced stepping as described in Fig. 3. There were 14/21 animals with MLR-induced locomotion and 11 of these were subjected to spinal hemisections. Four out of eleven rats showed persistent MLR-induced stepping after hemisection at a single thoracic site. There were 9/11 rats subjected to a second staggered, contralateral thoracic hemisection and only one showed persistent MLR-induced stepping after the second hemisection. . Animals not subjected to spinal hemisection showed alteration of the stepping rhythm in response to NMDA and 5-HT application. There were 7/21 animals without baseline MLR-induced stepping and they were subjected to pharmacological activation of the propriospinal neurons. These rats exhibited either enhancement of the motor activity evoked by descending MLR signals (n=3), or no change in responses to NMDA and 5-HT (n=4).

Excitation of thoracic propriospinal neurons induced hind-limb stepping

The ability of propriospinal neurons to influence locomotor-like activity in the hindlimbs was tested by applying excitatory drugs onto the thoracic spinal cord in adult rats (n=6). The application of NMDA alone or in combination with 5-HT led to lumbar motoneuronal activation, as monitored by hindlimb ENGs. Typically, in the absence of MLR stimulation, NMDA application resulted in brief bursts of tonic activity in one or more hindlimb nerves. However, when 5-HT was also applied or MLR stimulation was also used, nerve recordings showed rhythmic flexor-extensor alternations in response to NMDA, as shown in Figure 4. The application of NMDA and 5-HT in caudal (Fig. 4A) as well as in rostral (Fig. 4B) thoracic segments could lead to alternating hindlimb flexor and extensor activity. Subsequent application of 5-HT after NMDA resulted in enhanced locomotor-like output. This manifested as increased ENG burst duration and amplitude , and as more coordinated flexor-extensor alternation.

Excitation of thoracic propriospinal neurons facilitated MLR-induced stepping

A facilitatory effect of NMDA on MLR signal transmission similar to the illustrations in Fig. 5 was observed in three rats. Initially in these experiments, MLR stimulation alone resulted in absent or low amplitude, poorly coordinated, flexor and extensor output as shown by the top pair of traces in Fig. 5A. After NMDA was applied, MLR-induced stepping was observed within one minute of the drug application as shown by the bottom pairs of traces in Fig. 5B. Overall, the time required to observe this facilitatory effect ranged from one to five minutes. Stepping always ended when electrical stimulation of the MLR was stopped (data not shown). This type of effects

on the MLR-induced motor output was observed with both topical (Figs. 5A & 5C) and intraspinally injected NMDA application (Fig. 5B).

Suppression of synaptic transmission disrupts MLR-induced stepping

To test the role of propriospinal neurons in transmitting the descending locomotor command signal, indirect disruption of propriospional synaptic activity was attempted by adding Ca^{2+}-free, high Mg^{2+}, ethylene glycol tetraacetic acid (EGTA) solution over the T7-10 segments in two experiments. In the first experiment, the cocktail of these agents applied topically failed to show any effect of this solution. In the second experiment, alterations of rhythmic discharges were evident as shown in Fig. 6A and B after an intraspinal injection of the same mixture. After a washout-attempt over the targeted area with topically applied saline, a few rhythmic ENG bursts re-emerged (Fig. 6C).

Effects of unilateral and staggered, bilateral spinal hemisections on MLR induced stepping

The spinal hemisections were performed in an attempt to anatomically disrupt long, "direct" supraspinal tracts carrying the locomotor command signal from MLR and its supraspinal target cells to the lumbar motor circuitry. Experiments with hemisections at a single site, either rostral (C7-T2) or mid (T7-T9) thoracic segments verified previous results on locomotor activity developing by unilaterally intact descending systems activated by MLR stimulation (Steeves and Jordan 1980). This is illustrated in Fig. 7A and B, showing rhythmic fictive locomotion before and after unilateral hemisections. Experiments with staggered, bilateral hemisections at two different sites along the thoracic spinal levels were attempted in 9 rats. In 4 of these cases, the animals general physiological state declined after the second hemisection therefore conclusive results could not be obtained. In the other 5 experiments, after the second hemisection MLR induced locomotor activity could not be elicited in 4 rats. The hemisections performed in these 4 animals are illustrated in figure 8, and they show the maximal extent of damage resulting from the hemisections. One of four experiments with staggered, bilateral hemisections demonstrated bilateral hindlimb stepping, indicating that the acutely lesioned cord is capable of producing hindlimb stepping, even in the absence of time-dependant plasticity. This is illustrated in Fig. 9 by data from an experiment in which a hemisection was performed at the T2 level and it was followed by another contralateral hemisection at the T8 level. Bilateral hemisections were performed in nine animals but stepping persisted in only one. Post hoc histological analysis of the lesions revealed that the hemisections in the animal with persisting locomotor activity were incomplete. As illustrated in Fig. 9B, parts of the ventrolateral funiculi were spared bilaterally. The time required

for stepping to reappear after uni- or bilateral hemisections was variable ranging from a few minutes to one hour.

Figure 3. Summary of experiments

Total animals: 34

Experiments without useable results: 13

Experiments with results: 21

- Baseline MLR induced stepping: 14
  - First hemisection - no: 2
  - First hemisection - yes: 11
  - Second hemisection- no: 2
  - Second hemisection- yes: 9
    - Stepping persisted: 1
    - Stepping abolished: 8
  - Stepping unaltered? by Ca^{2+} free solution: 1
- Absent baseline MLR response
  - Drug enhanced MLR effect: 3*
  - No drug enhancement of MLR effect: 4

* Stepping pattern of one experiment of these three (experiment 30) was altered by zero Ca^{2+} solution application and not by enhancer drug application.

Figure 4.

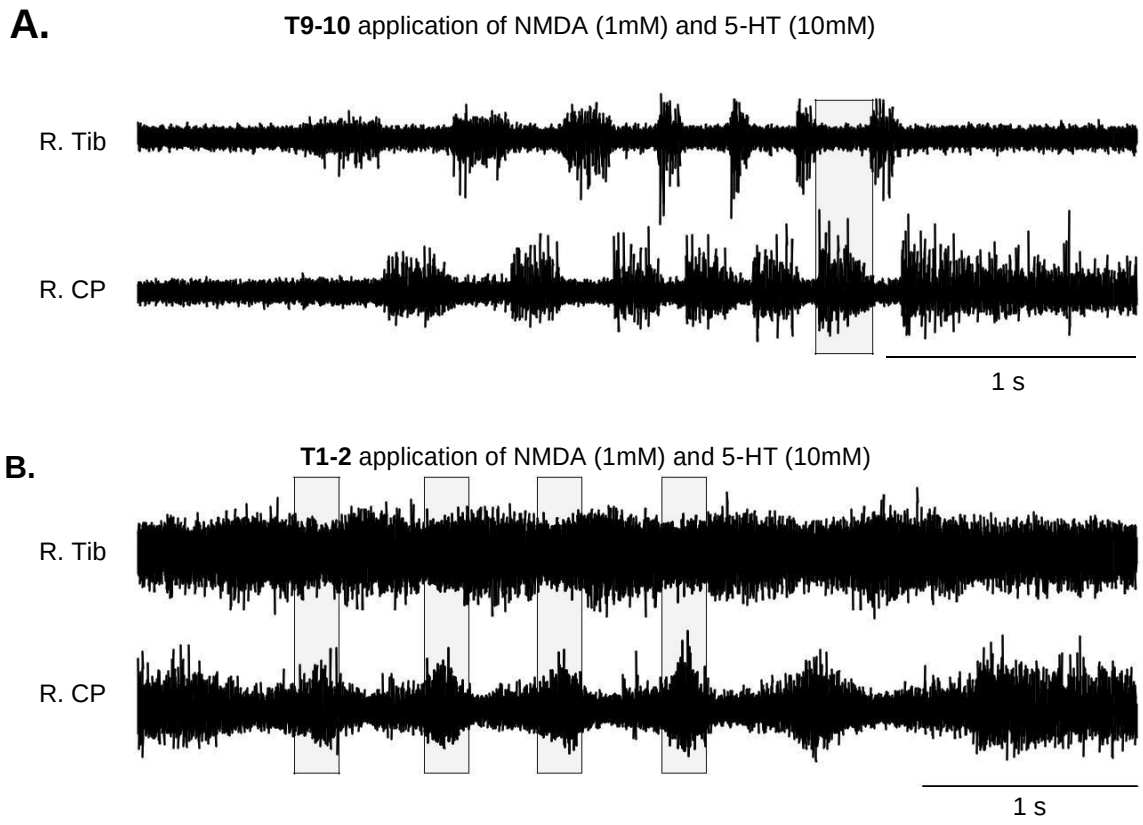
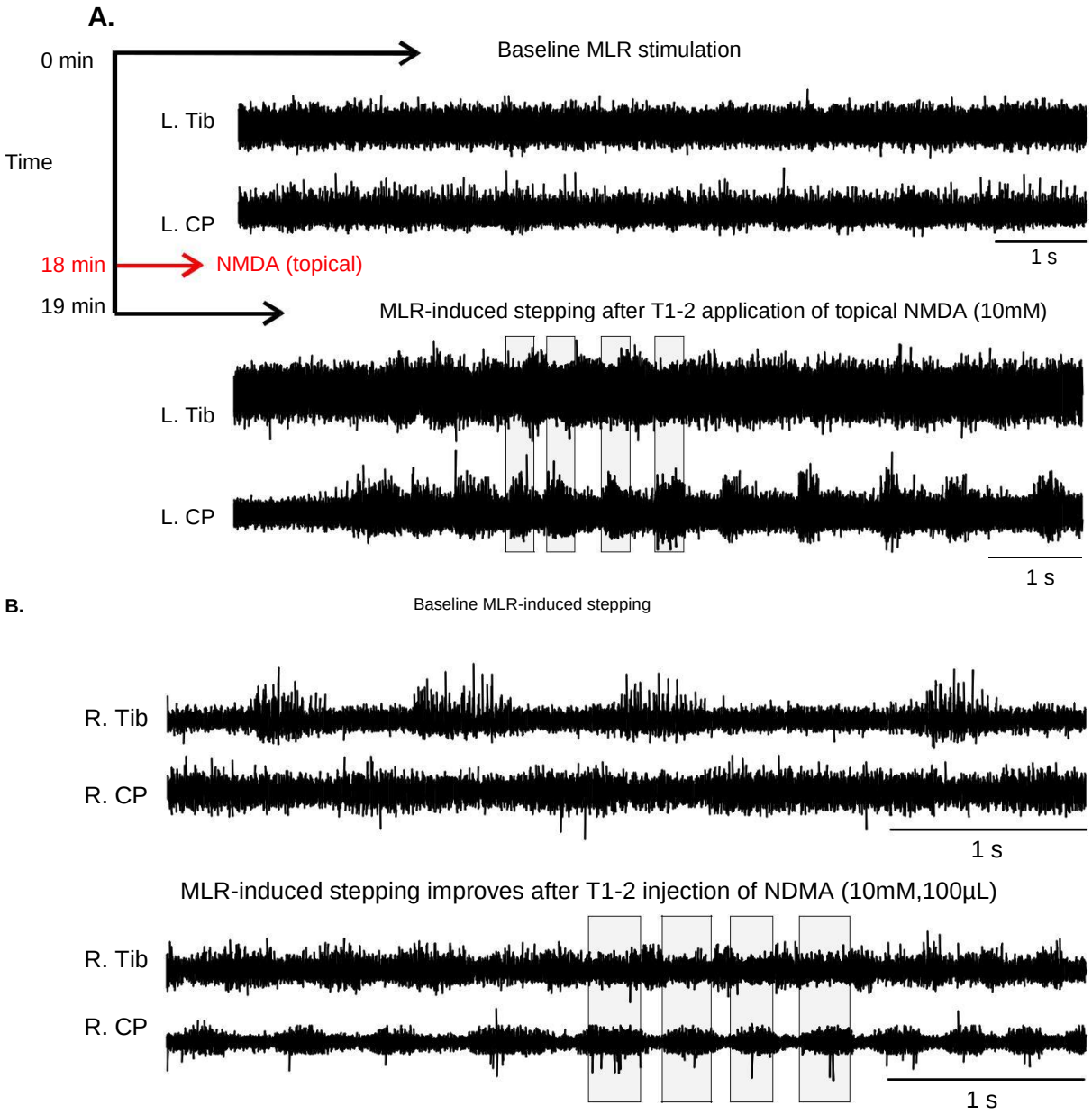


Fig. 4. Excitation of thoracic propriospinal neurons induced hindlimb stepping
Hindlimb ENG recordings from the flexor common peroneal (CP) and the extensor tibial (Tib) nerves showing rhythmic stepping-like fictive motor activity in a decerebrated rat after the topical application of NMDA and 5-HT on thoracic spinal cord segments.

- **A.** Both drugs were applied topically on the T9-10 vertebral levels. 100uL each of 1mM NMDA and 10mM 5-HT resulted in alternating right Tib and CP activity highlighted by grey shaded boxes over the flexor bursts.
- **B.** Similar effects as shown in A were observed when 100uL each of 1mM NMDA and 10mM 5-HT was applied over the T1-2 vertebral levels in the same experiment.

Figure 5.


A.
0 min
Baseline MLR stimulation
L. Tib
Time
L. CP
18 min
NMDA (topical)
1 s
19 min
MLR-induced stepping after T1-2 application of topical NMDA (10mM)
L. Tib
L. CP
1 s
B.
Baseline MLR-induced stepping
R. Tib
R. CP
1 s
MLR-induced stepping improves after T1-2 injection of NDMA (10mM,100µL)
R. Tib
R. CP
1 s

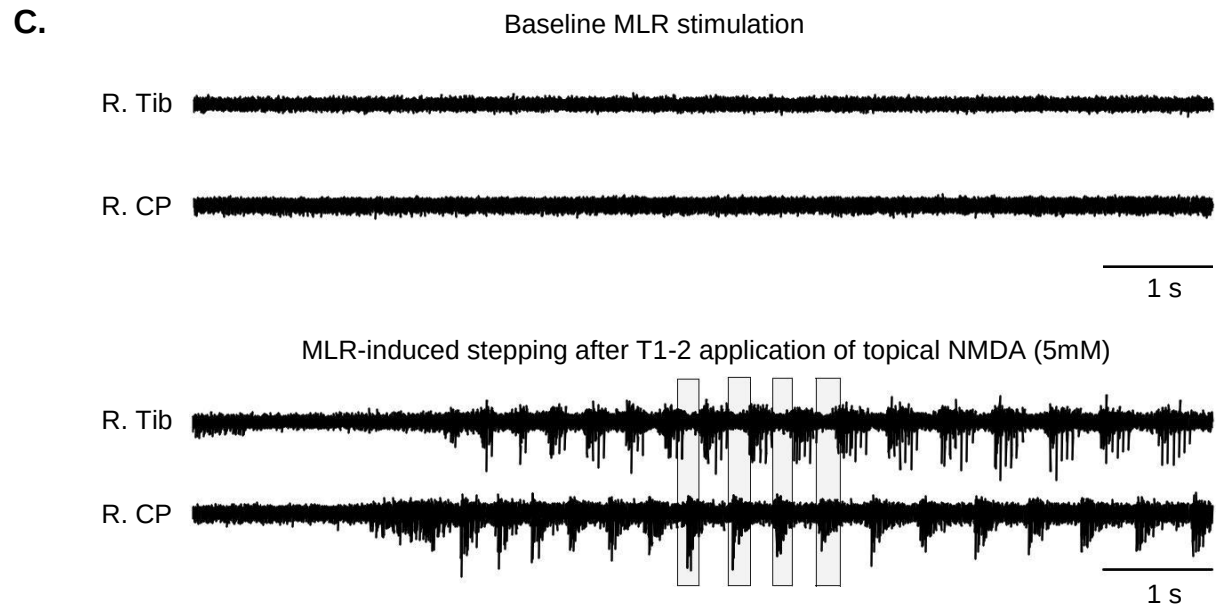


Fig. 5. Excitation of thoracic propriospinal neurons facilitated MLR-induced stepping. Hindlimb ENGs from flexor (CP) and extensor (Tib) nerves on the right (R) or left (L) sides following electrical stimulation of the mesencephalic locomotor region (MLR). In this set of experiments, the baseline MLR-induced stepping was either weak or absent.

A. A few low amplitude discharges of left CP were observed with the initial MLR stimulation (top traces). After the application of topical NMDA (100uL, 10mM) over T1-2 cord segments, the locomotor-like activity improved in the left CP previously displaying weak activity and also in the left Tib which had no activity before, as shown by shaded gray boxes over the flexor bursts. The time between NMDA application to recording the facilitated ENGs in this experiment is shown by black and red arrows. The top black arrow corresponds to the baseline MLR stimulation starting at 0 minutes. After 18 minutes, NMDA was applied over the high thoracic cord followed by MLR stimulation. Approximately one minute later the Tib and CP expressed rhythmic bursts.

B. In another experiment initial MLR stimulation induced low amplitude CP bursts (top traces). After the NMDA injection (5mM, 200uL) into the T1-2 segments rhythmic stepping cycle frequency increased and there was improved alternation of CP and Tib activity.

C. In a third experiment, baseline MLR stimulation failed to elicit an ENG response but after topical NMDA (5mM) application, alternating rhythmic stepping-like activity was observed in both extensor and flexor nerve ENGs. Note that rhythmic activity resulting from drug-induced facilitation of MLR-stimulation ceased immediately after the MLR stimulation has stopped and that in all of the experiments illustrated here, the position of the MLR electrode was kept the same before and after the drug application in order to ensure that same group of MLR neurons are being recruited to initiate the descending locomotor signal.

Figure 6.

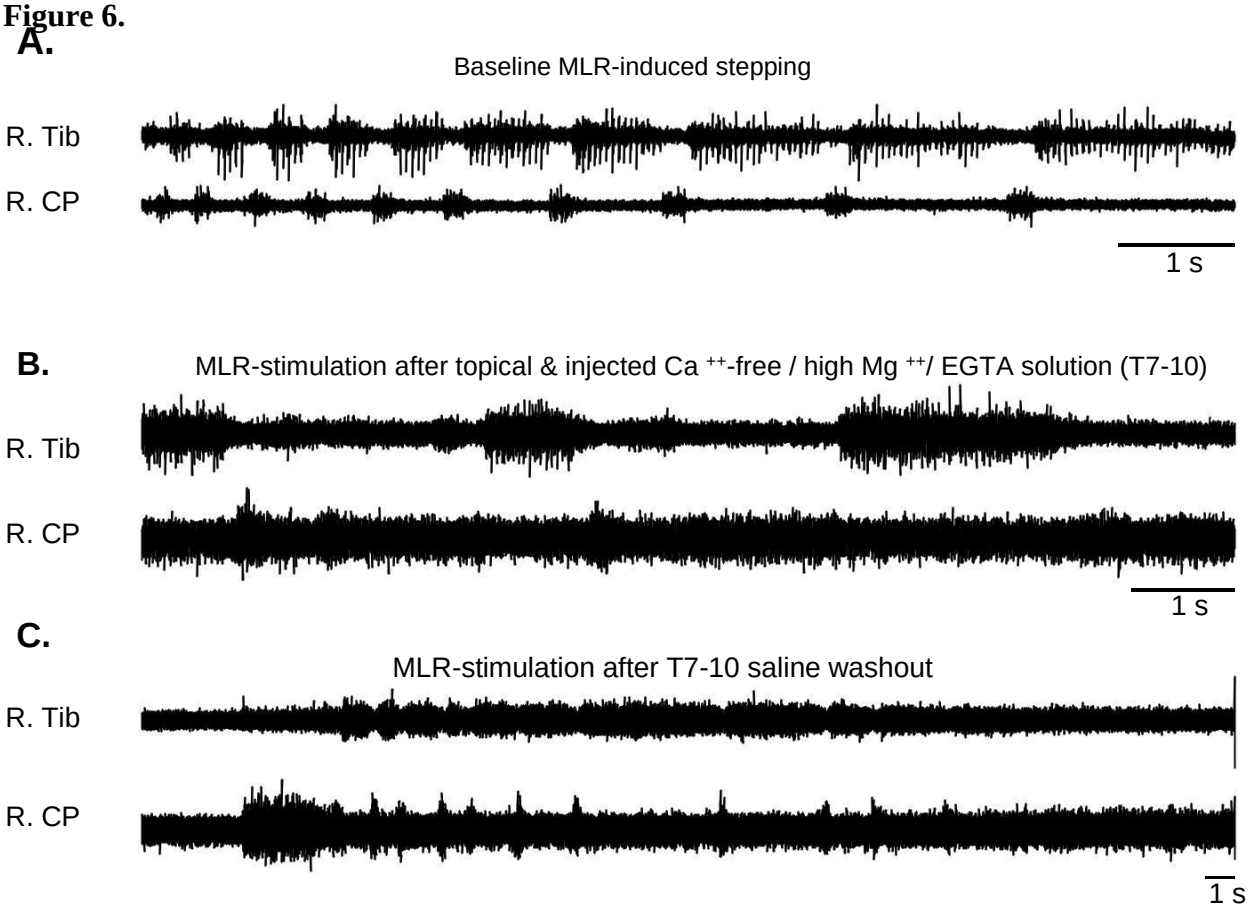


Fig. 6. Suppression of synaptic transmission disrupts MLR-induced stepping

Hindlimb ENGs from flexor (CP) and extensor (Tib) nerves on the right (R) or left (L) sides following electrical stimulation of the mesencephalic locomotor region (MLR).

A. Baseline MLR stimulation produced alternating activity in both flexor and extensor traces.

B. In order to block synaptic activity Ca^{2+}-free, high Mg^{2+}, (EGTA) solution was applied over T7-10 cord segmentsby intraspinal injections of 10µL at four different areas were done by a 33G Hamilton syringe, in addition to topical application over these segments which was done initially but it did not change the ongoing activity. The alternating flexor-extensor activity was replaced by long-lasting, weak ENG bursts in right Tib and low amplitude and/or absent bursts in right CP.

C. The T7-10 region was washed with copious amount of normal saline. A few minutes later, the alternating ENGs activity reappeared with variable rhythmicity. The time scale for this trace is relatively smaller than the for the data show in A and B in order to be able to show more steps (i.e. longer, continuous locomotor-like activity could be re-instated after washing out the mixture used for inducing synaptic blockade).

Figure 7.

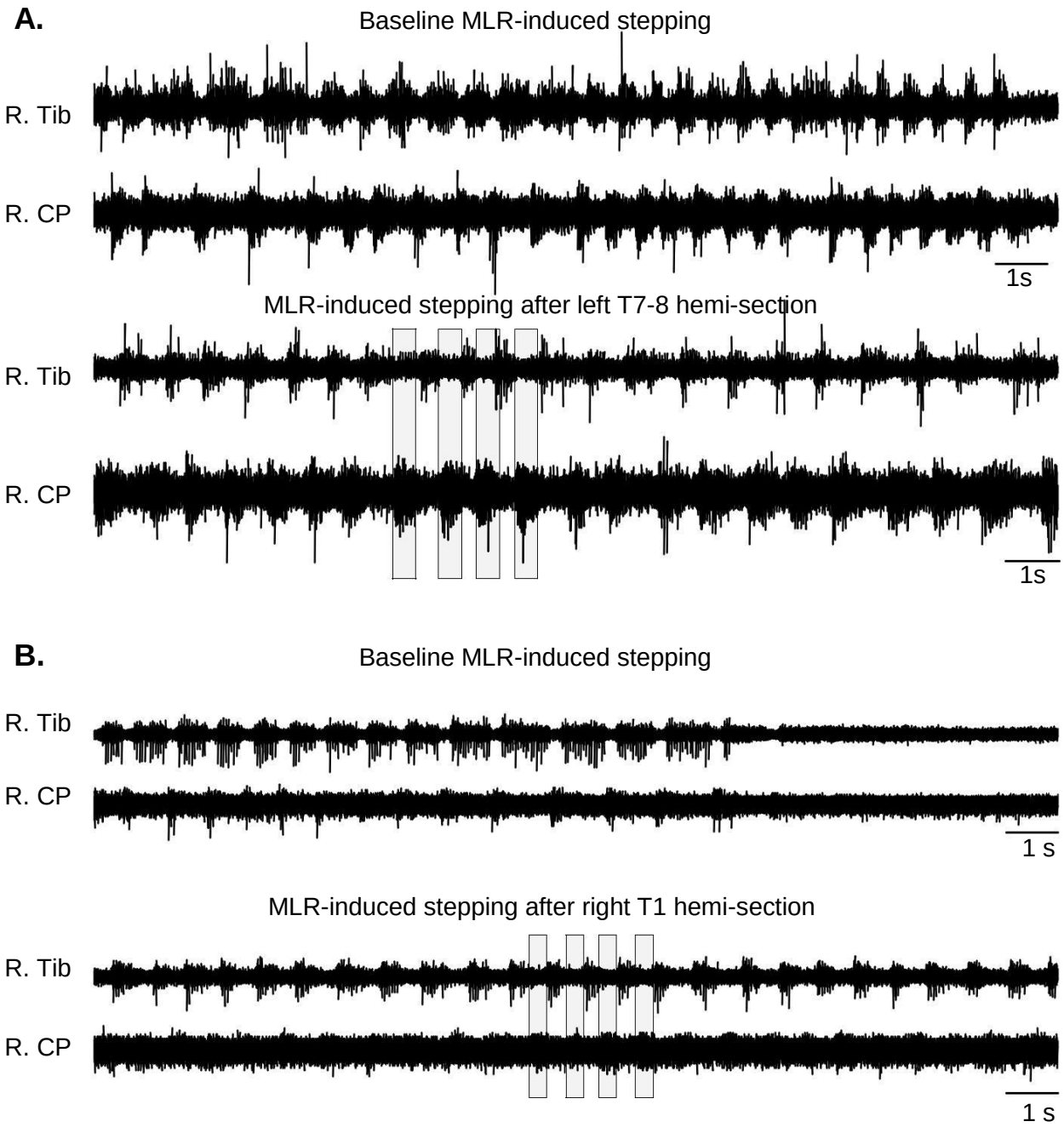


Fig. 7. MLR-induced stepping returned to normal after unilateral spinal cord hemisection
Hindlimb ENGs from flexor (CP) and extensor (Tib) nerves on the right (R) or left (L) sides following electrical stimulation of the mesencephalic locomotor region (MLR) in the top traces

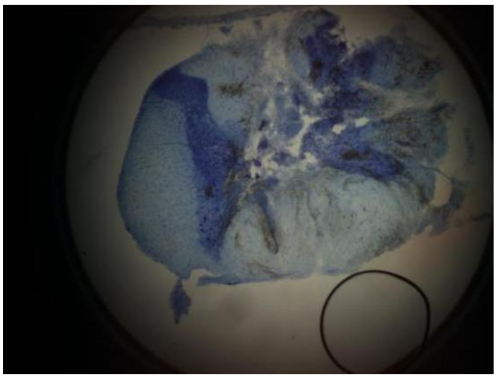
in each panel. The MLR-induced stepping persisted after acute unilateral hemisections (bottom traces in each panel).

A. Caudal thoracic spinal cord hemisection did not abolish locomotor-like activity after the spinal shock.)

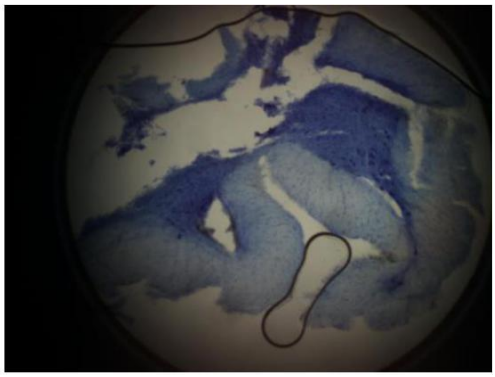
B. Rostral thoracic spinal cord hemisection did not abolish locomotor activity after the spinal shock. Following each hemisection, the rhythmic stepping returned to similar to the activity before the hemisections, as highlighted by grey shaded boxes over the flexor bursts.

Figure 8.

A

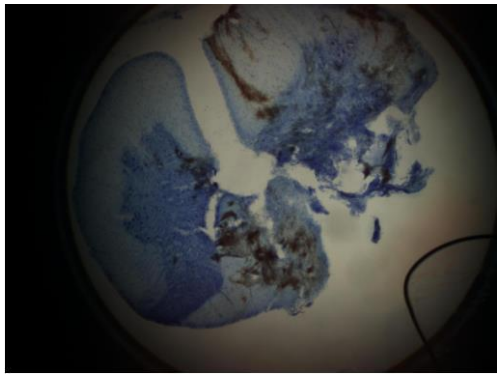


A1

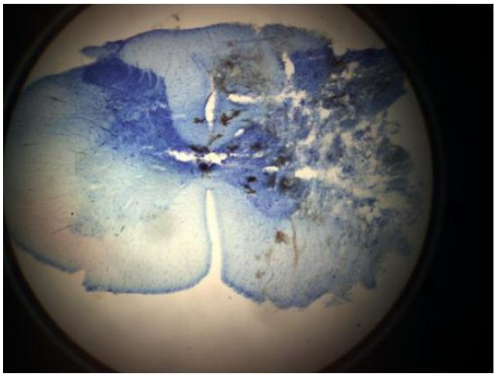


A2

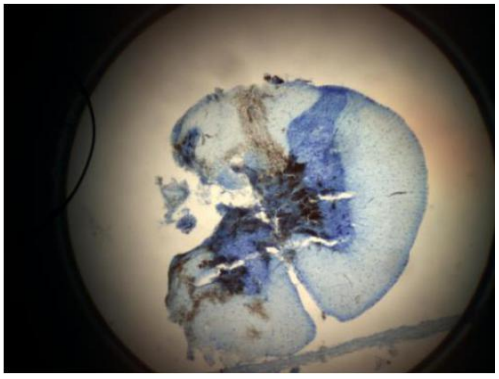
B



C

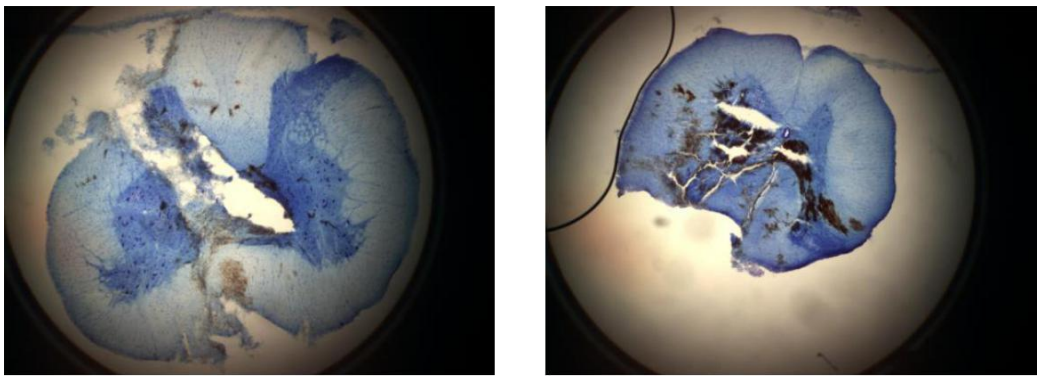


C1



C2

D



D1 D2

Fig. 8. Histological sections (Post fixed, Nissl stained, 80µm thick) of the experiments that received bilateral staggered hemisections.

- **A.** Sections from experiment 20. **A1** shows right sided T1 hemisection. **A2** shows left sided T7 hemisection. Stepping persisted after the first T1 hemisection but could not be elicited after the second T7 hemisection.
- **B.** Sections from experiment 22. Right sided T1 hemisection is shown and the left sided T7 hemisection could not be evaluated due to excessive damage caused while removing the cord from the animal after the experiment. Stepping persisted after the first T1 hemisection but could not be elicited after the second T7 hemisection.
- **C.** Sections from experiment 24. **C1** shows right sided T1 hemisection. **C2** shows left sided T7 hemiscetion. Stepping persisted after the first T1 hemisection but could not be elicited after the second T7 hemisection.
- **D.** Sections from experiment 26. **D1** shows left sided C7 hemisection. **D2** shows right sided T7 hemisection. Stepping did not reappear after the first and second hemisections.

Figure 9.

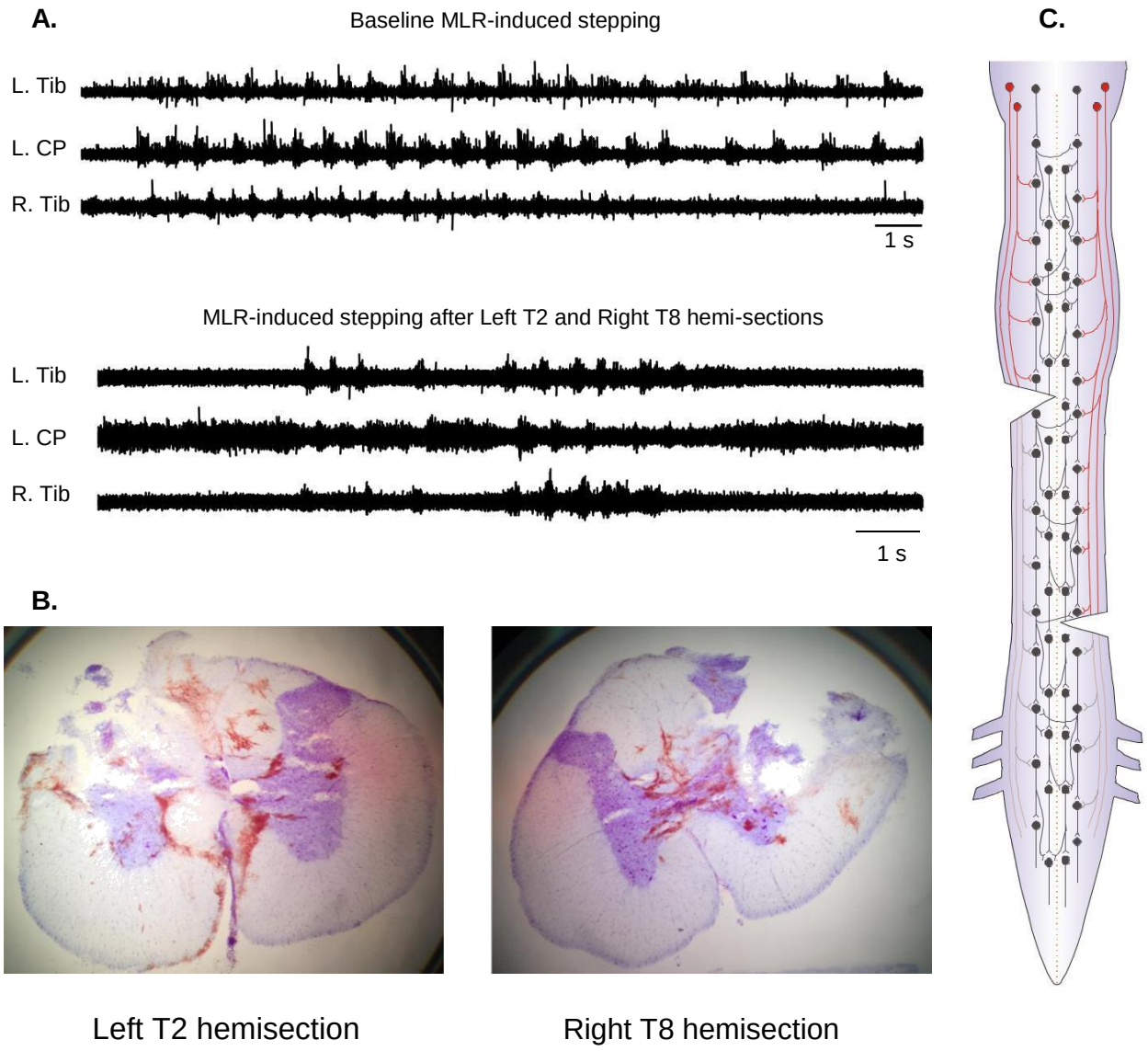


Fig. 9. MLR-induced stepping elicited after bilateral staggered hemisection

Hindlimb ENGs from flexor (CP) and extensor (Tib) nerves on the right (R) or left (L) sides following electrical stimulation of the mesencephalic locomotor region (MLR).

A. Rhythmic bursts were present in left flexor and extensor as well as a right extensor when MLR stimulation was applied initially (top three traces). Then a bilateral, stagerred hemisection was performed by pulling the spinal cord apart with forceps first at the T2 level on the left side and then at the T8 level in the right side. Then after a wait-period when MLR stimulation was applied again, persistent stepping-like activity could be evoked in spite of the left T2 and right T8 hemisections (bottom three traces).

B. Nissl stained spinal cord histological sections (80µm thick) showing incompleteness of the lesions. It is evident that a portion of ventrolateral funiculi were still intact bilaterally.

C. Spinal cord illustration depicting the ideal extent of bilateral staggered hemisections with discontinuation of long descending projections (in red) and the intervening redundantly distributed propriospinal neurons (in black) based on data from experiments performed on the neonatal preparations.

Discussion

Overall, the results of this work support the concept that propriospinal neurons influence locomotor circuitry in adult mammals. However whether or not the data supports the central hypothesis of this project, which states that propriospinal neurons in adult mammals contribute to transmission of the descending bulbospinal locomotor command signal, requires further discussion. At the end of introduction four aims/objectives of this study were stated. Third aim where neurochemicals were used to induce fictive locomotion was tested with the highest number of animals (6) and resulted in a most conclusive data set. Fourth aim which was targeted towards neurochemical facilitation of the locomotor signal was positive in 3 animals. Second aim of bilateral staggered hemisections was performed on 9 animals but partially successful in only 1. First aim to observe the effect of synaptic blockade was positive in one of two animals.

Evidence that propriospinal neurons activate hindlimb locomotor circuitry

Neurochemical excitation of thoracic propriospinal neurons elicited rhythmic stepping consistent with previous observations made using an *in vitro* neonatal rat spinal cord preparation (Zaporozhets et al. 2011). Neurochemicals topically applied and/or injected directly into the spinal cord influence cell bodies of propriospinal neurons and not the axons of passage. Excitatory drugs, NMDA in particular and 5-HT, were used for this purpose, using different concentrations and volumes. NMDA (10mM) produced a robust response but which rapidly led to a long-lasting loss of hindlimb flexor and extensor ENG discharge. Stepping induced by a lower concentration NMDA (5mM) was repeatable but with a longer delay to onset and eventual cessation of ENG discharge. The loss of ENG discharge was presumably related to NMDA-

induced depolarization block of the neurons. However more dose dependent tests would need to be performed as well as control experiments examining the excitatory blockade by evaluating forelimb motoneuronal activation, that is, high thoracic PS cells.

An inherent difficulty with this paradigm is that in order to achieve sufficient NMDA concentrations to influence as many propriospinal neurons as possible, the required NMDA concentration is unknown as the drug diffuses in the cord its concentration presumably decreases rapidly. Therefore, more distant neurons are exposed to lower concentrations of NMDA, potentially insufficient for receptor activation while other neurons, close to the application site, are initially appropriately activated by high concentrations but then may become tonically depolarized, which can even block action potential generation.

5-HT application alone was not effective in inducing stepping but rather acted as a modulator. In some instances it converted tonic NMDA-induced hindlimb ENG activity to rhythmic stepping. In other examples 5-HT increased the amplitude of weak NMDA-induced bursts. Given that brainstem electrical stimulation was not applied during these particular protocols, it may be reasonable to conclude that excitation of neurons in spinal cord segments located rostral (e.g. upper thoracic segments) to hindlimb motor centers in the lumbar cord are capable of facilitating hindlimb stepping. Such neurons are presumably propriospinal in nature. However, alternate explanations for the observations will now be considered.

In these experiments, it was not possible to accurately determine the extent of drug diffusion in the rostral and caudal directions. For instance, if the drugs diffused (either within the cord or in the subarachnoid space) as far as the hindlimb motor centers in the lumbar cord, the effect on locomotion could be due to direct effects on lumbar circuitry, rather than on propriospinal

neurons in more rostral cord regions. If rostral drug diffusion reached the brainstem it could excite cell bodies of long-direct brainstem projections to the lumbar cord. However, the lordotic positioning of the lower cervical-upper thoracic spinal cord in the experimental frame created a 'valley' for drug application which did not favor gravitational effects on long-distance rostral or caudal diffusion. At the end of two experiments, Chicago blue stain was added to the spinal segments that have received drugs. Dye did not diffuse beyond one or two segments in the rostral or caudal direction. However, due to possible differences in diffusion capacity of the dye versus drug, firm conclusions about the rostrocaudal extent of effective drug diffusion cannot be made based on analysis of this dye's dispersion alone. In addition, dye not detected by simple visual inspection of histological sections may have travelled more distally in the cord. Finally, the possibility that cell bodies neurochemically activated in the upper thoracic region project to brainstem neurons, which in turn project to the lumbar cord, cannot be excluded. Future experiments could address this issue by applying drugs to the rostral thoracic region, after a complete cervical cord transection.

Evidence for a propriospinal contribution to transmission of the brainstem locomotor command signal

Neurochemical excitation of thoracic propriospinal neurons improved previously absent or weak MLR-induced stepping. Stepping ceased when electrical stimulation of the MLR stopped. It means that neurochemical excitation did not elicit stepping alone (in these experiments) but facilitated the descending command signals. Therefore, in these experiments, neurochemical stimulation of the spinal cord on its own was subthreshold for activation of locomotion. These

observations suggest that propriospinal neurons contribute to and facilitate transmission of the descending locomotor command signal in the adult mammal. The observations are consistent with previous results observed in the *in vitro* neonatal rat spinal cord brain stem model (Zaporozhets et al. 2011). Because the preparation is acute, not chronic, suggests that propriospinal neurons contribute to locomotor command signal propagation in the intact animal. In contrast to previous work proposing that locomotor-related propriospinal neurons were called into play only as a time-dependent reaction to chronic injury (plasticity) (Courtine et al. 2008), these results suggest that without time dependent changes in PS networks the facilitation of locomotion is possible upon their activation.

NMDA was the only excitatory neurochemical used in this series. It was selected because it allows for a non-specific method of increasing neuron excitability, given that NMDA receptors are ubiquitous among neurons in the central nervous system. The facilitatory effect was observed using either topical or intraspinal micro-injection methods.

Response time was variable ranging from a few seconds to several minutes. There are limitations in the interpretation of the effects of cord drug administration, similar to those described above with respect to the effect of drugs applied in the absence of brain stem electrical stimulation. That is, the spread of drug to the lumbar region or to brain stem neurons, or activation of spinal neurons projecting to brainstem neurons, is not excluded, although perhaps unlikely. Another caution in the interpretation of the results is that although it appears neurochemical-activation of propriospinal neurons facilitates MLR-induced locomotion under experimental conditions, it is possible that during overground locomotion in an intact animal propriospinal support of the bulbospinal locomotor command signal is not essential. That is, although stimulation of propriospinal neurons facilitates stepping, just as does stimulation of tail

afferents in decerebrate preparations [personal observations, similar to that described in cats (Forssberg et al. 1980)] under normal intact conditions mammals may not require propriospinal system activation, just as tail stimulation is not required, in order to transmit an effective locomotor command signal from the brainstem to the lumbar region.

MLR-induced hindlimb stepping was altered by suppression of synaptic transmission. Unlike the case in using the *in vitro* neonatal rat model [42] the mixture was not completely suppressing synaptic transmission. Combined Ca^{2+}-free, high [Mg^{2+}], EGTA solutions were administered both topically and by injections to minimize the available calcium in the pre-synaptic terminals and promote block of the excitatory glutamatergic receptors (i.e. Mg^{2+} blockade of NMDA receptors). Major challenges to this approach *in vivo,* compared to *in vitro,* are the difficulties associated with perfusion of the larger tissue mass of an adult cord and the continuous physiological replacement of Ca^{+2} ions by the local microvasculature, making it virtually impossible to achieve a Ca^{+2}-free neuronal environment. More experiments are needed to see if this observation is repeatable. In addition, experiments should be repeated using normal artificial cerebrospinal fluid (but without Ca^{+2}) instead of normal saline, in order to more closely mimic interstitial fluid composition surrounding the spinal cord. An additional approach would be to administer a cocktail of glutamatergic, serotonergic, cholinergic and dopaminergic receptor antagonists to enhance the effectiveness of synaptic blockade.

What was learned from the spinal cord lesion experiments?

Staggered bilateral spinal cord hemisections have the potential to provide the most definitive evidence of a propriospinal contribution to descending transmission of the locomotor command

signal. However, these experiments are still challenging, despite the success we have achieved with this series. It was difficult to gauge the magnitude of "spinal shock", or the extent of the lesions. Only post hoc could the extent of lesions be confirmed. We were able to get MLR-induced stepping after the first hemisection in all instances if the animal's physiologic state did not decline. After the second contralateral staggered hemisection in only one of nine experiments did stepping occur in response to MLR stimulation. Subsequent analysis of the lesions in this experiment revealed that they spared 20-30% of ventrolateral funiculi bilaterally. Given the importance of the ventrolateral funiculi for transmission of brainstem-induced locomotion (Noga et al. 1991), sparing fibers in the ventrolateral funiculus may account for the successful induction of locomotion in this animal.

Another factor limiting our success eliciting locomotion after bilateral staggered hemisections is the unknown duration of spinal shock caused by the lesions, which theoretically would suppress overall spinal cord excitability. We typically intermittently tested MLR stimulation over the course of up to five to six hours, if the animal survived. However this practice itself was often limited by another issue, which was the declining health of the animal. Hemisections were often done late in the experiments and were associated with declining blood pressure and responsiveness of the animal to brainstem stimulation in 4 out of 9 animals that received bilateral staggered hemisections. In addition to the blood loss during surgery throughout the day, including during decerebration, dual laminectomies, and bilateral hindlimb nerve dissections, the rostral hemisection interrupted ipsilateral supraspinal drive to preganglionic sympathetic nuclei located in the intermediolateral nuclei in the thoracic and upper lumbar cord. This would further impair the animal's ability to mount a reflex sympathetic response to hypotension.

For future experiments, possible solutions to alleviate some of these problems include the use of ice to cool the spinal cord prior to hemisections and reduce the barrage of neural discharge acutely induced by the hemisections. Avoiding high (e.g. T1-2) rostral hemisections would leave intact supraspinal regulation of more thoracic sympathetic nuclei; perhaps this would prevent the acute decrease in blood pressure associated with lesioning. The effects of blood loss can be alleviated by infusion of plasma expanders or ideally by transfusion of whole blood.

Finally, although brainstem activation of locomotion in the presence of bilateral staggered hemisections has been well-demonstrated in the *in vitro* neonatal rat model (Cowley et al. 2008), it is perhaps surprising that this method should work in any preparation. That is, not only are the long direct projections lesioned, but a larger portion of the propriospinal system is also abolished by such lesions. An alternative method of selectively lesioning all direct long-projecting bulbospinal fibres while leaving the propriospinal system, or at least part of the propriospinal system, intact is not readily available.

Translational relevance of this research

The ultimate goal of this line of research study is to explore whether the locomotor-related propriospinal system might serve as an important target for the treatment of patients with spinal cord injury. The potential of propriospinal neurons in general to contribute to restoration of function after spinal cord injury has been demonstrated in earlier work by Schwab's group (Bareyre et al. 2004; Filli et al. 2014). Propriospinal neurons were shown to receive input from collaterals of severed descending corticospinal and bulbospinal neurons (Bareyre et al. 2004; Filli et al. 2014). Propriospinal neurons crossed from above the hemisection to the intact side

and then re-crossed below the lesion therefore bypassing the lesion site (Filli et al. 2014). This bridging function, across the lesion site, resulted in some recovery of locomotor function which suggests an important role of locomotor-related propriospinal neurons in relaying the locomotor command signal to the lumbar cord circuitry. However, these experiments were performed on chronically injured animals. It is already known that animals with chronic hemisections recover the ability to walk for example as reviewed in (Rossignol and Frigon 2011). It is possible that recovery of locomotion in these cases is a function of preserved long direct projections on the un-lesioned side, independent of any new propriospinal connections. Acute experiments provide the best insights into the organization of the normal substrate of this system in the intact animal, uninfluenced by time-dependent change (plasticity).

Another important reason to probe the locomotor-related propriospinal system is its potential implication in regeneration research. An understanding of this system would guide strategies aimed at restoring transmission in the spinal cord after injury. Regeneration of relatively short propriospinal projections instead of the long tract fibres is more feasible. In order to achieve this goal more studies are needed to ascertain the location and various types of synapses propriospinal neurons form, especially after an injury.

The propriospinal system as a novel target for developing clinical interventions

Pharmacological stimulation of neurons could be used clinically to alter their background excitability; this would enhance transmission of voluntary command signals. Drugs could be delivered to the appropriate spinal cord regions via intrathecal catheters, as presently done for the delivery of baclofen by implanted pump for managing spasticity (Ethans 2007).

Another approach is epidural electrical stimulation. This mode of stimulation has also been used in humans with favorable results where the patients with motor and sensory complete SCI were able to stand in response to lumbosacral epidural stimulation of different intensities as e.g. (Rejc et al. 2015).

In addition to descending propriospinal fibers, target of these therapies could also be ascending propriospinal neurons that relay afferent feedback critical in maintaining balance, and other sensory functions.

In conclusion, the locomotor-related propriospinal system is potentially very important in transmitting supraspinal command signals. In-depth investigation into its potential role in acute mammalian preparations, such as the one used in this project, can reveal critically important information. Further exploration of the propriospinal system, in both intact and acutely lesioned spinal cords, will not only clarify its physiological role but also demonstrate the potential of injured propriospinal neurons to establish new connection over time and enhance the functional capacity of the injured spinal cord.

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