Neurochemical excitation of propriospinal neurons facilitates locomotor command signal transmission in the lesioned spinal cord

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Previous studies of the in vitro neonatal rat brainstem-spinal cord showed that propriospinal relays contribute to descending transmission of a supraspinal command signal that is capable of activating locomotion. Using the same preparation, the present series examines whether enhanced excitation of thoracic propriospinal neurons facilitates propagation of the locomotor command signal in the lesioned spinal cord. First we identified neurotransmitters contributing to normal endogenous propriospinal transmission of the locomotor command signal by testing the effect of receptor antagonists applied to cervicothoracic segments during brainstem-induced locomotor-like activity. Spinal cords were either intact or contained staggered bilateral hemisections located at right T_{1/2} and left T_{10/11} junctions designed to abolish all direct long-projecting bulbospinal axons. Serotonergic, noradrenergic, dopaminergic and glutamatergic, but not cholinergic, receptor antagonists blocked locomotor-like activity. Approximately 73% of preparations with staggered bilateral hemisections fail to generate locomotor-like activity in response to electrical stimulation of the brainstem alone; such preparations were used to test the effect of neuroactive substances applied to thoracic segments (bath barriers place at T_3 and T_9) during brainstem stimulation. The percentage of preparations developing locomotor-like activity was as follows: 5-HT (43%), 5HT/NMDA (33%), quipazine (42%), 8-OH-DPAT (20%), methoxamine (45%), and elevated bath K^+ concentration (29%). Combined norepinephrine and dopamine increased the success rate (67%) compared to use of either agent alone (4% and 7%, respectively). NMDA, Mg^{2+} ion removal, clonidine, and acetylcholine were ineffective. The results provide proof-of-principle that artificial excitation of thoracic propriospinal neurons can improve supraspinal control over hindlimb locomotor networks in the lesioned spinal cord.

Key words: propriospinal, locomotion, brainstem electrical stimulation
INTRODUCTION

It is widely accepted that long direct bulbospinal projections, reticulospinal axons in particular, activate locomotor circuitry in the vertebrate spinal cord (for reviews see Grillner et al. 1997, Jordan et al. 2008). Using the in vitro neonatal rat brainstem-spinal cord preparation, we showed that propriospinal pathways also convey descending transmission of locomotor command signals originating in the brainstem (Zaporozhets et al. 2006). Reticulospinal and propriospinal systems are not likely independent in this function. Rather, reticulospinal collaterals, which are known to terminate diffusely, both ipsilaterally and contralaterally in cervical, thoracic, and lumbar segments (Jankowska et al. 2003; Matsuyama et al. 2004; Peterson et al. 1975; Reed et al. 2008) are well-positioned anatomically to distribute locomotor command signals to propriospinal neurons throughout the rostrocaudal extent of the spinal cord. During electrical stimulation of the brainstem, propriospinal transmission alone, independent of long-direct projections, was sufficient to activate locomotor-like activity in 27% of in vitro rat preparations (Cowley et al. 2008). Thus, regeneration of propriospinal connections and artificial enhancement of signal propagation through residual intact propriospinal pathways, even without re-growth of long-direct brainstem projections to the lumbar cord, are attractive strategies to pursue for restoring spinal cord function after injury.

Evidence of propriospinal system plasticity participating in the formation of functional bypass circuits in the lesioned cervical cord has been shown in rat (Bareyre et al. 2004) and cat (Fenrich and Rose 2009) preparations. Propriospinal mechanisms also contribute to motor recovery after spinal cord injury in the lamprey (McClellan 1994), chick embryo (Sholomenko and Delaney 1998), and rat (Arvanian et al. 2006a, b). Courtine et al. (2008) demonstrated that recovery of hindlimb stepping in mice with T7 and contralateral T12 hemisections was associated with an increased number of propriospinal neurons in the inter-lesion zone. Murray et al. (2010) also recently showed that rats with T6 and contralateral T12 hemisections improve their hindlimb locomotor scores over a period of several weeks.
The present study of lesioned spinal cord preparations examines whether neurochemical excitation focused specifically on thoracic propriospinal neurons (i.e. located rostral to hindlimb locomotor circuitry) facilitates descending propagation of the supraspinal command signal. This approach contrasts with previous studies that involved neurochemical activation of locomotor central pattern generating (CPG) circuitry in the lumbar segments through systemic administration of drugs or local application to the lumbar region. For instance, *in vivo* studies have shown that hindlimb stepping is promoted by L-3,4-dihydroxyphenylalanine (L-DOPA, Budakova 1971; Grillner and Zangger 1979; Jankowska et al. 1967a,b), clonidine (Barbeau et al. 1987; 2000; Forsberg and Grillner 1973), serotonergic agents (Antri et al. 2002, 2003, 2005; Barbeau and Rossignol 1990, 1991; Feraboli-Lohnherr et al. 1999; Fong et al. 2005; Guertin PA 2009; Hayashi et al. 2010; Ichiyama et al. 2008; Kim et al. 1999, 2001; McEwen et al. 1997; Viala and Buser 1971), and excitatory amino acid receptor agonists (Chau et al. 2002; Giroux et al. 2003; Douglas et al. 1993). Similarly, numerous reports using *in vitro* vertebrate preparations describe direct neurochemical stimulation of locomotor CPG circuitry, using excitatory amino acids, monoaminergic, and cholinergic agonists (Barry and O'Donovan 1987; Cazalets et al. 1992; Cohen and Wallen 1980; Cowley and Schmidt 1994, 1997; Dale and Roberts 1984; Gabbay and Lev-Tov 2004; Grillner et al. 1981; Guertin and Hounsgaard 1998; Harris-Warrick and Cohen 1985; Jovanovic et al. 1996; Jiang et al. 1999; Kiehn and Kjaerulff 1996; Kremer and Lev-Tov 1997; Kudo and Yamada 1987; Madriaga et al. 2004; McDiarmid et al. 1997; McLean and Sillar 2003; Panchin et al. 1991; Poon 1980; Smith and Feldman 1987; Sqalli-Houssaini and Cazalets 2000; Whelan et al. 2000). Cyproheptadine and clonidine promote stepping in humans with spinal cord injury (Fung et al. 1990; Remy-Neris et al. 1999; Stewart et al. 1991; Wainberg et al. 1990). Systemic administration of drugs *in vivo*, or whole cord applications of drugs *in vitro*, may influence locomotor-related cervicothoracic propriospinal neurons as well as lumbar CPG circuitry. Relatively little information is available as to which neurotransmitters may be involved in locomotor-related propriospinal transmission. However, assuming the locomotor command signal input to propriospinal neurons is delivered by reticulospinal...
projections, monoaminergic and/or glutamatergic mechanisms likely participate. A role for serotonin is also suggested by the observation that serotonin application to the lumbar cord will not elicit lumbar locomotor-like activity unless serotonin is also used to excite neurons in the cervicothoracic region (Cowley and Schmidt 1997). Similarly, combined serotonin/NMDA excitation of neurons in the cervical enlargement (Ballion et al. 2001; Cowley and Schmidt 1997) or rostral cervical segments (Cowley et al. 2008) can induce locomotor-like activity in the hindlimbs. Electrical stimulation of serotonin-containing reticulospinal neurons in the neonatal rat parapyramidal region evokes locomotion (Liu and Jordan 2005). In favor of a role for noradrenergic mechanisms is the observation that intraspinal injection of the alpha 2-noradrenergic receptor antagonist, yohimbine, into pre-CPG segments in the lower thoracic and upper lumbar region of the cat blocks spontaneous locomotion (Delivet-Mongrain et al. 2008).

Because of the ubiquitous nature of excitatory amino acid transmission in the nervous system it seems reasonable to speculate that glutamate activates propriospinal neurons and is released by them. Thus, monoaminergic and excitatory amino acid receptors on cervical and/or thoracic propriospinal neurons are logical targets for artificial stimulation in an effort to facilitate transmission of the locomotor command signal in the lesioned spinal cord.

The main goal of the present study was to determine whether neurochemically enhanced propriospinal transmission in the thoracic region improves supraspinal control over hindlimb locomotor circuitry located in the lumbar cord. We first screened a variety of receptor antagonists to determine which neurotransmitter systems contribute to locomotor-related propriospinal transmission in response to electrical stimulation of the brainstem in the neonatal rat preparation. Then we investigated the effect of neurochemically enhanced propriospinal excitation on locomotor command signal propagation using preparations with staggered bilateral hemisections in the thoracic region. The latter preparations were selected on the basis of failing to produce lumbar locomotor-like activity in response to brainstem stimulation alone. Some of the following data has been presented previously in abstract form (Cowley et al. 2009).
METHODS

Research protocols used in this study were in compliance with the Canadian Council on Animal Care and approved by the University of Manitoba Animal Protocol Review Committee.

Sprague-Dawley rats (1-5 days old) were anesthetized with isofluorane, decerebrated at the mid-collicular level, eviscerated, and placed in a bath chamber containing artificial cerebrospinal fluid (ACSF) composed as follows (in mM): NaCl 128, KCl 4.0, NaH₂PO₄ 0.5, CaCl₂ 1.5, NaHCO₃ 21, MgSO₄ 1.0, and glucose 30, equilibrated to pH 7.4 with 95% O₂/5% CO₂. The brainstem and spinal cord were then isolated ventral side up and bilaterally intact. Experiments were conducted at room temperature (ACSF approximately 22°C). Preparations were left in the bath solution, unstimulated, for approximately one hour before attempting to elicit locomotor-like activity via electrical stimulation of the brainstem.

Ventral root recordings, obtained using glass suction electrodes, were band-pass filtered (30-3000Hz), digitized and captured using Axoscope (v 9.0 Axon Instruments) software. Axoscope files were converted to an appropriate binary format for further analysis using special purpose software (developed by the Spinal Cord Research Centre, University of Manitoba).

In some preparations, hemisections were made in the rostral (right T₁/T₂) and contralateral caudal (left T₁₀/T₁₁) thoracic regions. The bath was then partitioned using thin plastic barriers sealed at cord contact edges with petroleum jelly. Barriers were placed such that spinal neurons in the inter-lesion zone (T₃ through T₉ inclusive) could be selectively exposed to neurochemicals. As noted in the Results section, intact spinal cords were used in some experiments, with barriers placed at C₁ and T₈/₉ for application of neurochemicals to the cervicothoracic region, or at T₁₁ for selective application of neurochemicals caudal to this level. Excitatory neurochemicals were applied at concentrations subthreshold for evoking locomotor-like activity in the
absence of brainstem stimulation. All neurochemical concentrations refer to final bath concentrations.

Electrical stimulation of the brainstem was performed as previously described (Zaporozhets et al. 2004). In brief, an ACSF-filled glass electrode, with a tip diameter of 200-300 μm, was placed in contact with the ventral surface of the brainstem. Bipolar stimulation was used to deliver monophasic rectangular current pulses (4 - 20 ms, 0.5 - 5 mA, 0.8-2.0 Hz). Stimulation was applied for a maximum of two to three minutes per test episode. For experiments involving application of neurotransmitter antagonists to thoracic segments in an effort to abolish brainstem-evoked locomotor activity, baseline rhythmic activity in the absence of drug application was first established. The selected antagonist was then applied using a range of concentrations as needed, and using the baseline brainstem stimulation parameters. If locomotor-like activity was abolished, higher stimulation current was administered in an effort to see if locomotor-like activity could break through. The antagonist was considered capable of blocking propriospinal transmission only if it blocked brainstem-evoked locomotor-like activity at all stimulation strengths. For experiments involving neurochemical excitation of thoracic propriospinal neurons in an effort to facilitate brainstem-evoked locomotor activity, it was first established that locomotor-like activity could not be elicited regardless of brainstem stimulation intensity. Excitatory neurochemicals were then applied using a range of concentrations as needed, while stimulating the brainstem at a range of current strengths (from 0.5 to 5 mA).

Criteria used to classify lumbar ventral root discharge as locomotor-like are in accordance with our previous work (e.g. Cowley et al. 2008). In particular, the ventral root discharge pattern was deemed locomotor-like if a) alternation was observed between the left and right sides at L2 and/or between left and right sides at the L5 level, and b) ipsilateral alternation was present between L2 (predominately flexor-related activity) and L5 (predominantly extensor-related activity) on at least one side. In some experiments, T8 ventral root activity was monitored instead of L5, which allowed monitoring of activity in the inter-lesion zone as well as the lumbar (L2) cord. However,
in these preparations without L5 recordings, we refer only to *rhythmic activity*, based on alternating left-right L2 discharge, rather than *locomotor-like* patterns, as full criteria could not be assessed.

**RESULTS**

The results are divided into two main sections. First, preparations with either intact or lesioned spinal cords, capable of generating lumbar locomotor-like activity in response to brainstem stimulation, were used to systematically screen the suppressive influence of a variety of neurotransmitter antagonists. This survey was used to determine which endogenous neurochemicals normally contribute to propriospinal transmission of the locomotor command signal. The second section used lesioned spinal cord preparations (staggered contralateral hemisections) that failed to produce lumbar locomotor-like activity in response to brainstem stimulation alone. Approximately 73% such preparations fail to display locomotion (Cowley et al. 2008). A variety of excitatory agents were thus applied to the thoracic cord to see if enhanced propriospinal excitation enabled the emergence of locomotor activity in the lumbar region, when combined with brainstem stimulation.

**Effect of neurotransmitter antagonists on propriospinal transmission in preparations producing lumbar locomotor-like activity during brainstem stimulation alone.**

a) Preparations with intact spinal cords.

The non-selective 5-hydroxytryptamine (5-HT) receptor antagonist mianserin (50-75 μM) was applied to the C1-T8 bath compartment while electrically stimulating the brainstem. Lumbar root locomotor-like discharge was abolished in 4/4 preparations (Fig. 1). Another non-selective 5-HT receptor antagonist, ketanserin (100 μM), had the same effect (n= 1/1). Of note, neither of these antagonists is entirely specific for 5-HT receptors as they also bind to α-noradrenergic and histamine receptors (Hoyer et al. 1994).
The dopamine receptor antagonist haloperidol (10-75 μM) blocked brainstem-evoked locomotor-like activity in the lumbar region when applied to the C₁-T₈ region of the intact spinal cord (n=6/6, Fig. 2A). When haloperidol (15-20 μM) was applied caudal to T₁₁, brainstem-evoked locomotor-like activity was also abolished (n=2/2; Fig. 2B). Thus dopaminergic receptor activation appears to contribute to cervicothoracic propriospinal signal propagation and to activation of locomotor circuitry in the hindlimb segments.

Application of the α₁,2 noradrenergic receptor antagonist prazosin (65 μM, n=2/2) or the α₂ noradrenergic receptor antagonist yohimbine (5 μM, n=6/6) to the C₁-T₈ region suppressed lumbar locomotor-like activity in response to brainstem stimulation (Figs. 3A and 4A, respectively). Similarly, both prazosin (65 μM, n=2/2) and yohimbine (15 μM, n=1/2) blocked brainstem induced rhythmic activity when applied on locomotor circuitry located caudal to T₁₁ (Figs. 3B and 4B, respectively).

b) Preparations with staggered bilateral hemisections.

A theoretical limitation of the intact brainstem-spinal cord preparation is that an inhibitory influence of receptor antagonists on propriospinal synaptic activity may be obscured by preserved transmission through bulbospinal axons projecting directly to lumbar locomotor circuitry. However, in the present series this may not have been a major factor because, as noted above, receptor antagonists with actions on all three monoamine systems tested (dopaminergic, noradrenergic and serotonergic) suppressed brainstem-induced lumbar locomotor-like activity. Nonetheless, this series included experiments using preparations with staggered contralateral hemisections made at the right T₁/₂ and left T₁₀/₁₁ junctions. These lesions abolish all direct-projecting bulbospinal input to the lumbar cord (Cowley et al 2008). Although propriospinal relays are also disrupted, at least partially, by these lesions, any brainstem signal reaching the lumbar region in such preparations must do so via at least one synaptic relay and cross projection. Thus, this model enables investigation of the effects of neurochemical manipulation of propriospinal transmission independent of the influence of long direct projections to the lumbar cord.
As was the case for intact spinal cord preparations, monoamine receptor antagonists (applied to the thoracic inter-lesion zone at T3-T9) blocked brainstem-induced locomotor-like activity in the lumbar region. More specifically, rhythmic activity was suppressed by: the 5-HT antagonist ketanserin (50-75 μM, n=3/7), the dopamine antagonist haloperidol (10-80 μM, n=6/9, Fig. 5), and α-1,2 noradrenergic antagonist prazosin (35-65 μM, n=4/7). On the other hand, SB269970 (10-40 μM), a serotonin antagonist that has relatively greater selectivity for 5-HT7 receptors, failed to suppress locomotor-like activity (n=3/3). Clozapine (1-30 μM), a nonspecific antagonist with high affinity for 5-HT7, but also actions on other 5-HT as well as alpha noradrenergic receptors (Svensson 2003) blocked brainstem-evoked locomotion in 6/9 preparations. In the example shown in Figure 5A, thoracic (T8) ventral roots were recorded along with L2 ventral roots. Alternating lumbar ventral root activity was restored after haloperidol washout while T8 recordings remained silent (Fig. 5A3). The reason for this is not known, but may reflect a greater capacity of the upper lumbar segments to generate rhythmic activity in response to brainstem stimulation, compared to thoracic segments, in the presence of incomplete washout of haloperidol.

The NMDA receptor antagonist AP-5 (20-80 μM) blocked locomotion in 4/4 preparations. Acetylcholine receptor blockade using atropine failed to abolish locomotor-like activity in the three preparations tested, consistent with earlier observations of this antagonist when applied to the cervicothoracic region of non-lesioned preparations induced using chemical or electrical stimulation of the brainstem (Zaporozhets et al. 2006).

Effect of neurochemical excitation of propriospinal neurons on locomotor command signal transmission in lesioned preparations unresponsive to brainstem stimulation alone.

The effect of neurochemical agents and manipulations was examined in preparations unresponsive to brainstem stimulation alone. Given the results using receptor antagonists, we were particularly interested in the potential of serotonergic, dopaminergic, noradrenergic and glutamatergic agonists to facilitate propagation of the
locomotor signal. In these experiments subthreshold concentrations of excitatory
agents were applied to the thoracic cord. That is, although neurochemical application
to the cervical and/or thoracic regions can sometimes induced rhythmic activity in the
lumbar region (Ballion et al. 2001; Cowley et al. 1997, 2008) in the present experiments
neurochemicals were applied at concentrations confirmed in each preparation to be
subthreshold for induction of rhythmic activity in the absence of brainstem stimulation.
The following results are summarized in Table 1.

During brainstem stimulation, bath application of 5-HT (10-50 μM) to the thoracic region
(T3-T9) of preparations with staggered bilateral hemisections, at the right T1/2 and left
T10/T11 junctions, enabled the emergence of locomotor-like activity in the lumbar region in
13/30 preparations (Fig. 6A). In three additional preparations with staggered lesions,
brainstem stimulation evoked rhythmic activity in one or two roots in the absence of 5-
HT application; subsequent 5-HT application to the thoracic region increased the
number of rhythmically active roots. Similarly the 5-HT receptor agonist quipazine (10
μM) promoted lumbar locomotor-like activity in response to brainstem stimulation in 3/7
preparations that initially failed to develop rhythmic activity in response to brainstem
stimulation alone. Application of the 5-HT1A receptor agonist 8-OH-DPAT (1-30 μM) to
the thoracic cord facilitated locomotor-like discharge in only 2/10 preparations that
failed to display locomotion in response to brainstem stimulation alone.

The effectiveness of brainstem stimulation depended on both the stimulus intensity and
concentration of 5-HT. Figure 6B shows results using 5-HT at 10, 30 and 50 μM in
seven preparations, at four different strengths of brainstem stimulation. At a
stimulation intensity of 1 mA only the high concentration of 5-HT (50 μM) facilitated
locomotor-like activity (n=1/7). At 2 mA stimulation strength, 30 and 50 μM 5-HT were
effective (n=2/7 and 4/7, respectively). At higher brainstem stimulus intensities (≥ 3
mA), 10 μM 5-HT was effective (n=3/7) in preparations otherwise unresponsive to the
same intensity of brainstem stimulation in the absence of 5-HT. However, even at the
maximum stimulation strength (4 mA) higher concentrations of 5-HT (30 and 50 μM)
were effective in more preparations (n=4/7 and 5/7, respectively).
The combination of 5HT/NMDA (10-50 / 2-5 μM) promoted locomotor-like activity in response to brainstem stimulation in 3/9 preparations unresponsive to either brainstem stimulation or neurochemical application (T3-T9) alone. It seems however, that the facilitatory effect on thoracic propriospinal transmission was mainly related to 5-HT actions because NMDA alone (2-4 μM) uniformly failed to enable locomotor-like activity (n=0/14, Figs. 7A1 & A2), despite the fact that the NMDA antagonist AP-5 consistently blocked locomotor-like activity evoked by brainstem stimulation (n=4/4). In addition, the percentage success rate using 5HT/NMDA (33%) was less than using 5HT alone (43%). In nine preparations unresponsive to brainstem stimulation alone an attempt was made to enhance NMDA receptor channel conductance, in the thoracic cord, using Mg2+-free bath solution (Mayer and Westbrook 1987). This approach also failed to facilitate propriospinal transmission of the locomotor command signal.

Norepinephrine application to the thoracic cord facilitated brainstem-evoked hindlimb rhythmic activity in only 1 of the 28 preparations tested. On the other hand, the noradrenergic receptor α-1 agonist methoxamine (40-100 μM) promoted rhythmic activity in 5/11 preparations that otherwise failed to develop rhythmic activity in response to brainstem stimulation alone (Fig. 7A3). The α-2 noradrenergic agonist clonidine (30-60 μM) was ineffective in all 5 preparations tested.

Dopaminergic (100-500 μM) stimulation of thoracic neurons promoted rhythmic discharge in response to brainstem stimulation in 3/15 animals. However, the pattern was locomotor-like in only one of these preparations. Interestingly, the combination of dopamine and norepinephrine more effectively facilitated locomotor-like output (n=6/9, Fig. 8) than either agent applied individually, suggesting a synergistic action.

Consistent with the failure of atropine to suppress brainstem-induced lumbar locomotor-like activity, muscarinic receptor activation using acetylcholine combined with the acetylcholinesterase inhibitor edrophonium (Ach/Edro 20-50 μM / 100 μM)
consistently failed to facilitate rhythmic output in response to brainstem stimulation (n=0/11).

In seven preparations the excitability of neurons was elevated in a nonspecific fashion by raising the concentration of K+ ions in the thoracic bath solution to 7-9 mM. This promoted lumbar locomotor-like activity in two preparations that otherwise failed to produce rhythmic activity during brainstem stimulation alone.

DISCUSSION

A major finding of this study is that, during brainstem stimulation, neurochemical excitation of propriospinal neurons located in the thoracic region facilitates the production of lumbar locomotor-like activity in lesioned preparations that fail to respond to brainstem stimulation alone. Because bath-applied receptor agonists and antagonists do not influence axons of passage, the results further support the concept that propriospinal relays contribute to propagation of the descending locomotor command signal (Zaporozhets et al. 2006; Cowley et al. 2008) in addition to long direct reticulospinal pathways.

The results of the first series of experiments, using receptor antagonists, suggest multiple neurotransmitter systems, including glutamatergic, serotonergic, dopaminergic, and noradrenergic participate in locomotor-related propriospinal transmission. This finding is not surprising, considering numerous studies, using a variety of vertebrate preparations, indicate that direct activation of locomotor circuitry isolated below a spinal cord transection can be achieved using a variety of neurochemical substances, alone or in combination (see Introduction). In addition, intrathecal injection of serotonergic and noradrenergic (alpha-1) agonists in the lumbar region has been shown to improve the voluntary locomotor pattern in cats with partial spinal cord injury at T13 (Brustein and Rossignol 1999). Unique to the present study, locomotor circuitry in the hindlimb enlargement was excluded from exposure to bath-applied agonists. Excitatory agents were applied exclusively to the thoracic region, which presumably contains
propriospinal neurons involved in descending transmission of the locomotor command signal to the lumbar region.

In the second part of the study, using neurochemical excitatory agents, monoaminergic receptor stimulation facilitated locomotor signal propagation, consistent with the ability of corresponding serotonergic, dopaminergic and noradrenergic antagonists to suppress such transmission. The fact the muscarinic receptor activation failed to enhance signal transmission is congruent with muscarinic receptor blockade having no effect on locomotor-like discharge in preparations responsive to brainstem stimulation alone. However, attempts to enhance NMDA receptor-mediated actions, either by application of NMDA or by increasing channel conductance (removal of Mg\(^{2+}\) ions) failed to facilitate bulbospinal transmission, despite the fact that NMDA receptor blockade in the thoracic region suppressed brainstem-induced rhythmic activity. This disparity may be due to interference by non-specific widespread neuronal excitation, given the ubiquitous distribution of NMDA receptors among spinal neurons. Therefore, it seems important to establish neuronal excitability at an appropriate level in order to facilitate locomotor command signal transmission. In the present series this was not successfully accomplished using NMDA application or Mg\(^{2+}\) ion removal. Similarly, in an earlier study involving neurochemical manipulation of the whole cord, Mg\(^{2+}\) ion removal failed to promote locomotor-like activity, an observation thought to be due to excessive activation of NMDA receptors (Cowley et al. 2005).

One limitation of the approach used in this study to facilitate bulbospinal transmission is the time course of receptor stimulation. Application of neurochemicals to the in vitro bath produces tonic long-lasting stimulation whereas during natural behavior endogenous release of neurochemicals has a physiological temporal profile which is no doubt quite different. Another limitation is that in addition to eliminating direct long projecting bulbospinal pathways staggered bilateral hemisections also interrupt, at least partially, the propriospinal system itself. Both of these factors may contribute to the observation that among agents showing a positive facilitatory effect on locomotor rhythm generation facilitation did not occur in all preparations exposed to such agents.
Selective neurochemical stimulation of cervical segments produces locomotor-like activity in both the cervical and lumbar regions of the \textit{in vitro} neonatal rat preparation (Ballion et al. 2001; Cowley et al. 2008; Cowley and Schmidt 1997; Juvin et al. 2005) as well as cervical segments of the mudpuppy (Wheatley and Stein 1992). Axial muscles supplied by thoracic segments are also rhythmically active during locomotion in rats (Gramsbergen et al. 1999), cats (Carlson et al. 1979; Koehler et al. 1984; Zomlefer et al. 1984) and humans (de Seze et al. 2008; Thorstensson et al. 1982). Earlier studies proposed that locomotor rhythm generators in the neonatal rat were restricted to a limited number of spinal segments in the lumbar and cervical regions, and suggested that rhythmic output of thoracic segments is passively driven by circuitry in the cervical and lumbar regions (Cazalets et al. 1995; Ballion et al. 2001). In contrast, we previously provided data suggesting that a 5-HT-sensitive oscillatory network, capable of producing a locomotor output, was distributed in the spinal cord, including the thoracic and cervical regions (Cowley and Schmidt 1997). Other studies support the concept of locomotor-related rhythmogenic elements distributed throughout the spinal cord, in vertebrates ranging from lamprey to humans (Ceccato et al. 2009; Grillner 1981; Hagevik and McClellan 1999; Falgairolle et al. 2006). Within such a longitudinally distributed system, gradients of enhanced rhythmogenesis that are centered in the forelimb and hindlimb enlargements is suggested by the available data (e.g. Cazalets et al. 1995; Kjaerulff and Kiehn 1996; Cowley and Schmidt 1997; Kremer and Lev-Tov 1997; Ballion et al. 2001). Assuming that oscillatory components are present throughout the spinal cord and that local circuits need to communicate with each other, the rhythm generating elements of a distributed locomotor network and the propriospinal neurons transmitting the descending command signal may be one and the same, or at least substantially overlap. Thus, during brainstem stimulation neurochemicals applied to the thoracic region may promote hindlimb stepping by enhancing propriospinal transmission of a tonic descending brainstem command signal or by recruiting activity in the thoracic portion of a widely distributed rhythmic network, or a combination of both mechanisms.
As highlighted by Rossignol et al. (2001) the effect of drug administration on locomotion in experimental animals depends on a variety of factors, not the least of which is the type of preparation. For example, the $\alpha_2$ noradrenergic receptor agonist clonidine promotes locomotion in the complete spinal cat (e.g. Forssberg and Grillner 1973; Marcoux and Rossignol 2000), has a deleterious effect in partial spinal cats (Giroux et al 1998; Brustein and Rossignol 1999) and fails to elicit locomotion in the in vitro neonatal rat spinal cord (Sqalli-Houssaini and Cazalets 2000) or mice with complete thoracic cord transection (Lapointe et al. 2008). Consistent with the previous reports using rodent preparations, clonidine failed to facilitate propriospinal transmission of the locomotor signal when applied to the thoracic region during brainstem stimulation. However, the $\alpha_2$ noradrenergic receptor antagonist yohimbine blocked locomotor-like activity in preparations capable of responding to brainstem stimulation alone. These incongruent observations, using $\alpha_2$ noradrenergic receptor agonists and antagonists, resemble the disparate results for NMDA receptor agonists versus antagonists noted above. In the case of yohimbine, 5HT$_{1A}$ receptor mediated inhibitory actions, in addition to $\alpha_2$ noradrenergic receptor blockade, might be considered. The proposal that yohimbine (when applied from C5 to the conus) suppresses brainstem-evoked locomotor-like activity through activation of 5HT$_{1A}$ receptors, which in turn exerts an inhibitory modulation of locomotor rhythm frequency (Beato and Nistri 1998), was suggested by Lui and Jordan (2005). They invoked this 5HT receptor-dependent mechanism to help explain why, unlike yohimbine, the more specific $\alpha_2$ noradrenergic receptor antagonist, RX 82002, failed to block locomotion. However, 5HT$_{1A}$ receptor activation alone does not adequately account for the effect of yohimbine observed in our experiments; in particular, the 5HT$_1$ receptor agonist 8-OH-DPAT facilitated propriospinal transmission in 2/10 preparations.

Complex actions of different noradrenergic receptor subtypes at pre and postsynaptic levels may also contribute to the incongruent results using the $\alpha_2$ noradrenergic receptor agonist (clonidine) versus antagonist (yohimbine). Agonists of $\alpha_2$ and $\beta$ noradrenergic receptors are known to presynaptically inhibit glutamatergic inputs to lumbar motoneurons in the neonatal rat (Tartas et al 2010) and decrease rhythm
frequency (Kiehn et al 1999; Sqalli-Housaini and Cazalets 2000). In contrast, agonists of all three major noradrenergic receptor subtypes (α-1, α-2 and β) increase motoneuronal membrane excitability (Tartas et al 2010). It is unclear whether these findings in lumbar motoneurons apply to other neurons such as thoracic propriospinal cells. Possibly clonidine does not increase thoracic propriospinal neuron excitability, or if increased excitability occurs it is insufficient to facilitate locomotor signal propagation and/or is counterbalanced by pre-synaptic inhibition (Tartas et al 2010). Similarly, norepinephrine application may be generally ineffective because of a dominance of presynaptic inhibitory actions (via α-2 and β noradrenergic receptors). On the other hand, the α-1 agonist methoxamine, unlike α-2 and β agonists, potentiates presynaptic excitatory glutamatergic drive, in addition to increasing membrane excitability (Tartas et al 2010). Thus, the combination of α-1 receptor-mediated pre and postsynaptic excitatory effects may explain why methoxamine facilitated locomotor-like activity in 45% of the preparations tested, while norepinephrine itself, which activates all three receptors (α-1, α-2, and β), was rarely (1/28 preparations) effective.

Why the combination of dopamine and norepinephrine was considerably more effective in facilitating propriospinal transmission of the locomotor command signal (6/9 preparations) compared to either substance alone (1/15 and 1/28, respectively) is unknown. Combinations of monoamines can have locomotor-promoting synergistic effects in the in vitro rodent spinal cord, as was demonstrated for 5HT and dopamine (Jiang et al 1999; Madriaga et al 2004). Studies of the ventral tegmental area have provided evidence of dopaminergic activation of noradrenergic receptors and vice versa (reviewed in El Mansari et al 2010), suggesting different monoamines can cross react at the receptor level (also see Madriaga et al 2004). When noradrenergic / serotonergic reuptake inhibitors and dopaminergic agonists are used in combination, at doses otherwise inactive when used separately, marked analgesic synergy is produced in a rodent model of pain (Munro 2007). The present data suggest synergy also exists between dopaminergic and noradrenergic stimulation of the locomotor-related thoracic propriospinal system. This observation is compatible with the results of the first series of experiments, using monoaminergic antagonists to block brainstem-induced
locomotion, which implicated a role for each of the three monoaminergic systems (serotonergic, noradrenergic or dopaminergic) tested.

Comparison with the results reported by Lui and Jordan (2005) further illustrate how the effects of neurochemical manipulation depend on the experimental preparation. Similar to our series, they employed brainstem electrical stimulation using an \textit{in vitro} neonatal rat preparation. However, they concluded neither dopaminergic nor noradrenergic receptors were critical for brainstem-induced locomotion. In contrast to the present study using macrostimulation of the brainstem, Lui and Jordan used microstimulation limited to the parapyramidal region of the medulla, which is an area containing a predominance of serotonergic neurons (Lui and Jordan 2005). In addition, their paradigm did not attempt to abolish long direct bulbospinal pathways, as the focus of their study was not propriospinal transmission. Therefore, when serotonergic bulbospinal neurons projecting to the lumbar cord are directly activated by focused stimulation, and remain intact, there appears to be sufficient serotonergic transmission to activate hindlimb locomotion independent of any requirement for dopaminergic or noradrenergic input. Under natural conditions, however, the other monoaminergic systems might contribute to descending network activation and/or modulation. This remains to be determined.

In summary, the present study provides proof-of-principle of a potential new approach for restoring function after spinal cord injury. Most cases of spinal cord injury, even those classified clinically as \textit{complete}, have some degree of residual anatomical continuity across the lesion area, which is likely to include at least part of the diffusely distributed propriospinal system (for review see Conta and Stelzner 2009). Technology allowing chronic sustained intrathecal delivery of neuroactive substances for management of pain and spasticity has been available in clinical practice for over two decades. The possibility of one day infusing neuroactive substances to improve voluntary motor function in individuals with spinal cord lesions seems plausible. An attractive feature of subthreshold facilitation of transmission is that activation of the desired motor behavior still depends on a supraspinal signal and therefore remains
under voluntary control. However, unlike *in vitro* preparations with acute lesions, a
different profile of responsiveness to neurochemicals is anticipated in the chronic state
because of compensatory changes in the nervous system (Rossignol 2006; Rossignol
et al. 2009). Other investigators have started to explore complementary methods of
facilitating locomotor-related propriospinal activity. These include the use of
neurotrophic factors to strengthen synaptic connections in the staggered contralateral
hemisected neonatal rat (Arvanian et al. 2006a,b) and tonic electrical stimulation of
propriospinal neurons caudal to complete chronic T8/9 transection in the rat
(Yakovenko et al. 2007). A combined approach may well offer the best chance of
improving function for patients with spinal cord injury, as discussed in recent literature
(e.g. Gerasimenko et al. 2007; Musienka et al. 2009).
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**Figure Legends**

**Figure 1**: Effect of 5-HT receptor blockade in the cervicothoracic region on brainstem-evoked rhythmic activity.  

- **A1**: A locomotor-like pattern of lumbar ventral root discharge was evoked by electrical stimulation of the brainstem.  
- **A2**: Application of mianserin to the C1-T8 bath compartment blocked rhythmic activity. Note, for this and subsequent figures regularly occurring spikes are artefacts due to brainstem electrical stimuli. Superimposed artefacts related to brief high frequency trains of stimulation are also noted, as seen six times in A2. The high frequency trains were applied in an attempt to induce rhythmic activity in preparations unresponsive to ongoing low frequency stimulation. (L= left, R= right, N-ACSF = normal artificial cerebrospinal fluid)

**Figure 2**: Effect of dopamine receptor blockade in the cervicothoracic and caudal spinal cord regions on brainstem-evoked rhythmic activity.  

- **A1**: A locomotor-like pattern of lumbar ventral root discharge was evoked by electrical stimulation of the brainstem.  
- **A2**: Application of haloperidol to the C1-T8 bath compartment blocked rhythmic activity, even in response to four attempts using high frequency trains of stimulation.  
- **B1**: In another preparation, a locomotor-like pattern of ventral root discharge was evoked by electrical stimulation of the brainstem.  
- **B2**: Application of haloperidol caudal to the T11 level suppressed brainstem-induced activity.  
- **B3**: Rhythmic activity reappeared in response to brainstem stimulation after washout of haloperidol.

**Figure 3**: Effect of α noradrenergic receptor blockade, using prazosin, in the cervicothoracic and caudal cord regions, on brainstem-evoked rhythmic activity.  

- **A1**: A locomotor-like pattern of lumbar ventral root discharge was evoked by electrical stimulation of the brainstem.  
- **A2**: Application of prazosin to the C1-T8 bath compartment blocked rhythmic activity, except for a few bursts in response to high frequency trains of brainstem stimulation.  
- **B1**: In another preparation, locomotor-like rhythm was induced by electrical stimulation of the brainstem.  
- **B2**: Application prazosin caudal to the T11
level suppressed rhythmic discharge. **B**3: Locomotor-like activity re-emerged in response to brainstem stimulation after washout of prazosin.

**Figure 4**: Effect of α noradrenergic receptor blockade, using yohimbine, in the cervicothoracic and caudal cord regions, on brainstem-evoked rhythmic activity. **A**1: A locomotor-like pattern of lumbar ventral root discharge was evoked by electrical stimulation of the brainstem. **A**2: Application of yohimbine to the C1-T8 bath compartment blocked rhythmic activity. **B**1: In another preparation, locomotor-like rhythm was induced by electrical stimulation of the brainstem. **B**2: Application yohimbine caudal to the T11 level suppressed the rhythmic discharge.

**Figure 5**: Effect of dopaminergic receptor blockade on thoracic cord segments located between staggered contralateral hemisections at the right T1/2 and left T10/11 junctions. **A**1: Haloperidol 40 μM failed to block rhythmic activity induced by electrical stimulation of the brainstem. Note that T8 ventral root rather than L5 recordings were used in this example, and that T8 and L2 recordings from the same side are normally co-active. **A**2: A higher concentration (80 μM) did block rhythmic activity. **A**3: After haloperidol washout lumbar rhythm activity reappeared, although rhythmic activity in the thoracic segment (T8) remained suppressed.

**Figure 6**: Facilitation of brainstem-evoked rhythmic activity in the lumbar region by stimulation of 5-HT receptors located on thoracic propriospinal neurons. **A**1: In this preparation electrical stimulation of the brainstem alone failed to elicit locomotor-like activity. **A**2: Application of 5-HT to thoracic cord segments (T3-T9) located between staggered contralateral hemisections (right T1/2 and left T10/11) enabled locomotor-like activity to appear in response to brainstem stimulation. **A**3: The facilitatory effect of 5-HT was abolished after washout. **B**: The ability to induce locomotor-like activity depended on both the brainstem stimulus intensity and concentration of 5-HT applied to the thoracic cord. Three different concentrations of 5-HT (10, 30 and 50 μM) were applied at four different levels of brainstem stimulus (1, 2, 3 and 4 mA) in seven preparations. At 1 mA only the high concentration of 5-HT (50 μM) was effective.
At high stimulation intensity (4 mA) all three 5-HT concentrations (10, 30, and 50 µM) enabled locomotor-like activity in preparations unresponsive to brainstem stimulation alone; however, higher 5-HT concentrations were effective in more preparations (n= 3/7, 4/7 and 5/7 for 10, 30 and 50 µM, respectively). The y-axis denotes the number of preparations displaying locomotor-like activity among the seven preparations tested at each concentration and stimulus strength.

**Figure 7**: Brainstem-evoked rhythmic activity was facilitated by stimulation of noradrenergic but not NMDA receptors located on thoracic propriospinal neurons. A1: Electrical stimulation of the brainstem alone failed to elicit rhythmic activity. A2: Application of NMDA to thoracic cord segments (T₃-T₉) located between staggered contralateral hemisections (right T₁/₂ and left T₁₀/₁₁) failed to facilitate any clear pattern of rhythmic activity in response to brainstem stimulation. A₃: The α₁ noradrenergic receptor agonist methoxamine promoted brainstem evoked rhythmic activity in thoracic and lumbar segments. Ipsilateral thoracic T₈ and L₂ ventral roots are usually co-active (see Fig 5).

**Figure 8**: Facilitation of brainstem-evoked rhythmic activity in the lumbar region by stimulation of dopaminergic and noradrenergic receptors located on thoracic propriospinal neurons. A₁: Application of dopamine and norepinephrine to thoracic cord segments (T₃-T₉) located between staggered contralateral hemisections (right T₁/₂ and left T₁₀/₁₁) promoted locomotor-like activity in response to brainstem stimulation. A₂: Electrical stimulation of the brainstem in the absence of dopamine and norepinephrine failed to elicit rhythmic activity in the same preparation.
Table 1
Summary of the effectiveness of neurochemical excitation of propriospinal neurons, in T₃-T₉ segments, on the capacity to generate lumbar locomotor-like activity during brainstem stimulation. All long direct bulbospinal projections were disrupted by staggered contralateral hemisections at the right T₁/₂ and left T₁₀/₁₁ junctions. All of these preparations were selected on the basis of failure to produce locomotor-like activity in response to brainstem stimulation alone.
Figure 1

A1  N-ACSF
L-L2
L-L5
R-L2
R-L5

A2  Mianserin 75 μM
L-L2
L-L5
R-L2
R-L5

10 s
Figure 2

A1: N-ACSF
L-L2
L-L5
R-L2

A2: Haloperidol 75 μM
L-L2
L-L5
R-L2

B1: N-ACSF
L-L2
L-L5
R-L2
R-L5

B2: Haloperidol 15 μM
L-L2
L-L5
R-L2
R-L5

B3: N-ACSF washout
L-L2
L-L5
R-L2
R-L5

5 s
Figure 3

A1
N-ACSF
L-L2
L-L5
R-L5

A2
Prazosin 65 μM
L-L2
L-L5
R-L5

B1
N-ACSF
L-L2
L-L5
R-L2
R-L5

B2
Prazosin 65μM
L-L2
L-L5
R-L2
R-L5

B3
NACSF
L-L2
L-L5
R-L2
R-L5
Figure 4
Figure 5
Figure 6

A1 N-ACSF
L-L2
L-L5
R-L2
R-L5

A2 5HT 50 µM
L-L2
L-L5
R-L2
R-L5

A3 N-ACSF
L-L2
L-L5
R-L2
R-L5

B

Number of preparations

Current, mA

[5HT]
- 50
- 30
- 10

Figure 6
Figure 8

A1

Dopamine 400 μM + NE 50 μM

L-L2
L-L5
R-L2
R-L5

A2

N-ACSF

L-L2
L-L5
R-L2
R-L5

5 s
<table>
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<th>Neurochemical agent or manipulation</th>
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<tr>
<td>5-HT + NMDA</td>
<td>(10 – 50) + (2 – 5)</td>
<td>3 / 9</td>
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<tr>
<td>NMDA</td>
<td>2 – 4</td>
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<td>13 / 30</td>
</tr>
<tr>
<td>Quipazine</td>
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<tr>
<td>8-OH-DPAT</td>
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<tr>
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<td>1 / 28</td>
</tr>
<tr>
<td>Methoxamine</td>
<td>40 – 100</td>
<td>5 / 11</td>
</tr>
<tr>
<td>Clonidine</td>
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</tr>
<tr>
<td>Dopamine (D)</td>
<td>100 – 500</td>
<td>1 / 15</td>
</tr>
<tr>
<td>D + NE</td>
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<td>6 / 9</td>
</tr>
<tr>
<td>ACh + Edrophonium</td>
<td>(20 – 50) + 100</td>
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