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

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Zaporozhets E, Cowley KC, Schmidt BJ. Neurochemical excitation of propriospinal neurons facilitates locomotor command signal transmission in the lesioned spinal cord. J Neurophysiol 105: 2818–2829, 2011. First published March 30, 2011; doi:10.1152/jn.00917.2010.—Previous studies of the in vitro neonatal rat brain stem-spi nal 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 brain stem-induced bulbspinal activity. Spinal cords were either intact or contained staggered bilateral hemisections located at right T1/T2 and left T10/T11 junctions designed to abolish direct long-projecting bulbspinal axons. Serotonergic, noradrenergic, dopaminergic, and glutamatergic, but not cholinergic, receptor antagonists blocked locomotor-like activity. Approximately 73% of preparations developing locomotor-like activity was as follows: 5-HT (43%), 5-HT/N-methyl-d-aspartate (NMDA; 33%), quipazine (42%), 8-hydroxy-2-(di-n-propylamino)tetralin (20%), methoxamine (45%), and elevated bath K+ concentration (29%). Combined norepinephrine and dopamine increased the success rate (67%) compared with the use of either agent alone (4 and 7%, respectively). NMDA, Mg2+ 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.

IT IS WIDELY ACCEPTED THAT long direct bulbspinal 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 brain stem-spi nal cord preparation, we showed that propriospinal pathways also convey descending transmission of locomotor command signals originating in the brain stem (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 brain stem, 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 regrowth of long direct brain stem 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 preparations (Fenrich and Rose 2009). 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, 2006b). 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 interlesion 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, 1967b); clonidine (Barbeau et al. 1987; Marcoux and Rossignol 2000; Forssberg and Grillner 1973), serotonergic agents (Antri et al. 2002, 2003, 2005; Barbeau and Rossignol 1990, 1991; Ferraboli-Lohnherr et al. 1999; Fong et al. 2005; Guertin 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; Douglas et al. 1993; Giroux et al. 2003). Similarly, numerous reports using in vitro vertebrate preparations describe direct...

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/N-methyl-D-aspartate (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 paraparamidal region evokes locomotion (Liu and Jordan 2005). In favor of a role for noradrenergic mechanisms is the observation that intraspinal injection of the α2-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 brain stem in the neonatal rat preparation. We then 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 brain stem stimulation alone. Some of the following data have 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 were approved by the University of Manitoba Animal Protocol Review Committee. Sprague-Dawley rats (1–5 days old) were anesthetized with isoflurane, decerebrated at the midcollicular level, eviscerated, and placed in a bath chamber containing artificial cerebrospinal fluid (ACSF) composed as follows (in mM): 128 NaCl, 4.0 KCl, 0.5 NaH2PO4, 1.5 CaCl2, 21 NaHCO3, 1.0 MgSO4, and 30 glucose, equilibrated to pH 7.4 with 95% O2-5% CO2. The brain stem and spinal cord were then isolated ventral side up and bilaterally intact. Experiments were conducted at room temperature (ACSF ~22°C). Preparations were left in the bath solution, unstimulated, for ~1h before we attempted to elicit locomotor-like activity via electrical stimulation of the brain stem.

Ventral root recordings, obtained using glass suction electrodes, were band-pass filtered (30–3,000 Hz), digitized, and captured using Axoscope software (version 9.0; Axon Instruments). Axoscope files were converted to an appropriate binary format for further analysis with the use of 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 interlesion zone (T3 through T9 inclusive) could be selectively exposed to neurochemicals. As noted in RESULTS, intact spinal cords were used in some experiments, with barriers placed at C1 and T8/T9 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 and bath concentrations. Subthreshold for evoking locomotor-like activity in the absence of brain stem stimulation. All neurochemical concentrations refer to final ventral cord bath concentrations.

Electrical stimulation of the brain stem 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 brain stem. 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 2–3 min per test episode. For experiments involving application of neurotransmitter antagonists to thoracic segments in an effort to abolish brain stem-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, using the baseline brain stem stimulation parameters. If locomotor-like activity was abolished, higher stimulation current was administered in an effort to determine whether locomotor-like activity could break through. The antagonist was considered capable of blocking propriospinal transmission only if it blocked brain stem-evoked locomotor-like activity at all stimulation strengths. For experiments involving neurochemical excitation of thoracic propriospinal neurons in an effort to facilitate brain stem-evoked locomotor activity, we first established that locomotor-like activity could not be elicited regardless of brain stem stimulation intensity. Excitatory neurochemicals were then applied using a range of concentrations as needed while the brain stem was stimulated 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 I alternation was observed between the left and right sides at L2 and/or between left and right sides at the L5 level,
and 2) ipsilateral alternation was present between L2 (predominantly 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 interlesion 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, because 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 brain stem 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 brain stem stimulation alone. Approximately 73% of such preparations fail to display locomotion (Cowley et al. 2008). A variety of excitatory agents were thus applied to the thoracic cord to determine whether enhanced propriospinal excitation enabled the emergence of locomotor activity in the lumbar region when combined with brain stem stimulation.

Effect of Neurotransmitter Antagonists on Propriospinal Transmission in Preparations Producing Lumbar Locomotor-Like Activity During Brain Stem Stimulation Alone

Preparations with intact spinal cords. The nonselective 5-hydroxytryptamine (5-HT) receptor antagonist mianserin (50–75 μM) was applied to the C1–T8 bath compartment while the brain stem was electrically stimulated. Lumbar root locomotor-like discharge was abolished in all preparations (n = 4/4; Fig. 1). Another nonselective 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, because they also bind to α-noradrenergic and histamine receptors (Hoyer et al. 1994).

The dopamine receptor antagonist haloperidol (10–75 μM) blocked brain stem-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, brain stem-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 brain stem stimulation (Figs. 3A and 4A, respectively). Similarly, both prazosin (65 μM, n = 2/2) and yohimbine (15 μM, n = 1/2) blocked brain stem-induced rhythmic activity when applied on locomotor circuitry located caudal to T11 (Figs. 3B and 4B, respectively).

Preparations with staggered bilateral hemisections. A theoretical limitation of the intact brain stem-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 brain stem-induced lumbar locomotor-like activity. Nonetheless, this series included experiments using preparations with staggered contralateral hemisections made at the right T1/T2 and left T10/T11 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 brain stem 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 interlesion zone at T3–T9) blocked brain stem-induced locomotor-like activity in the lumbar region. More specifically, rhythmic

![Fig. 1. Effect of 5-HT receptor blockade in the cervicothoracic region on brain stem-evoked rhythmic activity. A1: a locomotor-like pattern of lumbar ventral root discharge was evoked by electrical stimulation of the brain stem. A2: application of mianserin to the C1–T8 bath compartment blocked rhythmic activity. Note that for this and subsequent data, regularly occurring spikes are artifacts due to brain stem electrical stimuli. Superimposed artifacts related to brief high-frequency trains of stimulation are also noted, as shown 6 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.](http://jn.physiology.org/)

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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 the α1,2-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 receptors as well as actions on other 5-HT and α-noradrenergic receptors (Svensson 2003), blocked brain stem-evoked locomotion in six of nine preparations. In the example shown in Fig. 5A, thoracic (T8) ventral roots were recorded along with L2 ventral roots. Alternating lumbar ventral root activity was restored after haloperidol washout, whereas T8 recordings remained silent (Fig. 5A3). The reason for this is not known, but it may reflect a greater capacity of the upper lumbar segments to generate rhythmic activity in response to brain stem stimulation, compared with thoracic segments, in the presence of incomplete washout of haloperidol.

The NMDA receptor antagonist AP-5 (20–80 μM) blocked locomotion in four of four 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 nonlesioned preparations induced using chemical or electrical stimulation of the brain stem (Zaporozhets et al. 2006).

**Effect of Neurochemical Excitation of Propriospinal Neurons on Locomotor Command Signal Transmission in Lesioned Preparations Unresponsive to Brain Stem Stimulation Alone**

The effect of neurochemical agents and manipulations was examined in preparations unresponsive to brain stem 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 induce 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 brain stem stimulation. The following results are summarized in Table 1.

During brain stem 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/T2 and left T10/T11 junctions, enabled the emergence of locomotor-like activity in the lumbar region in 13 of 30 preparations (Fig. 6A). In three

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**Fig. 2. Effect of dopamine receptor blockade in the cervicothoracic and caudal spinal cord regions on brain stem-evoked rhythmic activity.**

**A1:** a locomotor-like pattern of lumbar ventral root discharge was evoked by electrical stimulation of the brain stem. **A2:** application of haloperidol to the C1–T8 bath compartment blocked rhythmic activity, even in response to 4 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 brain stem. **B2:** application of haloperidol caudal to the T11 level suppressed brain stem-induced activity. **B3:** rhythmic activity reappeared in response to brain stem stimulation after washout of haloperidol.
addition additional preparations with staggered lesions, brain stem 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 brain stem stimulation in three of seven preparations that initially failed to develop rhythmic activity in response to brain stem stimulation alone. Application of the 5-HT1A receptor agonist 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT; 1–30 μM) to the thoracic cord facilitated locomotor-like discharge in only 2 of 10 preparations that failed to display locomotion in response to brain stem stimulation alone.

The effectiveness of brain stem 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 brain stem 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 a stimulation strength of 2 mA, 30 and 50 μM 5-HT were effective (n = 2/7 and 4/7, respectively). At higher brain stem stimulus intensities (>3 mA), 10 μM 5-HT was effective (n = 3/7) in preparations otherwise unresponsive to the same intensity of brain stem 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 5-HT (10–50 μM) and NMDA (2–5 μM) promoted locomotor-like activity in response to brain stem stimulation in three of nine preparations unresponsive to either brain stem 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; Fig. 7, A1 and A2), despite the fact that the NMDA antagonist AP-5 consistently blocked locomotor-like activity evoked by brain stem stimulation (n = 4/4). In addition, the percentage success rate using 5-HT/NMDA (33%) was less than that using 5-HT alone (43%). In nine preparations unresponsive to brain stem stimulation alone, an attempt was made to enhance NMDA receptor channel conductance, in the thoracic cord, by 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 brain stem-evoked hindlimb rhythmic activity in only 1 of the 28 preparations tested. On the other hand, the noradrenergic receptor α₁-agonist methoxamine (40–100 μM) promoted rhythmic activity in 5 of 11 preparations that otherwise failed to develop rhythmic activity in response to brain stem stimulation alone (Fig. 7A3). The α₂-noradrenergic agonist clonidine (30–60 μM) was ineffective in all five preparations tested.

Dopaminergic (100–500 μM) stimulation of thoracic neurons promoted rhythmic discharge in response to brain stem stimulation in 3 of 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 brain stem-induced lumbar locomotor-like activity, muscarinic receptor activation using acetylcholine (20–50 μM) combined with the acetylcholinesterase inhibitor edrophonium (100 μM) consistently failed to facilitate rhythmic output in response to brain stem 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 brain stem stimulation alone.

DISCUSSION

A major finding of this study is that during brain stem 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 brain stem 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 (Cowley et al. 2008; Zaporozhets et al. 2006) 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, have indicated that direct activation of locomotor circuitry isolated below a spinal cord transaction can be achieved using a variety of methods.
The capacity of each agent or manipulation to facilitate brain stem-evoked rhythmic activity is indicated as the number of preparations out of the total in which locomotor-like activity developed. All long direct bulbospinal projections were eliminated by staggered contralateral hemisections at the right T1/T2 and left T10/T11 junctions. A1: haloperidol (40 μM) failed to block rhythmic activity induced by electrical stimulation of the brain stem. 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 active. A2: a higher concentration of haloperidol (80 μM) did block rhythmic activity. A3: after haloperidol washout, lumbar rhythm activity reappeared, although rhythmic activity in the thoracic segment (T8) remained suppressed.

of neurochemical substances, alone or in combination (see Introduction). In addition, intrathecal injection of serotonergic and noradrenergic (α1) 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 brain stem stimulation alone. However, attempts to enhance NMDA receptor-mediated actions, either by application of NMDA or by increasing channel conductance (removal of Mg2+ ions), failed to facilitate bulbospinal transmission, despite the fact that NMDA receptor blockade in the thoracic region suppressed brain stem-induced rhythmic activity. This disparity may be due to interference by nonspecific 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 to facilitate locomotor command signal transmission. In the present series this was not successfully accomplished using NMDA application or Mg2+ ion removal. Similarly, in an earlier study involving neurochemical manipulation of the whole cord, Mg2+ 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 that 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.

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 brain stem stimulation

<table>
<thead>
<tr>
<th>Neurochemical Agent or Manipulation</th>
<th>Concentration Range, μM unless otherwise stated</th>
<th>Brain Stem-Evoked Rhythmic Activity Facilitated</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT + NMDA</td>
<td>(10–50) + (2–5)</td>
<td>3/9</td>
</tr>
<tr>
<td>NMDA</td>
<td>2–4</td>
<td>0/14</td>
</tr>
<tr>
<td>Mg2+ removal</td>
<td>0</td>
<td>0/9</td>
</tr>
<tr>
<td>5-HT</td>
<td>10–50</td>
<td>13/30</td>
</tr>
<tr>
<td>Quinpirole</td>
<td>10–20</td>
<td>3/7</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>1–30 (2/10)</td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>15–60 (1/28)</td>
<td></td>
</tr>
<tr>
<td>Methoxamine</td>
<td>40–100 (5/11)</td>
<td></td>
</tr>
<tr>
<td>Clonidine</td>
<td>30–60 (0/5)</td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>100–500</td>
<td>1/15</td>
</tr>
<tr>
<td>Dopamine + NE</td>
<td>400 + 50</td>
<td>6/9</td>
</tr>
<tr>
<td>ACh + edrophonium (20–50) + 100</td>
<td>[K+] increase: Target: 7–9 mM</td>
<td>2/7</td>
</tr>
</tbody>
</table>

The capacity of each agent or manipulation to facilitate brain stem-evoked rhythmic activity is indicated as the number of preparations out of the total in which locomotor-like activity developed. All long direct bulbospinal projections were disrupted by staggered contralateral hemisections at the right T1/T2 and left T10/T11 junctions. All of these preparations were selected on the basis of failure to produce locomotor-like activity in response to brain stem stimulation alone. 5-HT, 5-hydroxytryptamine; NMDA, N-methyl-D-aspartate; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetrinal; NE, norepinephrine; ACh, acetylcholine; [K+]1, K+ concentration.
Selective neurochemical stimulation of cervical segments produces locomotor-like activity in both the cervical and lumbar regions of the in vitro neonatal rat preparation (Ballion et al. 2001; Cowley and Schmidt 1997; Cowley et al. 2008; 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...

Fig. 6. Facilitation of brain stem-evoked rhythmic activity in the lumbar region by stimulation of 5-HT receptors located on thoracic propriospinal neurons. A1: in this preparation, electrical stimulation of the brain stem alone failed to elicit locomotor-like activity. A2: application of 5-HT to thoracic cord segments (T3–T9) located between staggered contralateral hemisections (right T1/T2 and left T10/T11) enabled locomotor-like activity to appear in response to brain stem stimulation. A3: the facilitatory effect of 5-HT was abolished after washout. B: the ability to induce locomotor-like activity depended on both the brain stem 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 4 different levels of brain stem stimulus (1, 2, 3, and 4 mA) in 7 preparations. At 1 mA, only the high concentration of 5-HT (50 μM) was effective (n = 1/7). At high stimulation intensity (4 mA), all three 5-HT concentrations (10, 30, and 50 μM) enabled locomotor-like activity in preparations unresponsive to brain stem stimulation alone; however, higher 5-HT concentrations were effective in more preparations (n = 37, 4/7, and 3/7 for 10, 30, and 50 μM, respectively). The y-axis denotes the number of preparations displaying locomotor-like activity among the 7 preparations tested at each concentration and stimulus strength.

Fig. 7. Brain stem-evoked rhythmic activity was facilitated by stimulation of noradrenergic but not N-methyl-D-aspartate (NMDA) receptors located on thoracic propriospinal neurons. A1: electrical stimulation of the brain stem alone failed to elicit rhythmic activity. A2: application of NMDA to thoracic cord segments (T3–T9) located between staggered contralateral hemisections (right T1/T2 and left T10/T11) failed to facilitate any clear pattern of rhythmic activity in response to brain stem stimulation. A3: the α1-noradrenergic receptor agonist methoxamine promoted brain stem-evoked rhythmic activity in thoracic and lumbar segments. Ipsilateral thoracic T8 and L2 ventral roots are usually coactive (see Fig 5).

Selective neurochemical stimulation of cervical segments produces locomotor-like activity in both the cervical and lumbar regions of the in vitro neonatal rat preparation (Ballion et al. 2001; Cowley and Schmidt 1997; Cowley et al. 2008; 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 (Ballion et al. 2001; Cazalets et al. 1995). 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 rhythmicogenic elements distributed throughout the spinal cord in vertebrates ranging from lamprey to humans (Ceccato et al. 2009; Falgairolle et al. 2006; Grillner 1981; Hagevik and McClellan 1999). Within such a longitudinally distributed system, gradients of enhanced rhythogenesis that are centered in the forelimb and hindlimb enlargements are suggested by the available data (e.g., Ballion et al. 2001; Cazalets et al. 1995; Cowley and Schmidt 1997; Kjaerulff and Kiehn 1996; Kremer and Lev-Tov 1997). 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 brain stem stimulation, neurochemicals applied to the thoracic region may promote hindlimb stepping by enhancing propriospinal transmission of a tonic descending brain stem 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 α2-noradrenergic receptor agonist clonidine promotes locomotion in the complete spinal cat (e.g., Forsberg and Grillner 1973; Marcoux and Rossignol 2000), has a deleterious effect in partial spinal cats (Brustein and Rossignol 1999; Giroux et al. 1998), and fails to elicit locomotion in the in vitro neonatal rat spinal cord (Sqalli-Houssaini and Cazalets 2000) or in 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 brain stem stimulation. However, the α2-noradrenergic receptor antagonist yohimbine blocked locomotor-like activity in preparations capable of responding to brain stem stimulation alone. These incongruent observations, using α2-noradrenergic receptor agonists and antagonists, resemble the disparate results for NMDA receptor agonists vs. antagonists noted above. In the case of yohimbine, 5-HT1A receptor-mediated inhibitory actions, in addition to α2-noradrenergic receptor blockade, might be considered. The proposal that yohimbine (when applied from C5 to the conus) suppresses brain stem-evoked locomotor-like activity through activation of 5-HT1A 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 5-HT receptor-dependent mechanism to help explain why, unlike yohimbine, the more specific α2-noradrenergic receptor antagonist RX 82002 failed to block locomotion. However, 5-HT1A receptor activation alone does not adequately account for the effect of yohimbine observed in our experiments; in particular, the 5-HT1 receptor agonist 8-OH-DPAT facilitated propriospinal transmission in 2 of 10 preparations.

Complex actions of different noradrenergic receptor subtypes at pre- and postsynaptic levels may also contribute to the incongruent results regarding the α2-noradrenergic receptor agonist (clonidine) vs. antagonist (yohimbine). Agonists of α2- and β-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-Houssaini and Cazalets 2000). In contrast, agonists of all three major noradrenergic receptor subtypes (α1, α2, 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 presynaptic inhibition (Tartas et al. 2010). Similarly, norepinephrine application may be generally ineffective because of a dominance of presynaptic inhibitory actions (via α2- and β-noradrenergic receptors). On the other hand, the α1-agonist methoxamine, unlike α2- and β-agonists, potentiates presynaptic excitatory glutamatergic drive, in addition to increasing membrane excitability (Tartas et al. 2010). Thus the combination of α1-receptor-mediated pre- and postsynaptic excitatory effects may explain why methoxamine facilitated locomotor-like activity in 45% of the preparations tested, whereas norepinephrine itself, which activates all three receptors, 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 with 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 has been demonstrated for 5-HT 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 ineffective 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 brain stem-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 brain stem electrical stimulation using an in vitro neonatal rat preparation. However, they concluded neither dopaminergic nor noradrenergic receptors were critical for brain stem-induced locomotion. In contrast to the present study using macrostimulation of the brain stem, Lui and Jordan used microstimulation limited to the parapyramidal region of the medulla, which is an area containing a predominance of serotonergic neurons. In addition, their paradigm did not attempt to abolish long direct bulbo-spinal pathways, since the focus of their study was not propriospinal transmission. Therefore, when serotonergic bulbo-spinal 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 complete, have some degree of residual anatomic 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 long-term treatment with a 5-HT2 agonist. Eur J Neurosci 16: 467–476, 2002.


