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Initiation of segmental locomotor-like activities by stimulation of ventrolateral funiculus in the neonatal rat

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Abstract

Descending control is critically important for the generation of locomotor activities. Yet, our understanding of the descending control system of locomotion is limited. We hypothesized that stimulation of the ventrolateral funiculus (VLF) induces rhythmic activity in lumbar neurons that is correlated with locomotor-like activity in the neonatal rat. Intracellular recordings were conducted in the L2–L3 lumbar segments, while locomotor-like output was monitored in the L2 and L5 ventral roots. Stimulation of the VLF at thoracic segments induced locomotor-like activity in the L2 and L5 ventral roots in majority of the preparations (26/33). In a few midline split cord preparations (4/13), VLF stimulation induced rhythmic locomotor-like bursts in either L2 or L5 ventral root without alternating pattern between the ventral roots. The response latencies suggest that VLF stimulation induced antidromic activation (<1 ms, 8 cells), monosynaptic activation (1–3 ms, 18 cells), and oligosynaptic activation (3.5–5 ms, 14 cells) of segmental neurons in the lumbar region. VLF stimulation induced rhythmic membrane potential oscillations with or without bursting of action potentials in 9 of 40 putative interneurons. The membrane potential oscillations were in phase with the locomotor-like output of the L2 ventral root in 7 of the 9 cells while the other 2 cells oscillated in phase with the L5 ventral root activity. We have thus demonstrated that descending axons exist in the VLF which make synaptic connections with segmental neurons in the lumbar region that may be a critical element of the locomotor neural network for the initiation of locomotion.

Keywords

Spinal cord; Locomotion; Interneurons; Electrophysiology; Ventrolateral funiculus

Introduction

Descending pathways play a key role in the initiation and modulation of locomotor activities generated by the central pattern generators (CPGs) in the spinal cord (e.g. Jordan 1983, 1991, 1998; Armstrong 1988; Whelan 1996). Stimulating specific regions of the brainstem initiated locomotor-like activity in the cat (Shik et al. 1966a, b; Shefchyk and Jordan 1985), rat (Skinner and Garcia-Rill 1984), lamprey (McClellan and Grillner 1984), and mudpuppy

(Shik 1997). In mammals, this initiation is believed to be mediated by descending axons within the ventrolateral funiculus (VLF) of the spinal cord (Noga et al. 1991, 2003; Webster and Steeves 1991; Magnuson and Trinder 1997). Locomotion produced by the stimulation of the pontomedullary locomotor region was blocked by reversible cooling of the VLF in the cat (Noga et al. 1991). Field potential mapping of neurons in the lower lumbar spinal cord following stimulation of the mesencephalic locomotor region revealed cells in laminae VII and VIII that may be activated by descending axons in the VLF of the cat (Noga et al. 1995). The latencies of the field potentials suggest mono-, di-, and oligosynaptic connections. These results, although intriguing, have not established the descending pathways in the VLF due to limitations in the specificity of these techniques. For example, field potential mapping could not determine whether the cells, activated by descending axons in the VLF, are in fact interneurons involved in locomotion. It is necessary to demonstrate direct physiological evidence of such connections with spinal interneurons that are active during locomotor activities.

Cells that may be part of the CPG have been recorded during locomotor-like activity induced by bath application of NMDA and/or 5-HT in the in vitro preparations from the lamprey (Sigvardt et al. 1985; Wallén and Grillner 1987), the mudpuppy (Wheatley et al. 1994; Cheng et al. 2002), the neonatal rat (MacLean et al. 1995; Kiehn et al. 1996), and the tadpole (Reith and Sillar 1998). However, some of these cells may be activated non-specifically, and the connections between these cells and the descending locomotor pathways cannot be determined under these experimental conditions. It is therefore important to study the pattern of activation of individual cells in response to stimulation of descending pathways during initiation of locomotor-like activity.

In this study, we tested the hypothesis that stimulation of the VLF induces rhythmic activity in neurons from the lumbar region of the in vitro neonatal rat spinal cord preparation. We identified cells with response latencies that suggest antidromic, monosynaptic, and oligosynaptic connections between the VLF and the spinal interneurons. VLF stimulation elicited rhythmic and tonic changes in both the membrane potential and the firing of action potentials. The rhythmic changes were phasically related to locomotor-like output in the ventral roots. We have thus demonstrated for the first time that descending axons exist in the VLF that make synaptic connections with segmental neurons in the lumbar region that may be a critical element of the locomotor neural network for the initiation of locomotion. Parts of the results have been reported in abstract form (Cheng and Magnuson 1999, 2000).

Methods

Forty-six Sprague–Dawley rats, aged 2–6 days (3 P2, 11 P3, 15 P4, 14 P5, and 3 P6), were used for the experiments. The experimental procedures conformed to the NIH guidelines and were approved by the Animal Care and Use Committee of the University of Louisville.

The in vitro spinal cord preparation

The surgical procedures were similar to those reported previously (Smith and Feldman 1987; Atsuta et al. 1990; Magnuson and Trinder 1997). Briefly, the animals were anesthetized with halothane (Sigma, St. Louis, Mo). The heart was exposed and the animal was perfused transcardially with 2.5 ml of oxygenated (95% O₂/5% CO₂) artificial cerebrospinal fluid (ACSF) at 4°C that contained (in mM) 124.5 NaCl, 5.0 KCl, 26.0 NaHCO₃, 1.0 NaH₂PO₄, 2.0 MgSO₄, 2.0 CaCl₂, and 10.0 D-Glucose (pH 7.3–7.4). This modification has improved the viability of the spinal cord preparation in our pilot experiments. The animals were then quickly decapitated and eviscerated, and the spinal columns were exposed by removing the forelimbs, ribs, and surrounding tissues. The preparations were then placed in a dish containing continuously oxygenated ACSF at 4°C,

and were pinned down to the bottom of the dish coated with Sylgard® resin (Dow Corning) for further dissection under the microscope. The spinal cords were exposed ventrally, dissected free with the dura removed, and placed in an incubating chamber filled with oxygenated ACSF at 32°C. Particular care was taken to avoid damaging the lumbar ventral roots, of which 5–8 mm lengths were retained with the spinal cord. In 13 preparations, the spinal cord was split midsagittally caudal to T12 and the right half of the cord was used for recording. The split cords allowed better access to the interneurons located in the intermediate gray matter. After more than 40 min of incubation, the cords were transferred to a Sylgard-bottomed recording chamber that was continuously perfused with oxygenated ACSF at room temperature (20–22°C). The cords were stabilized with pins to the bottom of the chamber with the ventral side up (or the cut surface of midsagittally sectioned preparations with the assistance of additional pins that supported the rounded surface at the bottom). Care was taken to avoid pinning through any part of the lumbar and caudal thoracic segments where the networks for hindlimb locomotion are located (Cazalets et al. 1995; Kjaerulff and Kiehn 1996; Cina and Hochman 2000). A segment border is defined as midway between two adjacent ventral roots.

Electrophysiological recordings

Locomotor-like output is defined as rhythmic bursting activity that alternates between the ipsilateral L2 and L5 ventral roots or between the left and right L2 ventral roots for at least 5 cycles (Fig. 1a, b). Glass suction electrodes (about 150 µm in diameter) were used to monitor locomotor-like activity from the L2 and L5 ventral roots. The activity recorded from these roots corresponds to the hindlimb flexor and extensor activities, respectively (Cowley and Schmidt 1994; Kiehn and Kjaerulff 1996). Signals were filtered (bandpass 10–1,000 Hz), amplified, and digitized at 5 kHz (AI 401 headstages, Cyberamp 380, and Digidata 1200A digitizer, Axon Instruments, Inc.).

Intracellular recordings were made in preparations where locomotor-like output was induced by VLF stimulation. Intracellular recordings were initiated approximately 50 min after transfer of the spinal cord to the recording chamber. The recording microelectrodes (50–80 MΩ with 2 M KAc) were prepared on a microelectrode puller (P-87, Sutter Instruments, CA) from filament containing glass capillaries (borosilicate, 1.5 mm OD, 0.85 mm ID; AM Systems Inc, WA). For better penetration, the pia of the ventral surface of the L2 and L3 spinal cord segments was carefully removed using fine forceps under a dissecting microscope (Olympus 240). A hydraulic micromanipulator (SD Instruments, OR), which supported the recording electrode and headstage, was secured to a coarse manipulator (SD Instruments). The electrodes were lowered onto the ventral surface of the spinal cord in an area approximately 250–600 µm from the midline. The electrodes were driven through the ventral white matter (~90 µm) and into the gray matter (~90–350 µm deep). In the split cord preparations, the area around the central canal was identified under the microscope. Intracellular recordings were made from cells in a region about 100–350 µm lateral to the central canal. The recording electrodes were pointed to this area and driven directly into the gray matter. Intracellular potentials were amplified (Axoclamp 2B), digitized (Digidata 1200A), and stored (PClamp 7, Axon Instruments Inc., CA) in a computer for later analysis.

VLF stimulation

The stimulating techniques have been described previously (Magnuson and Trinder 1997). Briefly, the stimulating electrodes were made from filament containing glass capillaries and filled with 2 M NaCl (patch-clamp style pipettes with a resistance of 5–12 MΩ when filled with NaCl). Single or train stimuli were delivered as constant current bipolar square waves from an AM Systems 1,200 stimulator. The pulse durations were 0.2–1 ms. In a few cases, pulse durations of up to 3.0 ms were employed when the stimulus required current increase

that was limited by the high resistance of the stimulating electrode. Motor responses from the lumbar ventral roots were monitored for optimal stimulus intensity which was usually in the range of 8–24 μ A. In these experiments, the VLF ipsilateral to the recording site was stimulated between the T2 and T8 spinal cord segments (usually T5), approximately 1/3 of the distance from the ventral root exit point toward the dorsal root entry point. Small adjustments were usually required to find the most effective stimulation spot for each preparation. The electrode was placed on the surface of the cord, causing only slight dimpling. The distance between the stimulating and intracellular recording sites was approximately 6–12 mm depending on the level of the stimulating electrode and the age of the animal. The responses to 4–6 trains of VLF stimulation were recorded in each preparation, and the responses were consistent as long as the inter-stimulus intervals are greater than 2 min. With decreased intervals, the durations of the locomotor activity and the firing of the cells were progressively shorter.

Analysis

The recording electrodes were targeted to the intermediate gray matter (laminae VII and X) of the lumbar spinal cord where interneurons associated with locomotion are likely localized (MacLean et al. 1995; Kjaerulff and Kiehn 1996). However, this practice could not exclude the possibility of recording from motoneurons. The following techniques were therefore used to discriminate interneurons from motoneurons. First, action potential latency (APL) is defined as the time from the onset of single VLF stimuli to the onset of action potential recorded either intracellularly or extracellularly. The shortest motoneuron APLs to VLF stimulation were first determined in each preparation by ventral root recording (L2 and L5) with suction electrodes. They were always greater than 6.5 ms in all preparations tested (Fig. 2a). Cells that responded to VLF stimulation with APLs shorter than 5.0 ms were most likely interneurons. Those with APL longer than or equal to 6.0 ms could be either interneurons or motoneurons. Artifact subtraction technique was used to determine the APLs in cases where the stimulus artifacts prevented their direct measurement. Action potentials recorded from the cells were discriminated from the artifact by subtracting the artifact that was generated by subthreshold stimulation of the VLF. This technique is particularly useful in the analysis of cells that are antidromically activated with extremely short latencies (<1 ms, Fig. 3a). Artifact subtraction was accomplished using the Labtalk functionality (Origin 5, OriginLab Corporation, MA, USA).

Based on APLs, cells were considered interneurons if they responded to VLF stimulation with latencies ranging from 0.65 to 5 ms (Table 1). The response latency reflected the time that is required for both nerve conduction and synaptic transmission. The nerve conduction time was determined by measuring the time difference between the stimulus artifact and the onset of compound action potential recorded at the ipsilateral ventral lateral white matter. It was typically 0.65 ms over a distance of 8 mm within the VLF from the stimulating electrode at T5 segment to the recording electrode at L2 segments at room temperature (about 22°C). Synaptic transmission time of 1 ms was conservatively taken into account (Llinas 1982). We therefore categorized cells with APLs of <1 ms as antidromically activated interneurons, cells with APLs of 1.5–2.5 ms as monosynaptically activated interneurons, and cells with APLs of 3–5 ms as oligosynaptically activated interneurons.

The spike-triggered averaging (STA) technique was used to identify motoneurons. The STA (Wheatley et al. 1994; Cheng et al. 2002) involves averaging a number of responses from one recording electrode, each triggered from the action potential or extracellular spike recorded from a second recording electrode. This allows for the relationship between two recorded events to be estimated. Synchronization of intracellularly recorded action potentials with extracellularly recorded spikes from ventral roots L2 and L5 was considered to indicate a motoneuron (Fig. 4b). STA was achieved using specially written programs in Matlab

(Math Works, Inc., Natick, MA). Antidromic activation by stimulating the ventral roots could have been used to help identify motoneurons; however, the already tight configuration of the experimental setup prevented us from adding additional electrodes to stimulate all the ventral roots innervating the hind limb, from L2 to L5. Without the ability to independently stimulate each ventral root, antidromic stimulation would be inconclusive because a motoneuron could send its axon to any of these ventral roots. Cells that could not be identified were those that responded to VLF stimulation with a latency of 6.0 ms or longer and where STA did not reveal an extracellular spike in the ventral root recording.

Results

VLF stimulation-induced locomotor-like activity

VLF stimulation elicited rhythmic locomotor-like activity in 26 of 33 preparations. Each bout of the activity lasted about 10–30 s, consisting 5–14 cycles of alternating L2–L5 bursts. The burst durations were typically less than 1 s initially and gradually prolonged to 2–3 s with the progression of the activity. Similarly, the inter-burst intervals also prolonged from less than 1 to 2–3 s. This progressive lengthening of the burst duration and interburst interval is typical of such studies and may be related to increased reciprocal inhibition between the flexor and extensor centers. The responses were consistently reproducible with negligible variations for 4–6 trials when the preparation was allowed to recover from the excitation by using an inter-stimulus interval greater than 2 min. Figure 1 shows representative examples of the activity evoked by stimulating the ipsilateral VLF (50 Hz, 2 s duration, 0.5 ms pulses at 40 μ A). Simultaneous recordings from the L2 and L5 ventral roots revealed that rhythmic activity induced by VLF stimulation alternated between the two ventral roots (Fig. 1a). Activity recorded from the ipsilateral and contralateral L2 roots also alternated (Fig. 1b). In four of the 13 split cord preparations (see “Methods”), locomotor-like rhythmic output was induced in only one of the ventral roots, but not in both, after VLF stimulation. Figure 1c shows an example of such activity characterized by rhythmic output in the L5 ventral root and tonic discharge in the L2 ventral root.

Classification of cells that respond to VLF stimulation

A total of 93 cells were recorded from in the L2 and L3 segments, 76 of which were from preparations that exhibited rhythmic locomotor-like activity following VLF stimulation and the rest (17 cells) were from non-rhythmic preparations. The mean resting membrane potential was -58 mV (± 10 , S.D.). The membrane input resistance ranged from 30 to 170 M Ω with a mean of 84 (± 33 , S.D.). Classification of the cells is summarized in Table 1. Please note that although the split cord preparation allowed better access to intracellular recording, it also reduced the chance for robust locomotor-like activity. So large majority of the cells were recorded in the whole cord preparations.

The latencies from VLF stimulation to the onset of intracellularly recorded action potentials (APs) were used to estimate synaptic connections from VLF axons to neurons in the upper lumbar region. The latencies, measured from VLF stimulation to the earliest motoneuron discharges, were monitored in the ventral roots. Motoneuronal discharges recorded from the L2 ventral root had much shorter latencies than those of the L5 ventral root. This difference was usually 10–20 ms and was consistent over different preparations. The shortest latency ever recorded in these preparations was 6.5 ms, which provided a reference for the classification of connections (see “Methods”). Figure 2a demonstrates the measurement of the earliest ventral root outputs, indicated by the arrow in the upper trace, in response to VLF stimulation at the T5 level (single pulse at 1 Hz, 1 ms, 50 μ A). As described in “Methods”, cells with latencies shorter than 5 ms were likely interneurons. Forty cells fell into this category (Table 1).

Eight cells were recorded that responded to VLF stimulation with latencies that were less than 1 ms. As described in “Methods”, the conduction velocity of the fastest conducting axons in the VLF was calculated based on measurements of conduction time and the distance between the stimulating electrode at the T5 segment and recording electrode at the L2 segment where compound action potentials were recorded at the ipsilateral ventrolateral white matter. Figure 2b shows how the conduction time was measured and the conduction velocity of the VLF was calculated. Compound action potentials were averaged over 50 sweeps. The conduction time was 0.65 ms over a distance of 8 mm within the VLF. The velocity of the fastest conducting axons in the VLF was calculated to be 12 m/s at room temperature (8 mm/0.65 ms, 22°C). This was not different from the average conduction velocity from all preparations tested. It is noteworthy that this conduction velocity reflects the fastest conducting axon and should not be taken as an absolute value as it may vary due to developmental stage. Since it took at least 0.65 ms for even the fastest conducting axons in the VLF to conduct action potentials from the T5 level to the L2 level at room temperature, latencies of less than 1 ms did not allow sufficient time for synaptic transmission to occur in these young developing animals (Brocard et al. 1999; Vinay et al. 2000). Therefore, cells with latencies less than 1 ms are likely to be activated antidromically through their ascending axons in the VLF. This classification is further supported by the ability of these cells to follow high-frequency stimulation. Figure 3a shows an example of the raw traces of action potentials induced by high-frequency VLF stimulation (50 Hz). This cell was located in segment L3 about 500 μ m lateral to the midline and 120 μ m from the ventral surface. Stimulation of the VLF (50 Hz, 2 s duration, 1 ms pulse width at 50 μ A) induced action potentials (AP, stimulus/response ratio = 1) that were partially masked by the stimulus artifacts (the lower traces). Subtraction of the stimulus artifact (S) from the artifact-response complex (S + AP) revealed VLF-induced action potentials (AP in upper trace) with a latency of about 0.8 ms. This latency in response to high-frequency stimulation suggests that this cell was activated antidromically from an ascending axon in the VLF.

Excitatory responses were elicited by VLF stimulation in 18 cells with latencies between 1.5 and 2.5 ms. Responses were graded excitatory postsynaptic potentials (EPSPs) and action potentials with supra-threshold stimulation. Figure 3b shows an example of such recordings. This cell was recorded from L2 in a split cord preparation, about 100 μ m lateral to the central canal. Stimulation of the VLF (5 Hz, 3 ms pulse width at 11 μ A) 8 mm rostral to the recording site induced graded EPSPs up to 20 mV and APs of about 60 mV with hyperpolarizations of 5–10 mV. The APL, about 1.5 ms, was measured between the peak of the earliest AP (labeled as I) and a spontaneous AP (labeled as R) that happened to start at the onset of stimulation. Taking into account the conduction time (0.65 ms at 22°C, Fig. 2b), cells with APLs within 1.5–2.5 ms are likely activated monosynaptically by VLF stimulation.

Responses with latencies between 3 and 5 ms were induced by VLF stimulation in 14 cells. These cells are most likely activated by di or tri-synaptic connections from the VLF axons. The exact number of interposed synapses, however, could not be determined.

Responses with latencies greater than 5.5 ms were observed in 53 cells, six of which were identified as motoneurons by STA. The rest could be either interneurons or motoneurons. Figure 4a shows an example of the identification of a motoneuron by STA. This cell was located in the L2 segment and had intracellular spikes that were time-locked to the extracellular spikes recorded from the L2 ventral root (indicated by the vertical dotted lines). Cells with intracellular spikes that correlated with extracellular spikes recorded from a ventral root were considered motoneurons.

Rhythmic and tonic responses of cells to trains of VLF stimulation

Of the 93 cells tested, 91 responded to VLF stimulation. Long-lasting depolarizations with or without firing of action potentials occurred in interneurons ($n = 40$), motoneurons ($n = 6$), and unclassified cells ($n = 37$). Long-lasting hyperpolarizations (greater than 10 s) occurred in 6 unclassified cells. Two cells did not respond. The recordings were stable with little variation over repeated VLF stimulations of 4–6 trains as long as the inter-stimulus interval was greater than 2 min.

Rhythmic activity, determined by spike-triggered average, was recorded from 3 motoneurons, 9 putative interneurons, and 6 unclassified cells. Figure 4b shows the responses of a motoneuron to VLF stimulation (50 Hz, 2 s duration, 0.5 ms pulse width at 80 μ A). The recording was from a cell in the L2 segment, about 500 μ m lateral to the midline and about 100 μ m from the ventral surface. Depolarization and rhythmic membrane potential oscillations were induced in phase with the L2 ventral root motor output. Action potentials were generated in the depolarized phase of the oscillations. STA revealed motor responses in the L2 ventral root that was time-locked to firing of the cell (inset, average of 93 sweeps). The upward arrow indicates the onset of the spikes.

Of the 40 putative interneurons, 9 cells displayed rhythmic bursting of action potentials or rhythmic oscillations in membrane potential. Figure 5 shows an example of responses of interneurons activated antidromically from VLF stimulation. This cell was in the L2 segment about 550 μ m lateral to the midline and 180 μ m from the ventral surface. A train of stimulation (50 Hz, 2 s duration, 0.5 ms pulse width at 40 μ A) induced a depolarization and action potentials that, at first glance, did not appear to be rhythmic (upper trace). However, analysis of the instantaneous firing rate revealed a phasic modulation with increased discharges in the flexor phase (L2 active). The instantaneous mean firing rate is shown by the line that best fits the individual data points (generated using Origin5, OriginLab Corporation, MA, USA). This cell responded to single stimulus (1 ms pulse, 40 μ A) with a latency of 0.8 ms (inset) and was thus classified as an antidromically activated interneuron.

Figure 6a shows an example of membrane potential oscillations in a cell recorded in the L2 segment about 500 μ m from the midline and 250 μ m from the ventral surface. The cell was classified as a monosynaptically connected interneuron as it responded to single VLF stimuli with a latency of 1.5 ms and intracellularly recorded spikes were not phase-locked with extracellularly recorded spikes in the ventral roots. A train of stimuli induced rhythmic membrane potential oscillations that were in phase with the locomotor-like rhythm in the L2 ventral root (~ 0.7 Hz in this instance). On top of this “locomotor drive potential” are membrane potential changes at about 10 Hz. This activity appeared to be induced by synaptic inputs from other cells as a result of VLF stimulation. We observed that high-amplitude events (6–8 mV) were substantially more frequent in the depolarized phase of the locomotor rhythm than in the hyperpolarized phase, as shown in the inset of Fig. 6a. The arrows indicate the high-frequency membrane potential changes in the depolarized phase of the “locomotor drive potential”. It is noteworthy that all the 9 rhythmically active interneurons were recorded in the L2–L3 segments and that the rhythmicity was in phase with the L2 ventral root output in 7 of the 9 cells. The other 2 cells discharged in phase with the L5 ventral root output.

Tonic depolarization and/or firing without evidence of membrane potential oscillations or rate modulation were observed in 31 putative interneurons, 3 motoneurons, and 27 unclassified cells. Figure 6b shows an example of responses from an interneuron (upper trace). This cell responded to a train of VLF stimulation (50 Hz, 2 s duration, 1 ms pulse width at 18 μ A) with a depolarization of longer than 30 s. No evidence of locomotor-like rhythmic membrane potential oscillations could be identified from this response.

Discussion

This work demonstrates that pathways within the VLF are capable of activating neurons in the L2–L3 lumbar region, where the neuronal networks for locomotion reside. The descending pathways may therefore play a critical role in the initiation of locomotion. They make monosynaptic and oligosynaptic contacts with spinal interneurons that may be part of the central pattern generator. On the other hand, there are also interneurons in the L2–L3 region that send axons to more rostral structures. Previous studies have suggested that the neural networks for locomotion are primarily localized in the caudal thoracic and rostral lumbar segments of the isolated neonatal rat spinal cord (Cazalets et al. 1995; Kjaerulff and Kiehn 1996; Cowley and Schmidt 1997; Kremer and Lev-Tov 1997). Intracellular recordings show that lamina VII interneurons from the L2 and L3 segments are rhythmically active during NMDA-induced locomotor-like activity (MacLean et al. 1995), and the rostral lumbar segments appear to be more rhythmogenic than the caudal lumbar spinal cord (Tresch and Kiehn 1999). Our data demonstrated for the first time that locomotor-like activity of interneurons, as well as motoneurons, in the L2 and L3 segments could be generated by synaptic input from axons in the VLF, presumably rising from reticulospinal cells in the ventromedial medulla, an area known to receive descending input from the mesencephalic locomotor region (Garcia-Rill and Skinner 1987). In this work, we found that 45% (18/40) of the putative interneurons received monosynaptic inputs from the VLF, whereas 35% (14/40) had di- or tri-synaptic connections (Table 1). These data support the hypothesis that descending pathways from supraspinal sources make mono- and oligosynaptic connections with spinal interneurons associated with locomotion (Noga et al. 1995).

This study shows that there are interneurons in the L2 and L3 segments that project ascending axons or collaterals in the VLF to neuronal targets rostral to the T5 segments. This group of neurons accounted for 20% (8/40) of the putative interneurons. They presumably send rhythmic or tonic discharges to structures in the brainstem and/or the cervical region. Our previous labeling experiments have shown that cells in lamina VII, primarily of the L2 and L3 segments, were labeled when biocytin was pressure-applied to locomotor-related VLF sites (Magnuson et al. 1998; Antonino-Green et al. 2002). Collectively, these data provide electrophysiological and anatomical evidence that there are ascending axons in the VLF that originate from interneurons in lamina VII of the L2 and L3 segments. The physiological roles of these cells and their potential participation in the modulation of locomotion are not clear. They may be involved in the intersegmental coordination during locomotion as suggested by observations made in the chick embryo (Ho and O'Donovan 1993; Ritter et al. 1999). Alternatively, these ascending fibers may project to the lateral reticular nucleus in the brainstem (Magnuson et al. 1998) providing feedback about the activity of the spinal pattern generator for locomotion.

Stimulation of the VLF induced locomotor-like activity in the neonatal spinal cord preparation. In contrast, stimulation of the dorsolateral funiculus (DLF), ventral column (VC) or dorsal column (DC) failed to initiate locomotor-like activity. This observation is consistent with those reported previously (Magnuson and Trinder 1997; Antonino-Green et al. 2002) but different from the work on the mesencephalic cat where “stepping points” were found in the DLF of thoracic and cervical regions of the spinal cord (Kazennikov et al. 1985). It also differs from a lesion study in cats that suggested that the descending pathways for the activation of the spinal locomotor network are located ipsilaterally in the DLF as well as in the VLF (Yamaguchi 1986). This discrepancy may be due to differences between species or developmental stages. Recent work in the adult rat suggests some contribution to locomotor function may also come from DLF and VC (Loy et al. 2002a, b). While recognizing this discrepancy, it is clear from our experiments as well as others that the

medullary-spinal cord pathways within the VLF are essential for locomotor initiation, even though the other descending pathways may have roles in the maintenance or modulation of locomotion (Kazennikov et al. 1990; Yamaguchi 1986).

Stimulation of the VLF induced oscillations in the membrane potential of some interneurons that were in phase with locomotor-like ventral root activity (Fig. 6a). These oscillations resemble to the “locomotor drive potentials” recorded from cat motoneurons by Jordan (1983). They may result from intrinsic membrane properties of the neurons (Hochman et al. 1994) and/or synaptic interconnections between interneurons. It is worth noting that the role of interneurons with intrinsic membrane potential oscillation properties as pacemaker cells in the generation of locomotion remains speculative (Schmidt et al. 1998). In any case, it is less likely that the rhythmic membrane potential oscillations reflect non-specific local events resulting from motoneuron activity, such as cyclic potassium increases, since many neurons exhibited either tonic depolarization (Fig. 6b) or long-lasting inhibitory responses in the presence of locomotor-like activity. It is interesting to note that a faster rhythm of oscillations (10–13 Hz) was superimposed on top of the “locomotor drive potentials”. These oscillations have much higher amplitude in the depolarized phase than that in the hyperpolarized phase. This is similar to what was observed in motoneurons upon stimulation of the mesencephalic locomotor region in the cat where the EPSPs were largest during the depolarized phase of the step cycle in the large majority of motoneurons (Shefchyk and Jordan 1985). The changes we observed in interneurons appear to reflect synaptic interactions between spinal interneurons that contribute to the rhythmic bursting of action potentials in the excitation phase of the locomotor cycle.

The data of this study provide preliminary evidence supporting the notion of differential distribution of flexor and extensor interneurons in the spinal cord (Cheng et al. 1998, 2002). First, VLF stimulation produced rhythmic output in one of the ventral roots and tonic motor activity in the other in the split spinal cord preparations (Fig. 1c). This ability of the neurons in L5 to independently generate a locomotor pattern is consistent with the observation of distinct flexor and extensor centers in the mudpuppy (Cheng et al. 1998). A “half-center” (Brown 1911, 1914) appears to be capable of generating rhythmic motor output independent of the other “half-center”. Second, rhythmic activity in large majority of the putative interneurons (7/9) in the L2/L3 segments was in phase with the flexor output from the L2 ventral root, not the extensor output from the L5 ventral root. This is consistent with the observation of differential distribution of flexor and extensor interneurons in the mudpuppy neural networks for walking (Cheng et al. 2002). While intriguing, it has to be emphasized that the number of cells in this study is small to be conclusive. Recordings from interneurons in the L4/L5 segments would be a necessary addition for more decisive conclusions on this matter. Third, the significant difference in motor response latency between L2 and L5 ventral roots (Fig. 2a) cannot be explained on the basis of conduction delay. Instead, this difference may reflect a pattern of differential projection of descending axons to the flexor and extensor centers. Fibers in the VLF may project preferentially to the rostral lumbar region where a flexor center may reside. Our previous studies have clearly showed that initiation of walking-like activity induced by D-glutamate started with rhythmic flexor activity in the mudpuppy (Lavrov and Cheng 2004), consistent with observations on fictive locomotion and scratching in the cat, where initiation of locomotion usually starts with activation of the flexor muscles and the hip flexor activity precedes that of the ankle flexors (Quevedo et al. 2000). The flexor center typically has a faster rhythm than the extensor center when they are separated from each other in the mudpuppy (Cheng et al. 1998). Preferential projection of descending pathways to the flexor center would favor a prime control over the leading component of the central pattern generator for locomotion. Taken together, these observations indicate that distinct flexor and extensor centers for locomotion may exist in the mammalian spinal cord as well.

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Fig. 1. VLF-induced locomotor-like output. **a** VLF stimulation (*vertical square* stimulus artifact) elicited alternating locomotor-like activity in the ipsilateral L2 and L5 ventral roots. **b** VLF stimulation also produced locomotor-like bursts alternating between the ipsilateral and contralateral L2 ventral roots. **c** Locomotor-like bursts were induced in the L5 ventral root, while largely tonic output was induced in the L2 ventral root in a split cord preparation

Fig. 2.

Measurement of latencies of VLF-induced motor discharges and velocities of VLF conduction. **a** The latencies of the motor responses to VLF stimulation were about 6.5 ms in the L2 ventral root and 23 ms in the L5 ventral root, measured from the beginning of the stimulation to the earliest response indicated by the *arrows*. **b** Extracellular compound action potentials (average of 50 sweeps) were induced by stimulating the VLF at the T5 thoracic level and recorded in the ventrolateral white matter at the L2 lumbar segment at 22°C. The conduction time, between the *arrows*, was 0.65 ms over a distance of 8 mm and the conduction velocity of the fastest conducting fibers in the VLF was calculated (8 mm/0.65 ms = 12 m/s)

Fig. 3.

Determination of latencies of cell responses to VLF stimulation. **a** Example of antidromic activation of cells. VLF stimulation at T5 level elicited action potentials in a cell recorded in the L3 segment with an extremely short latency. The *lower* traces are raw recordings of the stimulus artifact-action potential complex (S + AP) and a trace of stimulus artifact only (S) from subthreshold VLF stimulation. The *upper* trace is the AP profile revealed by subtracting the artifact (S) from the S + AP complex. A latency of about 0.8 ms was estimated by measuring the time between the onset of the stimulus and the onset of the AP as indicated by the *arrows*. **b** Example of monosynaptic activation of cells. VLF stimulation at T5 level elicited graded excitatory postsynaptic potentials (EPSPs) and action potentials (APs) in a cell recorded in the L2 segment. A latency of 1.5 ms was estimated by measuring the peak-to-peak time between the earliest VLF-induced AP (labeled as *I*) and a spontaneous AP (labeled as *R*) that started at the onset of the stimulation. Stimulus artifacts are labeled as *S*.

Fig. 4.

VLF stimulation induced responses from motoneurons. **a** Spike-triggered average is used for the identification of motoneurons. Spontaneous action potentials of a cell recorded in the L2 segment were used to trigger averaging of activities in L2 ventral root (mean + standard deviation, average of 50 sweeps). The *vertical lines* indicate the synchronization of intracellular spikes with the extracellular activity recorded from the L2 ventral root (*Dark line* mean, *light line* SD). This time-locked relationship between the intra- and extracellular events suggests that this cell is likely a motoneuron with an axon projecting to the L2 ventral root. **b** VLF stimulation (50 Hz, 2 s duration, 0.5 ms pulse width at 80 μ A) elicited membrane potential oscillations and firing of actions potentials (truncated at *top*) in phase with the L2 ventral root motor output. This cell, recorded in the L2 segment, was identified as a motoneuron based on STA (93 sweeps) of the L2 ventral root activity, which is time-locked to the onset of the spikes of the cell (the *upward arrow* in the insert)

Fig. 5.

VLF stimulation induced discharges from an interneuron. VLF stimulation (50 Hz, 2 s duration, 0.5 ms pulse width at 40 μ A) elicited depolarization and discharge of action potentials (truncated at *top*) in a cell from the L2 segment. Instantaneous firing rates of the cell were modulated in phase with the L2 locomotor-like activity (flexor phase), as indicated by the interpolated *curve* (80 points derived from the beginning to end of the instantaneous firing data). The insert displays the latency of action potentials (AP) induced by single pulses of VLF stimulation (1 ms pulse width at 40 μ A). The trace is extracted from the stimulus artifact by the method illustrated in Fig. 3. The short latency (0.8 ms) indicates that the cell is activated antidromically by VLF stimulation

Fig. 6.

Rhythmic and tonic membrane potential changes. **a** VLF stimulation elicited rhythmic membrane potential oscillations in a cell from the L2 segment, which are in phase with the locomotor-like activity of the L2 ventral root. This cell was classified as an interneuron based on its APL (1.5 ms) in response to single VLF stimuli (50 Hz, 0.5 ms pulse width at 55 μ A). Membrane potential changes of ~ 10 Hz were superimposed on *top* of the locomotor-like rhythm. The *arrows* illustrate the large synaptic-like events in the expanded segment of the recording that were detected only in the depolarized phase. **b** VLF stimulation (50 Hz, 2 s duration, 1 ms pulse width at 18 μ A) produced long-lasting depolarization in a cell localized in the L2 segment. No evidence of membrane potential oscillations was found during the locomotor-like ventral root output

Table 1**Classification of cells**

Cell types	Tonic	Rhythmic	Total
Interneurons			
APL <1 ms	6	2	8
APL 1.5–2.5 ms	13 (3)	5	18
APL 3–5 ms	12 (2)	2	14
Motoneurons	4	2	6
Unclassified cells	41 (7)*	6	47
Total	76 (12)	17	93

Numbers in the parentheses indicate the number of cells recorded from the split cord preparations

* Indicates that this number includes 2 non-responsive cells and 6 cells with inhibitory responses (hyperpolarizations)