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Abstract

Reticulospinal neurons of the lamprey brain stem receive rhythmic input from the spinal cord during locomotor activity. The goal of the present study was to determine whether such spinal input has a direct component to reticulospinal neurons or depends on brain stem interneurons. To answer this question, an in vitro lamprey brain stem-spinal cord preparation was used with a diffusion barrier placed caudal to the obex, separating the experimental chamber into two baths. Locomotor activity was induced in the spinal cord by perfusion of d-glutamate or *N*-methyl-dl-aspartate into the spinal

cord bath. The brain stem bath was first perfused with normal Ringer solution followed by a high-Ca^{2+}, -Mg^{2+} solution, which reduced polysynaptic transmission. The amplitudes of membrane potential oscillations of reticulospinal neurons in the posterior and middle rhombencephalic reticular nuclei (PRRN and MRRN, respectively) recorded with sharp intracellular microelectrodes did not significantly change from normal to high-divalent solution. This finding suggests a large part of the spinal input creating the oscillations is direct to the reticulospinal neurons. Application of strychnine to the high-Ca^{2+}, -Mg^{2+} solution decreased membrane potential oscillation amplitude, and injection of Cl^- reversed presumed inhibitory postsynaptic potentials, indicating a role for direct spinal inhibitory inputs. Although reduced, the persistence of oscillations in strychnine suggests that spinal excitatory inputs also contribute to the oscillations. Thus it was concluded that both excitatory and inhibitory spinal neurons provide direct rhythmic inputs to reticulospinal cells of the PRRN and MRRN during locomotor activity. These inputs provide reticulospinal cells with information regarding the activity of the spinal locomotor networks.

INTRODUCTION

The spinal cord is capable of generating the basic neural pattern that activates limb and body muscles during locomotion. This basic spinal pattern is under the influence of the brain stem, which concurrently receives feedback from the spinal cord. Spinal feedback, as proposed in early cat studies, presumably serves to inform brain stem command centers about the locomotor activity of the cord (Lundberg 1971) and is conveyed by ascending spinal pathways. Specifically, the spinocerebellar pathway sends centrally generated information from the spinal cord to the brain stem via the cerebellum (Arshavsky et al. 1978a,b; Orlovsky 1970b). Work on spinal/brain stem interactions during locomotion has been hindered, however, due to the complexity of the mammalian system. Here, the synaptic interactions between the ascending spinobulbar pathway and reticulospinal neurons of the lamprey brain stem were investigated.

In lamprey, a lower vertebrate, the locomotor pattern consists of rhythmic contractions of body muscles that alternate from side to side and propagate down the length of the body. In the in vitro lamprey spinal cord preparation, where the muscles have been removed, this same pattern can be observed in ventral root discharges and is called fictive swimming (Cohen and Wallén 1980). Bath perfusion of an excitatory amino acid can be used to generate fictive swimming in the isolated lamprey spinal cord. In more intact preparations, the swim patterns of the spinal cord are under the control of sensory inputs as well as inputs originating from the brain stem (Buchanan and Cohen 1982; Guertin and Dubuc 1997; McClellan and Grillner 1983).

Several nuclei of the brain stem contain reticulospinal neurons that constitute the main descending control pathway in the lamprey nervous system (Ronan 1989; Rovainen 1967a). The reticulospinal system in lamprey is involved in the control and initiation of locomotion (McClellan and Grillner 1984; Viana Di Prisco et al. 1997) and in postural and steering control (Fagerstedt et al. 2001; McClellan 1984; Wannier et al. 1998). Some reticulospinal neurons, mainly those of the posterior and middle rhombencephalic reticular nuclei (PRRN and MRRN, respectively), exhibit rhythmic membrane potential oscillations during fictive swimming (Kasicki and Grillner 1986; Kasicki et al. 1989). These oscillations have been shown to originate from the spinal cord (Dubuc and Grillner 1989) and presumably come from spinal neurons with axons projecting to the brain stem (i.e., spinobulbar

neurons). Thus ascending spinal inputs could inform reticulospinal neurons of the state of locomotor spinal activity and thereby play a role in regulating the frequency of swimming and coordinating turning movements (Cohen et al. 1996; Vinay and Grillner 1993).

Anatomical studies in lamprey have revealed a population of spinobulbar neurons located primarily in the rostral spinal cord. The axons of these neurons project via the ventrolateral spinal lemniscal tracts (Ronan and Northcutt 1990; Vinay et al. 1998b), which mainly course ventrally and laterally through the basal plate of the brain stem, where they could potentially contact the distal dendrites of reticulospinal cells (Martin 1979). A few spinal lemniscal axons, however, travel medially where they could potentially contact the proximal dendrites and cell bodies of reticulospinal neurons (Ronan and Northcutt 1990). Extracellular stimulation of the lateral tracts in the rostral lamprey spinal cord, which contain spinobulbar axons, produced both excitatory and inhibitory responses in PRRN and MRRN cells (Vinay et al. 1998a). An early component of these responses persisted with twin pulses of 10–20 Hz, suggesting the existence of a fairly direct path from the lateral spinal columns to the reticulospinal neurons. However, this study could not rule out indirect inputs via local relay interneurons in the brain stem. This issue was addressed in the present study by reducing polysynaptic pathways in the brain stem with a high-divalent cation solution and observing membrane potential oscillations of PRRN and MRRN neurons during fictive locomotion in the spinal cord. We show that spinobulbar input to reticulospinal cells can be direct in lamprey, both excitatory and inhibitory, and thus provides a direct link between the brain stem and spinal cord.

METHODS

Adult silver lampreys (*Ichthyomyzon unicuspis*, *n* = 15, 28.5–34.0 cm long) and recently transformed sea lampreys (*Petromyzon marinus*, *n* = 5, 14.5–17.5 cm long) were used for these experiments. The experiments were conducted in conformity to the American Physiological Society's *Guiding Principles in the Care and Use of Animals* and were approved by the Marquette University Institutional Animal Care and Use Committee. The animals were anesthetized by immersion in a solution of tricaine methane sulfonate (~250 mg/l, Sigma) and were transected just caudal to the gills (*I. unicuspis*) or at the level of the anus (*P. marinus*). All muscles were cut away, and a longitudinal cut was made along the midline of the dorsal roof of the spinal canal to expose the spinal cord. The dorsal portion of the cranial casing was removed by two lateral cuts to expose the rostral spinal cord and brain stem. Dissection and storage of the tissue were done in cold Ringer solution (4–6°C) with the following composition (in mM): 91 NaCl, 2.1 KCl, 2.6 $CaCl_2$, 1.8 $MgCl_2$, 4.0 glucose, 20 $NaHCO_3$, 8 HEPES (free acid), and 2 HEPES (sodium salt). The solution was bubbled with 98% O_2-2% CO_2, pH = 7.4.

Experiments were conducted the next day after the dissection to ensure a consistent recovery time. The preparation was pinned to the silicone elastomer (Sylgard)-lined (Dow Corning) bottom of a cooled recording chamber perfused with normal Ringer solution (8–10°C, 1 ml/min). The typical preparation consisted of the brain stem and 10–15 spinal segments for *I. unicuspis* and 50–70 spinal segments for *P. marinus*. Longer lengths were used for *P. marinus* to determine if the amount of spinal cord had any impact on the results. No apparent differences were observed, however, from the two preparations consistent with anatomical findings regarding cell numbers after retrograde labeling (Vinay et al. 1998b). A barrier of one to two segments in thickness was constructed of dental caulk (Reprosil; Dentsply Caulk) (for *I. unicuspis*) or Vaseline (for *P. marinus*). The barrier was typically centered

between the second and third ventral roots to create a brain stem bath and a spinal cord bath (Fig. 1). For each experiment, the integrity of the diffusion barrier was tested by filling one bath with normal Ringer solution while the other bath was visually checked for leaks. Any leaks were repaired with Vaseline until no leakage was observed. In one test, when fast green dye (1%) was perfused in one bath, no diffusion of dye into the other bath was observed after 3 h. Once the barrier was made and tested, fictive swimming was induced in the spinal cord by perfusion of either 0.3 mM *N*-methyl-dl-aspartic acid (NMA) or 1 mM d-glutamate into the spinal cord bath. There was no significant difference observed in the results using d-glutamate versus NMA ($P > 0.05$, *t*-test).

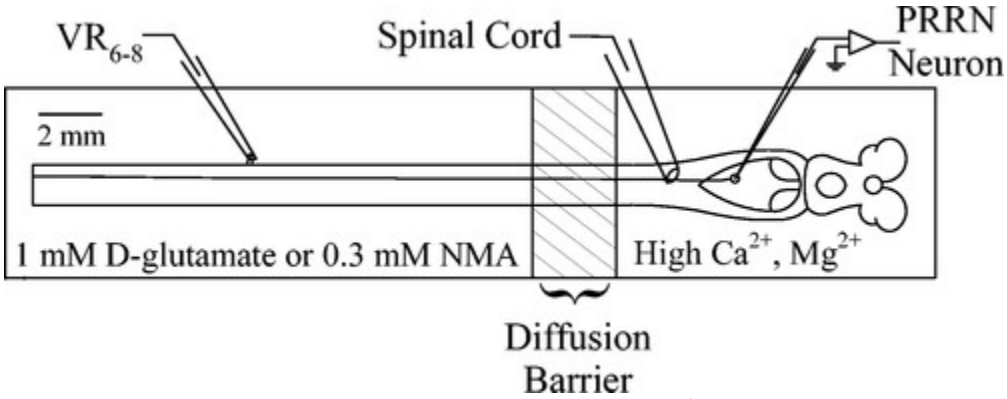

FIG. 1. The in vitro preparation of the lamprey brain stem/spinal cord. The brain stem and ~10–15 spinal segments were pinned out in a recording chamber, and the bath surrounding the brain stem was separated from the bath surrounding the spinal cord by a diffusion barrier consisting of either petroleum jelly (Vaseline) or dental caulk (Reprosil). Fictive swimming was induced by application of 1 mM d-glutamate or 0.3 mM *N*-methyl-dl-aspartate (NMA) into the spinal bath and was monitored by a suction electrode placed on a ventral root. To reduce polysynaptic pathways, a high-Ca^{2+}, -Mg^{2+} Ringer solution was applied to the brain stem while neurons in the posterior or middle rhombencephalic reticular nuclei (PRRN and MRRN, respectively) were recorded intracellularly with sharp microelectrodes. An extracellular electrode on the surface of the spinal cord near the obex region was used to detect orthodromic spikes of reticulospinal neurons.

Reticulospinal neurons of the PRRN and MRRN were impaled intracellularly with glass capillary microelectrodes pulled by a Flaming/Brown microelectrode puller (P-87, Sutter) and recorded using an AxoClamp 2A amplifier (Axon Instruments). The microelectrodes had resistances of 50–100 MΩ when filled with potassium acetate (4 M). In some experiments, potassium chloride (4 M) was used. To record ventral root activity, the tip of a glass suction electrode (0.1 mm inner tip diameter) was placed on the ventral root near its exit point from the spinal cord, typically on the sixth to eighth ventral root. A second suction electrode (0.3 mm inner tip diameter) was placed on the dorsal surface of the spinal cord near the obex to identify the axonal projection of PRRN and MRRN cells (Fig. 1). This identification was accomplished by eliciting an action potential in a neuron cell body with intracellular current injection and detecting a one-for-one orthodromic spike in the ipsilateral cord. In some cells, averaging was necessary to detect the projection (Signal software, Cambridge Electronic Design or CED). All cells included in the study were confirmed as reticulospinal neurons and had intracellular action potentials of ≥70 mV in base-to-peak amplitude [range = 70–120 mV; mean = 95 ± 16 (SD) mV]. The mean resting potential of all cells was –71 ± 9 mV, which was similar to resting potential in other studies done on reticulospinal neurons (Brocard and Dubuc 2003; Rouse et al. 1998; Wickelgren 1977). Intracellular signals were low-pass filtered at 3 kHz, and extracellular signals were filtered with a differential AC

amplifier (A-M Systems) at 100 Hz (high-pass filter) and 1 kHz (low-pass filter). Digitizing was done with a Micro1401 ADC converter (CED) at 6 kHz for intracellular and 2 kHz for extracellular recordings. The membrane potential of reticulospinal cells was recorded in ~2-min blocks with a personal computer (Spike2 software, CED) and stored on disk. Some recordings were stored on digital audiotape (Bio-Logic) and later converted to Spike2 files.

A high-divalent cation solution, composed of 20 mM Ca^{2+} and 5.8 mM Mg^{2+}, was applied to the brain stem. The high-divalent cation concentration raises spike threshold (Frankenhaeuser and Hodgkin 1957) and thereby reduces conduction in polysynaptic pathways. This effect has been observed in lamprey using 20 mM Ca^{2+} but was accompanied by an increase in monosynaptic responses (Rovainen 1974b). Therefore 5.8 Mg^{2+} was used in addition to 20 mM Ca^{2+} because this gave a reduction of polysynaptic responses without enhancing monosynaptic responses. Reticulospinal neurons in the PRRN and MRRN were recorded intracellularly before and after addition of the high-divalent cation solution. For six cells in the PRRN in three different preparations, the impalement was maintained while the normal Ringer solution surrounding the brain stem was replaced by the high-divalent cation solution. An additional four PRRN neurons, in three preparations of similar length, were maintained during perfusion of a high-divalent, strychnine solution. Other PRRN and MRRN neurons were randomly impaled before and after solutions were replaced. For these randomly impaled neurons, averages of membrane potential during fictive swimming were obtained by triggering from the manually marked beginnings of ventral root bursts of ~30 consecutive swim cycles for each neuron (Spike 2 software). The averages were then used to measure membrane potential oscillation amplitude by measuring the peak membrane potential of the crest and subtracting the potential obtained from the trough. For the PRRN cells that were continuously held before and after addition of high-Ca^{2+}, -Mg^{2+} to the brain compartment, measurements of amplitude were done cycle by cycle to make a statistical comparison. The mean membrane potential value for ~50 ms windows surrounding the peaks and troughs of 20 consecutive swim cycles were measured and subtracted to obtain membrane potential oscillation amplitudes for each neuron. This was done before and after high-divalent treatment.

All statistical analysis was done with SigmaStat software (SPSS). To compare two groups, such as with the pooled oscillation data, a *t*-test was applied. If a normality or equal variance test failed, a Mann-Whitney rank sum test was used (i.e., Fig. 4). Two groups were considered statistically different if one of the preceding tests was $P \leq 0.05$. For paired data, such as for the individually held PRRN neurons (as a group, bin 7 in Fig. 6), a paired *t*-test was used with the same stringency.

RESULTS

Efficacy of high-divalent cation solution

To determine whether locomotor-related spinal inputs to reticulospinal neurons are monosynaptic, a high-divalent cation solution (20 mM Ca^{2+}, 5.8 mM Mg^{2+}) was used to reduce conduction in polysynaptic pathways with relatively little alteration of monosynaptic potentials. In two types of experiments, the efficacy of the high-divalent solution in blocking polysynaptic activity was tested. In the first experiment, the synaptic potentials produced in a giant interneuron by a dorsal cell were tested using paired intracellular recordings. Rovainen (1967b, 1974a) demonstrated that dorsal cells,

which are primary mechanosensory cells, produce both mono- and polysynaptic excitatory potentials in giant interneurons. In the example of Fig. 2*A*, action potentials evoked in a dorsal cell by intracellular current injection produced excitatory postsynaptic potentials (EPSPs) in a nearby (3 mm) ipsilateral giant interneuron. The composite EPSP consisted of an early response (*) and a late response (Fig. 2*A1*). The early response was likely monosynaptic due to the short synaptic delay (2 ms) remaining after subtraction of the dorsal cell action potential conduction time (3 ms). The late response, beginning at ~20 ms, was polysynaptic due to the longer latency and the variable onset and trajectory. An additional late response sometimes occurred at ~100 ms. In the high-divalent cation solution, the response beginning at 20 ms was largely abolished (Fig. 2*A2*), while the amplitude of the early component was reduced by only 13%. A total of six dorsal cell-giant interneuron pairs in three preparations were tested in this manner. The amplitudes of the polysynaptic components for the six pairs were significantly different after application of high-divalent cation (paired *t*-test, $P = 0.02$; mean reduction = 62 ± 17%). The monosynaptic components were also significantly different (paired *t*-test, $P = 0.03$; mean reduction = 16 ± 3%) but by a smaller percentage. When compared with each other, the percent reductions for the polysynaptic components and the monosynaptic components were statistically different (paired *t*-test, $P = 0.002$).

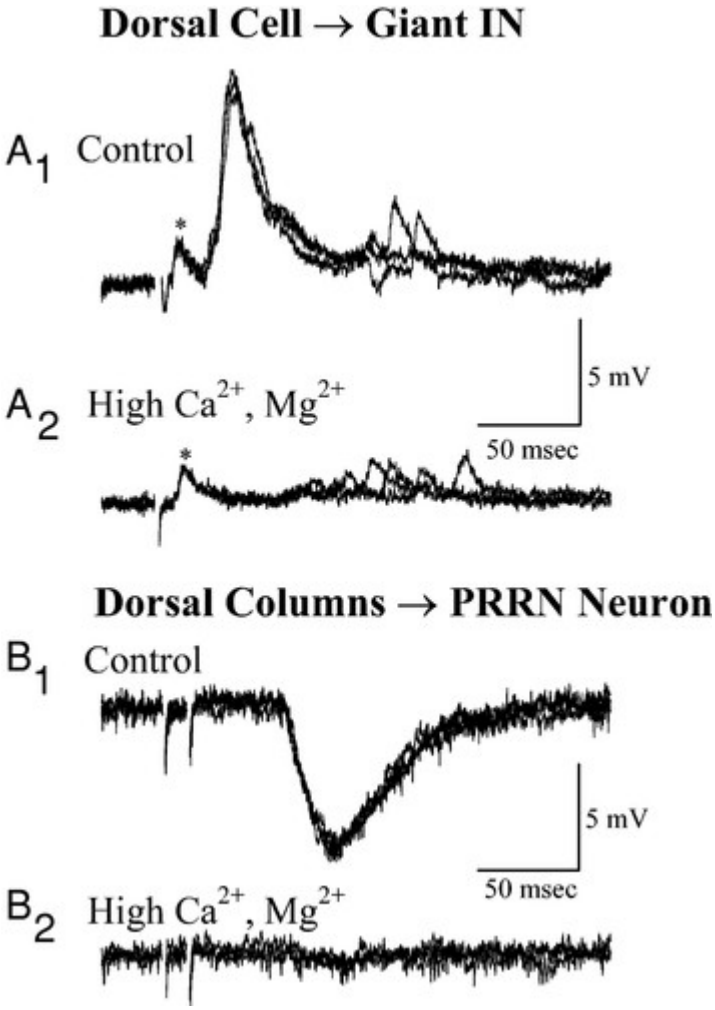

FIG. 2. Testing the efficacy of high-Ca^{2+} (20 mM), -Mg^{2+} (5.8 mM) solution in blocking polysynaptic activity. *A1*: 3 overlaid traces showing the response of a giant interneuron to action potentials in a dorsal cell (3 mm ipsilateral and caudal to the giant interneuron) consisting of a monosynaptic component (*) and a polysynaptic response.

A2: after perfusion of high-Ca^{2+}, -Mg^{2+} Ringer solution, the polysynaptic response was greatly reduced with little effect on the monosynaptic response. *B1*: response of a PRRN neuron while stimulating the spinal dorsal columns caudal to the diffusion barrier and 17 mm from the PRRN neuron. A double pulse (10-ms interval) elicited an inhibitory response of polysynaptic latency. *B2*: the response was greatly attenuated when the brain stem bath was perfused with high Ca^{2+}, Mg^{2+}.

To demonstrate the effectiveness of the high-Ca^{2+}, -Mg^{2+} solution on inputs to a PRRN cell, the dorsal columns, which contain axons of primary afferents, were stimulated caudal to the diffusion barrier and 17 mm from an impaled PRRN neuron. Using a stimulus intensity two times greater than the threshold value, a double pulse with a 10-ms interval produced an inhibitory postsynaptic potential (IPSP) in the PRRN cell (Fig. 2*B1*). The latency of this response (50 ms) was in agreement with previous studies indicating polysynaptic inputs from the dorsal columns (Dubuc et al. 1993). The amplitude of this response was reduced by 80%, from 7.0 to 1.4 mV, when the high-divalent cation solution was applied to the bath surrounding the brain stem (Fig. 2*B2*) with no change in the ascending volley rostral to the barrier (not shown).

Amplitude of oscillations in high-divalent cation solution

Neurons in the PRRN and MRRN have previously been shown to exhibit rhythmic membrane potential oscillations that are time-locked with ventral root bursting (Dubuc and Grillner 1989; Kasicki and Grillner 1986; Kasicki et al. 1989), but the directness of the spinal inputs producing these oscillations had not been tested. Because the high-divalent cation solution effectively reduced polysynaptic inputs, this solution was applied to the brain stem to test the directness of spinal inputs to reticulospinal cells during fictive locomotion. With fictive swimming activity in the spinal cord and high-divalent cation solution applied to the brain stem, PRRN (Fig. 3*A*) and MRRN (Fig. 3*B*) neurons still exhibit oscillations of membrane potential that are time-locked to the ventral root bursting.

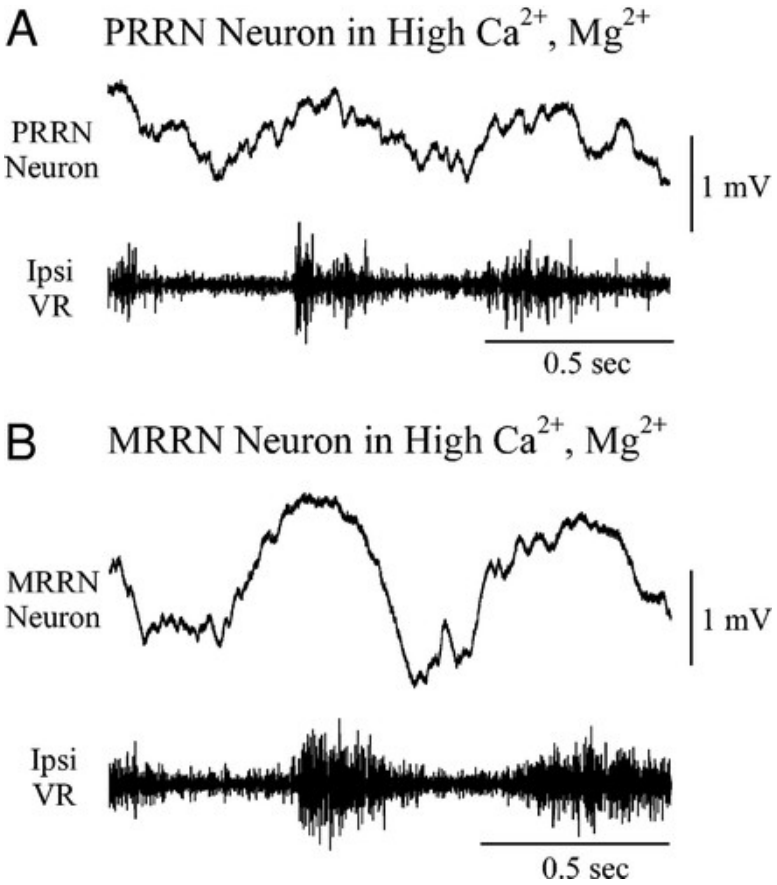

FIG. 3. Persistence of membrane potential oscillations in reticulospinal neurons in high-Ca^{2+}, -Mg^{2+} solution. *A*: example of membrane potential oscillations in a PRRN cell in high-divalent cation solution. *B*: example of oscillations in a MRRN cell in high-divalent cation solution. Membrane potential traces are averages of 30 consecutive swim cycles triggered at the beginning of ipsilateral ventral root bursts (the middle ventral root burst in figure). Because the high-Ca^{2+}, -Mg^{2+} solution largely reduces polysynaptic input, the persistent oscillations are likely caused by monosynaptic inputs from the spinal cord.

To determine whether the inputs are mainly direct, the amplitudes of the oscillations were compared in normal and in high-divalent cation solutions. In a sample of 18 PRRN neurons in normal Ringer solution, 17 (94%) exhibited membrane potential oscillations during fictive swimming induced with d-glutamate or NMA in the spinal cord bath. In a separate sample of 32 PRRN neurons in high-divalent cation solution, 28 (88%) showed clear oscillations during fictive swimming. These neurons were located throughout the rostral-caudal and medial-lateral extent of the PRRN. The mean membrane potential amplitude in normal Ringer solution (0.47 ± 0.36 mV, n = 18) as measured from the averages triggered by the beginning of the ventral root bursts, was not significantly different from the mean amplitude in high-divalent cation solution (0.36 ± 0.45 mV, n = 32; P = 0.09, Mann-Whitney rank-sum test; Fig. 4*A*). Thus the oscillations still occur in high-divalent cation solution and are not significantly smaller than those in normal Ringer, indicating that the locomotor-related spinal inputs to reticulospinal neurons are mainly direct.

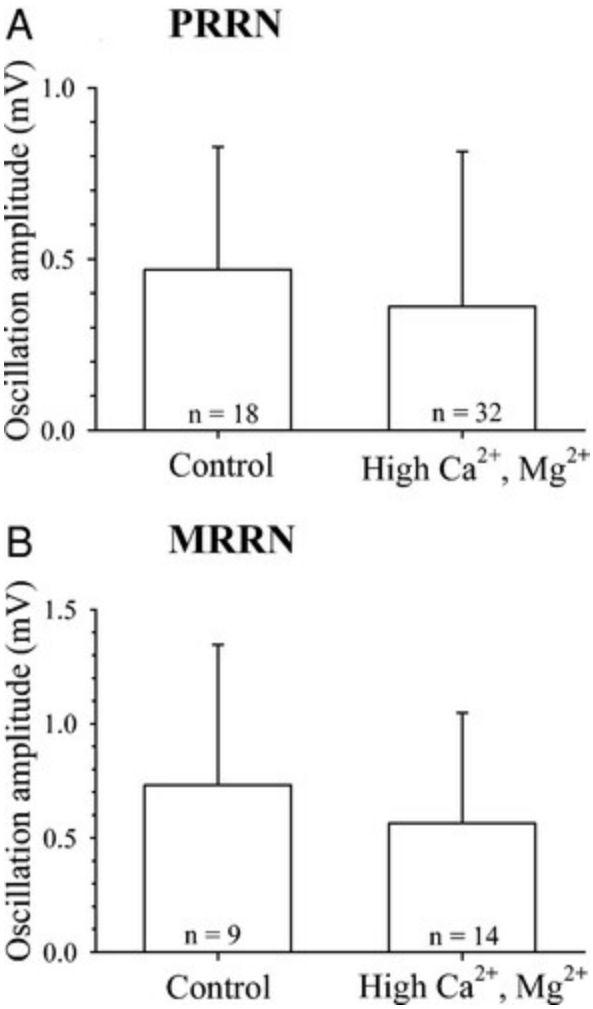

FIG. 4. Pooled measurements of mean membrane potential oscillation amplitude for cells in the PRRN (*A*) and MRRN (*B*). *A*: mean oscillation amplitude for a sample of PRRN neurons in normal Ringer was not significantly different from a sample of PRRN neurons in high-Ca^{2+}, -Mg^{2+} (Mann-Whitney rank-sum test, $P = 0.09$). *B*: a sample of MRRN neurons also did not show a significant difference in oscillation amplitude in normal vs. high-Ca^{2+}, -Mg^{2+} solutions (Mann-Whitney rank-sum test, $P = 0.6$).

Similar experiments were done on neurons in the MRRN. In a sample of nine MRRN neurons in normal Ringer solution, all nine had membrane potential oscillations during fictive swimming in the spinal cord (mean amplitude = 0.73 ± 0.61 mV, $n = 9$). In the high-divalent cation solution, 14 neurons were recorded, and all exhibited rhythmic membrane potential oscillations (mean amplitude = 0.56 ± 0.48 mV, $n = 14$). The oscillation amplitude was not significantly different in high-divalent cation solution ($P = 0.59$, Mann-Whitney rank-sum test; Fig. 4*B*). Also, the mean amplitude of membrane potential oscillations of MRRN neurons was not significantly different from that in PRRN neurons ($P = 0.20$, *t*-test).

In some experiments, PRRN neurons recorded in normal Ringer were held continuously during the change of the brain stem bath to high-divalent cation solution. Figure 5 shows the averaged membrane potentials of one such neuron before and after high-divalent cation solution. Of the six neurons recorded in this manner, four showed no significant change in membrane potential oscillation amplitude with the solution change (Fig. 6), whereas two neurons did show significant changes in

amplitude. One of these showed an increase in the amplitude of membrane potential oscillations from 3.22 ± 0.85 to 4.13 ± 0.87 mV (P = 0.002, *t*-test; *cell 2* of Fig. 5 and 6). In the second neuron, amplitude decreased from 0.73 ± 0.24 to 0.34 ± 0.13 mV (P < 0.001, *t*-test; *cell 6* of Fig. 6). As a group, the six neurons had a mean amplitude of 1.65 ± 1.15 mV in control Ringer solution and 1.66 ± 1.47 mV in the high-divalent cation solution, and these values were not significantly different (P = 0.97, paired *t*-test).

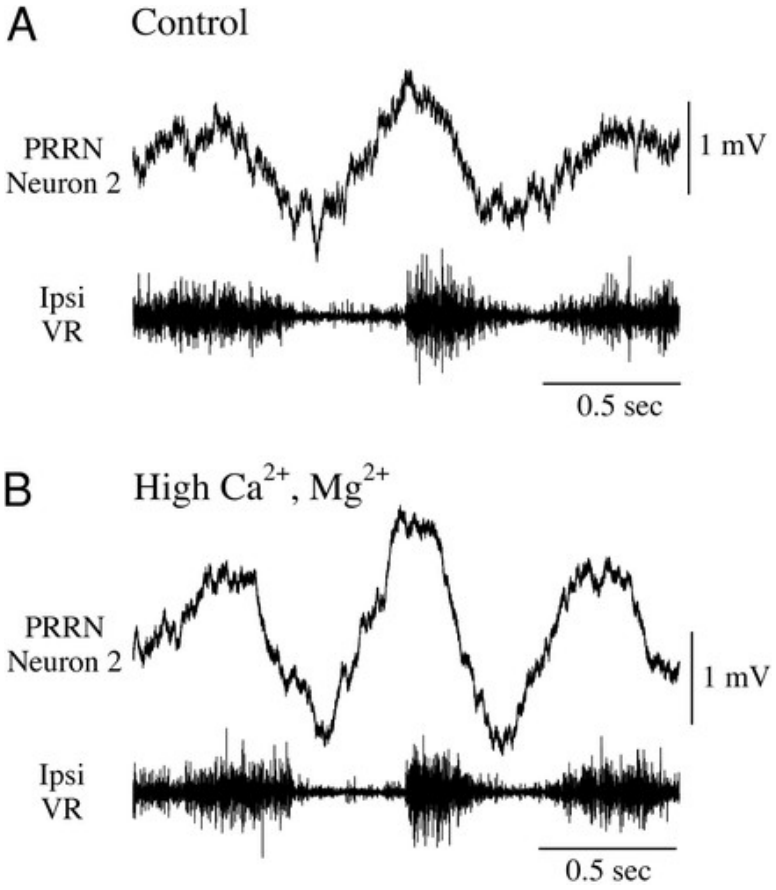

FIG. 5. Comparison of normal vs. high-divalent cation solutions on the same reticulospinal neuron. *A*: a PRRN reticulospinal neuron exhibiting oscillatory membrane potential activity from the spinal cord while the brain stem was in normal Ringer. *B*: the same neuron after perfusion of the brain stem with high-Ca^{2+}, -Mg^{2+} solution showing persistence of the oscillations. The traces are averages of ~30 consecutive swim cycles triggered at the beginning of ipsilateral ventral root bursts (the middle ventral root burst in figure). This neuron corresponds to *cell 2* in Fig. 6.

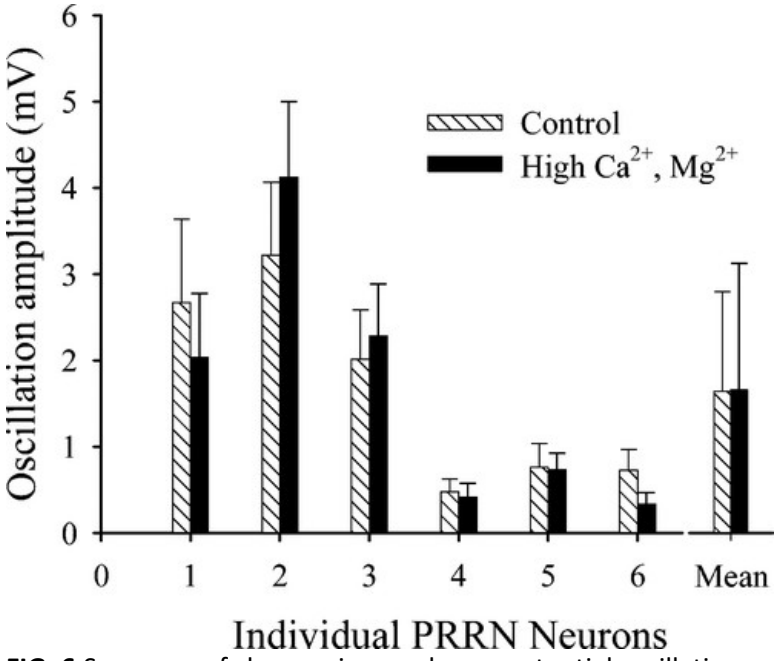

FIG. 6. Summary of changes in membrane potential oscillation amplitudes for 6 reticulospinal neurons in the PRRN in which the intracellular impalement was maintained from control Ringer solution to application of the high-Ca^{2+}, -Mg^{2+} solution to the brain stem bath. For all cells, 20 consecutive swim cycles were used for measuring oscillation amplitude from raw traces taken in each solution. *Cell 2* showed a significant increase in oscillation amplitude (mean = 3.22 ± 0.85 to 4.13 ± 0.87 mV; *t*-test, $P = 0.002$). *Cell 6* showed a significant decrease in amplitude (mean = 0.73 ± 0.24 to 0.34 ± 0.13 mV; *t*-test, $P < 0.001$). The mean for all cells is shown in last pair of bars.

Excitatory/inhibitory nature of spinal inputs

The presence of oscillations in PRRN and MRRN cells in high-divalent cation solution supports the hypothesis that these neurons receive synaptic inputs directly from the spinal cord during fictive swimming. To determine whether or not inhibitory inputs play a role in producing these oscillations, 4 M potassium chloride electrodes were used to inject Cl^- into four PRRN neurons in the high-Ca^{2+}, -Mg^{2+} solution. Chloride injection has previously been shown to reverse IPSPs in lamprey spinal neurons during fictive swimming (Kahn 1982; Russell and Wallén 1983) due to a shift in the equilibrium potential for Cl^-. In one PRRN neuron, a hyperpolarizing phase (Fig. 7*A1*) was reversed by hyperpolarizing current during chloride injection (Fig. 7*A2*) suggesting IPSPs. In another PRRN neuron, after Cl^- injection, there was a small depolarizing peak at the end of the ipsilateral ventral root burst that was similarly enhanced when the neuron was hyperpolarized. In two other PRRN neurons, there was a reduction in oscillation amplitude after 10 min of Cl^- injection (not shown) by ~20% in one cell and ~80% in the other, suggesting a Cl^--mediated component.

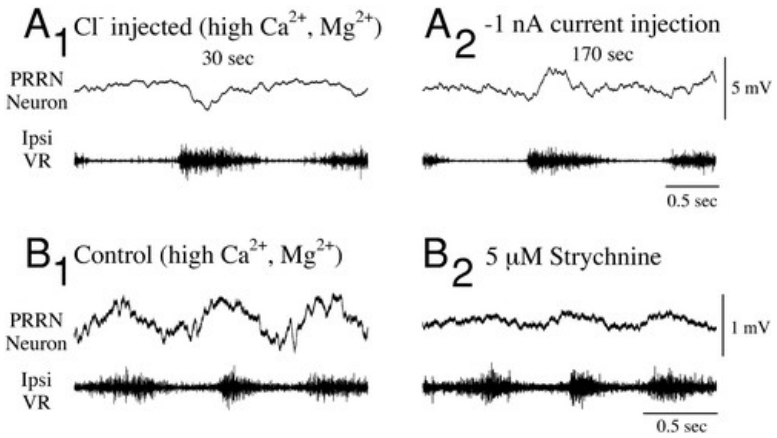

FIG. 7. Membrane potential oscillations of PRRN neurons during intracellular injection of Cl^- (*A*) or during strychnine (5 μM) application (*B*) to the brain stem. *A1*: membrane potential recorded after injection of Cl^- into a PRRN neuron in high-Ca^{2+}, -Mg^{2+} and averaged from the beginning of consecutive ventral root bursts during fictive swimming. *A2*: injecting the same neuron with –1 nA reversed a PSP at the beginning of the ipsilateral ventral root burst and is likely a reversed IPSP. *B1*: a PRRN neuron in a high-Ca^{2+}, -Mg^{2+} solution. *B2*: after application of 5 μM strychnine the amplitude was reduced indicating that glycinergic inhibition was involved in the oscillations.

To determine whether glycinergic inhibition is involved in the oscillatory inputs, strychnine was applied to the brain stem bath. Addition of strychnine (1 or 5 μM) to the high-divalent cation solution to block glycine-mediated IPSPs reduced membrane potential oscillation amplitude in all PRRN neurons tested (n = 4). The oscillation amplitude of the cell shown in Fig. 7*B* was reduced from 0.61 to 0.18 mV, a 70% reduction. In all cells tested, the mean amplitude in control (0.38 ± 0.18 mV) was significantly reduced after addition of strychnine (0.12 ± 0.05 mV; P = 0.03, n = 4, paired t-test). These results, along with the Cl^--injection findings, demonstrate the contribution of direct inhibitory inputs to PRRN neurons from spinal neurons. The persistence of membrane potential oscillations in 5 μM strychnine (Fig. 7*B2*) suggests that rhythmic EPSPs also contribute to the oscillations.

DISCUSSION

Reticulospinal neurons of the PRRN and MRRN in lamprey exhibit rhythmic membrane potential oscillations that are coordinated with the locomotor pattern of the spinal cord and persist with little or no change when the brain stem alone is bathed in a high-Ca^{2+}, -Mg^{2+} solution. This indicates that signals ascending from the spinal cord have direct inputs to the reticulospinal cells because the high-divalent solution was shown to reduce polysynaptic signals by 62% and monosynaptic signals by only 16%. Because strong disynaptic and some polysynaptic pathways may survive the blocking solution, paired recordings between spinobulbar and reticulospinal neurons will be needed to further clarify the directness of ascending inputs. The mean oscillation amplitude of the neurons decreased slightly in both the PRRN and MRRN with addition of high-divalent solution although not significantly. This could suggest the reduction of polysynaptic inputs but rather may reflect the slightly reduced monosynaptic inputs in high-divalent solution. In individual cells recorded continuously in both control and high-divalent cation solutions, most showed no change. One cell showed a decrease, suggesting the reduction of polysynaptic inputs; another cell increased in oscillation amplitude after divalent cation

solution, due perhaps to sealing of the membrane. In sum, the persistence of the oscillations in high-divalent cation is indicative of direct ascending inputs to reticulospinal neurons.

The oscillations observed in this study were small (~1 mV) when compared with rhythmic activity in PRRN and MRRN neurons during brain-stem-evoked fictive swimming. Under such conditions, i.e., when swimming was evoked by trigeminal nerve stimulation, PRRN and MRRN neurons had large membrane potential oscillations (>10 mV) and often exhibited action potentials during the depolarizing phase (Kasicki and Grillner 1986; Kasicki et al. 1989). The source of the rhythmic modulation was unclear, however, as inputs could have arisen from the brain stem and/or the spinal cord. To determine the source of rhythmic input to reticulospinal cells, Dubuc and Grillner (1989) pharmacologically activated fictive swimming in the spinal cord while keeping the brain stem quiescent as was done in the present study. Under these conditions, reticulospinal neurons showed rhythmic activity, although membrane potential oscillations were small (~2 mV) and nonspiking. The source of the additional input to reticulospinal neurons during brain-stem-evoked fictive locomotion is unknown at present, but there are at least two possibilities. First, local brain stem networks could be recruited to enhance rhythmic input to neurons of the PRRN and MRRN. Second, spinal circuits could be more strongly activated resulting in a stronger activation of reticulospinal neurons. A combination of these possibilities, however, is likely.

In addition to the reticular nuclei studied here, the lamprey brain also has an anterior rhombencephalic reticular nucleus (ARRN) and a mesencephalic reticular nucleus (MRN). Whether reticulospinal neurons in these more rostral nuclei receive rhythmic inputs from the spinal cord is not known and was not tested in the present study. An anatomical study of ascending spinal pathways in lamprey showed that relatively few spinal lemniscal fibers reach the rostral region of the rhombencephalon (Ronan and Northcutt 1990), making it unlikely that there are direct spinobulbar inputs to neurons in the ARRN. Moreover, no spinal lemniscal fibers reach beyond the isthmus and into the mesencephalon (Ronan and Northcutt 1990), making it less likely for MRN neurons to receive similar input. Direct spinal inputs cannot be entirely ruled out, however, because the large Müller cells located in these nuclei (I_1, $M_{1–3}$) showed some indication of rhythmic activity during brain-stem-evoked fictive locomotion (Kasicki and Grillner 1986). One putative source of this input is the spinal cord. Whether or not reticulospinal neurons in these rostral nuclei receive rhythmic signals from the spinal cord during fictive locomotion needs to be established before the directness of such input is proposed. Also, smaller reticulospinal neurons in the ARRN and MRN have not been tested.

Descending control pathways including the reticulospinal pathway were first well characterized in the cat. Unit recordings from reticulospinal (Drew et al. 1986; Orlovsky 1970a), vestibulospinal (Orlovsky 1972a), and rubrospinal (Orlovsky 1972b) neurons showed rhythmic activity during locomotion that was coordinated with one or more limbs. Furthermore, the rhythmic activity was not changed by the absence of peripheral sensory information (Arshavsky et al. 1978a,b), indicating that central nervous mechanisms were the source of the input. Lundberg (1971) advanced the idea that the ventral spinocerebellar tract could convey such centrally generated activity to centers of the brain stem and enable them to monitor the activity of spinal interneurons during locomotion. It was also found that the rhythmic activity of descending neurons during locomotion was completely dependent on the presence of the cerebellum (Orlovsky 1970b). This suggests that rather than a direct path to

descending systems, the ascending signals in cat are transmitted via the cerebellum, where they are integrated with other signals. In addition, Arshavsky et al. (1986) suggested that ascending information was greatly reduced by the cerebellum, thus informing descending control centers of only the critical components of the locomotor pattern. Supraspinal structures may then be continually informed of activity in the spinal cord yet utilize this information only when necessary. For example, if an obstacle is encountered, or a perturbation of locomotion occurs (e.g., changing surface), the brain stem would know the current activity of the spinal cord to appropriately adjust the motor pattern under the new circumstances.

In lamprey, ascending signals also relay information about the activity of the spinal locomotor network to the brain stem during fictive locomotion. Unlike in cat, however, this input can be direct to reticulospinal neurons. The directness of ascending inputs in lamprey is consistent with the lack of a clearly defined cerebellum and with the lack of affect on rhythmic activity in reticulospinal neurons on removal of the isthmic region of the cerebellum (Kasicki et al. 1989). As in cat, the presumed function of this ascending input in lamprey is to inform the descending systems about the current state of the locomotor network. Such information would be important in regulating speed and turning and in responding to unexpected perturbations during swimming. One approach to understanding the function of ascending spinal feedback is a detailed mapping of the connectivity and activity patterns between the spinal cord and brain stem that could provide a framework for studying the functional organization of ascending/descending systems. To this end, the relative directness of ascending inputs to the descending neurons in lamprey may offer an advantageous preparation for such studies.

The partial reduction of oscillation amplitude by strychnine in high-divalent cation solution supports the hypothesis that both direct excitatory and inhibitory spinal inputs create the membrane potential oscillations in lamprey PRRN neurons. Strychnine blocks Cl^--mediated IPSPs that are glycinergic in reticulospinal neurons (Dubuc et al. 1993; Matthews and Wickelgren 1979; Vinay et al. 1998a). Thus a partial reduction indicates that strychnine-sensitive IPSPs are involved but that a rhythmic excitatory component may also be present. Alternatively, GABA-mediated IPSPs could be involved. Although GABA has been shown to hyperpolarize reticulospinal cells and to presynaptically modulate synaptic inputs (Dubuc et al. 1993; Vinay et al. 1998a; Wickelgren 1977), Matthews and Wickelgren (1979) showed that inhibitory inputs to reticulospinal neurons were more sensitive to strychnine than to picrotoxin or bicuculline, suggesting that glycine is the main transmitter mediating inhibitory responses in reticulospinal neurons. This suggests that residual oscillations after strychnine application are caused by excitatory inputs and not by failure to block GABA-mediated IPSPs. Alternating excitatory and inhibitory inputs have been shown to underlie membrane potential oscillations in lamprey spinal neurons during fictive swimming (Kahn 1982). This input is conveyed directly to spinal neurons via identified premotor interneurons such as the contralateral, caudal inhibitory interneurons (CCIN) and the excitatory interneurons (EIN) (Buchanan 1982; Buchanan et al. 1989). The present study implicates a population of spinal neurons in providing inputs directly to reticulospinal neurons. These spinobulbar neurons may be similar to CCINs and EINs in that there are both inhibitory and excitatory neurons that are rhythmically active during fictive locomotion.

To summarize, the results of the present study support the conclusion that, in lamprey, nerve cells of the spinal cord connect directly with reticulospinal neurons in the PRRN and MRRN and thereby form a

direct spino-reticulo-spinal loop. Reticulospinal neurons constitute the main descending control pathway in lamprey and can affect the frequency of fictive locomotion and the intensity of ventral root bursting and are presumably involved in steering and postural control (Buchanan and Cohen 1982; McClellan and Grillner 1984; Wannier et al. 1998). The membrane potential oscillations of PRRN and MRRN cells that are created by spinal inputs provide information regarding the state of the spinal locomotor networks, thus putatively influencing reticulospinal neurons and their control of locomotion.

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FOOTNOTES

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AUTHOR NOTES

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