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# Spinobulbar Neurons in Lamprey: Cellular Properties and Synaptic Interactions

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# Abstract

An in vitro preparation of the nervous system of the lamprey, a lower vertebrate, was used to characterize the properties of spinal neurons with axons projecting to the brain stem [i.e., spinobulbar (SB) neurons)]. To identify SB neurons, extracellular electrodes on each side of the spinal cord near the obex recorded the axonal spikes of neurons impaled with sharp intracellular microelectrodes in the rostral spinal cord. The ascending spinal neurons (n = 144) included those with ipsilateral (iSB) (63/144), contralateral (cSB) (77/144), or bilateral (bSB) (4/144) axonal projections to the brain stem. Intracellular injection of biocytin revealed that the SB neurons had small- to medium-size somata and most had dendrites confined to the ipsilateral side of the cord, although about half of the cSB neurons also had contralateral dendrites. Most SB neurons had multiple axonal branches including descending axons. Electrophysiologically, the SB neurons were similar to other lamprey spinal neurons, firing

spikes throughout long depolarizing pulses with some spike-frequency adaptation. Paired intracellular recordings between SB and reticulospinal (RS) neurons revealed that SB neurons made either excitatory or inhibitory synapses on RS neurons and the SB neurons received excitatory input from RS neurons. Mutual excitation and feedback inhibition between pairs of RS and SB neurons were observed. The SB neurons also received excitatory inputs from primary mechanosensory neurons (dorsal cells), and these same SB neurons were rhythmically active during fictive swimming, indicating that SB neurons convey both sensory and locomotor network information to the brain stem.

# INTRODUCTION

A general organizational feature in the control of locomotion is a command system that acts on the locomotor networks and is kept informed of activity in those networks by feedback from ascending neurons (Orlovsky et al. 1999). This feedback may alter the output characteristics of the descending command neurons, transforming tonic input to phasic outputs, and provides timing information for the descending neurons with respect to the locomotor cycle. The goal of the present experiments was to characterize the ascending spinal neurons providing this feedback signal in the lamprey, a lower vertebrate, with respect to morphology, electrophysiological properties, and synaptic interactions with the descending reticulospinal neurons.

During fictive locomotor activity in mammals, neurons of various descending systems in the brain stem exhibit rhythmic modulations of their membrane potentials that are synchronous with either the flexion or extension phase of the step cycle (Arshavsky et al. 1978a,c; Orlovsky 1970a; Perreault et al. 1993). This rhythmic activity is largely dependent on the cerebellum because the rhythm is abolished in most descending neurons by cerebellar ablation (Arshavsky et al. 1978a,b; Orlovsky 1970b), although weak activity persists in some rubrospinal neurons (Arshavsky et al. 1978c). Similar to mammals, descending neurons of the lamprey brain stem exhibit rhythmic membrane potentials during fictive locomotor activity (Bussières and Dubuc 1992; Dubuc and Grillner 1989; Kasicki and Grillner 1986). Unlike most descending brain stem neurons in mammals, the lamprey cerebellum is not necessary for this rhythmic activity (Kasicki et al. 1989). It was recently demonstrated that the amplitude and timing of rhythmic activity in reticulospinal neurons during spinal activation are not changed when polysynaptic pathways in the brain stem are blocked by exposing the brain stem to a high-divalent cation solution (Einum and Buchanan 2004, 2005). Therefore it appears that the ascending projections from the lamprey spinal cord act directly on the descending cells without a cerebellar loop, whereas in mammals such a pathway may exist but is less extensive when compared with pathways involving the cerebellum. This suggests that the interactions between the brain stem and spinal cord occur in a more restricted set of neuronal structures in lamprey compared with higher vertebrates and that the overall system of brain stem-spinal cord communications is more simply organized, although still capable of complex interactions (Cohen et al. 1996; Jung et al. 1999; Vinay and Grillner 1993). This relative simplicity in lamprey will allow for more detailed analysis of synaptic connectivity and interactions between spinal cord and brain stem in attempting to understand the functions of ascending feedback.

Retrograde tracing has demonstrated that the rostral spinal cord contains large numbers of neurons projecting to the hindbrain and that the number of these cells per spinal segment falls rapidly with distance from the brain stem (Ronan and Northcutt 1990; Vinay et al. 1998b). Intraaxonal recordings of spinobulbar neurons previously demonstrated that these cells are rhythmically active during fictive

swimming (Vinay and Grillner 1992) and this was recently confirmed with intracellular recordings from the cell bodies of spinobulbar neurons during fictive swimming (Einum and Buchanan 2005). Extracellular axonal stimulation of presumed spinobulbar neurons demonstrated synaptic inputs to reticulospinal cells that are both monosynaptic and oligosynaptic (Vinay et al. 1998a).

To further investigate the ascending spinobulbar system in lamprey, the present study used sharp microelectrode intracellular recording techniques to characterize the properties of these cells including their morphology, electrophysiological properties, and synaptic interactions with reticulospinal neurons.

# METHODS

## Animals and dissection

Adult silver lampreys (*Ichthyomyzon unicuspis*, n = 34; 17–33 cm) and adult sea lampreys (*Petromyzon marinus*, n = 3; 14–34 cm) were used for these experiments. Experiments were conducted in conformity with 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. Before use in the experiments, the animals were kept at 5°C in aquaria containing aerated fresh water. Lampreys were anesthetized by immersion in water containing 250 mg/l tricaine (3-aminobenzoic acid ethyl ester) until response to tail pinch was lost. All chemicals were obtained from Sigma-Aldrich unless otherwise indicated. The dissection and experiments were done in physiological Ringer solution of the following composition (in mM): 91 NaCl, 2.1 KCl, 2.6 CaCl<sub>2</sub>, 1.8 MgCl<sub>2</sub>, 4 d-glucose, 20 NaHCO<sub>3</sub>, 8 HEPES (free acid), 2 HEPES (Na<sup>+</sup> salt), pH = 7.4, and bubbled with 98%  $O_2$ -2%  $CO_2$ . During anesthesia, the head and gill region was cut from the rest of the body just caudal to the last gill opening (about 20 spinal segments). Muscle and viscera were then removed from the notochord and cranial case. A midline cut was made along the dorsal aspect of the cartilaginous casing of the nervous system to expose the spinal cord and brain stem. The preparation was usually kept overnight at 4°C in Ringer solution before the experiment to provide a consistent recovery time after dissection and was used within 2 days of dissection.

In preparation for an experiment, the meninx primativa was stripped from the dorsal surface of the cord and, in some cases, the notochord was partially split down its ventral midline. The preparation was pinned to the Sylgard (Dow Corning)-lined floor of the recording chamber and the chamber was perfused continuously with Ringer solution (8–10°C). In some preparations a diffusion barrier was constructed with Vaseline between spinal segments 2–5 to divide the experimental chamber into a spinal cord compartment and a brain stem compartment (Fig. 1*A*). In the experiments with the diffusion barrier, locomotor activity was induced by perfusion of the spinal cord compartment with d-glutamate (0.5 or 1.0 mM), and the brain stem compartment was perfused with high-divalent cation solution (20 mM CaCl<sub>2</sub> and 5.8 mM MgCl<sub>2</sub>).



**FIG. 1.**Experimental configuration for recording spinobulbar neurons in the in vitro preparation of the lamprey brain stem and spinal cord. *A*: intracellular recording of membrane potential using sharp microelectrodes of reticulospinal (RS) neurons in the brain stem and spinobulbar (SB) neurons in the spinal cord. Ringer-filled glass electrodes were used for extracellular recordings made from the surface of each side of the spinal cord near the obex and from a ventral root (VR). *B*: example of the identification procedure for SB neurons. An intracellular recording of an SB neuron on the right side of the spinal cord is shown in the *bottom trace. Two top traces*: left and right surface recordings of the spinal cord near the obex. Averaging of the traces (*n* = 30) shows an extracellular spike in the right spinal cord (\*) but not the left spinal cord. Illustrated spike traveled 9.9 mm in 10.4 ms for a conduction velocity of 0.95 m/s. Presence of an ipsilateral ascending axon to the level of the obex identifies this cell as an ipsilaterally projecting (iSB) neuron.

#### Identification of spinobulbar neurons

As illustrated in Fig. 1, spinobulbar (SB) neurons were identified by their axonal action potentials recorded with an extracellular electrode. For this procedure, the tip of a glass suction electrode (tip diameter = 0.3-0.5 mm) was placed on the dorsal surface of the spinal cord at the level of the obex, one on each side of the midline (Fig. 1*A*). Spinal neurons were impaled with sharp microelectrodes and stimulated at 10 Hz with short (1–2 ms) depolarizing current pulses to elicit action potentials. A constant latency, one-for-one extracellular spike in one of the suction electrodes indicated that a spinal neuron projected to the brain stem (Fig. 1*B*). Averaging was sometimes used to confirm the presence of an extracellular spike (Signal, Cambridge Electronic Design, CED). Occasionally, extracellular spikes were detected by both surface spinal electrodes. In this case, if the two spikes had the same conduction time but were different in amplitude by a factor >2, it was concluded that there was only a single axon, and the smaller spike represented a recording of the axon spike from the opposite side of the cord.

# Intracellular recording and electrophysiological analysis

Microelectrodes for intracellular impalements were made from borosilicate capillary glass with filament (OD = 1.0 mm; ID = 0.58 mm; WPI) using a horizontal puller (Sutter, P-87). Electrodes were filled with 4 M potassium acetate or a biocytin solution (see following text). On impalement, nerve cells displayed either a steady resting membrane potential or injury-related spiking. Injured neurons were injected with continuous hyperpolarizing current that was slowly reduced as the cell recovered. If the action potential was ≤70 mV in base-to-peak amplitude, the cell was omitted from measurements of electrophysiological properties (see following text) but not from morphological analysis or from experiments that required only output from the neuron (e.g., synaptic interactions and conduction velocity).

Resting membrane potential was measured during a period of quiescence with no current injection through the microelectrode. The measured value was corrected for any electrode offset observed when the electrode was pulled out of the cell into the bathing solution. For the action potential, an average of >20 evoked action potentials was used to measure the peak, amplitude (from rest to peak), and duration at half-maximal amplitude of the action potential. In addition, these averages were used to measure the amplitude, latency to peak, and total duration of the slow afterspike hyperpolarization (sAHP) of the action potential. Input resistance ( $R_{in}$ ) was measured by injecting hyperpolarizing current pulses (200- to 300-ms duration at 1 Hz) in discontinuous current-clamp (DCC) mode using an Axoclamp-2A intracellular amplifier (Axon Instruments). For each neuron, about five pulses at each current level were averaged, and the change in voltage from the resting level was measured when the membrane potential reached a steady level (last 50 ms of pulse). The slope of the voltage versus current plot was used to determine  $R_{in}$ . Rheobase was the minimum current required to elicit an action potential during a long (300-ms) depolarizing current pulses. To measure the spiking frequency versus input current relationship (f/I), depolarizing current pulses (300- to 400-ms duration at 0.5 Hz) were injected while recording in DCC mode.

Intracellular recordings of SB neurons were also made during locomotor activity induced in the spinal cord by perfusing the spinal cord compartment in a split bath preparation with d-glutamate (0.5 or 1.0 mM). To characterize the timing of the membrane potential oscillations, >30 consecutive swim cycles were averaged by triggering on the ventral root burst onsets. Ventral root activity was recorded using a suction electrode placed on the dorsal surface of a nearby root. The timing of the peak depolarization ( $\Phi$ ) for SB neurons was expressed as a fraction of the normalized cycle period with 0 defined as the ventral root burst onset (Einum and Buchanan 2005).

# Intracellular labeling and morphological analysis

To reveal the morphology of SB neurons, cells were injected intracellularly with biocytin (example shown in Fig. 3). For this, a 4% solution of biocytin in 0.05 M Tris buffer was mixed 2:1 with 6 M potassium acetate and used to backfill the tips of the microelectrodes. The remainder of the microelectrode was filled with 4 M potassium acetate (final resistances ranged from 70 to 100 M $\Omega$ ). The biocytin was injected into the cell using pressure pulses (10–40 psi; 200- to 400-ms duration repeated at 1 Hz; Picospritzer) into the microelectrode for 10–20 pulses. Although this pressure injection procedure would provide labeling even in cells held for a short time (<5 min), it was found that the best labeling occurred in cells held for longer times (>1 h), presumably as a result of the

leakage of biocytin from the microelectrode into the cell. After dye injection, the tissue was kept overnight at 4°C and then fixed ( $\geq$ 2 h) by immersion in cold (4°C) 10% formalin. After fixation, the tissue was washed in phosphate-buffered saline (PBS) and then immersed for 4 h at 5°C in a solution of horseradish peroxidase (HRP)–avidin (Vector), which was diluted 1:1,000 in 0.1 M sodium bicarbonate– 0.15 M NaCl–1% Triton. After washing in PBS, the reaction was completed by processing with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB)–0.01% H<sub>2</sub>O<sub>2</sub> in 0.05 M Tris buffer for 5–10 min depending on the level of background staining of the preparation. After washing with PBS, the spinal cord–brain stem preparation was dehydrated by immersion in an ascending series of ethanol concentrations, cleared in methyl salicylate, and mounted on a slide using DEPEX mounting medium (BDH Laboratory Supplies). Myotomal motoneurons were also injected intracellularly with biocytin and processed in a similar manner for morphological comparison to SB neurons.



**FIG. 2.**Summary of conduction velocities of spinobulbar neurons. *A*: conduction velocity of iSB neurons (filled circles) increased significantly with distance of soma from obex (P = 0.034), whereas this trend for contralaterally projecting (cSB) neurons (filled triangles) was not significant (P = 0.078) (Pearson product moment). *B*: there was no significant correlation of conduction velocity vs. soma size for either iSB neurons (P = 0.33) or cSB neurons (P = 0.64).



**FIG. 3.**Example of an iSB neuron labeled by intracellular injection of biocytin and reacted with avidin conjugated to horseradish peroxidase in a wholemount preparation of the spinal cord. *A*: cell body of this neuron was located within the column of cell bodies in the 4th spinal segment. Three primary dendrites originated from the soma and the dendritic arbor extended to the lateral edge of the cord. In this neuron the dendrites were restricted to the ipsilateral side of the spinal cord. Axonal processes are labeled with "a" (axonal processes originating from another labeled SB neuron are labeled "a""). *B*: a higher magnification of the box in *A* to illustrate the origin of the axon from a primary dendrite (arrow). Axon narrowed, reexpanded, and then branched several times producing 3 ascending axons and one descending axon.

Morphological features of intracellularly stained SB neurons and motoneurons were measured either directly with a ×40 objective on a compound microscope (E-600, Nikon) or from a camera lucida tracing made with a drawing tube and a ×40 objective (examples in Fig. 4). Total dendritic length was calculated by adding the lengths of all dendritic branches as measured from the camera lucida drawing. Also measured from the drawings were the number of dendritic branch endpoints, soma width, and rostrocaudal extent of the dendritic spread. Soma width was measured at the widest part of the short axis of the neuron where no primary dendrites occurred using an eyepiece reticule. Photomicrographs were made with a digital camera (Spot; Diagnostic Instruments) and processed in Adobe Photoshop.



**FIG. 4.**Drawings of SB neurons from wholemount preparations. *A*: iSB neurons. One of these iSB neurons (*Ai*) had multiple axonal processes, both ascending and descending, and a single dendritic process crossing the midline; the other iSB neuron (*Aii*) had only a single axon process and no contralateral dendrites. *B*: bilaterally projecting (bSB) neuron with multiple axonal processes, both ascending and descending and descending, on both sides of the spinal cord. *C*: cSB neurons. About half of cSB neurons had contralateral dendrites (all of the illustrated except *Ciii*). Most cSB neurons also had both ascending and descending axonal processes as in *Ci* and *Civ*. Labeling and distance calibration bar in *Ai* applies to all drawings. Axonal processes are labeled with "a."

# Paired intracellular recordings

The presence of synaptic interactions between individual SB neurons and individual RS neurons of the brain stem was tested using simultaneous microelectrode impalements between pairs of these cells. The RS neurons were sampled mainly from the posterior and middle rhombencephalic reticular nuclei (PRRN and MRRN, respectively) but also from the anterior rhombencephalic nucleus (ARRN) and the mesencephalic reticular nucleus (MRN). In some cases the uniquely identifiable reticulospinal Müller cells (Buchanan 2001; Rovainen 1967a) were used in the paired recordings and were visualized by intracellular injection of Fast Green as previously described (Buchanan and Cohen 1982). In addition, dorsal cells, which are primary mechanosensory neurons with cell bodies in the spinal cord, were tested for synaptic outputs to SB neurons. To asses the directness of the postsynaptic potentials (PSPs), several criteria were considered: ability to follow 10-Hz presynaptic stimulation with constant latency, unitary shape, persistence in high-divalent cation solution, and an estimated synaptic delay of <3 ms. Synaptic delay was estimated by measuring the latency of the postsynaptic response from the peak of the presynaptic somatic action potential to the beginning of the response in the postsynaptic neuron using an average of >20 traces. To obtain synaptic delay, the estimated conduction time of the action potential to the postsynaptic cell was subtracted from this total postsynaptic latency. To estimate conduction time, the distance between the two neurons was divided by the conduction velocity of the presynaptic spike. As shown in Fig. 1 for SB neurons, conduction velocity was determined by recording extracellularly at the level of the obex. This procedure assumes that the conduction velocity does not change beyond the obex. For example, if the SB axon thinned as it entered the brain stem, its conduction velocity would slow in this region and the synaptic delay would be overestimated.

#### Data acquisition

Intracellular signals were low-pass filtered at 3 kHz using a CyberAmp 320 (Axon Instruments). Extracellular signals were filtered at 10 or 100 Hz (high-pass filter) and 1 kHz (low-pass filter) with a differential AC amplifier (A-M Systems). Digitizing was done with a micro1401 (CED) at >6 kHz for intracellular and >2 kHz for extracellular recordings to computer using Spike2 software (CED). Some experiments were also recorded with a modified digital audio tape recorder (Bio-Logic) and digitized with the 1401 and Spike2 at a later time.

## Statistics

Statistical comparisons were made using a Student's *t*-test unless indicated otherwise in the text. For all tests, a P < 0.05 was arbitrarily used to indicate that values were significantly different. For all comparisons, SigmaStat software (SPSS) was used.

# RESULTS

# Conduction velocity

Some 144 SB neurons were identified electrophysiologically by their axonal projection to the level of the obex (Fig. 1). Of these, 63 (44%) had projections to the ipsilateral side of the spinal cord with respect to the soma (iSB) and 77 (53%) had projections to the contralateral side (cSB). Six of these unilaterally projecting SB neurons had a double projection on one side (three iSB, three cSB). In addition, four SB neurons (3%) projected bilaterally (bSB). The mean conduction velocity of cSB neurons was  $0.79 \pm 0.41$  m/s (range = 0.10-2.1) and the mean conduction velocity of iSB neurons was  $0.74 \pm 0.31$  m/s (range = 0.19–1.6). Mean values were not significantly different (P = 0.47). For the bSB neurons, there was no significant difference between the mean conduction velocities of the ipsilateral versus contralateral axons (1.40  $\pm$  0.33 vs. 1.47  $\pm$  0.34 m/s, respectively; paired t-test, P = 0.81). However, both the ipsilateral and contralateral axonal conduction velocities were significantly different from iSB and cSB axon conduction velocities (one-way ANOVA, Tukey test). Comparatively, SB neurons conduct spikes with a mean velocity similar to that of smaller neurons in lamprey such as excitatory interneurons (EINs, 0.7 ± 0.3 m/s) (Buchanan et al. 1989). Conduction velocity of iSB neurons showed a weak but significant correlation with the distance of the soma from the obex (Pearson correlation coefficient = 0.27, P = 0.034, Fig. 2A, gray circles), with conduction velocity increasing with distance. A similar trend for cSB neurons was not significant (coefficient = 0.20, P = 0.078, Fig. 2A, black triangles). For those cells injected with biocytin, there was no significant correlation of conduction velocity versus soma size for either iSB neurons (coefficient = -0.40, P = 0.33, Fig. 2B, gray circles) or cSB neurons (coefficient = 0.15, *P* = 0.64, Fig. 2*B*, black triangles).

# Morphology

Intracellular injection of biocytin with subsequent HRP-avidin processing labeled 22 SB neurons well enough to discern their axonal projections in wholemount preparations of the spinal cord (Fig. 3A). The axon was identifiable by its origin from a dendrite, followed by a marked thinning and then widening to a smooth process extending out of the dendritic arbor (Fig. 3B). Of the 22 labeled neurons, eight (36%) were iSB neurons (Fig. 4A), 13 (59%) were cSB neurons (Fig. 4C), and one was a bSB neuron (5%) (Fig. 4B). In all cases, the morphological labeling confirmed the electrophysiological determination of axonal projection. Four of the 22 well-labeled neurons (18%) had only ascending axons with no axonal branch points. Axons of 18 of the 22 labeled cells (82%) exhibited at least one branch point. A single branch point always produced both an ascending and a descending axonal projection (Table 1). Multiple axonal branching was also common (12/22 with more than one branch point) producing multiple ascending and/or descending axons. The mean number of axonal branch points in iSB neurons was 3.4 ± 2.7 (n = 8 neurons; range = 0–7); the mean number of axonal branch points in cSB neurons was 1.3 ± 0.9 (n = 13 neurons; range = 0–3). The difference between the two median values (iSB = 3.5, cSB = 1.0) was not significant (P = 0.088; Mann–Whitney rank-sum test). For those cells with both an ascending and a descending axon (n = 18), three had fewer ascending than descending axons and seven had more ascending than descending axons. Overall, the mean ratio of the number of ascending axons to descending axons was  $1.45 \pm 0.96$  (Table 1). The difference between the median values for iSB and cSB (iSB = 1.5, cSB = 1.0) was not significant (P = 0.24; Mann–Whitney rank-sum test). Of the 57 axonal processes from the 22 labeled SB neurons, 54 (95%) were found in the ventral half of the spinal cord and the remainder in the dorsal half. About half of all axonal processes (31/57 or 54%) were localized lateral to the edge of the gray matter and the remaining axons were located medial to the lateral edge of the gray. However, axonal position within the cord was not constant because axons shifted position with distance from their origins. Because axonal staining became fainter with distance from the soma, it was usually not possible to follow the axon into the brain stem. However, in four cases (two iSB and two cSB), the axons could be followed rostrally beyond the obex. The ascending axons of both of the iSB neurons shifted dorsally as they neared the obex and entered the brain stem in a dorsolateral position. Both of these iSB neurons also had descending axons. The ascending axons of the cSB neurons entered the brain stem in the ventral tracts. One of the cSB neurons had a single ascending axon that entered the brain stem in a ventrolateral position. The other cSB neuron had two ascending axons and one descending axon. The two ascending axons of this cell entered the brain stem in a ventrolateral and a ventromedial position, respectively.

Cell	Number of	Caudal Axonal	Ratio of	>1 Axonal	Contralateral	Soma Width,
Туре	Cells	Projection	Asc:Desc	Branch Point	Dendrites	μm
			Axons			
iSB	8/22 (36%)	7/8 (88%)	1.82 ± 1.23	5/8 (63%)	2/8 (25%)	17.0 ± 4.0
			( <i>n</i> = 7)			(13.0–25.0)
cSB	13/22	10/13 (77%)	1.20 ± 0.75	6/13 (46%)	6/13 (46%)	17.3 ± 4.6
	(59%)		( <i>n</i> = 10)			(12.0–25.0)
bSB	1/22 (5%)	1/1 (100%)	1.33 ( <i>n</i> = 1)	1/1 (100%)	0/1 (0%)	12.0
	22 (100%)	18/22 (82%)	1.45 ± 0.96	12/22 (55%)	8/22 (36%)	17.0 ± 4.2
Total			( <i>n</i> = 18)			(12.0–25.0)

TABLE 1. Morphological characteristics of spinobulbar neurons

iSB, ipsilaterally projecting spinobulbar neuron; cSB, contralaterally projecting spinobulbar neuron; bSB, bilaterally projecting spinobulbar neuron; >1 Axonal Branch Point, those neurons with >2 axon branches; Soma Width, short-axis width means (±SD) (range). Ratio of Asc:Desc Axons = mean ratio (±SD) of number of ascending axons to number of descending axons for the cells with at least one descending axon.

The dendritic fields of SB neurons were either restricted to the ipsilateral side of the spinal cord or could have dendrites extending across the midline (Table 1). Although only two of the eight iSB neurons had contralateral dendrites (Fig. 4*Ai*), about half of the cSB neurons (6/13) had contralateral dendrites. Furthermore, some of the contralateral dendrites in cSB neurons could exhibit extensive branching (e.g., Figs. 4*C*, *i*, *ii*, and *iv* and 9*B*).



FIG. 5. Examples of inhibitory postsynaptic potentials (IPSPs) in reticulospinal neurons produced by 2 iSB neurons. Ai: experimental configuration for iSB (#234i1) showing location of intracellular recordings from the cell bodies of a uniquely identifiable reticulospinal Müller cell (B3) located in the middle rhombencephalic reticular nucleus (MRRN) and the iSB neuron, both on the right side. Distances between the iSB and the cord surface recording and the B3 neuron are indicated. Aii: the iSB was stimulated repetitively (10 Hz) with shortduration (1-ms) depolarizing pulses to generate action potentials (bottom trace). iSB conduction velocity to the cord recording was 1.3 m/s (top trace). Each action potential in the iSB neuron elicited an IPSP with constant latency and unitary shape as shown in 5 consecutive raw traces. Assuming the same conduction velocity from the cord recording to the B3 neuron, the synaptic delay was 2.6 ms. It was concluded that this was a monosynaptic connection. Bi: experimental configuration for another iSB neuron (#242i1). In this case, a diffusion barrier was constructed and high-divalent cation solution perfused into the brain stem bath. Bii: stimulation of the iSB at 10 Hz elicited IPSPs occurring at a constant latency and with unitary shape. These characteristics and the presence of the IPSP in high-divalent cation solution, which greatly reduces indirect PSPs, would indicate a direct synaptic connection. However, assuming the same conduction velocity from the cord electrode to the reticulospinal neuron yielded a synaptic delay of 8.8 ms. Taken together, these results suggest that the connection is direct but that conduction velocity of the iSB neuron slowed after passing the cord recording electrode and propagating into the brain stem. This could be explained in part by the presence of the high-divalent cation solution, which slows conduction velocity by raising axonal spike threshold, or by the possibility of axonal thinning as it nears its terminal field.



B cSB to non-RS PRRN Neuron



**FIG. 6.**Example of an excitatory postsynaptic potential (EPSP) produced by a cSB in an apparent nonreticulospinal (non-RS) neuron in the posterior rhombencephalic reticular nucleus (PRRN). *A*: experimental configuration showing arrangement of electrodes and the distances between them. *B*: 3 consecutive traces are shown for the extracellular recording of the contralateral spinal cord (with respect to the soma of the cSB) near the obex (*top traces*) and for the intracellular recording of a contralateral neuron in the PRRN (*bottom traces*) that did not show a projection to the spinal cord. Estimated synaptic delay was 0.2 ms.



**FIG. 7.**Summary of output PSPs from SB neurons to neurons of the reticular nuclei. Some 169 pairs were tested from SB to RS neurons and 4 pairs were tested from SB to non-RS neurons. PSPs in non-RS neurons are indicated

by enclosure with circles (n = 2). All PSPs followed 10-Hz presynaptic stimulation with constant latency and unitary shape except the EPSP enclosed in a square. All PSPs were recorded in normal solution except for 3 IPSPs recorded in high-divalent cation solution and indicated by enclosure in ellipses. A: amplitudes for excitatory (+) and inhibitory (–) PSPs as measured from averages. B: estimated synaptic delays. Note that the estimated synaptic delay for the bSB neuron was negative, which was likely attributable to an underestimation of the conduction velocity after the axon passed the obex.



**FIG. 8.**Two examples of interacting pairs of RS and SB neurons. *A* and *B*: mutual excitation. *A*: EPSP from an iSB neuron (#229i1) to an ipsilateral RS neuron located in the PRRN. *B*: same RS neuron provided excitation of the same iSB neuron. *C* and *D*: feedback inhibition. *C*: IPSP from an iSB neuron (#242i1) to the ipsilateral reticulospinal Müller cell B2. *D*: B2 reticulospinal neuron provided excitation of dual electrical/chemical nature to the same iSB neuron. Traces are averages of 30 sweeps.



**FIG. 9.**Example of input to a spinobulbar neuron from a primary mechanosensory neuron (dorsal cell). *A*: an ipsilateral dorsal cell (ipsi DC) (*bottom trace*) was stimulated repetitively at 1 Hz evoking an EPSP in a cSB (#233c2) located caudal to the dorsal cell. EPSP exhibited both an early monosynaptic component (synaptic delay = 3 ms) and a later, more variable polysynaptic component. *B*: drawing of the cSB in *A* exhibiting prominent contralateral dendrites reminiscent of giant interneurons in the caudal spinal cord.

Overall, the SB neurons tended to have small- to medium-size somata with a mean short-axis width of 17.0 ± 4.2  $\mu$ m (n = 22; range = 12–25  $\mu$ m) and with no significant difference in soma width between iSB and cSB neurons (Table 1). The mean soma width of SB neurons, however, was significantly smaller than the mean of a sample of motoneurons (n = 8, mean = 33.3 ± 7.0  $\mu$ m, P < 0.001).

In addition to the labeled iSB and cSB neurons, one SB neuron with axonal projections on both sides of the spinal cord (bSB) was also stained (Fig. 4*B*, Tables 1 and 2), consistent with the electrophysiological finding of bilaterally projecting SB neurons. This cell had ascending and descending axons on both sides of the spinal cord with seven axonal processes observed in the vicinity of the soma. This bSB had a small soma (12 µm) and lacked contralateral dendrites.

TABLE 2. Comparison of dendritic morphology of SB neurons versus motoneurons

Cell Type	Number of	Total Dendritic	Number of Dendritic	Dendritic Spread	
	Primary Dendrites	Length, mm	Branch Endpoints	(Rostrocaudal), μm	

iSB ( <i>n</i> = 5)	2.8 ± 0.8	5.1 ± 1.5	61 ± 27	527 ± 111
cSB ( <i>n</i> = 7)	2.9 ± 0.7	5.5 ± 2.4	71 ± 30	493 ± 202
bSB ( <i>n</i> = 1)	3	4.1	34	460
All SB ( <i>n</i> = 13)	2.8 ± 0.7	5.2 ± 2.0	64 ± 28	504 ± 158
Motoneurons	3.5 ± 0.9	8.3 ± 3.1*	110 ± 55*	351 ± 50*
( <i>n</i> = 8)				

Values are means  $\pm$  SD. The asterisk indicates a significant difference between mean values in motoneurons versus all SB neurons (P < 0.05) using Student's *t*-test. Statistical comparisons of means in iSB versus cSB revealed no significant differences.

To characterize dendritic morphology of SB neurons, 13 well-labeled and representative cells (five iSB, seven cSB, and one bSB) were traced with a drawing tube. From these drawings, measurements were made of total dendritic length, number of dendritic branch endpoints, and rostrocaudal extent of the dendrites. These data were compared with similar measurements done on myotomal motoneurons (MNs, n = 8; Table 2). From each SB cell body, two to four primary dendrites originated, and the mean number of primary dendrites was not significantly different from that in motoneurons (2.8 ± 0.7 in SB vs. 3.5 ± 0.9 in MN, P = 0.08). The mean total dendritic length of SB neurons was significantly shorter than that in motoneurons (5.2 ± 2.0 mm in SB vs. 8.3 ± 3.1 mm in MN, P = 0.011), the mean number of dendritic branch endpoints of SB neurons was significantly smaller (64 ± 28 in SB vs. 110 ± 55 in MN, P = 0.022), and the dendrites of SB neurons extended over a significantly larger rostrocaudal distance (504 ± 158 µm in SB vs. 351 ± 50 µm in MN, P = 0.016). Thus the dendrites of SB neurons were on average less extensive and less densely distributed than those of motoneurons. In comparing iSB versus cSB for these various morphological parameters, no significant differences were found (Table 2).

#### Synaptic outputs of SB neurons to RS neurons

To test for synaptic outputs of SB neurons to RS neurons, paired intracellular recordings were made between SB neurons (n = 42) and RS neurons. Postsynaptic potentials in RS neurons were observed in 14 of the 169 pairs tested (8%) (summarized in Fig. 7). The paired tests were made with RS neurons in the PRRN (4/71), MRRN (9/88), ARRN (1/4), and MRN (0/6). Among SB neurons producing at least one postsynaptic response, the rate of successful SB to RS pairs was 14 of 58 (24%). Of the nine SB neurons with demonstrated output to RS neurons, six were iSB neurons (three producing excitatory responses and three inhibitory), two were cSB neurons (one excitatory response and one inhibitory), and one had bilateral axons (excitatory). Examples of inhibitory outputs of SB neurons to RS neurons are shown in Fig. 5, and an example of an excitatory output of an SB neuron to a RS neuron is shown in Fig. 8A. The mean amplitude of excitatory postsynaptic potentials (EPSPs) to RS neurons was 0.5 ± 0.3 mV (n =5) and the mean amplitude of inhibitory postsynaptic potentials (IPSPs) to RS neurons was  $-0.9 \pm 0.8$ mV (n = 9) (individual values summarized in Fig. 7A).

In most cases, the postsynaptic responses in RS neurons followed 10-Hz stimulation of the SB neuron with constant latency and unitary shape and were thus consistent with monosynaptic connections. In some cases this conclusion was supported by the estimate of synaptic delay obtained by subtracting the estimated axonal conduction time from the total delay (individual values summarized in Fig. 7*B*). For example, Fig. 5*A* illustrates an example of an IPSP from an iSB to theipsilateral B3 reticulospinal Müller cell. Figure 5*Ai* shows the location of the iSB soma with respect to the surface recording of the

cord at the obex that was used to calculate the conduction velocity of the iSB neuron (1.3 m/s). The IPSPs in the B3 neuron followed one for one at constant latency and with unitary shape as shown in the overlay of raw traces (Fig. 5*Aii*). The synaptic delay was estimated to be 2.6 ms, consistent with a monosynaptic connection (Rovainen 1974b).

In several cases, the estimated synaptic delay was longer than expected for a monosynaptic connection, although otherwise appearing to be monosynaptic. For example, the IPSPs produced by the iSB illustrated in Fig. 5B followed 10-Hz stimulation with constant latency and unitary shape, but the estimated synaptic delay was 8.8 ms. In this experiment (n = 3 cell pairs, enclosed in ellipses in Fig. 7B), the paired recordings were made while the brain stem was perfused in high-divalent cation solution (20 mM Ca<sup>2+</sup> and 5.8 mM Mg<sup>2+</sup>), which was previously shown to reduce polysynaptic pathways (Einum and Buchanan 2004). Thus it seems unlikely that the SB neuron was exciting local inhibitory interneurons in the brain stem, although this possibility cannot be ruled out. A more likely explanation is that the conduction time from the obex to the postsynaptic neuron was underestimated and thus the synaptic delay overestimated. This underestimation of axonal conduction time would occur if the axon of the iSB neuron thinned after entering the brain stem, which might be expected if the axon terminated within the MRRN. Another contributing factor to conduction slowing in this case was the presence of the high-divalent cation, which slows conduction velocity in axons by roughly 25%, presumably as a result of the elevation of spike threshold (unpublished observations). In another case of long synaptic delay, the estimated delay was 22 ms with the brain stem in normal solution. Thus this EPSP was less likely to be the result of a monosynaptic connection (Fig. 7, enclosed in squares). This response was from a cSB (neuron 209c3 of Fig. 10) to a reticulospinal PRRN neuron and did not follow 10-Hz stimulation with constant latency or unitary shape.



**FIG. 10.**Example of an SB neuron (cell #209c3) that received direct dorsal cell input and input from the locomotor network. *A*: stimulation of an ipsi DC resulted in a early monosynaptic EPSP followed by a later, more variable polysynaptic EPSP. *B*: after d-glutamate was added to the spinal cord bath, fictive locomotion was induced. Averages of the contralateral ventral root (contra VR) and the corresponding membrane potential of the cSB were made by triggering from the onsets of 30 consecutive VR bursts. Peak of the oscillation in the cSB is out of phase with the contralateral VR burst and thus in phase with the ipsilateral VR ( $\Phi = 0.10$ ).

Synaptic outputs of SB neurons to nonreticulospinal neurons in the reticular nuclei In addition to synaptic outputs of SB neurons to RS neurons, occasionally neurons within the reticular nuclei that did not appear to have a spinal projection were encountered and tested for SB input (Fig. 7, enclosed in circles). In total, two SB neurons were tested for output to one nonreticulospinal (non-RS) neuron in the PRRN and three non-RS neurons in the MRRN. Postsynaptic potentials were observed in two of these four pairs by one of the two SB neurons. Specifically, a cSB neuron produced EPSPs in a neuron of the PRRN and a neuron of the MRRN. The EPSP to the PRRN neuron is shown in Fig. 6 and had an estimated synaptic delay of 0.2 ms.

## Synaptic inputs to SB neurons from RS neurons

During the paired recordings of RS and SB neurons, the inputs to SB neurons from RS neurons were also examined (Fig. 8). In general, outputs of RS neurons to SB neurons were found more frequently than were those from SB to RS neurons. Of the 108 pairs tested between ipsilateral RS neurons and SB neurons, 26 PSPs were observed (24%) in the SB neurons, all EPSPs. The paired tests were made with RS neurons in the PRRN (6/41), MRRN (20/60), ARRN (0/3), and MRN (0/4). Of these 26 EPSPs, 12 were clearly dual electrical/chemical EPSPs (Fig. 8*D*). Of the 14 that appeared to be only chemical, eight were monosynaptic on the basis of estimated synaptic delay. In addition to tests with ipsilateral RS neurons, some paired tests were made between contralateral RS neurons and SB neurons. Of the 58 pairs tested, five PSPs were observed in SB neurons (9%) (0/25 from PRRN, 5/31 from MRRN, and 0/2 from MRN), all IPSPs. These IPSPs had synaptic delays >4.5 ms and were likely mediated by inhibitory spinal commissural interneurons (Buchanan 1982).

In most cases, SB–RS pairs did not mutually interact. However, of the 40 pairs of SB and RS neurons that exhibited a PSP in at least one direction, three pairs did show bidirectional interactions (8%). In one case of mutual excitation, an iSB received an EPSP from the same RS neuron on which it produced an EPSP (Fig. 8, *A* and *B*). In two cases of feedback inhibition, two iSBs each received an EPSP from the RS neuron on which the iSB produced an IPSP (one example shown in Fig. 8, *C* and *D*).

# Synaptic inputs to SB neurons from mechanosensory dorsal cells

Sensory input to SB neurons was tested using dorsal cells (Figs. 9 and 10), which are primary mechanosensory neurons innervating the skin and with cell bodies in the spinal cord (Christenson et al. 1988a,b; Martin and Wickelgren 1971; Rovainen 1967b). Paired recordings were made between SB neurons (eight iSB and 14 cSB) and dorsal cells located between 0 and eight spinal segments from the SB neuron. Of the 22 tested SB neurons, dorsal cell input was observed in 11 (50%). Of the total 62 tested pairs, postsynaptic responses in the SB neurons were observed in 16 pairs (29%). The dorsal cells with demonstrated input to SB neurons were located between 0 and seven segments and were on either side of the spinal cord with respect to the postsynaptic SB neuron. All but one of the responses was excitatory and most of the responses (13/16) followed 1-Hz stimulation (mean latency =  $32 \pm 39$  ms). Two of these 13 responses followed 10-Hz stimulation with constant latencies that were short enough to be considered monosynaptic. One of these is shown in Fig. 9A where the initial excitatory response exhibited a constant latency and was followed by more variable polysynaptic PSPs. The postsynaptic neuron in this example was a cSB with extensive contralateral dendrites (Fig. 9B). These

three features of direct dorsal cell input, contralateral axon ascending to the brain stem, and extensive contralateral dendrites are shared with the giant interneurons in the caudal one third of the spinal cord (Rovainen 1974a). In three of the 16 responses between pairs of dorsal cells and SB neurons, trains of 10-Hz stimulation were required to evoke a response in the SB neuron (mean latency = 288 ± 177 ms).

Individual SB neurons receive both sensory and locomotor network inputs To determine whether SB neurons receive both mechanosensory input and input from the locomotor network, seven SB neurons that received dorsal cell input were also recorded during fictive swimming. All seven of the SB neurons also exhibited rhythmic membrane potential oscillations during fictive swimming. An example of one of these SB neurons is shown in Fig. 10. This cSB neuron received a constant-latency early EPSP from an ipsilateral dorsal cell followed by polysynaptic inputs (Fig. 10A). During fictive swimming, the oscillatory membrane potential activity reached a peak depolarization between the bursts of the contralateral ventral root (Fig. 10B). The peak depolarizations of this cell would therefore be occurring during the bursts of the ipsilateral ventral root burst ( $\Phi = 0.10$ ).

A previous study (Einum and Buchanan 2005) found that cSB neurons are equally divided between those that oscillate in phase and those that oscillate out of phase with the ipsilateral ventral root bursts. The morphological finding that about half of the cSB neurons have contralateral dendrites (Table 1) suggested that the out-of-phase cSB neurons might be those with contralateral dendrites, although this was not the case. For example, the cSB neuron in Fig. 10 oscillated in phase with the ipsilateral ventral root burst and had contralateral dendrites (Fig. 4*Civ*). In the four cSB neurons for which both morphology and fictive swim activity were available, all had contralateral dendrites. Two of these cSB neurons had peak depolarizations occurring in phase with the ipsilateral ventral root burst ( $\Phi$  values of 0.18 and 0.10, respectively, with the ventral root burst beginning at 0.0 and ending on average at 0.3). One of the cSB neurons with contralateral dendrites was clearly out of phase ( $\Phi$  = 0.58) and one cSB neuron was transitional ( $\Phi$  = 0.37). In addition, a bSB neuron had an out-of-phase peak depolarization during fictive swimming ( $\Phi$  = 0.50) yet lacked contralateral dendrites. Thus the presence of contralateral dendrites is not necessarily associated with out-of-phase swim activity.

#### Electrophysiological properties

Electrophysiological properties were characterized in 21 SB neurons with base-to-peak action potentials of >70 mV (Table 3). The mean resting membrane potential for these neurons was  $-70 \pm 8$ mV (n = 21), similar to that of other classes of lamprey neurons (Buchanan 1993). Mean input resistance, as measured in DCC with a series of hyperpolarizing pulses, was  $50.2 \pm 32.5$  M $\Omega$  (n = 9; range = 6.8-102.6 M $\Omega$ ). The mean input resistance for iSB neurons (n = 3) did not differ significantly from that of cSB neurons (n = 6):  $40.1 \pm 14.5$  versus  $55.3 \pm 38.9$  M $\Omega$ , respectively. The mean values of SB neurons are similar to those found for other small- to medium-size neurons in the lamprey spinal cord (Buchanan 1993).

Cell Type	V <sub>rest</sub> ,	<i>R</i> <sub>in</sub> , MΩ	AP <sub>peak</sub> ,	AP <sub>amp</sub> ,	AP <sub>dur</sub> , ms	sAHP <sub>amp</sub> ,	sAHP <sub>lat</sub> ,	sAHP <sub>dur</sub> ,	Rheobase,	<i>f/I</i> 1st Int. Slope,
	mV		mV	mV		mV	ms	ms	nA	Hz/nA
iSB	-73 ± 5	40.1 ±	16 ± 14	89 ± 16	2.0 ± 0.7	0.7 ± 1.2	60 ± 22	489 ± 123	0.95 ± 0.21	31 ± 22
		14.5								
	(9)	(3)	(8)	(9)	(9)	(8)	(3)	(3)	(2)	(2)
cSB	-67 ± 9	55.3 ±	18 ± 9	85 ± 11	1.5 ±	3.4 ± 2.9*	43 ± 13	334 ± 217	0.59 ± 0.53	68 ± 32
		38.9			0.3*					
	(11)	(6)	(11)	(11)	(11)	(10)	(8)	(8)	(3)	(3)
bSB	-64	_	6	70	2.4	0.4	—	426	—	—
	(1)		(1)	(1)	(1)	(1)		(1)		
	-70 ± 8	50.2 ±	17 ± 11	86 ± 14	$1.8 \pm 0.6$	2.2 ± 2.5	47 ± 17	380 ± 194	0.73 ± 0.44	53 ± 32
Total		32.5								
	(21)	(9)	(20)	(21)	(21)	(19)	(11)	(12)	(5)	(5)

 TABLE 3. Electrophysiological properties of spinobulbar neurons

Values are means  $\pm$  SD. Values in parentheses represent the number of cells for which that property was measured. The asterisks indicate a significant difference between iSB and cSB (*P* < 0.05). *V*<sub>rest</sub>, resting membrane potential; *R*<sub>in</sub>, input resistance; AP<sub>peak</sub>, membrane potential of the action potential peak; AP<sub>amp</sub>, base-to-peak amplitude of action potential; AP<sub>dur</sub>, duration of action potential at one half of amplitude; sAHP<sub>amp</sub>, absolute amplitude of slow afterspike hyperpolarization from resting membrane potential to peak sAHP; sAHP<sub>lat</sub>, time from peak of action potential to peak of sAHP; sAHP<sub>dur</sub>, total duration of sAHP from action potential peak to return of sAHP to resting membrane potential; rheobase, minimum current needed to induce an action potential using a 300-ms-duration pulse; *f*/*l* 1st int. slope, the slope of the initials portion of the *f*/*l* curve for the first spike interval.

With regard to spiking properties, the SB neurons had a mean action potential peak ( $17 \pm 11 \text{ mV}$ , n = 20) and mean base-to-peak amplitude ( $86 \pm 14 \text{ mV}$ , n = 21) similar to, although slightly lower than, that reported for other lamprey neurons ( $29 \pm 9$  and  $106 \pm 9 \text{ mV}$ , respectively; Buchanan 1993). The lower values likely arose from the less-stringent cutoff value for action potential amplitude used in the present study (70 vs. 90 mV). Action potential duration at half-peak amplitude for all SB neurons ( $1.8 \pm 0.6 \text{ ms}$ , n = 21) was also similar to previously reported values ( $1.9 \pm 0.4 \text{ ms}$ ; Buchanan 1993). The mean action potential duration of cSB neurons was significantly shorter than that of iSB neurons (Table 3). Another significant difference between cSB and iSB neurons was the amplitude of the slow afterspike hyperpolarization (sAHP). The sAHP amplitude of cSB neurons was significantly larger than that in iSB neurons ( $3.4 \pm 2.9 \text{ mV}$ , n = 10, vs.  $0.7 \pm 1.2 \text{ mV}$ , respectively, n = 8). However, this difference in sAHP amplitude may simply reflect the tendency of cSB neurons to have a more depolarized resting membrane potential compared with that of iSB neurons. ( $-67 \pm 9 \text{ vs.} -73 \pm 5 \text{ mV}$ , respectively). Examples of AHPs are shown in Fig. 11 for three SB neurons. The two cSB neurons (Fig. 11, *A* and *B*) had larger and somewhat earlier sAHP peaks than those of the iSB neuron (Fig. 11C), which exhibited a prominent early depolarization.



**FIG. 11.**Examples of afterspike hyperpolarization (AHP) in 2 cSB neurons and one iSB neuron. In general, the slow AHPs in cSB neurons were larger in amplitude and shorter in latency to peak than those in iSB neurons. iSB neurons often exhibited a prominent afterspike depolarization before the slow AHP. *A*: a cSB neuron exhibiting large-amplitude fast and slow AHPs. *B*: another cSB neuron with smaller fast and slow AHPs. *C*: an iSB neuron exhibiting a large early afterspike depolarization followed by a small slow AHP. Traces are averages of 30 consecutive sweeps. Calibration bars apply to all 3 traces.

Spike frequency versus current measurements were made on five SB neurons by injecting 0.3-sduration depolarizing current steps and plotting the instantaneous spike frequency of the first (circles) and second (triangles) spike intervals versus the injected current, and the results for three SB neurons are shown in Fig. 12, *Ai–Ci*. Sample spiking is shown for injected currents near threshold current (i.e., rheobase) for each neuron (Fig. 12, *Aii–Cii*) and for higher current levels (Fig. 12, *Aiii–Ciii*). As reported for other classes of lamprey neurons (Buchanan 1993), the SB neurons fired spikes throughout the depolarizing current pulses, increased their firing frequency with increasing current levels, and exhibited spike-frequency adaptation between the first and second spike intervals. The mean rheobase was  $0.73 \pm 0.44$  nA, similar to that of CC interneurons ( $0.58 \pm 0.41$  nA; Buchanan 1993). The mean slope of the first spike interval frequency versus current was  $53 \pm 32$  Hz/nA. There were no significant differences between iSB and cSB neurons with respect to their rheobase or mean slope of the first spike interval frequency (Table 3).



**FIG. 12.**Examples of the relationship of firing frequency vs. injected current (*f*/*I*) for 3 SB neurons for first (circles) and second (triangles) spike intervals. Each data point is the mean and SD of several current pulses at each current level. *Ai: f/I* relationship of a cSB neuron exhibiting a wide current range, high maximum frequency, and slight spike frequency adaptation. *Aii* and *Aiii*: sample spiking at 2 current levels as indicated in *f/I* plot (*Ai*). *Bi: f/I* plot of a second cSB with a more restricted current range, lower maximum firing frequency, and greater spike frequency adaptation. *Bii* and *Biii*: sample spiking at 2 current levels. *Ci: f/I* plot of an iSB neuron similar to *Bi* but showing greater variability of spike frequency from trial to trial. *Cii* and *Ciii*: sample spiking at 2 current levels.

# DISCUSSION

Spinal neurons in the rostral spinal cord with axons projecting to the brain stem were characterized electrophysiologically and morphologically using intracellular sharp microelectrode recording techniques in the lamprey. These spinobulbar neurons consisted of ipsilaterally projecting (iSB), contralaterally projecting (cSB), and a small population of bilaterally projecting neurons (bSB). Simultaneous intracellular recordings revealed that the SB neurons appeared to make direct excitatory

or inhibitory synaptic contacts on reticulospinal neurons. The SB neurons received input from 1) the locomotor network, 2) primary mechanosensory afferents, and 3) reticulospinal neurons. Morphologically, the SB neurons had small- to medium-size somata, often had descending axonal projections, and could have contralateral dendritic branches.

#### Identification of spinobulbar neurons

The method for identifying the spinobulbar neurons using surface recording of extracellular spikes at the level of the obex makes the assumption that the detected axons continue into the brain stem. In several cases, this assumption was confirmed by recording synaptic output of SB neurons to reticulospinal cells. In other cases, intracellular biocytin injection into SB neurons confirmed the projection of SB axons into the brain stem. The inability to confirm a brain stem projection for all neurons well-labeled by biocytin appeared to arise from the failure of the staining to diffuse along the axon far from the soma as the labeling tended to become lighter with distance. The biocytin labeling did, however, consistently confirm the electrophysiological determination of axon laterality and also revealed that most (82%) of the ascending cells identified as SB neurons also had descending axonal processes. The presence of the descending axonal processes suggests that the SB neurons also make synaptic connections within the spinal cord. Whether these spinal output connections are related to their ascending functions (such as recruitment or suppression of other ascending neurons) or other functional roles such as participation in rhythm generation remains to be determined.

## Synaptic interactions

Previous studies suggested that spinobulbar neurons make direct synaptic connections with reticulospinal neurons in the lamprey (Einum and Buchanan 2004, 2005). This conclusion was based on measurements of the amplitude and timing of locomotor-related oscillatory activity in reticulospinal neurons in a split-bath preparation when high-divalent cation solution was added to the brain stem bath. While this solution reduced polysynaptic pathways with little effect on monosynaptic potentials, the amplitude and timing of rhythmic spinal inputs to RS neurons were not affected. Another study using extracellular stimulation in the spinal cord while recording intracellularly from reticulospinal neurons demonstrated both direct and oligosynaptic inputs to RS neurons from presumed SB axons (Vinay et al. 1998a). The present study confirmed and extended these previous findings using paired intracellular recordings to demonstrate direct outputs from SB neurons to RS neurons. The conclusion of direct output was based on several criteria including constant latency with 10-Hz stimulation, unitary shape, persistence in high-divalent cation solution, and short synaptic delays. Synaptic delays in the present study were estimated based on the assumption that the conduction velocity of the presynaptic axon did not change after passing the recording electrode at the obex, located 1–5 mm from the postsynaptic cell. In several cases, estimated synaptic delays longer than monosynaptic connections were found. This could be attributable either to a slowing of the conduction velocity as the axon thinned near its target region or to intervening interneurons mediating the PSPs. In the present experiments, those PSPs tested persisted in the presence of high-divalent cation solution, which raises spike threshold (Frankenhaeuser and Hodgkin 1957) and reduces polysynaptic pathways (Einum and Buchanan 2004). However, strong excitatory synapses from SB neurons to local brain stem interneurons could potentially still fire interneurons in spite of this treatment. Strong excitatory connections are known to exist in lamprey. For example, the Müller cell 11 produces dual electrical and

excitatory chemical synapses on spinal neurons (Rovainen 1974b) and yet I1 was shown to elicit IPSPs in excitatory commissural interneurons in the spinal cord that followed high-frequency presynaptic stimulation with constant latencies and was presumably mediated by a local inhibitory interneuron (Buchanan and Cohen 1982). In addition, lamprey vestibular afferents conduct action potentials through electrical synapses to vestibulospinal neurons in a one-to-one fashion (Rovainen 1979). However, based on the usual criteria for monosynaptic connections, it seems reasonable to conclude that these connections are direct and that there are both excitatory and inhibitory representatives of SB neurons.

In addition to apparent monosynaptic connections, synapses of longer latencies were also found. The path of these longer connections is not known but one possibility is non-RS neurons within the reticular formation because direct excitatory input to these cells from SB neurons was shown in the present study. In general, the overall frequency of output connections from SB neurons to RS neurons was low (8%), suggesting that each SB neuron contacts only a small subset of RS neurons or, possibly, that some SB neurons have target areas within the brain stem other than the reticular nuclei.

Similar to other lamprey spinal neurons (Buchanan 1982; Rovainen 1974a), the SB neurons received direct excitatory inputs from ipsilateral reticulospinal neurons and this input was often dual electrical and chemical. Mutual interactions between SB and RS cells were found including both mutual excitation and feedback inhibition to the RS neurons from iSB neurons. A previous study found that during fictive swimming iSB neurons had rhythmic membrane potentials that were in phase with the nearby ipsilateral ventral root bursts as were most of the RS neurons (Einum and Buchanan 2005). Thus iSB neurons providing feedback inhibition would tend to inhibit the RS neuron during its depolarizing phase. This type of on-cycle inhibitory input has been observed in lamprey from lateral interneurons to commissural interneurons (Buchanan 1982), from small inhibitory interneurons to motoneurons (Buchanan and Grillner 1988), and from ascending inhibitory interneurons to sensory pathway interneurons in Xenopus spinal cord (Li et al. 2001, 2002). On-cycle inhibitory input may play a role in burst termination during locomotor activity (Buchanan 2001). Those iSB neurons with excitatory outputs to RS neurons likely provide a main component of excitatory drive to the RS neurons during locomotor activity. Both excitatory and inhibitory inputs to RS neurons were also found from cSB neurons. During fictive swimming, the cSB neurons were found to be equally divided between those with membrane potential oscillations in phase and those with oscillations out of phase with the nearby ipsilateral ventral root (Einum and Buchanan 2005). The present study showed no correlation of contralateral dendrites in cSB neurons with the phase of cSB neuron locomotor activity. However, it remains to be determined whether the sign of synaptic output in cSB neurons is correlated with the phase of their locomotor oscillations.

#### Comparison with other lamprey spinal neurons

The SB neurons were found to be small- to medium-size nerve cells on the basis of conduction velocity, input resistance, and soma size, and they showed most similarity in these values with the excitatory interneurons (EINs) and the commissural interneurons (CCINs) (Buchanan 1993). With regard to firing properties, the SB neurons were similar to other lamprey spinal neurons by their continuous spiking throughout long depolarizing pulses and showing spike-frequency adaptation. In a morphological comparison with spinal motoneurons, the dendrites of SB neurons were significantly smaller in total

length and had fewer dendritic branch endpoints, yet the SB dendritic arbor was more widely spread in the rostrocaudal dimension compared with motoneurons.

Many similarities exist between some cSB neurons and the previously described class of spinobulbar neurons, the giant interneurons (GINs). The GINs are large neurons in the caudal lamprey spinal cord with extensive contralateral dendrites and with axons crossing the midline and projecting to the brain stem, where they make excitatory synaptic connections with reticulospinal neurons (Rovainen 1967b). Morphologically, some labeled cSB neurons had features similar to those of GINs, such as the cSB neurons in Figs. 4*C*, *i*, *ii*, and *iv* and 9, with their extensive dendritic projections to the contralateral side of the spinal cord in addition to their contralateral axonal projection to the brain stem. Electrophysiologically, two cSB neurons received direct dorsal cell inputs as do GINs (Rovainen 1974a). Also, the GINs can exhibit rhythmic membrane potentials during fictive swimming (Buchanan and Cohen 1982) as do the cSB neurons (Einum and Buchanan 2005). Both GINs (Buchanan 1993) and cSB neurons tend to have large sAHPs compared with those of other cell types. These similarities suggest that cSB neurons with contralateral dendrites may be smaller, more rostral versions of GINs, although GINs lack descending axons, a common feature of the rostral cSB neurons.

#### Functional roles of SB neurons

Spinobulbar neurons provide the brain stem with information regarding both external mechanosensory signals and the activity of the locomotor network. This is similar to two major ascending pathways in mammals, the dorsal and ventral spinocerebellar tract neurons, although these pathways are more specialized for sensory and network signals, respectively (Arshavsky et al. 1972a, b, 1978b; Bosco and Poppele 1999; Lundberg 1971; Lundberg and Oscarsson 1960). In the case of mammals, these projections are first processed by the cerebellum before reaching the descending pathways (Arshavsky et al. 1978c; Orlovsky 1970b). In lamprey, the ascending paths appear to be more direct to the reticulospinal system, which is the only descending system in this animal to reach all levels of the spinal cord. As a result of the input from SB neurons, the RS neurons exhibit rhythmic activity during fictive swimming (Dubuc and Grillner 1989; Einum and Buchanan 2004). The timing of this rhythmic activity is not always in phase with the ipsilateral ventral root bursts but can also be out of phase in 25% of the RS neurons (Einum and Buchanan 2005). Locomotor rhythmic activity in the RS neurons would integrate with and modify other inputs, such as visual and vestibular signals, in the descending control of the spinal networks, particularly those in the rostral spinal cord where the specific timing signals in the RS neurons would still be appropriate. As a result of the slow segment-tosegment propagation of the locomotor oscillations (about 3 to 10 ms/segment for swim activity of 1 to 3 Hz) compared with the faster propagation of RS signals (0.5 to 2 ms/segment for RS axons of typical size range), precise timing in relation to the swim activity will be lost as the signals descend beyond the most rostral spinal cord segments.

It appears that there is specificity in the SB and RS synaptic interactions given that individual SB neurons synapse on only a subset of RS neurons and, similarly, any given SB neuron receives input from only a subset of RS neurons. Presumably, each RS neuron is receiving input from SB neurons that are providing information relevant to that RS neuron's output pattern to the spinal cord. In this regard, Zelenin et al. (2001, 2003) demonstrated that during fictive swimming in lamprey, individual RS

neurons have different patterns of excitatory and inhibitory effects on motoneurons innervating dorsal and ventral myotomes on the two sides of the cord.

In summary the present study provides the first thorough characterization of rostral spinobulbar neurons in the lamprey spinal cord. These neurons provide the reticulospinal neurons, which are the main descending system in lamprey, with rhythmic signals regarding the activity of the locomotor network and mechanosensory input from the skin. The spinobulbar neurons make direct synaptic contact with the reticulospinal neurons and receive direct input from the reticulospinal system and thus represent a simpler system for brain stem–spinal cord interactions than those found in higher vertebrates.

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# FOOTNOTES

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