

# Reticulospinal neurons controlling forward and backward swimming in the lamprey

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**Zelenin PV.** Reticulospinal neurons controlling forward and backward swimming in the lamprey. *J Neurophysiol* 105: 1361–1371, 2011. First published January, 19, 2011; doi:10.1152/jn.00887.2010.—Most vertebrates are capable of two forms of locomotion, forward and backward, strongly differing in the patterns of motor coordination. Basic mechanisms generating these patterns are located in the spinal cord; they are activated and regulated by supraspinal commands. In the lamprey, these commands are transmitted by reticulospinal (RS) neurons. The aim of this study was to reveal groups of RS neurons controlling different aspects of forward (FS) and backward (BS) swimming in the lamprey. Activity of individual larger RS neurons in intact lampreys was recorded during FS and BS by chronically implanted electrodes. It was found that among the neurons activated during locomotion, 27% were active only during FS, 3% only during BS, and 70% during both FS and BS. In a portion of RS neurons, their mean firing frequency was correlated with frequency of body undulations during FS (8%), during BS (34%), or during both FS and BS (22%), suggesting their involvement in control of locomotion intensity. RS activity was phasically modulated by the locomotor rhythm during FS (20% of neurons), during BS (29%), or during both FS and BS (16%). The majority of RS neurons responding to vestibular stimulation (and presumably involved in control of body orientation) were active mainly during FS. This explains the absence of stabilization of the body orientation observed during BS. We discuss possible functions of different groups of RS neurons, i.e., activation of the spinal locomotor CPG, inversion of the direction of propagation of locomotor waves, and postural control.

supraspinal commands; reticulospinal system; backward locomotion; forward locomotion; posture

MOST VERTEBRATES ARE CAPABLE of two forms of locomotion, forward and backward, strongly differing in the patterns of motor coordination. There is growing evidence that basic mechanisms generating these patterns are located in the spinal cord and that they are activated by supraspinal commands (Orlovsky et al. 1999). What, then, is the difference between the commands causing forward and backward locomotion? This question is related to one of the fundamental problems of motor control: encoding and decoding of supraspinal commands by the populations of descending neurons (Deliagina et al. 2002).

In the present study, this question was addressed for the lamprey (*Cyclostome*). This animal can display different forms of locomotion, including forward swimming (FS) and backward swimming (BS), as well as crawling (Archambault et al. 2001). FS in the lamprey is due to lateral body undulations propagating in the rostro-caudal direction (Grillner and Kashin 1976). These mechanical waves are caused by bursts of recip-

rocal activity of the right and left muscles (Williams et al. 1989). Spinal mechanisms generating FS movements have been analyzed in considerable detail (Grillner et al. 1995; Grillner et al. 2000). BS is also due to lateral body undulations, but they propagate in the caudo-rostral direction (Islam et al. 2006). During FS, the lamprey stabilizes its orientation in space (posture) on the basis of vestibular information (de Burlet and Versteegh 1930; Ullén et al. 1995). During BS, body orientation is not stabilized: the orientation is continuously changing, and no preferred orientation is observed (Islam et al. 2006).

The main route for descending motor commands that activate FS or BS spinal mechanisms in the lamprey is the reticulospinal (RS) pathway (Rovainen 1967; Nieuwenhuys 1972; Brodin et al. 1988), which originates from ~2,500 neurons (Ronan 1989; Bussières 1994). The majority of RS neurons project over long distances and affect different classes of spinal neurons (Rovainen 1974; Buchanan and Cohen 1982; Ohta and Grillner 1989). Previous experiments have shown that the activity of individual RS neurons increased during pharmacologically induced fictive FS *in vitro* (Kasicki and Grillner 1986; Dubuc and Grillner 1989) and in semi-intact preparations (Brocard and Dubuc 2003). In intact freely swimming lampreys, episodes of FS were accompanied by massive activation of RS neurons (Deliagina et al. 2000; Deliagina and Fagerstedt 2000). However, activity of individual RS neurons in freely behaving animals during FS has not been studied in detail, and the activity of RS neurons during BS has not been studied at all.

The aim of this study was the analysis of activity of individual RS neurons during FS and BS. The mass activity of RS neurons was recorded in freely behaving animals with the electrodes implanted in the middle body area. This method allows recording from larger RS axons with a high conduction velocity (Deliagina et al. 2000; Zelenin 2005). These large RS neurons constitute only a small part of the entire RS population, but they produce strong effects upon the spinal locomotor networks, usually along the whole extent of the axon (Zelenin et al. 2001). Activity of single neurons was extracted from mass activity using a spike-sorting procedure. Correlation of neuronal activity with locomotor activity of the animal was analyzed.

It is known that a portion of RS neurons transmit postural commands (Deliagina et al. 2000; Pavlova and Deliagina 2002; Karayannidou et al. 2007). To reveal these neurons, we tested their responses to vestibular input.

A brief account of this study has been published in abstract form (Zelenin et al. 2009).

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## MATERIALS AND METHODS

Experiments were carried out on 17 adult (25–35 cm in length) lampreys (*Lampetra fluviatilis*), which were kept in an aerated freshwater aquarium at 5°C, with a 12-h:12-h light-dark cycle. All experiments were approved by the local ethical committee (Norra Djurförsöksetiska Nämnden). Each animal was sequentially tested during forward and backward swimming, as well as by the vestibular, visual, and tactile stimulation.

**Recording of RS activity.** Activity of larger RS axons was recorded by chronically implanted electrodes (for details of the technique and discussion of the axons' identity see Deliagina et al. 2000). In short, the electrodes were made of silver wire (75 µm in diameter, 3 mm in length) and positioned on the dorsal surface of the spinal cord in parallel to RS axons (Fig. 1A). The electrodes were glued to a plastic plate (17 mm long, 2 mm wide, and 0.25 mm thick). Four electrodes were used for recording, the right and left rostral and the right and left caudal (Fig. 1B). The distance between the right and left electrodes was 1.5 mm; the distance between rostral and caudal ones was 10 mm. Two similar electrodes (rostral and caudal) on the opposite side of the plate were used for bipolar recording (Fig. 1B), which allowed subtraction of the artifacts caused by the electrical activity of the surrounding muscles (electromyogram; EMG). Several additional methods to reduce the EMG artefact were always used: 1) denervation of surrounding myotomes bilaterally throughout 5–10 spinal segments, symmetrically in relation to the site of the electrode implantation; and 2) electrical isolation of the electrodes from the surrounding muscles by wrapping them (together with the adjoining segments of the spinal cord) in a strip of thin (20 µm) plastic film (Fig. 1C). The electrodes had low resistance (<10<sup>3</sup> Ω) and low noise level (a few microvolts). As shown in the previous studies, such electrodes record almost exclusively spikes of larger RS axons, with the conduction velocity of higher than 2 m/s (Deliagina and Fagerstedt 2000; Deliagina et al. 2000). Recording from the same axon by electrodes positioned on the right and left sides allowed estimating the lateral position of the axon in the spinal cord, whereas recording with the rostral and caudal electrodes allowed measuring the axon conduction velocity (Fig. 1E). In contrast with previous studies, the rostral and caudal electrodes were placed not on separate plastic plates but on one plate, which reduced traumatic effects of their implantation.

Implantation of the electrodes was performed under tricaine methane sulfonate anesthesia (MS-222, 100 mg/l; Sigma, St. Louis, MO). The plate with electrodes was implanted through a longitudinal cut performed along the midline of the dorsal aspect of the body at the level of the 40th segment in front of the dorsal fins. The wound was then closed and sutured so that the connecting wires were tightly fixed between the sides of the wounds. Bipolar EMG electrodes were implanted bilaterally or unilaterally in the muscles at the level of the 35th spinal segment.

**Experimental design.** After full recovery from anesthesia (~3 h), the lamprey was positioned in an aquarium of 50 × 30 × 15 cm. The implanted electrodes for recording activity in RS pathways and EMGs were connected via a long flexible cable to the inputs of AC amplifiers.

Forward swimming appeared either spontaneously or was evoked by different sensory stimuli, i.e., pinching the head or the tail, illuminating the eye or tail photoreceptors (Ullén et al. 1993; Deliagina et al. 1995). The lamprey swam freely for periods of 20 to 100 s, randomly turning in different planes and changing the frequency of body oscillations. Episodes of FS (4–10) were recorded for each animal. Activity of the neurons was largely the same in all episodes of FS, no matter how the episode was initiated.

Backward swimming was evoked by continuous tactile stimulation. For this purpose, a thin elastic ring (a piece of toe protector, Scholl, 8 mm in diameter and 15–25 mm in length) was put over the gill region of the body or the head (Islam and Zelenin 2008). For each

animal, from 6 to 12 episodes of BS were recorded. The episodes lasted for 10 to 120 s.

To reveal the RS neurons that were not active during locomotion, each animal was tested with tactile and photo stimulation. These tests were performed in quiescent animals attached to a horizontal surface with their sucker mouth. Tactile responses of RS neurons were induced by light rubbing and tapping the skin in different parts of the body. To induce photo responses, the right or left eye, or the tail photoreceptors, were illuminated through a fiber optic system.

To reveal RS neurons related to the postural control system (this system in lampreys is driven by vestibular input) each animal was subjected to vestibular stimulation. A special device for natural stimulation of vestibular organs was positioned in the aquarium (Fig. 7A). The lamprey was placed in a tube that could be rotated around 1) the longitudinal axis of the animal through 360° (roll tilts), 2) the transverse axis through 360° (pitch tilts), or 3) the vertical axis through 120° (yaw turns). In the roll and pitch planes, two sequential full turns in opposite directions were performed, starting from the dorsal side down orientation. Rotation was performed in 45° steps, with a transition from one position to the next lasting ~1 s, and with each position maintained for ~2 s (Fig. 7B). For yaw turns, we used trapezoid 120° rotation to the left and to the right. Transition from one position to another lasted 0.5–1 s, and each position was maintained for 1–4 s. With each type of rotation, individual animals were tested at least three times. The neurons responding differently to rotation in opposite directions (at least in 1 plane) were considered as involved in the control of body orientation (Deliagina et al. 2000; Pavlova and Deliagina 2002; Karayannidou et al. 2007; Zelenin et al. 2007).

## Data Analysis

Signals from the four electrodes were band-pass filtered (300 Hz to 500 Hz), amplified, digitized with a sampling frequency of 10 kHz, and saved for further analysis with data acquisition software (Power1401, Spike2, version 4; CED, Cambridge, UK).

**Extraction of activity of individual RS neurons.** The multiunit spike trains recorded by each electrode (Fig. 1D) were separated into unitary waveforms (representing activity of individual axons) using a spike-sorting procedure in conventional data analysis software. The spikes with the same waveform were supposedly generated by the same RS neuron in all tests (both swimming and sensory stimulation). The spike extraction included several steps. First, one of the channels was used to build primary templates and to extract the groups of events that fitted them. Only spikes with amplitudes greater than 50 µV (typically, about 100 µV) were taken into account. The template width varied in the range from 10 to 25 µV, but it was always less than 20% of the spike amplitude. Second, for each of the groups, the corresponding waveforms from the other channels were extracted; they were then reclassified, i.e., secondary templates were built for each of the groups. Thus, for each RS neuron, there was a set of templates (primary plus secondary), with one template for each channel. An event was taken as a spike generated by an RS neuron, on condition that all its spike-like waveforms (extracted from all channels) fitted the corresponding templates. Any channel (right, left, rostral, or caudal) could be used to build the primary templates. At the beginning of the analysis of data recorded in a given animal, we looked through all recordings and compiled the templates corresponding to different neurons. After that, this common set of templates was used to extract the activity of single neurons from all these recordings. Thus we analyzed the activity of the same neurons in all conditions (rest, sensory stimulation, swimming). About 2–5% of high-amplitude events were discarded as not fitting any of the templates. Probably, these were the spike waveforms distorted by summation during intense activity in RS pathways.

**Analysis of the RS activity related to swimming.** The maximum of the rectified and smoothed EMG, recorded on the ipsilateral side of the RS axon, was taken as the swim cycle onset. For each cycle, an

average firing frequency was calculated. The frequency was then averaged across all cycles. We considered a neuron as active during locomotion if its average frequency was significantly higher than zero (*t*-test;  $P < 0.05$ ). For the active neurons, the phase dependence of the RS spike frequency was calculated with respect to the swim cycle. The Rayleigh test for directionality ( $P < 0.05$ ) was used to determine whether the activity of neuron was modulated in relation to the swim rhythm (Batschelet 1981; Fisher 1993).

The phase dependence of neuronal firing usually had a sine-like shape with one peak per cycle (Fig. 6A). We approximated this pattern using a Fourier image of the spike sequence:  $f(\varphi) = f_0 + f_1 \cos(\varphi - \varphi_1) + r(\varphi)$ , where  $\varphi$  is the phase of the locomotor cycle. The constant component  $f_0$  of the image provides the average frequency. The first harmonic  $f_1 \cos(\varphi - \varphi_1)$  is a sine approximation of the one-peak modulation. The phase of the peak of the first harmonic ( $\varphi_1$ ) indicates the preferred phase of neuronal discharge. This method of calculation of the preferred phase is by definition identical to circular statistics (Zar 1974). Finally,  $r(\varphi)$  is the remainder after the first two terms of the series (a sum of higher harmonics). The coefficient of modulation was estimated with a formula  $M = 2f_1/(f_0 + f_1) \times 100\%$ . This formula

allows avoiding ambiguity in determining the peak and the trough in noisy phase dependence. On the other hand, in the case of pure sine shape of phase dependence, it is equivalent to the commonly used assessment of the modulation value:  $(f_{max} - f_{min})/f_{max} \times 100\%$ , where  $f_{max}$  and  $f_{min}$  are the peak and the trough in the cyclic activity.

The activity of each neuron was tested for correlation with the frequency of locomotion. For each locomotor cycle of backward locomotion, the cycle duration  $T$  (in seconds) and the number of spikes  $N$  were measured. The locomotor frequency in the cycle was calculated as  $f_{loc} = 1/T$ , whereas  $f_{RS} = N/T$  provided the firing frequency in the cycle. The frequency  $f_{loc}$  was plotted against the frequency  $f_{RS}$ , and the correlation coefficient  $CC$  was calculated (Fig. 5, A and B). Statistical significance of correlation depends on the number of locomotor cycles that varied from 35 to 900 for different neurons. For neurons with 900 cycles recorded, the correlation coefficient as low as 0.05 is statistically significant, although this is obviously very weak correlation; that is why, instead of using statistical significance, we arbitrarily used the term significant correlation for the neurons with  $CC > 0.3$  (Fig. 5C). Such values would be statistically significant ( $P < 0.05$ ) if the number of points was about

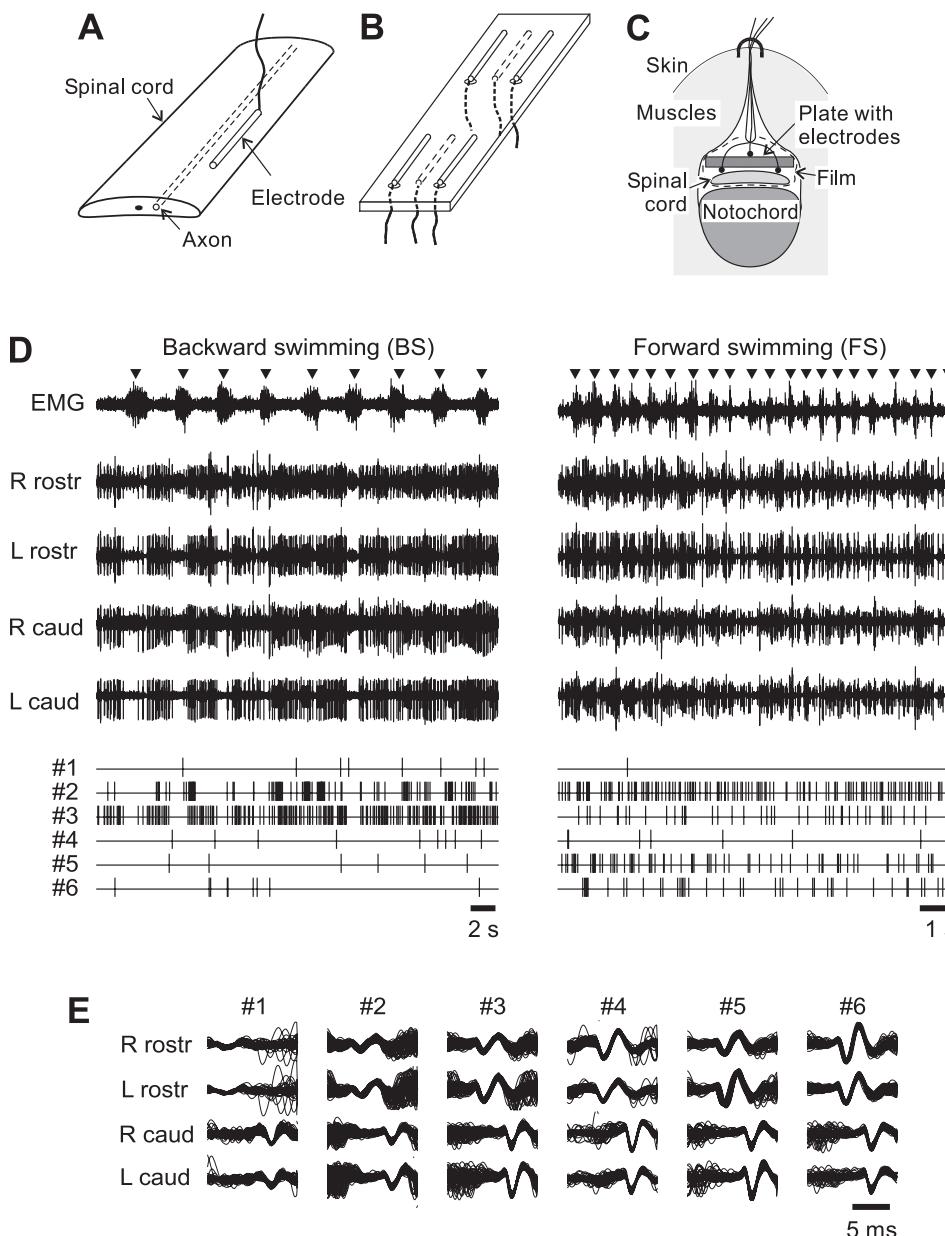


Fig. 1. Recording of activity of reticulospinal (RS) neurons. **A:** orientation of an electrode on the dorsal surface of the spinal cord in relation to the position of the RS axon. **B:** design of electrodes, view from below. Four electrodes that contact the spinal cord are visible. They recorded signals from RS axons contaminated by muscle artefacts. Two other electrodes on the opposite surface of the plate (shown with hatched lines) recorded these artefacts, which were then subtracted from the signals recorded by the main 4 electrodes. **C:** position of the implanted plate with electrodes as seen on the transversal section of the lamprey's body. The plate together with the spinal cord was isolated electrically from the muscles by a strip of plastic film. **D:** analysis of two episodes of swimming: backward swimming (BS) and forward swimming (FS) (note different time scales). EMG signal was recorded from the right muscles; arrowheads show the midpoints of EMG bursts. The activity of individual RS axons (No. 1–6) was extracted from the mass activity recorded by 4 electrodes: right rostral (R rostr), left rostral (L rostr), right caudal (R caud), and left caudal (L caud). **E:** superimposed spikes of the RS neurons extracted from the episodes shown in **D**. The voltage scale in **D** and **E** is the same for all channels. Note the difference in time scale in **D** and **E**. The neurons belonged to the following groups (see Fig. 2, etc.): 1, not active; 2, group FB; 3, group fB; 4, group FB; 5, group F; 6, group Fb.

30, that is, if the correlation was seen in an episode lasting for 30 cycles.

During FS, the RS firing frequency was comparable with the frequency of FS; the number of spikes in one cycle was small. This might cause underestimation of the correlation between locomotor frequency  $f_{loc}$  and firing frequency  $f_{RS}$ ; that is why for forward locomotion, instead of the data from a single cycle, we calculated the average firing frequency  $f_{RS}$  and the average locomotor frequency  $f_{loc}$  for five consecutive cycles (the current one, 2 preceding, and 2 following ones).

All average data are presented as means  $\pm$  SD.

## RESULTS

Activity of 146 axons was recorded in the spinal cord. The number of axons recorded in one animal ranged from 4 to 16, typically 8–10 (Fig. 1, D and E). In all axons, the spikes propagated in the caudal direction, with a conduction velocity ranged from 2.2 to 5.5 m/s (average  $3.7 \pm 0.8$  m/s; Fig. 4A), suggesting that these axons belonged to the larger RS neurons (see Deliagina and Fagerstedt 2000; Deliagina et al. 2000). Based on the relative amplitude of spikes recorded by the right and left electrodes (Fig. 1E), 70 axons were located on the right side of the spinal cord and 76 axons on the left side. The axon laterality estimated from the recorded spike amplitudes was in agreement with axon responses to vestibular stimuli (see below, e.g., Fig. 7).

### Activity of RS Neurons During Swimming

Of 146 recorded neurons, 123 were active during swimming at least in one direction. In the population of neurons active during FS ( $n = 119$ ), the mean firing frequency ranged from 0.3 Hz to 34 Hz, with the average of  $4.4 \pm 4.9$ . These data are in good agreement with those obtained previously (Zelenin 2005). In the population of neurons active during BS ( $n = 90$ ), the mean cycle frequency ranged from 0.2 Hz to 15 Hz, with the average of  $1.8 \pm 2.2$  Hz. Figure 2A shows, for each neuron, the mean frequency during backward swimming plotted against the mean frequency during forward swimming. Five groups of neurons could be distinguished.

**Group F.** These neurons were active during FS and silent (the average frequency was not significantly different from 0) during BS (Fig. 2, A and B, purple dots). We found 33 such neurons (27% of all neurons active during swimming). An example of the discharge pattern of group F neuron is shown in Fig. 3A (neuron F).

**Group B.** These neurons were active during BS and silent (the average frequency was not significantly different from 0) during FS (Fig. 2, A and B, dark blue dots). We found only four such neurons (3% of all neurons active during swimming). An example of activity of group B neuron is shown in Fig. 3A (neuron B). The neuron fired no spikes during FS. Activity of the neuron was strongly modulated in the locomotor rhythm during BS.

**Group FB.** These neurons were active during both FS and BS, and their mean firing frequencies were not significantly different during swimming in either direction ( $t$ -test;  $P > 0.05$ ; Fig. 2, A and B, green dots). There were 39 neurons in this group (32%). An example of such neuron is presented in Fig. 3A (neuron FB).

**Group Fb.** The neurons were active during both FS and BS, but the mean frequency was higher during FS ( $t$ -test;  $P < 0.05$ ;

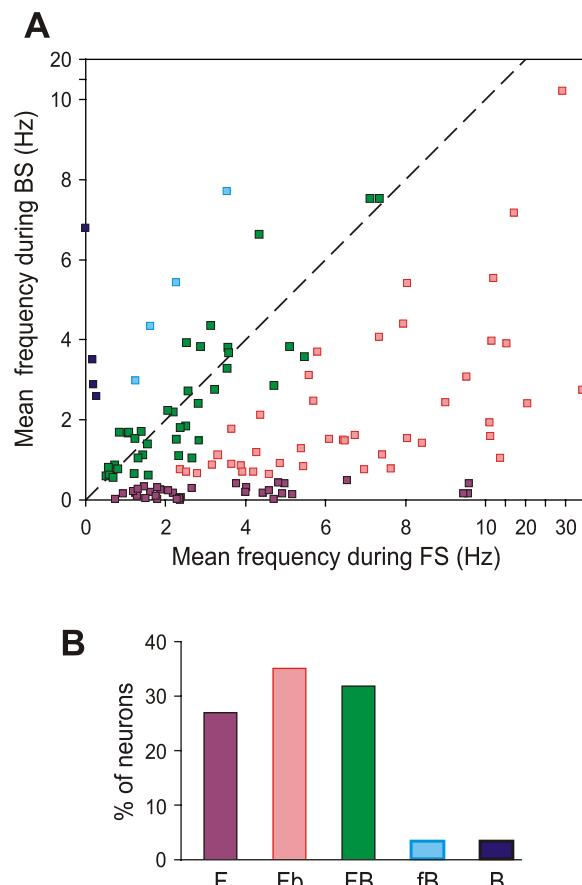


Fig. 2. Activity of RS neurons during FS and BS. A: in the scatter diagram, the  $x$  and  $y$  values of each dot show the mean firing frequency of an RS neuron during FS and BS, correspondingly. The RS neurons active during swimming were separated according to their activity into 5 groups: group F (purple dots), group Fb (pink dots), group FB (green dots), group fB (light blue dots), and group B (dark blue dots). B: percentage of neurons in these groups. Colors are the same as in A.

Fig. 2, A and B, pink dots). There were 43 neurons in this group (35%). An example of the discharge pattern of such neuron is shown in Fig. 3A (neuron Fb). Activity of the neuron varied from cycle to cycle. However, the average firing frequency was much higher during FS.

**Group fB.** The neurons were active both during FS and BS, but the mean frequency was higher during BS ( $t$ -test;  $P < 0.05$ ; Fig. 2, A and B, light blue dots). This group included four neurons (3%). The discharge pattern of one of such neurons is presented in Fig. 3A.

Thus the majority of RS neurons (groups fB, Fb, and FB;  $n = 86$ ), constituting 70% of all neurons active during swimming, are firing during swimming in both directions. Moreover, 32% of active neurons (group FB;  $n = 39$ ) do not discriminate the direction of swimming.

The neurons, which were active exclusively (group B;  $n = 4$ ) or preferably (group fB;  $n = 4$ ) during BS, constituted only 7% of the population. However, the neurons active during BS could be underrepresented in the population of recorded neurons, because our method of recording is biased toward the axons with higher conduction velocity and spike amplitude. To address this issue, we compared these characteristics of neurons from different groups. The average conduction velocity for the neurons active exclusively or preferably during BS

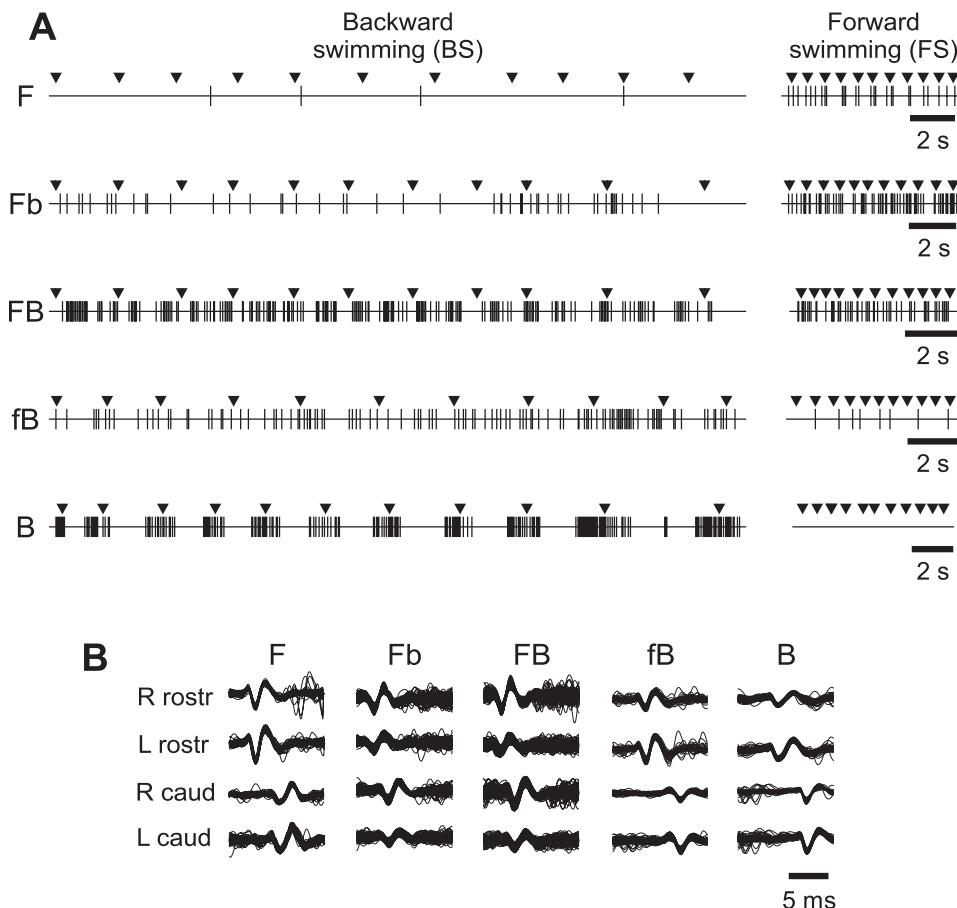


Fig. 3. Examples of discharge patterns of individual RS neurons from different groups. A: the discharge patterns of 5 RS neurons from 5 groups different in their activity during FS and BS: group *F*, group *Fb*, group *FB*, group *fB*, and group *B*. The spikes were extracted from the mass activity recorded by 4 electrodes (superimposed extracted spikes of the RS neurons are shown in *B*). Arrowheads indicate the midpoints of EMG bursts on the right side of the body. For each of the neurons the time scale is the same for the BS and FS episodes to facilitate comparison of the mean firing frequencies.

(combined groups *B* and *fB*) was indeed slightly (but not significantly) lower when compared with the neurons that did not discriminate the direction of swimming (*group FB*) or the neurons active exclusively or preferably during FS (combined groups *F* and *Fb*) (Fig. 4*B*). Average spike amplitude for combined groups *B* and *fB* was significantly lower than for combined groups *F* and *Fb* and not significantly lower than for *group FB* (Fig. 4*D*). This reduced average amplitude was due to the absence in groups *B* and *fB* of neurons with higher amplitude. These results suggest that RS neurons, which are active exclusively or preferably during BS, have smaller axons. This may also mean that our method of recording may have missed some of these neurons.

#### Correlation of Activity of RS Neurons with Swim Frequency

Swim frequency varied in a wide range, from 0.52 to 5.71 Hz for FS and from 0.13 to 1.50 Hz for BS. These values were similar to those observed in the previous studies (Islam et al. 2006; Islam and Zelenin 2008). Does the discharge frequency of RS neurons correlate with the swimming frequency? To answer this question, for each locomotor cycle we determined the instantaneous swimming frequency of the lamprey and the mean firing frequency of the neuron. The correlation coefficient between these values was then calculated. Two examples of such correlation analysis are presented in Fig. 5, *A* and *B*, where each dot corresponds to one locomotor cycle. The activity of a neuron shown in Fig. 5*A* was positively correlated with the FS frequency ( $CC = 0.41$ ). Activity of the neuron

from Fig. 5*B* was positively correlated with the BS frequency ( $CC = 0.71$ ).

It was found that, in the majority of neurons, their activity was correlated with the locomotor frequency (Fig. 5*C*). The correlation with  $CC > 0.3$  was considered as significant (see MATERIALS AND METHODS for justification of this choice). Such neurons constituted 79 out of 123 active ones. Of these neurons, the largest group (38/79) had their activity positively correlated to the frequency of BS and not correlated to the frequency of FS (area 2 on the plot in Fig. 5*C*). They included neurons of *group fB* (4/4), *group B* (4/4), *group FB* (16/39), and *group Fb* (14/43) (Fig. 5*E*). These neurons could be involved in the control of the frequency of BS.

Six RS neurons had their activity positively correlated to the frequency of forward locomotion and not correlated to the frequency of backward locomotion (area 6 in Fig. 5*C*). These neurons belonged to *group FB* (2/39) and *group Fb* (4/43) (Fig. 5*D*). They can be involved in the control of the frequency of FS.

Twenty-one RS neurons had their activity positively correlated to the frequency of locomotion in either direction (area 3 in Fig. 5*C*). These neurons belonged to *group Fb* (12/43) and *group FB* (9/39). These neurons could contribute to the activation of the locomotor CPG, as well as to the control of frequency of both FS and BS (see DISCUSSION).

Activity of 14 RS neurons was negatively correlated with the locomotor frequency (areas 1, 4, 7, 8, and 9 in Fig. 5*C*). These neurons were from *group Fb* (9/43) and *group FB* (5/39).

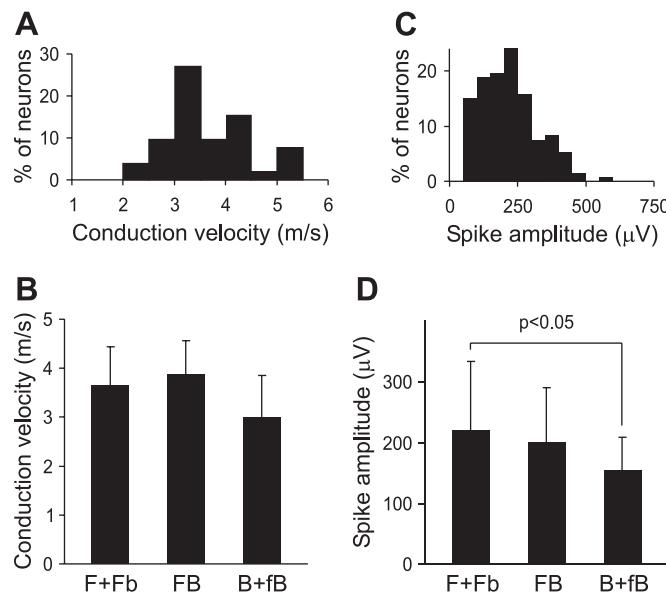


Fig. 4. Characteristics of RS neurons in different groups. *A*: distribution of conduction velocities of all recorded neurons. *B*: average conduction velocities in RS neurons more active during FS (*groups F + Fb*), equally active during FS and BS (*group FB*), and more active during BS (*groups B + fB*). *C*: distribution of spike amplitudes of all recorded neurons. *D*: average spike amplitudes in RS neurons more active during FS (*groups F + Fb*), equally active during FS and BS (*group FB*), and more active during BS (*groups B + fB*). Statistically significant difference was found between average spike amplitudes of RS neurons more active during FS and RS neurons more active during BS (*t*-test,  $P < 0.05$ ).

These neurons could be responsible for slowing down and termination of locomotion (see DISCUSSION).

In 44 neurons, activity did not correlate with the locomotor frequency (*area 5*). These neurons belonged to *group F* (33/33), to *group Fb* (6/43), and to *group FB* (5/39). These neurons are not involved in the control of swim frequency; they could be involved in the activation of the locomotor CPG (see DISCUSSION).

#### Modulation of RS Activity During Locomotion

Of the 123 neurons active during swimming, 81 neurons (66%) were modulated in the locomotor rhythm during swimming in at least one direction. Figure 6*A* shows an example of locomotor cycle-related modulation. This RS neuron was less active at the beginning of the cycle and more active in the middle of the cycle, with its preferred phase 157°. The RS neurons from *groups F* and *Fb* were modulated less often, whereas almost all neurons from *groups FB*, *fB*, and *B* were modulated (Fig. 6, *B* and *C*).

Figure 6*D* shows characteristics of all neurons modulated during FS. Each vector represents one RS neuron, the direction of the vector showing the preferred phase and the vector length showing the modulation coefficient. For the majority of neurons, their preferred phases were positioned around the middle of the cycle, the population average being  $214^\circ \pm 62^\circ$ .

The locomotor EMG-wave propagates in the caudal direction with a constant speed, and its length is approximately equal to the body length ( $\sim 100$  segments) (Williams et al. 1989). Thus the majority of RS neurons were active in phase with the ipsilateral muscles of rostral segments; the presumed phase of activity of these muscles is indicated with a dashed arc

in Fig. 6*D*. The modulation coefficient was in a range from 21% to 100% (mean,  $55 \pm 16\%$ ). These values were similar to those observed previously (Zelenin 2005).

Figure 6*E* shows characteristics of all neurons modulated during BS. Like for FS, the majority of RS neurons were active in phase with the ipsilateral muscles of rostral segments (the presumed phase of activity of these muscles is indicated with a dashed arc). The modulation coefficient was in a range from 28% to 100% (mean,  $63 \pm 22\%$ ).

Only 20 RS neurons were modulated during both FS and BS. For each neuron, the difference between the preferred phases in two forms of locomotion was determined. This difference was distributed randomly over the cycle (Fig. 6*F*;  $\chi^2$ -square test,  $P > 0.05$ ).

#### Revealing Neurons Participating in Postural Control

To reveal RS neurons related to the postural control system (these neurons are driven by vestibular input) (Deliagina et al. 2000; Pavlova and Deliagina 2002; Karayannidou et al. 2007) five animals were subjected to natural vestibular stimulation caused by rotation of the animal in different planes (see MATERIALS AND METHODS and Fig. 7*A*). Out of 44 tested RS neurons, 22 neurons responded differently to rotations in the opposite directions in at least one plane and thus were considered as involved in the control of body orientation. Some of them responded to rotation in all three planes, whereas others responded to rotation in one or two planes. Two of these neurons were not active during locomotion, 10 belonged to *group F*, four to *group Fb*, and six to *group FB* (Fig. 7*B*). Thus none of the recorded neurons involved in the control of body orientation was exclusively or preferably active during BS. (However, one cannot exclude that such neurons exist: *groups B* and *fB* constitute a small portion of the whole RS population, and the total number of recorded neurons with vestibular responses was small in our experiments.)

A population of the neurons involved in the control of body orientation was practically silent during BS, their population firing frequency being 0.84 Hz, which was four times lower than during FS (Fig. 7*C*). The absence of activity in RS neurons involved in the control of body orientation, observed during BS, is consistent with the observation that the lamprey does not stabilize its body orientation when swimming backward (Islam et al. 2006).

We checked a possibility of involvement of these 22 RS neurons in regulation of locomotor frequency. Only one neuron from *group Fb* and three neurons from *group FB* had their firing frequency strongly correlated with the frequency of BS ( $CC = 0.37, 0.35, 0.47$ , and  $0.82$ ). Only one neuron had its firing frequency strongly correlated with the frequency of FS ( $CC = 0.42$ ). It belonged to *group FB* and was one of the four neurons with activity correlated with the frequency of BS. These data suggest that the majority of RS neurons that are involved in postural control do not participate in the control of swim frequency.

#### DISCUSSION

##### Characterization of Recorded Neurons

In the present study, we extracellularly recorded the discharges in descending spinal axons of the lamprey. These

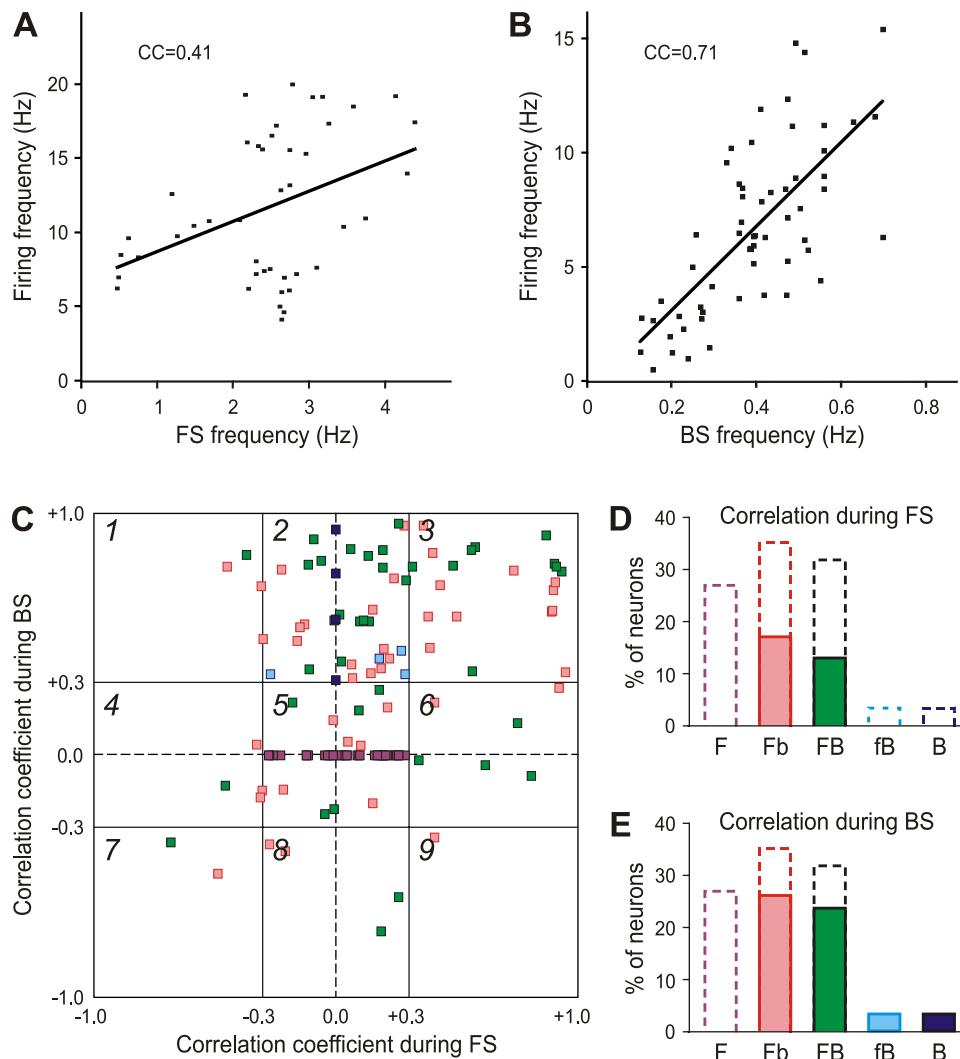


Fig. 5. Correlation of RS activity with locomotor frequency. *A* and *B*: examples of significant correlation of RS activity with locomotor frequency during FS (*A*; correlation coefficient  $CC = 0.41$ ) and BS (*B*;  $CC = 0.71$ ). Firing frequency is plotted against locomotor frequency (see details in MATERIALS AND METHODS). One dot corresponds to 1 locomotor cycle. Lines show the best linear fit of the dot clouds. *C*: correlation coefficients for individual RS neurons during FS and BS. Each dot shows data for one RS neuron. The dot colors are the same as in Fig. 2, thus indicating the group to which a neuron belongs. Cases with absolute  $CC$  values lower than 0.3 are considered noncorrelated (see MATERIALS AND METHODS). *D* and *E*: percentages of neurons in the 5 groups whose activity was correlated with the FS frequency (*D*) and with BS frequency (*E*). Dashed rectangles show percentage of all neurons in the groups (the same as in Fig. 2*B*). Note that all neurons of groups *fB* and *B* and no neurons of group *F* had their firing frequency correlated with the frequency of BS.

axons had high conduction velocities and apparently belonged to the larger RS neurons. In a previous *in vitro* study, the RS neurons with equally high conduction velocities were recorded intracellularly (Zelenin et al. 2001). All of them projected down and reached segment 75, and many of them projected more caudally (there are about 100 spinal segments in lampreys). In the present study, we recorded from the middle body area (around segment 40). Anatomical studies have shown that, from all 2,500 RS axons in the lamprey (Bussières 1994), about 900 axons reach segment 40; about 700 of them reach segment 57, and more than 250 of them reach segment 88. Several lines of evidence suggest that the RS neurons recorded in the present study, strongly differ in their characteristics and functions. First, the neurons had highly diverse activity levels and modulation patterns during FS and BS. Second, the neurons strongly differed in the patterns of vestibular input. Finally, similar RS neurons with high conduction velocities and long axons had highly diverse patterns of functional projections (patterns of influences on spinal networks) (Zelenin et al. 2001, 2007). Our recording method was biased to the axons with high conduction velocities. Thus the recorded population most probably included about 20 Müller cells, which have the largest axons. To determine whether other of the 900 RS neurons with axons reaching the middle of the body (besides Müller cells)

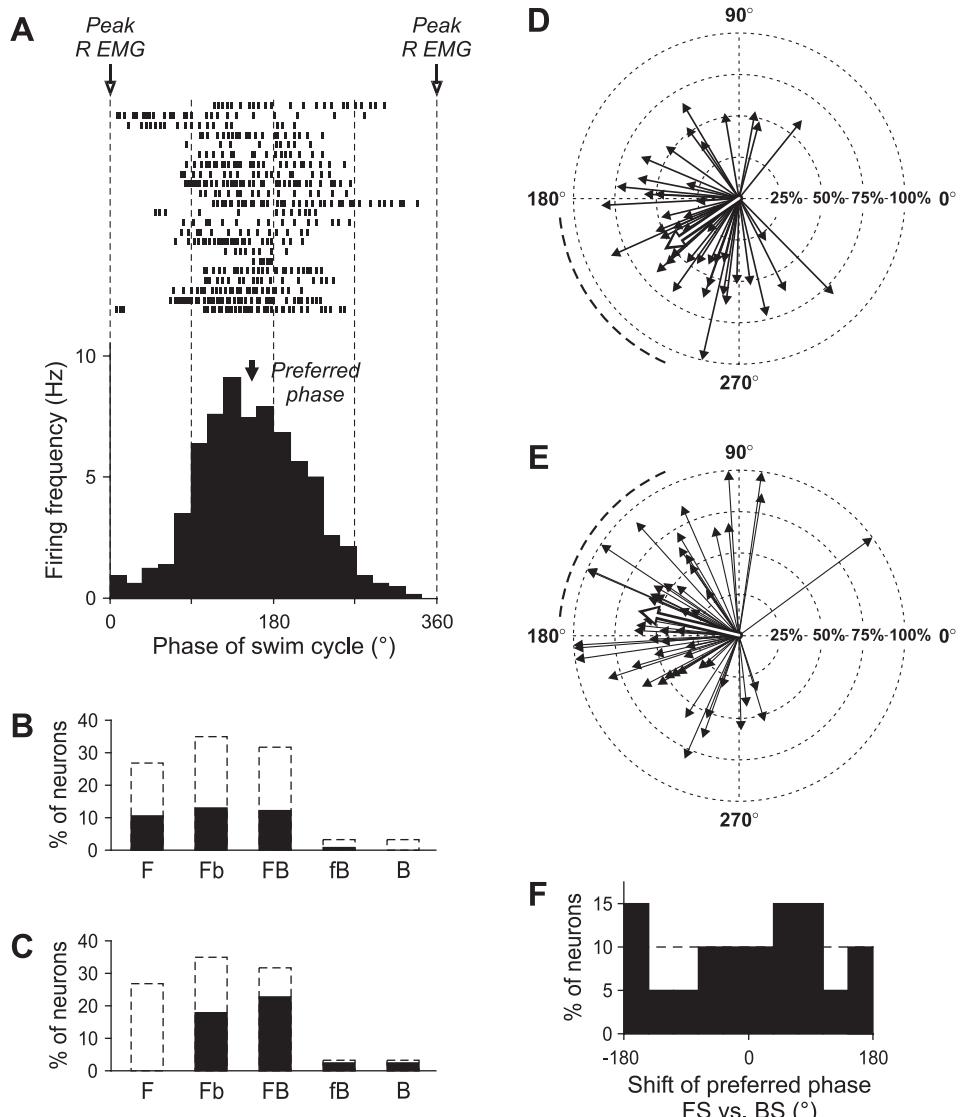
were represented in our sample, recording would need to be made from the RS cell bodies during FS and BS *in vitro*.

It is important to keep in mind that the RS neurons with thin axons, and with axons projecting only to rostral spinal segments (that constitute the majority of all RS neurons in the lamprey), have not been investigated. The lack of information on their activity during FS and BS could affect our estimation of the group sizes.

#### Possible Functions of Different Groups of RS Neurons During FS and BS

In this study, we analyzed the supraspinal control of two forms of locomotion in the lamprey: FS and BS. The neural mechanisms generating FS and BS are located in the spinal cord (Grillner et al. 1995). The supraspinal commands to these mechanisms are conveyed by the RS neurons (Grillner et al. 2000). These commands perform three main functions (Orlovsky et al. 1999): 1) activation of the spinal network (CPG) generating the basic pattern of either FS or BS. Involvement of individual RS neurons in this function during FS was studied by Di Prisco et al. (1997, 2000); 2) regulation of the locomotor frequency and the vigor of muscle contractions. Correlation of these characteristics of FS with activity of the RS population

**Fig. 6.** Periodic modulation of RS activity during FS and BS. *A*: an example of modulation of activity of an RS neuron during BS. The raster and phase histogram are shown for the right RS neuron ( $0^\circ$  corresponds to the peak of right EMG). The preferred phase was  $157^\circ$ , and the modulation coefficient was 97%. *B* and *C*: percentages of neurons in 5 groups, whose activity was modulated (filled histograms) during FS (*B*) and BS (*C*). The dashed rectangles show the percentages of neurons in each group compared with the total number of neurons (the same as Fig. 2*B*). *D* and *E*: distribution of preferred phases and modulation coefficients of RS neurons over the locomotor cycle during FS (*D*) and BS (*E*). Each vector represents 1 neuron. The direction of the vector represents the preferred phase, and the vector length indicates the modulation coefficient. The peak of EMG recorded from the ipsilateral muscles (in segment 35) was taken as the cycle onset. The dashed arc indicates the estimated position of the EMG burst generated by the ipsilateral muscles of segment 1. The white vector is the average for the whole population of neurons. *F*: distribution of the algebraic difference between the preferred phases of individual neurons during BS and FS. The hatched line indicates the level of random distribution of preferred phases.



was analyzed by Deliagina et al. (2000); 3) corrections of body orientation during swimming by modifying the basic locomotor pattern. The control of body orientation is observed during FS (de Burlet and Versteegh 1930; Ullén et al. 1995) but not during BS (Islam et al. 2006). Our goal was to reveal the RS neurons presumably responsible for each of these functions.

There are necessary (although not sufficient) conditions for assigning a particular functional role to an RS neuron:

1. The RS neurons performing the first function (activation of CPG either for FS or for BS) must be active during FS or BS, respectively. During FS, this criterion was met in all RS neurons except of *group B*. During BS, it was met in all neurons except of *group F* (Fig. 2*B*). Moreover, a considerable proportion of neurons (34%, *group FB*) did not discriminate between the directions of swimming; they were equally active both during FS and during BS.

2. The RS neurons performing the second function (regulation of the locomotor frequency) must have their activity correlated with this parameter. During FS, such correlation was found in a part of neurons of *groups Fb* and *FB*, whereas a large *group F* (27%), which was active exclusively during FS,

contained no neurons whose activity correlated with the locomotor frequency (Fig. 5*D*). During BS, the correlation was found in all neurons of *groups B* and *fB*, and in a part of neurons of *groups Fb* and *FB* (Fig. 5*E*).

3. The RS neurons performing the third function (corrections of body orientation) must have vestibular input, since this input is known to drive the body orientation system in the lamprey (Ullén et al. 1995). In the present study, vestibular input was found in a considerable proportion of neurons active during FS (*groups F, Fb, and FB*). By contrast, the neurons preferably active during BS (*groups fB and B*) typically had no vestibular input (Fig. 7*C*). These findings well correspond to the fact that the control of body orientation is observed only during FS.

Thus in this study we found that the majority of RS neurons discriminated between the swim directions (*groups F, Fb, B, fB*). In addition, we found RS neurons that were equally active during FS and during BS (*group FB*). One can suggest that the nondiscriminating neurons perform the functions common for different forms of locomotion, e.g., they could switch the spinal networks from the stationary state to the oscillatory state (see below). Similar results were obtained in our previous study, in which the activity of individual RS neurons in the

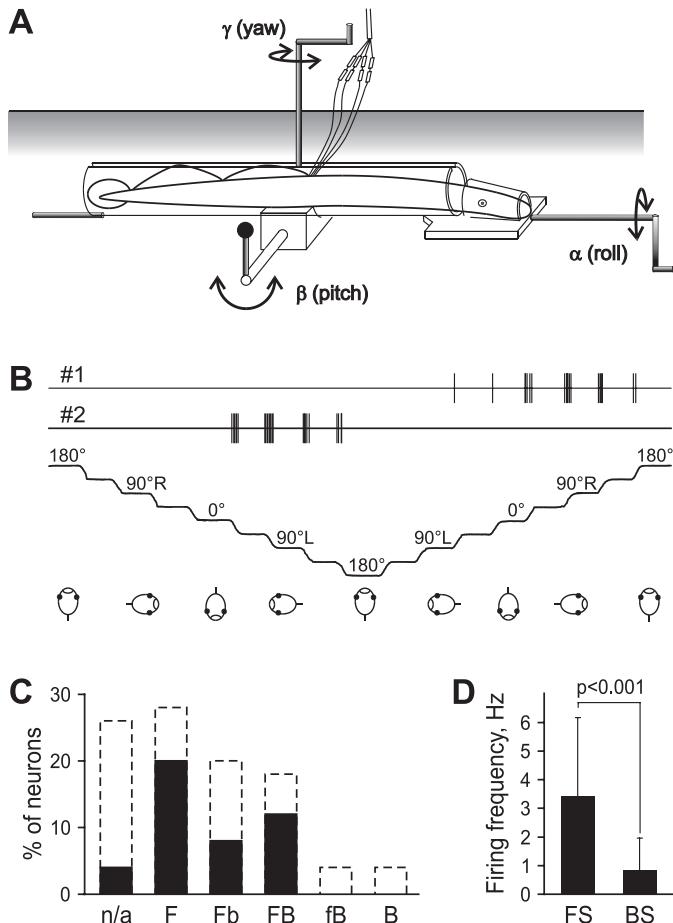


Fig. 7. Responses to RS neurons to vestibular stimuli. **A:** experimental setup for testing responses of the RS neurons to vestibular stimuli. **B:** an example of the left (1) and right (2) RS neurons that respond to turns in the roll plane. Two full turns in opposite directions were performed. The tilt angles are indicated (R, right; L, left). The angle of 0° corresponds to the normal orientation (dorsal side up). Successive positions in 90° steps in relation to the direction of gravity force are schematically shown. **C:** percentages of neurons in the 5 groups that respond to vestibular stimulation. The dashed rectangles show the percentages of all neurons tested for vestibular responses. **D:** mean firing frequency of the RS neurons that respond to vestibular stimulation during FS and BS.

lamprey was recorded during different forms of crawling, forward and backward (Zelenin 2005). It was found that some RS neurons discriminated between the forward and backward crawling, whereas others did not.

It is known that, during FS, many RS neurons exhibit periodic modulation of their firing frequency (Deliagina et al. 2000; Zelenin 2005). In the present study it was demonstrated that modulation in the rhythm of swimming was present also during BS (Fig. 6). In both FS and BS, the majority of RS neurons were active in phase with the ipsilateral muscles of rostral segments. It has been suggested that periodic modulation of RS neurons is important for coordination of different parts of the body during perturbations of regular swimming (Vinay and Grillner 1992).

#### Possible Spinal Targets of Different Groups of RS Neurons

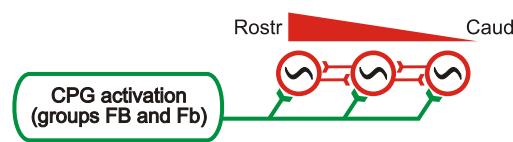
It is generally accepted that the locomotor rhythm in the lamprey and in some other vertebrates is generated by a chain of coupled segmental oscillators (Fig. 8A) (Grillner 1981, 1985). Each oscillator generates rhythmically alternating bursts

of activity in the right and left hemisegments and exerts influences on the neighboring oscillators. Due to these influences, a wave of neuronal activity propagates either in the rostro-caudal direction (that corresponds to FS) or in the caudo-rostral direction (that corresponds to BS).

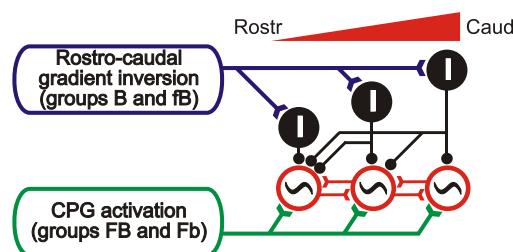
Two different models have been proposed to explain the change in the direction of locomotor waves: 1) the trailing-oscillator model with symmetrical intersegmental connections (Matsushima and Grillner 1990, 1992) and 2) the model with asymmetrical intersegmental connections (Kopell and Ermentrout 1986, 1988). We will consider the role of different groups of RS neurons in the frameworks of these two models.

The first model suggests that the direction of propagation is determined by a gradient of excitability of individual oscillators

#### A Command for forward locomotion



#### B Command for backward locomotion



#### C Integrated command system for locomotion and posture

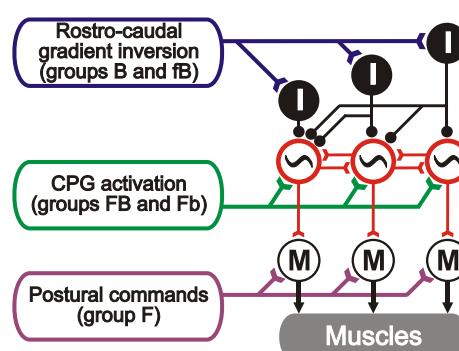


Fig. 8. A model of command system that controls FS and BS. A hypothetical model of the spinal locomotor CPG and of the descending commands controlling it is shown. The CPG consists of segmental oscillators (red circles) that excite one another and thus form a chain, along which the waves of activity propagate. **A:** elicitation of FS. The RS neurons of groups FB and Fb activate the chain of oscillators generating the swim rhythm. Because of an intrinsic gradient of excitability of oscillators (red ramp), the waves of activity propagate in the caudal direction. **B:** elicitation of BS. Like in FS, the RS neurons of groups FB and Fb activate the chain of oscillators generating the swim rhythm. At the same time, neurons of groups B and fB activate inhibitory neurons (I) that invert the gradient of excitability. This results in the caudo-rostral propagation of locomotor waves. **C:** integrated command system for FS, BS, and postural control. The RS neurons of group F send postural commands to motoneurons (M) and are active only during FS.

tors along the chain. Pharmacological activation of the locomotor CPG in the isolated spinal cord evokes waves of activity propagating in the caudal direction. These data suggest that the frequency of intrinsic oscillators is higher in the more rostral segments due to their higher excitability (red ramp in Fig. 8A), and these segments lead the caudal ones. To change the direction of the wave propagation, one needs to change the direction of the gradient (Fig. 8B).

We hypothesize that there is a special group of spinal interneurons responsible for the gradient inversion in the lamprey. Ascending inhibitory neurons (*I* in Fig. 8B) could collectively produce stronger inhibition of segmental oscillators in the more rostral segments. Such inhibitory interneurons have been found in the zebrafish; they were active only during BS (Liao and Fecho 2008). A similar inhibitory system was proposed for the BS of *Xenopus* tadpole (Green and Soffe 1998).

We suggest that the chain of segmental oscillators is activated, during FS and during BS, by the same RS neurons of *groups FB* and *Fb*, which are active in both forms of locomotion (CPG activation in Fig. 8, A-C). To produce BS, the gradient of excitability in the chain is reversed due to the activation of a population of inhibitory interneurons (*I* in Fig. 8B), which are excited by the RS neurons of *groups B* and *fB* (rostro-caudal gradient inversion in Fig. 8B). The suggestion that RS neurons of *groups Fb*, *FB*, *fB*, and *B* are involved in the control of locomotor CPG is supported by the fact that the majority of these neurons had their activity correlated with the frequency of locomotion.

Finally, we suggest that RS neurons of *group F*, whose activity does not correlate with the locomotor frequency, do not project to the segmental rhythm-generating circuits. Most of these neurons have vestibular input, and their likely function is correcting the body orientation (posture) during FS (postural commands in Fig. 8C). These commands come to motoneurons (*M* in Fig. 8C) either directly or through the interneurons that are not involved in the swim rhythm generation. Some neurons from *groups Fb* and *FB* (that receive vestibular inputs) may also participate in postural control.

Another possible mechanism for the reversal of swim direction in lampreys was suggested by Kozlov et al. (2009) within the framework of the trailing-oscillator model. In a simulation study these authors demonstrated that a chain of coupled oscillators, generating FS, could be switched to the generation of BS when excitability in only a few rostral segments was decreased. This could be done either by decreasing excitation or increasing inhibition coming from a special population of RS neurons with short axons. One can suggest that the two mechanisms of inversion of the excitability gradient (underlying BS) complement one another.

Another model of intersegmental coordination was proposed by Kopell and Ermentrout (1986, 1988). These authors have shown that FS and BS can be generated due to different ascending and descending coupling mechanisms. In the framework of this model one can expect that RS neurons of *groups F*, *Fb*, and partly *FB* boost the descending coupling and impede the ascending coupling, whereas RS neurons of *groups B*, *fB*, and partly *FB* do the opposite. The studies of the connections from RS neurons to the propriospinal neurons of the locomotor CPG are needed to test this hypothesis.

### Concluding Remarks

In the present study, for the first time the activity of individual descending neurons in the lamprey was recorded during rhythmic locomotion directed either forward or backward. Based on their activity, all neurons could be classified into several groups. In the framework of the chain-of-oscillators model of the spinal locomotor CPG, specific functions were suggested for different neuronal groups (activation of FS, activation of BS, etc.). Testing these hypotheses will be the goal of our future studies.

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### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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