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Swimming Rhythm Generation in The Caudal Hindbrain of The Lamprey

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Abstract

The spinal cord has been well established as the site of generation of the locomotor rhythm in vertebrates, but studies have suggested that the caudal hindbrain in larval fish and amphibians can also generate locomotor rhythms. Here, we investigated whether the caudal hindbrain of the adult lamprey (*Petromyzon marinus* and *Ichthyomyzon unicuspis*) has the ability to generate the swimming rhythm. The hindbrain-spinal cord transition zone of the lamprey contains a bilateral column of somatic motoneurons that project via the spino-occipital (S-O) nerves to several muscles of the head. In the brainstem-spinal cord-muscle preparation, these muscles were found to burst and contract rhythmically with a left-right alternation when swimming activity was evoked with a brief electrical stimulation of the spinal cord. In the absence of muscles, the isolated brainstem-spinal cord preparation also produced alternating left-right bursts in S-O nerves (i.e., fictive swimming), and the S-O nerve bursts preceded the bursts occurring in the first ipsilateral spinal ventral root. After physical isolation of the S-O region using transverse cuts of the nervous system, the S-O nerves still exhibited rhythmic bursting with left-right alternation when glutamate was added to the bathing solution. We

conclude that the S-O region of the lamprey contains a swimming rhythm generator that produces the leading motor nerve bursts of each swimming cycle, which then propagate down the spinal cord to produce forward swimming. The S-O region of the hindbrain-spinal cord transition zone may play a role in regulating speed, turning, and head orientation during swimming in lamprey.

NEW & NOTEWORTHY Although it has been well established that locomotor rhythm generation occurs in the spinal cord of vertebrates, it was unknown whether the hindbrain of the adult vertebrate nervous system can also generate the locomotor rhythm. Here, we show that the isolated hindbrain-spinal cord transition zone of adult lamprey can generate the swimming rhythm. In addition, the swimming bursts of the hindbrain lead the bursts occurring in the first segment of the spinal cord.

INTRODUCTION

The neural networks underlying the generation of rhythmic muscle activity for locomotion are referred to as central pattern generators (CPGs) because their activity does not require sensory feedback. Locomotor CPGs have been demonstrated in both invertebrates and vertebrates ([Arshavsky et al. 1985](#); [Büschges et al. 1995](#); [Cohen and Wallén 1980](#); [Grillner and Zangger 1984](#); [Mullins et al. 2011](#); [Orlovsky et al. 1999](#); [Roberts et al. 1981](#); [Wiggin et al. 2012](#)). In the lamprey, a lower vertebrate, the locomotor CPG has been localized to the spinal cord ([Cohen and Wallén 1980](#)) as it has in zebrafish ([Wiggin et al. 2012](#)), *Xenopus* ([Roberts et al. 1981](#)), and cats ([Grillner and Zangger 1984](#)). In lamprey, the swimming CPG exists as a chain of rhythm generators located throughout the rostrocaudal extent of the spinal cord. In the isolated spinal cord, each rhythm generator activates motoneurons in a left-right alternating pattern of bursting, and the rhythm generators are coupled to produce a rostral-to-caudal propagation of the bursts along the spinal cord, characteristic of forward swimming ([Cohen and Wallén 1980](#); [Wallén and Williams 1984](#)). In forward swimming, the most rostral rhythm generator begins first, and it has been tacitly assumed in lamprey that the first spinal segment serves as the lead generator ([Cohen et al. 1992](#); [Williams 1992](#)).

In the brainstem of lamprey, as in mammals, the mesencephalic locomotor region (MLR) plays a key role in the initiation of locomotion by activating the descending reticulospinal system ([Dubuc et al. 2008](#); [Ryczko et al. 2017](#)). These reticulospinal neurons often burst rhythmically during swimming activity because of input from ascending spinal neurons carrying swimming signals ([Antri et al. 2009](#); [Einum and Buchanan 2004, 2005, 2006](#)). Similar to lamprey, reticulospinal neurons in larval zebrafish and larval *Xenopus* exhibit rhythmic activity during swimming, but there is evidence that this rhythmicity persists in the absence of swimming input from the spinal cord, suggesting that there is a swimming rhythm generator in the caudal hindbrains of both species ([Chong and Drapeau 2007](#); [Li et al. 2009, 2010](#)). In the caudal hindbrain of the lamprey, there is a bilateral column of somatic motoneurons that innervate several muscles of the head via the spino-occipital (S-O) nerves ([Hardisty and Rovainen 1982](#); [Pombal et al. 2001](#); [Tretjakoff 1927](#)). However, it is unknown in lamprey whether the muscles of the head that are innervated by the S-O motoneurons are rhythmically active during swimming and whether the caudal hindbrain of lamprey contains a swimming rhythm generator.

The purpose of this study was to determine whether the S-O motoneurons, and the muscles they innervate, participate in swimming activity and whether the S-O region of the lamprey nervous system is capable of generating a swimming rhythm. We addressed this in brainstem-spinal cord-muscle preparations and in brainstem-spinal cord preparations using electrophysiological recording techniques. We found that the muscles of the head innervated by S-O motoneurons exhibited rhythmic electrical bursts and contractions during swimming activity. In the absence of muscles, the S-O motor nerves still exhibited rhythmic bursting in the swimming pattern (i.e., fictive swimming), and the S-O bursts preceded the bursts in the first ventral root of the

spinal cord. Finally, when the S-O region was physically isolated from the spinal cord and rostral brainstem, the S-O region could still generate rhythmic left-right bursting characteristic of swimming in the S-O motor nerves.

Thus the S-O region of the lamprey hindbrain-spinal cord transition zone contains a swimming rhythm generator with bursts that lead each burst in the spinal cord. This neural network in the caudal hindbrain may be a motor control point for the control of speed, turning movements, and head orientation during swimming.

MATERIALS AND METHODS

Animals.

Two species of lampreys were used for these studies, young adult *Petromyzon marinus* (120–180 mm in length) obtained from Acme Lamprey and adult *Ichthyomyzon unicuspis* (200–370 mm) obtained from DLM Aquatics. No significant differences between the two species were observed, and the data from the two species were pooled when used for the same experiments. The animals were kept in freshwater aquaria at 5°C until used for experiments. The animal protocol for these experiments was approved by the Marquette University Institutional Animal Care and Use Committee.

Dissection.

The typical experimental preparation was the brainstem-spinal cord preparation. For this dissection, the animal was first anesthetized by immersion in a solution of tricaine methanesulfate (250 mg/l, Tricaine-S; Western Chemicals) in buffered aquarium water until the reflex response to tail pinch was lost (5–10 min). After a transverse cut through the body just caudal to the gills, the oral disk, viscera, and ventral body muscles were cut off with scissors. The dissection was then continued with the preparation immersed in cold (4°C) lamprey Ringer's solution (in mM: 91.0 NaCl, 2.1 KCl, 2.6 CaCl₂, 1.8 MgCl₂, 4.0 glucose, 20.0 NaHCO₃, 8.0 HEPES free acid, and 2.0 HEPES Na salt) (all from Sigma-Aldrich). The skin was cut along the dorsal midline for the length of the preparation, and the body muscles were then cut off from the notochord with spring scissors. The spinal cord and brain were exposed by cutting off the dorsal portion of the cartilage surrounding the nervous system. A transverse section at the midbrain-forebrain junction was made with a no. 11 scalpel blade, and the forebrain was removed to create a brainstem-spinal cord preparation. Following dissection, the preparation was pinned to the Sylgard-lined bottom of the experimental chamber, which was continuously perfused with Ringer's solution (2–4 ml/min at 8°C).

The other preparation used in these experiments was the brainstem-spinal cord-muscle preparation. Following anesthesia, a transverse body cut just caudal to the last gill was made, and then the dissection was continued in cold Ringer's solution. The skin was cut down both the ventral and dorsal midlines and pulled off from the body. Then beginning at the caudal end of the preparation, the spinal cord was exposed by cutting off the overlying muscle, fat pad, and dorsal cartilage using spring scissors. This process continued rostrally until the spinal cord and brain were exposed. The forebrain was removed as described above. To splay the ventral muscles laterally for recording and observation, a cut was made along the ventral midline into the visceral and branchial cavities. Viscera and internal muscles were then removed by cutting. The brainstem-spinal cord-muscle preparation was transferred to the experimental chamber perfused with Ringer's solution and was securely pinned to the Sylgard-lined floor of the recording chamber to minimize movements.

Electrophysiological recordings.

Glass suction electrodes (tip diameter 100–300 µm) were used for extracellular recording of nerve and muscle activity. The signals were amplified (100–10,000×) and band-pass filtered (100 Hz to 1,000 Hz) with a differential AC amplifier (1620; A-M Systems). The conditioned signals were digitized using a micro1401 computer interface and Spike2 software (Cambridge Electronic Devices). Extracellular signals were digitized at 5,000 Hz.

Intracellular recording of membrane potentials of single nerve cells was done with sharp intracellular microelectrodes filled with 4 M potassium acetate or with 1 M lithium acetate plus 5% Lucifer yellow (Sigma-Aldrich). An AxoClamp 2B (Molecular Devices) amplifier was used with a CyberAmp320 signal conditioner (Molecular Devices) for the intracellular recordings. The intracellular signals were amplified 50×, low-pass filtered at 3,000 Hz, and digitized at 10,000 Hz using the micro1401 computer interface and Spike2 software. Motoneurons were identified electrophysiologically by the presence of a one-for-one spike in the extracellular recording of the S-O nerve.

Electrical stimulation of nerves and EMG recording.

For electrical stimulation of nerves to determine muscle innervation, a glass suction electrode with a tip diameter matching the nerve width was placed on the nerve between its emergence from the central nervous system (CNS) and its exit from the cranial or spinal cavities in the brainstem-spinal cord-muscle preparation. Single electric pulses (50 μ A, 0.1-ms duration, 2-s interval) were delivered via a stimulus isolator (A360; World Precision Instruments). To record electromyographic (EMG) responses, the tip of a glass suction electrode was placed on a muscle, and, at the same time, a stereomicroscope (Leica MZ10F) was used to visually determine whether individual muscles exhibited active contractions.

Induction of swimming activity.

In the brainstem-spinal cord and the brainstem-spinal cord-muscle preparations, electrical stimulation of the spinal cord was used to induce swimming activity. For this, a glass suction electrode was placed on the dorsal midline of the spinal cord in the caudal region of the preparation. Single pulses (0.1-ms duration) or short trains of pulses (2–5 pulses, interstimulus interval = $\sim$ 0.2 s) were used. If spinal cord stimulation alone was ineffective in inducing swimming activity, an excitatory amino acid was applied via the experimental bath perfusion system. Either d-glutamate (0.7 mM) (Tocris Bioscience) or NMDA (0.15 mM) (Sigma-Aldrich) was used ([Brodin et al. 1985](#)). In the experiments designed to test whether the S-O region can generate rhythmic swim bursts, only bath application of either d-glutamate or NMDA was used (i.e., no electrical stimulation).

Histology.

Histology was done only in *P. marinus*. Motoneurons were retrogradely labeled in vitro in the isolated brainstem-spinal cord preparation. Ringer's solution was removed from the preparation, and excess fluid was removed by blotting with tissue near the S-O nerves between their emergence from the CNS and their exit from the cranial cavity. The rostral and caudal S-O nerves were cut with a no. 11 scalpel blade, and crystals of dextran amine (10,000 MW, Molecular Probes) were immediately applied to the cut nerves. After 5–10 min, Ringer's was restored, and the preparation was kept for $\sim$ 24 h in Ringer's at 4°C. Tissue was then fixed by immersion in 10% formalin for $\sim$ 12 h at 4°C. Following fixation, the tissue was washed in Ringer's, and the hindbrain and several segments of spinal cord were removed from the cranial and spinal cavities and pinned to a Sylgard block. The isolated hindbrain and spinal cord were dehydrated in a series of increasing ethanol concentrations (50% to 100%, 2 min each) and then cleared in methyl salicylate for 1 h. The whole tissue was mounted on a depression slide in Depex mounting medium (Electron Microscopy Sciences) and coverslipped. The retrogradely labeled S-O motoneurons were imaged with a Nikon A1 confocal microscope with a $\times$ 10 objective lens.

In some experiments, motoneurons innervating the subocularis muscle were labeled in brainstem-spinal cord-muscle preparations to visualize the labeled cells in vitro. For this, the peripheral motor nerve innervating the subocularis muscle was accessed just rostral to the first gill in a muscle-free triangular region bounded dorsally by the probranchialis muscle and ventrally by the first hypobranchialis muscle (see [Fig. 1](#)). Ringer's was removed, and excess Ringer's was blotted with tissue. The nerve was cut with a scalpel blade or spring scissors, and dextran amine was applied to the cut nerve. After 5–10 min, Ringer's was restored, and the preparation was kept 24 h in a flask with $\sim$ 300 ml Ringer's on a rotating shaker at 5°C. A brainstem-spinal cord preparation was

then dissected for intracellular recording, and the labeled motoneurons were visualized with a fluorescence stereomicroscope (Leica MZ10F).

Fig. 1. Muscles of the spino-occipital (S-O) motor system and location of emergence of the S-O nerves from the central nervous system. **A:** lateral view of the head region of *P. marinus* after removal of the skin and staining with 0.3% methylene blue to enhance visibility of myosepta. The segmentally repeating epibranchialis (EB) and hypobranchialis (HB) muscles, as well as 3 nonrepeating muscles, cornealis (C), probranchialis (PB), and subocularis (SO), are innervated by the S-O nerves. 2 of the 7 gill openings (GO) are also labeled. **B:** isolated brain of *P. marinus* and 4 segments of spinal cord. The brain was tilted to the left to reveal nerves on the right side. The rostral and caudal S-O nerves (S-Or and S-Oc) emerge ventrally in the hindbrain-spinal cord transition zone. The 1st 4 ventral roots (VR) and several cranial nerves are labeled.

For anterograde labeling of S-O motoneuron axons, the S-O nerves were cut between their emergence from the CNS and their exit from the cranial cavity, and dextran amine was applied as described above in the brainstem-spinal cord-muscle preparation. Following a 24-h incubation period (as above), the brainstem and spinal cord were removed, and the muscle preparation was then cut along the midline into two halves. Internal muscles were trimmed, and the preparation was pinned to a Sylgard block and immersed in 10% formalin for 24 h. Following fixation, the tissue was washed in Ringer's, dehydrated in an ethanol series (10 min each), and cleared in methyl salicylate (12 h). The cleared muscle preparation was mounted on a depression slide in Depex and coverslipped for viewing in the confocal microscope with a $\times 4$ objective lens. Automated montages of muscle preparations were created using NIS-Elements software.

Photographs of in vitro preparations were made with a Leica DFC 295 camera on a Leica MZ10F stereomicroscope. For fresh muscle preparations, a 0.3% methylene blue solution was briefly applied to the muscles to reveal the myosepta. For imaging cranial nerves and ventral roots in the isolated CNS, the brain and spinal cord were removed from the cranial case and spinal canal and then fixed in 10% formalin for ~ 12 h at 4°C . Following the washing in Ringer's, the tissue was immersed for ~ 3 min in a 1% collagenase type 2 (Worthington) solution to facilitate removal of the meninges.

Phase angle measurements.

To measure the timing of swim bursts in the S-O nerves with respect to swim bursts in the first spinal ventral root (VR1), the isolated brainstem-spinal cord preparation was used from both *P. marinus* and *I. unicuspis*. Episodes of fictive swimming were induced with a brief electrical stimulation at the dorsal midline of the spinal

cord. This was usually done in the absence of bath perfusion of d-glutamate, but, if spinal cord stimulation was not effective at inducing swimming episodes, bath application of d-glutamate (0.7 mM) in combination with spinal cord stimulation was used. Three extracellular recording electrodes were used on the same side of the animal, S-Or nerve, VR1, and a more caudal VR (typically VR6 or VR7). For each preparation, 10 episodes of fictive swimming were analyzed. Each swim episode had on average ~20 bursts. To assess the timing of bursting between pairs of nerves, a cross-correlation of the rectified swim burst waveforms was performed using Spike2 software. The resulting cross-correlogram was a periodic function with a central peak slightly offset from 0 lag. This offset provided a measure of the relative timing of the bursts in the two nerves. A positive lag indicated that the rostral nerve preceded the caudal nerve; a negative lag indicated that the rostral nerve followed the caudal nerve. To objectively measure the time of occurrence of the central peak of the cross-correlogram, the amplitude midpoint of the central peak was determined (midpoint of the peak-to-trough amplitude), and then the time midpoint between the ascending and descending limbs of the peak at this amplitude midpoint was measured. This time of occurrence of the peak was normalized to the cycle period, which was measured as the time between peaks in the same cross-correlogram. This normalized time of occurrence of the central peak is referred to as the phase lag. The phase lag was converted to phase angle by multiplying by 360°, and circular statistics were used to calculate the mean phase angle for each animal. The population phase angle for all animals ($n = 12$) was then calculated, also using the circular mean ([Zar 1999](#)).

Rhythmic bursting in the isolated S-O region.

In the experiments to investigate whether the S-O region alone can generate rhythmic swim bursting, fictive swimming was first induced in a brainstem-spinal cord preparation with bath perfusion of either d-glutamate (0.7 mM) or NMDA (0.15 mM) ([Brodin et al. 1985](#)). Following recordings of the S-O nerves, the excitatory amino acid was washed out, and the S-O region was then physically isolated by making transverse cuts through the hindbrain and the spinal cord with a no. 11 scalpel blade. The same excitatory amino acid agonist was then reapplied to the bath perfusion, and the activity of the S-O nerves was recorded. In some cases, the isolated S-O region preparation was stored ~12 h in cold Ringer's before being tested with an excitatory amino acid. At the end of the experiment, the S-O motoneurons were retrogradely labeled as described above to determine the accuracy of the cuts with respect to the rostrocaudal extent of the S-O motor column.

For analysis, correlation techniques were used to assess the regularity of bursting, the cycle period, and the left-right burst timing. For the regularity of bursting and the cycle period, Spike2 software was used to convert nerve spikes to event times, and an autocorrelation of these event times was performed for each nerve. The autocorrelation resulted in a decaying periodic function. To quantify the regularity of the bursting, the amplitude of the second peak of the autocorrelation relative to the first peak ([Fig. 8B](#)) was measured and is referred to here as the quality of rhythmic activity (QRA) ([Buchanan 1999](#)). For each S-O nerve recording, this analysis was done on three to five episodes of swimming, each of at least 20 bursts in length, and these QRAs were averaged. The cycle period was also measured from the autocorrelograms as the time of occurrence of the second peak. For the left-right burst timing analysis, a waveform cross-correlation was performed on the rectified bursts of the left and right S-O nerves using the same data set. The phase relation of the left nerve bursts vs. the right nerve bursts was measured as the time midpoint of the first peak to the right of zero time lag. This time lag was normalized to cycle period, giving left-right phase lag. The left-right phase lag was converted to left-right phase angle by multiplying by 360°, and circular statistics were used to calculate the mean angle and the angular dispersion of the mean ([Zar 1999](#)).

Fig. 8. Timing analysis of swimming bursts in spino-occipital (S-O) nerve vs. first spinal segment. **A:** swim timing analysis was done in brainstem-spinal cord preparations with the following 3 ipsilateral nerve recordings: rostral S-O (S-Or) nerve, first ventral root (VR1), and a more caudal ventral root (VRc). Fictive swimming was induced by electrical stimulation of the spinal cord (SC stim). **B:** example of a fictive swimming episode in all 3 nerves in *P. marinus*. **C:** cross-correlation analysis of swimming episode in **B** to measure relative timing of the bursts in pairs of nerves. **C1:** cross-correlation function (CCF) of rectified traces of S-Or nerve vs. VR1 showed a 15-ms lag of central peak, indicating the S-Or nerve bursts led VR1 bursts by 15 ms. **C2:** VR1 bursts led VR10 bursts by 29 ms. These lag times were normalized to the cycle period and converted to phase angles by multiplying by 360° . **D:** phase angles of 10 swimming episodes plotted on an arc for the example preparation. Arrow indicates circular mean. **D1:** circular mean for this preparation was 10.1° . **D2:** 10 phase angles were normalized to 1 segment by dividing the phase angles by the number of segments (9 in this case) between the 2 electrodes. The circular mean per segment was 2.1° . **E:** summary of the mean phase angles for 12 preparations. **E1:** circular mean of the S-Or vs. VR1 phase angle is shown for each of the 12 preparations, and the r value for each mean is indicated by the distance of the symbol from the arc ($r = 1$ at arc). The mean S-Or vs. VR1 phase angle was 4.0° ($r = 0.997$). **E2:** mean VR1 vs. VRc phase angle per segment was 3.1° ($r = 0.999$). These 2 mean phase angles were not significantly different ($*P > 0.25$, Wheeler-Watson test, $n = 12$). The 95% confidence intervals are shown by the brackets. The lower end of the bracket is positive for both, indicating that there was forward swimming in the spinal cord and that the S-O region was, on average, leading the VR1 of the spinal cord.

Statistics.

For assessing the timing differences between bursting in S-Or nerve and VR1, the 95% confidence interval of the circular mean was used to evaluate whether the mean was significantly different than 0° . To test whether there was a significant difference in the timing between S-Or vs. VR1 and VR1 vs. more caudal ventral root (VRc), the Wheeler-Watson test from circular statistics was used (Zar 1999). For the experiments using the isolated S-O regions, the paired t -test (SigmaPlot, Systat Software) was used to determine whether there was a statistical difference in the mean QRA of fictive swimming in the brainstem-spinal cord preparations vs. the corresponding isolated S-O regions. For comparing the mean cycle periods in these two preparations, the Wilcoxon signed-rank test was used. To test whether the left-right phase lag was significantly altered after S-O region isolation, the Wheeler-Watson test from circular statistics was used. $P < 0.05$ was considered significant. Variance of means was indicated as $\pm$ SD. Variance for the circular means was expressed as $\pm$ angular dispersion (Zar 1999).

RESULTS

Spino-occipital motoneurons.

Previous anatomical studies (Hardisty and Rovainen 1982; Tretjakoff 1927) showed that there are somatic motoneurons in the hindbrain-spinal cord transition zone that innervate five muscles of the head region of the lamprey. Two of these (Fig. 1A) are segmentally repeating, i.e., the epibranchialis and hypobranchialis muscles, and three are single muscles, the subocularis, probranchialis, and cornealis muscles. The muscles receive their innervation via the S-O motor nerves, which emerge from the hindbrain-spinal cord transition zone ventrally as a pair, just rostral and caudal to the obex, referred to here as the S-Or and S-Oc nerves, respectively (Fig. 1B)

(Pombal et al. 2001). We observed that the S-Or and S-Oc nerves were distinct from the spinal ventral roots, not only in their location, but also in having greater widths and lengths than the ventral roots before they exit the cranial cavity.

We visualized the motoneurons that project out the S-O nerves by applying fluorescent dextran amine to the cut S-Or and S-Oc nerves in the in vitro brainstem-spinal cord preparation (Fig. 2A) to retrogradely label the S-O motoneurons. The labeled motoneurons form a bilateral column located near the midline (Fig. 2B). Rostral to the obex, the S-O motoneuron cell bodies lie just beneath the floor of the 4th ventricle, and the caudal end of each column is continuous with the motoneuron column of the first spinal segment. The medial side of each cell body tends to have sparse dendrites, whereas the bulk of the dendrites emerge laterally and extend to the edges of the CNS. Occasionally, medial dendrites cross the midline. The mean number of labeled S-O motoneurons (left and right sides combined) was 627 ± 81 per animal ($n = 5$).

Fig. 2. Spino-occipital (S-O) motoneurons. **A:** in vitro brainstem-spinal cord preparation of *P. marinus* shown with 2 suction electrodes on left and right rostral S-O nerves (S-Or n). Rectangle shows the location of the image in **B**. Labeled structures are as follows: mesencephalon (Mes), 3rd and 4th ventricles (3rd V and 4th V), otic capsule (OC), spinal cord (SC), and obex (*). **B:** retrogradely labeled S-O motoneurons. Motoneurons were labeled by cutting the S-Or and caudal S-O nerves on both sides of the in vitro brainstem-spinal cord preparation and applying fluorescein dextran amine to the cut nerves. The rostrocaudal midpoint of the S-O motor column lies at the obex in the hindbrain-spinal cord transition zone.

Muscle innervation.

Previous studies used anatomical methods to determine which muscles are innervated by the S-O nerves. To confirm the target muscles of the S-O motoneurons, we electrically stimulated the S-O nerves near their exit point from the cranial cavity while recording the EMG responses of the muscles and while observing muscle contractions with a stereomicroscope in brainstem-spinal cord-muscle preparations of *P. marinus*. In Fig. 3, stimulation of either the rostral or the caudal S-O nerve evoked electrical activity in each of the five indicated muscles, and this EMG activity was accompanied by visually observed, active contractions of each muscle. In contrast, electrical stimulation of the first ventral root (VR1) did not elicit EMG responses in any of the five muscles that was accompanied by visually observed, active contractions (Fig. 3). Similar results were observed in all nine preparations tested.

Fig. 3. Muscle innervation by spino-occipital (S-O) nerves shown by electromyographic responses from each muscle to electrical stimulation of the rostral or caudal S-O nerve (S-Or n, S-Oc n) or the first ventral root of the spinal cord (VR1) in a brainstem-spinal cord-muscle preparation of *P. marinus*. Recordings show a stimulus artifact followed by the electrical response of the muscle. Both the S-Or and S-Oc nerves innervate all 5 muscles of the head region, whereas the VR1 does not innervate these muscles. Similar results were observed in all 9 preparations.

When electrically stimulating the S-Or and S-Oc nerves, contractions and EMG responses were also observed in more caudal representatives of the segmentally repeating muscles, i.e., the epibranchialis and hypobranchialis muscles. In [Fig. 4A](#), the S-Or nerve was electrically stimulated while recording sequentially from the first five epibranchialis and hypobranchialis muscles. Both active contractions and EMG responses were observed in the first five epibranchialis muscles and in the first four hypobranchialis muscles. Similar responses were observed in all five preparations tested. Thus the electrophysiology demonstrated that S-O nerves innervate up to epibranchialis 5 and hypobranchialis 4 muscles.

Fig. 4. Spino-occipital (S-O) nerve innervation of segmentally repeating muscles of the head. **A:** electromyographic responses from epibranchialis (EB) and hypobranchialis (HB) muscles while electrically stimulating ipsilateral rostral S-O nerve (S-Or n) in a brainstem-spinal cord-muscle preparation of *P. marinus*. Muscles exhibited both electrical responses and visually observed contractions in EB1–5 muscles and HB1–4 muscles. Similar results were observed in all 5 preparations. **B:** muscle whole mount of left side of the head region of *P. marinus* with anterograde labeling of the S-Or n and caudal S-O nerves. Myosepta locations for the EB and HB muscles are shown with dashed white lines. Ventrally, labeled axons extended into the subocularis (SO) and the HB muscles. Both HB1 and 2 were heavily innervated, with fewer axons extending into HB3 and 4. Dorsally, labeled axons extended rostrally to potentially multiple EB muscles and more sparsely caudally to EB4 and 5. The large dorsal patch of fluorescence is nonspecific labeling of muscle tissue near the application site of the dextran amine. Although not visible in the micrograph, cornealis (C) and probranchialis (PB) muscles also exhibited labeled axons, but these axons were finer and fainter. Similar results were observed in all 4 preparations. GO, gill opening.

To confirm anatomically that the S-O motoneuron axons reach these more caudal muscles, we labeled the S-O motoneuron axons by applying fluorescent dextran amine to S-O nerves cut near their emergence from the CNS.

A photomontage of a whole-mount muscle preparation shows the distribution of the anterogradely labeled S-O motoneuron axons in [Fig. 4B](#). Ventrally, the labeled axons provided heavy innervation of the subocularis muscle and the first two hypobranchialis muscles (hypobranchialis 1 and 2). Sparser axons extended into hypobranchialis 3, with a few axons also visible in hypobranchialis 4. Dorsal to the eye, axons supplying the epibranchialis muscles extended rostrally ([Fig. 4B](#)), where they have the opportunity to innervate multiple epibranchialis muscles because of the rostromedial projections of these muscles. Caudal to the eye, heavy innervation can be seen in epibranchialis 2 and 3 muscles, with sparser labeling in epibranchialis 4 and 5. The cornealis and probranchialis muscles also received labeled axons, but, because of the small diameter and fainter labeling of these axons, they are not readily visible in the montage of [Fig. 4B](#). Similar anatomical results were observed in all four anterograde-labeling experiments. Thus both the electrophysiology and the anatomy showed that the S-O nerves not only innervated the three single muscles of subocularis, cornealis, and probranchialis but also innervated several of the segmentally repeating epibranchialis and hypobranchialis muscles.

Swimming activity in S-O-innervated muscles.

The swimming CPG of the spinal cord rhythmically activates body muscles in an alternating left-right bursting pattern during swimming activity. To determine whether the S-O-innervated muscles also exhibit rhythmic bursting during swimming activity, we used brainstem-spinal cord-muscle preparations of *P. marinus*, in which swimming activity was induced by a combination of bath application of d-glutamate (0.7 mM) and a brief electrical stimulation of the spinal cord. During swimming activity, all five of the S-O-innervated muscles exhibited rhythmic EMG bursts that alternated with rhythmic bursts occurring in the S-Or nerve on the opposite side ([Fig. 5](#)). In addition to the rhythmic EMG bursting, active rhythmic contractions of each muscle were confirmed visually with a stereomicroscope. Both rhythmic EMGs and active rhythmic contractions were observed in all five S-O-innervated muscles during swimming activity in each of the eight preparations tested. Neither rhythmic EMGs nor rhythmic contractions related to respiration were observed in any of the five S-O-innervated muscles.

Fig. 5. Electromyograms (EMGs) of spino-occipital (S-O)-innervated muscles during swimming activity. In a brainstem-spinal cord-muscle preparation of *P. marinus*, d-glutamate (0.7 mM) was applied to the bath, and electrical stimulation of the midline of the spinal cord induced swimming episodes. The preparation was pinned to reduce movements of the brainstem to allow suction electrode recording of the right rostral S-O nerve (r S-Or n). A second suction electrode recorded EMGs of head muscles on left side. Both nerve and muscles exhibited rhythmic bursting, with the left EMG bursts alternating with r S-O n bursts. Similar results were observed in all 8 preparations. Each of the muscles innervated by the S-O nerves were rhythmically active during swimming. l. SO m, left subocularis muscle; l. EB1 m, left epibranchialis 1 muscle; l. C m, left cornealis muscle; l. PB m, left probranchialis muscle; l. HB1 m, left hypobranchialis 1 muscle. All time bars = 1 s.

Fictive swimming in the S-O nerves.

When the isolated lamprey spinal cord is exposed to an excitatory amino acid, the ventral roots exhibit rhythmic bursting in a pattern similar to swimming, and this activity is referred to as fictive swimming ([Cohen and Wallén 1980](#)). To test whether the S-O nerves also exhibit rhythmic bursting in the absence of muscles, we used brainstem-spinal cord preparations ([Fig. 6B](#)) of both *P. marinus* and *I. unicuspis*. In these preparations, fictive swimming either occurred spontaneously or was induced by electrical stimulation of the dorsal midline of the spinal cord, with or without bath-applied d-glutamate. In [Fig. 6A](#), the membrane potential of an S-O motoneuron was recorded with a sharp intracellular microelectrode during a spontaneous episode of rhythmic bursting in the S-O nerves. In this episode, the S-O nerve bursts alternated between the left and right sides consistent with lamprey swimming ([Wallén and Williams 1984](#)). The membrane potential of the S-O motoneuron exhibited oscillations that produced occasional action potentials, and both the peak depolarizations of the oscillations and the action potentials occurred at the same time as the bursts in the ipsilateral S-Or nerve. The recorded motoneuron was visualized by injecting Lucifer yellow ([Fig. 6C](#)), and its cell body (green) was located near the rostral pole of the S-O motor column as indicated by the red fluorescent cells, which were retrogradely labeled subocularis motoneurons. The green axon of the recorded motoneuron along with several red retrogradely labeled axons can be seen exiting the hindbrain in the S-Or nerve ([Fig. 6C](#)). In all cases, when the S-O nerves exhibited fictive swimming, a simultaneously recorded S-O motoneuron also exhibited rhythmic membrane

potential oscillations (41 motoneurons in 17 preparations, *P. marinus* and *I. unicuspis*). Thus the S-O nerves were rhythmically bursting during swimming activity when muscles were contracting (Fig. 5), and also the S-O nerves and motoneurons were rhythmically bursting during fictive swimming when muscles were absent.

Fig. 6. Fictive swimming in the spino-occipital (S-O) region of the central nervous system. **A:** example of spontaneous episode of fictive swimming in the in vitro brainstem-spinal cord preparation of *P. marinus* characterized by alternating bursting in the left and right rostral S-O nerves (r. S-Or n and l. S-Or n). Simultaneous intracellular recording of S-O motoneuron (MN) showed oscillating membrane potential with occasional spiking during the swimming episode. Membrane potential oscillations and spiking were in phase with the ipsilateral S-Or n bursts. The resting membrane potential of the motoneuron was -73 mV. **B:** photograph of same brainstem-spinal cord preparation showing suction electrodes on l. and r. S-Or n and position of a Lucifer yellow intracellular microelectrode used to record and label MN. Square illustrates the location of the image in **C**. **C:** confocal image from a whole mount of the brainstem-spinal cord preparation with a green S-O MN labeled with Lucifer yellow. Nearby red cells were retrogradely labeled with Texas red dextran amine from peripheral motor nerve innervating subocularis muscle. Several red axons of these subocularis MNs were visible in S-Or n along with the green axon of the intracellularly recorded MN. The recorded MN lacked red labeling, indicating it was not a subocularis MN. The dendrites of the green MN extended to the lateral edges of the brainstem and, although not apparent in this image, the dendrites also extended to the ventral and dorsal edges of the hindbrain. Midline is indicated by dashed white line.

Fictive respiration in S-O nerves and S-O motoneurons.

In addition to fictive swimming, the S-O nerves could exhibit rhythmic bursts characteristic of fictive respiration (Fig. 7A) in the isolated brainstem-spinal cord preparation. Fast fictive respiration in the lamprey is characterized by synchronous bursts in the left and right VII, IX, and X cranial nerves with a cycle period of ~ 1 s (Missaghi et al. 2016). In preparations with intracellular recordings of S-O motoneurons, fictive respiratory bursting was present in the S-O nerves of 59% of these preparations (10 of 17 in *P. marinus* and *I. unicuspis*). Even in preparations with respiratory bursting in the S-O nerves, the activity was not continuously present. The intracellular recordings of S-O motoneurons ($n = 53$) revealed rhythmic membrane potentials related to fictive respiration in 26% of the motoneurons (14 of 53). These rhythmic membrane potentials were small (< 2 mV) and depolarizing, but they did not generate action potentials in any of the motoneurons (Fig. 7A). The presence of respiratory bursting in the S-O nerve improved the likelihood of observing related membrane potentials in the motoneurons (to 48%), but 14 of 27 motoneurons did not exhibit respiratory-related membrane potentials even though the S-O nerve showed respiratory bursting. The S-O motoneurons exhibiting respiratory inputs were also rhythmically active during fictive swimming ($n = 10$) (Fig. 7B). Thus individual S-O motoneurons can receive both respiratory and swimming rhythmic inputs.

Fig. 7. Respiratory and swimming inputs to spino-occipital (S-O) motoneurons (MN). **A:** fictive respiration was observed in the S-O nerves (n) in over half of the isolated brainstem-spinal cord preparations (10 of 17). In the illustrated example, respiratory activity was recognized by the simultaneous bursting in left and right rostral S-O n (l. S-Or n and r. S-Or n) and by the short duration of the bursts as a proportion of the cycle period. The S-O MN, recorded with a sharp intracellular microelectrode, exhibited small (1–2 mV) rhythmic depolarizations in phase with the respiratory nerve bursts. Similar respiratory activity was observed in 14 of 53 S-O MN recorded in 17 preparations. In none of these MN did the membrane potential reach spike threshold. **B:** same S-O MN shown in **A** also exhibited rhythmic activity during fictive swimming, which was induced in this preparation by a brief electrical stimulation of the spinal cord. In contrast to the respiratory activity, fictive swimming showed alternating left-right bursting in the S-O n, and the bursts had long durations as a proportion of the cycle period. Rhythmic inputs for both respiration and swimming were observed in 10 S-O MN in 7 preparations. The resting membrane potential of the illustrated motoneuron was –70 mV.

Timing of swimming bursts in S-O region vs. spinal cord.

In the lamprey spinal cord, any region of spinal cord of approximately two segments length is capable of generating the alternating rhythmic bursting characteristic of swimming activity ([Buchanan 1999](#)). These unit rhythm generators of the spinal cord are coupled to produce a coordinated pattern of bursting in the ventral roots, in which rostral spinal segments lead more caudal segments. This rostral-to-caudal propagation of bursts along the spinal cord produces forward swimming ([Wallén and Williams 1984](#)). Because the S-O nerves are located more rostrally than the first ventral root (VR1), it would be expected that the bursts in S-O nerves occur before the bursts in VR1. To test this, we used the isolated brainstem-spinal cord preparation (*P. marinus* and *I. unicuspis*) to compare the burst timing in the S-Or nerve vs. VR1, as schematically illustrated in [Fig. 8A](#). Episodes of fictive swimming were induced by electrical stimulation of the dorsal midline of the spinal cord, sometimes in the presence of bath-applied d-glutamate if electrical stimulation alone was ineffective.

An example of an episode of fictive swimming induced by electrical stimulation ([Fig. 8B](#)) shows nearly synchronous bursting in all three recorded nerves. Generally, the difference in the timing of bursts in adjacent spinal segments during swimming is ~1% of the cycle period or ~3.6° phase angle of the 360° swim cycle ([Wallén and Williams 1984](#)). We used cross-correlations of the rectified bursting waveform in pairs of nerves ([Fig. 8C](#)) (see materials and methods) to measure the phase angle between the S-Or nerve and VR1. For the swim episode of [Fig. 8B](#), the time difference between the bursts in S-Or nerve vs. VR1 was 15 ms, with S-Or leading ([Fig. 8C1](#)). These time differences were normalized to cycle period and converted to phase angle (see materials and methods). The measured phase angles for 10 swim episodes are plotted in [Fig. 8D](#) on an arc. Positive phase angles indicate that the rostral nerve preceded the caudal nerve. The measured phase angles between S-Or and VR1 for each of the 10 swim episodes for this example preparation ranged from 2.5° to 19.3° with a circular mean of $10.1 \pm 4.5^\circ$ ($\pm$ angular dispersion) as indicated by the arrow in [Fig. 8D1](#). The phase angle between VR1 and VRc was expressed as the phase angle per segment by dividing the phase angle by the number of intervening segments (9 in this example preparation). The measured phase angles per segment in the spinal

cord for this example preparation ranged from 1.4° to 2.9° per segment with a circular mean of $2.1 \pm 0.6^\circ$ per segment (Fig. 8D2).

Phase angle measurements of S-Or vs. VR1 in 12 preparations had phase angles ranging from -5.7° to 10.1° , with a population circular mean of $4.0 \pm 4.2^\circ$ ($n = 12$) (Fig. 8E1). In two preparations, the mean phase angle was negative. These two negative phase angles were not due to a condition of backward swimming because, in the spinal cord (Fig. 8E2), the phase angles ranged from 0.6° to 6.2° per segment, with a population circular mean of $3.1 \pm 2.1^\circ$ per segment ($n = 12$), indicating that the swimming in all 12 preparations was forward swimming. The circular mean for the S-Or vs. VR1 phase angles was not significantly different than that for VR1 vs. VRc ($P > 0.25$, Wheeler-Watson test, $n = 12$). To assess whether the circular mean for the S-Or vs. VR1 phase angle was significantly different than 0° , the 95% confidence interval for the mean was evaluated. As shown by the bracket in Fig. 8E1, the lower end of the 95% confidence interval was positive, supporting the conclusion that the circular mean for the 12 preparations was significantly positive. The two negative phase angles were likely the result of the large angular dispersion of the measurements between S-Or vs. VR1. Thus we conclude that the swim bursts of the S-O region lead the bursts of the spinal cord in the chain of coupled rhythm generators that extends throughout the spinal cord.

Rhythm generation in the isolated S-O region.

The S-O nerves burst rhythmically during swimming activity when muscles are present (Fig. 5) and when muscles are absent (Fig. 6). This rhythmic bursting could originate from within the S-O region of the hindbrain-spinal cord or could originate from the spinal cord, with transmission to the S-O region occurring via ascending interneurons (Einum and Buchanan 2006). To determine whether the S-O region of the hindbrain-spinal cord is itself able to generate the swimming pattern of alternating left-right bursting, the S-O region was physically isolated from both the spinal cord and the rostral brainstem by making transverse cuts of brainstem and spinal cord using a scalpel blade (Fig. 9A1) (*P. marinus* and *I. unicuspis*). The location of the cuts with respect to the S-O motor column was examined subsequently in each preparation using retrograde labeling of the S-O motoneurons with a fluorescent dextran amine (Fig. 9A2). For example, the preparation in Fig. 9A2 had additional tissue on each end after isolation of $\sim 20\%$ of the S-O motor column length. For all isolation experiments, an additional $19 \pm 9\%$ of spinal tissue remained caudally, and $12 \pm 8\%$ of brainstem tissue remained rostrally ($n = 9$). Thus the physical isolation was well restricted to the S-O region.

Before isolating the S-O region, we bath-applied d-glutamate to the brainstem-spinal cord preparation to induce fictive swimming, which was characterized by rhythmic bursting that alternated between the left and right S-O nerves (Fig. 9B1). Following removal of the d-glutamate, the S-O region was isolated by making the transverse cuts. Reapplication of d-glutamate to the bath again induced rhythmic bursting that alternated between left and right S-O nerves (Fig. 9C1), demonstrating that the isolated S-O region was capable of generating fictive swimming. To compare the bursting before and after isolation of the S-O region, we quantified the regularity of the bursting using an autocorrelation technique that measured the relative amplitude of the second peak in correlogram (Fig. 9, B2 and C2). The relative amplitude of the second peak varies with the regularity of the rhythm, with larger amplitudes indicating a more regular rhythm (Buchanan 1999). We refer to this amplitude as the QRA. As shown in Fig. 9, B2 and C2, the correlograms were quite similar before and after isolation of the S-O region.

Fig. 9. The isolated spino-occipital (S-O) region of the brainstem-spinal cord can generate fictive swimming. **A1:** S-O region after physical isolation from brainstem-spinal cord preparation of *I. unicuspis*. **A2:** confocal image of the retrogradely labeled S-O motoneurons in the same preparation as **A1** showing extra tissue on rostral and caudal ends of isolated S-O region (each ~20% of S-O motor column length). **B1:** before transverse cuts were made, fictive swimming was induced with bath application of d-glutamate and recorded from the left (l.) and right (r.) rostral S-O nerves (S-Or n) in the brainstem-spinal cord preparation. **B2:** autocorrelogram of bursting rhythm was used to measure the rhythm quality. The amplitude of the 2nd peak of the autocorrelation coefficient relative to the 1st peak quantified the rhythm regularity and is referred to as the quality of rhythmic activity (QRA). **C1:** removal of the spinal cord and the brainstem did not impair the ability of the S-O region to generate alternating left-right bursting in d-glutamate. **C2:** QRA from the autocorrelogram of the bursting was similar before vs. after isolation (0.39 vs. 0.43) although the cycle period was shorter before vs. after isolation (2.4 vs. 4.2 s). **D1:** summary of QRA in all preparations. Before vs. after isolation showed no significant difference of QRA ($P = 0.92$, paired t -test, $n = 11$). **D2:** summary of cycle period in all preparations. Before vs. after isolation showed a significant increase in cycle period after isolation ($*P = 0.032$, Wilcoxon signed-rank test, $n = 11$). Thus the isolated S-O region is capable of generating swimming activity although with a longer cycle period than the intact brainstem-spinal cord.

In the preparations with recordings before and after isolation of the S-O region, there was no significant difference in the mean QRA between the brainstem-spinal cord preparation vs. the S-O region only (Fig. 9D1) (0.40 ± 0.12 vs. 0.41 ± 0.12 , $P = 0.92$, paired t -test, $n = 11$). Thus removing the spinal cord and rostral brainstem did not eliminate or degrade the regularity of the rhythm. A separate group of animals with only the spinal cord removed also showed no significant difference in the QRA vs. those preparations with complete isolation of the S-O region [0.39 ± 0.14 ($n = 9$) vs. 0.41 ± 0.12 ($n = 11$), $P = 0.84$, t -test], suggesting that the rostral brainstem does not significantly contribute to the regularity of fictive swimming in the S-O region. The timing of the bursts on the left vs. the right also showed no significant change with isolation (Fig. 9, B1 and C1). The mean left-right timing angle in the brainstem-spinal cord preparations vs. the isolated S-O region was $183.6 \pm 7.7^\circ$ vs. $189.3 \pm 10.0^\circ$, $P > 0.25$, $n = 11$, (Wheeler-Watson test). One swimming rhythm parameter that did change was cycle period. In the example preparation of Fig. 9, the cycle period of the rhythm lengthened from 2.5 s to 4.2 s after isolation (Fig. 9, B and C). In all preparations with recordings both before and after isolation, there was a significant increase in the mean swimming cycle period after isolation (Fig. 9D2) (2.35 ± 0.93 s vs. 3.60 ± 2.44 s, $P = 0.032$, $n = 11$, Wilcoxon signed-rank test). Thus the S-O region was capable of generating rhythmic swimming activity in the absence of the spinal cord and the rostral brainstem, and the resulting rhythmic activity was as regular as the activity of the brainstem-spinal cord preparation. In addition, the left-right burst timing was not changed though the cycle period was significantly longer in the isolated S-O region.

DISCUSSION

This study showed that the S-O region at the hindbrain-spinal cord transition zone in lamprey has a swimming rhythm generator that activates several muscles of the head region. The presence of a swimming rhythm generator was shown by physically isolating the S-O region with transverse cuts and inducing rhythmic activity with bath application of d-glutamate. The regularity of the rhythmic activity and the left-right timing of the bursts were not significantly different than that observed before isolation in the brainstem-spinal cord preparation. The timing of swimming bursts in the S-O nerve was found to significantly precede the bursts in the first spinal ventral root.

Previously, it had been shown that the spinal cord of the lamprey contains a chain of coupled swimming rhythm generators, and isolated lengths of spinal cord taken from any rostrocaudal level can generate the left-right alternating bursting characteristic of swimming ([Cohen and Wallén 1980](#)). In a study examining the regularity of the swimming rhythm vs. the number of segments in isolated lengths of spinal cord, it was found that the regularity of the rhythm progressively decreases as the number of segments is progressively decreased; at ten or more spinal segments, the swimming regularity, as measured by the QRA (see materials and methods), is ~ 0.8 , and the QRA falls to 0.15 for two segments, the minimum number of segments that can generate left-right bursting ([Buchanan 1999](#)). When comparing these previous results on the spinal cord to the measurements here on the S-O region, we found that the S-O region has the same regularity as ~ 3.5 segments of spinal cord (QRA = 0.4). Thus, in terms of the ability to generate a regular swimming rhythm, the S-O region is similar to ~ 3.5 segments of spinal cord. This similarity in ability to generate a regular rhythm is paralleled by the number of motoneurons in the S-O region vs. the number of motoneurons in 3.5 segments of spinal cord; we found ~ 630 motoneurons in the S-O region, and [Rovainen and Dill \(1984\)](#) found ~ 200 motoneurons per spinal segment or 700 motoneurons in 3.5 segments (*P. marinus* for both).

Although the regularity of the rhythmic activity and the left-right burst timing of the S-O region did not change when it was isolated from the spinal cord and the rostral brainstem, there was a significant increase in the cycle period of the swimming rhythm of the S-O region after isolation. This finding is consistent with a previous study that demonstrated that, when the spinal cord is isolated from the brainstem, the spinal cord exhibits a shorter cycle period than when attached to the brainstem ([Vinay and Grillner 1993](#)). One explanation for this increase in cycle period is that the swimming rhythm generator of the S-O region has an intrinsic cycle period that is longer than the intrinsic cycle period of the spinal rhythm generators. It appears then that, when connected, the intrinsically slower S-O rhythm tends to slow the spinal rhythm, whereas the intrinsically faster spinal rhythm tends to speed the S-O rhythm.

If the S-O region has a longer intrinsic cycle period than the cycle period of the spinal cord, how can the S-O region produce bursts that lead the faster oscillators of the spinal cord? Mathematical modeling of coupled oscillators has shown that the relative timing of the oscillators depends both on the intrinsic cycle period of each oscillator and on the nature of their coupling ([Kopell and Ermentrout 1988](#); [Cohen et al. 1992](#)). For example, when two oscillators with different intrinsic frequencies are symmetrically coupled, the faster oscillator will be the lead oscillator. However, if the coupling is asymmetric, it is possible for the slower oscillator to be the lead oscillator if the signals from the slow oscillator can sufficiently delay the onset of each cycle in the faster oscillator, as would be characterized by a phase response curve. This outcome has been shown with computer simulations of the lamprey locomotor network in which asymmetric coupling allows an intrinsically slower oscillator to lead an intrinsically faster oscillator (Buchanan 1992). Experimentally, it has also been shown that the lamprey spinal cord does not have a rostral-to-caudal gradient of intrinsic cycle periods, suggesting that the intersegmental coupling among the swimming rhythm generators ensures the rostral-to-caudal phase lags despite both slower and faster oscillators along the chain of oscillators ([Cohen 1987](#)). Thus the intersegmental coupling signals in the lamprey appear to be asymmetric to promote the lead of more rostral oscillators, even if

the intrinsic frequency of the lead oscillator is slower. In the present study, ascending input from the spinal cord also appears to be present, acting to speed the SO oscillator. Therefore, it appears that both ascending and descending coupling are present between the S-O and the spinal cord. The coupling between S-O and spinal cord results in a speeding of the S-O (present study), a slowing of the spinal cord ([Vinay and Grillner 1993](#)), and a phase lead of the S-O region (present study) presumably attributable to an asymmetry in the strengths of the ascending and descending coupling.

We confirmed with electrophysiology and anterograde axon labeling that the S-O motoneurons innervate several muscles of the head region of the lamprey, subocularis, cornealis, probranchialis, epibranchialis 1, and hypobranchialis 1. This innervation had been demonstrated previously using anatomical techniques ([Hardisty and Rovainen 1982](#); [Tretjakoff 1927](#)). We further showed here that the S-O motoneurons innervate, not only epibranchialis 1 and hypobranchialis 1, but also several of their more caudal segmental representatives. Both electrophysiological recordings and anterograde labeling of S-O motoneuron axons showed innervation of the epibranchialis 1–5 and hypobranchialis 1–4 muscles. The strength of the innervation declines with distance, suggesting that there is a dual innervation of the more caudal muscles by the spinal cord. These two sets of muscles will likely be important in steering movements either in lateral turning by greater activation of both muscles on one side, or in upward or downward steering by bilateral activation of either epibranchialis or hypobranchialis muscles, respectively ([Zelenin et al. 2003](#)). The subocularis muscle likely functions similar to the hypobranchialis muscles, producing bending to the ipsilateral side and downwards. Less clear are the functions of the cornealis and probranchialis muscles during swimming. The cornealis muscle inserts on the cornea, and contraction of the cornealis muscle produces a flattening of the eye, which could serve as a protective withdrawal mechanism or could be involved in changing the refraction of the eye for focusing ([Sivakand and Woo 1975](#)). Why either of these functions would be invoked rhythmically during swimming is not known.

We also observed that the S-O nerves and some of the S-O motoneurons could exhibit fictive respiratory activity as well as fictive swimming activity. Fast fictive respiration is characterized by synchronous left-right, short-duration bursts in the VII, IX, and X cranial nerves with a cycle period of ~1 s ([Rovainen 1974](#); [Missaghi et al. 2016](#)). Motoneurons of these nerves innervate muscles that constrict the branchial baskets to expel water from the gill sacs ([Rovainen 1974](#)). Respiratory activity in the S-O nerves and in S-O motoneurons had not previously been reported in lamprey, nor had it been shown that individual motoneurons receive both respiratory and locomotor inputs. However, a link between respiratory and locomotor rhythms in lamprey has been reported by [Gariépy et al. \(2010, 2012\)](#), who found that stimulation of the MLR can increase the frequency of bursting in both rhythms. Although our results indicate that individual S-O motoneurons can receive inputs from both the respiratory and the locomotor CPGs, we do not know which muscles are innervated by these motoneurons or how these muscles contribute to respiratory movements.

In other vertebrates, there is also evidence for a locomotor rhythm generator within the caudal hindbrain. In larval zebrafish, rhythmic firing of reticulospinal neurons that is likely a swimming rhythm persists after a lesion separating the spinal cord from the hindbrain ([Chong and Drapeau 2007](#)). Similarly in larval *Xenopus*, reticulospinal neurons exhibit pacemaker-like activity that underlies swimming rhythm generation and occurs in the absence of spinal swimming activity ([Li et al. 2009, 2010](#)). In lamprey, reticulospinal neurons also exhibit rhythmic activity during fictive swimming ([Einum and Buchanan 2005](#); [Buchanan 2011](#)). However, in lamprey, much of this activity is abolished when ascending spinal inputs are blocked ([Antri et al. 2009](#)). It remains to be determined whether interneurons in the S-O region provide any swimming signals to reticulospinal neurons in lamprey.

In embryonic development, the neural tube of vertebrates displays segmental divisions referred to as rhombomeres, which develop into the hindbrain under the expression of *Hox* genes. The most caudal rhombomeres, 7 and 8, are associated with the hindbrain-spinal cord transition zone. It has been shown that the

regulatory network underlying *Hox* gene expression in lamprey is similar to the regulatory networks in all vertebrates ([Parker et al. 2014](#)). It has also been proposed that these caudal rhombomeres may have been the site of origin of several rhythmic circuits, including respiration, vocalization, electromotor, the inferior olive, and locomotion ([Bass and Baker 1997](#); [Bass et al. 2008](#); [Ma et al. 2010](#)).

In summary, this study showed in the adult lamprey that the S-O region at the hindbrain-spinal cord transition zone contains a swimming central pattern generator that provides rhythmic bursting to all of the head muscles innervated by the S-O somatic motoneurons. It was also shown that the rhythmic bursting of the S-O region is significantly phase advanced compared with the first ventral root of the spinal cord. Thus the swimming bursts in the S-O region lead each swimming burst in the spinal chain of swimming rhythm generators that descend the length of the spinal cord. This region of the nervous system may play a role in the regulation of speed, turning, and head orientation during swimming the lamprey.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

J.T.B. conceived and designed research; J.T.B. performed experiments; J.T.B. analyzed data; J.T.B. interpreted results of experiments; J.T.B. prepared figures; J.T.B. drafted manuscript; J.T.B. edited and revised manuscript; J.T.B. approved final version of manuscript.

AUTHOR NOTES

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