Cholinergic modulation of the locomotor network
in the lamprey spinal cord

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Abstract

Acetylcholine was found here to be a strong modulator of swimming activity in the isolated spinal cord preparation of the adult lamprey (*Ichthyomyzon unicuspis*). During fictive swimming induced with either D-glutamate or N-methyl-D-aspartate, addition of acetylcholine (200 µM) significantly reduced the cycle period of ventral root bursts to 54%, intersegmental phase lag to 32%, and ventral root burst proportion to 80% of control levels. Effects of acetylcholine were apparent at concentrations as low as 1 µM. Both nicotinic and muscarinic receptors are involved, as application of either nicotinic or muscarinic agonists alone significantly reduced cycle period. There is sufficient endogenous acetylcholine in the spinal cord to modulate ongoing fictive swimming as shown by application of the cholinesterase inhibitor eserine (physostigmine). Eserine (20 µM) significantly reduced cycle period to 78% and phase lag to 58% of control levels, and these effects were reversed with the addition of cholinergic blockers. Addition of only a nicotinic or muscarinic antagonist, mecamylamine (10 µM) or scopolamine (20 µM), respectively, to the spinal cord during fictive swimming produced significant increases in cycle period and phase lag, suggesting that both types of cholinergic receptors participate in endogenous cholinergic modulation. It is concluded that acetylcholine is an endogenous modulator of the locomotor network in the lamprey spinal cord and that acetylcholine may take part in the regulation of cycle period, intersegmental coupling, and ventral root burst duration.
Introduction

Neuronal networks are under the influence of both extrinsic and intrinsic neuromodulators which provide a mechanism for tuning and adapting networks to changing conditions (Marder and Thirumalai 2002). While invertebrate nervous systems have provided the best preparations for understanding neuromodulation at the cellular, synaptic, and network levels, the nervous system of the lamprey, a lower vertebrate fish, is an advantageous model for vertebrates. The isolated lamprey spinal cord expresses the neuronal correlate of swimming (fictive swimming), and many of the cellular, synaptic, and network mechanisms of the locomotor network have been described (Buchanan 2001; Grillner 2003). A variety of endogenous neuromodulators of locomotion have been investigated in the lamprey spinal cord including serotonin (Harris-Warrick and Cohen 1985; Wallén et al. 1989), dopamine (McPherson and Kemnitz 1994; Svensson et al. 2003), substance P (Parker et al. 1998; Parker and Grillner 1998), GABA (Tegner et al. 1993), and neurotensin (Barthe and Grillner 1995). We report here the effects of acetylcholine on fictive swimming.

Acetylcholine has been implicated in locomotor network function in many vertebrate preparations. In the embryonic *Xenopus* spinal cord, acetylcholine can change the frequency of occurrence of swim episodes via muscarinic receptors (Panchin et al. 1991). In addition, collateral motoneuron axons from *Xenopus* motoneurons provide nicotinic excitatory postsynaptic potentials (EPSPs) upon other motoneurons and locomotor interneurons in a positive
feedback mechanism that may help sustain the swim episode. However, this feedback is not necessary for rhythm generation as the rhythm continues in the presence of a nicotinic antagonist (Perrins and Roberts 1994, 1995a-c; Zhao and Roberts 1998). In the isolated spinal cord of the mudpuppy, application of acetylcholine, acting via muscarinic receptors, disrupts locomotor activity (Fok and Stein 2002). In turtles (Guertin and Hounsgaard 1999) and in the neonatal rat spinal cord (Cowley and Schmidt 1994; Kiehn et al. 1996), acetylcholine can induce or facilitate the induction of rhythmic activity in motoneurons. In the decerebrate cat, cholinergic commissural spinal neurons located in the intermediate gray of the lumbar spinal cord are rhythmically active during fictive locomotion (Huang et al. 2000).

In the lamprey, cholinergic neurons have been mapped in the brain using choline acetyltransferase (ChAT) immunohistochemistry (Pombal et al. 2001; LeRay et al. 2003). In addition to somatic motoneurons, ChAT immunoreactivity reveals numerous groups of interneurons. One such group is located near the mesencephalic locomotor region (MLR), and it has been proposed that these cholinergic neurons provide a major output of the MLR to the reticular formation (LeRay et al. 2003). In support of this proposal, stimulation of the MLR produces excitatory nicotinic synaptic potentials upon reticulospinal neurons, and activation of reticulospinal neurons with ACh can induce locomotor activity (LeRay et al. 2003). Less is known about cholinergic actions in the lamprey spinal cord. Spinal motoneurons stain for acetylcholinesterase (Wachtler 1974) and ChAT
immunohistochemistry (Pombal et al. 2001), and there are some spinal neurons that stained positive for ChAT yet have a morphology and localization that does not match that of the motoneurons (Wallén et al. 1985, Pombal et al. 2001). As in *Xenopus*, locomotor activity in the isolated lamprey spinal cord is not blocked by application of cholinergic antagonists (Grillner et al. 1981).

In the present study we have examined the effects of acetylcholine upon locomotor activity induced in the isolated lamprey spinal cord by D-glutamate or N-methyl-D-aspartate. We have found powerful modulatory effects of acetylcholine on cycle period and intersegmental phase lag acting via both nicotinic and muscarinic receptors. In addition, there is sufficient endogenous acetylcholine within the isolated spinal cord to provide an ongoing modulation of the lamprey locomotor network.

**Materials and Methods**

Seventy-eight adult silver lampreys (*Ichthyomyzon unicuspis*) between 18 and 38 cm in length were used for these experiments. Prior to use, the animals were kept in aerated and filtered fresh water aquaria at 5 °C. The experiments were conducted in conformity to the American Physiological Society’s *Guiding Principles in the Care and Use of Animals* and were approved by the Marquette University Institutional Animal Care and Use Committee. The animals were anesthetized by immersion in a solution of tricaine methane sulphonate (~250 mg/l) until responses to tail pinch were lost. The spinal cord / notochord preparation
was dissected as previously described (Rovainen 1974) using the midbody region from the caudal gills to the beginning of the first dorsal fin. Care was taken to remove muscle fibers from the notochord by initially stripping or cutting the bulk of the muscle from the notochord and then by scraping the surface of the notochord with the tips of microscissors. Two types of preparations were used for the experiments (Fig. 1): 1) short spinal cord preparations (7 – 20 segments) with fictive locomotion induced by N-methyl-D-aspartate (NMDA) and 2) long spinal cord preparations (30 – 40 segments) with fictive locomotion induced by D-glutamate. Ringers solution contained (in mM): 91 NaCl, 2.6 CaCl₂, 2.1 KCl, 1.8 MgCl₂, 4.0 glucose, 20 NaHCO₃, 8 HEPES (free acid), 2 HEPES (sodium salt) (pH = 7.4). Ringers solution in the experimental chamber was kept cooled (7 – 10 °C). When not in use, the tissue was stored in Ringers at 4 °C and was used within three days of dissection. All chemicals were obtained from Sigma-Aldrich.

Short Spinal Cord Preparations

In the short spinal cord / notochord preparations (7 – 20 segments), the notochord was cut down its ventral midline to optimize visibility, spread laterally, and pinned to the Sylgard-lined (Dow Corning) floor of the experimental chamber. One or two ventral root recordings were made from the middle region of the spinal cord piece using glass suction electrodes (Fig. 1A₁). To induce fictive swimming, the bath was perfused with Ringers solution containing NMDA (0.15 – 0.2 mM). After the swim rhythm had stabilized (~1 hr after start of
perfusion), the perfusion of the bath was stopped for 5 to 10 minutes, and the experiments were then performed using the non-perfused, static bath. After obtaining control recordings, acetylcholine was applied to the bath by adding a small volume (0.001X the bath volume) of Ringers containing ACh at a concentration 1000X the final desired concentration (1 – 1000 µM). The bath was then rapidly stirred by drawing the fluid of the experimental chamber into a syringe and then expelling the volume back into the chamber. This stirring was accomplished in 5 – 10 seconds, thus providing a rapid concentration step compared to the time required for adding ACh via the perfusion system. The slower perfusion application produced less robust and consistent effects due to desensitization. Stirring the bath without adding ACh produced no significant change in the swim cycle period (Fig. 2). In these preparations, nicotine was applied in the same manner as ACh because it showed desensitization similar to ACh. Mecamylamine, dihydro-β-erythroidine hydrobromide, scopolamine, and eserine (physostigmine) were applied by bath perfusion (1-4 ml/min). Oxotremorine was applied either to the static bath or by perfusion.

Long Spinal Cord Preparations

Long spinal cord preparations (30 – 40 segments) were used to make accurate measurements of phase lag and to assess endogenous ACh modulation of the swim rhythm. In these preparations, continuous perfusion (2-3 ml/min) of D-glutamate (0.5 mM) was used to induce fictive locomotion because D-glutamate gave more regular rhythms in long spinal cord preparations than
NMDA. The long spinal cord / notochord preparation was pinned to the Sylgard chamber floor. The notochord was not split in these preparations as a matter of convenience due to the long lengths used. Ventral root recordings (2 – 4 roots) were made from ventral roots on one side of the spinal cord (Fig. 1B1). As described above for the short preparations, a small volume of ACh at a high concentration was added to the bath followed by rapid stirring to achieve the final desired concentration. In the long preparations, however, bath perfusion was continuous and upon addition of acetylcholine the perfusion was switched immediately to D-glutamate Ringers containing the same final ACh concentration. In contrast to the NMDA experiments, stirring of the bath in the absence of ACh when D-glutamate was used as the excitatory amino acid agonist did produce a significant decrease in swim cycle period, though no effect on phase lag (Fig. 9C,D). However, this decrease was smaller and of shorter duration than that produced by the addition of ACh. Other cholinergic drugs in the long preparation were applied by perfusion (eserine, mecamylamine, scopolamine, and atropine).

Recording Techniques

Ventral root recordings were made with glass suction electrodes filled with Ringers solution. The tip of the electrode was placed on a ventral root near its exit point from the spinal cord. The signals were amplified (10,000X) and filtered (10 Hz high pass; 1 kHz low pass) with an AC differential amplifier (A-M Systems). The signals were digitized (2 kHz) using a micro1401 computer
interface and Spike2 data acquisition software (Cambridge Electronic Design, CED) and stored on disk for later analysis.

Data Analysis

Data analysis was done using Spike2 software (CED). Two types of data analysis were used: 1) cycle-by-cycle analysis for the short spinal cord preparations (Fig. 1A3) and 2) cross-correlational analysis for the long spinal cord preparations (Fig. 1B3). For cycle-by-cycle analysis, the ventral root bursts were digitally rectified, and ventral root spikes detected by thresholding (Fig. 1A3,4). Thresholds were set above the noise level of the ventral root recording, and were kept constant throughout each experiment. Event times of the spikes were then used to mark the onsets and offsets of ventral root bursts using a custom script in Spike2 software. The marked onsets and offsets of each burst were then checked manually for accuracy. Cycle period was measured as the time interval from the onset of one burst to the onset of the subsequent burst (Fig. 1A3). Burst duration was the time from burst onset to offset and was normalized to its cycle period to obtain burst proportion. Burst intensity was defined as the product of mean instantaneous spike frequency within the burst and the mean amplitude of the peaks of the rectified spikes (Fig. 1A4). The peaks were detected as the highest data point between the time when voltage ascended through a threshold level until it descended back through the threshold level. Beginning at the maximal effect, at least 30 successive cycles of swimming were used to provide
means and standard deviations of cycle period, burst proportion, and burst intensity under each condition.

The long spinal cord preparations were used to measure the effect of acetylcholine on cycle period and phase lag. For these measurements, Spike2 (CED) software was used to create cross-correlograms of spike event times between two ventral roots separated by 15 to 25 segments (Fig. 1B_{2,3}). Windows of data 10 to 20 seconds in duration were analyzed to produce each cross-correlogram. The cross-correlograms provided data on both cycle period and the rostral-caudal delay between bursts in two ipsilateral ventral roots (Fig. 1B_{3}). For phase lag, the delay between bursts was normalized to the cycle period and to one segment (by dividing delay by the number of segments between the ventral root recordings), and the result expressed as a percentage.

**Statistical Analysis**

SigmaStat software (SPSS) was used for statistical analysis. Mean values of cycle period before, during, and after ACh application were analyzed for significance using a students’ paired t-test. \( P \) values of 0.05 and under were considered significant, and data were checked for normality \( (P > 0.01) \) and equal variance. To analyze the effects of increasing ACh concentrations on cycle period (Fig. 3D), a one-way analysis of variance (ANOVA) using Tukey’s procedure was used with a \( P \) value of 0.05 to determine significance. Mean percent change in cycle period and phase lag calculated in Fig. 11B_{3,4} and 11A_{3}
also used ANOVA with Tukey’s procedure. Since the data of percent change in phase lag for Fig. 11A4 did not pass the normality test for Tukey’s procedure, a Student-Newman-Keuls method ANOVA was used to determine significance.

**Results**

*Short Spinal Cord Preparations*

Our initial experiments applied ACh via bath perfusion (data not shown). While the results of these first experiments were consistent with those described here, the effects were weaker and more variable due to strong desensitization. Therefore, a more rapid application technique for ACh was used as described in Materials and Methods. Control experiments of stirring the bath during NMDA-induced fictive swimming in the absence of added ACh were done in short spinal cord preparations. An example experiment (Fig. 2A) showed little or no change in mean cycle period after the stir. Combined results from 10 preparations (Fig. 2B) showed no significant change in mean cycle period ($P = 0.56$).

When the bath was stirred after the addition of ACh, there was a significant reduction in the cycle period. An example experiment is shown in Fig. 3A-C. As can be seen in the cycle-by-cycle plot of cycle period in Fig. 3A, addition of 1 µM ACh to the bath induced a small but clear reduction in the cycle period (to ~85% of control). The peak reduction in cycle period occurred about 20 sec following the stir, and there was a slow return toward control levels over several minutes which may have been due to ACh breakdown by cholinesterases.
as well as receptor desensitization. Desensitization could also be demonstrated by a diminished response to a second application of ACh within the next two hours, although the swim parameters returned to normal within the next hour. Therefore, to determine the dose responsiveness of the effects of ACh on cycle period, each ACh concentration was applied to a previously unexposed spinal cord preparation in each experiment. As shown in Fig. 3D, maximal effects on cycle period occurred with 200 \( \mu M \) (cycle period reduced to 54% of control).

To examine the effects of ACh on other swim parameters, 200 \( \mu M \) ACh was applied, and an example experiment is shown in Fig. 4. The cycle-by-cycle plots of swim parameters are shown in Fig. 4A, and samples of rectified ventral root recordings are shown in Fig. 4B-D. With the addition of 200 \( \mu M \) ACh, cycle period decreased to half of its control level, from 0.6 sec to 0.3 sec. In the continued presence of ACh, the cycle period returned toward the control level, ACh’s effect diminishing to half-maximal in about 60 seconds. The swim rhythm also began to undergo a slow modulation with a period of about 10 to 20 seconds, marked by asterisks in Fig. 4A. A sample of raw data undergoing slow modulation is shown in Fig. 4D. Induction of slow modulation of the rhythm by ACh was observed in about half of the preparations. With the addition of ACh, burst proportion and burst intensity showed increased variation (Fig. 4A), and the mean burst proportion decreased slightly while the mean burst intensity showed little or no change. A summary of 7 experiments in which 200 \( \mu M \) ACh was applied is shown in Fig. 5. Acetylcholine significantly reduced cycle period to an
average of 54% of control \( (P < 0.001) \) (Fig. 5A1) and burst proportion to an average of 80% of control \( (P = 0.044) \) (Fig. 5B1), while burst intensity did not show a significant change (Fig. 5C1). The effects of ACh on cycle period and burst proportion generally returned to control levels after washing for one hour. To quantify the increased variability of the swim parameters, the coefficient of variation was measured for 1 min following peak ACh effect, and the results are summarized in Fig. 5A2, B2, and C2. There were significant increases in the coefficient of variation for all three parameters.

To examine the cholinergic pharmacology of the ACh effects (Fig. 6 and 7), we restricted our analysis to cycle period because this parameter was most strongly altered by ACh. The experiments indicate that both nicotinic and muscarinic cholinergic receptors contribute to the ACh effects on cycle period. Application of nicotine \( (200 \mu M) \) to the static bath followed by stirring produced a significant \( (P = 0.03) \) decrease in cycle period to 72% of control (Fig. 6A). This effect showed a decay similar to that of ACh, returning towards control levels soon after it was stirred into the bath. With prior application of a nicotinic antagonist, either 20 \( \mu M \) mecamylamine \( (n = 4) \) or 20 \( \mu M \) dihydro-\( \beta \)-erythroidine hydrobromide \( (n = 3) \), this effect was attenuated (cycle period only reduced to 92% rather than 72% of control with nicotine addition) but the effect was still statistically significant compared to control \( (P = 0.015) \) (Fig. 6B). Application of these nicotinic antagonists prior to 200 \( \mu M \) ACh application also attenuated, but did not eliminate, the reduction of cycle period produced by ACh (cycle period
only reduced to 79% rather than 54% of control with ACh application) (Fig. 6C), suggesting that muscarinic receptors are also involved.

To test the muscarinic receptor contribution to ACh’s effects, a muscarinic agonist, oxotremorine (20 µM), was added to the static bath followed by stirring. This resulted in a small (to 89% of control) but non-significant reduction in cycle period (Fig. 7A). Because effects induced by activation of muscarinic receptors might have longer latency than nicotinic actions and drugs were applied in the static bath for only 5 to 10 minutes, bath perfusion of oxotremorine was performed over a longer time period. With this method of application, oxotremorine again produced a small (to 92% of control) but, in this case, statistically significant reduction in cycle period ($P = 0.017$) after 20 minutes of perfusion (Fig. 7B). This reduction was reversed by addition of a muscarinic antagonist (10 µM scopolamine or 2 µM atropine) (Fig. 7B). The reduction of cycle period by ACh was also attenuated by prior treatment with a muscarinic antagonist (cycle period reduced to 71% of control in the presence of the antagonist compared to 54% of control with just the addition of ACh) (Fig. 7C).

While neither nicotinic nor muscarinic antagonists alone could completely block the effect of ACh (Fig. 6C and Fig. 7C), when added together they eliminated the cholinergic reduction of cycle period (Fig. 7D). To look for evidence of endogenous ACh, the cholinesterase inhibitor eserine (20-100 µM) was also stirred into the static bath of short preparations, and a slight (97% of control) but non-significant ($P = 0.14$) decrease in cycle period was observed ($n = 9$, data not
shown). As with oxotremorine, the duration of the drug application in the static bath was likely insufficient to produce the significant changes which were seen in longer preparations with perfusion of the drugs over a longer period.

**Long Spinal Cord Preparations**

In order to accurately determine the effect of ACh on intersegmental phase lag, longer spinal cord preparations were used (30 to 40 segments). Since NMDA did not always produce regular bursting in these preparations, D-glutamate (0.5 mM) was used to induce fictive swimming. As in the short preparations using NMDA to induce fictive swimming, rapid application of ACh in the longer D-glutamate preparations significantly decreased the cycle period. In addition, ACh produced a significant decrease in the intersegmental phase lag. These effects can be seen in an individual experiment in Fig. 8. Figures 8A and B show the decrease in cycle period and phase lag, respectively, produced by the addition of 200 µM ACh to the bath. These decreases can also be seen in the sample ventral root records of Fig. 8C and D. At the extreme, the phase lag in the experiment shown here became negative, indicating a reversal of the normal rostral-to-caudal propagation of the bursts. Because glutamate-induced fictive swimming was more sensitive to pauses in bath perfusion than NMDA-induced fictive swimming, perfusion of ACh-Ringers was initiated immediately after stirring the bath in the long preparations. Little desensitization, if any, is seen in the time course of the experiment shown in Fig. 8. However, in other experiments using the long preparation a decay in the response was observed so
that after 5 minutes of ACh exposure only half of the maximal effect remained. The longer time course of desensitization seen in long preparations could be due to the continued perfusion of ACh during the experiments, in contrast to the static bath experiments in which the applied ACh could be broken down locally by esterases without being replenished. Figure 9A and B summarize the results of 12 experiments with the application of ACh to longer spinal cord preparations. Addition of ACh (200 µM) reduced the average cycle period to 45% of control ($P = 0.002$) and reduced average phase lag to 32% of control ($P = 0.002$). Stirring the bath without the addition of ACh also significantly decreased the cycle period of fictive swimming induced with D-glutamate ($P < 0.001$; $n = 14$) but to a significantly lesser degree than with addition of ACh, to 88% of control without ACh vs. to 45% of control with ACh ($P < 0.001$) (Fig. 9C). Phase lag, however, was not significantly changed from control by stirring without addition of ACh ($P = 0.46$) (Fig. 9D).

**Endogenous ACh Modulation**

Ventral root bursting during D-glutamate fictive swimming in the longer spinal cord preparations could be quite regular and thus offered the possibility to reliably measure small changes in the cycle period and phase lag. Therefore, this preparation was used to determine whether there is a sufficient level of acetylcholine within the spinal cord to produce ongoing modulation of the locomotor rhythm. If ACh is being released endogenously, then application of an acetylcholinesterase inhibitor would be expected to have similar effects as the
addition of exogenous ACh. In an example experiment, when eserine (20 µM) was added to the bath perfusion, the cycle period (Fig. 10A₁) and the phase lag (Fig. 10A₂) were both clearly decreased. For all experiments (n = 7), eserine significantly decreased cycle period to 73% of control ($P = 0.007$) (Fig. 10A₃) and decreased phase lag to 58% of control ($P = 0.009$) (Fig. 10A₄). While eserine was still present, the nicotinic and muscarinic antagonists, mecamylamine (10 µM) and scopolamine (20 µM), were added together to the bath perfusion. The antagonists generally not only reversed the effects of eserine but increased both cycle period and phase lag beyond the control levels before adding eserine (Fig. 10A₁₋₄). These observations suggest that in the control condition, the swim rhythm is under the influence of endogenous ACh. To test this possibility, the combined antagonists were bath perfused without the addition of eserine. As shown in an individual experiment, the combined blockers increased both the cycle period (Fig. 10B₁) and the phase lag (Fig. 10B₂). In 5 experiments, perfusion of the combined blockers significantly increased cycle period to 119% of control ($P = 0.034$) and phase lag to 143% of control ($P < 0.001$) (Fig. 10B₃₋₄).

To determine the relative contribution of nicotinic versus muscarinic receptors to the endogenous ACh effects, the cholinergic antagonists were added separately. In these experiments, eserine (20 µM) was applied first followed by increasing concentrations of one of the antagonists. An example experiment is shown for mecamylamine in Fig. 11A₁,₂ in which step changes in cycle period and phase lag are discernible with the step increases in
mecamylamine concentration. The summary of 6 experiments (Fig. 11A₃,₄) shows that 10 µM mecamylamine significantly increased both cycle period and phase lag to 170% and 130% of the eserine levels, respectively. In an example experiment for scopolamine, the effects of step increases in scopolamine concentration can be seen in the cycle period and phase lag (Fig. 11B₁,₂). The summary of 6 experiments (Fig. 11B₃,₄) shows that at 25 µM scopolamine, both cycle period and phase lag increased to 144% of the eserine levels. Atropine (2 and 10 µM) produced similar effects as scopolamine (n = 2) (data not shown).

**Discussion**

This study has shown for the first time that acetylcholine is a powerful modulator of the locomotor rhythm in the isolated lamprey spinal cord. Bath application of acetylcholine produced significant reductions of cycle period, burst proportion, and phase lag. These effects were similar in short spinal cord preparations (down to 7 segments) vs. long spinal cord preparations (up to 40 segments) and whether using NMDA or D-glutamate as the excitatory agonist to elicit fictive locomotion. The effects were mediated by both nicotinic and muscarinic receptors, though nicotinic receptors appear to be more effective. Application of cholinergic blockers without the addition of acetylcholine also produced significant changes in cycle period and phase lag suggesting that there is sufficient acetylcholine within the spinal cord to provide an ongoing modulation of the locomotor network.
Possible sources of endogenous acetylcholine

The endogenous acetylcholine producing the effects observed here could potentially originate from either motoneurons or from cholinergic interneurons. Acetylcholinesterase histochemistry (Wachtler 1974) and ChAT immunohistochemistry (Pombal et al. 2001) revealed brain and spinal neurons with morphology consistent with somatic motoneurons. Furthermore, muscle activity is blocked by the nicotinic antagonist curare (Teräväinen 1971), confirming that lamprey motoneurons are cholinergic. If endogenous acetylcholine originates from motoneurons, two possible sites of origin would be the neuromuscular junction in the periphery or synaptic outputs of motoneuron axons within the spinal cord. The release of acetylcholine from neuromuscular junctions seems unlikely as the source of endogenous acetylcholine because muscle fibers were carefully removed from the notochord in the spinal cord / notochord preparation (see Materials and Methods), and the preparation was continuously perfused with Ringers solution in the cholinergic blocker experiments (2 – 3 ml/min). As for output synapses of lamprey motoneuron axons within the spinal cord, collateral axon branches have been observed in lamprey (Wallén et al. 1985), and antidromic stimulation of ventral roots produces synaptic potentials within spinal neurons that are blocked by cholinergic antagonists (Buchanan 1999). Although antidromic stimulation of up to 3 ventral roots did not change the swimming rhythm (Wallén and Lansner 1984), perhaps firing of the motoneurons from only 3 ventral roots was not of sufficient strength
to modify the rhythm of the entire spinal cord preparation (11-29 segments) during fictive swimming. A second possible source of endogenous acetylcholine is cholinergic interneurons. Labeling of spinal neurons with ChAT immunohistochemistry revealed cells that by their size and location do not appear to be motoneurons (Pombal et al. 2001), although further investigation into the possible existence of non-motoneuron cholinergic cells needs to be done. It is also possible that axons from cholinergic brain neurons could contribute to spinal acetylcholine as descending cholinergic fibers have been followed into the rostral spinal cord (Pombal et al. 2001). These populations of cholinergic neurons, alone or in combination, could be the source of endogenous ACh.

Possible mechanisms of cholinergic modulation of fictive locomotion

Acetylcholine is known to have a variety of cellular and synaptic effects within the central nervous system. In lamprey, it is known that acetylcholine has an excitatory effect on neurons via nicotinic receptors (LeRay et al. 2003), and may also have presynaptic inhibitory actions on EPSPs via muscarinic receptors (Quinlan and Buchanan 2003). In other preparations, both presynaptic facilitation of glutamatergic synapses by presynaptic nicotinic receptors (Girod et al. 2000) and presynaptic inhibition via muscarinic receptors (Fernandez de Sevilla and Buno 2003) have been shown. In addition, various cholinergic effects on firing properties have been described. In the turtle spinal cord, for example, acetylcholine can regulate firing frequency via the M-current (Alaburda et al.)
and can up-regulate L-type calcium currents to promote plateau potentials (Perrier et al. 2000). Thus, acetylcholine could be altering locomotor networks via several possible cellular and synaptic mechanisms.

In lamprey, the locomotor network has been proposed to consist of a pair of half centers on either side of the spinal cord coupled by reciprocal inhibition (Buchanan 2001). An increase of the speed of the swim rhythm could be accomplished by any mechanism that reduces the duration of the activity of the half-center generators on each side. In the proposed model of the lamprey locomotor network (Buchanan and Grillner 1987), rhythmicity is generated in large part by reciprocal inhibition of commissural interneurons (CC INs) so that mechanisms which decrease the excitability of CC INs or weaken their synaptic outputs would lead to a speeding of the network (Buchanan 1992). An additional feature of the proposed model is an off-switch mechanism by which the duration of CC IN activity on one side is limited by inhibition from a class of ipsilaterally-projecting inhibitory interneurons, the lateral interneurons. Intracellular recordings of CC INs during fictive swimming demonstrated that they have an earlier peak depolarization compared to nearby motoneurons (Buchanan and Cohen 1982) and an earlier beginning of their inhibitory phase (Kahn 1982). These two features are consistent with inhibition of CC INs from lateral interneurons. In addition to lateral interneurons there are small glycinergic interneurons that have local inhibitory input to motoneurons (Buchanan and Grillner 1988). These local inhibitory interneurons could be functioning as an off-
switch to the half center on one side of the spinal cord. If cholinergic interneurons or cholinergic feedback from motoneurons provided part of the excitation of such off-switch cells, this could account for the shortening of cycle period and for the reduced burst proportion that is observed with the addition of acetylcholine.

Less is known about the cellular mechanisms of intersegmental coupling underlying the delay of ventral root bursting in more caudal segments during forward swimming. Like burst duration, this delay is normally a constant fraction of the cycle period over a wide range of cycle periods during forward swimming (Wallén and Williams 1984) and is presumably regulated to maintain efficient swimming form. Acetylcholine has been shown here to modulate phase lag, and increasing the presence of ACh, either through addition of exogenous ACh or by blocking its normal breakdown with eserine, causes intersegmental phase lag to decrease from its normal value. Alteration of phase lag by acetylcholine could be accomplished by changes in the excitability of the locomotor neurons or the coupling neurons or by changes in the synaptic strengths of the coupling signals among the rhythm generators. The powerful modulatory effect of acetylcholine on phase lag and the ongoing modulation of phase lag by endogenously-released acetylcholine suggest that acetylcholine may be part of the mechanism responsible for controlling phase lag, maintaining it within certain limits and adjusting it to varying swimming conditions found in a lamprey’s natural environment, such as swimming upstream against a current vs. swimming
downstream with a current, as well as changing behavioral needs such as forward swimming vs. backward swimming.

Previous studies of acetylcholine actions in lamprey

As confirmed in the present study, activation of cholinergic receptors is not necessary for the expression of fictive swimming. In the isolated spinal cord preparation, swimming activity continues in the presence of combined nicotinic and muscarinic blockers (Grillner et al. 1981) and in many past experiments on lamprey the nicotinic antagonist curare was added to Ringers to block muscle contractions during in vitro experiments (Teräväinen 1971). Based on the results of the present study, one would expect that addition of curare to block muscle contractions would increase cycle period and phase lag. In addition, acetylcholine application alone is not capable of inducing locomotor activity (unpublished observations). At the brainstem level, however, acetylcholine may be playing an important role in the initiation of locomotor activity. Using ChAT immunohistochemistry, cholinergic neurons have been found in the vicinity of the mesencephalic locomotor region (MLR) of lamprey (LeRay et al. 2003). In the presence of nicotinic antagonist, locomotor activity induced by MLR stimulation is greatly depressed as are the monosynaptic EPSPs produced in reticulospinal neurons by this stimulation. Local application of acetylcholine to reticulospinal cells can induce swimming in a semi-intact preparation or fictive swimming in the isolated brain / spinal cord preparation, and it can speed ongoing swimming (LeRay et al. 2003).
Fictive swimming has been elicited in lamprey with a variety of glutamatergic agonists and in spinal cord preparations that range widely in length. In this study NMDA and D-glutamate were used in short and long preparations, respectively, because of previous observations that these combinations of agonists and spinal cord length produced the most regular fictive swimming activity (T.L. Williams, personal communication). The basic finding of a decreased cycle period of fictive swimming in both preparations and agonists indicates that the results are not dependent on these experimental differences. There were, however, some differences between the two preparations. With fictive swimming induced by NMDA, the swimming rhythm was not sensitive to stirring the bath, while with D-glutamate-induced fictive swimming, there was some sensitivity to stirring (Fig. 2B vs. Fig. 9C). The mechanism of this difference is not known but may reflect differences in agonist uptake and resulting changes in local agonist concentration. Another difference in the two preparations was the slower decay of the ACh effect in the long preparations. This may in part reflect the action of endogenous acetylcholine esterase. The continuous perfusion of ACh in the long preparation may serve to replenish ACh but the static bath short preparation may experience local ACh concentration decreases.
Locomotor network actions of acetylcholine in other vertebrates

Acetylcholine is involved in locomotion in other vertebrates although no consistent pattern of involvement has emerged. In the embryonic *Xenopus* spinal cord, acetylcholine can change the frequency of occurrence of swim episodes via a muscarinic receptor (Panchin et al. 1991). In addition, *Xenopus* motoneuron axons provide excitatory nicotinic EPSPs upon other motoneurons and locomotor interneurons in a positive feedback mechanism that may help sustain the duration of an episode of swimming. However, this feedback is not necessary for rhythm generation as the rhythm continues to be expressed in the presence of a nicotinic antagonist (Perrins and Roberts 1994,1995a-c; Zhao and Roberts 1998). In the isolated spinal cord of the mudpuppy, walking activity induced with NMDA is disrupted by application of acetylcholine while not changing the overall level of activity in flexor and extensor motoneurons. This action of acetylcholine was blocked by atropine, suggesting that muscarinic receptors mediate the disruption, while atropine alone had no effect (Fok and Stein 2002). In turtles, rhythmic activity in motoneurons can be induced by muscarine, and this action appears to be dependent upon enhancement of L-type calcium currents (Guertin and Hounsgaard 1999; Perrier et al. 2000). In an isolated preparation of the embryonic mouse spinal cord, cholinergic positive feedback from motoneurons is essential for the generation of spontaneous rhythmic activity (Hanson and Landmesser 2003). In isolated neonatal rat spinal cord, acetylcholine application can induce or facilitate the induction of rhythmic activity in motoneurons (Cowley and Schmidt 1994; Kiehn et al. 1996). In
decerebrate cat preparations, combined c-fos labeling and ChAT immunohistochemistry revealed cholinergic neurons of the intermediate gray of the lumbar spinal cord that are active during fictive locomotion. Electrophysiology demonstrated that these cells are active in phase with ipsilateral extension and that they project axons to the contralateral side of the spinal cord (Huang et al. 2000).

**Conclusions**

The present study demonstrated that acetylcholine provides an ongoing modulation of swimming activity in the lamprey spinal cord and that these actions are mediated by both nicotinic and muscarinic acetylcholine receptors. Acetylcholine speeds the rhythmic output of the network and may thus contribute to the regulation of rhythm generation. Acetylcholine has other locomotor network effects as well. Normally, burst duration and burst delay between segments are maintained as constant fractions of the cycle period, but acetylcholine alters these parameters, suggesting that acetylcholine plays a role in the regulation or in the modification of burst proportion and phase lag as an adaptive response to changes in swimming conditions.
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References


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Figure Legends

Fig. 1. Experimental setup and data analysis. A₁: short spinal cord preparations consisted of 7 – 20 segment lengths of isolated spinal cord perfused with 0.15 – 0.2 mM NMDA in Ringers. A₂: sample of rhythmic bursting recorded with a glass suction electrode on a ventral root. A₃: ventral root activity was digitally rectified and spike event times detected by voltage rising through a threshold level. The time between onsets of consecutive bursts was the cycle period (CP) and the time from burst onset to offset was the burst duration (BD). A₄: burst intensity was defined as the product of the mean instantaneous spike frequency within the burst and the mean amplitude of the detected peaks. Peaks were detected as the highest point between the time of voltage rising through a threshold and falling back below the threshold level. B₁: long spinal cord preparations consisted of 30 – 40 segment lengths of isolated spinal cord perfused with 0.5 mM D-glutamate. B₂: bursting activity recorded in two ipsilateral ventral roots separated by 23 segments. B₃: cross-correlograms were made between the event times of the two ventral root recordings in 10 to 20 second windows. The time between adjacent correlogram peaks is the cycle period (CP) and the delay (D) of the main peak from zero lag was used to determine the phase lag as described in the Material and Methods.

Fig. 2. In NMDA-induced fictive swimming, stirring the static bath does not significantly alter the cycle period. A: cycle-by-cycle plot of cycle period with the time of stirring indicated by the arrow. Dotted line indicates pre-stir mean of
cycle period.  

**B:** summary of 10 preparations showing mean and SD of cycle period before and after stirring. There was no significant change in cycle period ($P = 0.56$).

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Fig. 3. Acetylcholine produces a dose-dependent decrease in fictive swimming cycle period.  

**A:** when stirred into the bath during fictive swimming, 1 µM ACh decreased cycle period as shown in the cycle-by-cycle plot of cycle period. Dotted line represents the mean of the control cycle period.  

**B:** sample of rectified ventral root bursting before ACh was added in region indicated in **A**.  

**C:** sample of bursting during ACh application in region indicated in **A** showing shortened cycle periods.  

**D:** summary of results of addition of various concentrations of ACh in different preparations. Using one-way ANOVA, concentrations of 200 µM and 1000 µM ACh were found to elicit significant decreases in cycle period ($P < 0.001$).

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Fig. 4. Example of the effects of acetylcholine on fictive swim parameters.  

**A:** cycle-by-cycle plot of cycle period (top), burst proportion (middle), and burst intensity (bottom) with the addition of 200 µM ACh to the bath during fictive swimming. Dotted lines indicate the pre-ACh means. Asterisks mark the troughs of a slow modulation of the cycle period.  

**B:** sample of ventral root activity before ACh in region indicated in **A**.  

**C:** sample of ventral root activity at the peak of the ACh effect in region indicated in **A**.  

**D:** sample of slow modulation of ventral root bursting that appeared after addition of ACh.
Fig. 5. Summary of changes in fictive swim parameters produced by 200 µM ACh in 7 preparations. 

A1: cycle period decreased significantly to 54% of control ($P < 0.001$). A2: variability of cycle period also increased significantly ($P = 0.004$) as indicated by the mean of the coefficient of variation (CV). B1: burst proportion decreased significantly to 80% of control ($P = 0.044$). B2: burst proportion variability increased significantly ($P = 0.014$). C1: burst intensity did not change significantly with ACh ($P = 0.46$). C2: variability of burst intensity increased significantly ($P = 0.001$). Significance was determined by paired t-tests where $\alpha < 0.05$.

Fig. 6. Nicotinic contribution to changes in cycle period. A: addition of 200 µM nicotine significantly decreased cycle period to 72% of control ($P = 0.03$). B: prior 30 min perfusion of a nicotinic antagonist, mecamylamine (20 µM) or dihydro β-erythroidine hydrobromide (20 µM), resulted in less nicotine-induced decrease of cycle period (to 92% of control vs. 72%), though the decrease was significant ($P = 0.015$). C: prior 30 min perfusion of a nicotinic antagonist resulted in an ACh-induced decrease of cycle period to 79% of control ($P = 0.019$), compared to a reduction to 54% of control with ACh alone (see Fig. 5A1).

Fig. 7. Muscarinic contribution to changes in cycle period. A: addition of 20 µM oxotremorine, a muscarinic agonist, reduced cycle period to 89% of control when stirred into a static bath but the effect was not statistically significant ($P = 0.099$).
B: bath perfusion of oxotremorine (20 μM) produced a significant reduction of cycle period to 92% of control ($P = 0.017$). This decrease was reversed with the additional perfusion of a muscarinic antagonist, scopolamine (10 μM) or atropine (2 μM) ($P = 0.016$). C: when scopolamine or atropine were bath perfused first, the addition of ACh decreased cycle period to 71% ($P = 0.012$) with the addition of ACh compared to a reduction to 54% of control with ACh without blocker (see Fig. 5A1). D: bath perfusion of both a nicotinic and a muscarinic antagonist together prevented ACh from inducing any significant changes in cycle period ($P = 0.86$).

Fig. 8. Example of ACh effects on cycle period and phase lag in a long spinal cord preparation. A: rapid addition of ACh (200 μM) to the bath during D-glutamate-induced fictive swimming reduced cycle period from 2.4 sec to 0.4 sec. B: phase lag also decreased dramatically with ACh, from 2.0% to 0.4% per segment. C: sample of ventral root bursting before addition of ACh. D: sample of ventral root bursting after addition of ACh showing that the bursting in the widely separated ventral roots became nearly synchronous.

Fig. 9. Summary of effects of ACh (200 μM) in D-glutamate-induced fictive swimming in long spinal cord preparations. A: cycle period was reduced to 45% of control in 12 preparations ($P = 0.002$). B: phase lag was reduced to 32% of control ($P = 0.002$). C: stirring the bath of D-glutamate-induced fictive swimming resulted in a significant decrease of cycle period to 88% of control ($P < 0.001$).
This is in contrast to the lack of stirring effect in NMDA-induced fictive swimming (see Fig. 2).  _D:_ phase lag was not changed significantly by stirring (_P_ = 0.46).

**Fig. 10.** Acetylcholinesterase inhibition and ACh receptor antagonism indicate endogenous release of ACh and ongoing modulation of fictive swimming.  _A_1: in an example experiment, the ACh esterase inhibitor eserine (20 µM) was added to the D-glutamate perfusion producing a small but clear reduction in cycle period.  Addition of both nicotinic and muscarinic antagonists (10 µM mecamylamine and 20 µM scopolamine) to the perfusion increased cycle period beyond the pre-eserine levels.  _A_2: the same experiment shows similar effects of eserine and the blockers on phase lag.  _A_3: summary of the effects of eserine and combined blockers on cycle period in 7 preparations.  Cycle period decreased to 73% of control with eserine (_P_ = 0.007) and then increased to 154% of eserine levels with the addition of the blockers.  _A_4: summary of the effects of eserine and combined blockers on phase lag in 6 preparations.  Phase lag decreased to 58% of control with eserine (_P_ = 0.009) and then increased to 230% of the eserine level with the addition of the blockers.  _B_1: sample experiment showing that the addition of combined mecamylamine and scopolamine to the D-glutamate perfusion produced a small but clear increase in cycle period.  _B_2: the same experiment shows a similar effect on phase lag.  _B_3: summary of the effects of combined blockers on cycle period in 5 preparations showing a significant increase to 119% of control (_P_ = 0.034).  _B_4: summary of the effects of combined...
blockers on phase lag, showing a significant increase to 143% of control ($P < 0.001$).

Fig. 11. Nicotinic and muscarinic contributions to endogenous ACh modulation.  
$A_1$: example experiment of adding increasing concentrations of mecamylamine after pre-treatment with eserine. Each increase in mecamylamine concentration produced a clear increase in cycle period.  
$A_2$: same experiment showing the effect of mecamylamine on phase lag.  
$A_3$: summary of 6 preparations showing that 10 $\mu$M mecamylamine produced a significant increase of cycle period to 170% of eserine levels ($P = 0.003$).  
$A_4$: summary of 6 preparations showing that 10 $\mu$M mecamylamine produced a significant increase of phase lag to 130% of eserine levels ($P = 0.043$).  
$B_1$: example experiment of adding increasing concentrations of scopolamine on cycle period after pre-treatment with eserine.  
$B_2$: same experiment showing effect on phase lag.  
$B_3$: summary of 6 preparations showing that 25 $\mu$M scopolamine significantly increased cycle period to 144% of eserine levels ($P < 0.001$).  
$B_4$: summary of 6 preparations showing that 25 $\mu$M scopolamine significantly increased phase lag to 144% of eserine levels ($P < 0.001$).  
Significance was determined by $\alpha < 0.05$ using one-way ANOVA with Tukey’s procedure ($A_3$, $B_3$, $B_4$) and SNK method ($A_4$).
FIGURE 1

A1  Short Preparations

A2  raw VR4

A3  rect. VR4

A4  mean threshold

B1  Long Preparations

B2  D-Glutamate

B3  Cross Corr. Function

CP

BD

VR4

Rostral

50 msec
FIGURE 2

A

B

Cycle Period (sec)

Time (sec)

0 30 60 90 120 150

0.2 0.4 0.6

Stir

n = 10

Control

Stir

Cycle Period (sec)

n = 10

0.0 0.2 0.4 0.6 0.8
FIGURE 3

D

Percent Reduction of Cycle Period

[Acetylcholine] (µM)

n = 4 n = 4 n = 4 n = 7 n = 4

A

B Control

C 1 µM Acetylcholine

D

Acetylcholine (1 µM)
FIGURE 4

A

Acetylcholine (200 µM)

Cycle Period (sec)

0.0

0.2

0.4

0.6

0.8

B

C

D

** ** * * * *

Burst Proportion

0.0

0.2

0.4

0.6

0.8

Acetylcholine (200 µM)

Burst Intensity

0

100

200

300

400

Time (sec)

0

30

60

90

120

150

180

210

B Control

C 200 µM Acetylcholine

D 200 µM Acetylcholine

1 sec

2 sec
FIGURE 5

A1: Cycle Period (sec) for Control, ACh, and Wash conditions. 
A2: Cycle Period CV for Control, ACh, and Wash conditions. 
B1: Burst Proportion for Control, ACh, and Wash conditions. 
B2: Burst Proportion CV for Control, ACh, and Wash conditions. 
C1: Burst Intensity for Control, ACh, and Wash conditions. 
C2: Burst Intensity CV for Control, ACh, and Wash conditions. 

n = 7 for each condition.
FIGURE 6

A

B

C

n = 7

n = 6

Cycle Period (sec)

Cycle Period (sec)

Cycle Period (sec)

Control

Nicotine

Wash

Control

Nicotine

Wash

Control

ACh

Wash

n = 7

* * *
**FIGURE 7**

A. 

- *n = 6*
- Cycle Period (sec)
- Control, Oxo, Wash

B. 

- *n = 7*
- Cycle Period (sec)
- Control, Oxo, Oxo+, Wash

C. 

- *n = 4*
- Cycle Period (sec)
- Control, ACh++, Wash

D. 

- *n = 5*
- Cycle Period (sec)
- Control, M.Ant., N.Ant., Ant., ACh, Wash
FIGURE 8

A

![Graph showing cycle period vs. time with Acetylcholine (200 µM) indicated.]

B

![Graph showing phase lag vs. time with Acetylcholine (200 µM) indicated.]

C

Before ACh

1.VR$_{08}$

1.VR$_{28}$

D

During ACh

1.VR$_{08}$

1.VR$_{28}$

FIGURE 8
FIGURE 9

A

B

C

D

Cycle Period (sec)

Phase Lag (% CP/seg)

Control
ACh
Wash

Control
ACh
Wash

Control
Stir

Control
Stir

n = 12

n = 14

*
FIGURE 10
FIGURE 11

A1

Eserine

Mecamylamine

5 µM | 10 µM | 25 µM

Cycle Period (sec)

0 60 120 180

Time (min)

2.0

2.5

3.0

3.5

4.0

4.5

A2

Eserine

Mecamylamine

5 µM | 10 µM | 25 µM

Phase Lag (% CP/seg)

0 60 120 180

Time (min)

0.6

0.8

1.0

1.2

1.4

1.6

A3

Eserine

Scopolamine

5 µM | 10 µM | 25 µM

Mean CP (% Control)

0 50 100 150

n = 6

A4

Eserine

Scopolamine

Scopolamine

5 µM | 10 µM | 25 µM

Mean PL (% Control)

0 50 100 150

n = 6

B1

Eserine

Scopolamine

5 µM | 10 µM | 25 µM

Cycle Period (sec)

0 60 120 180

Time (min)

2.0

2.5

3.0

3.5

4.0

4.5

B2

Eserine

Scopolamine

5 µM | 10 µM | 25 µM

Phase Lag (% CP/seg)

0 60 120 180

Time (min)

0.6

0.8

1.0

1.2

1.4

1.6

B3

Eserine

Scopolamine

Scopolamine

5 µM | 10 µM | 25 µM

Mean CP (% Control)

0 50 100 150

n = 6

B4

Eserine

Scopolamine

Scopolamine

5 µM | 10 µM | 25 µM

Mean PL (% Control)

0 50 100 150

n = 6

*