NEUROMUSCULAR ORGANIZATION IN THE SWIMMING SYSTEM OF THE PTEROPOD MOLLUSC CLIONE LIMACINA

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Accepted 19 April 1993

Summary

Swim motor neurons of the pteropod mollusc Clione limacina were identified by a combination of electrophysiological and morphological characteristics. Two types of motor neurons were found, including small motor neurons which are active during both slow and fast swimming and which innervated restricted fields of the ipsilateral wing. General excitor motor neurons have large cell bodies, innervate widespread fields and are recruited into activity for fast swimming. Small motor neurons monosynaptically innervate slow-twitch muscle cells, whereas general exciters monosynaptically innervate both slow-twitch and fast-twitch muscle cells. Activity in general exciters can centrally enhance that in small motor neurons because the neurons are electrically coupled. Neuromuscular recordings and lesion experiments indicate that a peripheral nerve network does not appear to play an important role in the spread of excitation throughout the muscle fields.

Introduction

An important feature of most locomotory systems is the ability to change propulsion speed. The need for variability in locomotory speed has created interesting problems for central control systems and peripheral musculo-skeletal systems. One such problem is maximizing energetic and mechanical efficiency of muscular activity over a wide range of contraction speeds. Every muscle cell operates most efficiently at a particular shortening velocity (for reviews, see Goldspink, 1977, 1981). Outside this range of optimal shortening velocities, each muscle cell will be energetically and mechanically inefficient. This problem has been ‘resolved’ in many locomotory systems through the use of a two- or three-geared system of muscle activation. Geared systems make use of muscle cells (and motor units) with different biochemical and contractile properties, giving rise to differences in speed of contraction, fatigue resistance and optimal shortening velocities. As locomotory speed increases (by an increase in the rate of movement of locomotory appendages), it is necessary simply to ‘shift gears’ (in some

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Key words: swimming, locomotion, motor neuron, mollusc, Clione limacina.
cases, change gait) to change from activation of primarily one muscle cell type to activation of another cell type, and thus to maximize the mechanical and energetic efficiency of the movement.

The problem of how different peripheral effectors are recruited during changes in locomotory speed has received a great deal of attention over the past few decades, primarily with the use of vertebrate preparations, and has produced fundamental rules of neuromuscular organization (e.g. the Henneman size principle; see Henneman et al. 1965; Henneman and Mendell, 1981; Stuart and Enoka, 1983). Despite this, relatively little is known about the central aspects of locomotory speed changes, including changes in central pattern generator organization, motor neuron activity and the role of neuromodulators in changing both central and peripheral activities.

The pteropod mollusc *Clione limacina* is a holoplanktonic opisthobranch that exhibits two distinct forms of forward locomotion in addition to a ballistic startle response (Arshavsky et al. 1985a,b,c; Satterlie et al. 1985; Satterlie, 1991a,b). Within both slow and fast forms of swimming, swimming speed is variable so that the entire range of swimming speeds represents a continuum. Animals occasionally accelerate smoothly from slow to fast swimming, although the more typical form of speed change is more abrupt, as is seen, for example, following stimulation of the tail or during the initiation of feeding reactions. The change between the two swimming speeds can be considered to be a ‘change-in-gears’ because (1) the pattern generator is reconfigured to accomplish the increased frequency of appendage movements (Arshavsky et al. 1985c,d) and (2) two types of skeletal muscle cells are used during the two forms of swimming, including slow-twitch fatigue-resistant and fast-twitch fatigable fibers. The two types of muscle fibers have been characterized structurally, histochemically and electrophysiologically (Satterlie et al. 1990; Satterlie, 1991a). Similarly, swim motor neurons have been electrophysiologically and morphologically identified in the pedal ganglia of *Clione*. They can be divided into d-phase and v-phase neurons, which initiate dorsal and ventral bending of the wings respectively. Furthermore, within each of these subgroups, there are two size classes of motor neurons. One cell of each subgroup is several times larger than its compatriots. The two large motor neurons (one d-phase and one v-phase) are referred to here as ‘general excitor motor neurons’, and the remaining motor neurons are called ‘small motor neurons’. Both types of motor neuron receive two-phase synaptic inputs from the swim pattern generator, but neither participates in the generation of the motor pattern (Arshavsky et al. 1985b,c; Satterlie and Spencer, 1985). Despite our detailed knowledge of pattern generator interneuron and skeletal muscle activities, and information about connectivity between interneurons and motor neurons in the *Clione* swimming system, detailed information on motor neuron activities during changes in locomotory speed is lacking. Most notably, it is not known whether the motor neuron assemblage is arranged in a two-geared system as seen in the pattern generator and peripheral musculature.

Because all the cells in the swimming system are available for microelectrode recording during swimming activity in dissected preparations, the present study was conducted to describe the organization of the motor neuron pool and the changes in motor
neuron activity that accompany recruitment of fast-twitch muscles during the change from slow to fast swimming speed in Clione.

Materials and methods

Adult specimens of Clione limacina (Phipps) (1–2.5cm body length) were collected from the breakwater at Friday Harbor Laboratories, Friday Harbor, Washington, and held in 4-l jars partially submerged in sea tables. Animals were anesthetized in a 2:1 mixture of isotonic MgCl₂ (0.33mol⁻¹) and sea water, dissected and pinned in Sylgard-coated (Dow Corning) Petri dishes containing sea water using cactus spines (Opuntia sp.). Reduced preparations included the central ring of ganglia (cerebals, pleurals and pedals) connected to the wings by the pedal wing nerves. Ganglionic sheaths were partially removed with a 1mgml⁻¹ solution of protease (Sigma, type XIV) followed by several washes in fresh sea water.

Intracellular recordings from wing muscle cells were accomplished by tightly pinning a circle of wing tissue with cactus spines. Care was taken to avoid pinning through a branch of the wing nerve. If movement was noticed, the tissue could be tightened by pushing the barbed shafts of the cactus spines further into the Sylgard. No significant differences were noted in motor neuron activities in preparations in which the wings were tightly pinned and those in which the wings were left unpinned.

Intracellular recordings were made with microelectrodes filled with 3mol⁻¹ KCl or 2mol⁻¹ potassium acetate. Electrode resistances ranged from 10 to 35 MΩ. Current was injected through recording electrodes using amplifier bridge circuits. All experiments were conducted at 16–18˚C.

Wing movements were detected using a Cambridge Technology 400A force transducer system. A sealed glass microelectrode was waxed to the transducer arm and positioned against the wing tissue using a micromanipulator. Because the microelectrode was not firmly attached to the wing tissue, and the angle of attachment was not in line with the generation of muscle force, the transducer system was used merely as an uncalibrated movement detector. The sensitivity of the movement detector was sufficient to pick up movements that were barely detectable at a magnification of 84× in a Nikon dissecting microscope. Swimming activity was nearly continuous, even in reduced preparations. In almost all cases, recorded swimming activity, with associated changes in speed, was spontaneous.

For morphological data, neurons were injected with one of the following dyes/markers: Lucifer Yellow CH (4%, Sigma), 5(6)-carboxyfluorescein (5%, Sigma), Neurobiotin (2%, Vector Laboratories), 4% horseradish peroxidase (HRP type VI-A, Sigma). Lucifer Yellow and carboxyfluorescein preparations were immersed in a 1:1 mixture of sea water and 0.33mol⁻¹ MgCl₂ and photographed live in the recording dish with a Nikon epifluorescence microscope fitted with an FITC filter package. Neurobiotin-filled neurons were fixed in phosphate-buffered glutaraldehyde (2%) for 2h, washed in phosphate buffer containing 0.1% Triton X-100 or Tween 20, and treated with the avidin/peroxidase (ABC) reagent of a Vectastain ABC kit (Vector Laboratories). The preparations were then treated with diamino-benzidine (DAB) and H₂O₂ to achieve the dark brown reaction
product. HRP preparations were treated similarly, but without the avidin/peroxidase step. The stained preparations were then dehydrated and embedded in EPONATE 12 plastic (Ted Pella). Thick sections (1–2 μm) were cut with glass knives on an ultramicrotome, mounted on slides and examined unstained in a Nikon microscope. Stained processes appeared brown with normal illumination.

Some Lucifer Yellow preparations were treated with an anti-Lucifer-Yellow antibody and then with the avidin/biotin/peroxidase technique (Vectastain kit, Vector Laboratories) to make the filled cell processes light-opaque and electron-dense. Preparations were fixed as described above, washed in phosphate buffer followed by phosphate buffer containing 0.1% Triton X-100 and then phosphate buffer/Triton X-100 with 5% goat serum. They were then incubated in a 1:500 dilution of a polyclonal anti-Lucifer-Yellow antibody (Chemicon) in phosphate/Triton X-100/goat serum for 48h. After 12h of washing, the preparations were processed according to the Vectastain ABC kit instructions, reacted with DAB–H₂O₂, dehydrated, embedded and prepared for microscopy as described above. Of the fluorescent dyes, carboxyfluorescein gave the best results. For electron microscopical preparations, Neurobiotin injections were superior, although the Lucifer Yellow immunohistochemical preparations were also useful.

Results

Swim motor neurons were identified by a combination of morphological characteristics, patterned synaptic inputs and motor effects. Motor neurons are not components of the swim pattern generator, but receive alternating excitatory and inhibitory synaptic inputs from pattern generator interneurons (Figs 1, 4). According to Arshavsky et al. (1985a,b,c) and Satterlie and Spencer (1985), each motor neuron receives excitatory input from agonistic interneurons and inhibitory input from antagonistic interneurons. This has been confirmed in this study and extended to show that both excitatory and inhibitory inputs persisted when the preparations were bathed in high-Mg²⁺, high-Ca²⁺ sea water, indicating that the contacts are monosynaptic (Fig. 1B,C,D). In addition, synaptic delays were short (2–4ms) and constant in any one recording session. Because of the reciprocal inhibitory connections between antagonistic interneuron pools, the subthreshold activity of motor neurons appeared as a two-phase alternation of inhibitory and excitatory postsynaptic potentials (PSPs) (see Figs 1A, 4).

Initial identification of putative swim motor neurons was therefore made by searching for cells that exhibited electrical activity that was phase-linked to swimming movements of the wings. Candidate motor neurons were then intracellularly stimulated during non-swimming periods or in preparations bathed in high-Ca²⁺, high-Mg²⁺ sea water. The latter treatment halted swimming movements. Under these conditions, stimulation of swim motor neurons induced contractions of the wing musculature in direct relation to the duration of spike activity in the injected cell. Occasionally, penetration of a swim interneuron produced false-positive motor neuron identifications due to their electrical coupling with swim motor neurons; however, the threshold for the peripheral responses was very high, particularly in high-Ca²⁺, high-Mg²⁺ sea water.

To confirm the identification of motor neurons, and to eliminate interneuron
penetrations, central neurons that satisfied the first two criteria were either ionophoretically or pressure-injected with Lucifer Yellow, carboxyfluorescein, horseradish peroxidase (HRP) or Neurobiotin. The cell bodies of all swim motor neurons were located in the pedal ganglia (based on over 150 motor neuron fills). Each motor neuron sends a single axon to the ipsilateral wing through the pedal wing nerve. Upon entering the wing, the wing nerve divides into four major branches. Each branch divides repeatedly so that the entire wing is innervated (Fig. 2; see also Fig. 11). One primary branch (branch 1) runs nearly perpendicular to the longitudinal axis of the body in the anterior one-third of the wing (parallel to the leading edge of the wing). The second primary branch (branch 2) runs posterio-laterally within the wing and forms several major secondary branches that fan out across the central region and trailing edge of the wing (Fig. 2B). The third primary branch of the wing nerve (branch 3) bends medially and joins with a similar branch of the contralateral wing nerve (Fig. 2A). This connection forms an extraganglionic commissure that will be referred to as the parapodial connective. The fourth primary branch (branch 4) originates from either branch 2 or
branch 3 and runs posteriorly, parallel to the long axis of the body, to innervate the medial regions of the wing (Fig. 2A). Motor neurons send axon branches into one, two, three or all four primary wing nerve branches as discussed below.

Based on maps of cell body positions from many preparations, and individual experiments in which many motor neurons were identified, the total number of swim motor neurons in each pedal ganglion was estimated to be between 20 and 30. These motor neurons could be divided into two groups on the basis of cell body size. All but two of the motor neurons had small cell bodies (15–20 μm in diameter). These motor neurons, which will be referred to as ‘small motor neurons’, were divided between d-phase (dorsal bending) and v-phase (ventral bending) neurons. The remaining two motor neurons had large cell bodies, up to 80 μm in diameter (neurons 1A and 2A in Arshavsky et al. 1985a,b). One innervates the dorsal swim musculature and the other the ventral swim musculature. For reasons described below, these two neurons are given the more descriptive name of ‘general excitor motor neurons’. Small motor neurons and general excitors received similar synaptic inputs from the swim pattern generator. This similarity was seen during slow and fast swimming.

Small motor neurons

Most of the small motor neuron somata were found on the dorsal and ventral surfaces of the pedal ganglion near the emergence of the wing nerve (Fig. 3 shows the locations of some small motor neuron cell bodies). A few additional somata were found on the lateral margin of the ganglion midway between the wing nerve and the origin of the cerebro-pedal connective. At least one soma was found in the middle of the dorsal surface of the ganglion. Small motor neuron axons typically followed one or two of the primary branches of the wing nerve (see Fig. 2C), so that each cell had a restricted innervation field in the ipsilateral wing. Fig. 3 shows the locations of the somata of seven small motor neurons and their innervation fields in the wing as determined by visual observation of twitch activity following intracellular depolarization of the somata. These preparations were bathed in high-Mg²⁺, high-Ca²⁺ sea water so that spontaneous swimming was halted, and the stimulating current was adjusted to be just above spike threshold to minimize the possibility of stimulating electrically coupled motor neurons. The observation that contractions persisted with this treatment suggests that the neuromuscular connections are monosynaptic.

Small motor neurons produced a single spike or, more typically, a burst of spikes with each depolarizing input from the swim pattern generator during both slow and fast swimming.

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Fig. 2. Dye-fills of swim motor neurons. (A) Lucifer Yellow fills of the d-phase general excitor motor neurons from the two pedal ganglia. The axon of each motor neuron sends four primary branches into the ipsilateral wing (labelled 1–4), which run in the four major branches of the wing nerve. Branch 3 runs medially and passes close to branch 3 of the contralateral cell. (B) Carboxyfluorescein fill of the d-phase general excitor motor neuron of the right pedal ganglion. The extensive branching pattern of the axon can be seen. Branch 3, from which branch 4 originated, was accidentally severed (arrowhead). (C) Carboxyfluorescein fill of a d-phase small motor neuron. This cell has a single primary axon branch which runs in nerve branch 1 of the wing. Scale bars, 100 μm.
swimming (Fig. 4; based on recordings from well over 200 motor neurons). Typical bursts included 3–10 action potentials with a maximum firing frequency of 130Hz. In some motor neurons, individual spikes could not be seen, as the cell appeared to have a single, long-duration action potential similar to those seen in interneurons (compare the motor neuron recording in Fig. 4B with the interneuron recording in Fig. 1A). Dye-injections of these cells revealed that they were indeed motor neurons. Burst termination in small motor neurons may be due at least partially to their endogenous membrane properties because the cells produced bursting activity when subjected to either a long-duration suprathreshold or a ramp depolarization (Fig. 4C). The frequency of bursts in these cases was between 5 and 7Hz, which is the upper limit of recorded wingbeat frequencies.

Simultaneous recordings from small motor neurons and muscle cells within their fields of innervation indicated that these neurons innervate slow-twitch muscle cells, but do not activate fast-twitch muscle cells (Fig. 5: N=23; for criteria for identification of muscle cells types, see Satterlie, 1991a). For slow-twitch muscle cells, either an EJP or a spike-like response occurred 1:1 with each spike burst in the motor neuron. The response had a short, constant latency with repetitive stimuli in a single preparation (4–10ms depending on the location on the wing of the muscle cell and the particular motor neuron; Fig. 5A). Slow-twitch muscle cells showed a variety of activity thresholds to small motor neuron

Fig. 3. Schematic representation of a pedal ganglion (top) and the ipsilateral wing (two lower drawings). The locations of the cell bodies of eight motor neurons are shown in the drawing of the ganglion, including the d-phase general excitor (cell 8). The innervation fields of the eight cells are shown in the bottom drawings. Note that the small motor neurons innervate restricted fields, whereas the general excitor innervates the entire area of the wing. For all three drawings, medial is to the left and lateral is to the right. Not drawn to scale.
inputs: some muscle cells produced only excitatory junctional potentials (EJPs), whereas others produced a spike-like response with every motor neuron input. Neuromuscular connections were blocked when the preparation was bathed in high-Mg\textsuperscript{2+} saline, but persisted when the preparation was bathed in high-Mg\textsuperscript{2+}, high-Ca\textsuperscript{2+} saline, suggesting that the neuromuscular contacts are chemical and monosynaptic (Fig. 5; \(N=14\)).

Small motor neurons provided axon branches to one or more wing nerve branches including branches 1, 2 and 4 described above. No small motor neurons with axon branches in the parapodial connective (wing nerve branch 3) have been found. Light microscopic examination of 1–2 μm plastic sections of Neurobiotin-filled and Lucifer-Yellow-filled/immunoreacted small motor neurons revealed that putative terminals were located exclusively in the region of slow-twitch muscle cells (Fig. 6C,D; \(N=5\)).

**General excitor motor neurons**

The two large motor neurons (general exciters) were found on the dorsal surface of each pedal ganglion. The d-phase general excitor (1A of Arshavsky, 1985\textsuperscript{b}) was always found near the lateral margin, among a cluster of small motor neurons near the origin of the wing nerve (see Fig. 3). The v-phase general excitor (2A of Arshavsky, 1985\textsuperscript{b}) was found slightly anterior and medial to the d-phase cell. Each general excitor sent a single axon into the ipsilateral wing and into each of the four primary branches of the wing nerve.
From there, the axons branched repeatedly so that they innervated the entire wing. The d-phase general excitor innervated the dorsal swim musculature, whereas the v-phase cell innervated the ventral swim musculature. Each general excitor sent one

Fig. 5. Simultaneous recordings from small motor neurons and muscle cells of the ipsilateral wing. All recordings were conducted in high-Mg\(^{2+}\), high-Ca\(^{2+}\) sea water. Bursts of spikes in motor neurons were elicited by injection of depolarizing current. (A) At high sweep speed, a burst of action potentials in the motor neuron (sm) produces a single EJP in a slow-twitch muscle cell (st). (B) Repetitive bursting in a small motor neuron produces repetitive EJPs in slow-twitch muscle cells. Each spike burst looks like a single action potential at this slow sweep speed. (C) Spike bursts in small motor neurons do not elicit EJPs in fast-twitch muscle cells (ft). Scale bar, A, 20mV, 15ms; B,C, 20mV, 1s.

(Fig. 2A,B). From there, the axons branched repeatedly so that they innervated the entire wing. The d-phase general excitor innervated the dorsal swim musculature, whereas the v-phase cell innervated the ventral swim musculature. Each general excitor sent one
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branch to the contralateral wing via the parapodial connective (wing nerve branch 3) to innervate the swim musculature along the anterior margin of the contralateral wing. Light microscopic examination of plastic sections of filled general excitor motor neurons revealed processes and putative terminals among both slow-twitch and fast-twitch muscle cells (Fig. 6E,F; \( N = 4 \)).

The two general excitor motor neurons exhibited similar activity patterns, but were in antiphase with one another. During slow swimming, the general exciters did not usually produce spikes, but only exhibited rhythmic membrane potential oscillations (Fig. 4A; based on over 250 recordings). In some cases, general exciters produced single action potentials with each depolarizing input during slow swimming or spiked intermittently. During periods of fast swimming, these cells produced a burst of spikes with each excitatory input from the pattern generator. Bursting activity typically coincided with increases in swim cycle frequency (Fig. 7B; see also Fig. 4A) and produced enhanced contractions of the entire ipsilateral wing (Fig. 7B) and of the leading edge of the contralateral wing. Similar widespread contractions were triggered when general exciters were depolarized in non-swimming preparations (Fig. 7A). When directly depolarized

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**Fig. 7.** Dual recordings from general excitor motor neurons (ge) and an uncalibrated force transducer (f). (A) In high-Mg\(^{2+}\), high-Ca\(^{2+}\) sea water, bursting induced in the motor neuron produces widespread contractions of the ipsilateral wing. (B) During spontaneous swimming activity in normal sea water, a change of general excitor activity from the non-spiking mode to the spiking mode (arrowheads) results in contractions of increased force. Note that at the arrowheads there is an increase in cycle frequency accompanying the recruitment of the motor neuron. Scale bars, A, 20mV, 0.5s; B, 40mV, 2s.
with rectangular or ramp currents, they exhibited ‘bursty’ activity similar to that of small motor neurons (Figs 7A, 8).

Spikes in general excitor motor neurons produced 1:1 EJPs or spike-like responses in both slow-twitch and fast-twitch muscle cells (Fig. 8). Synaptic delays were short, 4–7ms, and constant in any one recording. Neuromuscular activity was blocked by high-Mg$^{2+}$ saline, but persisted in high-Mg$^{2+}$, high-Ca$^{2+}$ saline, suggesting that these motor...
neurons make monosynaptic connections with both types of muscle cells throughout the
wing. In these experiments, it is possible that excitation of slow-twitch muscle was due to
activation of small motor neurons that are electrically coupled to the general excitors.
Although this may indeed occur, direct connections between general excitors and slow-
twitch muscle cells were suggested by four pieces of evidence. First, morphological
evidence described above indicates that putative terminals of general excitors are found
in both fast-twitch and slow-twitch muscle regions. Second, in preparations bathed in
high-Mg²⁺, high-Ca²⁺ sea water, the decreased excitability of central neurons prevented
or greatly decreased firing of small motor neurons during induced spiking in general
excitor motor neurons. Third, each general excitor spike produced an individual EJP or
spike-like response in both slow-twitch and fast-twitch muscle cells (see Fig. 8A), in
contrast to small motor neurons in which a burst of spikes was required to produce a
single muscle response. Finally, in dual recordings from a general excitor motor neuron

Fig. 9. Dual recording from a general excitor motor neuron (ge) and a slow-twitch muscle cell
(st). All records are from the same cell pair and represent spontaneous activity in normal sea
water. In A, the general excitor does not spike until intracellularly depolarized (upward
arrowhead denotes onset of current pulse, downward arrow denotes release from
depolarization). Spiking in the general excitor produces enhanced spike-like responses in the
muscle cell. In B, high-frequency swimming resulted in intermittent spiking in the general
excitor. When the general excitor dropped out of the spiking mode, the spike-like responses of
the muscle cell were unaffected.
and a slow-twitch muscle cell during slow swimming (general excitor not spiking), slight depolarization of the general excitor was sufficient to shift it from the non-spiking to the spiking mode. Such induced spiking resulted in enhancement of the EJPs or spike-like responses of slow-twitch muscle cells (Fig. 9A). General excitor input thus had an additive effect with small motor neuron input. This experiment was repeated in five preparations, four of which were from the d-phase general excitor.

As previously noted by Arshavsky et al. (1985b), not all d-phase small motor neurons were found to be electrically coupled to the d-phase general excitor. Furthermore, in most preparations this electrical coupling, when found, was extremely weak. Because injected currents in these experiments were small, it is unlikely that the enhancement of muscle cell electrical responses was due solely to electrical coupling between motor neurons; it was probably the result of direct innervation of the muscle cells by the general excitor motor neuron. The converse experiment, in which an ‘active’ general excitor was hyperpolarized so that its spiking was blocked, or in which the general excitor ceased firing spontaneously, did not significantly change the size of spike-like responses of slow-twitch muscle cells, provided that the non-firing period did not last for more than five or six cycles and that the cycle frequency did not drop appreciably (Fig. 9B). Care was taken to duplicate these experiments with muscle cells from all parts of the wing to ensure that the leading-edge branch of the contralateral general excitor did not influence the muscle responses.

Central interactions between motor neurons

As documented by Arshavsky et al. (1985b), electrical coupling has been confirmed between general excitor motor neurons and some of their synergistic small motor neurons, particularly within the v-phase motor neuron group. This electrical coupling appeared to be functionally rectifying, because spikes in general excitors sometimes produced spikes in small motor neurons, whereas spike bursts in small motor neurons produced no detectable changes or, at most, small subthreshold depolarizations in the general excitors. Spike activity in the general excitors thus had a significant influence on the activity of some of the small motor neurons. This influence is illustrated in Fig. 10. In this case, electrical coupling was confirmed between the general excitor and the small motor neuron (Fig. 10A,B). The small motor neuron produced only a single spike with each depolarization during spontaneous swimming activity so long as the synergistic general excitor was not spiking (Fig. 10C). An increase of spiking activity of the general excitor, caused either by intracellular depolarization or by spontaneous recruitment of spiking in the general excitor, directly added spikes to the activity of the coupled small motor neuron in a 1:1 manner: when the general excitor produced two spikes, the small motor neuron produced three spikes and when the general excitor produced a single spike, the small motor neuron produced two spikes (Fig. 10D). The second and third spikes in the small motor neuron occurred simultaneously with the general excitor spikes. In the particular case illustrated in Fig. 10, there was no significant increase in cycle frequency, suggesting that the change in small motor neuron spiking was not due to an obvious change in pattern generator activity.

General excitor motor neurons were recruited into activity during fast swimming.
Because they innervate both slow-twitch and fast-twitch muscle cells throughout the entire ipsilateral wing (and leading edge of the contralateral wing), and directly excite coupled synergistic small motor neurons centrally, their influence over the generation of contractile force of the wings during fast swimming is greater than would have been expected simply as a result of recruitment of fast-twitch muscles. It is due to this widespread excitatory influence that the cells are referred to as general excitors.

**Does a peripheral nerve net play an important role in the swimming system?**

Three pieces of evidence suggest that the spread of excitation throughout the swim musculature of *Clione* does not involve a widespread peripheral nerve network. First, in any one neuromuscular recording, including both small motor neurons and general excitors, motor neuron spikes or spike bursts were followed by a junctional potential or spike-like response with a constant, short latency. These contacts have been shown to be monosynaptic. Second, all motor neurons have dense branching patterns within the area that they innervate. Third, the results of lesion experiments discount the possibility of significant lateral spread of excitation from the primary nerve branches of the wing. These experiments involved isolated wing preparations in which an intracellular recording from a muscle cell was performed while the surface of the wing was stimulated by a suction electrode placed in a line with the recording electrode, which was parallel to
the longitudinal axis of the body (the organization of electrodes and the position of cuts are shown in Fig. 11A). Recordings were conducted in the vicinity of the medial branch of the wing nerve, with the stimulating electrode placed near the anterior branch of the wing nerve, or with the reverse configuration. In the uncut, isolated wing, increases in stimulus strength revealed a threshold above which a single EJP was produced in the muscle cell recording (similar to that shown in Fig. 11B). This was characteristic of both recording/stimulating configurations. Unfortunately, the type of muscle cell being recorded could not be determined in the isolated wing preparations. Cuts were made in the wing tissue between the recording and stimulating electrodes, followed by a repeat stimulus series (three preparations). EJPs were recorded in those preparations in which the cut was extended well beyond the direct alignment of the recording and stimulating electrodes, so long as neither the anterior nor the medial branch of the wing nerve was cut (Fig. 11B). If the cut was extended through either branch of the wing nerve, EJPs could not be elicited. The same effect (disappearance of EJPs) could be obtained by making a small incision that transected either nerve branch medial to the recording and stimulating electrodes, even though no cut was made directly between the two electrodes, and the stimulus voltage was increased 10-fold above the original threshold value (Fig. 11C). This suggests that there is no significant lateral spread of activity between the primary

Fig. 11. Lesion experiments using isolated wing preparations. In A, the locations of the stimulating (star) and recording (square) electrodes are shown. (Ai) An incision was made through the wing tissue as indicated by the dashed line. (Aii) A cut was made through wing nerve branch 1 (broken line). The recording shown in B was obtained following a cut similar to that shown in Ai (similar records were typical of pre-cut control recordings). Two stimuli were delivered from the suction electrode (arrowheads). The first was just below threshold, the second just above threshold. The second stimulus produced an EJP. The restricted cut shown in Aii blocked production of the EJP (as shown in C), despite stimulus strengths several times greater than the control threshold. In C, the stimulus artifacts exceeded the travel of the pen recorder.
nerve branches of the wing and that EJPs recorded in the intact wings were probably produced by antidromic stimulation of motor neuron axons in one branch of the wing nerve that was then conducted to the other branches.

**Discussion**

Previous work of Arshavsky *et al.* (1985a,b,c) and Satterlie and colleagues (Satterlie *et al.* 1985, 1990; Satterlie and Spencer, 1985; Satterlie, 1989, 1991a,b) has provided basic information about putative motor neurons in the swimming system of *Clione*. These investigations included descriptions of the morphology and sub- and supra-threshold electrical activity of motor neurons during swimming activity, as well as descriptions of the connectivity between motor neurons and pattern generator interneurons. This report extends these observations in four important ways. First, the connections between interneurons and motor neurons are confirmed, as is evidence for the monosynaptic nature of the majority of the non-electrotonic connections, including inhibitory connections. Although all possible combinations of interneurons and motor neurons have not been tested, all observations to date suggest that the connections are direct.

Second, motor neurons can be separated into two morphological classes on the basis of cell body size and the extent of their peripheral innervation of the wing. Small motor neurons have small cell bodies and innervate restricted fields within the ipsilateral wing. Large motor neurons (general excitors) have cell bodies that are up to five times larger than those of small motor neurons and innervate the entire ipsilateral wing and the leading edge of the contralateral wing. One general excitor innervates the dorsal swim musculature, whereas the other innervates the ventral swim musculature. Similar variation in motor neuron innervation fields has been reported for the parapodia of a swimming species of *Aplysia* (*Aplysia brasiliana*), although a clear dichotomy in motor neuron types, as seen in *Clione*, was not evident (McPherson and Blankenship, 1991b). Swim-related motor neurons were, however, clustered in functional groups in *Aplysia*.

Third, the peripheral connectivity of the two types of motor neurons is described (see Fig. 12). Small motor neurons innervate slow-twitch, fatigue-resistant muscle cells exclusively, as shown by a combination of physiological and morphological evidence (see Satterlie *et al.* 1990; Satterlie 1991a, for a description of muscle cell types in *Clione*). General excitors, in contrast, make excitatory connections with both slow-twitch and fast-twitch fibers. In all cases, neuromuscular connections were judged to be monosynaptic as shown by constant, short synaptic latencies and the persistence of transmission in sea water containing elevated levels of calcium and magnesium ions. In a number of molluscs, effectors have been found to be polynervously innervated (e.g. Carew *et al.* 1974; Cohen *et al.* 1978; Kobayashi, 1972; Orkand and Orkand, 1975; Peters, 1979; Peters and Altrup, 1984; McPherson and Blankenship, 1991a). In *Clione*, physiological evidence suggests that the slow-twitch musculature is polynervously innervated, although multiple inputs to individual muscle cells cannot be confirmed because of the electrical coupling between slow-twitch fibers (Satterlie, 1991a). Ultrastructural evidence supports polynervous innervation: slow-twitch muscle is innervated by three types of neuron terminals, each with a distinct population of vesicles (Neal, 1988).
Fourth, the roles of the two motor neuron groups in producing the dramatic change in swimming speed noted when \textit{Clione} accelerates from slow to fast swimming have been described. There are two primary means of increasing swimming speed: by increasing the frequency of movements of locomotory structures and by increasing the force of muscle contractions in the locomotory structures. Additionally, biomechanical changes, such as changes in the angle of attack and changes in wing thickness/turgor, can influence the degree of wing bending. In \textit{Clione}, an increase in wingbeat frequency is produced by pattern generator interneurons and is imposed upon the motor neurons through the interneuron-to-motor neuron connections (Arshavsky \textit{et al.} 1985\textit{c,d}; Satterlie, 1991\textit{b}). Because this report describes motor neurons, the primary concern is with the method of increasing contractile strength in the swim musculature, which is the province of the activity of motor neurons and muscle cells (and any modulatory inputs). The relationship between the activities of the two types of motor neurons, the activities of the two types of swim muscle cells and recorded wing movements, taken together, indicates that slow swimming movements are produced primarily through the activity of small motor neurons and slow-twitch muscle. During slow swimming, general excitor motor neurons receive alternating depolarizing and hyperpolarizing inputs from pattern generator interneurons, but either do not produce spikes or intermittently produce one or two spikes with each depolarization. Since both small motor neurons and general excitors must produce spike bursts to generate significant tension in the whole wing, the lack of spike burst activity in the general excitors at slow swimming speeds ensures that very little or no tension will be generated in fast-twitch muscles during slow swimming. During the switch to fast swimming, general excitors are recruited and produce a burst of spikes with each depolarizing input. This increased activity results in recruitment of fast-twitch...
muscle, enhancement of the size of junctional potentials and spike-like responses in slow-twitch fibers, and enhancement of the central excitatory inputs in at least some of the small motor neurons that are electrically coupled to the general excitors.

The additive effect of general excitor inputs to slow-twitch muscle is clearly evident, as both EJPs and spike-like responses increase in amplitude when general excitors are artificially or spontaneously recruited (see Fig. 9). Furthermore, repetitive activity in muscle cells results in nonsynaptic facilitation of EJPs and spike-like responses (Satterlie, 1991a). Once facilitated to near-maximal spike size through a combination of small motor neuron inputs and general excitor inputs, slow-twitch fiber spikes do not significantly decrease in size when general excitors stop producing spikes, provided that this ‘silent’ period does not last more than a few swim cycles. Thus, to maintain slow-twitch fibers in the facilitated state during fast swimming, general excitors do not need to fire on a regular basis, but rather can intermittently produce bursts of spikes. Fast-twitch fibers, which also exhibit nonsynaptic facilitation (Satterlie, 1991a), are more dependent on regular general excitor input to maintain a facilitated state, because the general excitors are their sole source of synaptic drive during normal fast swimming. With repetitive firing at frequencies characteristic of fast swimming, contractions of Clione wings show a force profile that includes an initial increase in force followed by a decline and eventual steady state (Satterlie et al. 1990). The steady-state contraction strength can be influenced directly by the activation and fatigue properties of the fast-twitch (fatigable) fibers and the level of activity in the slow-twitch (fatigue-resistant) fibers. Both are strongly influenced by the activity of the general excitor motor neurons.

One important question regarding the peripheral organization of molluscan neuromuscular systems concerns the distribution of motor output throughout a muscle field. Three ways to achieve widespread muscle activation are (1) direct activation by a few motor neurons with wide innervation fields and/or by many motor neurons with restricted innervation fields, (2) electrical coupling between muscle cells, and (3) peripheral distribution of excitation in a peripheral motor nerve net. Innervation fields of Clione motor neurons have been discussed above. Electrical coupling within muscle fields has been described in many molluscs (e.g. Cohen et al. 1978; Florey and Kriebel, 1969; Orkand and Orkand, 1975; Peters and Altrup, 1984; Tareskevich et al. 1977) and is limited to slow-twitch muscle fibers in Clione (Satterlie, 1991a). The significance of this coupling between slow-twitch fibers, but not between fast-twitch fibers, is not clear. Peripheral nerve nets have been found in motor fields of several molluscs (Bailey et al. 1979; Hoyle and Willows, 1973; Lukowiak and Peretz, 1977; Peretz, 1970; Peretz and Estes, 1974; Peretz and Moller, 1974; Prior, 1972). Although these are generally considered to be distributor networks, they can act in parallel with central motor neurons (Bailey et al. 1979; Carew et al. 1974). Electrophysiological evidence, including crude cutting experiments, does not support the involvement of a peripheral network in the swimming system of Clione. Furthermore, neuromuscular contacts are monosynaptic, muscle EJP latencies are short and constant in any one neuromuscular recording, and EJPs and spike-like responses mirror spike activity in motor neurons. Finally, all motor neurons have relatively dense arrays of putative axon terminals within their innervation fields. Monosynaptic innervation is further supported by morphological evidence,
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presented here at the light microscope level, which is currently being confirmed in an electron microscopical investigation of intracellularly marked motor neurons.

On behavioral, morphological, histochemical and electrophysiological grounds, the swimming system of Clione is judged to operate as a two-geared system (Arshavsky et al. 1985b,c,d; Satterlie et al. 1985, 1990; Satterlie and Spencer, 1985; Satterlie, 1991a,b). Here, this two-geared organization has been confirmed at the level of motor neurons. These results strongly support the contention that the dramatic increase of swimming speed during the change from slow to fast swimming is accomplished not only by a change in pattern generator cycle frequency, but also by activation of fast-twitch muscle and enhancement of activity in slow-twitch muscle through recruitment of the pair of general excitor motor neurons. Although this is a relatively simple system, the motor neuron recruitment pattern observed when Clione changes locomotory speed appears to follow some of the basic ‘rules’ of neuromuscular function, such as the Henneman size principle (Henneman et al. 1965; Henneman and Mendell, 1981; Stuart and Enoka, 1983), described for more phylogenetically complex animals. With solid background knowledge of the organization of pattern generator interneurons, swim motor neurons and the swim musculature, and a description of the changes that occur during dramatic changes in speed, the Clione preparation can now be used to investigate more subtle forms of speed change, such as those resulting from central and peripheral modulatory inputs and changes in sensory inputs to the swimming system; in other words, changes of speed within gears rather than changes of speed between gears.

I thank Dr A. O. D. Willows, Director of Friday Harbor Laboratories, for providing space and generous assistance, Dr T. Norekian and S. Jordan for critically reading the manuscript and Lou and Alison Satterlie, as well as Dr Claudia Mills and the rest of the Friday Harbor dock-walkers, for help in collecting animals. This study was supported by NIH grant R01 NS27951.

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