The role of postinhibitory rebound in the locomotor central-pattern generator of *Clione limacina*

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Synopsis In animals, networks of central neurons, called central-pattern generators (CPGs), produce a variety of locomotory behaviors including walking, swimming, and flying. CPGs from diverse animals share many common characteristics that function at the system level, circuit level, and cellular level. However, the relative roles of common CPG characteristics are variable among different animal species, in ways that suit different forms of locomotion in different environmental contexts. Here, we examine some of these common features within the locomotor CPG in a model system used to investigate changes in locomotory speed—the swim system of the pteropod mollusk, *Clione limacina*. In particular, we discuss the role of one cellular characteristic that is essential for locomotor pattern generation in *Clione*, postinhibitory rebound.

A quick scan of the animal kingdom reveals a variety of examples in which body appendages are moved rhythmically to produce forward locomotion. This includes animals that move in air, in water, and on land.

In some of these cases, movements of the appendages involve an active stroke, whereby some form of biomechanical advantage is gained, and a recovery stroke which may, or may not, offer some locomotory advantage. In animals with jointed skeletons (internal or external), multi-joint appendages undergo a complicated mix of coordinated movements of this nature, frequently with rotational components included. In other animals, and within some individual joints in multi-jointed appendages, the movements may be more symmetrical.

Despite variability in types of appendages, the way appendages move, and the environmental medium through (against) which they move, a basic need to coordinate antagonistic sets of muscles is found in many of these animals, and underlies locomotory movements. This requires rhythm-generating neural circuitry that produces a patterned output with at least two phases, either for the entire appendage or for some of the individual joints within a multi-jointed appendage.

Delcomyn (1980), Pearson (1993), Selverston et al. (1997), and Marder et al. (2005) discussed the features of rhythmic motor system control, including common features found at three important levels: system level, circuit level, and cellular level. From these reviews, we have selected an important feature at each organizational level, and discuss how each one relates to the neural control of a relatively simple locomotory system, the swim system of the pteropod mollusk, *Clione limacina*, and how these features are related to the body form and behavioral ecology of this unique animal.

Clione is a shell-less, holoplanktonic opisthobranch that is found in open ocean and coastal regions, where it is believed to undergo a daily vertical migration, descending to more than 100 m during the day and up to surface waters at night (Mackie and Mills 1983; Mackie 1985). It has two lateral wing-like parapodia, bent in such a way that they produce flapping, swimming movements. Two aspects of *Clione's* swimming behavior hold interest for this discussion. First, three distinct forms of swimming are known, both behaviorally and in terms of the identity and activity of neurons that generate the appropriate wing movements (Arshavsky et al. 1985b; Satterlie et al. 1985). *Clione* is negatively buoyant, and must swim more-or-less continually to maintain its position in the water column or to move upward. This is accomplished with wing movements showing a cycle

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frequency of 1–2 Hz (called slow swimming). When nonnociceptive stimuli are delivered to the tail or body wall, the wings show a sudden increase in both the strength and frequency of contractions; this acceleration increases wing-beat frequency to up to 5 Hz (called fast swimming). Fast swimming is also triggered during stimulation by food, in which the animal turns in fast loops (called hunting behavior), or during the acquisition phase of feeding, which also involves fast swimming. If nociceptive stimuli are delivered, the animal produces a ballistic startle response of only one or two extremely strong wing cycles that blend into a variable period of fast swimming and propel the animal up to 20 body lengths from its position when stimulated (Satterlie et al. 1997). A similar lunge is seen during the initiation of food acquisition.

The second aspect that is important to this discussion centers on the slow swimming mode only. Animals observed in the water column are nearly always swimming in the slow mode, and do so for extended periods, so that slow swimming appears to be a continuous behavior. If an animal touches the water surface, or bumps into something with the head end, swimming is inhibited resulting in passive sinking. Sinking is usually terminated within a short distance by arching the body (the tail acts as a rudder), asymmetrically altering wing contractility, and actively swimming back to a head-upward posture (Panchin et al. 1995a; Deliagina et al. 1998, 1999). This makes the *Clione* locomotory system somewhat unique in that slow swimming can be considered a background behavioral activity through much of the animal's daily activity cycle. This is an important distinction considering the construction of the neural circuit underlying swimming. Superimposed on this background activity are the other forms of swimming, including fast swimming, startle responses, and the swimming modifications seen during feeding behavior. We will focus on this second aspect of swimming to illustrate how the ecology of this particular animal impacts the basic principles of rhythmic motor control.

At the system level, rhythmic drive to locomotory muscles originates in a central- pattern generator (CPG) that continues to produce a normal rhythm when all peripheral nerves are cut (isolated central ganglia; Delcomyn 1980; Pearson 1993; Selverston et al. 1997). In many animals, particularly those with hard skeletal elements associated with locomotory appendages, a CPG exists but proprioceptive feedback may modify its output so that patterning of muscle activity emerges from this combination of central drive and sensory modification (Pearson 1993). Proprioceptive feedback has not yet been found to be important in *Clione* swimming. If muscle activity is blocked in slow swimming preparations by the cholinergic antagonist, hexamethonium, the CPG output is identical to that seen before hexamethonium application (Satterlie, manuscript in preparation). The wing skeleton is hydrostatic, which may complicate discovery of proprioceptive elements. This structural property of the *Clione* wings may require, or allow, less proprioceptive feedback as compared to the appendages of animals with hard skeletons. If this lack of proprioceptive modification turns out to be true, patterned muscle activity may retain more of the ''pure'' CPG output than in other locomotory systems.

The apparent lack of significant proprioceptive shaping of CPG activity does not rule out the existence of sensory inputs as major cycle-by-cycle modifiers of swimming in *Clione*. A tilt from the normal vertical orientation results in asymmetric alteration of muscle activity in the two wings and bending of the tail. Asymmetric muscle contractions and tail bending brings the animal back to the proper body orientation. A pair of statocysts, associated with the central ganglia, provides the sensory contribution for this spatial orientation (Panchin et al. 1995a, 1995b; Deliagina et al. 1998, 1999).

Wing movements in *Clione* during slow swimming are nearly symmetrical with both half-strokes producing forward propulsion (Satterlie et al. 1985). At least theoretically, this simplifies the neuronal drive necessary to coordinate the dorsal and ventral contractions of the wings, so CPG output approximates a simple and symmetrical two-phase activity pattern. At the circuit level, a common feature found in a variety of CPGs (both locomotory and nonlocomotory) is reciprocal inhibition between CPG neurons (Arshavsky et al. 1985c; Satterlie and Spencer 1985; Satterlie 1985; Satterlie and Norekian 2001). In CPGs with complex drive patterns, reciprocal inhibition may be embedded within more complex circuitry (Selverston et al. 1997). On the other hand, a simple two-phase activity pattern could emerge from two neurons or neuronal groups that interact exclusively via reciprocal inhibition. The *Clione* CPG for slow swimming includes two populations of pedal interneurons which interact through reciprocal inhibitory connections, reflecting the simplicity of the behavioral output. We note that the swim CPG is reconfigured during the change to fast swimming. This involves recruitment of additional neurons and results in an increase in the

complexity of interneuronal interactions, as compared to slow swimming (Arshavsky et al. 1985d, 1989; Pirtle and Satterlie 2006).

The output of any CPG is a mixture of synaptic and modulatory influences between component neurons and the cellular properties of each of those neurons (Pearson 1993; Selverston et al. 1997). Any active ion channels that influence the basic firing properties or shape the output of these cells, not only have major influences on the pattern of muscle activity, but also represent possible modulation targets for altering rhythmic motor drive. At the cellular level, two common features of many CPG circuits are the presence of endogenous rhythmic activity and postinhibitory rebound (PIR) in component neurons (Arshavsky et al. 1997; Selverston et al. 1997). Experiments conducted by Arshavsky et al. (1985c), Arshavsky et al. (1986), Panchin et al. (1995c), and Panchin et al. (1996) indicate that rhythmic endogenous activity of swim interneurons is important to locomotor rhythm generation in *Clione*. Our experiments with physically isolated swim interneurons (Satterlie et al. 2000) and with chemically isolated swim interneurons here and elsewhere (Pirtle and Satterlie 2004) do not substantiate a role for endogenous rhythmic activity in *Clione* swim interneurons. This discrepancy may result from differences in experimental technique. For example, Arshavsky et al. (1985c) used 15 mM $CoCl_2$ to show endogenous rhythmic activity in swim interneurons. However, as indicated in these experiments the ganglia were covered with a layer of agar so that the exact concentration of $CoCl_2$ applied to the ganglia remained unknown. Thus, the primary emphasis here is to show that PIR plays an important role in phase transitions in the *Clione* locomotor CPG.

Postinhibitory rebound (PIR) produces an immediate excitation following imposed hyperpolarizations, and is believed to contribute to phase transitions in rhythmic motor systems. PIR is an important property in slow-swim CPG neurons in *Clione* (Satterlie 1985). Each swim interneuron produces a single, broad action potential in its appropriate half-cycle, which in turn, produces an inhibitory synaptic potential in the antagonistic CPG neurons (Arshavsky et al. 1985b, 1985c; Satterlie and Spencer 1985; Satterlie et al. 2000; Satterlie and Norekian 2001). This gives rise to PIR in the antagonists, which can directly lead to generation of an action potential and return inhibition to the original neurons. The strength of PIR in these cells is such that, once swimming is initiated, the CPG can cycle in the absence of tonic drive for variable periods of time (Satterlie 1985). This does not suggest that tonic drive to the slow-swim CPG is unimportant. On the contrary, several pathways have been found that initiate CPG activity in quiescent animals, and that modify the output of ongoing activity (Panchin et al. 1995d; Satterlie and Norekian, 1995). This includes the switch from slow to fast swimming and all of the circuit and cellular modifications that go with it.

An additional property of PIR in *Clione* swim interneurons is illustrated when examining its ionic basis. Current clamp experiments substantiate the hypothesis that a calcium conductance underlies PIR in these cells. Application of the inorganic calcium channel blockers $CdCl_2$ or $NiCl_2$ prevented PIR in synaptically isolated swim interneurons with Cd^{2+} being the more potent. PIR was also reduced in Ca^{2+}-free seawater (Fig. 1). Similarly, use of a sodium-free saline decreased the amplitude of PIR in swim interneurons, suggesting that the ionic basis of PIR may include both calcium and sodium components (data not shown).

In addition to calcium and sodium contributions, a hyperpolarization-activated inward current, I_h, and its resultant sag potential, influences the timing of PIR in swim interneurons. Previous experiments have shown that serotonin enhanced PIR in physically isolated interneurons (Satterlie et al. 2000). An inhibitor of I_h, Cs^+ inhibits the sag potential and concurrently increases the latency to PIR without altering its amplitude (Pirtle and Satterlie 2004). Similarly, a specific inhibitor of I_h, ZD7288, consistently inhibited the sag potential and increased the latency to peak PIR (Fig. 2; latency before ZD7288 is 47.8 ± 8.17 ms and latency with ZD7288 is 75.3 ± 8.14 ms; $n = 4$; $P = 0.0116$).

Serotonin, and identified serotonergic neurons in the cerebral ganglia of *Clione*, initiate swimming in quiescent preparations and produce the change from slow to fast swimming in active preparations (Arshavsky et al. 1985a, 1992; Panchin et al. 1995d; Satterlie and Norekian 1995). Serotonin enhanced the sag potential and significantly decreased the latency to peak PIR in swim interneurons (Fig. 3; latency before serotonin is 81.7 ± 15.1 ms and latency with serotonin is 46.8 ± 6.01 ms; $n = 7$; $P = 0.0142$; paired *t*-test), thus contributing to the increase of cycle frequency characteristic of serotonin-induced acceleration of swimming.

These observations suggest that at least one ionic component of PIR in *Clione* swim interneurons is a modulatory target for acceleration-inducing inputs from identified higher-order neurons, and that this modulation may affect both the strength of PIR and

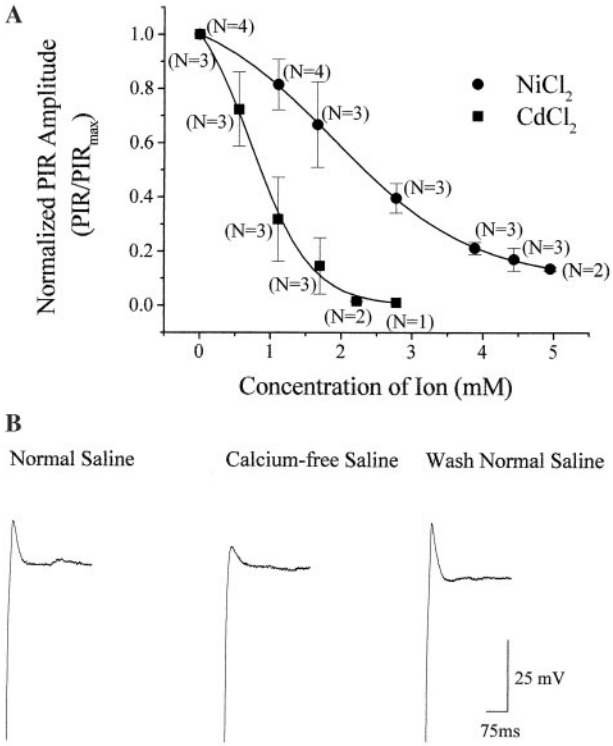

Fig. 1 The effects of inorganic calcium channel blockers and calcium-free saline on PIR. (**A**) $NiCl_2$ (circles) and $CdCl_2$ (squares) block PIR in current clamp experiments in which the swim interneurons were synaptically isolated using chemical means by applying tetrodotoxin (10 μM), atropine (10 mM), and CNQX (10 μM). The $NiCl_2$ or $CdCl_2$, prepared from isotonic stock solutions, were administered at different concentrations to show the relative potency of these calcium channel antagonists on inhibiting PIR. Dividing the PIR amplitude in experimental saline containing $NiCl_2$ or $CdCl_2$ by the PIR amplitude in control saline normalized PIR amplitude in separate experiments. Normalized PIR amplitudes from these experiments were averaged and are expressed as the mean ± SEM. Normalized PIR amplitude is plotted as a function of $CdCl_2$ and $NiCl_2$ concentration and fitted using a sigmoid function. The numbers of replicated experiments for each concentration of $NiCl_2$ and $CdCl_2$ are given in parentheses next to each data point. This experiment demonstrates that $CdCl_2$ is more potent at blocking PIR. The EC_{50} for $CdCl_2$ and $NiCl_2$ are 0.667 mM and 1.92 mM, respectively. (**B**) Calcium-free saline-inhibited PIR in synaptically isolated interneurons further demonstrating the calcium dependency of PIR. Applying normal saline reverses the effect. PIR was evoked in each recording by injecting a 1s duration, −1nA current.

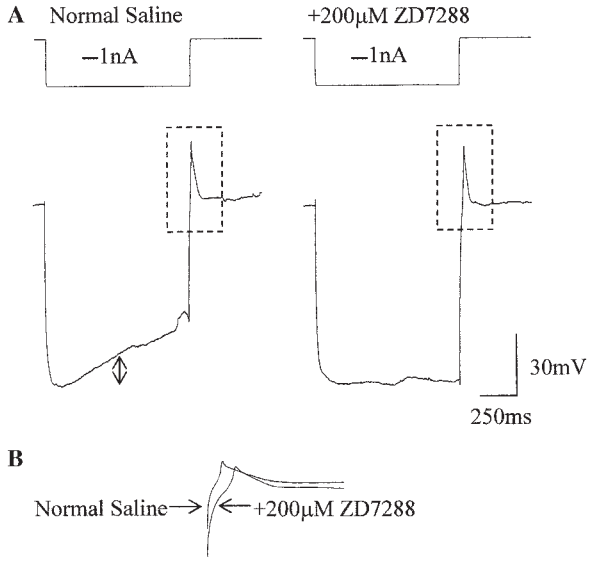

Fig. 2 The effects of ZD7288 on the sag potential and latency to PIR. (**A**) Application of ZD7288, an I_h antagonist, blocks the sag potential characteristic of cells having I_h (changes in sag amplitude were measured at the midpoint of the hyperpolarizing current injection and calculated by taking the difference between control and experimental values of sag amplitude; double headed arrow). ZD7288 also hyperpolarizes the cell. (**B**) Expanded part of the record outlined by the dashed boxed-in area in (A) shows that ZD7288 also significantly increases the latency to peak PIR from 47.8 ± 8.17 ms before ZD7288 to 75.3 ± 8.14 ms with ZD7288 (arrows; $n = 4$; $P = 0.0116$; paired t-test). Normal saline consisted of seawater plus 10 μM TTX, 10 mM Atropine, and 10 μM CNQX to suppress synaptic activity.

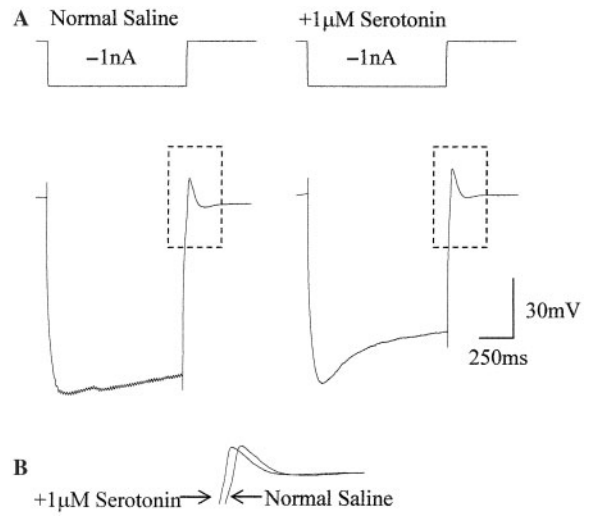

Fig. 3 The effects of serotonin on the sag potential and latency to PIR. (**A**) Application of serotonin enhances the sag potential and significantly decreases the latency to peak PIR from 81.7 ± 15.1 ms before serotonin to 46.8 ± 6.01 ms with serotonin is ($n = 7$; $P = 0.0142$; paired t-test). (**B**) Expanded part of the record outlined by the dashed boxed-in area in (A) shows detail of serotonin's effect on the latency to peak PIR (arrows). Normal saline consisted of seawater plus 10 μM TTX, 10 mM Atropine, and 10 μM CNQX to suppress synaptic activity.

its latency, thus reinforcing phase transitions, and contributing to increases in cycle frequency during acceleration of swimming.

The overall locomotory system of *Clione limacina* can be considered simple in comparison to that of most other animals, and this can be traced to the phylogenetics and natural history of this animal group.

The lack of significant proprioceptive feedback (thus far) can be attributed to the organization of the body of these soft-bodied opisthobranchs, and to the hydrostatic nature of their skeleton, particularly of the wings.

The importance of reciprocal inhibition and PIR in the slow swim CPG of *Clione* seems greater than in other CPGs, in terms of its influence on the overall rhythmic output. This strong dependence on this property of the circuit (reciprocal inhibition) and of the cellular property (PIR) presumably results from the behavioral ecology of the animal. Slow swimming is a near-symmetrical two-phase activity. Furthermore, it can be considered a baseline behavior during much of the animal's daily activity cycle. This is presumably reflected in an experimental challenge for studying many CPGs—the need to reliably turn on the rhythmic behavior so it can be examined. The opposite problem is often the case with swimming in *Clione*. The challenge is to turn off swimming so aspects of its neuronal underpinnings can be tested. The application of the antagonists, atropine (10 mM), 6-cyano-7-nitroquinoxaline-2, 3-dione disodium salt (CNQX, 10 μM) and tetrodotoxin (TTX, 10 μM) were used to suppress synaptic activity, thus silencing the rhythmic output of swim interneurons, and thereby make investigation of cellular properties (PIR) possible.

Neuronal circuits that produce a long-term or continuous behavior as a background activity would be expected to have characteristics that support a robust expression of its cyclic motor drive. In *Clione's* case, strong PIR may serve as a back-up to tonic drive and thereby ensure long bouts of swimming activity even with central drives that are variable in strength or intermittent in occurrence. Add to this the possibility that ion currents underlying PIR may serve as a modulatory target, helping alter the frequency of swim cycling, and PIR becomes an important player in the behavioral output and plasticity of this locomotory system.

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