Coordination of Startle and Swimming Neural Systems in the Pteropod Mollusk *Clione limacina*: Role of the Cerebral Cholinergic Interneuron

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Norekian, Tigran P. Coordination of startle and swimming neural systems in the pteropod mollusk *Clione limacina*: role of the cerebral cholinergic interneuron. J. Neurophysiol. 78: 308–320, 1997. The holoplanktonic pteropod mollusk *Clione limacina* has a unique startle system that provides a very fast, ballistic movement of the animal during escape or prey capture behaviors. The startle system consists of two groups of large pedal motoneurons that control ventral or dorsal flexions of the wings. Although startle motoneurons innervate the same musculature used during normal swimming, they are independent of the swim central pattern generator and swim motoneurons. This study demonstrates that a cerebral startle (Cr-St) interneuron, which provides prominent excitatory inputs to startle motoneurons, plays a very important role in coordination of the startle and swimming neural systems. The Cr-St interneuron produces, simultaneously with monosynaptic excitatory inputs to dorsal startle motoneurons, monosynaptic inhibitory inputs to all types of swim neurons, including interneurons of the central pattern generator, general excitor motoneurons, small motoneurons, and modulatory pedal serotonergic wing neurons. The inhibitory synaptic transmission between the Cr-St interneuron and swim interneurons and motoneurons, as well as excitatory transmission between the Cr-St interneuron and startle motoneurons, appears to be cholinergic because it is blocked by the cholinergic antagonists atropine and d-tubocurarine, mimicked by exogenous acetylcholine in very low concentrations, and enhanced by the cholinesterase inhibitor eserine (physostigmine). The Cr-St-neuron-mediated inhibitory inputs to the swimming system are strong enough to completely terminate swimming activity while the Cr-St interneuron is active. Mechanosensory inputs are capable of triggering Cr-St neuron firing at rates sufficient to suppress fictive swimming in reduced preparations. Thus the Cr-St interneuron can temporally remove the swimming system from the control over the swim musculature while simultaneously activating the startle system to produce a powerful, short-latency response.

INTRODUCTION

Coordination of the activities of different neuronal centers is always crucially important for the normal initiation of a particular behavior, whether these centers underlie mutually exclusive behaviors or sustain the same behavioral goal. Understanding how interactions between different neuronal systems are organized has always been an important goal for neurobiological investigations.

The typical mechanism of coordination between motor centers, underlying mutually exclusive behaviors, includes strong inhibitory connections between these centers. In the crayfish *Procambarus*, the lateral giant tailflip escape system is inhibited by feeding (Krasne and Lee 1988) and by the backward walking neural programs (Beall et al. 1990). In the mollusk *Pleurobranchaea*, the feeding network inhibits the withdrawal command neuron (Kovac and Davis 1980) and is inhibited by neural elements that mediate escape swimming (Jing and Gillette 1995). In the pteropod mollusk *Clione limacina*, pleural neurons controlling whole body withdrawal behavior strongly inhibit swimming activity (Norekian and Satterlie 1996), and pedal interneurons of the local wing retraction system produce monosynaptic inhibition of swim interneurons and motoneurons (Huang and Satterlie 1990).

Many behaviors sustain similar functional goals and can be activated together. However, a coordination of their underlying neural networks is also required. One example of such closely related behaviors is swimming and the startle response in *Clione*. *Clione* spends most of its time swimming with the help of two winglike parapodia (Lalli and Gilmer 1989; Wagner 1885). The swim central pattern generator consists of two antagonistic groups of interneurons localized in the pedal ganglia (Arshavsky et al. 1985a,b; Satterlie 1985). Synergistic interneurons are electrically coupled, whereas interneurons from opposite phases of the swim cycle (dorsal and ventral) interact via reciprocal inhibitory connections. All swim motoneurons are also located in the pedal ganglia and divided into d-phase and v-phase neurons, which initiate dorsal and ventral bending of the wings, respectively (Arshavsky et al. 1985a,b; Satterlie and Spencer 1985).

Animals can accelerate from slow to fast swimming, smoothly or abruptly, for example during escape swimming or during initiation of feeding behavior (Litvinova and Lory 1985; Norekian 1995; Sakharov and Kabotyansky 1986; Satterlie et al. 1985). The difference between slow and fast swimming involves an increase in wingbeat frequency, and includes reconfiguration of the swim pattern generator and recruitment of additional types of motoneurons (Arshavsky et al. 1985a,b; Satterlie 1993). Nevertheless, wing movements during all these modifications of swimming are controlled by the same neural network—swim interneurons of the central pattern generator and swim motoneurons.

Occasionally, *Clione* exhibits a startle response, which is quite different from a regular fast or slow swimming activity. The startle response involves one or two powerful wingbeats, which propel the animal forward at an extrapolated rate of nearly 18 body lengths per second, compared with 3–6 body lengths per second during fast swimming (Satterlie et al. 1997). The startle response can be induced by tactile stimulation of the tail and is typically followed by a prolonged...
period of regular fast swimming (Satterlie et al. 1997). It can be triggered in nonswimming, slowly swimming, or fast-swimming animals at any phase of the swim cycle, temporarily overriding regular swimming activity.

The startle response is controlled by two pairs of large pedal motoneurons that have very high membrane potentials and high thresholds for spike generation (Satterlie et al. 1997). One type of startle motoneurons produces dorsal flexion of the wings (d-phase motoneurons), and other type produces ventral flexion of the wings (v-phase motoneurons). Each startle motoneuron directly innervates swim musculature in the ipsilateral wing, the same musculature used during normal swimming (Satterlie et al. 1997). Nevertheless, startle motoneurons do not form synaptic connections with the swim pattern generator and swim motoneurons, nor are connections found in the reverse direction (Satterlie et al. 1997).

A bilaterally symmetrical interneuron that receives appropriate excitatory mechanosensory inputs and activates startle motoneurons has been recently identified in the cerebral ganglia of Clione [cerebral startle (Cr-St) interneuron] (Norekian and Satterlie 1997). The Cr-St interneuron strongly activates ipsilateral d-phase startle [ST(d)] motoneurons by producing monosynaptic, fast excitatory postsynaptic potentials (EPSPs) of unusually high amplitudes (up to 50 mV). Thus the Cr-St interneuron provides an essential excitatory input to startle motoneurons and appears to be an important element of the startle response circuitry.

The present study demonstrates that the role of the bilaterally symmetrical Cr-St interneuron is much more important than simply transferring the excitatory inputs to the startle motoneurons. It is found to be involved in coordination of the activities of both startle and swimming systems during initiation of a startle response. This is accomplished by producing strong, but temporary, inhibitory inputs to the swimming system, which temporarily terminate swimming while simultaneously activating startle motoneurons.

METHODS

Adult specimens of C. limacina, 2–3 cm in body length, were collected from the breakwater of Friday Harbor Laboratories and held in 1-gallon jars in a refrigerator at 5°C. Animals were anesthetized in a 1:1 mixture of sea water and isotonic MgCl₂ and dissected in a Sylgard (Dow Corning)-coated Petri dish. Electrophysiological experiments were performed on reduced preparations consisting of the CNS, head, and wings. All nerves running from the central ganglia to the head and wings remained intact, whereas body nerves were cut. Tactile stimulation of the lips and buccal cones was provided by a thin polymeric filament 0.2 mm diam. The filament was attached to a plastic stick, which was hand held. The elasticity and length of the filament (4 cm) allowed a relatively consistent strength of stimulation from trial to trial.

Before recording, the sheaths of the ganglia were softened by bathing the preparation in a 1 mg/ml solution of protease (Sigma, type XIV) for ~5 min, followed by a 30-min wash. Standard electrophysiological techniques were used for intracellular recording. Glass microelectrodes were filled with 2 M potassium acetate and had resistances of 10–30 MΩ. When putative chloride-mediated inhibitory postsynaptic potentials (IPSPs) and acetylcholine (ACh)-induced hyperpolarizations were investigated, electrodes were filled with 3 M potassium chloride; chloride leakage from the electrodes caused the required increase in intracellular chloride concentration. To test for monosynaptic connections, a high-Mg²⁺/high-Ca²⁺ solution (110 mM MgCl₂, 25 mM CaCl₂) was used. Intracellular staining of neurons was achieved via recording electrodes filled with a 5% solution of carboxyfluorescein (Molecular Probes), which was iontophoresed with the use of negative current pulses. Semi-intact preparations were then immobilized in MgCl₂/sea water and observed live in the recording dish with the use of a fluorescence microscope (Nikon) with a B2 filter cluster.

ACh chloride (Sigma), its agonist carbachol (Sigma), antagonists (atropine sulfate, Sigma; d-tubocurarine chloride, Sigma; hexamethonium chloride, USBC), and the cholinesterase inhibitor eserine (Sigma) were applied with the use of a graduated 1-ml pipette. The final concentration was estimated from the volume of injected solution and the volume of saline in the recording dish. To test whether a particular neuron was directly sensitive to ACh, two methods were used. The first involved application of ACh to neurons that were chemically isolated by a high-Mg²⁺ solution consisting of a 1:3 mix of 0.33 M MgCl₂ in sea water superfused into the preparation dish. The second method involved local application of ACh directly onto the cell body of the recorded neuron by microiontophoreses. Glass micropipettes with tip diameters of 1–2 mm were filled with a 10 mM solution of ACh prepared in distilled water. ACh was iontophoresed with the use of positive current pulses with an amplitude of 100 nA and a duration of 300 ms. To measure the reversal potentials of IPSPs and ACh-induced hyperpolarizations, two microelectrodes were used to penetrate the neuron, one for current injection and the other for voltage recording. Measurements of IPSPs were made in a high-Mg²⁺/high-Ca²⁺ saline to eliminate spontaneous synaptic inputs, and the high-amplitude swim rhythm in swim interneurons and motoneurons, which masked individual IPSPs. Measurements of ACh-induced changes of membrane potential were carried out in high-Mg²⁺ saline.

RESULTS

Cr-St interneuron activated both ipsilateral ST(d) motoneurons

The Cr-St interneuron has been previously shown to produce monosynaptic excitatory inputs to the ipsilateral ST(d) motoneuron (Norekian and Satterlie 1997). In this investigation, an additional ST(d) motoneuron was identified in each pedal ganglion, and was named the ST2(d) neuron. The originally described startle motoneuron was labeled as the ST1(d) neuron. The ST2(d) neuron had a somata 40 ± 3 cm in body length, were cut. Tactile stimulation of the lips and buccal cones was provided by a thin polymeric filament 0.2 mm diam. The filament was attached to a plastic stick, which was hand held. The elasticity and length of the filament (4 cm) allowed a relatively consistent strength of stimulation from trial to trial.

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Induced spike activity in a Cr-St interneuron produced strong activation of both ipsilateral ST(d) motoneurons (Fig. 1C). Each spike in a Cr-St interneuron produced a single EPSP in both ST1(d) and ST2(d) neurons (n = 7, Fig. 1C). EPSPs in ST2(d) neurons were identical to previously described EPSPs in ST1(d) neurons (Norekian...
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In addition to activation of the ST(d) motoneurons, the Cr-St interneuron was found to produce a strong inhibition of swimming activity (n = 54, Figs. 2A, 3A, and 4A). Inhibition of swimming was brief and lasted as long as Cr-St interneuron was spiking, simultaneously producing brief activation of the ST(d) motoneurons (Figs. 2A, 3A, and 4A). When induced firing of a Cr-St interneuron was terminated, ST(d) motoneurons instantly returned to their resting, silent mode and the swimming system resumed fictive swimming activity.

The Cr-St interneuron could act by targeting all or only some elements of the swimming system. For example, it could have differential influence on the d-phase and v-phase neurons. The latter situation is feasible because the Cr-St interneuron activates only d-phase motoneurons (Norekian and Satterlie 1997). In this investigation, it was found that the Cr-St interneuron produced widespread, inhibitory inputs to all neuron types of the swimming system, including d-phase and v-phase general excitor (GE) motoneurons, small motoneurons, and interneurons of the central pattern generator. There are two GE swim motoneurons in each pedal ganglion that are recruited into activity during fast swimming; one of the d-phase and one of the v-phase of the swim cycle (Satterlie 1993). The Cr-St interneuron produced inhibitory inputs to both ipsilateral GE swim motoneurons (n = 32, Fig. 2). Each spike in a Cr-St interneuron initiated a single IPSP in each GE motoneuron along with a single EPSP in ipsilateral ST2(d) motoneuron. Fast IPSPs persisted in high-Mg²⁺/high-Ca²⁺ saline and demonstrated the stable one spike:one IPSP ratio, suggesting monosynaptic connections (Fig. 2, B and C). Monosynapticity was also confirmed by the very short and constant delay between spikes in Cr-St neurons and IPSPs in ST2(d) neurons (2 ms, n = 12). In a few experiments (n = 3) the ST1(d) neuron had its axon severed and could not trigger motor output. Nevertheless, stimulation of the Cr-St interneuron still initiated a strong contraction of the ipsilateral wing musculature via activation of the remaining ST2(d) neuron.

Cr-St interneuron terminated swimming activity by directly inhibiting all neuron types of the swimming system.
The central pattern generator, which generates the swim rhythm and drives the activities of swim motoneurons, consists of two antagonistic groups of pedal interneurons (Arshavsky et al. 1985a,b; Satterlie 1985). The Cr-St interneuron produced direct inhibition of all recorded swim interneurons of both phases in all studied preparations (n = 18 neurons in 12 preparations, Fig. 4). Each spike in a Cr-St interneuron produced a single IPSP in each interneuron (n = 16, Fig. 4, B–D). IPSP amplitudes varied between 2 and 8 mV. IPSPs persisted in high-Mg$^{2+}$/high-Ca$^{2+}$ saline and demonstrated a stable one spike:one IPSP ratio, suggesting monosynaptic connections (n = 16, Fig. 4, B–D). One of the most noticeable features of swim interneurons is their ability to generate a strong postinhibitory rebound, which has been proposed as a mechanism contributing to pattern generation in the Clione swimming system (Satterlie 1985). IPSPs in swim interneurons, produced by Cr-St interneuron spiking, were capable of triggering postinhibitory rebound (Fig. 4D). A few individual IPSPs or a group of IPSPs with small amplitudes were usually not effective. However, when inhibition produced by the Cr-St interneuron was strong enough, it always triggered a postinhibitory rebound. This postinhibitory rebound could be clearly seen in high-Mg$^{2+}$/high-Ca$^{2+}$ saline as slow depolarization of the membrane potential after an induced burst of IPSPs in a swim interneuron (Fig. 4D).

IPSPs produced by Cr-St interneurons in swim interneurons and motoneurons of different phases and types were similar in their amplitudes and durations. They also appeared to be similar in their ionic mechanisms. The reversal potentials for IPSPs in swim interneurons and motoneurons were around −70 mV (−70 ± 1.5 mV, mean ± SD, n = 7, 1985).
Fig. 4. Cr-St interneuron inhibited interneurons of swim central pattern generator. A: induced burst of spikes in a Cr-St interneuron produced activation of an ST(d) motoneuron and inhibition of swim central pattern generator as traced via recording d-phase swim interneuron SI(d) activity. Each spike in an SI(d) interneuron is correlated with dorsal flexion of wings. Scale bars: 10 mV, 1 s. Each spike in a Cr-St interneuron produced an individual IPSP in a d-phase swim interneuron (B) and a v-phase swim interneuron (C) in high-Mg²⁺/high-Ca²⁺ sea water. D: prominent Cr-St-interneuron-mediated inhibition of swim interneurons triggered a postinhibitory rebound. Arrow: brief depolarization of a swim interneuron membrane potential induced by a group of Cr-St-neuron-mediated IPSPs. Scale bars: 10 mV, 200 ms.

Fig. 5. A). When the recording electrodes filled with 2 M potassium acetate were replaced with electrodes filled with 3 M potassium chloride, IPSPs in swim interneurons and motoneurons slowly decreased in amplitude and reversed 5–10 min after penetration (n = 9, Fig. 5, B and C). Leakage of chloride ions from the tips of such electrodes caused an increase of intracellular chloride concentration and a shift in the reversal potential of chloride-mediated responses in the depolarizing direction. Thus these experiments indicate that an increase in membrane permeability to chloride ions was involved in generating IPSPs produced by Cr-St interneurons in swim interneurons and motoneurons.

One additional group of pedal neurons involved in the regulation of swimming activity is the cluster of serotonergic cells that innervate the wings and produce a temporary increase in swim muscle contractility without direct motor effects [pedal serotonergic wing (Pd-SW) neurons] (Satterlie 1995). The firing pattern of these modulatory neurons is closely correlated with the activity of the swim central pattern generator and swim motoneurons, with synchronous periods of inhibition and acceleration. Cr-St interneurons produced direct inhibitory inputs to the ipsilateral Pd-SW neurons (n = 18, Fig. 6A). Each spike in a Cr-St interneuron produced a single IPSP in each Pd-SW neuron (n = 14, Fig. 6B). IPSPs persisted in high-Mg²⁺/high-Ca²⁺ saline and demonstrated the stable one spike:one IPSP ratio (n = 14, Fig. 6B). IPSP amplitudes varied between 2 and 6 mV and were sufficient to inhibit spontaneous spike activity in Pd-SW neurons (Fig. 6A). When inhibitory inputs to Pd-SW neurons had amplitudes >5 mV and lasted >0.5 s, they were usually followed by a brief period of Pd-SW neuron activation (n = 10, Fig. 6A). Postinhibitory rebound was found to be a mechanism of this increased spike activity in Pd-SW neurons after Cr-St-neuron-induced inhibition. Negative current pulses injected in a Pd-SW neuron, which produced hyperpolarizations similar to the Cr-St-neuron-induced inhibition, triggered similar postinhibitory activation (n = 12, Fig. 6C).

Mechanosensory inputs: Cr-St interneuron coordinated activities of the startle and swimming systems

Tactile stimulation of the tail, which triggers a startle response in intact animals, has been previously shown to produce excitatory inputs to Cr-St interneurons and startle motoneurons (Norekian and Satterlie 1997). In addition, tactile stimulation of the lips or buccal cones, which is known to initiate prey capture reactions with increased swimming activity in intact animals, has been shown to produce excitatory inputs to Cr-St interneurons, startle motoneurons, and the swimming system (Norekian 1995; Norekian and Satterlie 1997). In this investigation, it was found that tactile stimulation of the buccal cones or lips was the most stable and reliable source of excitatory mechanosensory inputs to both the startle and swimming systems in reduced preparations. Therefore this type of stimulation was used in the experiments in which the role of the Cr-St interneuron in coordination of the activities of the two systems was studied.

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When tactile stimulation failed to initiate spike activity in Cr-St interneurons and startle motoneurons, generating instead only subthreshold depolarizing inputs, immediate acceleration of swimming occurred without any preceding inhibitory inputs \( (n = 28\) in 7 preparations, Fig. 7B) . This period of fast swimming lasted up to 20 s. In some experiments, tactile stimulation of the lips and buccal cones produced strong inhibitory inputs to the swimming system without triggering activity in Cr-St interneurons and startle motoneurons \( (n = 17\) in 4 preparations) . This indicated the existence of inhibitory pathways to the swimming system other than the Cr-St interneuron pathway.

One of the important questions concerning sensory inputs is the importance of a single Cr-St interneuron in swimming inhibition. It appeared that Cr-St interneuron spiking, induced by tactile stimuli, was sufficient to trigger a strong swimming inhibition. Bursts of Cr-St neuron spikes induced by mechanosensory inputs were similar in strength and duration to intracellularly induced bursts that were sufficient to produce a strong inhibition of fictive swimming in reduced preparations. In four additional experiments, preparations were used in which tactile stimulation of the lips and buccal cones produced swim acceleration and subthreshold depolarizing inputs to Cr-St interneurons (Fig. 8, A and C). Depolarization of a Cr-St neuron close to firing threshold enabled tactile stimuli to trigger a burst of spikes in the Cr-St interneuron that produced a prominent inhibition of swimming activity (Fig. 8B). These experiments indicated that a single Cr-St interneuron was sufficient to trigger a noticeable behavioral reaction in the reduced preparations—termination of fictive swimming along with initiation of the startle response, thus coordinating activities of the two neuronal systems.

Cholinergic nature of the inhibitory inputs from Cr-St neurons to swim neurons

Because it has been previously concluded that the transmission between Cr-St interneurons and ST(d) motoneurons was cholinergic (Norekian and Satterlie 1997) , it appeared logical to suggest that inhibitory transmission from Cr-St interneurons to motoneurons was cholinergic.

![Figure 5](http://jn.physiology.org/)

**Figure 5.** A: injection of a negative current into GE(d) motoneuron changed its membrane potential in several steps from resting level of \(-52\) mV to \(-85\) mV. Reversal of IPSPs to EPSPs occurred at a membrane potential around \(-70\) mV. Electrical activity of a Cr-St interneuron whose spikes produced postsynaptic potentials in GE(d) motoneuron is not shown. Experiment was conducted in high-Mg\(^{2+}\)/high-Ca\(^{2+}\) saline to prevent swim rhythm and other synaptic inputs from masking Cr-St-interneuron-induced postsynaptic potentials. B: when recording electrode in a GE(d) motoneuron was filled with 2 M potassium acetate (KAcet), normal IPSPs were induced by spike activity in a Cr-St interneuron. C: when electrode was replaced with one containing 3 M potassium chloride (KCl), normal Cr-St-interneuron-induced IPSPs became reversed 15 min after penetration. Note that GE(d) neuron membrane potential remained same after electrode replacement \((-54\) mV). Scale bars: 10 mV, 200 ms.

Tactile stimulation of the buccal cones or lips produced two types of reactions in most cases. When mechanosensory inputs triggered a burst of spikes in a Cr-St interneuron and a subsequent activation of ST(d) motoneurons, a brief period of inhibition occurred in the swimming system immediately followed by a period of fast swimming that lasted for the next 10–20 s \( (n = 21\) in 5 preparations, Fig. 7A). Inhibition of swimming activity lasted as long as Cr-St interneurons and ST(d) motoneurons were active, usually <1 s.

![Figure 6](http://jn.physiology.org/)

**Figure 6.** Cr-St interneuron inhibited serotonergic modulatory Pd-SW neurons. A: induced burst of spikes in a Cr-St interneuron produced prominent hyperpolarization and inhibition of a Pd-SW neuron. Note brief excitation in Pd-SW neuron after inhibition. Scale bars: 10 mV, 1 s. B: each spike in a Cr-St interneuron produced an individual IPSP in a Pd-SW neuron in high-Mg\(^{2+}\)/high-Ca\(^{2+}\) sea water. Scale bars: 10 mV, 200 ms. C: hyperpolarization of a Pd-SW neuron via intracellular injection of a negative current pulse triggered postinhibitory rebound and brief excitation of a Pd-SW neuron after termination of stimulus. Scale bars: 10 mV, 1 s.
Mechanosensory inputs. A: tactile stimulation of the buccal cones (\( f \)) induced strong bursts of spikes in a Cr-St interneuron and an ST(d) motoneuron, and brief inhibition of swimming recorded in a GE(d) motoneuron. Swimming inhibition was immediately followed by a period of fast swimming. B: tactile stimulation of the buccal cones (\( f \)) produced subthreshold excitatory inputs to a Cr-St interneuron and an ST(d) motoneuron, and strong acceleration of swimming recorded in a GE(d) motoneuron. Note absence of swimming inhibition before its acceleration observed in A. Scale bars: 10 mV, 1 s.

The cholinergic antagonist \( d \)-tubocurarine was found to reversibly block transmission from Cr-St interneurons to swim motoneurons and interneurons (\( n = 11 \), Fig. 9, A and C). The Cr-St-neuron-induced IPSPs in all studied GE swim motoneurons, small motoneurons, and interneurons of the central pattern generator were blocked by \( d \)-tubocurarine. The concentration of \( d \)-tubocurarine necessary for the complete blockade of IPSPs was 50 \( \mu M \). IPSPs were usually blocked 3–5 min after \( d \)-tubocurarine application and were restored after a 10-min wash in sea water (Fig. 9, A and C). Atropine was also very effective, and reversibly blocked IPSPs induced by spike activity in a Cr-St interneuron in all types of swim interneurons and motoneurons (\( n = 9 \), Fig. 9B). The complete blockade of IPSPs required atropine concentrations of \( \geq 20 \mu M \), occurred 3–5 min after atropine application, and was washed out after 10 min in sea water (Fig. 9B). Hexamethonium was much less effective and produced only partial, reversible blocking of synaptic transmission from Cr-St neurons to swim neurons at concentrations of \( \geq 500 \mu M \) (\( n = 3 \)).

Eserine (physostigmine), a known inhibitor of cholinesterase activity, had a dramatic effect on postsynaptic potentials induced by Cr-St interneuron spikes (\( n = 9 \), Fig. 10). It did not significantly change the amplitudes of the IPSPs in swim interneurons and motoneurons, but increased the duration of hyperpolarizing responses by \( \sim 10 \)-fold (Fig. 10A). Similarly, EPSPs in ST(d) motoneurons were broadened by eserine 5–10-fold without a significant change in their amplitudes (Fig. 10B). Eserine had a dramatic effect at concentrations as low as 10 \( \mu M \). The changes occurred after 2–3 min of eserine application, and persisted even after a 1-h wash in sea water.

All swim motoneurons and interneurons were found to be very sensitive to exogenous ACh, responding to its application with a prominent hyperpolarization. However, when ACh was bath applied in sea water, the result was a strong activation of swimming activity due to the parallel activation of some excitatory inputs to the swimming system (\( n = 5 \)). A similar excitatory effect of ACh, when applied to the cerebral ganglia of \textit{Clione}, on locomotion was described...
COORDINATION OF STARTLE AND SWIMMING NEURAL NETWORKS

FIG. 8. Tactile stimulation of the buccal cones (↓) produced only subthreshold excitatory inputs to Cr-St interneuron and acceleration of swimming recorded in a GE(d) motoneuron (A). When Cr-St interneuron was depolarized close to spike threshold so that tactile stimulation induced a strong burst of spikes in Cr-St interneuron, an episode of swim inhibition appeared in GE(d) neuron followed by acceleration (B). Returning Cr-St interneuron to its normal resting potential removed strong burst of spikes in Cr-St interneuron along with prominent inhibition of swimming as a response to tactile stimulation of the buccal cones (C). Scale bars: 10 mV, 1 s.

by Arshavsky et al. (1993). Even in high-Mg$^{2+}$/high-Ca$^{2+}$ saline, ACh application caused a brief, initial hyperpolarization that was then overridden by strong excitatory synaptic inputs ($n = 7$). Only in experiments with high-Mg$^{2+}$ saline, which completely blocks chemical transmission in the CNS, was the direct sensitivity of swim interneurons and motoneurons to ACh established. ACh in concentrations as low as 1 μM produced hyperpolarization of d-phase and v-phase interneurons of the swim central pattern generator, GE swim motoneurons, small motoneurons, and the serotonergic modulatory Pd-SW cells ($n = 32$, Figs. 11, A and B, and 12, A and B). ACh-induced hyperpolarizations reached amplitudes up to 20 mV. In preparations with desheathed ganglia, swim interneurons and motoneurons responded to ACh concentrations as low as 0.2 μM. Carbachol, a cholinergic agonist, also produced hyperpolarization of swim interneurons, motoneurons, and Pd-SW neurons, although it appeared to be less potent than ACh because it required a concentration of $\approx 10$ μM to induce a noticeable hyperpolarization ($n = 7$, Fig. 11, C and D).

In addition to the experiments with bath application of ACh in high-Mg$^{2+}$ saline, local microiontophoretic application of ACh was used to establish the direct sensitivity of swim neurons. This technique was used mostly for investigation of the membrane sensitivity of GE swim motoneurons that have cell bodies large enough to accommodate an intracellular electrode and an iontophoretic micropipette in the middle of the cell body surface without noticeable diffusion of neurotransmitter to the surrounding neurons. Local iontophoretic release of ACh triggered prominent hyperpolarizing responses in GE motoneurons with amplitudes up to 15 mV and durations of 0.5–1 s ($n = 7$, Fig. 12C).

Additional confirmation of the cholinergic nature of synaptic transmission between Cr-St interneurons and swim interneurons and motoneurons came from experiments suggesting that the ionic mechanisms of ACh-induced hyperpolarizations and Cr-St-neuron-induced IPSPs are the same. The reversal potentials for ACh-induced hyperpolarizations in GE swim motoneurons were measured (Fig. 12A) and found to be around $-70$ mV ($-70 \pm 2$ mV, $n = 4$), a value
FIG. 9. Blocking effect of cholinergic antagonists on inhibitory synaptic transmission between a Cr-St interneuron and swim neurons. A: 50 μM d-tubocurarine completely blocked IPSPs in a GE(d) motoneuron induced by spike activity in a Cr-St interneuron. Transmission was restored after a 10-min wash. B: 20 μM atropine blocked Cr-St-interneuron-induced IPSPs in a GE(d) motoneuron. Effect was washed out after 15 min. C: 50 μM d-tubocurarine blocked Cr-St-interneuron-induced IPSPs in an SI(d) interneuron. Transmission was restored after a 10-min wash. Experiments were conducted in high-Mg²⁺/high-Ca²⁺ saline to eliminate swim rhythm and to prevent synaptic inputs from masking Cr-St-interneuron-induced IPSPs. Scale bars: 10 mV, 200 ms.

similar to reversal potentials of Cr-St-neuron-induced IPSPs in the same neurons (Fig. 5A). Experiments with electrodes filled with 3 M potassium chloride demonstrated that as with Cr-St-neuron-induced IPSPs, ACh-induced hyperpolarizations in swim interneurons and motoneurons were generated by increased permeability to chloride ions. When recording electrodes filled with 2 M potassium acetate were replaced with electrodes filled with 3 M potassium chloride, ACh-induced membrane responses in swim interneurons and motoneurons reversed 5–10 min after penetration. ACh began to produce a prominent depolarization in these neurons, whether bath applied or iontophoretically released (n = 8, Fig. 12, B and C).

DISCUSSION

The startle response and swimming in Clione are not mutually exclusive behaviors. They both sustain similar functional goals and can be activated together, as for example during escape swimming (Satterlie et al. 1997). However, both the startle and swimming motor systems innervate the same swim musculature, and therefore a strict coordination of their activities is required when a startle response is elicited in swimming animals. Several other animals that have fast escape responses do not have this problem. For example, in the bat-avoidance system of crickets, the initial response to ultrasonic stimuli is a unilateral metathoracic leg kick that alters the downstroke of the ipsilateral hindwing to initiate a turn away from the stimulus (May and Hoy 1990). This kick can occur any time in the wingbeat cycle and is not directly linked to flight pattern generator activity. The independence of these two systems is important for avoiding phase-locking delays and is affordable because both systems control different body parts. This is not the case with startle and swimming systems in Clione, which compete for the same swimming musculature. One possible solution to this problem could be coordination of the activities of the same-phase startle motoneurons and swim motoneurons. However, the existence of phase-locking delays in this model makes it unacceptable, because the main purpose of having a separate neural system for the startle response is to provide extremely short response latencies. A more realistic solution would be to temporarily remove one system from control of common elements while the second system is being activated.

Behavioral observations show that the startle response is not incorporated into swimming activity; it overrides swimming occurring at any time during the wingbeat cycle (Satterlie et al. 1997). Startle motoneurons themselves are completely separated from phase-locked swimming activity (Satterlie et al. 1997). The present study demonstrates that, in addition to strong excitatory inputs to ST(d) motoneurons (Norekian and Satterlie 1997), the Cr-St interneuron produced strong inhibition of the swimming system. All neuron types of the swimming system received monosynaptic inhibi-
It is interesting to compare the startle system in Clione with the analogous startle system in fish (for reviews see Eaton and Hackett 1984; Fetcho 1991; Nissanov and Eaton 1989; Zottoli et al. 1995). One of the most striking similarities between the two very distinct species is found in the interactions that exist between the neural networks for startle and swimming. Fish can produce C-start bends while swimming, using the same axial muscles for both behaviors. Mauthner cell activity, which underlies the C-start response, overrides the swimming motor output regardless of the phase of swimming and also resets the swimming rhythm (Fetcho 1992; Jayne and Lauder 1993; Svoboda and Fetcho 1996). Such interactions between startle and swimming systems in fish appear to be extremely similar to those in Clione. However, unlike startle motoneurons in Clione, Mauthner cells are descending interneurons and produce their effects via local spinal neurons that are shared by both startle and swimming systems (for review see Fetcho 1991). The ability of Mauthner cells to reset swimming is attributed to their powerful monosynaptic inputs into the local spinal network, which override any inputs from the swimming circuits (Fetcho 1991, 1992; Svoboda and Fetcho 1996). In Clione, startle and swimming networks are completely separate systems that do not share any neural elements.
FIG. 12. A: bath application of 5 μM ACh produced hyperpolarization of a GE(d) motoneuron. Injection of negative current into GE(d) motoneuron changed its membrane potential in several steps from resting $-56 \text{ mV}$ to $-80 \text{ mV}$. Reversal of ACh-induced hyperpolarization to ACh-induced depolarization occurred at a membrane potential between $-68$ and $-72 \text{ mV}$. B: when recording electrode was filled with 2 M potassium acetate, a normal hyperpolarizing response was induced by ACh in a GE(d) motoneuron. Fifteen minutes after potassium-acetate-filled electrode was replaced with one containing 3 M potassium chloride, normal ACh-induced hyperpolarization turned into an ACh-induced depolarization. Note that GE(d) neuron membrane potential remained same after electrode replacement ($-60 \text{ mV}$). C: local iontophoretic application of ACh onto somata of GE(d) motoneuron produced a prominent hyperpolarizing response. ACh was released from tip of a glass micropipette by positive current pulses 100 nA in amplitude and 300 ms in duration. Fifteen min after potassium-acetate-filled recording electrode was replaced with KCl-filled electrode, hyperpolarizing response to iontophoretic application of ACh turned into a depolarizing response. Experiments were performed in high-Mg$^{2+}$ sea water, which blocks all chemical synaptic transmission in CNS. Scale bars: 10 mV, 2 s.

Because the startle system in fish uses the same neural elements that are involved in swimming, it cannot simply inhibit the activity of the swimming system. It needs to modify the output of the shared spinal network by producing coordinated inhibitory and excitatory inputs to the reciprocal left and right neurons. When one Mauthner axon fires, it monosynaptically excites ipsilateral descending interneurons and large primary motoneurons while blocking the activity of the interneurons on the opposite side via excitation of inhibitory commissural interneurons (for review see Fetcho 1991). The interesting feature of Cr-St-neuron-induced inhibition of swimming in Clione is its disregard for the phase of wing contractions. The Cr-St interneuron activated only ST(d) motoneurons; v-phase startle motoneurons remained silent. Therefore the real problem at the moment of Cr-St neuron activation would be created only by the activity of v-phase swim motoneurons. Continuous activity of d-phase swim motoneurons might even be helpful for increasing the force of wing muscle contractions. Thus Cr-St interneurons could theoretically inhibit only v-phase swim motoneurons, leaving d-phase swim motoneurons unaffected or even activating them, to reach the same goal—to coordinate the functioning of the two systems. Cr-St interneurons, however, inhibited d-phase swim motoneurons and interneurons as efficiently as v-phase motoneurons and interneurons. One possible explanation for this is that startle motoneurons of both the d- and v-phases do not require the participation of swim motoneurons to induce the startle response. They are motoneurons themselves and are capable of producing extremely strong wing muscle contractions without the assistance of swim motoneurons (Satterlie et al. 1997).

Despite the fact that the startle system, when active, inhibits its swimming, both startle and swimming systems are pursuing similar behavioral goals and are usually active, but separated in time, during the same behaviors. Escape behavior, which can be induced by tactile stimulation of the tail, includes a brief startle response at its initial phase followed by a prolonged period of fast swimming (Satterlie et al. 1997). Prey capture behavior, which is always characterized by a dramatic acceleration of swimming, might also recruit the startle system. The system of startle neurons may be involved in triggering a forward lunge during prey capture in Clione, thus increasing the success of capturing prey. A similar dual function has recently been suggested for Mauthner cells in goldfish, which fire when the fish performs a C-shaped flexion in association with the terminal phase of prey capture (Canfield and Rose 1993).

In escape or prey capture, the initial startle response is immediately followed by a period of fast swimming. This sequence can be induced in either swimming or resting animals. The main mechanism for triggering fast swimming following the startle response appears to be very simple: sensory inputs. The same stimulus that triggered startle system activity produced excitatory inputs to the swimming system. However, during the initial period, powerful inhibitory inputs from Cr-St interneuron to swim interneurons and motoneurons overrode these excitatory sensory inputs and produced brief inhibition of swimming instead of its activation. When the inhibitory influence from Cr-St interneurons was terminated, excitatory sensory inputs immediately activated the swimming system, triggering a period of fast swimming. This sequence of events was confirmed by experiments demonstrating that if mechanosensory inputs to the startle system were not strong enough to bring Cr-St inter-
neurons and startle motoneurons to spike thresholds, they induced an immediate acceleration of swimming without its initial inhibition (Fig. 7B). There is, however, one additional mechanism that could contribute to the swimming acceleration after the startle response. An important characteristic of interneurons of the swim central pattern generator of *Clione* is their ability to produce postinhibitory rebound (Satterlie 1985). Swim motoneurons and Pd-SW neurons also developed depolarizing responses and excitation after strong membrane hyperpolarizations. Such hyperpolarizations in the form of Cr-St-interneuron-induced IPSPs were found to be effective in triggering afterdepolarizations and excitation in swim neurons. Thus inhibitory inputs from Cr-St interneurons themselves contributed to the excitation of the swimming system following initial activation of the startle system due to postinhibitory rebound in swim neurons.

One animal that exhibits a similar sequence of events between fast startle response and swimming is the crayfish. When crayfish escape is initiated by a sudden tactile stimulus to the abdomen, an initial stereotyped short-latency tailflip is mediated by a pair of giant axons, which is commonly followed by subsequent tailflips (called ‘‘swimming’’) produced by a nongiant system (for review see Krasne and Wine 1984). It has been found that there are several different mechanisms involved in triggering nongiant-mediated swimming after the giant-mediated tailflip. These include the excitatory effect on the nongiant central pattern generator produced by giant axons themselves and peripheral afferent feedback from the original giant flip (Krasne and Wine 1982, 1983). It is interesting that solutions to the problem of possible interference between two separate competing systems are quite different in *Clione* and crayfish. In *Clione*, strong inhibitory inputs from the high-order interneurons that activate the startle system prevent the swimming system from interfering. In crayfish, the key element is different reaction times in two separate neural systems: the latency for the giant-mediated response is <7 ms, whereas that for the nongiant-mediated response is ~250 ms (Reichert and Wine 1982, 1983). The duration of the giant-mediated tailflip itself, including flexion and reextension, is ~200 ms. Thus it is clear that the typical latency to the beginning of nongiant-mediated tailflips (swimming) is long enough to allow the giant-mediated tailflip to occur in the interim (Reichert and Wine 1982, 1983). There is no need for inhibitory inputs from giants to the nongiant swimming system. When interaction between two different behavioral responses controlled by separate neural systems is discussed, one important question is how to organize a smooth transition between them. In teleost fish, Mauthner cell spiking, which underlies the C-start response and overrides the swimming motor program, also resets the swimming rhythm dramatically in a way that leads to a smooth transition from the C-start bend into subsequent swimming (Fetchco 1992; Jayne and Lauder 1993; Svoboda and Fetchco 1996). It apparently occurs because startle and swimming systems share the same interneurons and motoneurons, and the Mauthner axon is tapping into some of the same premotor interneurons that are important for generating swimming (Fetchco 1991). Behavioral observations in *Clione* also demonstrate that a smooth, phase-dependent transition exists between one or two wingbeats of the startle response and the following fast swimming rhythm. However, how the swim central pattern generator is being reset by the startle neuronal system to have this smooth transition remains unknown.

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