Swim Initiation Neurons in *Tritonia diomedea*¹

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SYNOPSIS. Two groups of interneurons, Tr1 and DRI, have been identified in the escape swim circuit of the marine mollusc *Tritonia diomedea* that have important roles in behavioral initiation. DRI functions as a command neuron, receiving direct excitatory input from the afferent neurons, and in turn directly exciting the DSI neurons of the central pattern generator. DRI fires throughout the swim motor program, and activity in DRI is both necessary and sufficient for sensory input to elicit the swim motor program. Tr1 is an excitatory interneuron that fires briefly in response to sensory input and then remains silent during the motor program. Tr1 excites DRI with an excitatory connection that has fast and slow components and thus appears to have a role in converting brief afferent neuron activity to long-lasting firing in downstream circuit elements. These neurons complete the description of a continuous synaptic pathway from afferent to flexion neurons in the *Tritonia* swim circuit. Their identification should facilitate studies of motor program initiation, as well as of how various forms of experience, including simple forms of learning, act to influence neuronal decision-making processes.

INTRODUCTION

The marine mollusc *Tritonia diomedea* initiates a rhythmic escape swim upon contact with a sufficiently aversive stimulus, such as the seastar *Pycnopodia helianthoides* (Fig. 1a). The neural program underlying this behavior can be elicited by briefly stimulating any one of a number of peripheral nerves in the isolated brain preparation, indicating that the motor program is centrally generated, with little involvement of sensory feedback ((Dorsett *et al.*, 1969; Frost, 1999) and Fig. 5). Previous work has described much of the circuitry mediating the *Tritonia* escape swim response, including the afferent neurons (S-cells) (Getting, 1976; Slawsky, 1979), the central pattern generator (CPG) neurons (Getting, 1983), and the swim flexion neurons (Willows *et al.*, 1973; Hume *et al.*, 1982) (Fig. 1b). While these findings made it possible to study pattern generation, motor program initiation could not readily be addressed due to a gap in our knowledge of the circuit elements between the afferent neurons and the swim CPG. Here we fill-in this gap, by describing two recently discovered pre-CPG interneurons, Tr1 (Trigger-type 1) and DRI (Dorsal Ramp Interneuron), that convey the output of the afferent neurons to the CPG. An initial description of DRI has been published previously (Frost and Katz, 1996); Tr1 is described here for the first time.

METHODS

Animals

*Tritonia diomedea* were collected from the waters of Puget Sound, Washington, and were maintained in natural running seawater (10–12°C) at the University of Washington’s Friday Harbor Laboratories and in artificial seawater aquaria (Instant Ocean, 10–12°C) in Houston and Chicago.

Recording preparations

For experiments where skin stimulation was used to elicit the swim motor program, animals were first anesthetized by injecting 60 ml of a solution composed of half 350 mM MgCl₂ and half artificial seawater (In-
FIG. 1. The *Tritonia* escape swim and its underlying circuit. A. Upon contact with a suitably aversive stimulus, such as the tube feet of the seastar *Pycnopodia helianthoides*, *Tritonia* respond with an escape swim consisting of a series of alternating ventral and dorsal whole-body flexions. The photograph shows an animal at a moment of maximum dorsal flexion. B. The known swim circuit. Solid lines represent direct, monosynaptic connections, broken lines represent indirect connections, or connections not yet confirmed to be monosynaptic. Synaptic symbols: lines = excitatory, black circles = inhibitory, lines and circles = multiple component monosynaptic connections. "VSI" represents both VSI-A and VSI-B; the exact connectivity shown is for VSI-B only. The known number of neurons of each type on each side of the brain are: S-cells, 80; Tr1, 1; DRI, 1; DSI, 3; C2, 1; VSI, 2; FNs, 55 (Getting, 1983; Frost and Katz, 1996).

Electrophysiology

Neurons were impaled with glass micro-electrodes (20–40 MΩ) filled with 3 M K-acetate and inserted into chucks containing 3 M KCl. Swim neurons were identified based on their location, size, color, synaptic connections with other identified neurons, and their activity during the swim motor program (Getting, 1976; Getting *et al.*, 1980; Frost and Katz, 1996). Normal saline composition for both preparations was (in mM): 420 NaCl, 10 KCl, 10 CaCl₂, 50 MgCl₂, 11 D-glucose and 10 HEPES, pH 7.6. High divalent cation saline composition was (in mM): 285 NaCl, 10 KCl, 25 CaCl₂, 125 MgCl₂, 11 D-glucose, and 10 HEPES, pH 7.6. Data were initially stored on videotape using a Vetter 3000A PCM recording adapter, and were then digitized and analyzed offline using a Biopac MP100 data acquisition system. Tr1 was iontophoretically injected with 5, 6-carboxyfluorescein and photographed in the live preparation using an Optronics DEI-750D CCD camera.
RESULTS

DRI

For many years, a key missing element in the *Tritonia* swim network was the interneuronal population responsible for producing the long-lasting, declining “ramp” of depolarization in the dorsal swim interneurons (DSIs) of the CPG in response to a swim-eliciting stimulus (Lennard et al., 1980). Prior work (Getting and Dekin, 1985) concluded that this depolarization has an important role in initiating the swim motor program. Using a dye filled electrode, we searched for the missing ramp interneurons among the axons in the central commissure. This procedure led to the discovery of DRI, an interneuron that receives direct excitatory synaptic input from the afferent neurons (see below), and in turn makes strong, direct excitatory connections onto the DSIs of the CPG (Frost and Katz, 1996). The available data are consistent with there being just one DRI on each side of the brain (Frost and Katz, 1996). The discovery of DRI completed the description of a continuous synaptic pathway from the afferent to the efferent neurons of the swim circuit (Fig. 1b).

An early report (Getting, 1977) suggested that the S-cells made a monosynaptic connection to CPG neuron Cerebral Cell 2 (C2). (3 of 8 cells were reported to do so.) In the present experiments we observed several indirect, but no direct connections between 58 S-cell–C2 pairs tested in 11 preparations using either saline or a high divalent cation saline solution effective at blocking polysynaptic pathways in *Tritonia* (Katz and Frost, 1995b). We therefore suggest that the connections between the S-cells and C2 are indirect.

In contrast to their connections to C2, we found that the majority of S-cells made direct excitatory connections to DRI (Hoppe, 1998). In 10 preparations, 44 of 62 S-cells made 1-for-1, constant latency EPSPs onto DRI. In one of these preparations, 9 of 11 S-cells produced EPSPs onto DRI in high divalent cation saline.

As reported previously (Frost and Katz, 1996), directly activating DRI using intracellular stimulation elicits the swim motor program (Fig. 2), while hyperpolarizing a single DRI prevents the nerve shock-elicited motor program. These findings satisfy the most stringent criteria for inclusion of DRI as a command neuron (see Discussion). In our prior work we only tested the ability of DRI hyperpolarization to block motor programs elicited by stimulation of Pedal Nerve 3. Here we report that hyperpolarizing DRI also blocks the initiation of motor programs elicited by salt stimuli applied to the skin (Fig. 3, n = 3 preparations)—the stimulus used in many of our behavioral studies (Frost et al., 1996; Monguio and Frost, 2000).

Although DRI satisfies the formal criteria for assignment as a command neuron, the fact that it receives feedback from the CPG and its firing oscillates with the motor program raised the issue of whether it might actually function as a CPG member. Previous work had shown that injecting sufficient hyperpolarizing current to suppress firing in one of the two DRIIs completely
blocks the ability of a nerve stimulus to elicit the motor program (Frost and Katz, 1996). We reasoned that if DRI functions as a CPG member, suppressing its firing via hyperpolarization should also block oscillation initiated from within the CPG, such as can be elicited via intracellular stimulation of one or more DSI neurons (Fickbohm and Katz, 2000). On the other hand, if DRI functions more as a gating-command neuron that drives the CPG and also receives feedback from it, then suppressing DRI firing should not prevent DSI-driven CPG activity. We found that hyperpolarizing DRI failed to prevent the DSI-driven motor program (Fig. 4, n = 5 preparations). This result supports our categorization of DRI as a pre-CPG command neuron that functions more to initiate and sustain the motor program than as part of the mechanism generating the cycle-by-cycle mechanics of the oscillation itself.

**Tr1**

Although the discovery of DRI established a continuous afferent-to-flexion neuron synaptic pathway, the firing patterns of the cells suggested the existence of an as-yet unidentified circuit element. Brief sensory stimulation elicits a correspondingly brief S-cell firing response, yet all previously identified downstream neurons in the swim circuit typically fire for tens of seconds (Figs. 3, 5). Since the S-cells excite DRI with fast EPSPs, something else seemed needed to mediate this brief-to-long firing transformation.

We describe here a new neuron that appears to contribute to this process. The Tr1 neuron (n = 20 preparations, Figs. 1B, 5) is located below the surface in the dorsal pleural ganglion, near DRI and the statocyst (Fig. 6A). Its defining features are: 1) the location of its cell body and its characteristic axonal morphology (Fig. 6A), 2) it receives direct EPSPs from the S-cells (28 of 28 cells tested in 10 preparations, Fig. 6B), 3) it fires briefly, like the S-cells, at the onset of the swim motor program (Fig. 5), 4) it makes an indirect excitatory connection to DSI (Fig. 6C), and 5) direct intracellular stimulation of a single Tr1 neuron is often sufficient to activate the motor program (Fig. 6D). Such Tr1-elicited motor programs typically continue 1 to 3 cycles beyond the end of intracellular stimulation (Avg. 1.5 ± 0.3 extra cycles, n = 8 preparations). This latter property suggests that Tr1 may con-
Fig. 4. DRI does not appear to function as a CPG element. DRI, C2 and 2 DSIs were impaled with intracellular electrodes. The two DSIs were stimulated to fire at 20 Hz for 20 sec (hatched bar) while DRI was hyperpolarized to prevent its spiking. This procedure failed to prevent the swim motor program, consistent with the view that DRI is not part of the cycle generating mechanism, but instead functions as a pre-CPG command neuron. During the swim motor program, C2, DSI and DRI received inhibition during the ventral phase, which was sufficient to phasically block firing in one of the two DSIs (DSI-1), leaving just the stimulus artifacts between the bursts. Calibration bars: All 20 mV, 10 sec.

Fig. 5. The S-cells and Tr1 fire briefly at the onset of the swim motor program. A brief stimulus (10 Hz, 1 sec) applied via suction electrode to Pedal Nerve 3 (arrow) elicited a brief firing train in the S-cell and Tr1 cell, followed by a three cycle swim motor program in CPG neurons DSI and C2. Calibration bars: All 20 mV, 10 sec.

To date, only one Tr1 neuron has been encountered on each side of the brain. However, hyperpolarizing Tr1 does not block the ability of nerve stimulation to elicit the swim motor program (n = 2 preparations), nor does Tr1 stimulation elicit motor program activity as long lasting as that elicited by nerve stimulation, suggesting that there may be additional Tr1 neurons, or an additional class of Tr-like neurons on each side of the brain.

We next tested whether Tr1’s indirect excitation of DSI (Fig. 6C) occurred via recruitment of DRI, the only known excitatory input to DSI originating from outside the CPG. This was a difficult experiment because Tr1 and DRI are both located out of sight, below the surface of the pleural ganglion. We were successful in penetrating both DRI and Tr1 in two preparations, and found that Tr1 produced 1-for-1, constant latency EPSPs onto DRI (Fig. 7A). Significantly, brief Tr1 trains produced long-lasting firing in DRI (Fig. 7B). Hyperpolarizing DRI in normal saline revealed that the connection had both fast and slow components (Fig. 7A, C). These preliminary observations suggest that a key location for the brief-to-long firing transformation in the swim circuit may be a dual component fast/slow excitatory synaptic connection between Tr1 and DRI. However further work is needed to test this hypothesis. Such dual component fast/slow synaptic connections mediate brief-to-long lasting firing transitions in other circuits as well (Brodfuehrer and Friesen, 1986b; Cleary and Byrne, 1993; Lieb and Frost, 1997).

DISCUSSION

While significant progress has been made in elucidating the neural basis of rhythmic pattern generation (Marder and Calabrese, 1996), less is known about the mechanisms
of motor program initiation—the decision-making process that determines whether or not a particular behavioral act will occur. The *Tritonia* escape swim response is an attractive model system for investigating the neural mechanisms of behavioral initiation. The swim is an all-or-none response—the animal either swims or it doesn’t in response to an appropriate stimulus—so the nature of its decision is unambiguous. Also, the animal takes a variable amount of time (up to 20 sec) when deciding whether or not to swim, consistent with decision-making processes in higher animals.

Progress toward understanding the swim-initiation process in *Tritonia* has come with the identification of the circuitry conveying sensory input to the swim CPG. A key question concerns how brief S-cell firing brings about a sustained activation of the CPG. Before the pre-CPG interneurons were identified, this was addressed by comparing the duration of depolarization in the DSI neurons when the nerve-elicited CPG oscillation was either allowed or prevented by hyperpolarizing C2 (Getting and Dekin, 1985). It was concluded that sensory input initiates a slowly declining ramp of depolarization in the DSIs that sustains the ini-
Fig. 7. Tr1 to DRI connection. A. Tr1 connects to DRI with 1-for-1 constant latency EPSPs. B. A Tr1 train (2.5 sec, mean = 22.6 Hz) produced several seconds of firing in DRI. C. Hyperpolarizing DRI during a Tr1 train revealed a dual component fast/slow EPSP in DRI. Calibration bars: A. DRI, 5 mV; Tr1, 20 mV; 1 sec. B. DRI, 10 mV; Tr1, 20 mV; 10 sec. C. DRI, 4 mV; Tr1, 20 mV; 1 sec. All records in normal saline.

tial 10–15 sec of oscillation. After that, excitatory interactions among the CPG neurons were suggested to sustain the remaining duration of the motor program.

DRI was recently identified as the major source of the excitatory synaptic input to the DSIs (Frost and Katz, 1996). When CPG oscillation is suppressed by hyperpolarizing both C2 neurons, a brief nerve stimulus elicits a gradually slowing train of action potentials in DRI, and a corresponding declining ramp of depolarization in the DSIs (Frost and Katz, 1996). The excitatory ramp in the DSIs was found to be reduced or eliminated when DRI was hyperpolarized to prevent its firing. DRI was furthermore found to be individually necessary and sufficient for afferent input to activate the swim motor program (Figs. 3, 4 and (Frost and Katz, 1996)).

These features satisfy the most stringent criteria for command neuron status—a cell that fires in response to sensory input and whose activity is both necessary and sufficient for the activation of a motor program by that input (Kupfermann and Weiss, 1978). A number of individual neurons have now been identified that satisfy these criteria (Rock et al., 1981; Fredman and Jahan-Parwar, 1983; Nolen and Hoy, 1984; Frost and Katz, 1996; Xin et al., 1996; Edwards et al., 1999; Jing and Gillette, 1999; Hedwig, 2000). In practice, however, the term “command neuron” has frequently been applied to neurons whose activity is simply sufficient to elicit a motor program,
without the requirement of individual necessity. Stein divided command neurons into two functional classes, trigger and gating neurons (Stein, 1978). Trigger neurons fire at the outset, and are then silent during the sensory-elicited motor program. Their role is therefore to initiate motor program activity, often by exciting gating command neurons. Gating command neurons, such as DRI, fire throughout the motor program and are important for sustaining rhythmic motor program generation. When driven directly, these neurons elicit a motor program that ceases shortly after the end of the eliciting train (see Fig. 2).

Tr1 has several properties common to trigger command neurons, such as those described in the leech (Brodfuehrer and Friesen, 1986a) and crayfish (Murchison and Larimer, 1990). It fires briefly at motor program onset, due to direct, highly convergent, fast excitatory connections from the S-cells, and is then silent during the program itself. When driven directly, a single Tr1 can often elicit motor program activity, and this activity continues for a period of time after the end of the train. Unlike DRI, however, Tr1 fails the test of individual necessity—hyperpolarizing Tr1 does not block motor program activation by sensory input (nerve stimulation). Tr1’s ability to elicit a motor program that outlasts the Tr1 train is also modest, unlike the trigger neurons that initiate leech swimming (Brodfuehrer and Friesen, 1986a). These results suggest that there are additional, as yet unidentified Tr1-type neurons that work together and reinforce one another in initiating the swim, perhaps functioning as a trigger “command system.”

Tr1 appears to have a role in converting brief S-cell firing into long lasting DRI firing, which in turn strongly excites all DSI neurons of the swim CPG. The DSI neurons contain 5HT and produce neuromodulatory enhancement of the excitability and synaptic efficacy of CPG neuron C2 (Katz et al., 1994; Katz and Frost, 1995a, b, 1997). The synaptic enhancement involves all chemical synaptic connections made by C2, including those within the CPG and an excitative feedback connection from C2 to DRI. The modulatory enhancement of this positive feedback connection from the CPG to its own command neuron may have an important role in initiating and sustaining the swim rhythm (Katz et al., 1994; Katz and Frost, 1997).

The overall organization of the swim initiation circuitry in Tritonia appears to be similar to that described for swimming in the leech (Brodfuehrer et al., 1995). Both species direct sensory input to trigger-type neurons that in turn excite gating command neurons that activate and sustain CPG activity. In both cases, as in others (Gillette et al., 1978; Fredman and Jahan-Parwar, 1983; McCrohan, 1984; Nolen and Hoy, 1984; Delaney and Gelperin, 1990; Jing and Gillette, 1999) the gating neurons also receive synaptic feedback from the pattern generator during the rhythm. These similarities across species reinforce the idea that this circuit structure for the control of motor program initiation may be commonly employed for the control of many stimulus-elicited, rhythmic motor programs, such as those mediating escape responses.

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REFERENCES


