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J Neurophysiol 105:130-144, 2011. First published 27 October 2010;
doi: 10.1152/jn.00507.2010

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Local-Distributed Integration by a Novel Neuron Ensures Rapid Initiation of Animal Locomotion

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Submitted 4 June 2010; accepted in final form 25 October 2010

Mullins OJ, Hackett JT, Friesen WO. Local-distributed integration by a novel neuron ensures rapid initiation of animal locomotion. *J Neurophysiol* 105: 130–144, 2011. First published October 27, 2010; doi:10.1152/jn.00507.2010. Animals are adapted to respond quickly to threats in their environment. In many invertebrate and some vertebrate species, the evolutionary pressures have resulted in rapidly conducting giant axons, which allow short response times. Although neural circuits mediating escape behavior are identified in several species, little attention has been paid to this behavior in the medicinal leech, a model organism whose neuronal circuits are well known. We present data that suggest an alternative to giant axons for the rapid initiation of locomotion. A novel individual neuron, cell E21, appears to be one mediator of this short-latency action in the leech. In isolated nerve cord and semi-intact preparations, cell E21 excitation initiates and extends swimming and reduces the cycle period. The soma of this cell is located caudally, but its axon extends nearly the entire length of the nerve cord. We found that cell E21 fires impulses following local sensory inputs anywhere along the body and makes excitatory synapses onto the gating cells that drive swimming behavior. These distributed input–output sites minimize the distance information travels to initiate swimming behavior, thus minimizing the latency between sensory input and motor output. We propose that this single cell E21 functions to rapidly initiate or modulate locomotion through its distributed synaptic connections.

INTRODUCTION

To effectively escape predators and catch prey, animals must respond rapidly to sensory inputs that indicate threats. The speed of the response is crucial; a delay lasting a fraction of a second can be the difference between death and survival. Neuronal circuits mediating these responses must therefore relay information quickly and efficiently to initiate locomotion. In leeches, a common locomotion used in escape and predation is swimming; however, despite extensive research into swim circuits, there has been little focus on how this behavior might be rapidly initiated. Among other sensory modalities, swim episodes are initiated by touch (Kristan Jr et al. 1982) and surface water waves (Friesen 1981). Water waves might indicate the nearby presence of a suitable host to feed on (Sawyer 1986) and touch may indicate a threat from a predator. Rostral touch often initiates a shortening response, whereas caudal touch usually initiates swimming. If such input is noxious, swimming may be preceded by shortening, curling, or writhing (Kristan Jr et al. 1982). These behaviors remove the leech from the offending stimulus and parallel escape responses observed in other animals, where initial retreats or turns from noxious stimuli are followed by rhythmic locomotion (Domenici et al. 2008; Sillar 2009). Escape circuits in species such as crayfish (Edwards et al. 1999), teleost fish (Eaton and Hackett 1984; Korn and Faber 1996), cockroaches (Ritzmann and Eaton 1998), rats (Depoortere et al. 1990; Mitchell et al. 1988), and cats (Mori et al. 1989) have been identified and elucidated to various degrees. In many invertebrates, the rapid escape responses are mediated by large interneurons with high impulse conduction velocities, such as the medial and lateral giant fibers in the crayfish (Wiersma 1947), the Mauthner neurons in teleost fish (Sillar 2009), and the giant interneurons in the cockroach (Kolton and Camhi 1995). In the leech, the large axon of the S-cell was once thought to mediate a shortening escape response, however this intersegmental interneuron is neither sufficient nor necessary for this behavior (Sahley et al. 1994). Since it lacks high conduction velocity axons, the leech must use alternative strategies to minimize its response latency to sensory signals.

The latency of a motor response will be reduced if the distance between the input site and motor system is minimized. For segmental animals, local sensory input might activate segmental sensory interneurons that, in turn, activate local elements of the motor system. Alternatively, multisegmental interneurons with distributed input–output sites could perform a similar function. Neurons with multiple spike initiation zones have been identified in many species. The dorsal gastric (DG) motoneuron in the stomatogastric system of *Cancer borealis*, for example, possesses a secondary spike initiation zone important for the persistent firing evoked by some stimuli (Le et al. 2006). Multisegmental tactile interneurons in the crayfish, stomatogastric and cardiac neurons in the lobster, and the heart interneurons in the leech can also initiate spikes at multiple locations, either inter- or intrasegmentally (Calabrese and Kennedy 1974; Hartline and Cooke 1969; Moulins et al. 1979; Thompson and Stent 1976). Several interneurons in the crayfish especially have been shown to have distributed input sites (Wiersma and Bush 1963; Wiersma and Hughes 1961; Wiersma and Ikeda 1964; Wiersma and Mill 1965). A similar organization is also seen in the lateral giant neurons in the crayfish and in the S-cell of the leech; these make up networks of tightly electrically coupled segmental cells that have multiple segmental inputs and outputs (Edwards et al. 1999; Kristan Jr et al. 2005).

Here, we introduce an excitatory neuron, cell "E21," that broadly integrates sensory input from most, and perhaps all, body segments to trigger swim initiation in the leech nerve cord. During ongoing fictive swimming, depolarization of cell E21 decreases the cycle period and prolongs swim episodes.

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The soma of cell E21 resides in the caudal-most midbody ganglion but projects to the rostral nerve cord. Through electrophysiological studies on semi-intact and isolated preparations of *Hirudo verbana*, we show that cell E21 interactions link mechanosensory cells with swim-gating neurons. Furthermore, we found that cell E21 receives sensory input, excites swim-gating cells, and has spike-initiation zones in many segmental ganglia. We conclude that sensory input anywhere along the leech body could rapidly initiate or modulate swimming locomotion via the local-distributed structure of cell E21. This synaptic pathway could prove to be critical in mediating rapid swim initiation in leeches.

METHODS

Preparations

Experiments were performed on adult medicinal leeches, *Hirudo verbana*, supplied by Niagara Leeches (Cheyenne, WY) or Leeches USA (Westbury, NY). Leeches were maintained in aquaria in a temperature-controlled room on a 12-h light/12-h dark cycle at 18–21°C. Prior to dissection, leeches were anesthetized with 4°C leech saline, containing (in mmol/l) 115 NaCl, 4 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES buffer (pH 7.4) (Friesen 1981). During experiments, preparations were superfused with this normal saline, with saline containing 50 μM serotonin to enhance swim initiation (Willard 1981) or with a high divalent cation solution containing (in mmol/l) 91 NaCl, 4 KCl, 10 $CaCl_2$, 10 $MgCl_2$, and 10 HEPES buffer (pH 7.4).

Experiments were performed on isolated nerve cords, on nearly isolated nerve cord preparations or on semi-intact preparations (Kristan Jr et al. 2005). Isolated nerve cord preparations comprised a chain of ganglia extending from M2 (the second segmental ganglion) through the caudal (tail) brain (T). The rostral (head) brain (H) and M1 inhibit swim initiation (Brodfuehrer and Friesen 1986a) and thus they were excluded from most preparations. Preparations, with one or more segmental ganglia desheathed, were pinned onto a glass-bottom dish that was covered by a thin layer of resin (<1 mm). To permit quasi-natural stimulation, we used nearly isolated preparations that included a flap of tissue from the caudal sucker innervated by the otherwise isolated nerve cord (M2–T). In semi-intact preparations, the middle and caudal sectors of the body wall were removed (posterior to M10 or M11), leaving the posterior nerve cord exposed for extracellular and intracellular recording. The rostral ganglia, H and M1, were either disconnected or the anterior body wall was denervated via a small window in the body wall. The preparation was then placed either in the flat glass-bottom dish or in a dish incorporating a well to immerse the intact portion of the leech. For both dishes, threads were attached to the rostral sucker and to the denervated midbody, permitting undulatory swimming movements by the anterior half of the preparation, but limiting movement in the caudal end to allow stable recordings. The threads attached to denervated portions of the leech were taped to the sides of the dish, so the leech was suspended in the minitrough. In all semi-intact preparations, a petroleum jelly dam placed between intact and isolated sections allowed independent manipulation of saline in anterior and posterior compartments. In semi-intact preparations, swimming was monitored both by visual inspection of the intact portion of the leech and by electrophysiological recording from nerves in the isolated portion of the preparation.

Electrophysiology

To monitor swimming activity, extracellular suction electrodes recorded axonal impulses from several dorsal-posterior (DP) nerves. DP nerves contain the axon of a dorsal excitatory motoneuron, cell DE-3, whose rhythmic bursting reports swimming (Kristan Jr and Calabrese 1976). The bursting pattern of cell DE-3 is often called "fictive" swimming, although here, for convenience, the terms "swim," "swim episode," "swimming," and "swim activity" are used to describe the neuronal activity occurring in isolated or nearly isolated preparations that underlies swimming in intact animals. Suction electrodes on DP nerves were also used to provide electrical shocks to initiate swimming. Impulses traveling in the intersegmental connectives were detected through en passant suction electrode recordings.

Sharp microelectrodes for intracellular recording were pulled using a P-87 Flaming Brown Micropipette Puller (Sutter Instrument, Novato, CA) and when filled with 2.7 M KAc and 20 mM KCl had a measured resistance of 30–60 MΩ. Intracellular recording and current injection were accomplished by Axoclamp2A amplifiers (Axon Instruments, Sunnyvale, CA) in bridge mode. Although the bridge was balanced immediately before penetration, it often became unbalanced over the course of the experiment. Extracellular signals were amplified by a preamplifier and then, along with intracellular signals, were further amplified and digitized with PowerLab, then displayed and stored with Chart software (AD Instruments, Colorado Springs, CO). Intracellular recordings were obtained from the somata of neurons identified by location, size, and electrical and functional properties. We used a microscope reticule to measure cell E21 soma diameter.

Procedures

To initiate swimming, we either shocked a caudal DP nerve with a train of 2- to 4-V, 5-ms pulses at 25 Hz (referred to as nerve shock) or injected depolarizing current into cell E21. For experiments to compare stimulus duration with swim duration, the level of current injection was kept constant, although this sometimes led to a variable impulse frequency. To examine swim duration in terms of impulse frequency, we varied the current injection amplitude and used a constant stimulus duration within each experiment.

We evaluated the effects of sensory stimulation in nearly isolated preparations by applying electrical shocks to body wall flaps. Pulses of 2, 5, or 7 V, lasting about 1.5 ms, were delivered at about 6 Hz via silver wires attached to a stimulator. In semi-intact preparations, we stroked, poked, or pinched innervated regions of the body wall with a small probe or a forceps.

Morphology

To visualize the neurite and axon of cell E21, we injected the soma with Alexa Fluor hydrazide 568 (Molecular Probes, Eugene, OR). Glass micropipettes were backfilled with the dye dissolved in 100 mM lithium chloride (Fan et al. 2005). Dye injection was accomplished by applying hyperpolarizing current pulses (−0.8 nA) superimposed on a constant depolarizing current (~0.1 nA), with a duty cycle of about 1 s for 30–70 min. Ganglion M21 was then placed in saline on a glass slide under a coverslip and visualized under green fluorescent light with a Zeiss fluorescence microscope. Pictures were obtained with a MagnaFire SP digital camera (Olympus America, Melville, NY).

Data analysis

Chart software (AD Instruments, Mountain View, CA) was used to store and analyze physiological data. Swim duration was measured by counting the number of spike bursts from cell DE-3 that occurred during a swim episode. Cycle periods were obtained by exporting data from Chart for analysis with Matlab (The MathWorks, Natick, MA) and the Rhythm Analysis System (RAS) (Hocker et al. 2000), which uses the median spike in each burst as a reference point.

There is large variability in swim duration among preparations; consequently, we normalized swim durations by expressing values as a fraction of the average swim duration in a given experiment. This

normalized value was then plotted against either stimulus duration or cell E21's impulse frequency. To determine the effect of cell E21 impulse frequency on cycle period, we injected single 400-ms depolarizing current pulses after the fifth or sixth burst in a series of swim episodes. Results are reported as relative differences in the cycle periods following and those immediately preceding the current injection. To detect cell E21 spikes in the suction electrode recordings from the intersegmental connectives, averaged records were triggered on soma spikes. We also used spike-triggered averaging to analyze synaptic transmission from sensory neurons to cell E21. We estimated excitatory postsynaptic potential (EPSP) duration from the exponential decay of these averaged data.

Statistical analysis was performed using Prism5 (GraphPad, La Jolla, CA). Prism5 was also used to generate all graphs. Results are reported as means with SE. Unless otherwise noted, the "*n*" value refers to the number of leech preparations from which the data were collected.

RESULTS

Here, we introduce an excitatory, command-type neuron, cell E21, whose soma is located in the caudal midbody ganglion 21 (M21) of the leech nerve cord. Excitation of this cell powerfully influences swimming activity, suggesting that this cell may be important for the rapid onset of locomotion.

Cell E21 triggers swimming

To investigate cell E21's swim-initiating properties, we recorded from its soma in isolated nerve cord preparations while monitoring swimming activity via motoneuron output with suction electrode recordings from DP nerves. Cell E21 displays high-frequency firing during and just after a swim-initiating DP nerve shock (Fig. 1*A1*). This property is shared by previously identified trigger neurons Tr1 and Tr2 in the rostral brain (Brodfuehrer and Friesen 1986b). Trigger neurons are so-called because swim duration is independent of their initiating intensity. We found that like these cephalic trigger neurons, brief excitation of cell E21 by depolarizing current injections effectively initiated swim episodes similar to those elicited by DP nerve shock (Fig. 1, *A2* and *A3*). Pulse durations of 350–400 ms initiated swimming if the impulse frequency of cell E21 was relatively high (~50–60 Hz), whereas lower impulse frequencies (<30 Hz) were effective when injected current pulses were longer (~1 s). Swimming was more reliably initiated as we increased the frequency or number of impulses in cell E21. However, unlike the cephalic trigger neurons, cell E21 exhibits small membrane potential oscillations that are phase-locked with DP bursts (Fig. 1*A2*, *inset*). These often give rise to impulses, indicating that cell E21 receives some feedback from the swim oscillator circuit.

We next investigated whether the swim duration was independent of stimulus intensity, as it is for the previously identified trigger neurons. We injected current pulses into cell E21 with either fixed duration and varying amplitude or fixed amplitude and varying duration, and observed the length of swim episodes elicited by these pulses. Figure 1, *A2* and *A3* illustrates that brief (0.36-s) and longer (1.48-s) injected current pulses elicit swim episodes with similar durations. Overall, swim length was not correlated with either the duration (Fig. 1*B1*) or the impulse frequency (Fig. 1*B2*) of the initiating stimulus in the parameter range tested. These data show that cell E21 is a trigger neuron by the criteria used to classify trigger neurons Tr1 and Tr2. The small depolarization and membrane potential oscillations observed in cell E21 suggest, however, that there are interesting differences in circuit interactions and potentially in functional properties between cell E21 and the cephalic trigger neurons.

In isolated nerve cord preparations swimming is often initiated by peripheral nerve stimulation, which activates the tactile

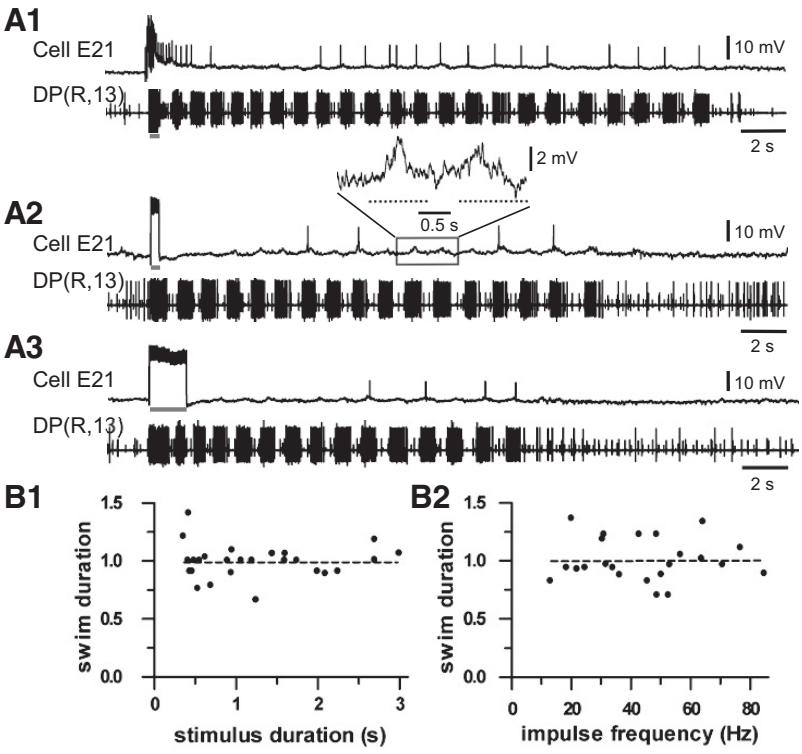

FIG. 1. Cell E21 triggers swimming. *A*: swim initiation. *A1*: swimming activity initiated by electrical stimulation (gray bar) of dorsal-posterior (DP) nerve (R,17). Cell E21 fires in response to the stimulus and, at a reduced rate, during the swim episode. *A2*: brief current injection into cell E21 (1.2 nA, 0.36 s, gray bar) elicits a swim episode like episodes initiated by DP nerve shock, but with shorter duration. *Inset*: enlargement of data within the gray rectangle; dotted lines indicate timing of DP bursts. *A3*: prolonged current injection (1.2 nA, 1.70 s, gray bar) into cell E21 elicits a swim episode with a duration similar to that of a brief current pulse (*A2*). *B*: cell E21 is a swim trigger neuron. *B1*: swim duration is not altered by the duration of depolarizing current pulses injected into cell E21; linear regression; slope is not different from zero; $P = 0.91$. *B2*: there is no correlation between cell E21 firing frequency and swim duration; dashed line is the linear regression; slope is not different from zero; $P = 0.96$.

mechanoreceptors whose excitation can initiate swimming (Debski and Friesen 1987). We tested whether the mode of swim-initiation affected swim properties by comparing the durations of cell E21-triggered swims to those elicited by DP nerve-shock in isolated preparations. Our results show that swims elicited by DP nerve shock are significantly longer than those elicited by cell E21 excitation (22.8 ± 6.0 vs. 14.8 ± 2.9 bursts per episode [BPE], respectively [mean, SE, $n = 8$]; paired t-test, $P = 0.04$; cf. Fig. 1*A1* with Fig. 1, *A2* and *A3*). The longer duration of DP-evoked swims suggests that there is differential recruitment of swim-maintenance circuits by these two modes of swim initiation.

E21 has gating properties

The swim maintenance system comprises a set of neurons that provide the excitation that underlies the expression of swimming. Segmental cells 204 and 205 are important elements of this system and are termed "gating neurons" since their continuous excitation is thought to be required for swim expression (Weeks and Kristan Jr 1978). These neurons receive direct synaptic inputs from the cephalic trigger neuron Tr1 and the cephalic swim-excitatory neuron, cell SE1. However, prolonged excitation of trigger neurons and cell SE1 has only weak effects on extending swim duration (Brodfuehrer and Friesen 1986b; Brodfuehrer et al. 1995). Unlike these rostral cells, prolonged excitation of cell E21 via depolarizing current injections was found to extend swim episodes indefinitely. As an example, Fig. 2*A* shows a swim extended to 57 BPE by continuous cell E21 depolarization; control swims triggered by brief cell E21 excitation in this preparation averaged 13 BPE. Note that swimming did not continue beyond the end of the current injection. One swim was extended to 242 bursts (not shown), which terminated only because we stopped the depolarizing current. Even continuous current injections that elicited low-frequency impulse activity in cell E21 (7–20 Hz) significantly extended swim duration. We also examined the effects of repeated transient, rather than continuous, current injection (Fig. 2*B*). Here, depolarizing current pulses (400 ms) were injected every 2–3 s during swims initiated via DP nerve shock. These current pulses elicited impulse frequencies in cell E21 ranging from 23 to 70 Hz. We found that these brief depolarizations of cell E21 increased swim duration from 20.8 ± 3.9 to 38.6 ± 10.4 BPE (mean ± SE) (Fig. 2, *B1* and *B2*; paired

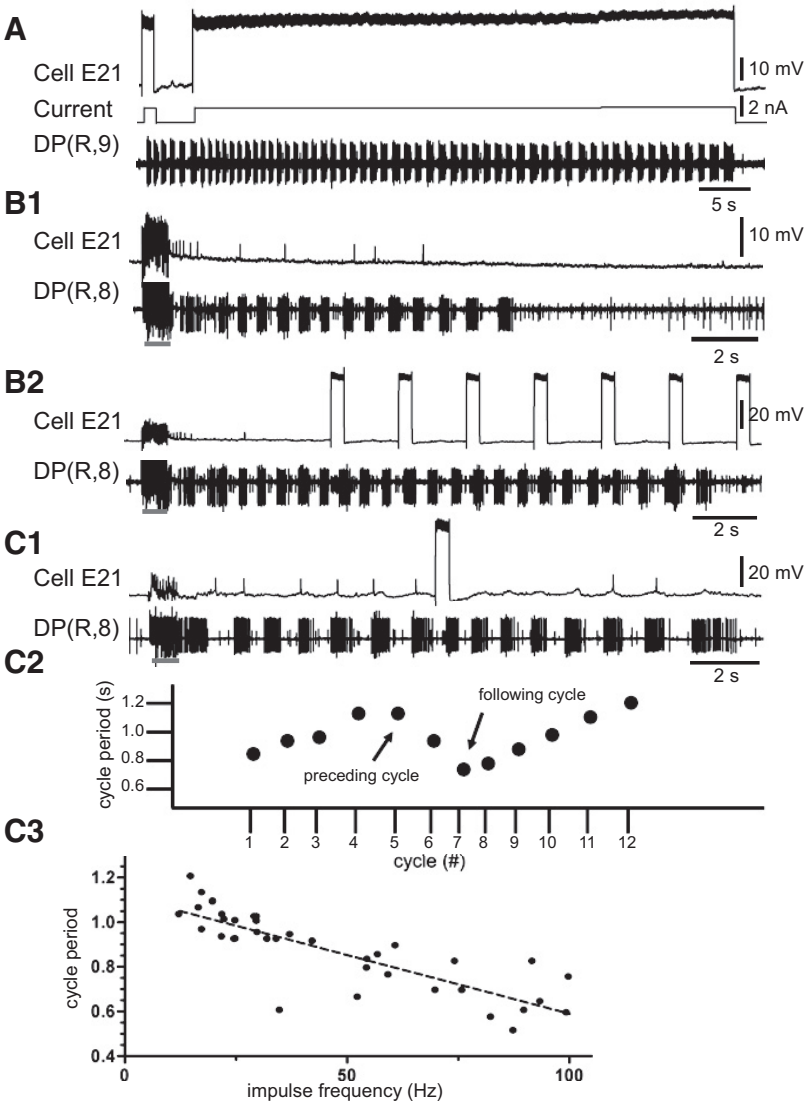

FIG. 2. Cell E21 exhibits gating cell properties *A*: swim duration is extended by cell E21 depolarization. The swim episode was initiated by brief cell E21 depolarization and then extended (to 57 bursts) by prolonged current injection (average impulse frequency was 43.3 Hz). The swim ends at current termination. Control swim episodes averaged 13 bursts. *B*: swim episode maintenance. *B1*: control swim episode initiated by DP (R,16) nerve shock (gray bar). *B2*: swim extension by repeated depolarization of cell E21. Cell E21 was injected with 400-ms, 0.7-nA (from a −0.5-nA hyperpolarization) current pulses at 2.0-s intervals. *C*: cell E21 depolarization caused a prolonged decrease in cycle period. *C1*: brief depolarization (400 ms) of cell E21 during an ongoing swim episode (initiated by DP nerve stimulations, gray bar). *C2*: graph of swim cycle period vs. ordinal cycle number for swim episode shown in *C1*. Arrows indicate the cycles before and after stimulus. *C3*: cycle period is inversely correlated with cell E21 impulse frequency. The ordinate is the normalized cycle period (the cycle period following stimulation divided by the cycle period preceding it, *C2*); dashed line is the linear regression; slope differs significantly from zero, $P < 0.0001$; $r^2 = 0.70$; $n = 38$ observations. Data in *A*, *B*, and *C* are from separate preparations.

t-test, $P = 0.055$; $n = 5$). Note that in Fig. 2*B2*, nearly two cycles occurred between each cell E21 excitation.

Our data show that cell E21 excitation has strong effects on sustaining swimming. To interpret the influence of cell E21 on swim maintenance, we note that swim duration varies widely between preparations. Within each preparation, we observed many control swim episodes to establish the average control swim duration. As demonstrated earlier, transient cell E21 activity during this control swim duration was capable of exciting the swim-maintenance system and extending the period of the swim episode, indicating that, during this time, cell E21 activity has modest effects *beyond* the period of its own excitation. In contrast, when continuous cell E21 depolarization drove swimming activity well beyond its control duration, the swims terminated quickly after cell E21 was allowed to repolarize. These results suggest that for intervals close to the control swim duration, cell E21 activity can enhance the swim-maintenance system, but during prolonged depolarization cell E21 excitation becomes the sole driver of the swim-maintenance system. We also note that hyperpolarizing cell E21 by current injection has no obvious effects on swim duration. This finding is not surprising because cell E21 is not very active during swimming and, further, as we show in the following text, hyperpolarizing the soma of cell E21 does not control impulse activity in remote processes of this neuron.

Animal locomotion exhibits a broad range of cycle periods in response to the level of excitation that drives the underlying neuronal circuits. Excitation levels in the swim-maintenance circuit can modulate cycle periods; thus the mechanisms that affect cycle period can be considered swim-maintenance properties. To determine the effects of cell E21 on cycle period, we gave a single 400-ms depolarizing current pulse to cell E21 following burst 5 or burst 6 in swim episodes elicited by DP nerve shock (Fig. 2*C1*). The amplitude of the depolarizing pulses varied. In typical swim episodes, cycle period increases gradually as swimming progresses (cycles 1–4 in Fig. 2*C2*). To reduce the confounding effects of period variability, we evaluated the effects of cell E21 depolarization by computing fractional changes in cycle periods (Fig. 2*C2*). We found that the cycle period following the perturbation is inversely correlated with the impulse frequency of cell E21 (Fig. 2*C3*), demonstrating that cell E21 excitation decreases swim cycle period in a graded fashion. An interesting observation is that the swim period is often reduced for two to five cycles after cell E21 stimulation, indicating that the brief depolarization of cell E21 engenders several seconds of elevated excitation in the swim circuit. Three cycles after stimulation, cycle periods remain significantly shorter following cell E21 excitation (includes all impulse frequencies) than in control swims in which current was not injected (*t*-test, $P = 0.007$; $n = 38$ observations). These results are consistent with data showing that transient, repeated cell E21 depolarizations can increase swim duration (Fig. 2*B*).

These experiments demonstrate that cell E21 combines the gating features of cells 204 and triggering properties of cells Tr1 and Tr2. It is therefore of particular interest to compare the morphology and circuit properties of cell E21 with these neurons.

Identification

The soma of cell E21 is about 40 μM in diameter and is located on the ventral aspect of the most caudal segmental midbody ganglion, M21 (Fig. 3*A*). The positions of previously identified neuronal somata in the leech are highly stereotyped, although their precise coordinates may vary a bit. However, the location of cell E21 somata in our preparations was rather variable (Fig. 3*B*). In 65 preparations, the soma was most often found in the anterior medial glial packet ($n = 51$). Sometimes, however, it was located outside of the anterior medial packet ($n = 14$): in the right-lateral ($n = 4$), left-lateral ($n = 6$), or posterior ($n = 4$) packets (Fig. 3*B*). When not in the anterior medial packet, the cell E21 soma was usually found adjacent to it although rarely it was one cell body away. The packet margins in ganglion M21 sometimes appear distorted, which might account for some of the variability, although cell E21 was sometimes found outside the anterior medial packet even in preparations with "typical" packet margins (Fig. 3*B*). Searches for cell E21 homologs in ganglia M18–M20 ($n = 5$) never revealed a cell in or near the anterior medial packet with the properties of cell E21, whereas in >90% of our experi-

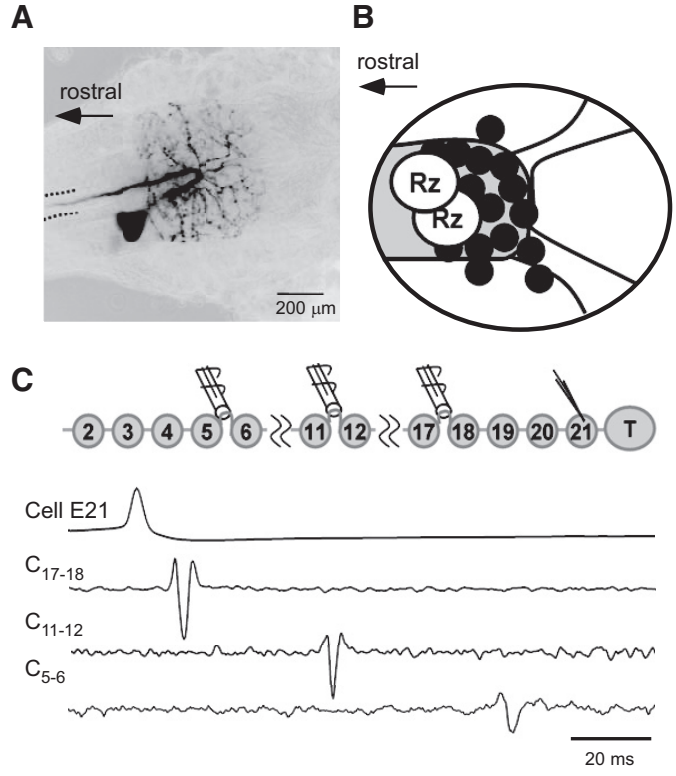

FIG. 3. Cell E21 morphology. *A*: cell E21 dyed with Alexa 568. The soma of this neuron is located on the ventral aspect of segmental ganglion M21. The neurite has rough symmetry around the midline of the central neuropile, with an axon that projects rostrally through the medial Faivre's nerve (indicated by dotted lines). Rostral is to the *left*. Color has been inverted. Soma appears enlarged due to long exposure time. *B*: locations of the soma. Filled circles indicate representative locations of cell E21 soma in preparations with discernable typical packet margins. Cell E21 is usually found in the anterior medial packet (gray) but also in adjacent packets at the positions indicated relative to the Retzius (Rz) cells. *C*: physiological extent of the cell E21 axon. *Inset*: recording configurations for the intracellular and extracellular recordings shown below. *Top trace*: cell E21 spike recorded in the soma. *Bottom 3 traces*: suction electrode records of cell E21 spikes from the intersegmental connectives (averaged data from 198 spikes). Note that extracellular spike size decreases progressively in more rostral recordings.

ments we found cell E21 in ganglion M21. Further, in >30 yr of cell surveys of midbody ganglion, no cell with cell E21 properties has ever been identified. We therefore conclude that within the M2–M21 nerve cord cell E21 is unique. However, we have not closely examined the caudal brain, which might contain cell E21 homologs among its 2,000 neurons.

Several medium-sized somata are colocalized with cell E21 in the anterior medial glial packet and thus electrical and functional properties also need to be considered for positive identification of cell E21. The resting membrane potential of cell E21, as measured 10 min following penetration, is approximately −42 mV ($n = 11$), with an impulse threshold at approximately −40 mV.

It should be noted that there is a cell of similar size with similar firing patterns found anterior to the Rz neurons that is capable of initiating swimming. This cell can be differentiated from cell E21 by the presence of postinhibitory rebound, greater latency for swim initiation, and the lack of inputs from sensory neurons (see following text). Further, visualization of this otherwise unidentified neuron with injected Alexa 568 dye revealed that its axon travels in the lateral connectives. We conclude that cell E21 is an easily identifiable neuron in M21 whose relatively large soma allows stable recordings.

Cell E21 has extensive processes and a rostrally projecting axon

An examination of neuronal morphology can aid in understanding the functional and circuit properties of a neuron. Neuronal processes may be local (limited to a segment) or intersegmental and may have either extensive ipsilateral, contralateral, or bilateral branching or more modest branching (Fan et al. 2005; Friesen et al. 1976, 1978; Masino and Calabrese 2002). Injection of Alexa 568 dye into the soma of cell E21 revealed a large neurite with extensive bilateral projections. Images of cell E21 show that the main neurite extends into the center of the neuropile regardless of the soma location. The axon, which is a continuation of the initial neurite segment, passes rostrally in the medial intersegmental connective, Faivre's nerve (FN; Fig. 3*A*). Its main axon does not project caudally. At least six secondary branches arise from the main neurite; these give rise to numerous tertiary branches. Although we used prolonged injection times, the dye never spread far into the intersegmental connective, preventing us from visualizing axonal processes rostral to M21. The bilateral neuritic arbor of cell E21 is similar to that of gating cell 204 (Weeks and Kristan Jr 1978) and the excitatory oscillator neuron cell 208 (Weeks 1982b), although cell 208's axon projects through a lateral connective, whereas the axons of cell 204 and cell E21 travel in the FN.

To determine how far the axon of cell E21 projects, we penetrated its soma with a microelectrode and placed suction electrodes on several intersegmental connectives. Axonal spikes were visualized through spike-triggered averaging (from soma spikes) and appeared at constant latencies with respect to spikes observed in the soma. These connective spikes were seen throughout the neuroaxis, including the connective between M1 and M2 (C_{1-2}), suggesting that cell E21 could have interactions throughout the nerve cord. We measured conduction velocity by simultaneous suction electrode recordings from three connectives ($n = 4$; Fig. 3*C*). Intersegmental travel times were less in the caudal nerve cord (7.6 ms/segment; $n = 4$; Fig. 3*C*) than those in the anterior end (8.5 ms/segment). Converted to conduction velocity, given an interganglionic spacing of about 5 mm, the values are about 0.7 m/s in the caudal cord and 0.6 m/s more rostrally. We observed a reduction in the amplitude of averaged spikes as the impulses travel rostrally (Fig. 3*C*), which is consistent with the observed changes in intersegmental conduction times. These observations suggest a thinning of the axon as it travels rostrally.

Cell E21 displays two spike waveforms

Many interneurons in arthropods and annelids receive inputs, initiate spikes, and have outputs in more than one segment (Calabrese 1980; Kennedy and Mellon Jr 1964; Moulins et al. 1979). Intracellular recordings from such neurons show prepotentials preceding spikes initiated near the recording site, but rise from the baseline without a noticeable inflection when the spike is initiated remotely, such as in a distant segment. We observed two differently shaped spikes in intracellular recordings from cell E21 (Fig. 4*A*). One waveform, an "A" spike (Fig. 4*A1*), has a prepotential, and thus was considered likely to originate within M21. Further, the "A" spike was elicited by depolarizing current injections into the soma and blocked by hyperpolarizing current injections. The second, "B" spike (Fig. 4*A2*), appears to be initiated remotely (from the soma) because of its abrupt rise from baseline and because it was not elicited by depolarizing current injections into the soma. Furthermore, "B" spikes were elicited by the DP nerve or sensory cell stimulation and could not be blocked by hyperpolarization.

To determine whether these two spike types were indeed initiated at different locations, we placed suction electrodes on two intersegmental connectives, C_{16-17} and C_{17-18}, while recording from the soma of cell E21. When we injected depolarizing current into cell E21 and elicited the "A" spike, we observed the cell E21 connective spikes with progressive caudal to rostral delays (Fig. 4*B1*), confirming that it had initiated near the soma and was traveling rostrally. We first attempted to elicit "B" spikes by stimulating rostral DP nerves in normal saline, however because of the massive activity evoked in the connectives we were unable to discern the cell E21 connective spikes. To reduce excitation we placed a petroleum jelly dam around M14 and filled the well thus created with saline. All other ganglia in the preparation were then bathed in a high divalent cation solution (HiDi: leech saline modified to include 10 mM Mg^{2+} and 10 mM Ca^{2+}) to raise the impulse threshold (Nicholls and Purves 1970). Under these conditions, we stimulated a pressure (P) sensory cell in M14, which elicited "B" spikes in the soma of cell E21. Here, we observed spikes first in C_{16-17}, then in C_{17-18}, and finally in the soma, indicating that "B" spikes are initiated rostral to the soma and travel caudally (Fig. 4*B2*). The increased travel time in the remotely elicited spikes in this figure was due to the HiDi saline; the "A" spikes showed similar intersegmental travel times during HiDi application.

The "B" remotely initiated spikes, but not the "A" locally initiated spikes, were abolished by cutting the medial FN between ganglia M20 and M21 ($n = 2$; not shown), providing confirmation that "B" spikes are not initiated in the caudal brain or in M21 and that the cell E21 axon runs in the FN. Although these local and remotely initiated waveforms are

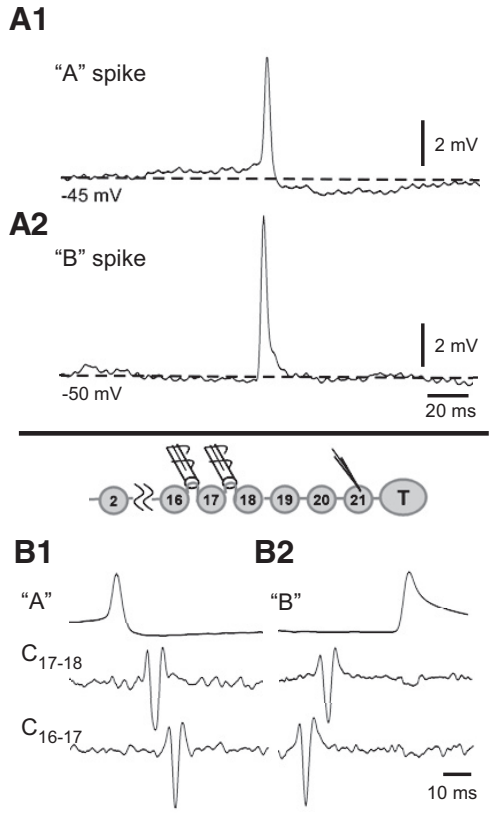

FIG. 4. Cell E21 displays 2 spike waveforms. *A:* soma spikes. *A1*: the "A" spike waveform is preceded by a depolarizing prepotential and exhibits an undershoot. *A2*: the "B" spike rises abruptly from baseline, with no prepotential, and has a depolarizing afterpotential. Amplitudes of spikes in *A1* and *A2* range from 5 to 8 mV. Spike durations measured at half-maximum amplitude are about 3 ms. *B*: source of soma spikes. *Inset*: locations of the intracellular and suction electrode recording sites. *B1*: simultaneous recordings of soma and axon spikes when soma is depolarized. Cell E21 spikes initiated by soma depolarizations appear first in the soma recording, with sequential delays in the connective records. These soma spikes have the "A" spike waveform. *B2*: cell E21 spikes recorded when activity is initiated in the rostral nerve cord. Caudally traveling impulses give rise to soma spikes with the "B" spike waveform. Suction electrode records were obtained from intersegmental connectives between ganglia M16 and M17 (C_{16-17}) and between M17 and M18 (C_{17-18}). Data are averaged traces of 82 spikes in *B1* and 129 spikes in *B2*.

usually easily distinguished, a high rate of activity in cell E21 can make spike identity ambiguous by obscuring any prepotential. Amplitudes of both soma spikes range from 5 to 8 mV and have a duration, at half-maximum amplitude, of about 3 ms. Locally initiated "A" spikes typically have an undershoot (Fig. 4*A1*). The "B" spikes observed when the soma of cell E21 was hyperpolarized usually have a brief depolarizing afterpotential (Fig. 4*A2*). These results demonstrate that cell E21 has at least two spike initiation zones: one that is electrically near the soma and another, perhaps several, electrically distant, in more rostral ganglia.

Cell E21 receives mechanosensory input

Body wall stimulation elicits cell E21 activity. Cell E21 activity has strong effects on swim properties but only exhibits a small increase in firing during swimming, raising the question of what inputs excite cell E21 to trigger or modulate swimming. Because mechanosensory inputs are known to elicit swimming in intact (Kristan Jr et al. 1982) and semi-intact animals (Kristan Jr et al. 1974), as well as in nearly isolated nerve cord preparations with a flap of body wall attached (Debski and Friesen 1985), we checked whether such inputs might excite cell E21. Our semi-intact preparations comprised a nearly intact rostral half of the leech with the caudal nerve cord isolated. In four of six semi-intact preparations, we detached the rostral brain by severing the nerve cord between M1 and M2; in two other preparations the rostral brain and ganglion M1 remained attached, but the body wall was denervated. We stroked and poked, and in one preparation pinched, the rostral leech body wall. These mechanical inputs to the body wall elicited spiking activity in intracellular recordings from the soma of cell E21 in all six preparations (Fig. 5*A*). Prolonged stroking or pinching (>300 ms) elicited spiking, with average frequencies up to nearly 35 Hz; spike bursts (four to seven impulses) achieved 55 Hz. Pinching elicited the highest impulse frequencies (Fig. 5*A*, *bottom trace*). The spike waveforms following these sensory inputs were mostly those of remotely evoked "B" spikes.

Noxious pinching elicited higher E21 firing frequencies than stroking; further, gentle stroking seemed to elicit less intense spiking then firmer contact. To quantitatively test whether the intensity of the input influences the strength of the cell E21 response, we used nearly isolated preparations in which only a small flap of the caudal sucker was attached to an otherwise isolated nervous system. We used electrical stimulation of the body wall, which activates the mechanosensory neurons and has behavioral effects similar to mechanical sensory stimulation (Kristan Jr et al. 1982). Stimulus trains (five to seven pulses, with durations of ~1.5 ms at 5.9 Hz) with three different amplitudes, 2, 5, or 7 V, were delivered every 60 s in random order while recording from the soma of cell E21 ($n = 4$). The strength of cell E21 responses to this stimulation was correlated with the intensity of the shocks (Fig. 5*B*). Minimal depolarization and spiking activity occurred in response to 2-V shocks, whereas large depolarizations with several spikes occurred following each 7-V pulse. Quantitative results are shown in Fig. 5*C*, which is a graph of the total number of spikes evoked in cell E21 by the first five pulses at each stimulus intensity. Clearly, higher voltages elicited significantly stronger responses (repeated-measures ANOVA, $P < 0.001$, $n = 4$, Newman–Keuls posttest). These results show that mechanical stimulation of both rostral and caudal body wall elicits activity in cell E21 and that the level of this response increases with stimulus intensity.

Mechanosensory cell stimulation elicits cell E21 activity. Mechanical or electrical stimuli applied to the body wall activate local T (touch), P (pressure), and N (nociceptive) cells, which are located in all ganglia (Kristan Jr et al. 1982; Nicholls and Baylor 1968). We undertook paired recordings ($n = 4$) of cell E21 and sensory neurons in normal saline to determine whether excitation of individual sensory neurons could elicit activity in cell E21. We stimulated individual identified sensory neurons in M10, remote to the cell E21 soma, and sensory neurons in M21, local to the cell E21 soma, with current pulses of varying amplitudes and observed the response in cell E21. Excitation of all remote (Fig. 6*A*) and local (Fig. 6*B*) T, P, and N cells elicited excitation in cell E21. The EPSPs evoked by stimulation of sensory cells in M21 were usually larger than those evoked by stimulation in M10. Cell E21 excitation had no effect on sensory cell activity (not shown). These data

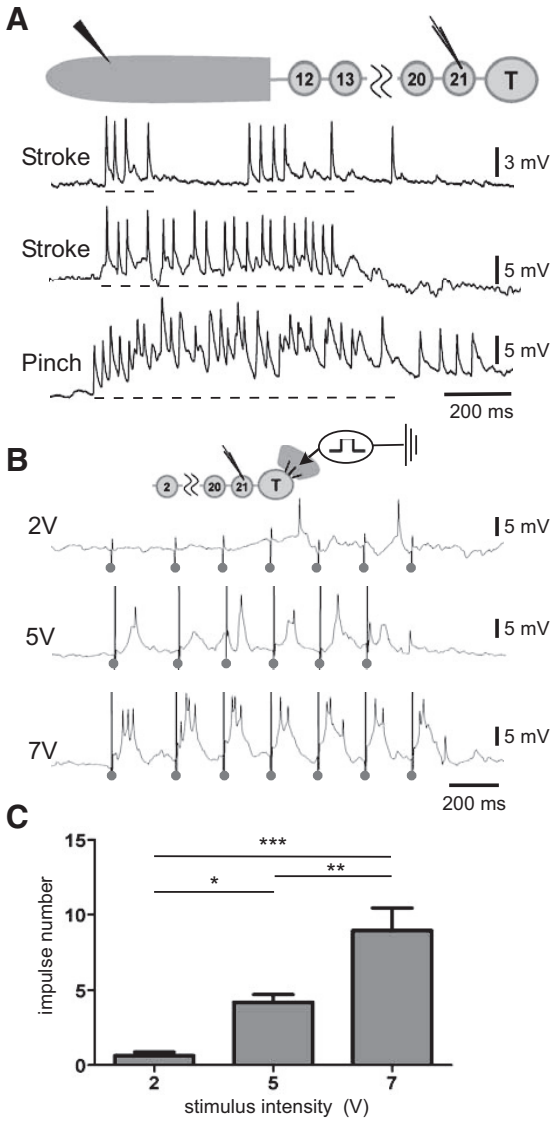

FIG. 5. Body wall stimulation activates cell E21 *A*: activity in cell E21 during stroking and pinching of the rostral body wall in a semi-intact preparation. *Inset*: experimental preparation; the shaded rostral body wall is nearly intact. Dashed line denotes the approximate timing of the stimulus. Note that the more noxious stimuli (pinching) elicited a higher impulse frequency. Responses are from 3 separate preparations. Data in the *top trace* were obtained from preparations bathed in saline containing serotonin (50 μM) on the isolated portion. *B*: nearly isolated preparation with a small flap of caudal body wall (*inset*). The body flap was stimulated by 2-, 5-, or 7-V electrical shocks, which activated cell E21 in a graded fashion. The gray dots mark the timing of the shocks. *C*: quantified data from *B*. Bars show the average number of impulses elicited by the first 5 stimulus pulses in each sequence. Shocks were delivered every 60 s. Repeated-measures ANOVA, $P < 0.001$, followed by Neuman–Keuls multiple-comparison posttests; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

indicate that the excitatory response in cell E21 following mechanical and electrical inputs to the body wall is mediated by the mechanosensory neurons.

Mechanosensory neurons directly contact cell E21. Our data show that excitation of individual, identified sensory neurons activates cell E21, but are these connections direct? Leech mechanosensory neurons synapse with other swim-initiating cells, including the cephalic trigger neurons and cell SE1 (Brodfuehrer and Friesen 1986c; Brodfuehrer et al. 1995). To test whether cell E21 receives similar inputs, we repeated the paired recording from sensory neurons and cell E21 in HiDi saline. HiDi raises the impulse threshold to block most spiking activity, thereby blocking polysynaptic pathways (Nicholls and Purves 1970), including those mediated by electrical junctions (Crisp 2009). The presence of discrete, constant-latency EPSPs

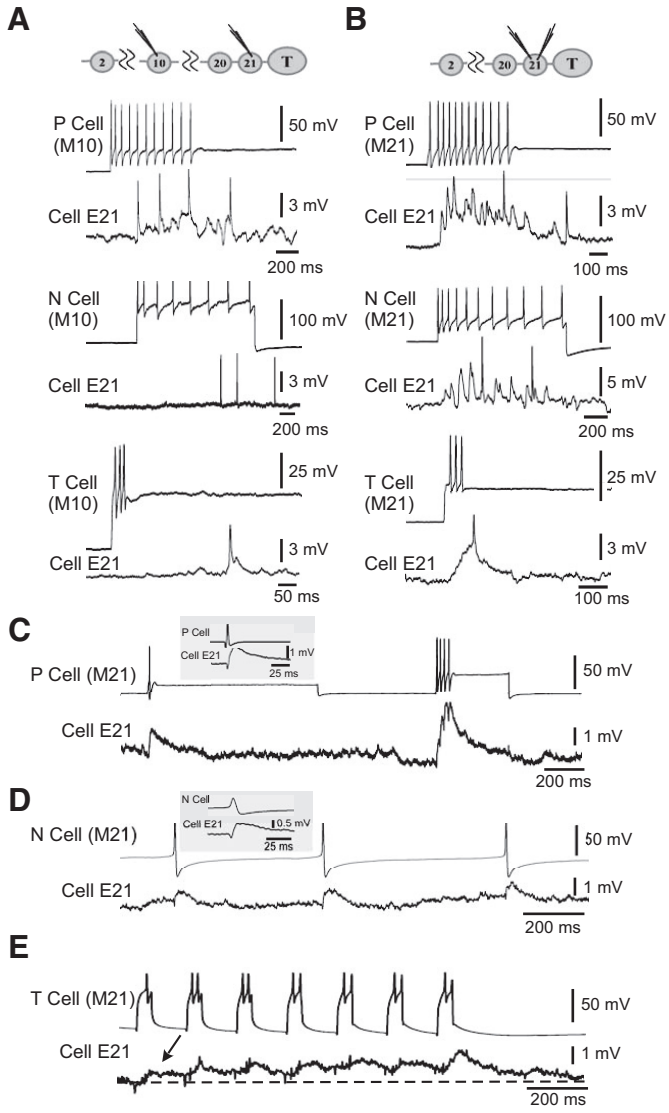

FIG. 6. Mechanosensory neurons excite cell E21. *A*: responses of cell E21 to excitation of remote mechanosensory cells, P (pressure; r), N (nociceptive; r), and T (touch; l) cells. The sensory neurons and cell E21 are in different ganglia (*inset*). *B*: responses of cell E21 to excitation of local (within M21) mechanosensory cells P, N, and T (*inset* shows recording configuration, all from the l side). The evoked synaptic excitation is stronger from sensory cells in M21, whereas spiking activity is comparable following distant and local sensory cell activity. Data in *A* and *B* are from 4 preparations. *C–E*: monosynaptic tests. *C*: in high divalent (HiDi) cation solution, an individual P cell (r) spike elicits an excitatory postsynaptic potential (EPSP) in cell E21; a P cell spike burst leads to EPSP summation. (Recording configuration is that of *inset* in *B*.) *Inset*: averaged data from 6 spikes recorded from another P cell in the same ganglion. These short and constant-latency (~3–4 ms) cell E21 EPSPs following single P cell spikes implicate a direct P cell to cell E21 input. *D*: in HiDi solution, EPSPs follow individual N cell (l) spikes (~4 ms). *Inset*: averaged data from 10 traces in the same preparation. Data from *C* and *D* are from the same preparation. *E*: a small EPSP follows an individual T cell (l) spike; larger, more easily discernable EPSPs are evoked by 2-spike trains. Dashed line shows baseline. r, l refer to the laterality of the sensory neurons.

with brief synaptic delay in HiDi strongly suggests a direct synaptic connection. However, it is unlikely that EPSPs evoked at sites outside M21 would be visible in our recordings due to electrotonic decay; sensory cells have only local projections (Nicholls and Baylor 1968). We examined the responses of cell E21 evoked by individual sensory neurons both in a remote ganglion (M10) and in M21 in HiDi ($n = 6$). We found that single P cell spikes evoked in M21 were usually followed by discrete EPSPs ($\leq$1.5 mV); the decay of these EPSPs had a time constant of around 100 ms (Fig. 6*C*, *inset*). (Occasionally, no distinct EPSP was visible following individual local P cell spikes.) P cell spike trains, elicited by longer current pulses, resulted in temporal summation (Fig. 6*C*). Results were more variable following stimulation of M21 T and N cells. Recordings from two of five N–E21 pairs revealed no discrete EPSPs in cell E21 following N cell spikes. However, in three of the five, small ($\sim$0.6 mV) but unambiguous EPSPs were visible after individual N cell spikes (Fig. 6*D*). When we elicited single spikes in M21 T cells ($n = 2$), small EPSPs ($<$0.4 mV) were sometimes visible (Fig. 6*E*, arrow), although these EPSPs did not occur reliably. However, when stimulated to generate trains of spikes all local T cells ($n = 7$) elicited discrete cell E21 EPSPs in preparations bathed in HiDi saline (Fig. 6*E*).

Because HiDi saline elevates the impulse threshold, spikes were absent from cell E21 recordings following local sensory cell excitation in HiDi saline, but discrete EPSPs were clearly discernible. As expected, excitation of M10 sensory cells with the preparation in HiDi generated neither spikes nor visible EPSPs in the soma recordings of cell E21 (not shown). These results neither confirm nor deny the presence of direct connections between remote sensory neurons and cell E21. However, because leech circuitry is highly stereotyped (Kristan Jr et al. 2005) and because sensory neurons in M21 directly contact cell E21, it is likely that the sensory inputs in ganglia anterior to M21 are also direct.

Firing rates elicited from sensory stimulation have functional effects

After determining the range of cell E21 impulse frequencies elicited by mechanosensory stimulation (Fig. 5), we examined the impact of such realistic impulse frequencies on swim properties. We reexamined our earlier data, focusing on the effects of cell E21 impulse frequencies of $\leq$40 Hz. First, we note that swims can be initiated by impulse frequencies in cell E21 well under 35 Hz (Fig. 1*B2*; see also Fig. 10*A*). We then reanalyzed the effect of cell E21 activity on cycle periods. In that experiment, we had given 400-ms depolarizing current pulses into cell E21 following burst 5 or burst 6 of a swim initiated by DP nerve shock. We binned those data into three groups based on the firing rate of cell E21 during the current pulse: group 1: 21–30 Hz; group 2: 31–40 Hz; and group 3: 0 Hz (no stimulation). With 0-Hz stimulation, the ratio of the cycle periods preceding the "stimulation" to those directly following it was 1.11; that is, cycle periods increased by an average of 11%. In the 21- to 30-Hz bin the period increased only 4%. In the 31- to 40-Hz bin the ratio was 0.88; that is, the cycle period *decreased* by 12%. A comparison of the cycle periods in the three groups showed that they were all significantly different from each other (one-way ANOVA with Neuman–Keuls posttest, $P < 0.05$). We also compared the cycle period of bursting later, two cycles after the stimulus, to the cycle period prior to the stimulus. Here, a one-way ANOVA test between the three groups showed significance ($P = 0.047$); however, posttests (Newman–Keuls) yielded significant differences only between the 0-Hz and 31- to 40-Hz groups ($P < 0.05$). Our results thus demonstrate that physiologically appropriate firing rates in cell E21, such as are elicited by mechanosensory stimulation, can initiate swimming and decrease cycle periods.

Cell E21 controls swimming in semi-intact animals

Isolated nerve cord preparations retain important aspects of the swimming motor program observed in intact animals (Kristan Jr and Calabrese 1976; Pearce and Friesen 1984); however, sensory feedback clearly has powerful influences on swim behavior (Cang and Friesen 2000; Yu et al. 1999). To get a more complete picture of the effects of cell E21 excitation in the intact animal, we used semi-intact preparations to observe behavioral responses to this cell's activity. The rostral portion of the animal was left nearly intact, whereas the body wall was dissected from the caudal nerve cord. Suction electrodes were placed on the caudal DP nerves. The anterior ganglia, H and M1, were detached by severing the nerve cord between M1 and M2 so that the intact nerve cord comprised ganglia M2–T, comparable to our M2–T isolated preparations. We then placed an intracellular electrode into cell E21 and monitored behavior both by visual inspection and through DP nerve recordings. In the first set of these experiments, the preparation was placed in a flat-bottom dish ($n = 3$). In all these semi-intact preparations, cell E21 depolarizations elicited swim episodes (Fig. 7). Importantly, "physiological" cell E21 impulse frequencies (up to $\sim$35 Hz) were sometimes able to initiate or prolong swimming. Note that in Fig. 7, swim episodes were elicited by cell E21 activity $<$30 Hz; these low firing frequencies also seemed to maintain the swim episodes because swims terminated shortly after the termination of current injection. However, in these preparations, the responses to cell E21 stimulation were more variable than those observed in isolated nerve cords. Swimming was less reliably initiated and maintained and sometimes cell E21 stimulation elicited shortening, thrashing, or swim–thrash hybrid behavior that was usually accompanied by a nonrhythmic or semirhythmic increase in impulse activity in the caudal DP nerves.

It was unclear whether this response variability was caused simply by the presence of the body wall. Alternatively, the variability could have arisen from the relatively low level of the saline ($\sim$6 mm deep) around the intact portion of the leech or from contact of the body wall with the dish, both of which can be swim-inhibitory factors (Esch et al. 2002; Gray et al. 1938; Puhl and Mesce 2010). We therefore generated a semi-intact preparation in which the rostral intact portion of the leech was suspended in deep water ($>$10 mm; $n = 3$), eliminating the confounding swim-inhibitory factors. Under these conditions, cell E21 depolarization triggered swimming and decreased cycle periods in ongoing swim episodes. Further, prolonged cell E21 excitation reliably maintained swim episodes, even at impulse frequencies $<$20 Hz. In sum, the responses to cell E21 activity in suspended, semi-intact leeches were similar to those we observed in isolated nerve cord preparations. The variability of cell E21 responses in the

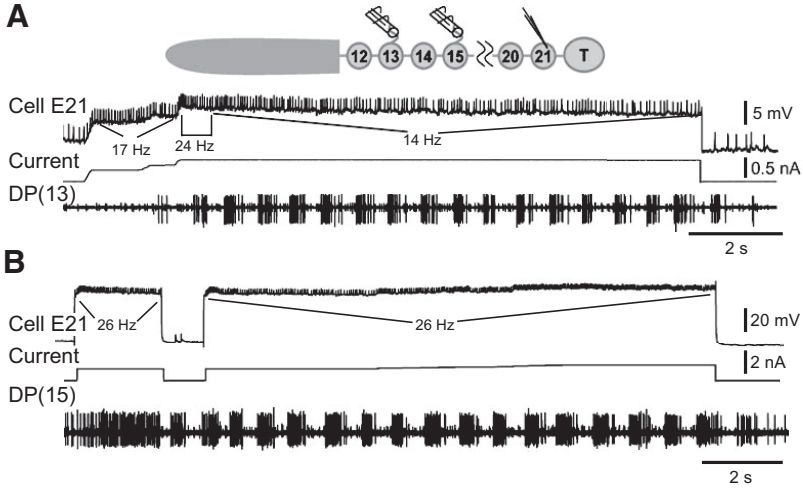

FIG. 7. Physiologically appropriate cell E21 activity elicits swimming in semi-intact preparations. *Inset*: experimental setup for both *A* and *B*. *A*: impulse frequencies comparable to those elicited from body wall stimulation initiated (24 Hz) and maintained (14 Hz) swimming. *B*: moderate frequency cell E21 activity (26 Hz) initiated and extended swimming. This swim was 80% longer than the longest control swim in this preparation.

flat-bottom dish appears to have been caused by swim-inhibitory inputs to the body wall, not the presence of the body wall itself. These data show that cell E21 activity can initiate and maintain swimming in a semi-intact animal, providing support for the idea that E21 may have this function in the intact animal. However, with swim-inhibitory factors present, it is possible that cell E21 might also mediate escape behaviors that precede or replace escape swimming, such as shortening or thrashing.

Cell 204 is a mediator of cell E21 output

The question arises: Which cells mediate cell E21's strong functional effects? We have described several functions of cell E21 that are similar to gating cells 204, including the ability to extend swimming and decrease the cycle period. Cell 204 somata are located in midbody ganglia M10–M16, with the soma of a functionally similar homolog, cell 205, in M9 (Weeks 1982a). We investigated whether these gating neurons are postsynaptic to cell E21 by conducting paired intracellular recordings from the somata of cells 204/205 and cell E21 in isolated nerve cord preparations. These experiments showed that cell E21 excitation depolarizes these gating neurons (Fig. 8*A*). In the example shown, a 32-Hz (average during stimulation) cell E21 excitation lasting 1.5 s increased the impulse frequency in cell 204 by a factor of 5, from 4 to 20 Hz; cell 204 excitation occurred even if a swim episode was not initiated (not shown). In normal saline, cell E21 impulse activity elicited a strong excitatory depolarization and increase in impulse frequency in all cells 204 tested in M10 ($n = 3$), M11 ($n = 1$), M15 ($n = 1$), and in cell 205, in M9 ($n = 1$). Because leech circuitry is highly stereotyped among ganglia, these data strongly suggest that cell E21 excites all cell 204/205 homologs in segments M9–M16. Because firing of a single gating neuron can sometimes drive swimming, these multiple outputs to eight gating cells can account for the highly reliable swim initiation observed in response to cell E21 firing.

When cell 204 is hyperpolarized, cell E21 spikes elicit constant-latency, unitary EPSPs with amplitudes $\leq$1.5 mV and a decay time constant of roughly 28 ms (Fig. 8*B*). These EPSPs persisted in HiDi in four cells 204 tested (located in M10, M11, M14, and M15), and two cells 205, indicating that this connection is monosynaptic (Fig. 8*B*, *inset*, shows averaged data). Thus the strong functional effects of cell E21 depolarization on swim initiation and maintenance could occur through direct excitatory interactions with all gating neurons, cells 204 and cell 205.

Cell E21 can initiate spikes throughout the nerve cord

Our data show that cell E21 can initiate axon spikes in ganglia remote from M21 in response to sensory inputs (Figs. 5*A* and 6) and that cell E21 makes direct synaptic contacts with midbody cells 204 (Fig. 8). We sought to determine the location of these remote impulse initiation sites. Can sensory input at any segmental ganglion activate cell E21 and could cell E21 therefore serve as a direct link from sensory input to gating neurons *anywhere* along the ventral nerve cord? To answer these questions, we used the following protocol. A petroleum jelly dam was placed around one midbody ganglion (the "target ganglion") and the inside was filled with saline (Fig. 9*A*). Cell E21 was penetrated in M21 while one sensory neuron (usually a P cell) was penetrated in the target ganglion. A HiDi solution was superfused over the entire nervous system

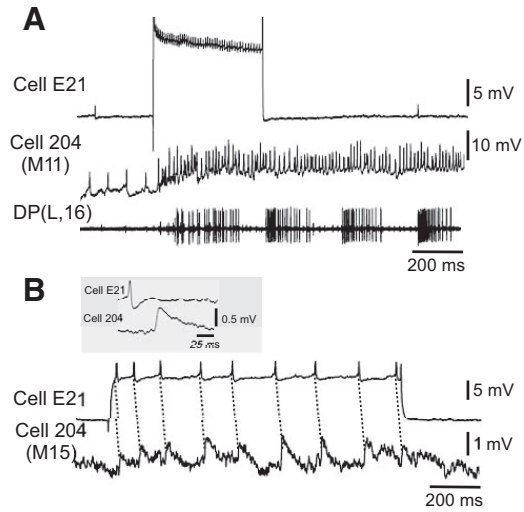

FIG. 8. Cell E21 provides excitatory input to cell 204. *A*: cell E21 excitation increases impulse frequency and membrane potential in cell 204, initiating swimming. *B*: cell E21 impulses elicit 1:1 constant latency (here, ~32 ms) EPSPs in cell 204. These persist in HiDi (*inset*), suggesting that this interaction is direct. *Inset*: averaged data from 10 cell E21 impulses and the corresponding EPSPs in cell 204, bathed in the HiDi solution.

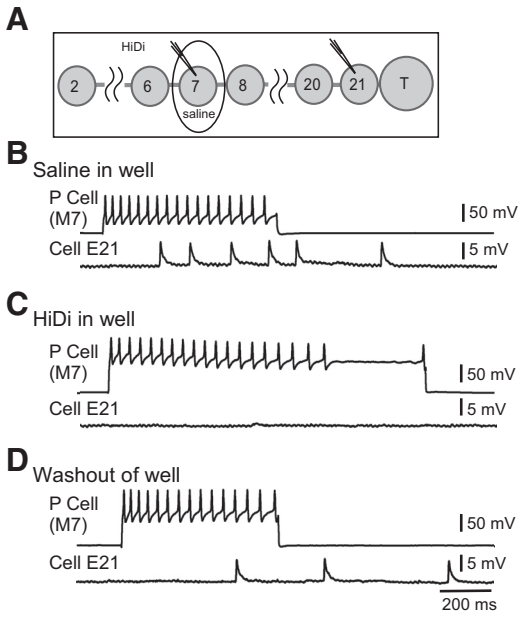

FIG. 9. Cell E21 has spike initiation sites throughout the nerve cord. *A*: experimental setup. A petroleum jelly dam was used to create a saline well for the "target" ganglion. High-divalent cation (HiDi) saline was bath-applied to the remaining nerve cord to raise impulse thresholds throughout the nerve cord except at the target ganglion. *B*: normal saline in the well at M7. Excitation of a P cell in M7 elicited a barrage of impulses in cell E21. *C*: HiDi saline in well. With impulse thresholds elevated, P cell activation did not elicit spikes in cell E21. *D*: following washout of the HiDi saline with normal saline, P cell activity again elicited spiking in cell E21.

except for the target ganglion. Recall that HiDi blocks cell E21 spiking following sensory cell excitation (see Fig. 6, *C–E*). We found that impulse activity in sensory neurons in all target ganglia tested ($n = 4$; M4, M7, M10, and M14) elicited spikes in cell E21 (Fig. 9*B*), demonstrating that a cell E21 spike initiation site is present in each of these ganglia. Cell E21 spiking did not occur after the HiDi solution replaced the normal saline in the well (Fig. 9*C*); washout of the HiDi with saline restored cell E21 spiking in response to sensory cell stimulation (Fig. 9*D*). We also tested for the presence of cell E21 spike initiation in M20 by simply cutting the intersegmental connectives between M19 and M20, leaving an M20–T preparation ($n = 2$). With cell E21 hyperpolarized to block local spike initiation, stimulation of the M20 DP nerve elicited "B" spikes, which are initiated outside of M21. We conclude that these spikes are initiated in M20 and not the caudal brain (T) because cutting FN rostral to M21 abolishes all "B" spikes. Because cell E21 spike initiation occurred in all five midbody ganglia tested, we conclude that cell E21 has impulse initiation sites in most, perhaps all, midbody ganglia. These distributed activation sites, together with local synaptic inputs from sensory neurons, should allow cell E21 to provide a direct, short-distance pathway between sensory inputs anywhere along the body to all gating cells 204/205.

Cell E21 effects are conveyed exclusively via Faivre's nerve

Our data suggest that excitatory actions arising from cell E21 are mediated by direct excitation of cell 204. However, Alexa dye images show that cell E21 has extensive local processes and thus the potential for outputs within M21 (Fig. 3*A*). To test whether cell E21 exerted any of its effects on swimming through local neurons that then project through the lateral intersegmental connectives (LCs), we made selective partial lesions in the C_{20-21} connective in the isolated nerve cord. We either cut both LCs, which each contain around 2,700 axons (Wilkinson and Coggeshall 1975), leaving the FN intact ("LC-cut"), or we severed the FN nerve, which contains about 100 axons, leaving the LCs intact ("FN-cut"). In LC-cut preparations, cell E21 excitation initiated swimming and decreased cycle periods as in intact preparations (Fig. 10*A*; $n = 2$). However, in FN-cut preparations, even high-frequency stimuli (up to 67 Hz) did not increase DP nerve activity, initiate swimming, decrease cycle period, or extend swim duration (Fig. 10*B*; $n = 4$). These data suggest that cell E21 acts on swimming only via the rostral-projecting axon and not through local outputs to secondary neurons. Alternatively, cell E21 could modulate swimming through local neurons if their axons also exit M21 in the medial FN.

DISCUSSION

We have described the electrophysiological and morphological properties of a novel neuron, cell E21. This neuron has strong effects on fictive swim initiation, duration, and cycle periods. Cell E21 receives mechanosensory inputs throughout the body and, in turn, directly excites swim-gating neurons, cells 204 and 205. We found that cell E21 impulses can be initiated in all midbody ganglia examined, and travel both rostrally and caudally through its axon located in the medial Faivre's nerve. We conclude that cell E21 serves as a local (within segmental ganglia) and distributed (interactions all along the nerve cord) nexus for sensorimotor integration with broad control over swim initiation and excitatory drive. We emphasize that cell E21 is the first trigger neuron of segmental origin identified in the leech and also the first identified whose cell body is at the caudal end.

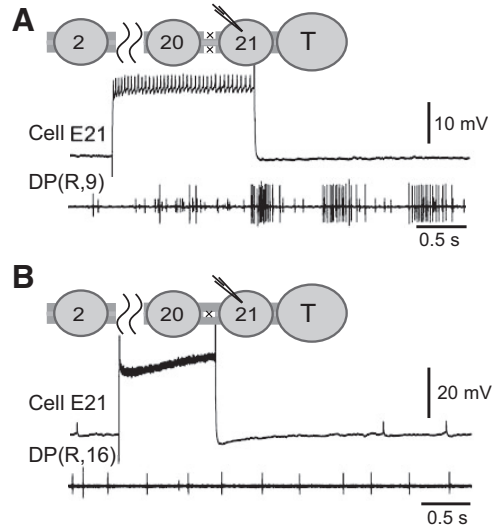

FIG. 10. Cell E21 acts through Faivre's nerve. *A*: lateral intersegmental connective nerves severed. With only the medial Faivre's nerve intact (*inset*) cell E21 activity at 25 Hz reliably initiated swimming. *B*: with Faivre's nerve severed, but lateral intersegmental connective nerves intact (*inset*), high-frequency excitation of cell E21 (67 Hz) failed to elicit swimming. Note the absence of any response in DP (R,16). These data show that cell E21 drives rostral nerve cord activity only via Faivre's nerve.

Proposed model: anatomy and circuitry

Like most invertebrate neurons, cell E21 is monopolar, but with the unusual characteristic of rough bilateral symmetry of its processes around the ganglion midline. Although we were able to visualize its processes only in M21, we found that cell E21 receives bilateral sensory inputs along much of the nerve cord. We thus hypothesize that cell E21 has repeated bilateral processes in all midbody nerve cord ganglia (Fig. 11*A*). The proposed composite input–output structure for cell E21 comprises direct synaptic inputs from the tactile mechanosensory neurons and excitatory output synapses to cells 204/205 (Fig. 11*B*).

Initiation and maintenance

Cell E21's excitation of the gating neurons excites multiple homologs of the excitatory interneuron cell 208 (Weeks 1982a,b). Each cell 208 provides monosynaptic excitation to at least two pairs of oscillator interneurons, cells 115 and 28, in most, and perhaps all, midbody ganglia (Nusbaum et al. 1987). Overall, there exists an interneuronal amplifier cascade, with cell E21 at the apex, so that excitation of this unique cell rapidly diverges to excite gating neurons and then oscillator interneurons in a strong avalanche of activity. The consequence is reliable swim initiation and, when cell E21 is driven during swimming, a marked decrease in cycle period and prolonged swim durations. The initiation of this cascade by a brief cell E21 stimulation is consistent with a role as a trigger neuron. Neurons that initiate behaviors that outlast the stimulus, trigger command cells, are particularly prominent in stereotyped escape behaviors such as crayfish tailflips (Kramer and Krasne 1984; Olson and Krasne 1981) and the startle response coupled to escape swimming in teleost fish (Sillar 2009; Zottoli 1977), but they also initiate flight in the locust (Bicker and Pearson 1983) and swimming in the mollusk (Arshavsky et al. 1998) and the leech (Brodfuehrer and Friesen 1986b). Excitation of *gating* command neurons can also initiate locomotion, but their continued excitation is necessary, or at least important, for sustaining the behavior. Identified gating interneurons contribute to rhythmic behaviors in the cricket, sea slug, *Aplysia*, and leech (Bohm and Schildberger 1992; Gamkrelidze et al. 1995; Jing and Gillette 1999; Weeks and Kristan Jr 1978). Reticulospinal neurons in the brain stem of the lampreys have a similar function (Di Prisco et al. 1997, 2000; McClellan and Grillner 1984). Injections of depolarizing current into command neurons, and especially gating neurons, during locomotion can increase the frequency of the locomotor rhythm and prolong the behavior, properties shared by cell E21. Although continuous excitation of this cell could usually sustain swimming indefinitely, repeated pulses of cell E21 activity eventually became ineffective. When swims were extended well beyond their control duration by continuous cell E21 excitation, swimming ceased when the current was terminated. Continuous high-frequency stimulation of cell E21 is almost certainly nonphysiological, based on its low firing frequency during swimming. However, the strength of these effects is remarkable and may provide future insight into how the maintenance system functions. Further, the fact that cell E21 stimulation has effects that outlast its stimulation likely does have a physiological function. Mechanosensory inputs during an ongoing swim episode would cause activation of cell E21, which could lead to a faster, more vigorous swim. Cell E21 may also be activated by unknown inputs, to have effects on maintenance system. Based on these considerations, cell E21 is best labeled as a "trigger command neuron," but with strong gating properties.

Comparison with previously identified swim neurons

Similarities between cell E21 and cells Tr1 and 204 led us to ask whether any of these neurons could be homologs. The ability to trigger swimming by soma depolarization is similar in Tr1 and cell E21; further, the axons of both cells project the length of the nerve cord, both respond to distributed sensory inputs, and both excite the swim-gating neurons (Brodfuehrer and Friesen 1986b,c). However, cell Tr1 occurs as a mirror-image pair with its homolog, has ipsilateral neurite processes, and its soma is located in the rostral brain. Further, cell E21, unlike Tr1, appears to receive feedback from the swim–oscillator system. These differences show that cells E21 and Tr1 are not homologs.

Cell E21 shares features with cell 204 beyond gating functions. Both neuronal somata are located in the ventral anterior medial packet, both exhibit roughly symmetric bilateral processes, and both have axons that project intersegmentally through the medial Faivre's nerve. Further, neither has a homolog within its ganglion. However, cell E21's soma diameter is almost 50% larger than the soma of cells 204. Importantly, unlike cell E21, cells 204 exhibit sustained depolariza-

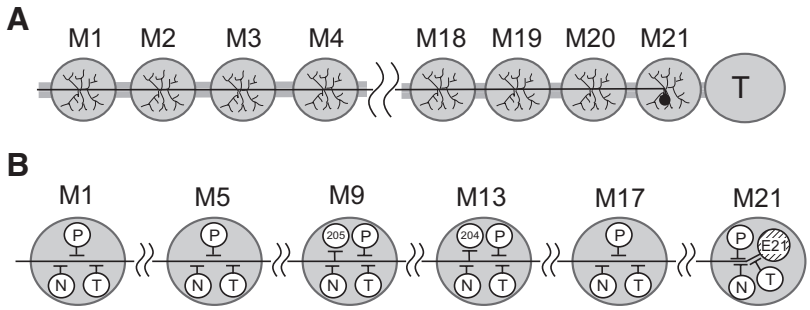

FIG. 11. Summary. *A*: conjectural anatomy of cell E21. The processes in ganglion M21 are based on images from dye injection into the soma. Processes in more anterior ganglia are assumed to resemble those in M21 because of the identified input–output connections of cell E21 in many ganglia of the nerve cord. *B*: circuit diagram of demonstrated and proposed cell E21 inputs and outputs. Every fourth ganglion is shown. The axon of cell E21 projects down the nerve cord via the medial Faivre's nerve. Using the criterion of constant latency, 1:1 EPSPs that persist in HiDi, there are direct synaptic inputs to cell E21 from local (M21) mechanosensory cells and direct synaptic outputs to cell 205, and all tested cells 204. We propose that cell E21 also receives direct synaptic inputs from remote mechanosensory neurons and directly contacts all cells 204, in M10–M16. T-bars indicate excitatory connections.

tion and large increases in impulse frequency during swimming, only initiate spikes near the soma, are not directly postsynaptic to mechanosensory neurons and do not directly synapse with other cell 204 homologs (Weeks and Kristan 1978). Because of these numerous differences, cell E21 seems unlikely to be a cell 204 homolog.

Role in the intact animal: escape?

In isolated preparations and suspended semi-intact preparations, cell E21 excitation elicited a stereotyped swim response. However, in semi-intact preparations where swim-inhibitory factors were present, the responses were variable and included shortening, thrashing, or writhing, which were sometimes, but not always, followed by swimming. Noxious shocks to leech body wall elicit similar escape-type behaviors such as "tensing" and "writhing" followed by escape swimming (Kristan Jr et al. 1982). The similarity of these responses suggests that cell E21 might mediate escape movements. Although there are often long latencies (>1 s) preceding fictive swimming, the latency to increased motor activity in the DP nerve is usually much shorter (<200 ms; cf. Fig. 2). This unspecified activity may correspond to one of these withdrawal behaviors that precedes the swim. In other species, high-speed rhythmic locomotor escapes are often preceded by some preliminary, nonrhythmic movements. Examples include the C-start turn that precedes swimming in teleost fish (Sillar 2009), turns away from the predator that precede running in cockroaches (Domenici et al. 2008), and freezing that often precedes darting in rats (Mitchell et al. 1988). It is currently unknown whether cell E21 responds to other sensory modalities such as water waves, light, or chemotactic factors that can elicit rapid onset of predatory or exploratory swimming. Determining whether other stimuli can activate cell E21 will clarify the range of inputs, outputs, and functions mediated by this neuron.

Importantly, in our experiments, cell E21 impulse frequencies in the range of those elicited by mechanical stimulation initiated and modulated swimming. These physiologically appropriate activity levels in cell E21 generate a range of outputs that might function in escape behavior. Moreover, noxious mechanical stimuli, which are likely to elicit swimming in quiescent leeches (Kristan Jr et al. 1982), elicited strong responses in cell E21 (Fig. 5). Clearly cell E21 activation does not occur in isolation and parallel pathways are involved in swim control. Sensory inputs also activate cephalic trigger neurons and cell SE1, which also excite gating cells 204/205 (Brodfuehrer and Friesen 1986b; Brodfuehrer et al. 1995). Most likely, cell E21 acts in concert with these cells during the initiation of escape swimming, which raises the possibility that, in conjunction with these additional neurons, low-frequency cell E21 activity might have an even larger role in swim initiation than described here. Alternatively, cell E21 may be the major CNS element in rapid swim initiation.

Distributed input–output

Rapid onset of locomotion requires short-latency neuronal pathways between sensory stimuli and muscle activation. Minimizing the distance that signals must travel or minimizing impulse conditions times are two means toward achieving this end. Neurons in several species can initiate spikes in areas remote from their somata (Chen et al. 1997; Funch and Faber 1980; Le et al. 2006; Mathy et al. 2009; Meyrand et al. 1992; Palani et al. 2010; Thompson and Stent 1976). Interneurons B and C in the crayfish have similar multisegmental organizations and are able to initiate spikes in at least three segments (Wiersma and Hughes 1961; Wiersma and Ikeda 1964). However, cell E21 is the first neuron identified in the leech with such widely repeating sites that combine input and output. Because cell E21 initiated spikes in all six nonconsecutive midbody ganglia examined, it is likely that it is capable of initiating spikes in all 21 midbody ganglia. This organization allows cell E21 to integrate sensory inputs in all midbody ganglia and to transmit this information directly to the gating cells in ganglia 9–16 whose multisegmental output sites throughout the nerve cord contact oscillator neurons to initiate swimming. This organization is reminiscent of the lateral giant fibers in the crayfish and the S-cell in the leech. These cells have somata present in multiple ganglia with tight electrical coupling of their axons, allowing them to function as one unit (Bagnoli et al. 1975; Watanabe and Grundfest 1961; Wiersma 1947). Here, the cells can receive inputs and have outputs at multiple segments (Hughes and Wiersma 1960; Shaw and Kristan Jr 1995; Wiersma and Hughes 1961). Cell E21 serves a functional role similar to that of these cells, while reducing the number of cell bodies present.

GRANTS

This research was supported by National Science Foundation Grant IOS-0615631.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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