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A cephalic projection neuron involved in locomotion is dye coupled to the dopaminergic neural network in the medicinal leech

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Summary

It is widely appreciated that the selection and modulation of locomotor circuits are dependent on the actions of higher-order projection neurons. In the leech, *Hirudo medicinalis,* locomotion is modulated by a number of cephalic projection neurons that descend from the subesophageal ganglion in the head. Specifically, descending brain interneuron Tr2 functions as a command-like neuron that can terminate or sometimes trigger fictive swimming. In this study, we demonstrate that Tr2 is dye coupled to the dopaminergic neural network distributed in the head brain. These findings represent the first anatomical evidence in support of dopamine (DA) playing a role in the modulation of locomotion in the leech. In addition, we have determined that bath application of DA to the brain and entire nerve cord reliably and rapidly terminates swimming in all preparations exhibiting fictive swimming. By contrast, DA application to nerve cords expressing ongoing fictive crawling does not inhibit this motor rhythm. Furthermore, we show that Tr2 receives rhythmic feedback from the crawl central pattern generator. For example, Tr2 receives inhibitory post-synaptic potentials during the elongation phase of each crawl cycle. When crawling is not expressed, spontaneous inhibitory post-synaptic potentials in Tr2 correlate in time with spontaneous excitatory post-synaptic potentials in the CV motor neuron, a circular muscle excitor that bursts during the elongation phase of crawling. Our data are consistent with the idea that DA biases the nervous system to produce locomotion in the form of crawling.

Key words: central pattern generator, swimming, crawling, neuromodulation, serotonin, fictive locomotion, *Hirudo medicinalis*, leech.

Introduction

It is widely appreciated that the maturation, selection and modulation of locomotor circuits are dependent on the actions of higher-order projection neurons (Harris-Warrick and Marder, 1991; Kupfermann and Weiss, 2001; McLean et al., 2000). In vertebrate and invertebrate animals, transmitter molecules associated with such descending neurons include the catecholamines and other biogenic amines (McLean et al., 2000). In the rat, for example, pharmacological lesion of dopamine (DA) neurons with the neurotoxin 6-OHDA profoundly reduces locomotion (Koob et al., 1978). In more recent studies using the neonatal rat preparation, DA has been shown to activate locomotor pattern-generating networks in the spinal cord (Barriere et al., 2004; Kiehn and Kjaerulff, 1996). In addition, knockout mice defective in the gene encoding tyrosine hydroxylase (TH), the rate-limiting enzyme for the synthesis of DA, have reduced locomotor activity; this reduction is reversed by administration of L-DOPA (Zhou and Palmiter, 1995).

Dopamine often modulates the expression of locomotion and other rhythmic motor patterns, including feeding-related programs, by inducing both short- and long-lasting changes in the activity of neural circuits known as central pattern generators (CPGs) (Barriere et al., 2004; Harris-Warrick et al., 1998; Kabotyanski et al., 2000). In the leech, disruption of DA signaling induced simply by bathing intact leeches in the DA receptor antagonist haloperidol (Sakharov et al., 1994) disrupts crawling behavior, thus supporting the possibility that DA is important for the modulation of locomotion in this preparation. The leech (in particular, *Hirudo medicinalis*) has long served as a key invertebrate model of the neural bases underlying rhythmic motor pattern generation (Marder and Calabrese, 1996). In the present study, we present anatomical and physiological data documenting the role of DA in the control of locomotion in the leech.

Locomotion in the leech is modulated by a number of cephalic projection neurons that descend from the subesophageal ganglion (SEG) in the head. For example, cell Tr1, a command-like interneuron, can activate the swimming motor rhythm (Brodfuehrer and Friesen, 1986). Another descending brain interneuron, swim-inhibiting neuron SIN-1,

terminates swim episodes (Brodfuehrer and Burns, 1995). Descending brain interneuron, R3b1, is a state-dependent neuron (Esch et al., 2002) shown to activate swimming (when the leech is in deep water) or activate crawling activity (when the leech is in shallow water). These examples suggest that descending information from the head brain of the leech plays important roles in behavioral choice, and the selection of which form of locomotion is expressed.

Although swimming behavior has previously been shown to be modulated by the biogenic amines in preparations lacking their head brain (Hashemzadeh-Gargari and Friesen, 1989; Willard, 1981), more recent studies have demonstrated the importance of the brain and its chemical modulation (Crisp and Mesce, 2003; Mesce et al., 2001). Furthermore, descending brain interneurons, which are modulated by the biogenic amines (Crisp and Mesce, 2003), have the potential to influence the activity of down-stream aminergic cells. For example, stimulation of Tr1 excites serotonergic Retzius cells in all segments of the central nervous system (CNS) that have been examined to date (Brodfuehrer and Friesen, 1986). Together, these data suggest a tight coupling between the activity of descending brain interneurons and the serotonergic system, which is associated with swimming behavior.

In this study, we determined whether the catecholaminergic system is coupled to cephalic descending interneurons. Here, we describe coupling between the DA-synthesizing neurons of the leech head brain (Crisp et al., 2002) and the descending brain interneuron, Tr2, shown previously to terminate (O'Gara and Friesen, 1995; Taylor et al., 2003) or trigger (Brodfuehrer and Friesen, 1986) swimming. Our report is significant to the field of locomotion, because no other studies have previously documented the physiological actions of DA on the swim or crawl neural networks of the leech. Data are consistent with DA biasing the nervous system to produce crawling. In addition, we show that Tr2 receives rhythmic feedback from the crawl CPG. We also demonstrate that DA inhibits the fictive motor rhythm for swimming, but does not inhibit the crawling motor pattern, indicating that Tr2 is most likely involved in both swimming and crawling.

Materials and methods

Animal preparations and anatomical methods

Adult *Hirudo medicinalis* L. leeches were obtained from Leeches USA (Westbury, NY, USA) and dissected in normal leech saline (115.0 mmol l^{-1} NaCl, 1.8 mmol l^{-1} $CaCl_2$, 4.0 mmol l^{-1} KCl, 10.0 mmol l^{-1} Tris-maleate; Nicholls and Baylor, 1968). The leech cephalic ganglion, also called the 'head brain', is composed of the supraesophageal ganglion and subesophageal ganglion (SEG).

Intracellular impalements, for iontophoretic injection of Neurobiotin or physiological recordings, were performed using glass micropipettes with a resistance of 40–60 MΩ; pipettes were tip filled with 5% Neurobiotin (Vector Laboratories, Burlingame, CA, USA) dissolved in 2 mol l^{-1} potassium acetate and back-filled with 2 mol l^{-1} potassium acetate. Cells were filled with Neurobiotin by iontophoretic injection using 500 ms pulses of 1–2 nA positive current delivered at a rate of 1 Hz for a minimum of 15 min. Cephalic ganglia were then fixed in 4% paraformaldehyde for 1 h at room temperature and rinsed in iso-osmotic Millonig's buffer (13 mmol l^{-1} NaH_2HPO_4, 86 mmol l^{-1} Na_2HPO_4, 75 mmol l^{-1} NaCl, pH 7.8). Tissues were incubated for 30–60 min in type IV collagenase (Sigma, St Louis, MO, USA; 0.5 mg ml^{-1} in phosphate-buffered saline with 1 mmol l^{-1} $CaCl_2$, pH 7.4) and then placed in a blocking solution (containing 10% normal goat serum and 1% Triton X-100) for a minimum of 2 h. Tissues were incubated overnight at 4°C in a 1:100 dilution of streptavidin [conjugated to the cyanine fluorophore Cy3 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA)] in hypo-osmotic Millonig's buffer (13 mmol l^{-1} NaH_2HPO_4, 86 mmol l^{-1} Na_2HPO_4, pH 7.8).

Labeling DA-synthesizing neurons in the leech CNS was conducted according to the methods of Crisp et al. (2002). Briefly, ganglia (in which neurons had been filled with Neurobiotin) were incubated for 48 h in a mouse monoclonal antiserum raised against TH, the rate-limiting enzyme in the synthesis of DA, diluted to 1:100 in hypo-osmotic Millonig's buffer (containing 3% normal goat serum and 0.3% Triton X-100). This antibody was shown previously to stain selectively only leech neurons that expressed immunoreactivity to DA (Crisp et al., 2002). Tissues were then incubated for 48 h in a donkey anti-mouse antiserum conjugated to the cyanine fluorophore Cy5 (Jackson ImmunoResearch) diluted at 1:100 in hypo-osmotic Millonig's buffer (containing 3% normal goat serum and 0.3% Triton X-100).

Physiological methods and analysis

Fictive crawling was monitored by recording simultaneously from the dorsal posterior (DP) nerve, with extracellular electrodes (Crisp and Mesce, 2003), and from the segmentally repeated circular muscle excitor motor neuron CV, using intracellular electrodes. Fictive crawling activity was diagnosed using established criteria whereby the largest unit in the DP nerve, motor neuron DE-3, fired in alteration with CV with a cycle period between 7–22 s over multiple cycles within the same ganglion (Eisenhart et al., 2000). Although crawling can be evoked by electrically stimulating the nerves of the tail brain (Eisenhart et al., 2000), this technique was not used to elicit any of the crawling reported here. In our study, all fictive crawling activity occurred spontaneously (i.e., without deliberate intervention).

In only one set of experiments was electrical stimulation used to evoke locomotion, which was in the form of fictive swimming. During these experiments, the DP nerve (ganglion 16) was electrically stimulated once every 5 min, with a 1 s train of 20 pulses (10 ms, 5 V), to induce fictive swimming (Mesce et al., 2001). Nerve cords had their head brains removed, and were treated for 30 min in saline, 30 min in 50 μmol l^{-1} DA, and 30 min in saline wash. No electrical stimuli were presented for the first 10 min of a given treatment period and a total of five stimuli were presented for each

condition. DP stimulation was deemed to cause swimming if swim bursts occurred within 10 s of the shock. A paired Student's *t*-test was used to test the null hypothesis that DA had no influence on the probability of shock-induced swimming. Reported are the means ± the standard error of the means. For all other statistical analyses, contingency tables were tested using the Fisher Exact Test for Independence (Rees, 1985).

Extracellular recordings were obtained using a Grass P15 amplifier (Grass Instruments, Quincy, MA, USA), and displayed and recorded digitally (at a sampling rate of 2 kHz) on a Macintosh Performa 5200 using the PowerLab data acquisition system (ADInstruments, NSW, Australia) and associated PowerLab Chart v3.6.3/s software. Intracellular signals were obtained using a Cornerstone IX2-700 electrometer (Dagan Corporation, Minneapolis, MN, USA) and recorded digitally in the same way as the extracellular signals. To maintain a fairly accurate measure of a cell's membrane potential, care was taken to balance the bridge circuit inside the cell while injecting current.

Results

Iontophoretic injection of Neurobiotin into Tr2 cells revealed Neurobiotin coupling between Tr2 and the dopaminergic system of the head brain (Fig. 1), a network of cells that was recently described by Crisp et al. (2002). Examination of all head brains (*N*=7) in which cell Tr2 was filled with Neurobiotin revealed a collection of dye-coupled neurons in the SEG. Brains containing only a single Neurobiotin-labeled Tr2 cell (Fig. 1A) contained dye-filled cells on both the ipsi- and contralateral regions of the SEG (Fig. 1A). These bilaterally paired dye-filled cells expressed immunoreactivity to TH (Fig. 1B–D). Neurons possessing Neurobiotin (Cy3) and TH-immunoreactivity (Cy5) are indicated by arrows (Fig. 1C). In addition to the TH-immunoreactive (TH-ir) network, Tr2 was dye coupled to two or three small, unidentified neurons of the SEG (Fig. 1A). All of these cells were in positions of the SEG medial to the TH-ir neurons, and none were in locations corresponding to previously identified neurons of the head brain.

Dye transfer to the dopaminergic network was specific to Tr2. For example, intracellular Neurobiotin fills of other descending cephalic interneurons, including swim-trigger neuron Tr1 (*N*=9) and swim-inhibiting neuron SIN-1 (*N*=5), revealed very few or no dye-coupled neurons. Importantly, none of these dye-filled Tr1 or SIN-1 cells resulted in the transfer of Neurobiotin to any of the dopaminergic neurons.

Our observation that the command-like cell Tr2 was dye coupled to the DA system, suggested to us that DA plays a role in the modulation of locomotion in the leech. Thus, the following experiments were conducted to determine whether DA could activate fictive swimming. We found that a 30 min bath application of 50 μmol l^{-1} DA to the leech head brain and nerve cord did not induce any swim episodes (*N*=11). This result sharply contrasted with what we had found previously

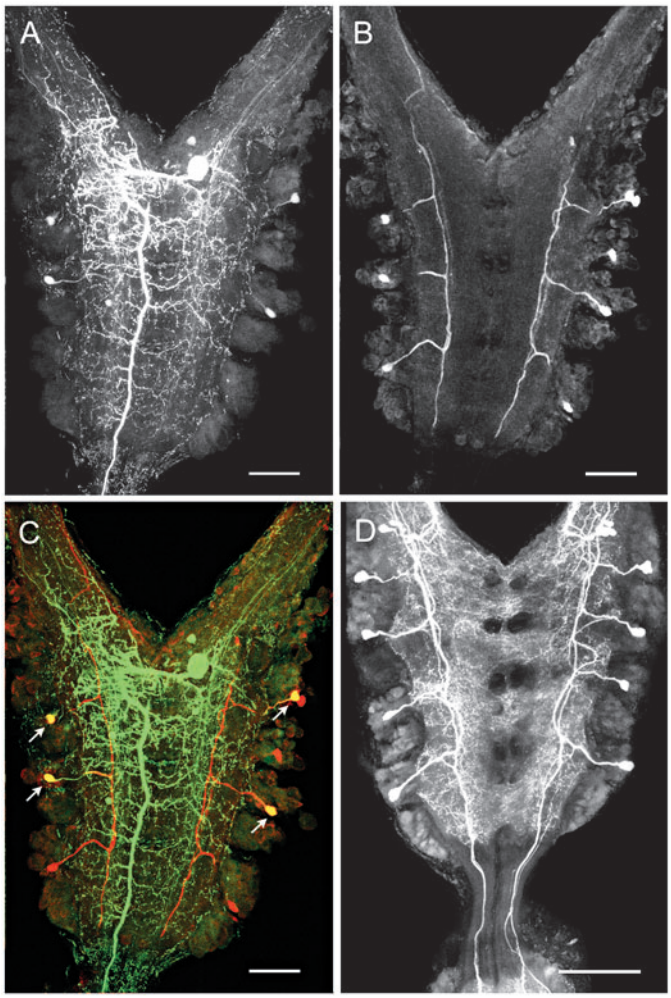

Fig. 1. Confocal photomicrographs showing Neurobiotin transfer from cell Tr2, in the subesophageal ganglion (SEG), to neurons identified as synthesizing DA. (A) Shown are Tr2 and Neurobiotin-containing neuronal populations visualized with Cy3-streptavidin. The soma of only one Tr2 neuron was iontophoretically injected with Neurobiotin using 500 ms pulses of positive current (1.5 nA) at a rate of 1 Hz for about 30 min. Cell Tr2 is shown crossing the midline and descending out of the SEG. (B) Same sample as in (A) showing Cy5 fluorescence from the network of dopaminergic neurons stained with an antibody against tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of dopamine (DA). (C) Merged two-color fluorescence showing neurons (yellow) that contain both Neurobiotin (green) and TH (red). In this sample, arrows indicate the presence of four TH-immunoreactive (TH-ir) cells that are dye coupled to Tr2. (D) In another sample, the processes and projection patterns of the TH-ir neurons are displayed. Scale bars, 100 μm.

in response to the bath application of serotonin (5-HT) or octopamine (OA; Mesce et al., 2001). Our new data thus suggested that DA might inhibit rather than induce swimming activity.

To study the potential inhibitory effects of DA, we first needed to activate the swim motor pattern. We used the application of a mixture of 50 μmol l^{-1} 5-HT and 50 μmol l^{-1} OA, followed by a saline wash, as this was shown to be a robust

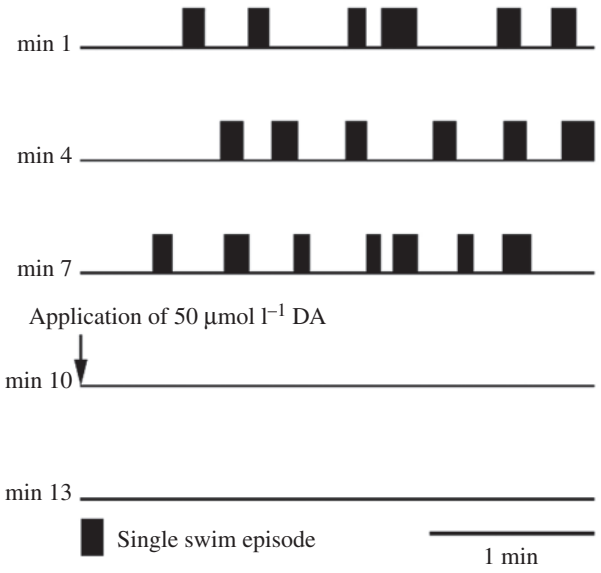

Fig. 2. Dopamine inhibits the expression of fictive swimming. Robust swimming was induced by a combination of a 30 min bath application of the amine mixture (50 µmol l^{-1} serotonin and 50 µmol l^{-1} octopamine) administered to the brain and nerve cord, followed by a 30 min saline 'washout' (Mesce et al., 2001). At the end of washout, 50 µmol l^{-1} DA was applied to the brain and nerve cord for 30 min, thus terminating the expression of fictive swimming. The entire last 9 min of washout (labeled min 1, to arrow) and first 6 min of DA application (min 10 to end of record) are depicted here. Black boxes depict entire swim episodes, defined as a series of uninterrupted swim motor neuron (DE-3) bursts in the dorsal posterior (DP) nerve. Swim inhibition by DA occurred within 1 min, and swimming was never observed in any preparations treated with DA (N=5).

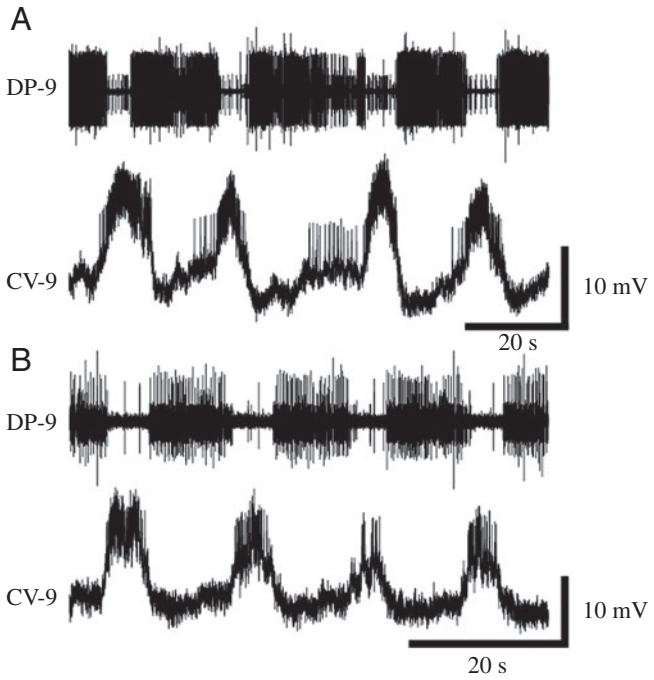

Fig. 3. Fictive crawling is not inhibited by bath application of 50 µmol l^{-1} DA. The fictive crawling motor rhythm is characterized by alternating bursts of action potentials in the dorsal longitudinal muscle excitor DE-3 (the largest unit in the DP nerve) and the circular muscle contractor CV in ganglion 9, with a cycle period of 7–20 s (Eisenhart et al., 2000). (A) Spontaneous fictive crawling activity after a 10 min bath application of 50 µmol l^{-1} DA. (B) Spontaneous fictive crawling activity in saline without the amine.

and reliable method for inducing swim episodes (Crisp and Mesce, 2003; Mesce et al., 2001). This 5-HT/OA mixture was applied to the entire CNS for 30 min, followed by a 30 min washout. Then, 50 µmol l^{-1} DA was bath applied to the preparation (both head brain and nerve cord) to determine if DA could inhibit swimming.

We observed that swim episodes abruptly ceased within 1 min of DA application in 100% of preparations examined (Fig. 2; N=5). By contrast, swimming persisted in four of the six control preparations in which the mixture washout was followed by saline without DA. The interaction between perfused salines (with or without DA) and the number of preparations that stopped swimming was statistically significant (P<0.05).

To demonstrate that DA can decrease the probability of swimming in other contexts, we induced swimming by electrically shocking the DP nerve of preparations perfused in saline (30 min) and in 50 µmol l^{-1} DA (30 min) (see Materials and methods). Because this protocol works especially well in nerve cords lacking the head brain (Hashemzadeh-Gargari and Friesen, 1989), we removed the head brain and ganglion 1 to maximize the amount of swimming induced by DP nerve shock. By maximizing swim production, we increased the rigorousness of our experiments testing whether DA can limit fictive swimming. Among five preparations tested, we observed that 18 of 25 DP nerve shocks initiated fictive swimming in saline (baseline control), whereas only 6 of 25 shocks caused swimming in nerve cords perfused with DA. The mean number of shock-evoked swims was 3.60±0.18 (saline) *vs* 1.20±0.43 (saline with DA). Using a paired Student's t-test this difference was deemed statistically significant P=0.024.

The inhibitory effects of DA on swimming were not reversed within the 30 min washout period following DA application. For example, DP nerve-evoked swims occurred in only 3 of 25 stimuli (N=5), even though DP nerve activity persisted and preparations appeared viable. Although longer washes may result in greater reversibility, it is our perception that the viability of the preparation then comes into question by about 2 h, limiting us from accurately assessing the effects of extensive washings. In preparations with head brains intact, during washout periods (30–60 min), fictive crawling or crawl-like patterns (see below) were expressed to the exclusion of spontaneous swimming.

The fictive crawling motor rhythm, which shares some motor neurons and interneurons with the swimming neural network (Baader, 1997; Eisenhart et al., 2000; Esch et al., 2002; Kristan et al., 1988), was expressed in 10 of 11 preparations to which 50 µmol l^{-1} DA was bath applied

(Fig. 3A). We found no evidence that DA inhibited crawling. Whether DA induced crawling was more difficult to determine, as four out of five control animals expressed fictive crawling in saline that did not contain DA (Fig. 3B).

Although Tr2 has not previously been shown to be involved in crawling, it has been shown to be excited by stimuli leading to another behavior, shortening (Shaw and Kristan, 1997), suggesting that Tr2 has the potential of functioning in more than one behavior. Here, we observed that Tr2 receives inhibitory post-synaptic potentials (IPSPs) that are correlated in time with the fictive crawling rhythm. Fig. 4 shows simultaneous recordings from Tr2 in the SEG, the CV motor neuron, and DE-3 (largest unit in the DP nerve) in ganglion 9. Fig. 4A demonstrates that hyperpolarizing potentials in Tr2 were visible during the elongation phase of each fictive crawling cycle, and correspond approximately to the peak of each CV motor neuron burst. When the membrane potential of Tr2 was at –68 mV, these IPSPs appeared as negative deflections. Fig. 4B shows that when Tr2 was hyperpolarized to –98 mV, the IPSPs appeared as positive deflections, indicating the reversal of putative synaptic activity. Intracellular stimulation of cell Tr2 did not cause any notable changes in the membrane potential of CV, nor did stimulation of CV visibly alter the membrane potential of cell Tr2 (data not shown). In addition, Tr2 stimulation did not initiate crawling in any preparations examined (N=11), nor was it observed to reset or alter any parameters of the crawl rhythm (e.g., cycle period, data not shown). Recalling that Tr2 can inhibit or trigger swimming, this finding underscores that Tr2 is linked to swim-based circuits. When the crawl CPG was not active (Fig. 4C), depolarizing potentials in CV were correlated with IPSPs in Tr2. (Note: Tr2 is hyperpolarized to –98 mV in Fig. 4C, causing the IPSPs to resemble excitatory PSPs.) The correlation of post-synaptic activity in CV and Tr2 while the crawl CPG is inactive suggests that the rhythmic activity Tr2 received during fictive crawling is due to a pre-synaptic input shared by CV and Tr2 (N=3).

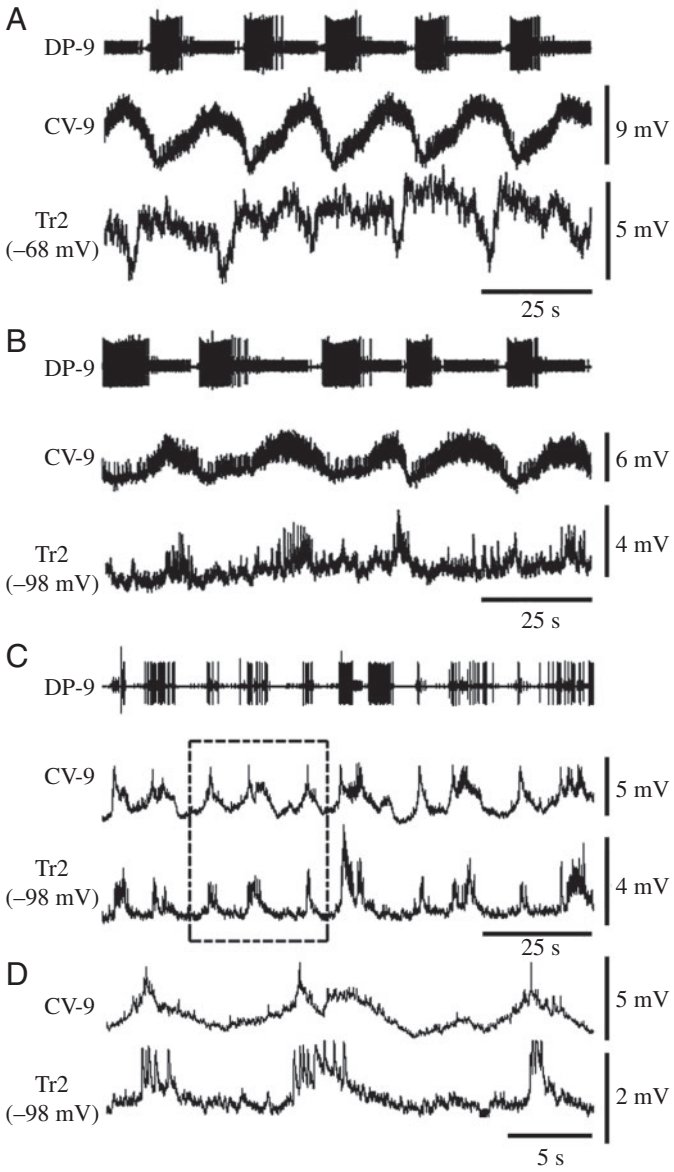

Fig. 4. Descending brain interneuron Tr2 receives rhythmic inhibition in phase with the elongation phase of the fictive crawling motor rhythm. The CV neuron shows bursting during the elongation phase of crawling and the DE-3 motor neuron (DP nerve) bursts during the contraction phase. (A) At a resting membrane potential of –68 mV, inhibitory post-synaptic potentials (IPSPs) ranging from 3–5 mV in amplitude are apparent during the peak of CV burst activity during each crawl cycle. (B) When the membrane potential of Tr2 is hyperpolarized to –98 mV, by an injection of –0.2 nA of constant current, the IPSPs are evident as positive deflections of the membrane potential, ranging in amplitude from 2-4 mV. (C) Between crawl episodes, synchronous activity is observed between DE-3 (the largest unit in the DP nerve), circular contractor motor neuron CV, and Tr2, suggesting a shared synaptic input between these three neurons. During this recording, Tr2 is once again hyperpolarized to –98 mV with a constant current injection of –0.2 nA. (D) Portions of the CV and Tr2 traces shown in C (see rectangle in C) are expanded in time to resolve whether individual PSPs are 1:1. Although the summed potentials are clearly phase locked, unitary PSPs are not.

Discussion

Compared with the literature base documenting the physiological actions of DA on feeding-related circuits (Ayali et al., 1998; Flamm and Harris-Warrick, 1986; Kabotyanski et al., 2000; Quinlan et al., 1997), and the behavioral ramifications of genetically altered DA synthesis (Neckameyer, 1996; Pendleton et al., 2002), surprisingly little is known about the physiological actions of DA on invertebrate locomotor networks (Barthe et al., 1989). In this present study on the leech, we have established that DA does indeed play a role in the control of locomotion, and that a dopaminergic neural network is coupled to the command-like cephalic projection neuron, Tr2. This association may provide novel insights into mechanisms of DA regulation that are potentially conserved in mammals and, ultimately, may help stimulate novel approaches to the functional recovery of spinal cord injury in humans (Barriere et al., 2004; Rossignol, 2000).

Of the cephalic projection neurons examined, we found that

Tr2 was the only command-like neuron that transferred dye to the dopaminergic neural network of the CNS. These DA cells in the SEG were labeled using an antiserum raised against TH, the rate-limiting enzyme in the synthesis of DA. Crisp et al. (2002) previously showed that these same TH-immunoreactive (TH-ir) neurons also immunostained with an antiserum raised against DA. Because DA, but not noradrenaline, was shown to be the final synthetic end product of the TH-ir cells (Crisp et al., 2002), we can conclude that the TH-ir cells are indeed dopaminergic.

The cessation of swimming by DA demonstrated here is quite remarkable, because the swim-inducing paradigm we used (amine mixture removal) has been shown to cause robust and persistent fictive swimming for up to hours (Mesce et al., 2001; Crisp and Mesce, 2003). This paradigm based on amine mixtures also has a physiological basis, which stems from the observation that sets of OA and 5-HT-containing neurons are co-activated by sensory neurons known to promote swimming (Gilchrist and Mesce, 1997). Without such manipulations, nerve cords with intact brains rarely exhibit spontaneous swimming (Mesce et al., 2001), a phenomenon likely due to the presence of descending swim-inhibitory inputs (Brodfuehrer and Burns, 1995).

To demonstrate that DA can terminate fictive swimming induced by multiple methods, we initiated swimming by electrically shocking the DP nerve in preparations in which the brain was detached and the descending inhibition of swimming was removed. In the presence of DA, such shock-induced swimming was significantly inhibited. Aside from showing that DA inhibits swimming within multiple contexts, such experiments indicate that the swim-related targets of DA modulation may, in part, be located in the segmental nerve cord. Segmentally repeated monosynaptic follower neurons of Tr2 have recently been identified that inhibit swimming; these cells likely constitute an anti swim-gating network (Taylor et al., 2003). Future studies are warranted to determine whether DA and its release, by way of Tr2 coupling, can influence these newly identified targets. Dopamine's inhibitory actions, however, are likely to involve more than segmental targets because our preliminary experiments indicate that DA (100–500 μmol l^{-1}) applied to the brain alone can limit swimming activity.

In the leech, consistent dye transfer between neurons is associated with electrical coupling (Davis, 1989; Wolszon et al., 1995). Although electrical coupling between neurons can be accompanied by the absence of dye coupling if the neuronal tracer is too large (e.g., Lucifer Yellow does not transfer between electrically coupled Retzius neurons, K.M.C. and K.A.M., personal observation), to our knowledge, the reverse has not been documented. Thus, there is no reason to doubt that Tr2 and the DA neurons are electrically coupled. Cell coupling appears to be an important element for locomotor activity in the leech. For example, the swim-gating neuron 204, a segmental command-like interneuron (Shaw and Kristan, 1997), is weakly electrically coupled to the serotonergic swim-initiating interneuron 61 (Nusbaum and Kristan, 1986). Cells 204 and 61 contribute in parallel to the activation of the swim CPG, and may coordinate their efforts through electrical coupling. Tr2 may likewise contribute to the inhibition of swimming through coordinated efforts with the DA system, which clearly has a strong inhibitory influence (Fig. 2). The potential activation of the DA system by Tr2 may, in turn, help to coordinate locomotor activities with other behaviors. For example, dopaminergic innervation of the stomatogastric nervous system in the medicinal leech (Crisp et al., 2002) and other behavioral studies (O'Gara et al., 1991) suggest that DA may also regulate feeding-related behaviors. Possibly, DA suppresses swimming while coordinating the consumption of a blood meal, just as DA inhibits locomotion in the nematode *Caenorhabditis elegans* during encounters with food substrates, thus prolonging feeding (Horvitz et al., 1982; Sawin et al., 2000). Because we obtained no evidence that DA inhibits crawling, DA could promote crawling behavior while the leech explores the surface of its prey looking for a point from which to feed (Lent and Dickinson, 1984).

Even though we demonstrated here that the swim-based Tr2 cell does not trigger crawling under our conditions, we have shown that Tr2 is linked to the crawling motor rhythm. This is because Tr2 shows rhythmic neural activity that is matched to the fictive crawling rhythm. Additionally, Tr2 shares synaptic input with the CV motor neuron, a cell responsible for the elongation phase of crawling (Eisenhart et al., 2000). This implies that Tr2 shares circuitry with networks involved in the production of swimming, as well as crawling. The swim-gating cell 204 also shares circuitry with crawling because its activity is often time-locked to the elongation phase of crawling (Baader, 1997; Kristan et al., 1988). Because of Tr2's coupling with the DA network, and its synaptic links to the crawl pattern generator, both Tr2 and DA have the potential to bias the locomotor system in favor of crawling as opposed to swimming.

It remains to be established whether bath application of DA is sufficient to induce fictive crawling. Although crawling is clearly promoted in the presence of DA, control preparations also expressed fictive crawling when the applied bath solution contained no DA. One explanation is that we may have inadvertently activated the segmental DA neurons. With the exception of the head and tail brains, the somata of all DA-synthesizing neurons in the leech nerve cord reside in nerve roots (specifically, within the anterior root ganglia; Lent et al., 1983). Each monopolar DA cell projects a single axon that projects centrally and ramifies throughout multiple ganglia within the CNS (Crisp et al., 2002). Thus, by freeing the nerve cord from the body, the axons from these cells become transected. Such lesions may have led to a trauma-induced release of DA throughout the CNS. In a preliminary study, we pretreated several leeches with reserpine to eliminate the influence of endogenous DA (and other amines; O'Gara et al., 1991). In normal saline, we did indeed observe that the level of spontaneous fictive crawling was greatly reduced, further supporting a role for the amines.

Because of the intimate association between Tr2 and the dopaminergic neural network, these neural elements, in particular, provide a fruitful area in which to examine the cellular mechanisms of motor pattern selection. Perhaps, studies in vertebrate systems may soon reveal a similar coupling between command-like locomotor projection neurons and aminergic networks. Such studies are certainly ripe for investigation, now that DA projection neurons have been identified in the mammalian diencephalon and spinal cord (Bjorklund and Skagerberg, 1979; Ridet et al., 1992), and electrical coupling among mammalian spinal neurons has been shown to be of significance for locomotor control (Kiehn and Tresch, 2002).

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