The Journal of Experimental Biology 209, 1746-1756
Published by The Company of Biologists 2006
doi:10.1242/jeb.02204

Beyond the central pattern generator: amine modulation of decision-making neural pathways descending from the brain of the medicinal leech

Kevin M. Crisp[1,2] and Karen A. Mesce[1,2,*]

[1]*Graduate Program in Neuroscience and* [2]*Departments of Entomology and Neuroscience, University of Minnesota, St Paul, MN 55108, USA*

*Author for correspondence (e-mail: mesce001@umn.edu)

Accepted 23 February 2006

Summary

The biological mechanisms of behavioral selection, as it relates to locomotion, are far from understood, even in relatively simple invertebrate animals. In the medicinal leech, *Hirudo medicinalis*, the decision to swim is distributed across populations of swim-activating and swim-inactivating neurons descending from the subesophageal ganglion of the compound cephalic ganglion, i.e. the brain. In the present study, we demonstrate that the serotonergic LL and Retzius cells in the brain are excited by swim-initiating stimuli and during spontaneous swim episodes. This activity likely influences or resets the neuromodulatory state of neural circuits involved in the activation or subsequent termination of locomotion. When serotonin (5-HT) was perfused over the brain, multi-unit recordings from descending brain neurons revealed rapid and substantial alterations. Subsequent intracellular recordings from identified command-like brain interneurons demonstrated that 5-HT, especially in combination with octopamine, inhibited swim-triggering neuron Tr1, as well as swim-inactivating neurons Tr2 and SIN1. Although 5-HT inhibited elements of the swim-inactivation pathway, rather than promoting them, the indirect and net effect of the amine was a reliable and sustained reduction in the firing of the segmental swim-gating neuron 204. This modulation caused cell 204 to relinquish its excitatory drive to the swim central pattern generator. The activation pattern of serotonergic brain neurons that we observed during swimming and the 5-HT-immunoreactive staining pattern obtained, suggest that within the head brain 5-HT secretion is massive. Over time, 5-HT secretion may provide a homeostatic feedback mechanism to limit swimming activity at the level of the head brain.

Key words: gating neuron, command neuron, neuromodulation, locomotion, octopamine, serotonin, behavioral choice, leech, *Hirudo medicinalis*.

Introduction

The medicinal leech *Hirudo medicinalis* L. is a well-recognized animal model of locomotory control and the decision-making processes that govern the selection of one mode of locomotion over another (i.e. swimming *vs* crawling) (Briggman et al., 2005; Esch et al., 2002; Garcia-Perez, 2005; Kristan, Jr et al., 2005). In the leech, central neural networks and sensory-to-motor pathways underlying swimming have been mapped to an unparalleled level of cellular detail (Ort et al., 1974; Willard, 1981; Friesen, 1989; Hashemzadeh-Gargari and Friesen, 1989; Eisenhart et al., 2000; Brodfuehrer and Thorogood, 2001) (for a review, see Kristan, Jr et al., 2005). The neuronal control of swimming is regulated by hierarchically organized circuitry that parallels the top-down neural architecture of vertebrate locomotory systems (Friesen, 1989). As with other rhythmic behaviors, swimming oscillations are generated by segmentally reiterated central pattern generators (CPGs), which can produce motor patterning independent of patterned sensory input (Marder and Calabrese, 1996; Kristan, Jr et al., 2005). Yet, in spite of the relative simplicity of the leech nervous system, it is difficult to predict if any given sensory stimulus will initiate swimming or crawling from one trial to the next (Grobstein, 1994; Cellucci et al., 2000; Brodfuehrer and Thorogood, 2001; Briggman et al., 2005).

The decision to swim appears to be distributed across competing populations of swim-activating and swim-inactivating brain interneurons, each of which exerts only a small influence on the initiation and maintenance of swimming (Cellucci et al., 2000). A number of these identified cephalic projection neurons have been shown to integrate and transfer swim-related information to down-stream gating neurons that generate an excitatory drive to the segmental CPGs (Weeks and Kristan, Jr, 1978; Weeks, 1982; Brodfuehrer and Friesen, 1986a; Brodfuehrer and Friesen, 1986b; Brodfuehrer and Friesen, 1986c; Brodfuehrer and Friesen, 1986d; Brodfuehrer and Friesen, 1986e).

At any given time, this distributed brain network is under the influence of circulating and local biogenic amines, which modulate behavior in all animals (Libersat and Pflüger, 2004). Only recently, however, have studies begun to examine the neuromodulation of command-like systems in the brain of the leech and how chemical modulation influences the initiation and termination of swimming (Crisp and Mesce, 2003; Crisp and Mesce, 2004). Such limited information contrasts with what has been accumulated at the level of the segmental swim circuits (Willard, 1981; Hashemzadeh-Gargari and Friesen, 1989; O'Gara et al., 1991; Angstadt and Friesen, 1993a; Angstadt and Friesen, 1993b; Mangan et al., 1994a; Mangan et al., 1994b; Mesce et al., 2001).

Swimming activity is clearly modulated by serotonin (5-HT) (Willard, 1981; Hashemzadeh-Gargari and Friesen, 1989; O'Gara et al., 1991). and blood levels of 5-HT can quickly rise to a 100 nmol l^{-1} concentration during bouts of swimming (Willard, 1981). Large amounts of 5-HT are secreted directly from the voluminous somata of the segmental Retzius neurons, so relatively high concentrations of 5-HT can be established locally within the CNS (for a review, see De-Miguel and Trueta, 2005). In addition, swim-related mechanosensory inputs can activate octopamine (OA)-containing interneurons in parallel with serotonergic cells (Gilchrist and Mesce, 1997). When considering the aminergic modulation of swimming across the entire CNS, however, the modulatory systems appear somewhat complex. Although 5-HT promotes swimming at the level of the segmental swim networks (i.e. swim gating and CPG cells), it suppresses swimming when focally applied to the brain (Crisp and Mesce, 2003). Furthermore, 5-HT or OA can promote swimming when administered to the entire nervous system, but a mixture of the two suppresses it. Subsequent removal of this mixture, however, induces robust and repeated bouts of swimming that can last for hours (Mesce et al., 2001). The brain-specific inhibitory effects of 5-HT help to explain some of the non-additive effects of the 5-HT and OA mixture, and underscore the fact that the brain is, indeed, an important site of aminergic modulation that contributes to decision-making processes (Crisp and Mesce, 2003).

Here, we present the results of a study that examined how 5-HT, and a mixture of 5-HT and OA, can influence a population of identified command-like interneurons that form a decision-making pathway from the brain to swim-gating neurons and segmental oscillators responsible for swimming. Specifically, we aimed to determine if 5-HT inhibited the descending swim-activation pathway and excited the swim-inhibitory one. In addition, we describe the firing patterns of 5-HT-containing neurons in the brain when swimming is activated or initiated spontaneously, a pattern consistent with the somatic and paracrine-like secretion of 5-HT.

Materials and methods

Animal preparations

Adult *Hirudo medicinalis* L. were purchased from Leeches USA (Westbury, NY, USA) and maintained at room temperature in spring water containing *Hirudo*-salt (0.5 g l^{-1}; Leeches USA). Embryonic *Hirudo* were a generous gift from John Jellies, and reared in a breeding colony at the University of Western Michigan, MI, USA. Rearing, maintenance and dissection of these animals have been described elsewhere (Jellies et al., 1987; Jellies et al., 1993). Adult animals were anaesthetized on ice for 15 min before dissection in normal saline, containing (in mmol l^{-1}): 115.0 NaCl, 4.0 KCl, 1.8 $CaCl_2$, 1.5 $MgCl_2$, 10.0 dextrose, 10.0 Tris-maleate, pH 7.4 (Nicholls and Baylor, 1968). Embryonic leeches were anaesthetized in leech saline containing 10% ethanol for 15 min and dissected minimally; cuticle was splayed from the dorsal midline and the enteric tract was removed. The leech nervous system consists of 21 unfused segmental ganglia and two compound 'brains,' each composed of several fused neuromeres. The supraesophageal ganglion (SPEG) and subesophageal ganglion (SEG) comprise the cephalic nervous system. Although the tail (posterior) brain was included in all preparations described in this report, experiments focused on pharmacological manipulations of the head brain. As such, the words 'head brain' and 'brain' are used interchangeably.

Extracellular recordings and focal amine perfusion to the head brain

Neuronal activity descending from the brain was recorded from the cut end of the posterior connective of preparations consisting of just the head brain and ganglion M1. Electrical activity was recorded from both lateral connectives and Faivre's nerve using a suction electrode, and neural activity was amplified on a P-15 AC pre-amplifier (Grass Instruments, Quincy, MA, USA). Every 10 s, a 1-s recording was filtered with a LPF202 low-pass bessel filter (Warner Instruments LLC, Hamden CT, USA), and digitalized (at a sampling rate of 20.8 kHz) using the Digidata 1322A interface and associated Axoscope data acquisition hardware (Axon Instruments, Union City, CA, USA). In several experiments, recordings were obtained from a cut hemiconnective between M1 and M2 while descending brain interneurons were recorded from with intracellular electrodes.

Fictive swimming was recorded extracellularly from the segmentally-repeated dorsal posterior (DP) nerve, and fictive swimming was defined as three or more consecutive bursts of action potentials in the dorsal longitudinal muscle exciter, motor neuron DE-3, with a cycle period of 0.4–2.0 s (Ort et al., 1974). A petroleum jelly well was built around the cut end of a DP nerve to isolate it electrically from the grounded perfusion bath. One Teflon-covered silver wire was placed inside the well and another outside in the bath. The two signals were amplified differentially by a P-15 amplifier (Grass Instruments), digitalized at a sampling rate of 2 kHz using the MacLab 4/s data acquisition hardware and associated Chart v 3.6.3/s software (ADInstruments, NSW, Australia) on a Macintosh Performa 5200. A similar set-up was used to evoke swimming with electrical excitation of the DP nerve. In these experiments, a petroleum jelly well was built around a more posterior DP nerve [e.g. DP(19)]. A 1-s electrical stimulus

consisting of a train of 100 ms, 3–5 V pulses was delivered using a Grass S88 stimulator and a Grass SIU5 stimulus isolation unit (Grass Instruments).

Petroleum jelly wells also isolated head brains from the main bath to allow focal perfusion of normal and amine-containing saline over the brain when recordings were made from cell 204. The connective between segmental ganglion 1 (M1) and M2 passed through the petroleum jelly barrier; no attempt was made to isolate the head brain from M1 due to the short length of the connective in that region of the nervous system. Although M1 was usually covered in petroleum jelly, the potential exposure of M1 to amines could not be ruled out. Saline was perfused into and out of the well containing the head brain at a rate of 1 ml min^{-1} as described elsewhere (Crisp and Mesce, 2003). Cell 204 is found only in M10–M16 (Weeks, 1982) and its axon projects anteriorly and posteriorly (Weeks and Kristan, Jr, 1978; Nusbaum, 1987). No evidence, however, supports its projection to the head brain, making it unlikely that cell 204 was directly affected during the amine perfusion. All recordings from cell 204 were made in M11.

In a typical experiment, normal saline was first perfused for a 30-min 'baseline' period, followed by a 30-min perfusion of saline containing 50 μmol l^{-1} 5-HT; 50 μmol l^{-1} OA or a mixture of 50 μmol l^{-1} 5-HT and 50 μmol l^{-1} OA (Sigma-Aldrich Corporation, St Louis, MO, USA). Preparations were subsequently perfused with saline for a 30-min 'washout' period. Amines were used at a concentration of 50 μmol l^{-1}, as this is the standard concentration most often used for physiological studies of the leech (Kristan, Jr et al., 2005). In all analyses of swimming activity, the standard error of the means (s.e.m.) is reported.

Intracellular recordings, cell identification and microscopy

Intracellular recordings were obtained using glass micropipettes with a resistance of 40–60 MΩ; glass electrodes were filled to their tips with 5% Neurobiotin tracer (Vector Laboratories, Burlingame, CA, USA) and back-filled with 2 mol l^{-1} potassium acetate. Intracellular signals were amplified and recorded digitally using the MacLab/4s, as described above. At the end of each experiment, cells were filled with Neurobiotin by iontophoresis and identified unambiguously using a variety of physiological and morphological characteristics unique to each cell (Crisp and Mesce, 2003; Crisp and Mesce, 2004). Methods for processing ganglia containing Neurobiotin-filled neurons have been described elsewhere (Crisp and Mesce, 2003; Crisp and Mesce, 2004).

Methods used for the 5-HT immunostaining of leech embryos have been described previously in detail (Gilchrist et al., 1995). Briefly, a goat anti-5-HT antiserum (gift from Dr Robert Elde, University of Minnesota) was used at a dilution of 1:200. The specificity of this antiserum has been previously characterized (Maley and Elde, 1982; Wessendorf and Elde, 1985; Wessendorf and Elde, 1987). The primary antibody was recognized by a donkey anti-goat secondary antibody conjugated to Cy5 (Jackson ImmunoResearch, West Grove, PA., USA).

Following staining, all tissues were washed in hypo-osmotic Millonig's buffer (Gilchrist et al., 1995), dehydrated through a graded ethanol series, and specimens were mounted between coverslips in Depex mounting medium (Electron Microscopy Sciences, Fort Washington, PA, USA). An MRC 1024 Laser Scanning Confocal Microscope (Bio-Rad, Hercules, CA, USA) mounted on an AX70 microscope equipped for epifluorescence (Olympus, Lake Success, NY, USA) was used to view and image mounted samples as described (Mesce et al., 1993).

Results

Activity of 5-HT-immunoreactive cells during swimming

Because 5-HT can act locally at the level of the brain to inhibit swimming (Crisp and Mesce, 2003), we wanted to determine the conditions under which 5-HT is released by individual neurons identified as serotonergic. The distribution of serotonergic somata has been described previously in the head brain using a variety of histological stains (e.g. Neutral Red or the Falck–Hillarp method) (Marsden and Kerkut, 1969; Rude, 1969; Lent, 1982). We were unable, however, to locate any published reports containing the immunocytochemical identification of the 5-HT neurons in the brain and the projection pattern of serotonergic fibers within and surrounding the brain. Because 5-HT stands to play a significant modulatory role affecting the decision to swim, we wanted to determine the extent to which an array of serotonergic fibers would be found associated with the compound cephalic ganglia comprising the 'head brain'.

The brain of the leech consists of the supraesophageal ganglion and the subesophageal ganglion (SEG and SPEG, respectively). A laser scanning confocal micrograph of the leech brain is shown in Fig. 1. Using the same 5-HT antibody we reported on previously in segmental ganglia (Gilchrist et al., 1995), we immunolabeled a population of somata and their associated processes (Fig. 1). A dense network of 5-HT-immunoreactive (5-HT-ir) fibers was associated with the SEG and SPEG (N=6). The neuropil of the SEG was densely filled with serotonergic arbors, emphasizing the potential importance of serotonergic neuromodulation in the head brain. Although there were no serotonergic somata located in the SPEG (Fig. 1B), this most anterior portion of the leech brain was richly innervated by 5-HT-ir fibers projecting from cells in the SEG and possibly more posterior ganglia (Fig. 1B). At least one serotonergic neuronal pair in neuromere 1 (labeled '1' in Fig. 1A), the large lateral (LL) cell pair, was observed to project contralaterally and anteriorly toward the SPEG. Because we examined brains in filleted leeches (see Materials and methods), we were also able to determine that none of the processes within the brain region originated from somata within the periphery; in fact, no 5-HT-ir somata were observed outside the CNS. In addition, a number of 5-HT-ir fibers in the SPEG resided in a region previously identified as a neurohemal release site (Fig. 1B) (Webb, 1980); these 5-HT fibers contained

varicosities and represent possible sites of neurohemal secretion.

The LL and Retzius cells of the SEG, being the largest serotonergic neurons in the brain (Fig. 1A), were targeted as the best candidates for playing a potential neurosecretory role during swimming. Thus, we recorded the activity of these neurons with intracellular electrodes during bouts of swimming.

The activity of the LL and Retzius cells was altered during electrically evoked and spontaneous swimming (Fig. 2). The LL cell was indirectly excited by the electrical shock of a posterior DP(19) nerve (3–5 V, 100 ms pulses for 1 s) (Fig. 2A left; *N*=5). A DP nerve shock is a standard way to induce swimming (Hashemzadeh-Gargari and Friesen, 1989). During spontaneous swimming (i.e. in the absence of electrical stimulation), the LL cell displayed rhythmic excitatory depolarizations that were phase locked to individual bursts in the DE3 swim motor neuron (Fig. 2A right; *N*=5). These excitatory potentials suggest that the LL cell receives rhythmic feedback from the swim CPG.

The Retzius cells in all four neuromeres of the SEG (Fig. 2B) were excited by electrical shock of a DP nerve, and excitation persisted for the duration of the swim episode (Fig. 2B left; *N*=5). The swim trigger neurons are likely candidates for the pathway leading to this excitation, as cell Tr1 makes direct chemical synapses onto the Retzius cells of

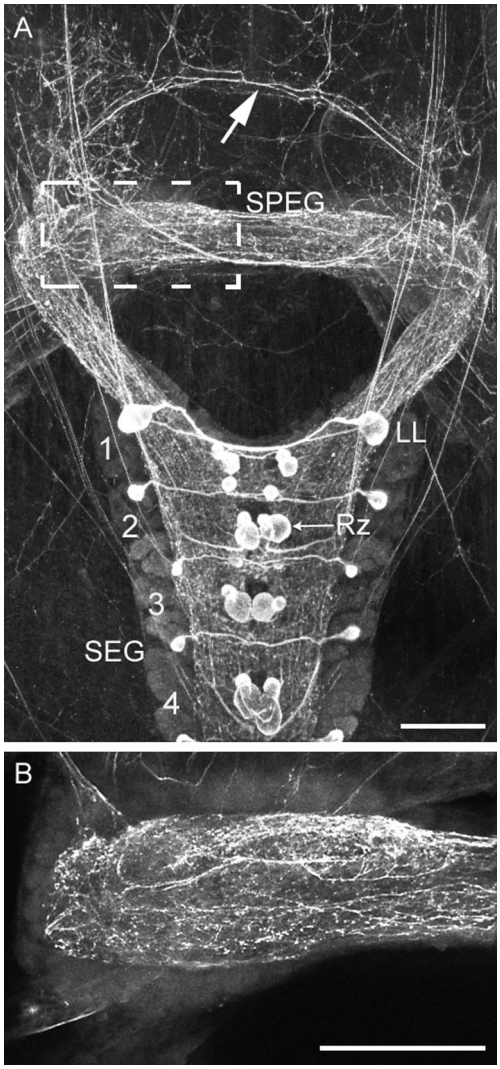

Fig. 1. Distribution of 5-HT-immunoreactive (5-HT-ir) neurons and associated processes in the subesophageal (SEG) and supraesophageal (SPEG) ganglia comprising the leech head brain. The brain of an embryonic leech at stage embryonic day 20 (E20) is shown, which was immunostained and imaged *in situ* (*N*=6). (A) The neuropil of the SPEG is filled with a dense array of 5-HT-ir fibers, probably originating from within the CNS because no peripheral 5-HT-ir cell bodies were observed. The four neuromeres of the SEG (marked 1–4) contain sets of large 5-HT-ir somata, some of which project toward the SPEG, e.g. the large lateral (LL) cells in neuromere 1. Several 5-HT-ir fibers exit the brain, such as those projecting throughout the stomatogastric nerve ring (large arrow points to one half of nerve ring). The rectangular broken box outlines the image shown at higher magnification in B. (B) Punctate 5-HT-ir fibers were visible in a putative neurohemal region of the SPEG. The terminations of varicose 5-HT-ir fibers appear to be concentrated in the upper region of the image shown, which lies in the approximate location of a neurohemal release site (Webb, 1980). Optical sections/image, 39 (A); 9 (B). Section intervals, 2 μm (A); 1.5 μm (B). Scale bars, 100 μm.

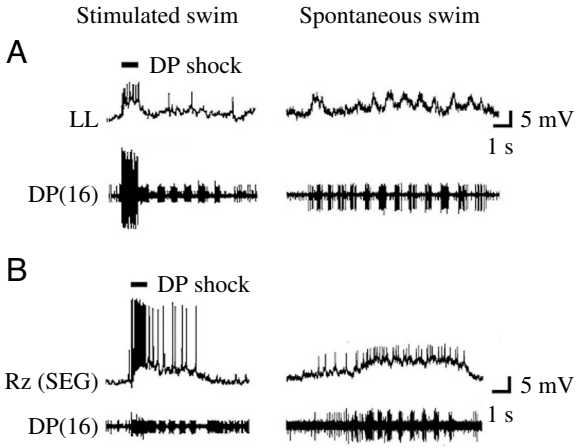

Fig. 2. Serotonergic neurons of the head brain were activated by swim-initiating stimuli and remained active throughout swimming. An electrical stimulus (100 ms, 3–5 V pulses delivered for 1 s) delivered to a posterior DP nerve has previously been shown to initiate swimming (Hashemzadeh-Gargari and Friesen, 1989). (A) Electrical shock (black bar) delivered to a DP(16) nerve excited the serotonergic LL cells of the first neuromere of the SEG (left). In addition, LL received rhythmic synaptic input correlating with individual swim-motor bursts during the expression of a spontaneous swim episode (right). (B) SEG Retzius cell (Rz) is excited by a swim-initiating electrical shock to a DP(16) nerve (left), and was persistently depolarized throughout the duration of the swim episode. During a spontaneous bout of swimming, the RZ fired above 1 Hz at the onset of swimming and remained active at a relatively high firing frequency (5 Hz or greater) (right). These data are consistent with 5-HT being secreted in the brain during both swim-initiation and swim episode maintenance *via* the somatic release of 5-HT (De-Miguel and Trueta, 2005).

the head brain (Brodfuehrer and Friesen, 1986b). Furthermore, during the occurrence of spontaneous swim episodes, the Retzius cells were correlated with a slow depolarization and corresponding increase in spiking frequency that persisted for the duration of the swim episode (Fig. 2B right; *N*=5). Together, these data imply that 5-HT is released within the brain during bouts of swimming, probably acting as a local neurohormone. This effect could contribute to the termination or long-term inhibition of swimming activity especially if levels were to become differentially elevated across the CNS.

Net effect of 5-HT on the brain

The question remains, however, whether 5-HT, acting solely at the level of the brain, is capable of biasing decisions made by the swim-gating neurons. Specifically, can a local elevation of 5-HT in the brain indirectly affect the firing rate of swim-gating neurons? Cell 204 is segmentally repeated and integrates monosynaptic excitatory input from Tr1 (Brodfuehrer and Friesen, 1986a; Brodfuehrer and Friesen, 1986b) and polysynaptic inputs from Tr2 (Brodfuehrer and Friesen, 1986b). It also integrates inputs from SIN1 (Brodfuehrer and Burns, 1995) and other descending brain interneurons. How cell 204 determines its state of excitation or inhibition based upon a population of descending swim-activating and swim-inactivating neurons remains unknown, but it is clear that cell 204 plays an integral role in the swim-initiation pathway (Weeks, 1982; Friesen, 1989). Thus, the level of excitation in cell 204 reflects changes in the net contributions of both swim-activating and swim-inactivating descending interneurons. By recording from cell 204, a site of convergence, one can gain some insight into how decisions to swim are regulated when the brain is influenced by 5-HT, an amine mixture (e.g. a 5-HT/OA mixture), or other biogenic amines. Because of the location and structure of the segmental 204s, inputs can be modulated independently of any direct actions on cell 204 so as not to confound the aminergic effects observed (see Materials and methods).

Because 5-HT, but not OA, applied to the brain inhibits swimming (Crisp and Mesce, 2003), we predicted that application of 5-HT, but not OA, would indirectly inhibit 204. To test this hypothesis, intracellular recordings from cell 204 were obtained while the amines were each applied individually to the head brain (Fig. 3). During saline perfusion over the brain (Fig. 3, top), cell 204 fired action potentials at a relatively constant rate. After a 30-min perfusion of 50 μmol l^{-1} 5-HT over the brain (Fig. 3, middle), cell 204 became hyperpolarized by an average of 5±4 mV in 10±3 min (mean ± s.e.m.) and fired no action potentials (*N*=5). In contrast, after a 30-min perfusion of 50 μmol l^{-1} OA over the brain only (Fig. 3, bottom), no change in the activity of cell 204 was observed (*N*=5).

Effects of 5-HT on descending brain interneurons

Because it is not known how swim circuitry forms the decision to swim based on parallel swim-activating and swim-inactivating pathways, we aimed to determine if 5-HT inhibits the swim-activation system while exciting the swim-inhibitory one. Swim-trigger neuron Tr1 is the most reliable swim-activator yet to be identified in the leech brain (Brodfuehrer and Friesen, 1986b). Thus, we examined the effects of 5-HT on swim-trigger neuron Tr1 and another swim-related interneuron, Tr2. All the Tr1 and Tr2 cells recorded were observed to hyperpolarize when 50 μmol l^{-1} 5-HT was focally perfused on the brain. Tr1 hyperpolarized by 5±2 mV within minutes of bath application of 5-HT (*N*=5; Fig. 4). Tr2 also hyperpolarized by a mean of 8±3 mV, within 5–10 min of 5-HT application (*N*=5; data not shown). Because no evidence was found that swim-inhibitor neuron SIN1 was activated by the 5-HT/OA mixture (see below), the effects of 5-HT on SIN1 were not formally examined.

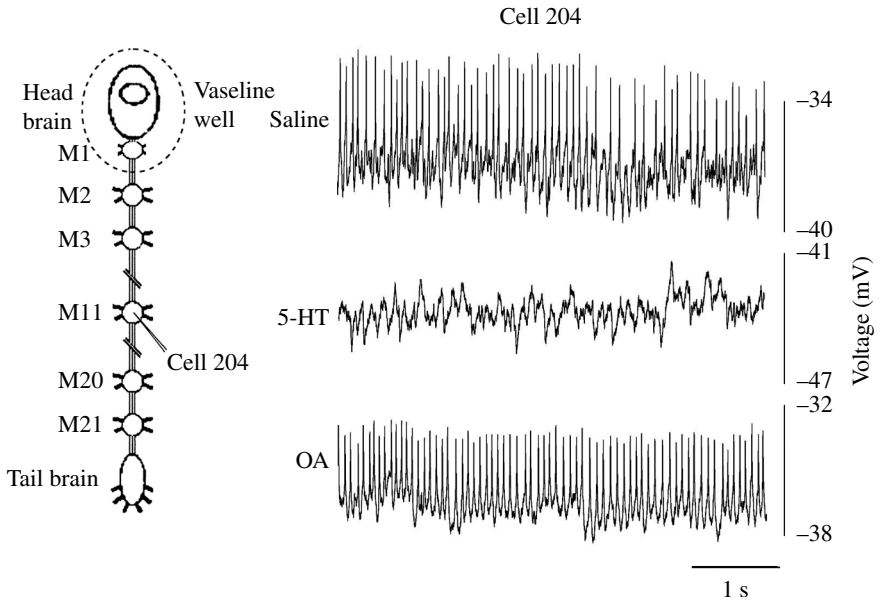

Fig. 3. Focal brain application of 5-HT indirectly inhibits the swim-gating neuron 204 (recorded in M11). Cell 204 becomes hyperpolarized and its tonic firing rate is suppressed in response to the brain application of 5-HT (5 μmol l^{-1}) (middle), but not by octopamine (50 μmol l^{-1}) (OA; bottom). During perfusion of amine-free saline (top), cell 204 fired action potentials at a constant rate of about 10 Hz. A schematic of the experimental preparation, cell 204 recording site, and placement of amine is shown to the left.

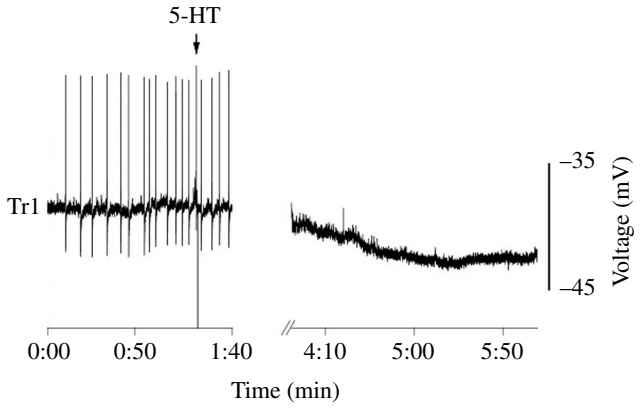

Fig. 4. Brain application of 50 μmol l^{-1} 5-HT hyperpolarized the swim-triggering neuron Tr1. 5-HT caused a gradual change of the resting membrane potential of Tr1, resulting in a 6 mV hyperpolarization after approximately 3 min of the application. At this hyperpolarized membrane potential, Tr1 ceased firing action potentials.

Effects of the 5-HT/OA mixture on descending brain interneurons

The focal delivery of a mixture of 5-HT and OA produces an even more dramatic inhibition of swimming than 5-HT alone (Crisp and Mesce, 2003). Thus we predicted that the mixture application would inhibit cell Tr1. Tr1 indeed hyperpolarized during application of the amine mixture (Fig. 5A). Intracellular recordings from cell Tr1 were obtained simultaneously with extracellular recordings from DP(16). During baseline perfusion of saline (Fig. 5A left), Tr1 spontaneously (no electrical stimulation) fired a train of action potentials prior to the expression of a swim episode, which also occurred spontaneously (no electrical stimulation). This represents an event rarely observed under baseline conditions and in the absence of amines (Hashemzadeh-Gargari and Friesen, 1989; O'Gara et al., 1991; Mesce et al., 2001; Crisp and Mesce, 2003). The swim episode, or bout, is defined as a collection of swim motor neuron DE-3 bursts recorded extracellularly from the DP nerve. During the initial and spontaneous depolarization of Tr1, the tonic firing activity of DE-3 was suppressed. After a latency of several seconds, swimming was initiated. Although it was previously demonstrated (Brodfuehrer and Friesen, 1986a; Brodfuehrer and Friesen, 1986b) that swimming activity could be evoked by intracellular stimulation of Tr1, the behavior of Tr1 prior to the expression of a spontaneous swim episode has not been reported until now.

After a 30-min perfusion of the 5-HT/OA mixture (Fig. 5A middle), cell Tr1 became hyperpolarized by a mean of 5±2 mV and all spike activity ceased (N=9). A close examination of its membrane potential also revealed a notable decrease in synaptic activity following mixture perfusion compared with that observed during baseline. In addition, it became more difficult to drive Tr1 sufficiently to provoke a spike train during perfusion of the mixture as compared to baseline (data not shown). Recovery of the membrane potential of Tr1 was gradual (and often incomplete) during washout (Fig. 5A right). It is unclear to what degree the spontaneous spike in Tr1 triggered the swim episode shown in washout. Possibly, in washout conditions, Tr1 had an enhanced effect on triggering swimming, such that less Tr1 activity was required to induce swimming. Furthermore, as only one of the two Tr1 cells was recorded, the activity of the contralateral Tr1 may have contributed more strongly to the swim bout shown. Washout of the 5-HT/OA mixture from Tr1 also revealed a return of synaptic activity (Fig. 5A right).

Descending brain neuron SIN1 is a potent swim-inhibiting neuron, and aside from cell Tr2, represents the only identified

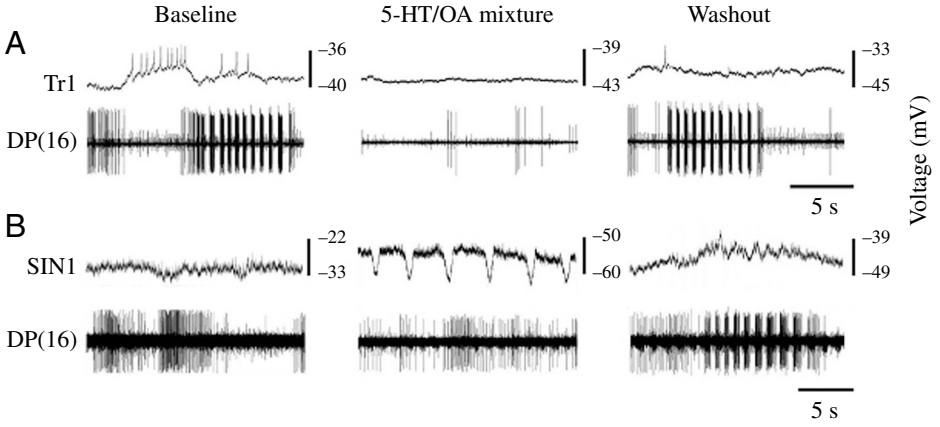

Fig. 5. Amine modulation of command-like neurons known to activate or inhibit swimming. Bath application of a mixture of 5-HT and OA (50 μmol l^{-1}) caused the inhibition of cell Tr1 (Brodfuehrer and Friesen, 1986b) and SIN1 (Brodfuehrer and Burns, 1995). (A) During perfusion of a saline baseline (left), swim-trigger neuron Tr1 fired a train of action potentials just before a swim episode (see swim motor neuron bursts in the DP nerve extracellular recording). After a 30-min application of the 5-HT/OA mixture (middle), Tr1 became hyperpolarized (5 mV) and fired no action potentials. Furthermore, Tr1 appeared to receive less synaptic activity (i.e. fewer small and rapid fluctuations in membrane potential) following mixture application. During washout of the mixture (with saline, right), Tr1 partially repolarized and synaptic inputs to Tr1 resumed. Tr1 spiked once preceding the onset of a swim bout. (B) During perfusion of a saline baseline (left), swim-inhibitory neuron SIN1 fired action potentials at a constant rate. After a 30-min application of the mixture (middle), the membrane potential of SIN1 became inhibited (18 mV), and large, rhythmic inhibitory inputs were observed. During washout of the mixture (right), SIN1 partially repolarized and rhythmic membrane potential fluctuations were phase-locked with swim-motor bursts in the DP nerve.

source of descending swim inhibition from the brain (Brodfuehrer and Burns, 1995; O'Gara and Friesen, 1995; Taylor et al., 2003). As such, we predicted that the 5-HT/OA mixture, which inhibits swimming (Crisp and Mesce, 2003; Mesce et al., 2001), would correlate with an elevated level of SIN1 excitation. By contrast, application of the mixture was found in all preparations to hyperpolarize SIN1 (Fig. 5B). During baseline (Fig. 5B left), SIN1 showed tonic spike activity. After a 30-min perfusion of the mixture (Fig. 5B middle), SIN1 was hyperpolarized by a mean of 18±4 mV (N=5). In addition to this hyperpolarization, rhythmic inhibitory post-synaptic potentials were observed in three of the SIN1 cells. Although one might expect SIN1 to remain inhibited throughout amine washout to facilitate swimming, the membrane potential partially recovered during washout (Fig. 5B, right). No dramatic changes in the firing frequency of SIN1 were apparent in the mixture or washout. Thus, we concluded that SIN1 makes little contribution to either the inhibiting effects of the 5-HT/OA mixture or the promoting effects of washout on swimming activity. These data suggest that the inhibition of descending brain neurons by the mixture is widespread, affecting neurons that activate and inhibit swimming.

In addition to affecting the firing frequency of command-like brain interneurons, the amine mixture also changed the way unidentified units (recorded from a cut hemiconnective between M1 and M2) responded to the intracellular stimulation of command-like neurons (Fig. 6A,B). Depolarization of cell Tr1 can lead to a prolonged period of elevated impulse activity in the connective that outlasts, by several seconds, the spike train in cell Tr1 (Cellucci et al., 2000). This increase may correspond to a recruitment of other descending fibers that also play a role in mediating the decision to swim or not to swim. Similarly, we found that depolarization of cell Tr2 caused a prolonged increase in connective activity (Fig. 6A). After a 30-min treatment with the amine mixture (50 μmol l^{-1}), the basal level of activity in the connective was elevated (discussed above), which was also observed in connective recordings during perfusions of the brain with 50 μmol l^{-1} 5-HT (Fig. 6C). The delayed and long-lasting increase in connective activity observed following stimulation of Tr2 during baseline was absent after the mixture was applied (Fig. 6B). From the seven Tr2 cells in which intracellular depolarization induced this characteristic response, five Tr2 cells subsequently failed to show this response in the presence of the amine mixture. This suggests that the mixture not only affects the impulse activity of descending command-like interneurons (as demonstrated above), but also modulates the way neural elements within the brain respond to inputs from cell Tr2 (and perhaps other brain interneurons).

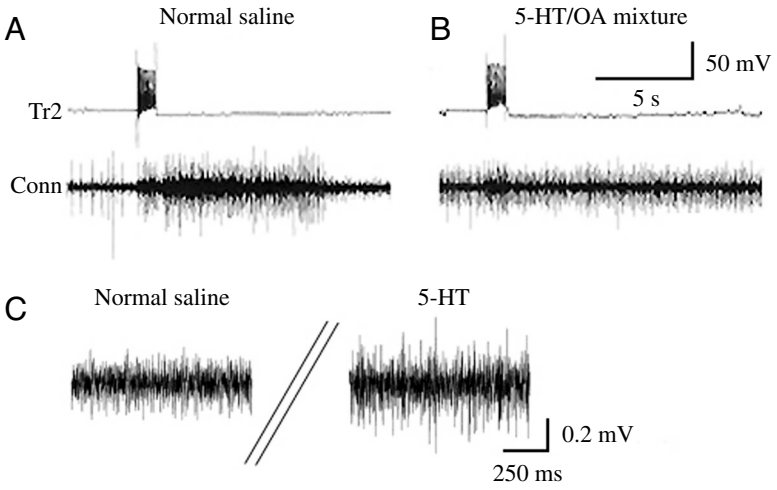

Fig. 6. Application of the 5-HT/OA mixture (50 μmol l^{-1} for 30-min) or 5-HT (50 μmol l^{-1} for 30-min) changed the activity profile of fibers in the descending connectives (Conn) and altered the way in which this activity was recruited by brain command-like cells. (A) As is the case for cell Tr1 (Cellucci et al., 2000), intracellular depolarization of cell Tr2 triggered an increase in activity descending through a single hemiconnective (ca. 2800 fibers between M1 and M2) (Wilkinson and Coggeshall, 1975). This elevated activity long outlasted (by several seconds) the train of action potentials in cell Tr2, and appeared to represent the recruitment of other descending fibers in the brain that may also be involved in mediating the decision to swim or not to swim. (B) During treatment with the amine mixture, the basal activity in the hemiconnective increased. In addition, the delayed response of units in the connective to a spike train in cell Tr2 was no longer present. (C) A representative 1-s recording of descending activity from the brain in normal saline (left) and 1-s sampling of activity after 3 min of a 50 μmol l^{-1} 5-HT exposure. In all preparations examined (N=5), there was a recruitment of larger amplitude fibers during 5-HT application.

Net effect of 5-HT/OA mixture on the brain

Because the amine mixture on the brain has such a robust effect on swimming, we determined its net and indirect action on the activity of cell 204 (Fig. 7). During baseline (Fig. 7B, top), cell 204 maintained a tonic level of spike activity. After a 30-min perfusion of the amine mixture over the head brain (Fig. 7B, middle), cell 204 was indirectly hyperpolarized (15±5 mV in 14±10 min) and all spiking activity ceased. During washout of the mixture (Fig. 7B, bottom), the membrane potential of cell 204 partially recovered. When swim episodes subsequently occurred, oscillations in cell 204 became phase-locked with individual swim motor bursts in the DP nerve. These data indicate that, although mixture application inhibited both swim-activating and swim-inactivating neurons, the net effect of mixture application to the head brain was the inhibition of cell 204 (N=5).

Discussion

Activity of cephalic 5-HT neurons during swimming

Locomotion-related changes have been correlated to the activity of serotonergic neurons in several species (Willard, 1981; Jacobs and Fornal, 1993). Serotonergic neurons in the

nucleus raphe pallidus display rhythmic bursts during locomotion in the cat (Jacobs and Fornal, 1993), and serotonergic neurons in the dorsal and median raphe nucleus become tonically active during rhythmic (i.e. CPG-mediated) movements, such as chewing and grooming (Ribeiro-do-Valle, 1997). The activity of serotonergic neurons in the raphe nucleus may function to coordinate motor output and corresponding autonomic demands (Jacobs and Fornal, 1999).

In the present study, we have demonstrated that the serotonergic LL and Retzius neurons of the brain (SEG) were activated during swim initiation and during the progression of swim episodes. Rhythmic fluctuations in membrane potentials were observed in cell LL (Fig. 2A right), indicating that this cell probably receives feedback from the swim CPG. During swimming, the Retzius neurons fired trains of impulses at relatively high frequencies. This profile of higher firing frequency (e.g. 5–10 Hz *vs* 1 Hz) has been shown to promote the secretion of large amounts of 5-HT from the somata of the Retzius cells (De-Miguel and Trueta, 2005). In addition, the cellular mechanisms underlying this somatic secretion, such as L-type calcium channels and calcium-induced calcium release, are common to excitable endocrine cells (De-Miguel and Trueta, 2005). At higher firing rates, it has been proposed that the Retzius cells influence entire circuits, as opposed to single synaptic targets (De-Miguel and Trueta, 2005). Thus, 5-HT at a concentration of 50 μmol l^{-1} that we bath-applied to the brain is most likely physiological, mimicking the paracrine-like mode of 5-HT secretion that occurs naturally during swim activation (Bruns et al., 2000; De-Miguel and Trueta, 2005; Dierkes and Schlue, 2005). Clearly, a differential upregulation of 5-HT secretion from the Retzius neurons in the head brain could account for the swim-inhibitory effects that we observed during focal application of 5-HT to the brain. Because brain neurons Tr1 and Tr2 can also induce a gradual and sustained excitation of the serotonergic Retzius cells (Brodfuehrer and Friesen, 1986b), descending command-like neurons also have the potential to contribute to the release of 5-HT and other amines in the brain that, in turn, may modulate the state of decision-making systems for on-going and subsequent behaviors (Crisp and Mesce, 2004; Garcia-Perez et al., 2005).

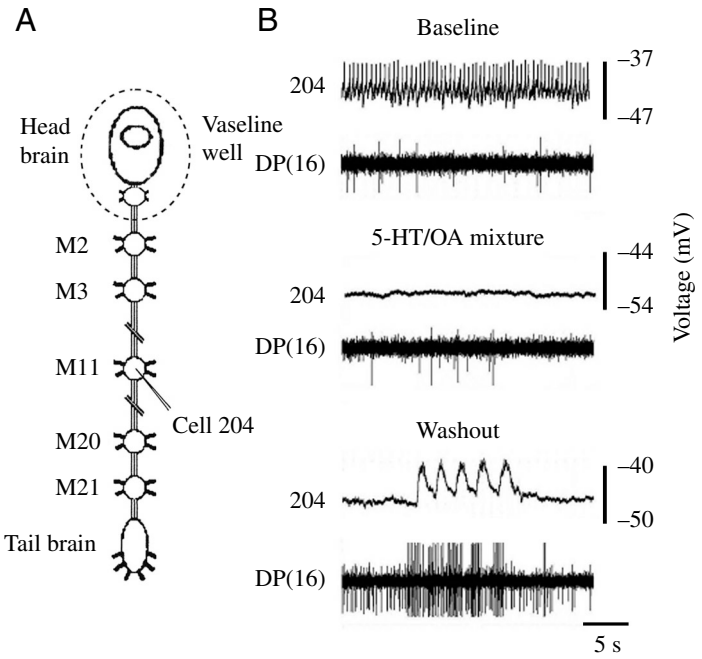

Fig. 7. Swim-gating neuron 204 (in M11) is indirectly hyperpolarized in response to a 30-min focal brain application of the 5-HT/OA mixture (50 μmol l^{-1}). (A) A schematic of the experimental preparation, emphasizing that cell 204 was not directly exposed to the amine mixture. (B) During brain perfusion of the saline baseline (top), cell 204 fired action potentials at a constant rate. After a 30-min application of the mixture (middle), 204 became hyperpolarized (6 mV) and all action potentials ceased. This depression translated into a decrease in the excitatory drive to the swim CPG and no swimming was observed. During washout of the mixture (bottom), 204 partially repolarized and showed rhythmic depolarizations phase-locked with individual swim motor bursts in the DP nerve.

Several other lines of supportive evidence point to a connection between the brain, 5-HT, swim episode length and overall swimming activity. First, preparations with the head brain attached generate shorter duration swim episodes than brainless nerve cords, and at least one cell in the brain, cell SRN1, can reset the swim rhythm (Brodfuehrer and Friesen, 1986b; Brodfuehrer and Friesen, 1986e). Secondly, depletion of 5-HT with reserpine increases the duration of swim episodes in intact animals, but reserpine blocks swimming in isolated nerve cords that lack the head brain (Hashemzadeh-Gargari and Friesen, 1989; O'Gara et al., 1991). Finally, application of 5-HT to the brain is inhibitory and is associated with shorter duration swim episodes (during washout) as compared to trials in which the brain is treated with OA or the removal of an OA/5-HT mixture (Crisp and Mesce, 2003). Collectively, these observations suggest that the modulatory state of the brain may contribute not only to the frequency or probability of swim initiation, but also to the quality (duration and rhythmicity) of the episodes expressed.

One additional issue to address is how quickly the system might change. Firing of the LL and Retzius cells could be achieved with a single swim-activating stimulus, which supports the idea that the modulatory state of brain pathways may change from one swim-initiating stimulus to the next. While we have demonstrated more long-term effects of 5-HT over a period of 30 min (Crisp and Mesce, 2003), 5-HT can cause more fast-acting changes in leech neurons on the order of seconds to minutes (Leake and Koubakanis, 1995; Burrell et al., 2001). Such a mechanism could explain previous observations of trial-to-trial variability in decision-making and swim initiation (Briggman et al., 2005).

Modulation of brain pathways, gating neurons and the decision to swim

Extracellular recordings revealed an increase in descending unit activity during focal application of 5-HT or the 5-HT/OA mixture to the brain (data not shown) (Fig. 6). Because of this observation, the amine mixture was initially postulated to exert its nonadditive and inhibitory effects on swimming by simultaneously activating a population of descending swim-inhibitory neurons and suppressing the swim-trigger cells. Contrary to expectations, 5-HT alone or the amine mixture inhibited Tr2 and SIN1, both of which are swim inactivating cells. Not surprisingly, swim-trigger neuron Tr1 was inhibited by 5-HT (Fig. 4) and the amine mixture (Fig. 7). Identification of the complete population of descending neurons activated by 5-HT remains incomplete, although it may consist of unidentified swim-inactivating neurons, or possibly ones that play roles in the expression of other behaviors. For example, cell R3b1, which activates swimming or crawling in a context-specific way (Esch et al., 2002). Nevertheless, the net and indirect effect of 5-HT to the brain caused an overall decrease in the excitation fed to the swim CPG *via* cell 204. Furthermore, this decreased drive was correlated with changes in the basal activity level of descending fibers (Fig. 6), and the recruitment of fibers in response to stimulation of identified command-like interneurons (Fig. 6). Because we found no evidence that swim-terminating neurons were excited by application of the 5-HT/OA mixture, we conclude that 5-HT inhibits swimming at the level of the brain by decreasing the net tonic excitation of cell 204 *via* the descending brain neurons. Possibly, the paradoxical increase in descending activity in response to brain modulation (Fig. 6) resides in the contribution of downstream segmental anti-swim gating cells (Taylor et al., 2003), cells that may contribute to the net suppression of cell 204.

Gating neurons, such as cell 204, play a vital role in transferring information from higher-order or command-like circuits into an excitatory drive to neural networks controlling motor behavior (Reichert and Rowell, 1985). Neural circuits in the lamprey notochord, for example, gate sensory information to directionally appropriate motor neurons that mediate the tail fin withdrawal reflex (McClellan and Grillner, 1983). Accordingly, swim-gating neuron 204 integrates indirect sensory information and descending excitation (Brodfuehrer and Friesen, 1986a) and inhibition (Brodfuehrer and Burns, 1995) from the brain and drives swim oscillatory neurons (Weeks, 1982; Nusbaum et al., 1987). Intracellular stimulation of cell Tr1, as well as swim-exciting brain interneuron SE1 (Brodfuehrer et al., 1995), excites cell 204 *via* a monosynaptic connection (Brodfuehrer and Friesen, 1986b; Brodfuehrer and Thorogood, 2001). Cell 204 also receives polysynaptic inhibitory input from cells SIN1 (Brodfuehrer and Burns, 1995) and Tr2 (Brodfuehrer and Friesen, 1986b), possibly by way of the newly described segmental anti-swim-gating interneurons 24 and 256 (Taylor et al., 2003).

Because the amine mixture decreased the activity of cells SIN1 and Tr2, we can conclude that the depression in cell 204 activity is probably due to an inhibition of the descending swim-activating population rather than an excitation in the swim-inactivating one. Although direct bath application of 5-HT to 204 does not alter the basal firing rate or membrane potential of 204 (Willard, 1981; Hashemzadeh-Gargari and Friesen, 1989), 5-HT enhances the intrinsic excitability of cell 204, thus lowering its response threshold to swim-promoting stimuli (Angstadt and Friesen, 1993a; Angstadt and Friesen, 1993b); this helps to explain why 5-HT on brain-less preparations is highly stimulatory for swimming. In contrast to the effects of challenging cell 204 directly with 5-HT, we have shown here that focal application of 5-HT to the leech head brain indirectly hyperpolarizes the resting membrane potential of cell 204 and decreases its firing frequency. This indirect modulatory effect likely decreases the excitation that 204 feeds to cells of the swim CPG and explains why we have observed that 5-HT to the brain inhibits swimming (see Crisp and Mesce, 2003).

Modulation and decision-making processes

It is not yet known whether the effects of 5-HT (or the amine mixture) on command-like brain interneurons are due to direct modulation by the amines, or to changes in synaptic activity from even higher-level decision-making neurons or other circuits. For example, Tr2 has been shown to receive rhythmic input from the crawl CPG (Crisp and Mesce, 2004). During application of the 5-HT/OA mixture, recordings of Tr1 and SIN1 revealed interesting alterations in presynaptic activity (Fig. 5). The only known inputs to cell Tr1 are sensory neurons (Brodfuehrer and Friesen, 1986b), including the pressure-sensitive P cells that are inhibited by 5-HT (Sanchez-Armass et al., 1991; Ali et al., 1998). In Fig. 5A, spontaneous depolarizations of Tr1 were observed prior to swim initiation, perhaps due to excitation from higher-order brain neurons. Because most (if not all) of the descending command-like interneurons (and cell 204) are mulitfunctional [e.g. involved in crawling and shortening (Kristan, Jr et al., 1988; Baader, 1997; Shaw and Kristan, Jr, 1997; Crisp and Mesce, 2004)], altering the probability of swimming will likely influence the expression of other behaviors as well.

During spontaneous swimming, or when a swim-related stimulus arrives, the decision to swim or not to swim appears to depend on the ‘state’ of the brain and segmental swim-related neural networks (Briggman et al., 2005; Garcia-Perez et al., 2005). What defines this state is complex, although our studies of aminergic modulation have attempted to contribute some insights. Because locomotory behaviors tend to be expressed on the order of tens of seconds, the operation of these networks may define their own and subsequent states (Garcia-Perez et al., 2005), and may influence the operation of related circuits over short and longer time periods. Altogether, our observations support the conclusion that a full understanding of the dynamics underlying decision-making processes will require a greater understanding of the

modulatory processes that occur during the activation of individual behavioral routines.

List of abbreviations

5-HT	serotonin
CNS	central nervous system
Conn	connective
CPG	central pattern generator
DP(X)	dorsal posterior [nerve] of segmental ganglion (X)
ir	immunoreactive
MX	segmental ganglion X
OA	octopamine
SEG	subesophageal ganglion
SIN1	swim-inhibitor neuron SIN1
SPEG	supraesophageal ganglion cell

We would like to thank Kathleen Klukas for help with the confocal microscopy and figure production. This research was funded, in part, by grants from the National Science Foundation (IBN-9813995 and IOB-0523959) to K.A.M., and through Fellowships from the University of Minnesota's Graduate School and NSF-IGERT Training Grant (IBN-9870633) awarded to K.M.C.

References

Ali, D. W., Catarsi, S. and Drapeau, P. (1998). Ionotropic and metabotropic activation of a neuronal chloride channel by serotonin and dopamine in the leech *Hirudo medicinalis*. *J. Physiol.* **509**, 211-219.

Angstadt, J. D. and Friesen, W. O. (1993a). Modulation of swimming behavior in the medicinal leech. I. Effects of serotonin on the electrical properties of swim-gating cell 204. *J. Comp. Physiol. A* **172**, 223-234.

Angstadt, J. D. and Friesen, W. O. (1993b). Modulation of swimming behavior in the medicinal leech. II. Ionic conductances underlying serotonergic modulation of swim-gating cell 204. *J. Comp. Physiol. A* **172**, 235-248.

Baader, A. P. (1997). Interneuronal and motor patterns during crawling behavior of semi-intact leeches. *J. Exp. Biol.* **200**, 1369-1381.

Briggman, K. L., Abarbanel, H. D. and Kristan, W. B., Jr (2005). Optical imaging of neuronal populations during decision-making. *Science* **307**, 896-901.

Brodfuehrer, P. D. and Burns, A. (1995). Neuronal factors influencing the decision to swim in the medicinal leech. *Neurobiol. Learn. Mem.* **63**, 192-199.

Brodfuehrer, P. D. and Friesen, W. O. (1986a). From stimulation to undulation: a neuronal pathway for the control of swimming in the leech. *Science* **234**, 1002-1004.

Brodfuehrer, P. D. and Friesen, W. O. (1986b). Initiation of swimming activity by trigger neurons in the leech subesophageal ganglion. I. Output connections of Tr1 and Tr2. *J. Comp. Physiol. A* **159**, 489-502.

Brodfuehrer, P. D. and Friesen, W. O. (1986c). Initiation of swimming activity by trigger neurons in the leech subesophageal ganglion. II. Role of segmental swim-initiating interneurons. *J. Comp. Physiol. A* **159**, 503-510.

Brodfuehrer, P. D. and Friesen, W. O. (1986d). Initiation of swimming activity by trigger neurons in the leech subesophageal ganglion. III. Sensory inputs to Tr1 and Tr2. *J. Comp. Physiol. A* **159**, 511-519.

Brodfuehrer, P. D. and Friesen, W. O. (1986e). Control of leech swimming activity by the cephalic ganglia. *J. Neurobiol.* **17**, 697-705.

Brodfuehrer, P. D. and Thorogood, M. S. E. (2001). Identified neurons and leech swimming behavior. *Prog. Neurobiol.* **63**, 371-381.

Brodfuehrer, P. D., Parker, H. J., Burns, A. and Berg, M. (1995). Regulation of the segmental swim-generating system by a pair of identified interneurons in the leech head brain. *J. Neurophysiol.* **73**, 983-992.

Bruns, D., Riedel, D., Klingauf, J. and Jahn, R. (2000). Quantal release of serotonin. *Neuron* **28**, 205-220.

Burrell, B. D., Sahley, C. L. and Muller, K. J. (2001). Non-associative learning and serotonin induce similar bi-directional changes in excitability of a neuron critical for learning in the medicinal leech. *J. Neurosci.* **21**, 1401-1412.

Cellucci, C. J., Brodfuehrer, P. D., Acera-Pozzi, R., Dobrovolny, H., Engler, E., Los, J. and Thompson, R. (2000). Linear and nonlinear measures predict swimming in the leech. *Phys. Rev. E Stat. Phys. Plasmas Fluids Relat. Interdiscip. Topics* **62**, 4826-4834.

Crisp, K. M. and Mesce, K. A. (2003). To swim or not to swim: regional effects of serotonin, octopamine, and amine mixtures in the medicinal leech. *J. Comp. Physiol. A* **189**, 461-470.

Crisp, K. M. and Mesce, K. A. (2004). A cephalic projection neuron involved in locomotion is dye-coupled to the dopaminergic neural network in the medicinal leech. *J. Exp. Biol.* **207**, 4535-4542.

De-Miguel, F. F. and Trueta, C. (2005). Synaptic and extrasynaptic secretion of serotonin. *Cell Mol. Neurobiol.* **25**, 297-312.

Dierkes, P. W. and Schlue, W.-R. (2005). Ca^{++} influx into identified leech neurons induced by 5-hydroxytryptamine. *J. Neurobiol.* **62**, 106-120.

Eisenhart, F. J., Cacciatore, T. W. and Kristan, W. B., Jr (2000). A central pattern generator underlies crawling in the medicinal leech. *J. Comp. Physiol. A* **187**, 631-643.

Esch, T., Mesce, K. A. and Kristan, W. B., Jr (2002). Evidence for sequential decision making in the medicinal leech. *J. Neurosci.* **22**, 11045-11054.

Friesen, W. O. (1989). Neuronal control of leech swimming movements. In *Neuronal and Cellular Oscillators* (ed. J. W. Jacklet), pp. 269-316. New York: Marcel Dekker.

Garcia-Perez, E., Mazzoni, A., Zoccolan, D., Robinson, H. P. C. and Torre, V. (2005). Statistics of decision making in the leech. *J. Neurosci.* **25**, 2597-2608.

Gilchrist, L. S. and Mesce, K. A. (1997). Coactivation of putative octopamine- and serotonin-containing interneurons in the medicinal leech. *J. Neurophysiol.* **78**, 2108-2115.

Gilchrist, L. S., Klukas, K. A., Jellies, J., Rapus, J., Eckert, M. and Mesce, K. A. (1995). Distribution and developmental expression of octopamine-immunoreactive neurons in the central nervous system of the leech. *J. Comp. Neurol.* **353**, 451-463.

Grobstein, P. (1994). Variability in brain function and behavior. In *Encyclopedia of Human Behavior* (ed. V. S. Ramachandran), pp. 447-458. New York: Academic Press.

Hashemzadeh-Gargari, H. and Friesen, W. O. (1989). Modulation of swimming activity in the medicinal leech by serotonin and octopamine. *Comp. Biochem. Physiol.* **94C**, 295-302.

Jacobs, B. L. and Fornal, C. A. (1993). 5-HT and motor control: a hypothesis. *Trends Neurosci.* **16**, 346-352.

Jacobs, B. L. and Fornal, C. A. (1999). Activity of serotonergic neurons in behaving animals. *Neuropsychopharmacology* **21**, 9S-15S.

Jellies, J., Loer, C. M. and Kristan, W. B., Jr (1987). Morphological changes in leech Retzius neurons after target contact during embryogenesis. *J. Neurosci.* **7**, 2618-2629.

Jellies, J., Kopp, D. M. and Geisert, E. E. (1993). Developmental regulation of a glycolipid epitope on actively extending growth cones and central and peripheral projections in the medicinal leech. *Dev. Biol.* **159**, 691-705.

Kristan, W. B., Jr, Wittenberg, G., Nusbaum, M. P. and Stern-Tomlinson, W. (1988). Multifunctional interneurons in behavioral circuits of the medicinal leech. *Experientia* **44**, 383-389.

Kristan, W. B., Jr, Calabrese, R. L. and Friesen, W. O. (2005). Neuronal control of leech behavior. *Prog. Neurobiol.* **76**, 279-327.

Leake, L. D. and Koubanakis, M. (1995). Central and peripheral 5-HT receptors in the leech (*Hirudo medicinalis*) redefined. *Gen. Pharmacol.* **26**, 1709-1717.

Lent, C. M. (1982). Fluorescent properties of monoamine neurons following glyoxylic acid treatment of intact leech ganglia. *Histochemistry* **75**, 77-89.

Libersat, F. and Pflüger, H. J. (2004). Monoamines and the orchestration of behavior. *BioScience* **54**, 17-25.

Maley, B. and Elde, R. P. (1982). Immunocytochemical localization of putative neurotransmitters within the feline nucleus tractus solitarii. *Neuroscience* **7**, 2469-2490.

Mangan, P. S., Cometa, A. K. and Friesen, W. O. (1994a). Modulation of swimming behavior in the medicinal leech. IV. Serotonin-induced alteration of synaptic interactions between neurons of the swim circuit. *J. Comp. Physiol. A* **175**, 723-736.

Mangan, P. S., Curran, G. A., Hurney, C. A. and Friesen, W. O. (1994b). Modulation of swimming behavior in the medicinal leech III. Control of cellular properties in motor neurons by serotonin. *J. Comp. Physiol. A* **175**, 709-722.

Marder, E. and Calabrese, R. L. (1996). Principles of rhythmic motor pattern generation. *Physiol. Rev.* **76**, 687-717.

Marsden, C. A. and Kerkut, G. A. (1969). Fluorescent microscopy of the 5HT- and catecholamine-containing cells in the central nervous system of the leech *Hirudo medicinalis*. *Comp. Biochem. Physiol.* **31**, 851-862.

McClellan, A. D. and Grillner, S. (1983). Initiation and sensory gating of 'fictive' swimming and withdrawal responses in an in vitro preparation of the lamprey spinal cord. *Brain Res.* **269**, 237-250.

Mesce, K. A., Klukas, K. A. and Brelje, T. C. (1993). Improvements for the anatomical characterization of insect neurons in whole mount: the use of cyanine-derived fluorophores and laser scanning confocal microscopy. *Cell Tissue Res.* **271**, 381-397.

Mesce, K. A., Crisp, K. M. and Gilchrist, L. S. (2001). Mixtures of octopamine and serotonin have nonadditive effects on the CNS of the medicinal leech. *J. Neurophysiol.* **85**, 2039-2046.

Nicholls, J. G. and Baylor, D. A. (1968). Specific modalities and receptive fields of sensory neurons in CNS of the leech. *J. Neurophysiol.* **31**, 740-756.

Nusbaum, M. P., Friesen, W. O., Kristan, W. B., Jr and Pearce, R. A. (1987). Neural mechanisms generating the leech swimming rhythm: swim-initiator neurons excite the network of swim oscillator neurons. *J. Comp. Physiol. A* **161**, 355-366.

O'Gara, B. A. and Friesen, W. O. (1995). Termination of leech swimming activity by a previously identified swim-trigger neuron. *J. Comp. Physiol. A* **177**, 627-636.

O'Gara, B. A., Chae, H., Latham, L. B. and Friesen, W. O. (1991). Modification of leech behavior patterns by reserpine-induced amine depletion. *J. Neurosci.* **11**, 96-110.

Ort, C. A., Kristan, W. B., Jr, and Stent, G. S. (1974). Neuronal control of swimming in the medicinal leech. II. Identification and connections of motor neurons. *J. Comp. Physiol.* **94**, 121-154.

Reichert, H. and Rowell, C. H. F. (1985). Integration of nonphaselocked exteroceptive information in the control of rhythmic flight in the locust. *J. Neurophysiol.* **53**, 1201-1218.

Ribeiro-do-Valle, L. E. (1997). Serotonergic neurons in the caudal raphe nuclei discharge in association with activity of masticatory muscles. *Braz, J. Med. Res.* **1**, 79-83.

Rude, S. (1969). Monoamine-containing neurons in the central nervous system and peripheral nerves of the medicinal leech, *Hirudo medicinalis*. *J. Comp. Neurol.* **136**, 349-372.

Sanchez-Armass, S., Merz, D. C. and Drapeau, P. (1991). Distinct receptors, second messengers and conductances underlying the dual responses to serotonin in an identified leech neurone. *J. Exp. Biol.* **155**, 531-547.

Shaw, B. K. and Kristan, W. B., Jr (1997). The neuronal basis of the behavioral choice between swimming and shortening in the leech: control is not selectively exercised at higher circuit levels. *J. Neurosci.* **17**, 786-795.

Taylor, A. L., Cottrell, G. W., Kleinfeld, D. and Kristan, W. B., Jr (2003). Imaging reveals synaptic targets of a swim-terminating neuron in the leech CNS. *J. Neurosci.* **23**, 11402-11410.

Webb, R. A. (1980). Intralamellar neurohemal complexes in the cerebral commisure of the leech *Macrobdella decora* (Say, 1824): an electron microscope study. *J. Morphol.* **163**, 157-165.

Weeks, J. C. (1982). Synaptic basis of swim initiation in the leech. I. Connections of a swim-initiating neuron (cell 204) with motor neurons and pattern-generating 'oscillator' neurons. *J. Comp. Physiol. A* **148**, 253-263.

Weeks, J. C. and Kristan, W. B., Jr (1978). Initiation, maintenance and modulation of swimming in the medicinal leech by the activity of a single neurone. *J. Exp. Biol.* **77**, 71-78.

Wessendorf, M. W. and Elde, R. P. (1985). Characterization of an immunofluorescence technique for the demonstration of coexisting neurotransmitters within nerve terminals. *J. Histochem. Cytochem.* **33**, 984-994.

Wessendorf, M. W. and Elde, R. P. (1987). The coexistence of serotonin- and substance P-like immunoreactivity in the spinal cord of the rat as shown by immunofluorescence labeling. *J. Neurosci.* **7**, 2352-2363.

Wilkinson, J. M. and Coggeshall, R. E. (1975). Axonal numbers and sizes in the connectives and peripheral nerves of the leech. *J. Comp. Neurol.* **162**, 387-396.

Willard, A. L. (1981). Effects of serotonin on the generation of the motor program for swimming by the medicinal leech. *J. Neurosci.* **1**, 936-944.