

RESEARCH ARTICLE

The stomatogastric nervous system of the medicinal leech: its anatomy, physiology and associated aminergic neurons

Karen A. Mesce^{1,*}, Magda Alania², Quentin Gaudry³ and Joshua G. Puhl¹

ABSTRACT

Blood feeding is an essential and signature activity of the medicinal leech species *Hirudo verbana*. Despite keen interest in understanding the neuronal substrates of this behavior, a major component of the nervous system associated with feeding has remained overlooked. In this study, for the first time, we report on the presence and characteristics of five stomatogastric ganglia (STGs) comprising the visceral stomatogastric nervous system (STN) of the leech. Although a brief report was published by Ruth Hanke in 1948 indicating that a ring of three ganglia (not five) was associated with the cephalic ganglia, this information was never integrated into subsequent neurobiological studies of feeding. Here, the anatomical features of the STGs are described, as are the morphological and electrophysiological characteristics of neurons originating in them. We also determined that two of the five STGs (STG-1 and STG-3) each contained two relatively large (ca. 40 µm diameter) serotonergic neurons. The STN was also enriched with dopaminergic and serotonergic arborizations; however, no intrinsic dopaminergic somata were observed. The trajectory of the serotonergic large lateral (LL) neuron, a command-like cell for feeding, was documented to project directly to the STN and not to the jaw and pharyngeal musculature as previously reported, thus reopening the important question of how the LL cell activates and coordinates biting activity with pharyngeal swallowing. Additional studies revealed that the LL cell is excited by blood serum applied to the lip and is strongly inhibited by dopamine. These findings provide a new foundation for understanding the regulation and modulation of neural networks involved in feeding.

KEY WORDS: Feeding, Serotonin, Dopamine, Central pattern generator, Decision making, Neuromodulation

INTRODUCTION

For many decades, the European medicinal leech (*Hirudo verbana*) has served as an accessible animal preparation in which to understand the physiological underpinnings of specific neuronal phenotypes, enabling researchers to determine how the connectivity among individual cells leads to naturalistic behaviors (Muller et al., 1981; Kristan et al., 2005). From decision-making processes and sensorimotor integration to centrally generated fictive locomotor behaviors in the isolated nerve cord, cellular ‘connectomes’ have

been identified that can explain many of the key behaviors expressed by the leech (Kristan et al., 2005; Briggman and Kristan, 2006; Lamb and Calabrese, 2011; Wagenaar, 2015).

Sanguivorous (blood) feeding is often the first thought that comes to mind when one thinks about the behavioral repertoire of a leech. Before a leech can begin to consume or swallow the blood of its prey, however, it must first use its three serrated and razor-sharp teeth, each attached to a muscular jaw, to bite (i.e. rasp) into the flesh of its host. In comparison to other invertebrates, surprisingly little is known about the neural bases of biting behavior in the medicinal leech or any leech species. Only one report exists, originally published 70 years ago, which briefly describes the visceral stomatogastric nervous system (STN) associated with the muscular jaws and pharynx of the leech (Hanke, 1948). These findings were again presented by Sawyer (1986) in his encyclopedic ‘Leech Biology and Behavior’ series, but no new information was added.

In the study reported here, we have taken a fresh look at the original findings of Hanke and have significantly expanded upon them using contemporary methods for cell labeling and imaging, and electrophysiological recording. Although the original study first described the locations of some of the visceral ganglia comprising the STN, not all of the ganglia were detected and the nature of the neurons originating in them were never examined, nor were any neural inputs to the STN revealed.

While describing the STN more fully here, we also focused our attention on whether the STN was associated with serotonergic and dopaminergic modulation by way of projection neurons and novel endogenous neurons that might express aminergic immunoreactivity. The biogenic amine serotonin, in particular, was targeted for study because it has been shown previously to play an important role in leech feeding behavior, acting to help orchestrate a wide range of feeding and locomotor behaviors to ensure that a successful blood meal is achieved (Lent and Dickinson, 1984; Lent, 1985; Lent et al., 1991; Groome et al., 1995; Gaudry and Kristan, 2009). Lastly, the previously studied serotonergic neuron, the large lateral (LL) cell (Lent and Dickinson, 1984, 1987; Lent, 1985; Crisp and Mesce, 2006), located (as a pair) in the first neuromere of the subesophageal ganglion (SEG), was examined in the context of the STN, bringing to light a new association that redefines the operation of this important feeding-related neuron.

The medicinal leech feeds only intermittently throughout its life, consuming a large meal of mammalian blood every 6–18 months, yet many of its physiological and behavioral activities are devoted to the detection, consumption and subsequent satiation of its blood meal (Lent and Dickinson, 1988). Understanding the role of the STN and its coordination with other regions of the central nervous system (CNS), and how it operates in the larger context of feeding and non-feeding behaviors, necessitates the type of examination we have performed here. Furthermore, based on our demonstration that the somata intrinsic to the STN are accessible to intracellular

¹Departments of Entomology and Neuroscience, University of Minnesota, St Paul, MN 55108, USA. ²Department of Biology, Faculty of Exact and Natural Sciences, Ivane Javakishvili Tbilisi State University, Tbilisi, Georgia. ³Department of Biology, University of Maryland, College Park, MD 20742, USA.

*Author for correspondence (mesce001@umn.edu)

 K.A.M., 0000-0002-7544-1500

recording, this numerically small neural system has the potential to inform us about the underlying circuits and neuromodulators that generate rhythmic and stereotypical movements associated with chewing and swallowing, similar to some arthropod and molluscan preparations, which have revealed elegant neural solutions to such problems (Ayali, 2004; Cropper et al., 2004; Marder and Bucher, 2007; Marder et al., 2014).

MATERIALS AND METHODS

Animals

Medicinal leeches (*Hirudo verbana* Carena 1820), weighing between 1.3 and 3.0 g, were procured from either Niagra Medical Leeches Inc. (Niagara Falls, NY, USA) or from Leeches USA (Westbury, NY, USA). They were kept in 5 gallon buckets containing distilled water supplemented with Instant Ocean salts (Spectrum Brands, Atlanta, GA, USA; 1.89 g gallon⁻¹) or with fresh local pond water stored at the University of Minnesota, MN, USA.

Histological procedures

Animal preparation and neuronal staining

For all protocols, leeches were first anesthetized on ice until they became immobile. Under a dissecting microscope, a dorsal midline incision was made at the anterior region of the leech to reveal the compound cephalic ganglion (the supra- and subesophageal

ganglia) and muscular jaws. The STN is not immediately obvious but comprises a five-ganglion ring that parallels the face of the supraesophageal ganglion as it circumscribes the pharynx (Fig. 1). The STN and other targeted tissues were dissected in ice-cold normal leech Ringer's solution, which contained (in mmol l⁻¹): 116.0 NaCl, 4.0 KCl, 1.8 CaCl₂, 1.5 MgCl₂, 10.0 dextrose, 10.0 Trizma pre-set crystals, pH 7.4 (Sigma-Aldrich, St Louis, MO, USA) (adapted from Nicholls and Baylor, 1968). Note: staining the STN *in situ* enables one to become familiar with its location for subsequent extirpation with or without staining. To prepare mounted specimens of the STN, tissues were dissected from the animal and placed in a Methylene Blue solution (30 mg ml⁻¹ of leech Ringers), at room temperature, for 4–6 h (Macagno, 1980). The tissue was then fixed in 4% paraformaldehyde (made up in leech Ringers) for 15–30 min. After fixation, the tissue was placed in 30% acetone until the neuropile showed a clearing of its bluish color. The tissue was then dehydrated in an acetone series (60, 70, 80, 90 and 3×100%) for 1–2 min at each step, cleared with methyl salicylate and mounted in DePeX (Electron Microscopy Sciences, Fort Washington, PA, USA) between two coverslips.

As a secondary method to obtain accurate cell counts of somata in each STN ganglion, we used the nuclear stain propidium iodide (Sigma-Aldrich). The compound cephalic ganglion and STN were dissected out of the leech and placed in phosphate buffered saline

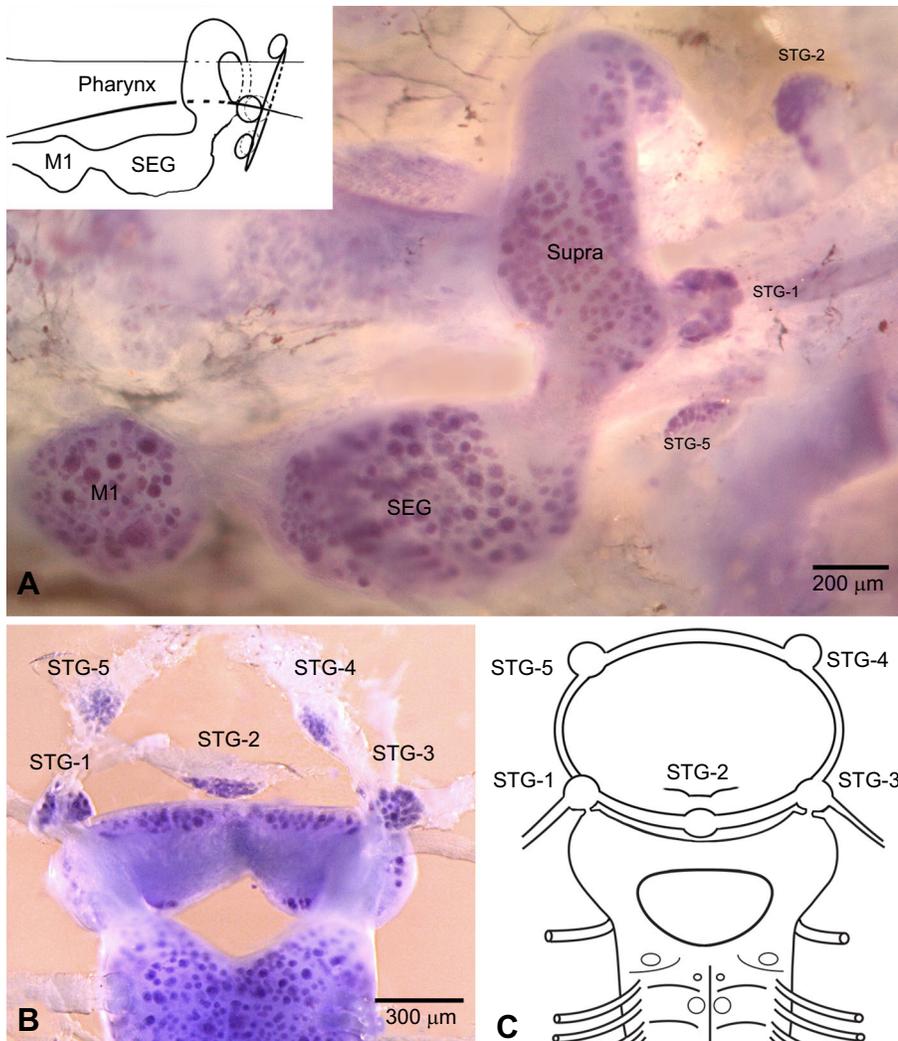


Fig. 1. Methylene-Blue-stained stomatogastric nervous system (STN) presented in association with other anatomical structures. (A) Three of the five ganglia of the STN [stomatogastric ganglion (STG)-1, STG-2 and STG-5], stained with Methylene Blue, are shown *in situ* and in relation to the pharynx and the cephalic ganglia, which comprise the supraesophageal ganglion (Supra) and the subesophageal ganglion (SEG). The first segmental or midbody ganglion (M1) of the ventral nerve cord is also shown. Note that STG-2 is the dorsal-most STG when viewed *in situ*. Neuronal somata in the STN and CNS appear dark blue-purple. The upper left inset provides a diagrammatic perspective of how the STGs form a ring around the pharynx. (B) Whole-mounted specimen of the stained cephalic ganglia with the ventral surface of the SEG facing up and the STN ring flipped upwards such that STG-5 and STG-4 are unnaturally positioned above STG-2. This configuration enables one to view all five of the STGs with their interconnected nerves (light blue). (C) This diagram depicts the configuration of the five STGs and their interconnecting nerves in whole-mounted specimens, highlighting our convention of numbering the STG from 1 to 5 in the counter-clockwise direction.

(PBS), and then transferred into 70% ethanol, at 4°C, for 30 min. The tissue was then washed in PBS (2×1 min). A stock solution of propidium iodide was initially made in distilled water at a concentration of 1 mg ml⁻¹ and diluted at 1:100 in PBS. Incubation of the tissue for 30 min was done in the dark at room temperature. The tissue was then cleared in PBS with 80% glycerol, 20% PBS, 2% (w/v) *n*-propyl-gallate (to reduce fading).

Labeling of aminergic neurons

To facilitate the identification of the ventrally located large serotonergic LL neurons for electrophysiological studies, the vital stain Neutral Red (Sigma-Aldrich) was used according to the methods of Gilchrist et al. (1995). Individual leeches were placed in an 8 oz plastic cup containing a solution of 0.001% Neutral Red (in pond water). Leeches were able to swim and crawl overnight, during which time the stain was able to be transported through the skin and into the CNS.

For serotonin immunolabeling, we relied on our previously published protocols using a highly specific rabbit anti-serotonin antiserum (ImmunoStar, Inc., Hudson, WI, USA) (Crisp and Mesce, 2006). Briefly, the STN and associated cephalic ganglia were first fixed in 4% paraformaldehyde (in PBS) overnight followed by three 10 min washes in PBS. Washed tissue was then placed in a buffered solution of collagenase (Sigma type IV; 0.5 mg ml⁻¹) for 10–30 min, followed by incubation in normal goat serum (10%) and Triton X-100 (0.6%) for 2 h to reduce background labeling. The serotonin rabbit antibody (ImmunoStar) was diluted 1:200 in PBS and the tissue remained in this solution for 48 h. The tissue was rinsed in PBS and then placed in a 1:200 or a 1:500 dilution of secondary antibody (Cy-3-conjugated goat or donkey anti-rabbit) for 48 h.

To describe the dopaminergic profile of the stomatogastric nerve ring, antisera to dopamine's rate-limiting synthetic enzyme, tyrosine hydroxylase (TH) (DiaSorin, Stillwater, MN, USA), was used. In previous reports (Crisp et al., 2002), we substantiated the specificity of this antiserum and confirmed that only dopamine-synthesizing neurons express immunoreactivity to this monoclonal anti-TH antibody. Briefly, the cephalic ganglia and STN nerve ring were dissected and fixed in 4% paraformaldehyde for 50 min at room temperature. After fixation, tissues were washed in PBS and then placed in a buffered solution of collagenase (Sigma type IV; 0.5 mg ml⁻¹) for 10–30 min. To reduce background staining, tissues were incubated for 24 h in a blocking buffer containing 10% normal goat serum and 1% Triton X-100. Tissues were incubated in a 1:50 dilution of the mouse monoclonal anti-TH antibody (DiaSorin) for 48 h. After incubation in the primary antibody, tissue was washed in PBS–Triton-X-100 solution three times for 1 h and subsequently incubated with a 1:200 dilution of a donkey anti-mouse IgG secondary antibody conjugated with the cyanine-3.18 (Cy3) fluorophore (Jackson ImmunoResearch, West Grove, PA, USA).

After their incubations in secondary antisera, all samples were washed thoroughly before they were pinned out and then processed through an ethanol series (70, 80, 90 and 3×100%); each step timed for 15 min. After dehydration, methyl salicylate was used to clear the tissue, which was subsequently mounted between two coverslips in DePeX mounting medium. A Nikon C1 spectral imaging laser scanning confocal microscope was used to view the mounted specimens (1 μm optical sections).

Electrophysiological studies

General methods

For some of the electrophysiological experiments conducted (see figure legends), the thin sheath surrounding the SEG and STN was

digested with a brief 5 min application of collagenase–dispase at 2 mg ml⁻¹ (Roche catalog number 269638 lot 10050102, Basel, Switzerland). After washing in leech saline for ca. 30 min, recordings of STN and LL neurons were made using standard sharp microelectrode electrophysiological recording techniques and dark-field microscopy. The electrophysiological data shown in the final two figures, however, were collected from preparations not exposed to the digestion procedure. Sharp microelectrodes were fabricated and filled with 2 mol l⁻¹ potassium acetate and 20 μmol l⁻¹ KCl to a final resistance of 20–40 MΩ. For Neurobiotin cell labeling, 3% Neurobiotin was added to the electrode and a positive current of about 0.5–1.0 nA was used for the iontophoretic injection of the tracer. For some of the LL Neurobiotin fills, small current injections were continued for up to several hours to obtain excellent visualization of distal projections into the STN.

Isolated lip preparations

These preparations were performed as previously described (Groome et al., 1995; Zhang et al., 2000). The leech was pinned down into a wax dissection tray and anesthetized using ice-cold leech saline. The CNS was isolated along with the prostomial lip, making sure not to sever the connecting cephalic nerves. The CNS and lip were pinned ventral side up in a Sylgard-coated recording chamber and petroleum jelly was used to divide the chamber in two, isolating the lip from the CNS. Warmed bovine serum (~35°C) was applied to the lip via a transfer pipette and removed using constant perfusion through the portion of the chamber containing the lip. Contact between the transfer pipette and bath solution resulted in an electrical artifact that we used to determine the onset of the lip stimulation. Recordings of the LL cells were made in bridge mode using an Axoclamp-2B amplifier and analyzed using custom MATLAB software. The LL neuron was identified based on its large soma size and position within the SEG. Dye fills were conducted using iontophoresis of Alexa-Fluor-488–dextran (10,000 MW) or Neurobiotin and visualized using confocal microscopy to confirm the identity of the neuron.

RESULTS

Fig. 1 shows the location and composition of the STN stained with Methylene Blue. It is composed of five individual ganglia, which we have designated stomatogastric ganglion 1 through 5 (STG-1–STG-5). Because the entire STN is not only associated with the buccal cavity but the pharynx as well, we have opted to use the more general 'stomatogastric' terminology. In contrast to the study of Hanke (1948), we have identified a total of five STGs, not three – STG-1 and STG-3 were not mentioned previously. In Fig. 1A, the position of STG-1 can be viewed as it sits laterally in relationship to the dorsally positioned supraesophageal ganglion. In Fig. 1B, a Methylene-Blue-stained STN is shown dissected out of the leech and shown with its associated SEG and supraesophageal ganglion, which have been flipped ventral side up to highlight the STN. The commissures and lateral nerves that connect all of the five ganglia together, forming a ring, are shown diagrammatically in Fig. 1C. We observed that STG-2, STG4 and STG-5 each have nerve roots that innervate the three jaws and the pharyngeal walls.

The number of neurons and sizes of somata contained within each STG were counted using several staining methods: the vital stain Methylene Blue and the nuclear dye propidium iodide. Similar cell counts were obtained using each of these methods; however, in practice, recounting the cells in the same ganglion often led to slightly different numbers each time the whole-mounted specimen was inspected. In STG-1 and STG-3, we observed an average of

25 neurons ganglion⁻¹ with the sizes of somata ranging from ca. 20 to 60 μm . STG-2 had, on average, 60 cells, and STG-4 and STG-5 contained an average of ca. 70 neurons, with somata ranging in size from 10 to 30 μm across these three ganglia. Maximal ranges for all cell counts were ± 3 cells ganglion⁻¹ preparation⁻¹; $n=3$ preparations for each protocol. The propidium iodide method also re-confirmed the existence of five ganglia within the STN and the positions of the cells using confocal microscopy (Fig. 2).

As mentioned above, the neurons residing in the leech STN have never been described prior to the current study. Thus, we went beyond assessing the somatic sizes and numbers of the STG cells, and explored their morphological and physiological profiles as well.

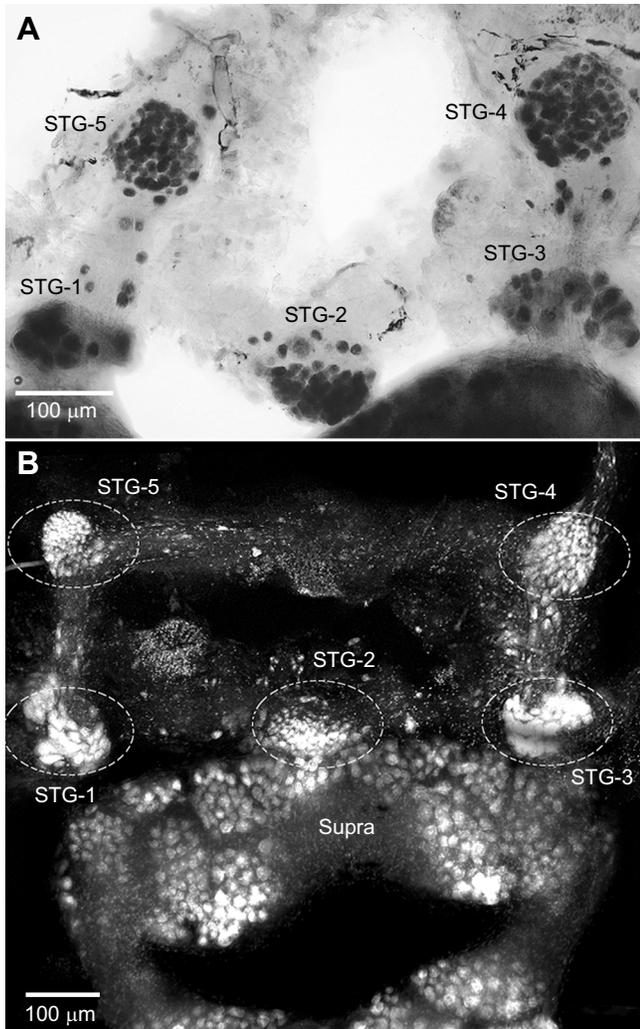


Fig. 2. Complementary staining protocols of Methylene Blue and propidium iodide used to reveal the composition of neurons within the five STGs. (A) Brightfield micrograph of the somata within each of the five STGs stained with Methylene Blue. Such protocols were used to facilitate measurements of the numbers and sizes of the somata located within each ganglion. The bilaterally paired STG-1 and STG-3, which reside in closest proximity to the supraesophageal ganglion, have not been reported in the literature previously. (B) The nuclear stain propidium iodide labeled a similar complement of cells in the STN. The various STGs are labeled STG-1 to STG-5 and outlined by dashed circles. Note that a few of the stained cells are sometimes found positioned beyond the boundaries of their home STG. Propidium iodide staining is also seen in the supraesophageal ganglion (Supra), where the nuclei of neuronal somata can be seen grouped by their individual glial packets, which are formed by only a few giant glial cells.

Most importantly, we wanted to establish whether these neurons were accessible and amenable to intracellular recording with sharp electrodes. In Fig. 3, we demonstrated that, indeed, the neurons in the STG can be individually labeled with Neurobiotin iontophoretic injection and their electrophysiological signatures obtained; standard dark-field microscopy was used to locate cells visually. In Fig. 3A–C, the morphology of two of the larger cells (ca. 60 μm), in two different preparations, are shown. Both neurons have the typical unipolar morphology with numerous arborizations that were observed projecting to other STGs, and the supraesophageal ganglion. The soma of the cell depicted in Fig. 3C is shown flopped over its primary neurites, thus falsely revealing a multipolar-like morphology. In Fig. 3A,B, the ascending processes from the serotonergic LL cell in the SEG can be seen in association with the labeled cell in STG-1. In Fig. 3C (bottom), this cell's corresponding spontaneous action potentials are shown. The magnitude of the voltage response (ca. 6 mV) compared very favorably with other leech interneurons and motor neurons: neurons whose action potentials are typically in the 3–6 mV range.

Because there is evidence that the biogenic amines play a role in feeding behavior and, possibly, biting motor activity (Lent, 1985; Lent et al., 1991; O'Gara et al., 1991), we examined whether the STGs were associated with dopamine and serotonin expression. Fig. 4A shows the presence of dopaminergic fibers and their varicosities as characterized by antisera raised against TH, the rate-limiting synthetic enzyme for dopamine. The TH antiserum used was shown previously in the leech to label dopaminergic neurons exclusively (Crisp et al., 2002). Although all of the STGs were richly innervated by TH-immunoreactive (TH-ir) fibers, as were the commissures linking the STGs, in no samples ($n=6$) were we able to detect any TH-ir somata (Fig. 4A). TH-ir somata were present, however, in the SEG (Fig. 4A) and likely contributed to the numerous TH-ir fibers observed throughout the STN. In contrast, when we immunolabeled the STGs with an antibody raised against serotonin, we could detect a number of serotonin-positive somata that were intrinsic to the STGs. Fig. 4B,C show that STG-1 and STG-3 each contain two relatively large serotonin-ir neurons. In addition, all five of the STGs contained dense serotonin-ir neuropiles. Serotonin-ir fibers were also observed projecting out of the fine nerves associated with STG2, STG4 and STG5, nerves that innervate the jaw muscles (Fig. 4B). Lastly, the ascending axons of the large serotonin-ir LL cells, located in the anterior-most neuromere of the SEG (Fig. 4B), can be seen projecting up and throughout the entire STN (i.e. stomatogastric nerve ring). This specific LL-projection pattern stands in sharp contrast to previous descriptions wherein the LL cell was perceived to exit out one of the lateral nerves (i.e. the cerebrobuccal nerve root) of the supraesophageal ganglion (Lent et al., 1991).

The serotonergic LL neuron is one of the most influential feeding-related interneurons identified to date. For example, intracellular stimulation of the LL cell can directly activate biting motor activity (Lent, 1985). Thus, we aimed to establish the precise route by which the LL activates its targets. We labeled the LL neuron with Neurobiotin via intracellular iontophoretic injection and examined its complete morphology with the STG nerve ring intact and attached to the compound cephalic ganglion. In addition, we conducted extracellular electrophysiological recordings from the supraesophageal nerve in which Lent et al. (1991) had judged the LL to exit (cerebrobuccal nerve root). Note: it appears that Lent and colleagues were not aware of the existence of the STG nerve ring when any of the reports on LL-mediated feeding were published.

Fig. 5A shows clearly the precise projection pattern of the LL cell, and illustrates that it does not exit through any of the lateral

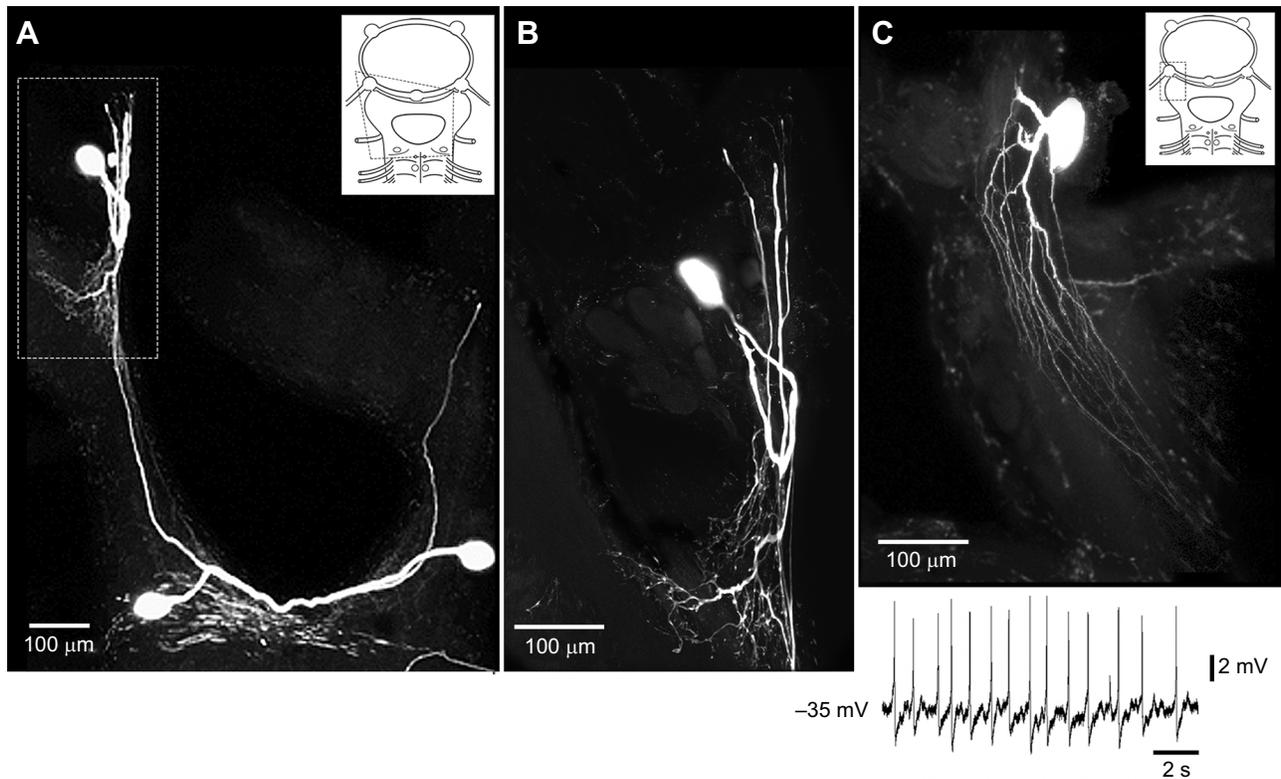


Fig. 3. First ever look at the anatomical and physiological features of neurons originating in the STN. (A) Confocal micrograph showing the morphology of a monopolar neuron located in STG-1 (within dashed box). This neuron was labeled with an intracellular iontopheretic injection of Neurobiotin. Each of the two large lateral (LL) neurons located in the SEG was also injected with Neurobiotin to determine whether there were overlapping neuronal processes between the LL and STG-1 cells. A diagram of the relative positions of the labeled cells is shown in the upper-right inset. (B) Higher-magnification view of the STG-1 neuron. Fine processes can be seen emanating from the primary neurite, which could be observed to project anteriorly to STG-5 and posteriorly to the supraesophageal ganglion. The ascending axon of the contralateral LL cell can be followed to regions of fine arbors associated with the STG-1 cell. (C) A different STG-1 neuron in a second preparation labeled with Neurobiotin. The location of this cell is shown in the upper-right inset. Although this neuron appears to have a multipolar morphology, by viewing each of the confocal optical sections carefully there appeared to be only one primary neurite, albeit short. The processes of this particular cell were observed to project to the supraesophageal ganglion and within the commissure to STG-2. The corresponding electrophysiological activity of this neuron is shown in the trace below. The cell displayed spontaneous and tonically active spiking activity and synaptic potentials similar to that found in other leech neurons located in the segmental ganglia.

roots of the supraesophageal ganglion. Furthermore, when the LL cell was injected with positive current and spikes were elicited (Fig. 5B,C), no extracellular spikes were detected in the nerve root that were phase-locked with the LL spike-triggered average (Fig. 5C). In contrast, in control recordings where the nerve-exiting mechanosensory touch sensory cell (T cell) was recorded (SEG-neuromere 1, ventral-ipsilateral side), an extracellular spike was evoked and phase-locked to the intracellular spikes (Fig. 5D).

Based on our findings that the pair of serotonergic LL neurons do not project directly to the jaw musculature or to any other peripheral targets, and that there is an intimate association of the LL cells with the STGs, we subsequently wanted to establish whether the LL cells were integrators of feeding-related stimuli that could then drive potential circuits in the STGs. We thus used a semi-intact head preparation that enabled us to stimulate the lip regions of the leech with a blood serum while simultaneously recording from the LL cells in the SEG (Fig. 6A). We found that blood serum application to the dorsal lip, indeed excited the LL cells (Fig. 6B). As a control, saline was applied to the lip: no increase in cell firing was observed (Fig. 6C,D). The LL cells recorded in this semi-intact preparation ($n=5$) were quiet 10–15 min prior to blood serum delivery. During the 5 s period before stimulation, two cells each exhibited one spike and three cells exhibited zero spikes, and increased their firing rates

with a variable delay. LL neurons depolarized an average of 2.7 mV during blood application and increased their firing rate from 0.08 to 9.44 Hz, a statistically significant amount ($P<0.05$) (Fig. 6D).

Finally, while exploring the physiological properties of the LL cells, we noted that they were electrically coupled and fired synchronously. This coupling was revealed through the transfer of Neurobiotin between the two cells (see the dye-coupled soma in Fig. 5A), the non-rectifying transfer of hyperpolarizing current (maximum coupling coefficient of 0.8) and the highly synchronous bursting activity shown across the two cells (Fig. 7A). We also discovered that the application of dopamine to spontaneously active cells caused a reversible inhibition of the LL's firing activity (Fig. 7B), which may provide insights into how feeding-related sub-routines might be orchestrated and coordinated with other behavioral events.

DISCUSSION

Overview

Despite numerous reports describing the neural bases of feeding in the medicinal leech, the role of the STN has been overlooked for essentially all of this research. Ironically, networks within the STGs are likely the most essential for the feeding-related movements observed during biting and pharyngeal pumping when the leech

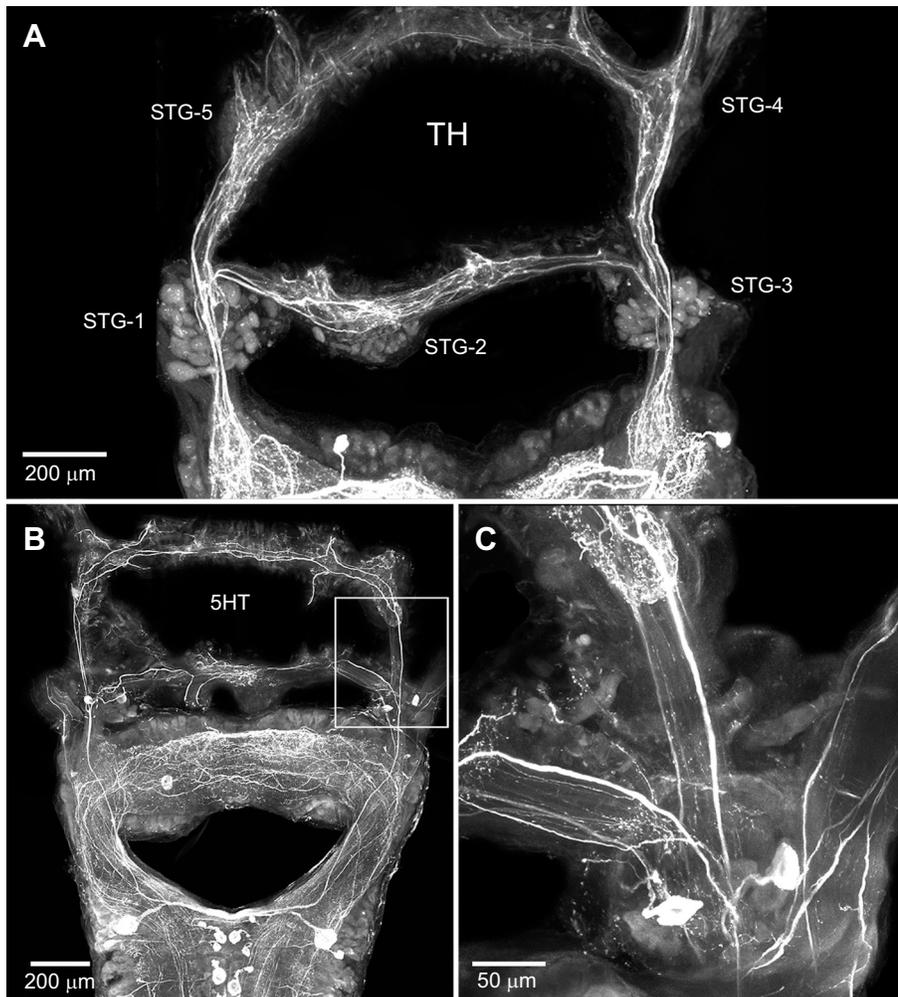


Fig. 4. Immunolabeling of the STN and cephalic ganglia with antisera raised to detect dopaminergic and serotonergic neurons.

(A) Confocal micrograph of the STN and anterior portion of the supraesophageal ganglion labeled with an anti-tyrosine hydroxylase (TH) antibody, which recognizes the rate-limiting synthetic enzyme for dopamine. A rich supply of TH-immunoreactive (ir) processes can be observed ramifying throughout all five of the STGs and throughout all of their interconnecting nerves and commissures. No TH-ir somata, however, were observed to be present in any of the STGs, only labeled fibers, which likely arose from the TH-ir somata present in the SEG. (B) Serotonin (5HT) immunolabeling of cells and arborizations in the STN, supraesophageal ganglion and SEG. The known serotonergic LL cells are clearly visible in the SEG, as are the medial Retzius neurons. The drawn box outlines STG-3, which houses two 5HT-ir somata. Note that 5HT-ir fibers are present in the fine nerves exiting STG-2, STG-4 and STG-5, nerves that innervate the jaw musculature. (C) Higher-magnification view of the STG-3 shown within the box in panel B. In STG-3 (and STG-1), two 5HT-ir somata are revealed. These monopolar serotonergic neurons send their processes to STG-2; resolution of other projections was inconclusive.

consumes its blood meal. In insects and crustaceans, for example, the ganglia associated with the STN are important not only for generating the rhythmic motor patterns associated with mastication and swallowing (foregut-related activity), but for their inter-coordination and communication with higher brain centers (Bräunig, 1990; Miles and Booker, 1998; Ayali et al., 2002; Marder and Bucher, 2007).

In 1948, Ruth Hanke published the first and only report, to date, that has formally described the location and arrangement of the STGs in the leech (Hanke, 1948). She compared two species of Hirudinea, *Hirudo* and *Haemopis*, and provided a hand-drawn illustration of the cephalic nerve ring observed in each species. For the jaw-less *Haemopis*, a nerve ring was detected, but no notable ganglia within the ring were mentioned. In contrast, in the jawed *Hirudo*, Hanke illustrated three ganglia associated with the nerve ring and included each ganglion's nerve roots, which she found to project to each one of the leech's three jaws, as did we. This species-comparative aspect of Hanke's work supports a potential link of the STGs with the regulation of the jaws and thus biting. However, only three ganglia were visible, not the five that we have described here. Based on our current nomenclature, she omitted STG-1 and STG-3 (Figs 1 and 2), which would have been difficult to visualize with the histological methods and lower microscope magnifications (10 \times) used back in that day. Not until decades later was Hanke's study recognized in the biology community by Sawyer (1986), who re-published the original stomatogastric illustrations.

Feeding behavior and the potential role of the STN

Much of the pioneering research on leech feeding behavior and its neural correlates were conducted by Charles Lent and his colleagues in the 1980s and 1990s, with a bulk of the work conducted prior to Sawyer's (1986) re-reporting of the STN. Studies of the large serotonergic LL cell, in particular, played a key role in how Lent and others viewed how feeding-related movements were initiated, executed and modulated. Unfortunately, Lent and his group (Lent and Dickinson, 1984, 1987; Lent et al., 1991) mistakenly concluded that the LL cell projected out the cerebrobuccal nerve to innervate the muscles of the jaws and pharynx directly. We have shown in this study, however, that the LL cell projects directly to the ganglia of the STN, and not to the periphery via any nerve roots. We used serotonin immunolabeling, Neurobiotin intracellular cell labeling and electrophysiological recordings to substantiate this conclusion (Figs 4B, 5). It is understandable, however, that the reason why Lent and his colleagues had concluded that the LL cell projected out one of the cephalic nerves was as follows: when the nerve ring is inadvertently cut during dissection, the stomatogastric commissures often become draped along the lateral edge of the supraesophageal ganglion and any stained LL axon would appear to exit in what would be perceived to be a peripheral nerve root.

In our current study, we have revealed that the two serotonergic LL cells are synchronously active by way of electrical coupling, and often display rhythmic bursting activity (Fig. 7). Furthermore, we have demonstrated that blood serum, a natural stimulus present in

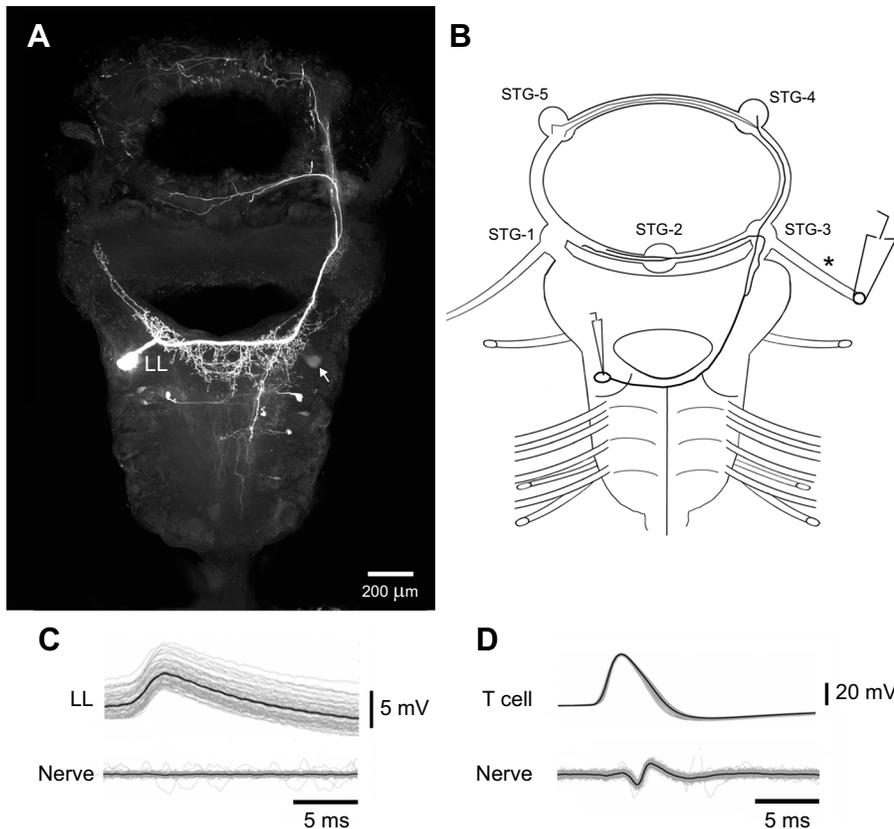


Fig. 5. Complete morphology of the serotonergic LL cell and verification that it projects to the STN only, not to the peripheral musculature of the jaws and pharynx.

(A) Intracellular iontophoretic injection of Neurobiotin into a single LL cell. The brightly labeled LL soma (left) is seen sending its axons contralaterally to eventually ascend through the supraesophageal ganglion and into all of the STGs but for STG-1. Note that there is no anatomical evidence that the axonal projections exit out the STG-3-associated nerve root. The arrow points to the contralateral homolog of the LL cell, which is faintly labeled due to its dye coupling. A collection of very small neurons also show Neurobiotin dye coupling. (B) Schematic diagram outlining the branching pattern of the LL cell and the extracellular-recording site of the STG-3 nerve root (asterisk). (C) Electrophysiological verification that the axon of the LL cell does not project out the nerve root associated with STG-3. The spike in LL (intracellular recording, top trace) was never locked with the spike-triggered averages of extracellular potentials in the nerve (bottom traces). (D) In contrast to panel C, spikes evoked in the touch sensory cell (T cell), known to project out the STG-3 nerve, were always phase-locked to the extracellular action potential in that nerve, thus substantiating that the recording configuration was adequate to test nerve root sensitivity to evoked spikes in the SEG. The soma of the recorded T cell was located in neuromere 1 of the SEG, close to the arrow in panel A.

the leech's mammalian meal, causes an increase in the firing frequency of the LL cell (Fig. 6) upon its delivery to the lip. These results reconfirm the role of the LL in feeding-related processes (Lent and Dickinson, 1984). However, in contrast to previous reports, we support the idea that the LL-induced pharyngeal muscle contractions and jaw movements that others have observed (Lent, 1985) are not a direct consequence of LL activity acting directly on the periphery. Possibly, the coupled LL cells act as a decision-making or arousal element for biting and pharyngeal peristalsis, acting on the circuitry within the STN. Additional support for this idea comes from a study by O'Gara et al. (1999), wherein they found another feeding-related interneuron in the SEG – neighboring the LL cell – that was shown to induce what was described as pharyngeal swallowing behavior. This cell, named SW-1, was found to be FMRFamide (Phe-Met-Arg-Phe)-like immunoreactive. Most relevant to the current study, however, was their mention that SW-1 projected through the stomatogastric (or stomatodael) nerve and on to what was briefly mentioned as a 'pharyngeal ganglion', with a rare reference to Hanke's (1948) paper; unfortunately, no further details of what constituted a pharyngeal ganglion was mentioned. This group also reported that at no time were they able to observe a 1:1 correlation between the action potentials recorded in the SW-1 cell and the excitatory postsynaptic potential (EPSPs) obtained in the pharyngeal muscle cells. Thus, they concluded that the effects of SW-1 were likely indirect, and put forth the idea that the SW-1 neuron functions as an upstream 'command element' for pharyngeal muscle control. Although SW-1 was not confirmed to be both necessary and sufficient for swallowing, their ideas match with ours that the LL initiates biting and pharyngeal motor activity, presumably, by acting on pattern-generating circuits residing in the STN. Although direct confirmation is needed, we propose that the

rhythmic movements of the three jaws and their associated chitinous teeth are produced by central pattern generators within STG-2, STG-4 and STG-5; nerves emanating from each of these ganglia likely contain the axons of motor neurons and modulatory fibers. Neurons residing in STG-1 and STG-3 possibly participate in the generation of pharyngeal peristalsis and its coordination with biting.

In addition to establishing that the SW-1 cell was FMRFamide immunopositive, O'Gara et al. (1999) reported that about half of the cells in one of the 'pharyngeal' ganglia was FMRFamide immunoreactive. Although no details were provided about which of the STGs were studied, based on the sizes of the labeled neurons reported (ca. 40–50 μm) and the number of total cells found within a given ganglion (ca. 20), one can safely conclude that the ganglia in question were STG-1 and STG-3. They also mentioned that some of the processes emanating from the FMRFamide-immunolabeled cells projected back into the CNS (O'Gara et al., 1999), a similar trajectory pattern to what we observed for one of the STG-1 neurons that we labeled with Neurobiotin (Fig. 3C). Because the study of O'Gara et al. (1999) did not provide photomicrographs of FMRFamide-immunolabeled neuronal projections emanating from somata intrinsic to the STGs, we were unable to make any further comparisons.

The STN and the modulatory roles of the biogenic amines

Our current studies provide a framework for subsequent studies that can now expand upon the potential motor and modulatory roles of the STN. As with other STNs in crustaceans, insects and oligochaetes (Beltz et al., 1984; Klemm et al., 1986; Barna et al., 2001; Pulver et al., 2003), we observed the STN in the leech to be enriched with dopaminergic and serotonergic fibers. Although a dense array of dopaminergic fibers was present in all five STGs and their commissures (Fig. 4), no TH-ir somata were detected. In

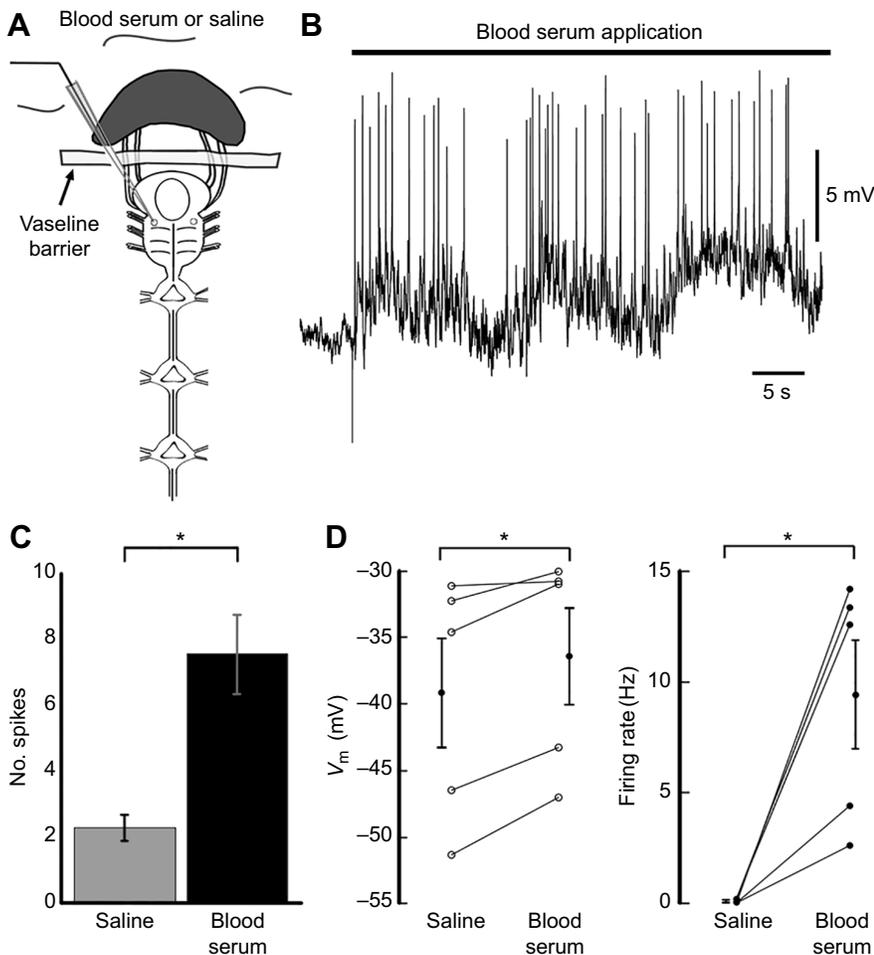


Fig. 6. Use of a semi-intact lip and cephalic ganglia preparation to establish whether the LL cells, which communicate with the STN, are themselves sensitive to feeding-related stimuli in the environment. (A) Diagram of the semi-intact lip and CNS preparation that enabled the lip regions of the leech (shaded area) to be stimulated with a blood serum while simultaneously recording from the LL cells in the SEG. Note that a Vaseline barrier was used so that there was no direct contact of the blood serum with the CNS. (B) Sample electrophysiological recording showing that blood serum application to the dorsal lip caused excitation in the LL cells; in contrast, control saline application did not increase the firing rate. (C) Histogram showing the increase in the number of LL spikes (within 10 s intervals) after application of blood serum as compared with saline ($n=5$ animals). LL neurons were quiet prior to serum application and increased their firing rate with a variable delay. (D) LL neurons depolarized an average of 2.7 mV during blood serum application (left panel) and increased their firing rate from 0.08 to 9.44 Hz (right panel). V_m , membrane potential. * $P<0.05$. Error bars are \pm s.e.m.

contrast, we observed two serotonin-ir neurons in STG-1 and STG-3 (Fig. 4C), which appeared to project to neighboring ganglia in the STN. In some respects, the limited number and distributions of aminergic somata in the leech appear to be most aligned with that of the four STGs in the decapod crustaceans versus the STNs of insects; for example, the adult lobster STN is richly invested with dopaminergic fibers despite the STG having at most one intrinsic dopaminergic neuron and the commissural ganglia possessing 3–4 dopaminergic cells (Pulver et al., 2003). In the lobster, during embryonic development, the large dopaminergic L cell migrates from the cephalic ganglion and into the commissural ganglia. In the adult leech, however, the brain and the large paired dopaminergic neurons it houses (depicted in Fig. 4A, bottom) remains in close proximity to the STN, possibly precluding the need for dopaminergic neurons to develop in the STN.

Although a number of serotonin-ir fibers can be observed exiting the nerves of STG-2, STG-4 and STG-5 to the jaws and possibly pharynx (Fig. 4B), and serotonergic fibers are known to innervate the jaw musculature (Crisp and Mesce, 2006), we cannot conclude with certainty whether these processes emanate from somata within the STN and/or from the SEG. Regardless, identifying a new group of serotonergic neurons originating in the STN is, indeed, notable because this neurotransmitter, neuromodulator and neurohormone is inextricably linked to events involved in the feeding process across a variety of invertebrate and vertebrate animals (Klemm et al., 1986; Lange et al., 1989; Lent et al., 1989; Gillette, 2006; Voigt and Fink, 2015; Okumura et al., 2017; Gaudry and Kristan, 2009).

Serotonin and the neurons that release it contribute to a plethora of events that, at first glance, might seem unrelated or even incompatible, but ultimately facilitate the achievement of a singular goal – to ingest a blood meal (Lent and Dickinson, 1988). The act of feeding itself, although intermittent, trumps all other behaviors, and thus has served as an excellent model of behavioral choice (Gaudry and Kristan, 2009, 2012). During a singular feeding, the medicinal leech increases its body size by 10-fold or more, and thus a single meal can sustain a leech for 6 months to a year (Dickinson and Lent, 1984; Lent et al., 1989). Hungry leeches are known to have elevated levels of serotonin, and become highly responsive to the visual, mechanosensory, thermal and chemical cues associated with its mammalian prey and blood (Dickinson and Lent, 1984). For example, the serotonergic LL cell that we recorded from here was significantly excited when blood serum was applied to the lip of the leech (Fig. 6). Upon contact with a warm substrate, a rise in serotonin also helps to link the CNS with actions at the periphery; for example, the excitation of salivary glands (Marshall and Lent, 1988). Although serotonin is typically associated with the activation of swimming (Willard, 1981), and both the serotonergic LL and Retzius neurons (Groome et al., 1995) are excited during feeding, swim activation would be highly disadvantageous during biting and blood ingestion. A solution to this apparent paradox comes from the work of Crisp and Mesce (2006), who showed that serotonin applied exclusively to the region of the compound cephalic ganglion resulted in an inhibition of fictive swimming. In addition, Gaudry and Kristan (2009) have shown that, during feeding, and similarly

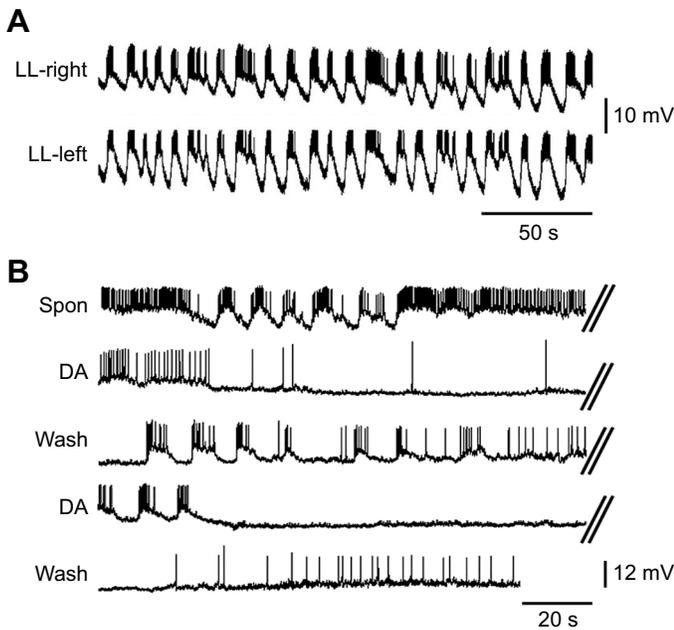


Fig. 7. Synchronization of LL cell activity and its modulation by dopamine (DA). (A) Simultaneous intracellular recordings of both left and right LL cells in the SEG. These electrophysiological traces showcase not only the synchronicity of firing across the two cells, but indicate that the LL cells are capable of patterned activity in the form of fairly regular bursting activity that can last for minutes to over an hour ($n=6$ animals). The activity shown here was induced by application of collagenase–dispase (2 mg ml^{-1}) applied locally to the SEG for ca. 7 min and then washed out; rhythmic bursting typically began within 15 min after application. (B) Spontaneous activity (Spon) in the LL cells is reversibly inhibited by $100 \mu\text{mol l}^{-1}$ DA bath application. In 100% of preparations tested ($n=6$; minimum of two reversal washes per preparation), DA caused either a complete cessation of all firing activity or a maximum of 1–2 spikes during the 30 s period prior to washout.

upon bath application of serotonin to the CNS, the EPSP amplitudes of the pressure-sensitive mechanosensory neurons (P-cells) are reduced; this reduction then prevents tactile stimulation across the body from activating local bend responses and P-cell-activated swimming behavior. In parallel with these events, Gaudry and Kristan (2010) demonstrated that a distension of the body wall, presumed to activate body-wall stretch receptors, caused a robust termination or decline in swim maintenance. Curiously, during and after feeding, leeches have been reported to crawl. This particular form of locomotion has been shown previously to be activated by dopamine (Puhl and Mesce, 2008), which we observed to inhibit the spontaneous firing of the serotonergic LL neuron (Fig. 7B). Furthermore, we observed here that numerous dopaminergic arborizations were present within the STN (Fig. 4A), in addition to their extensive ramifications throughout the cephalic ganglia (Fig. 4A; Crisp et al., 2002). We have shown previously that dopamine application to the CNS serves as a potent blocker of swimming, even when electrical swim-inducing stimuli are provided (Crisp and Mesce, 2004). Thus, the data presented in this study support the potential role of dopamine as a neuromodulator that counters or limits the serotonergic-mediated actions that maintain the serotonin-biased feeding state, and restores locomotion in the form of crawling, but not swimming. Possibly, an early uptick in the levels of dopamine during host location could act eventually to help coordinate both a switch in locomotor state (Mesce and Pierce-Shimomura, 2010) and the eventual cessation of pharyngeal pumping by way of LL inhibition (Fig. 7B). Based on the rich

dopaminergic investiture in the STN and the cephalic CNS, we propose that both dopamine and serotonin play significant and orchestral roles in coordinating feeding-related activities.

In retrospect, it is quite remarkable that the leech STN has been overlooked for so long, especially because the nervous system of the leech has been scrutinized for many decades, serving as one of the best-studied invertebrate preparations to elucidate the neural underpinnings of behavior. Incorporating our new information, studies of the STN promise to provide fertile ground in which to explore the regulation, modulation and generation of rhythmic feeding movements associated with the jaws and pharynx, and may help to reveal how feeding is coordinated with other behavioral routines. Because the annelid leech and molluscs share the same phylogenetic clade, Lophotrochozoa, characterizing the similarities and differences between the molluscan buccal ganglia and the leech STN may be fruitful. For example, might we identify an evolutionary relationship between the functionally similar serotonergic LL cells in the leech and the large feeding-inducing serotonergic neurons found in the cerebral ganglia across numerous molluscan species (Weiss and Kupfermann, 1976)? Adding an experimentally accessible annelid animal preparation to comparative studies of STN networks may help to illuminate how evolutionary and developmental factors shape the structural and functional architecture of neural centers that govern feeding-associated movements (Ayali, 2009), including those in mammals (Lund, 1991).

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.A.M., M.A., Q.G., J.G.P.; Methodology: K.A.M., M.A., Q.G., J.G.P.; Validation: K.A.M., Q.G., J.G.P.; Formal analysis: K.A.M., M.A., Q.G., J.G.P.; Investigation: K.A.M., M.A., Q.G., J.G.P.; Resources: K.A.M.; Data curation: K.A.M., J.G.P.; Writing - original draft: K.A.M.; Writing - review & editing: K.A.M., M.A., Q.G., J.G.P.; Visualization: K.A.M., Q.G., J.G.P.; Supervision: K.A.M., J.G.P.; Project administration: K.A.M.; Funding acquisition: K.A.M., M.A.

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