Serotonergic pathways in the Drosophila larval enteric nervous system

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1. Introduction

Feeding requires coordination of different parts of the body. In many invertebrates and vertebrates, a distinct part of the peripheral nervous system, the enteric nervous system (ENS), exists to regulate specific phases of feeding (Gershon, 2008; Penzlin, 1985; Selverston and Moulins, 1987). In insects, the nerves comprising the ENS were shown to interconnect the central nervous system (CNS) with the neuroendocrine organs and foregut structures (Kirby et al., 1984; Willey, 1961). Physiologically the ENS has been shown to be important for a wide range of feeding and metabolic related processes (Penzlin, 1985). What has been generally lacking in these studies, however, is the cellular resolution and the projection patterns of identified neurons that comprise the ENS.

Genetic tools available in Drosophila melanogaster provide an opportunity to complement and extend the analysis of the ENS at a cellular and axonal level. Genetic approaches have already been used in the analysis of ENS and foregut development during embryogenesis (Gonzalez-Gaitan and Jäckle, 1995; Pankratz and Hoch, 1995). Subsequently, the basic neuroanatomy of the ENS in association with the foregut structures have been characterized in the Diptera larva (Spieß et al., 2008). The Drosophila larval ENS shows similar overall structure as other insects. The ENS is connected to the CNS through the antennal nerve (AN). The bilateral AN fuses with the frontal nerve junction via the frontal connectives, which bifurcates into the anteriorly projecting frontal nerve (FN) and the posteriorly projecting recurrent nerve (or nervus recurrens, RN). The FN innervates the pharyngeal muscles, whereas the RN innervates the esophagus. The RN is connected to the proventricular nerve (PVN) by the hypocerebral ganglion (HCG), from which a separate nerve (NCS: nervi cardiostomatogastrici) branches out to innervate the ring gland (RG). One notable difference between Drosophila larval ENS and most other insect studied is that Drosophila larva lack a frontal ganglion (FG). Instead, it is replaced by a nerve junction devoid of neurons, and the motor neurons that are located in FG in most insects are presumably found in the brain (Spieß et al., 2008). The most posterior component of the ENS is the proventricular ganglion (PVG), which innervates the proventriculus (PV), a valve-like organ which gates the passage of food from the esophagus to the midgut.

Here we identify a cluster of four serotonergic cells that innervates all the major target organs of the ENS, including the pharynx, esophagus, proventriculus and the ring gland. Their cell bodies are located in the brain and send their axons through all the nerves of the ENS. Calcium imaging and electrophysiological studies suggest their role in regulating gut movement.

2. Materials and methods

2.1. Flies

The following Gal4 driver and UAS effector lines were used: TRH-Gal4 (Alekseyenko et al., 2010), UAS-dTrpA1 (Bloomington #26263), UAS-eYFP (Bloomington #6659), UAS-H134R-ChR2-mCherry (Bloomington #28995), UAS-GCaMP3 attp2 (Bloomington #32236) and UAS-10X-mCD8-GFP (Bloomington #32184). In control experiments OregonR (wildtype) was used. For ChR2 experiments a stable homozygous line of TRH-Gal4 and UAS-H134R-ChR2-mCherry was generated.

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Adult flies and larvae were reared on standard fly-food and kept at 25°C. 4 h egg collections were made on apple juice-agar plates with yeast-water paste. After 48 h, hatched larvae were transferred into vials containing standard fly food. For ChR2 experiments, 48 h old larvae were transferred into vials containing 100 µM all-trans retinal (ATR). Vials with retinal were darkened with aluminum foil to protect the retinal from degradation. After additional 48 h, larvae were used for experiments.

All experiments were performed with third instar larvae 98 ± 2 h AEL (after egg laying).

2.2. Immunohistochemistry

Dissected larval brains of third instar larvae were fixed in paraformaldehyde (4%). For the antibody staining of TRH > 10X-mCD8-GFP, a FITC conjugated goat anti-GFP antibody (1:500, Abcam plc) was used. The simultaneous antibody staining of serotonin, primary antibody was rabbit anti-5HT (1:1000, Sigma–Aldrich) and secondary antibody was anti-rabbit Alexa568 (1:200, Invitrogen).

For immunofluorescence staining of serotonin and elav, we used third instar larvae of OregonR (wildtype). The primary antibodies were rabbit anti-5HT (1:1000, Sigma–Aldrich) and rat anti-elav (1:500, DSHB). Secondary antibody was anti-rabbit Alexa488 (1:200, Invitrogen) and anti-rat Alexa568 (1:200, Invitrogen). Nuclei were counter stained with DAPI. Labeled larval brains were mounted in Moviol. Imaging was carried out using Laser Scanning Microscope (ZEISS LSM780). The obtained images were arranged using Zen LE and Corel DrawX5 (for detailed staining procedures see (Bader et al., 2007)).

2.3. Fluorescence microscopy

All images were obtained by using a confocal microscope Zeiss LSM 780. Images were acquired using Zen 2011 (Zeiss).

2.4. Calcium imaging

For calcium imaging the genotype: TRH > GCaMP3 was used. Neural activity of neurons in the CNS was studied in isolated CNS of third instar larvae. Calcium imaging was performed with a Laser Scanning Microscope (ZEISS LSM780) using a ZEISS LCI “Plan-Neofluar” 25×/0.8 Imm Korr DIC M27 objective dipped into the saline solution. For excitation an argon laser with a wavelength of 488 nm was used. Images were acquired using Zen 2011 (Zeiss). Data was analyzed using custom made script for Fiji (ImageJ).

2.5. Electrophysiology

For the electrophysiological experiments, a reduced semi-intact preparations were made of TRH-Gal4/TRH-Gal4; UAS-H134R-ChR2-mCherry/UAS-H134R-ChR2-mCherry larva consisting of the CNS, cephalopharyngeal skeleton (CPS), antennal nerve and the enteric nervous system with the innervation targets (ring gland and proventriculus). Detailed description of the dissection has been described earlier (Schoofs et al., 2010). All dissections and

Fig. 1. Four serotonergic neurons projecting through antennal nerve. (A) Overview of larval CNS (third instar) showing the expression pattern TRH > eYFP, SOG marked by dashed lines, arrows indicate the serotonergic cell cluster, (B and C) images of the larval subesophageal ganglion (TRH > eYFP) revealing a cluster four cells whose axons project through the antennal nerve; inlaid image shows the four cells by red numbers in the SOG region marked by dashed box, (D–F) 5HT antibody staining of TRH > eYFP: fluorescence expression driven by TRH-Gal4 (E), cells (3–4) and arborizations labeled by 5HT antibody (F), merge of E and F (D). Abbr.: AN, antennal nerve; H, brain hemispheres; SOG, subesophageal ganglion.
Experiments were performed in saline solution composed of (in mM): 140 NaCl, 3 KCl, 2 CaCl$_2$, 4 MgCl$_2$, 10 sucrose and 5 HEPES (Rohrbough and Broadie, 2002). Neural activity was measured by *en passant* extracellular recording of the respective nerves with a preamplifier connected to a four channel amplifier/signal conditioner (Model MA 102/103; Ansgar Büschges group electronics lab, University of Cologne). For *en passant* extracellular recording, the nerve was insulated with a surrounding petroleum jelly border on a piece of Parafilm. Recording electrodes were made of silver wire (diameter: 25–125 μm, Goodfellow). All recorded signals were amplified (amplification factor: 5000) and filtered (bandpass: 0.1–3 kHz). The recordings were sampled at 20 kHz. Data was acquired with Micro3 1401 or Power 1401 mk2 A/D board (Cambridge Electronic Design) and Spike2 software (Cambridge Electronic Design). Analysis of the double recordings was performed with the Spike2 analysis tools.

![Diagram of the enteric nervous system](image)

**Fig. 2.** Serotonergic projection of the enteric nervous system. (A) Schematic drawing of the larval enteric nervous system and associated foregut and midgut structures, (B) overview of a dissected third instar larva showing the fluorescence expression driven by TRH-Gal4 in the enteric nervous system, (C) magnification of the posterior portion of the pharynx and anterior part of esophagus; note that axons of the antennal nerve cross the frontal nerve junction and project to frontal nerve and recurrent nerve, (D) innervation of the ring gland via nervus cardio stomatogastricus; note the projections on the ring gland and aorta, (E) focus on hypocerebral ganglion; the projections pass the hypocerebral ganglion to proventricular nerve and nervus cardio stomatogastricus, (F) focus on the proventricular ganglion; note the projection to the proventriculus which extend into the midgut (H), (G) double antibody staining of elav and 5-HT indicating neurons in the hypocerebral ganglion (14–15 somata, n = 3) and a 5-HT positive neural plexus, (I) double antibody staining of elav and 5-HT revealing neurons in the proventricular ganglion (9–13 somata, n = 3) and 5-HT positive projections onto the proventriculus, (J) schematic drawing 5-HT positive somata which send out projections through the enteric nervous system and larval endocrine organ. Abbr.: AN, antennal nerve; A, aorta; CNS, central nervous system; DO, dorsal organ; E, esophagus; FN, frontal nerve; FNJ, frontal nerve junction; HCG, hypocerebral ganglion; MG, midgut; NCS, nervi cardio stomatogastrici; P, pharynx; PV, proventriculus; PVG, proventricular ganglion; PVN, proventricular nerve; RG, ring gland; RN, recurrent nerve (Scale bars: B – 200 μm; F, H – 50 μm; D, E – 20 μm G, I – 10 μm).
For the light stimulation during extracellular recordings, a mounted ultrabright blue LED with collimated lens and heatsink with a wavelength of 470 nm (M470L2, Thorlabs) was used for all ChR2-experiments. LED was regulated via the A/D board which was connected to voltage-controlled LED power supply (LED1B, Thorlabs). LED was mounted on a custom built LED holder. Optical multimode fiber (AFS200/220Y, Thorlabs) was placed into the LED holder in front of the LED. Distal end of the optical fiber was then placed directly over the ventral side of CNS. Timing of the stimulus was given through stimulus protocols in Spike2 software. All stimuli were applied with the highest intensity (1 mA). Optical fiber had a length of approx. 90 cm with a transmission efficiency of >99.8%/m. All experiments with ChR2 were done in dark conditions.

2.6. Behavioral experiments

Movements of the esophagus, proventriculus and midgut were studied in semi-intact larvae. The preparation consisted of the CNS and associated enteric nervous system, CPS, foregut and midgut. Thermal stimulation was applied directly to the CNS of TRH > dTrpA1 and OrgeonR (control) crossed to UAS-dTrpA1 or TRH-Gal4.

For the temperature stimulation, a custom-made stimulator consisted of a silver wire (diameter: 4 mm) which was attached to a Peltier element by thermally conductive adhesive. Peltier element was driven by a voltage-regulated power supply (VSP 2405,Voltcraft) connected to an A/D board. End of the thermal stimulator was filed to a tip and insulated with nail polish. Applied temperature was measured by digital thermometer (GMH 3210, Greisinger electronic). Sensor for the thermometer was placed 5 mm from the tip of the thermal stimulator. Before the experiments the thermal stimulator was calibrated to ensure constant thermal stimuli to the CNS. Temperature signals were acquired with the A/D board. Thermal stimulator was regulated by a script-based feedback loop via the A/D-board.

Consecutive videos of 5 min at 18 °C and 5 min at 32 °C were recorded using a digital camera (Quickcam 9000 Pro, Logitech) mounted on a binocular (Stemi 2000-CS, Zeiss). Movements of esophagus, proventriculus and midgut were counted at 18 °C and 32 °C. The measurements were performed using the software ImageJ (Fiji). All behavioral experiments were tested for significance with the Mann–Whitney-Rank-Sum-test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

3. Results and discussion

3.1. Four central serotonergic neurons innervate the entire enteric nervous system

During our analysis on the role of the central neurons in regulating Drosophila larval feeding behavior (Schoofs et al., 2014; accepted for publication), we noticed that a small cluster of serotonergic cells located in the subesophageal ganglion (SOG) sent their axons out to the periphery through the AN (Fig. 1). This was visualized by using TRH-Gal4 line to drive expression of eYFP (n = 10, Fig. 1A–C). The soma of these four serotonergic cells are located near the point where AN extends out from the CNS (Fig. 1B and C). Staining with antibodies to serotonin revealed three to four labeled cells (n = 3, Fig. 1D–F).

The projections from these cells extend throughout the ENS (Fig. 2A and B). They project anteriorly to the pharyngeal muscles as part of the FN, as well as posteriorly along the esophagus as part of the RN (Fig. 2C). Branching of the projections at the HCG can also be seen (Fig. 2E), as well as its innervation of the ring gland via the NCS (Fig. 2D) and proventriculus via the PVN (Fig. 2F). The projection ends at the anterior region of the midgut (Fig. 2H). We could not detect any serotonergic positive cell bodies within HCG or PVG (Fig. 2G and I; based on stainings with antibody to serotonin and additionally elav, which labels post-mitotic neurons, we counted approximately 14–15 cells in the HCG, and 9–13 cells in the PVG; n = 3 in both cases). These results indicated that the
serotonergic projections within the entire ENS derive from the cluster of four cells located in the brain. (See Fig. 2J for scheme).

3.2. Electrophysiological analysis of serotonergic pathways in the ENS

Based on the anatomical analysis, we next investigated whether the different branches of the serotonergic projections from the CNS were functionally connected. The experimental strategy was to activate the central serotonergic neurons and perform double extracellular recordings from the different branches. Therefore, we performed double recordings of AN and RN (Fig. 3A), AN and NCS (Fig. 3B) and AN and PVN (Fig. 3C). For technical reasons, we could not record from the frontal nerve. Neuronal activation was achieved with light after expressing Channelrhodopsin (ChR) in the serotonergic cells (Nagel et al., 2003; Pulver et al., 2009; Schroll et al., 2006). We could detect a neuronal unit in the recordings for all cases (Fig. 3). The unit could be temporally correlated with the onset of the light stimulus, supporting the view that the serotonergic axons of the AN are functionally connected to those of RN, NCS and PVN (Fig. 3).

3.3. Calcium imaging of serotonergic AN axons

We then wanted to monitor the activity of these neurons using the genetically encoded calcium indicator, GCaMP3 (Tian et al., 2009). Therefore, we expressed GCaMP3 in serotonergic cells and recorded from the axons of the AN (Fig. 4). To quantify the recordings, we measured the $\Delta F/F$-peak frequency. Due to the low temporal resolution of the GCaMP3, a detected peak can reflect one action potential or up to series of action potentials. Two types of activity patterns were observed, a slower acting (Fig. 4A and B) and a faster acting (Fig. 4C and D). However, these patterns were not observed in the majority of the larvae. Only 16 out of 34 GCaMP3-recordings exhibited activity patterns. The slow acting pattern showed $\Delta F/F$-peak frequency range of 0.003–0.028 Hz (occurred in 24% of the total recordings), whereas the fast acting pattern had a $\Delta F/F$-peak frequency range of 0.04–0.15 Hz (detected in 18% of the total recordings). As the recordings are performed over a maximum time span of about 10 min, the low frequency may reflect a slower acting signal that modulates post-ingestive movements.

Fig. 4. GCamp analysis of TRH positive projections leaving the CNS via the antennal nerve. (A, C) Thermal color representation of calcium activity in projections of TRH positive neurons leaving the CNS via the AN in inactive (left) and active (right) state. Region of interest is marked as rectangular field. The activity of the whole axon bundle is shown in (A). The activity of a single axon is shown in (C). (B, D) Fluorescence change plotted as delta $F/F$ in a 600 s time period. Red arrows mark the time points seen in the pictures below. Analysis of the axon bundle is shown in (B), whereas the analysis of a single axon is shown in (D). Abbr.: AN, antennal nerve. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3.4. Central serotonergic regulation of esophagus movements

To determine if serotonergic neurons in the CNS, in particular the four cell cluster projecting within the ENS, modulated post-ingestive movements associated with passage of food through the esophagus and the proventriculus, we activated these central serotonergic neurons and monitored the movements of three different gut structures: esophagus, proventriculus and midgut. For this, we used the temperature dependent dTrpA1 activation (Pulver et al., 2009) of serotonergic neurons (Fig. 5A). Via the Gal4-UAS system we directed the expression of dTrpA1 to the central serotonergic system. This temperature- and voltage-gated channel induces a cation influx at a restrictive temperature of above 27°C, which can be used to remotely activate specific neuronal circuits. In our experiments we measured the fold change in movements per min between 18°C (inactivated dTrpA1 state) and 32°C (activated dTrpA1 state).

The esophagus showed two types of peristaltic waves which propagate from anterior to posterior: (1) a circular, local relaxation traveling over the contracted esophagus and (2) a local contraction propagating over the relaxed esophagus. For the proventriculus, we observed a ring-like contraction restricted to the anterior part; midgut showed local squeezing and bidirectional peristaltic waves. (C) Bar graph represents the fold change in movements/min between 18°C and 32°C for OregonR crossed with TRH/dTrpA1 and TRH > dTrpA1, result exhibit a significant increase of the esophagus movements during TrpA-induced activation of central serotonergic neurons, but not of the proventriculus and midgut.

Taken together, our anatomical and physiological analyses suggest that a four-cell cluster of serotonergic cells in the brain

**Fig. 5.** Activation of central serotonergic neurons affects esophagus and proventriculus movements. (A) Experimental setup: semi-intact larvae of OregonR crossed with TRH/dTrpA1 and TRH > dTrpA1 with exposed foregut and midgut were video-recorded to monitor the movements of the esophagus, proventriculus and midgut at 18°C and 32°C, note temperature stimulus was applied to CNS, (B) esophagus showed local extension and local contraction propagating from anterior to posterior; proventriculus showed ring-like contraction restricted to the anterior part; midgut showed local squeezing and bidirectional peristaltic waves, (C) Bar graph represents the fold change in movements/min between 18°C and 32°C for OregonR crossed with TRH/dTrpA1 and TRH > dTrpA1, result exhibit a significant increase of the esophagus movements during TrpA-induced activation of central serotonergic neurons, but not of the proventriculus and midgut.
innervates the entire ENS and may be involved in coordinating motor and endocrine activities of the foregut.

3.5. Brain–gut neural pathways

Our results indicate that the four-serotonergic neurons play a special role by acting as a conduit through which the brain communicates with the gut. At this point, we cannot exclude the possibility that other serotonergic neurons within the CNS participate in the modulation of post-ingestive movements. The uniqueness of these cells can be seen in the context of the different serotonergic neurons that have been analyzed in previous studies (Huser et al., 2012; Neckameyer, 2010; Vallés and White, 1988): of all the serotonergic neurons in the brain, this cluster is the only one that leaves the CNS and extends into the gut. Furthermore, we consistently observed only two of the four axons showing rhythmic activity in the calcium imaging studies, suggesting that there may be further functional subdivision within this cluster (we have termed these neurons as SEA neurons, for serotonergic-antennal; some of these may correspond to the SEO neurons of Huser et al., 2012). Based on the innervation of the ring gland by the SEA neurons, a possible function is in regulating hormonal release in response to nutrient or digestive cues. These neurons may also be analogous to the “SNS neurons” (satellite nervous neurons) in the locust (Schachtner and Bräunig, 1993), which are serotonergic neurons in the SOG that project out into the periphery.

In vertebrates, there is a major nerve that also connects the brain to the gut, namely the vagus nerve (Travaglì et al., 2006). The vagus nerve projects from the brainstem to innervate the pharynx, the stomach and the midgut. It also innervates the pancreas. In Drosophila, the nerves that extend from the brain to the periphery have different names (AN, FN, RN, NCS and PVN); however, the projections of the SEA cluster of serotonergic neurons illustrate that there is a direct projection from the brain to the different foregut structures. Thus, the projections of the AN to the gut may be functionally analogous to the vagus nerve of vertebrates (Fig. 6).

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