Characterization of the octopaminergic and tyraminergic neurons in the central brain of *Drosophila* larvae

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Running head: the larval octopaminergic/tyraminergic system

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Abstract

Drosophila larvae are able to evaluate sensory information based on prior experience, similar to adult flies, other insect species and vertebrates. Larvae and adult flies can be taught to associate odor stimuli with sugar reward and prior work has implicated both the octopaminergic and dopaminergic modulatory systems in reinforcement signaling. Here we use genetics to analyze the anatomy, up to the single-cell level, of the octopaminergic/tyraminergic system in the larval brain and suboesophageal ganglion. Genetic ablation of subsets of these neurons allowed us to determine their necessity for appetitive olfactory learning. These experiments reveal that a small subset of about 39 largely morphologically distinguishable octopaminergic/tyraminergic neurons is involved in signaling reward in the *Drosophila* larval brain. In addition to prior work on larval locomotion, these data functionally separate the octopaminergic/tyraminergic system into two sets of about 40 neurons. Those situated in the thoracic/abdominal ganglion are involved in larva locomotion, whereas the others in the suboesophageal ganglion and brain hemispheres mediate reward signaling.

Introduction

Drosophila larvae are able to evaluate sensory information from their environment based on prior experience (Apostolopoulou et al., 2013; Gerber and Stocker, 2007; Gerber et al., 2009; Michels et al., 2005). This allows them to select an appropriate behavioral response by assessing the consequences of its own action or an upcoming external event.

There is a convincing amount of data across diverse insect species demonstrating that the biogenic amines dopamine (DA) and octopamine/tyramine (OA/TA) are involved in processing specific aspects of reward (Burke et al., 2012; Honjo and Furukubo-Tokunaga, 2009; Liu et al., 2012; Schroll et al., 2006; Selcho et al., 2009; Unoki et al., 2006). In his classic experiment, Hammer (1993) had shown that in honeybees a single OA/TA neuron called VUM_{mx1} mediates reward during classical conditioning (Hammer, 1993). The role of OA/TA in reinforcement signaling was later confirmed by pharmacological and genetic interference in *Drosophila* larvae and flies, honeybees and crickets (Burke et al., 2012; Hammer and Menzel, 1998; Kim et al., 2013; Schroll et al., 2006; Schwaerzel et al., 2003; Unoki et al., 2005; 2006). Thus, the participation of the OA/TA system in appetitive reinforcement signaling in insectsis widely accepted.

Interestingly, several groups recently reported that sugar reinforcement includes at least two different componentsin *Drosophila*, sweet taste and nutrient value both contribute reinforcing value (Burke and Waddell, 2011; Fujita and Tanimura, 2011; Miyamoto et al., 2012; Rohwedder et al., 2012). Moreover, in flies non-nutritious sugars – like arabinose and xylose –form a less robust memory reinforced by OA (Burke and Waddell, 2011). Thus, OA seems to specifically signal the sweet component of the multi-factory sugar reward signal.Therefore, it was suggested that

OA/TA neurons involved in processing of sweet taste during conditioning, signal onto DA neurons which target the mushroom bodies (Burke et al., 2012). However, this was recently questioned as the alpha1-like octopamine receptor OAMB was shown to be sufficient for reward learning in α/β and γ mushroom body neurons indicating direct OA signaling onto the mushroom bodies (Kim et al., 2013). Nevertheless, it is clear that different functions of sugar reinforcement signaling in adult *Drosophila* depend on combined processing of the DA and OA/TA systems.

Immunohistochemical work showed that OA is mainly synthesized in unpaired median (UM) neurons of insects whose cell bodies are located either ventrally (VUM neurons) or dorsally (DUM neurons) in the suboesophageal ganglion (SOG) and ventral nerve cord (VNC)(Braunig and Burrows, 2004; Busch et al., 2009; Busch and Tanimoto, 2010; Cole et al., 2005; Monastirioti et al., 1995; Monastirioti et al., 1996; Nagaya et al., 2002; Selcho et al., 2012; Sinakevitch and Strausfeld, 2006; Vömel and Wegener, 2008). It was also reported that UM neurons of the thoracic ganglion send efferents to most organs and muscles, whereas those of the SOG broadly innervate nearly all parts of the brain (Braunig and Burrows, 2004; Busch et al., 2009; Selcho et al., 2012). The *Drosophila* larval CNS consists of only about 80 OA/TA neurons (Monastirioti et al., 1995; Selcho et al., 2012). Therefore, in terms of numbers the organization is simpler than the adult system that covers more than 100 OA/TA positive neurons in the brain hemispheres and SOG while not taking into account OA/TA positive neurons of the thoracic and abdominal ganglia (Sinakevitch and Strausfeld, 2006). In a prior study, the detailed single cell morphology for a set of about 40 OA/TA neurons having their cell bodies located in the thoracic and abdominal ganglion has been demonstrated (Selcho et al., 2012). These OA/TA neurons broadly innervate the muscles of their respective segment. Only larval

muscles 6, 7 and potentially 28 seem to lack OA/TA neuron arborizations (Hoang and Chiba, 2001; Monastirioti et al., 1995; Selcho et al., 2012). In the same study it was shown that neuronal output of these OA/TA neurons is important to trigger proper larval locomotion (Selcho et al., 2012).

Using a similar anatomical and functional approach we analyzed the role of the remaining set of about 39 OA/TA neurons in the brain hemispheres and SOG in more detail. First, we provide a comprehensive anatomical description of the OA/TA neurons on the single cell level. Thus – in combination with our earlier study on larval locomotion – the OA/TA system of the entire larval CNS is now comprehensively described on the single cell level. Second, we show that this set of OA/TA neurons is neither involved in locomotion, nor in odor and sugar sensation (Selcho et al., 2012). Moreover, we confirm the role of these neurons in sugar reward learning and potentially refine their role to non-nutritious sugar reward processing necessary for establishing appetitive memories.

Based on multiple roles of OA/TA proven in distinct insect systems, such as locomotion, learning and memory, stress induced behaviors or the regulation of the energy state, we allocate distinct functions to two different subsets within this multipart circuit that modulates specifically larval learning (Honjo and Furukubo-Tokunaga, 2009; Roeder, 2005; Saraswati et al., 2004; Schroll et al., 2006; Selcho et al., 2012). Thus, our findings might help to understand how specific behavioral functions are executed within distinct subcircuits of a complex neuronal network.

Materials and Methods

Fly strains

Flies were cultured according to standard methods (for details, see (Selcho et al., 2012; Selcho et al., 2009)). For the behavioral experiments UAS-*Hid,rpr* (Kurada and White, 1998; White et al., 1996) were crossed to *Tdc2-GAL4*;*tsh-GAL80* (kindly provided by J. Simpson, HHMI, Janelia Farm, Ashburn, VA; Selcho et al., 2012) driver line. Heterozygous controls were obtained by crossing GAL4-driver and UAS-effector to w^{1118}. For visualizing neurons, we used the UAS-*Cameleon2.1* reporter (Diegelmann et al., 2002; RRID: BDSC_6901). For creating single-cell flp-out clones, y,w,hsp70-flp; Sp/CyO; UAS>CD2>mCD8::GFP/TM6b virgins (Wong et al., 2002; kindly provided by Gary Struhl, Columbia University, New York, NY) were crossed to *Tdc2-GAL4* males (Cole et al., 2005; RRID: BDSC_9313). A single heat shock was applied by placing vials containing eggs or larvae in a water bath at 37°C for 17.5 minutes. For the onset of heat shock, we chose different times from 0 to 200 hours after egg laying.

Immunofluorescence

Immunostaining

The staining protocol of third instar larvae CNS' is described in detail in Selcho et al., 2012. To detect TA we used a modified staining protocol of Sinakevitch and Strausfeld (2006; Busch et al., 2009), also described in detail in Selcho et al., 2012.

Antibodies

To visualize the total expression pattern of *Tdc2-GAL4* and the innervation patterns of single *Tdc2-GAL4*-positive neurons, we applied a polyclonal serum against green fluorescent protein (anti-GFP, A6455, Molecular Probes, Eugene, OR; 1:1000, RRID: AB_221570; Table 1) in combination with two different mouse antibodies labeling the neuropil (anti-ChAT, ChAT4B1, anti-Cholineacetyltransferase; DSHB, Iowa City, IA; 1:100; RRID: AB_528122; Table 1) and axonal tracts (anti-FasII, 1d4, anti-Fasciclin II; DSHB; 1:55, RRID: AB_528235; Table 1), respectively. anti-GFP with an antibody against tyramine b-hydroxylase produced in rats (anti-TbH; Monastirioti et al., 1996; kindly provided by M. Monastirioti, IMBB, FORTH, Greece; 1:75; Table 1) was used to analyze whether all *Tdc2-GAL4*-positive neurons are octopaminergic. TA was labeled via a polyclonal antibody against glutaraldehyde-coupled p-TA (anti-TA, AB124, Chemicon International, Temecula, CA; 1:200; Table 1) in combination with a chicken anti-GFP antibody (anti-GFPch, AB16901, Chemicon; 1:150 and 1:170, RRID: AB_90432; Table 1). As secondary antibodies, goat anti-rabbit IgG Alexa Fluor 488 (A11008, Molecular Probes, 1:200, RRID:AB_143165), goat anti-rat IgG Alexa Fluor 568 (A11077, Molecular Probes; 1:200, RRID:AB_141874), fluorescein (FITC)-conjugated donkey anti-chicken (703-095-155, Jackson ImmunoResearch, West Grove, PA; 1:150), goat anti-rabbit IgG DyLight 488 (111-486-003, Jackson; 1:250), Cy3 goat anti-rabbit IgG (111-165-003, Jackson; 1:100), Cy3 goat anti-mouse IgG (A10521, Molecular Probes, 1:100, RRID:AB_1500665 or 115-166-003, Jackson; 1:250) were used.

Antibody characterization

Anti-GFP

The rabbit anti-GFP antibody gave the same staining pattern in the CNS of the *Tdc2-*

GAL4/UAS-*Cameleon2.1* larvae as the anti-GFP antibody produced in chicken. Additionally, staining was not observed in the CNS of larvae expressing only *Tdc2-GAL4* or only UAS-*Cameleon2.1* (data not shown).

Chicken anti-GFP

The anti-GFPch antibody detects a band of a molecular weight around 30 kDa in lysates prepared from *E. coli* expressing GFP on western blot. No band was detected in lysates of *E. coli* that do not express GFP.

ChAT4B1

The anti-ChAT antibody was shown to label a single band at a position of about 80 kDa in crude fly head samples (Takagawa and Salvaterra, 1996).

1D4 anti-Fasciclin II

The anti-FasII antibody labeled a 97 kDa band in western blot, which was gone in FasII null mutants (Grenningloh et al., 1991; Mathew et al., 2003). The staining pattern observed in this study is identical to previous reports (Grenningloh et al., 1991; Landgraf et al., 2003; Mathew et al., 2003).

TbH

In immunoblots of protein extracts from *Drosophila* heads and bodies a single band corresponding to the 76-kDa protein was observed using the anti-TbH antibody (Monastirioti et al., 1996). TbH-immunoreactivity was nearly abolished in larval brains of *TbH* mutants (Monastirioti et al., 1996).

Anti-p-tyramine

The anti-TA antibody was used to characterize tyraminergicneurons in *Drosophila* and locust (Busch et al., 2009; Kononenko et al., 2009). The specificity of the antibody was tested by competition experiments in equilibrium dialysis (Geffard et al., 1984). The crossreactivity ratio at half displacement of the labeled ligand and different unlabeled catecholamine conjugates (including TA conjugate) was determined. The best displacement was observed with the TA conjugate, while the OA conjugate was 42 times less immunoreactive (Geffard et al., 1984).

Microscopy and figure production

CNS preparations were scanned using a confocallight scanning microscope (LeicaTCS SP5, LeicaMicrosystems, Wetzlar, Germany). The images scanned with a step size of 1 µm or 0.8 µmthickness were analyzed with the software program ImageJ (NIH, Bethesda, MD, RRID: nif-0000-30467). Contrast, brightness, and coloring were adjusted with Photoshop (Adobe Systems, San Jose,CA).

Behavioral experiments

Appetitive olfactory learning and naive preferences to tastants applied were tested using standardized, previously described assays (Apostolopoulou et al., 2013; Gerber and Stocker, 2007; Michels et al., 2005; Pauls et al., 2010a; Rohwedder et al., 2012; Scherer et al., 2003; von Essen et al., 2011). In detail, all learning experiments were conducted on assay plates filled by a thin layer of agarose solution containing either pure 2.5% agarose or 2.5% agarose plus fructose, sorbitol, or arabinose, respectively,

at concentration of 2 M. As olfactory stimuli, we used 10 µl amyl acetate (AM, Fluka cat. no.: 46022; diluted 1:250 in paraffin oil, Fluka cat. no.: 76235) and benzaldehyde (BA, undiluted; Fluka cat. no.: 12010). Odorants were loaded into custom-made Teflon containers (4.5-mm diameter) with perforated lids as described in Gerber and Stocker (2007). Learning ability was tested by exposing a first group of 30 animals to BA while crawling on agarose medium containing sugar as a positive reinforcer. After 5 min, larvae were transferred to a fresh Petri dish in which they were allowed to crawl on pure agarose medium for 5 min while being exposed to AM. A second group of larvae received the reciprocal training. Immediately, after three training cycles, larvae were transferred onto test plates on which AM and BA were presented on opposite sides. After 5 min, individuals were counted on the AM side (#AM), the BA side (#BA), and in a neutral zone (for further details there is a video available at Apostolopoulou et al., 2013). By subtracting the number of larvae on the BA side from the number of larvae on the AM side divided by the total number of counted individuals (#TOTAL), we calculated a preference index for each training group:

$$PREF_{AM+/BA} = (\#AM - \#BA) / \#TOTAL$$

$$PREF_{AM/BA+} = (\#AM - \#BA) / \#TOTAL$$

We then compiled a performance index (PI):

$$PI = (PREF_{AM+/BA} - PREF_{AM/BA+}) / 2$$

Negative PIs represent aversive learning, whereas positive PIs indicate appetitive learning.

Gustatory preference

For gustatory preference tests, 2.5% agarose solution (Sigma Aldrich) was boiled in a

microwave oven and filled as a thin layer into Petri dishes (85-mm diameter; Greiner). After cooling, the agarose was removed from half of the plate. The empty half was filled with 2.5% agarose solution containing arabinose (2 M). Assay plates were used on the same day or stored at 4 °C until the day of experiments. Groups of 30 larvae were placed in the middle of the plate, allowed to crawl for 5 min, and then counted on the sugar side, the sugar-free agarose side, and the neutral zone. By subtracting the number of larvae on the pure agarose side (#nS) from the number of larvae on the sugar side (#S) divided by the total number ofcounted larvae (#TOTAL), a preference index for the respective sugar was calculated:

$$PREFs = (\#S - \#nS) / \#TOTAL$$

Negative PREF values indicate sugar avoidance, whereas positive PREF values represent sugar attractiveness.

Statistical methods

For the comparison between genotypes, Wilcoxon rank sum test was used. To compare single genotypes against chance level, we used the Wilcoxon signed ranked test. All statistical analyses and visualizations were done with R version 2.13.2 (RRID: nif-0000-10474). Figure alignments were done with Adobe Photoshop. Data are presented as box plots, 50% of the values of a given genotype being located within the box, the entire data set being represented by the whiskers and the median performance index by the bold line within the box plot. Outsiders are given as open circles. Significance levels between genotypes shown in the figures refer to the p values obtained in the statistical tests.

Results

Anatomy of the octopaminergic/tyraminergic neurons in the larval brain and suboesophageal ganglion

To identify the OA/TA neurons in the larval central nervous system (CNS), we used the *Tdc2-GAL4* line (Cole et al., 2005). Tyrosine decarboxylase (Tdc) catalyzes the synthesis of tyramine, the precursor of octopamine; therefore, *Tdc2-GAL4* expression should include all tyraminergic and octopaminergic neurons. To precisely describe the OA/TA neurons, we labeled the GAL4 expressing cells with Cameleon2.1 (Diegelmann et al., 2002) and double stained them with antibodies directed towards tyramine β-hydroxylase (TβH) and TA, respectively, to examine the neurotransmitter/-modulator of the cells (Fig. 1). Cameleon2.1 was used as it provides a stronger and more stable expression compared to UAS-GFP similar to the results published in a prior study on the thoracic and abdominal OA/TA system (Selcho et al., 2012).

Tdc2-GAL4/UAS-*Cameleon2.1* shows GAL4 expression in around 83 (83.10 ± 1.20, n=10) cells in the whole larval CNS. Approximately 44 (43.80 ± 0.66, n=10) cell bodies are located in the ventral nerve cord (VNC)(Selcho et al., 2012), while ~39 (39.36 ± 0.68, n=11) are labeled in the hemispheres and SOG (Fig. 1A-B). Three paired cell-clusters are situated in the hemispheres: lCA (larval calyx); lDMPa (larval anterior dorsomedial protocerebrum); lAL (larval antennal lobe) (Fig. 1E-G). The lCA cells (1.00 ± 0.00, n=11 right hemisphere) lie basomedial to the calyx. Cluster lDMPa (4.18 ± 0.12, n=11 right hemisphere) is located in the dmp, anterior to the vertical lobes of the mushroom bodies (MB). In addition, about 2 cells (1.82 ± 0.26, n=11 right hemisphere) per side are labeled dorsomedial to the antennal lobe (lAL cluster).

Most prominent are three sVM (sog ventral median) clusters in the SOG characterized by their cell body position along the ventral midline (Fig. 1C). Like in adults, these clusters seem to be located in the mandibular (md), maxillary (mx) and labial (lb) neuromeres (Busch et al., 2009; Busch and Tanimoto, 2010). In total 24.45 ± 0.34 cells are located in the sVM clusters (sVMmd 9.09 ± 0.34, sVMmx 8.00 ± 0.00, sVMlb 7.36 ± 0.15; n=11). Another *Tdc2-GAL4*-positive cell is located in the posterior SOG at the margin to the thoracic ganglion (1.00 ± 0.00, n=11; data not shown)(Vömel and Wegener, 2008).

Using an antibody against the TβH enzyme, which catalyzes the step from TA to OA, it is in principle possible to discriminate between OA/TA and TA neurons (Monastirioti, 1999). Interestingly, from the neurons described above only those in the SOG were recognized by the antibody, while in none of our stainings the neurons in the hemispheres were labeled (Fig. 1I-J; n=27). Thus, our data suggests that the neurons having their soma located in the hemispheres are likely only TA positive and lack OA. Notably, also in flies some of the *Tdc-GAL4*-positive neurons are not labeled by anti-TβH (their soma are located in the ASM cluster) (Burke et al., 2012).

In detail, all *Tdc2-GAL4*-positive cells of the sVM clusters were TβH-positive (n=11), but in the sVMlb cluster two additional cells were recognized by the TβH antibody (n=11) indicating that the GAL4 line is not covering all of the OA/TA neurons.

To confirm that the *Tdc2-GAL4*-positive cells of the hemispheres are indeed tyraminergic, we double stained with TA and GFP antibodies (Fig. 1K-L). As reported for the OA antibody, the TA antibody is also known to show a high variation in the staining between specimens (Busch et al., 2009). The cells of the brain hemispheres showed TA-immunoreactivity, as the sVM cells did. Again, we observed in some specimens two additional TA-positive cells in the sVMlb cluster that were

not labeled in the GAL4 line. Therefore, we conclude that all of the *Tdc2-GAL4* expressing neurons are tyraminergic, while those in the SOG additionally contain OA. However, it may also be possible that the TβH levels within the neurons of the hemispheres are only strongly reduced and therefore not detectable with our antibody staining. Nevertheless, our data suggests significant difference in TβH levels between sVM cells and brain hemisphere neurons.

Anatomy of single octopaminergic/tyraminergic neurons in the larval SOG

To better understand the potential neuronal network underlying sugar rewardreinforcement, we studied the arborization patterns of the larval OA/TA neurons at the single-cell level. Using the Flp-out technique (Wong et al., 2002) in combination with *Tdc2-GAL4*, we were able to characterize fourteen different OA/TA or TA cell types (summarized in Table 2 and Fig. 7).

The three sVMclusters consist of paired and unpaired neurons, called ventral paired median (VPM) and ventral unpaired median (VUM) neurons (Busch et al., 2009; Busch and Tanimoto, 2010). All cell types identified show a characteristic projection of their primary neurites: they extend dorsally, either via the midline (VUM neurons; Fig. 1D, arrow) or next to it (VPM neurons; Fig. 1D, arrowhead), and bifurcate while reaching the dorsal end of the SOG/tritocerebrum (Figs. 2 and 3 second row).

We identified four types of sVPM neurons, i.e., two in the mandibular neuromere and one each in the maxillary and labial neuromeres (Fig. 2). The two types of $sVPM_{md}$ neurons are descending neurons, while those of the $sVPM_{mx}$ and $sVPM_{lb}$ clusters are of the ascending type. Like in adults (Busch et al., 2009), the six sVUM neuron types can also be subdivided into ascending and descending cells (Fig. 3). Ascending sVUM neurons belong to the md and mx clusters, while descending VUM cells are

located in the lb cluster. Interestingly, all ascending cell types of the md cluster were also identified in the mx cluster. In contrast, the cell types of the lb cluster were never found in the other two clusters.

Ventral paired median neurons of the suboesophageal ganglion

sVPM$_{md}$1 innervates the SOG and VNC (Fig. 2A-D) and sends a process around the oesophagus (Fig. 2B,C). Arborizations are formed bilaterally on the lateral edges of the ventralmost SOG (Fig. 2C). An ipsilateral, descending fiber innervates ventral levels of thoracic and abdominal neuromeres (Fig. 2D). **sVPM$_{md}$2** innervates bilaterally the SOG and the basomedial protocerebrum (Fig. 2E-G). However, at ventral SOG levels, contralateral arbors are stronger than ipsilateral ones (Fig. 2G). In addition, sVPM$_{md}$2 innervates the contralateral VNC, as far as the fourth abdominal neuromere (Fig. 2A,H). In contrast to the sVPM$_{md}$ cells, the **sVPM$_{mx}$** neuron is of the ascending type. It innervates the SOG, the basomedial protocerebrum and - contralaterally - the MB vertical lobe, as well as the dorsolateral and dorsomedial protocerebrum (Fig. 2I-L). A secondary neurite (arrowheads Fig. 2J,K) projects laterally toward the oesophagus and ramifies in the basal vertical lobe and adjacent dorsal protocerebrum (Fig. 2K). The **sVPM$_{lb}$** cell type, which is also an ascending neuron, innervates - almost entirely contralaterally - the SOG and various protocerebral regions (Fig. 2M-P). For example, neurites ramify dorsal to the MB medial lobe and peduncle, innervating the dorsal and basolateral protocerebrum. The medial basomedial protocerebrum is also innervated (Fig. 2P). In contrast to other sVM neurons, the primary neurite of sVPM$_{lb}$ sends ramifications into the contralateral SOG *before* - rather than *upon* - reaching its dorsal part (Fig. 2O). Minor ipsilateral arborizations are present in the most anterior/medial SOG (2O).

Ventral unpaired median neurons of the suboesophageal ganglion

The arborizations of VUM neurons are bilaterally identical. Mandibular and maxillary VUM neurons share their anatomy, but differ from the labial VUM neurons. The sVUM1 cell type of the mandibular (**sVUM$_{md}$1**) and maxillar (s**VUM$_{mx}$1**) VM clusters (Fig. 3A-D) innervates the SOG (Fig. 3A,B), the antennal lobes (Fig. 3D), the basomedial and basolateral protocerebrum and - via the inner antennocerebral tract - the MB calyces (Fig. 3C). The sVUM2 cell type, residing also in the mandibular (**sVUM$_{md}$2**) and maxillar (**sVUM$_{mx}$2**) VM clusters (Fig. 3E-H), innervates the SOG, the basomedial, dorsomedial (Fig. 3G) and dorsolateral protocerebrum - close to the MB calyx - as well as the larval optic neuropil (Fig. 3G,H). The sVUM3 cell type, found again in mandibular (**sVUM$_{md}$3**) and maxillar (**sVUM$_{mx}$3**) VM clusters (Fig. 3I-L), innervates the SOG and the posterior basomedial and basolateral protocerebrum (Fig. 3K). Terminals are also established posterior to the MB medial lobe and medial appendix, and in the dorsomedial protocerebrum medial to the MB vertical lobe (Fig. 3L). The descending **sVUM$_{lb}$1** cell type (Fig. 3M-P) in the labial VM cluster innervates the SOG and thoracic neuromeres, mostly at lateral levels (Fig. 3O,P). A neurite runs lateral to the oesophagus (3O). The second cell type of the labial VM cluster, **sVUM$_{lb}$2** (Fig. 3Q-T), innervates the SOG (Fig. 3R-T), the basolateral protocerebrum, as well as medial and lateral areas of thoracic and abdominal neuromeres (Fig. 3S,T). A third labial VM cell type, **sVUM$_{lb}$3** (Fig. 3U-X), densely innervates the SOG (Fig. 3V,W) and the posterior basolateral and basomedial protocerebrum (Fig. 3W). Moreover, a single axon per side extends - close to the midline - to the third thoracic neuromere and then follows a more lateral pathway to ramify in the third thoracic and all abdominal neuromeres (Fig. 3X).

Anatomy of single octopaminergic/tyraminergic neurons in the larval brain hemsipheres

In the brain hemispheres, we were able to identify four different cell types. The descending **lCA** cell type (Fig. 4A-D) innervates ipsilaterally the dorso- and basolateral (Fig. 4B) and basomedial protocerebrum. A neurite extends anterior to the MB vertical lobe in the dorsomedial protocerebrum (Fig. 4B). The primary neurite crosses the midline posteriorly and extends into the contralateral basomedial protocerebrum (Fig. 4C). The descending process passes to the SOG - arborizing *en route* in the posterior basomedial protocerebrum (Fig. 4C) - and further into the VNC, as far as to the ninth abdominal neuromere (Fig. 4D). Arborizations remain mostly ipsilateral but to some extent also cover the contralateral SOG and VNC (Fig. 4D). Among the four cells of the lDMPa cluster, we identified two cell types, both of which innervate the two brain hemispheres. At least two cells belong to the lDMPa1 type, one to the lDMPa2 type, while the identity of the fourth cell remains unclear. The **lDMPa1** type innervates many regions in the dorsal and basal protocerebrum (Fig. 4E-H), including the contralateral side (Fig. 4F). Prominent arborizations in the posterior and anterior protocerebrum are shown on Fig. 4G and Fig. 4H, respectively. The **lDMPa2** cell type exclusively innervates the basomedial protocerebrum in both hemispheres (Fig. 4I-J). The only identified type of lAL cells, **lAL1**, innervates the SOG and both antennal lobes (Fig. 4K-L).

The role of octopaminergic/tyraminergic neurons in reward processing in larval associative learning

A recent study in adult *Drosophila* reported OA/TA neurons to be important in

mediating sweet taste onto dopaminergic neurons, to reinforce odor-sugar learning (Burke et al., 2012). In our previous work we were able to show that dopaminergic neurons are required both for reward and punishment learning in larvae (Selcho et al., 2009). This offers the possibility that the behavioral function of OA/TA neurons may be conserved between developmental stages. Thus, to test whether OA/TA neurons mediate different aspects of the sugar reward, we used a standardized two-group, reciprocal training regime for larval chemosensory learning (Michels et al., 2005, Gerber and Stocker, 2007) and tested fructose (sweet and nutritive), sorbitol (not sweet but nutritive) and arabinose (sweet but not nutritive) as rewarding US, respectively (Rohwedder et al., 2012). Ectopic expression of both UAS-*Hid* (*head involution defective*) and UAS-*rpr* (*reaper*) leads via DNA-fragmentation and chromatin condensation to apoptosis (Kurada and White, 1998; White et al., 1996). As shown by our previous work, OA/TA neurons within the VNC are necessary for proper larval locomotion (Selcho et al., 2012). To restrict the induction of cell apoptosis to OA/TA neurons in the brain and SOG and thus exclude thoracic or abdominal circuits to circumvent locomotory defects, we used *Tdc2-GAL4;tshGAL80*. *tshGAL80* (kindly provided by J. Simpson) specifically inhibits GAL4 activity in cells of the VNC (Selcho et al., 2012). Selective genetic ablation of OA/TA neurons in the brain and SOG did not lead to any deficit in appetitive olfactory learning using fructose as a reward (Fig. 5A). *Tdc2-GAL4;tshGAL80*/UAS-*Hid,rpr* experimental larvae showed significant learning scores ($p=6.104x10^{-5}$) and performed on control level (p=0.1456 compared to *Tdc2-GAL4;tshGAL80*/+ and p=0.5125 compared to UAS-*Hid,rpr*/+). Additionally, *Tdc2-GAL4;tshGAL80*/UAS-*Hid,rpr* larvae exhibited robust appetitive memory in sorbitol learning, comparable to genetic controls (Fig. 5B; p=0.1186 for *Tdc2-GAL4;tshGAL80*/+ and p=0.0566 for UAS-*Hid,rpr*/+). This

indicates that OA/TA neurons in the hemispheres and SOG are not necessary for mediating the nutritional value-dependent reinforcing function as it is present in both fructose and sorbitol. In contrast, as *Tdc2-GAL4;tshGAL80*/UAS-*Hid,rpr* larvae failed to show significant learning scores in arabinose-odor learning (Fig. 5C; p=0.285). Experimental larvae performed significantly reduced compared to *Tdc2-GAL4;tshGAL80*/+ (p=0.0070) and UAS-*Hid,rpr*/+ (p=0.0278) controls. Thus, OA/TA neurons in the brain and SOG seem to be required in processing the sweetness of a sugar during associative learning. To confirm the requirement of OA/TA neurons in mediating reinforcing sweet taste, we examined the perception of arabinose in experimental and control larvae (Fig. 5D). Notably, the perception of both odors AM and BA seems to be unaffected as learning scores were indistinguishable from controls using fructose and sorbitol as rewarding US (Fig. 5A,B). *Tdc2-GAL4;tshGAL80*/UAS-*Hid,rpr* showed normal attraction behavior towards arabinose (p=0.0089) and performed indistinguishable from genetic controls (Fig. 5D; p=0.8034 for *Tdc2-GAL4;tshGAL80*/+ and p=0.7088 for UAS-*Hid,rpr*/+).

Taken together, our data suggest that OA/TA neurons are important in mediating sugar reinforcement. The conditions used in our studies suggest that this effect may be based rather on the sweetness of the sugar than on nutrition-dependent information. A result that is in line with recent data published in adult flies (Burke et al., 2012; Burke and Waddell, 2011).

Discussion

Architecture of the larval OA/TA system on the single-cell level compared to other insects

In invertebrates and vertebrates, the activity of distinct neuronal networks within the CNS and consequently the final behavioral output is modulated by biogenic amines like OA and TA - or epinephrine and norepinephrine (the respective vertebrate counterparts) (Monastirioti, 1999). In *Drosophila* and other insects OA and TA affect a nearly endless list of behaviors including for instance sleep, egg-laying, learning, locomotion and stress-dependent behaviors such as fight-or-flight responses, which in vertebrates are regulated by the adrenergic system (Baier et al., 2002; Claassen and Kammer, 1986; Crocker et al., 2010; Erion et al., 2012; Hoyer et al., 2008; Lee et al., 2003; Monastirioti, 1999; Roeder, 2005; Saraswati et al., 2004).

In *Drosophila* larvae about 39 OA/TA neurons are located within the larval brain and SOG. While most of the neurons situated in the brain seem to have normal TA levels but lower OA level (or even no OA), all remaining neurons (about 25) within the three suboesophageal clusters are tyraminergic and octopaminergic. The basic organization within the SOG shows remarkable similarities with the adult system (Busch et al., 2009; Busch and Tanimoto, 2010): 1) all unpaired neurons in the mandibular and maxillary cluster have ascending fibers; 2) all unpaired neurons in the labial cluster have descending neurites; 3) the morphology of the mandibular and maxillary cluster suggests a duplication from a common ancestor; 4) the labial cluster is morphologically different; 5) as far as our data allows to conclude, the larval and adult OA/TA neurons in the SOG have a similar organization in terms of numbers and assembling in paired and unpaired neurons; 6) several neurons in adults and larvae

show remarkable morphological similarities with respect to their innervation patterns. For example, the two adult VUM-a2 type neurons have their cell bodies located either in the mandibular or maxillary cluster and innervate in a similar way the SOG, antennal lobes, MB calyces and lateral horns, both ipsi- and contralaterally. The same is true for the two sVUM1 neurons in the larva (Fig. 3, Table 2).

Given the massive reorganization of the brain during metamorphosis such conservation on the level of specific cells is remarkable and suggests a well-preserved function of these neurons in different developmental stages. The larval anntenal lobe, for instance, that is innervated by sVUM1 and lAL1 neurons, degenerates during puparium formation and is replaced by an adult specific structure (Jefferis et al., 2004). Also the larval born MB neurons innervated by sVPMmx neurons prune during early puparium formation and only retain their main processes in the peduncle. In contrast, the basic axon projections of the later-born larval MB neurons (α'/β') are preserved during metamorphosis (Lee et al., 1999). Unfortunately, there is no data available on the development of these OA/TA neurons over metamorphosis. Thus, based on the similarties that we describe between the larval and adult OA/TA neurons we can only speculate that the neurons persist during different developmental stages. Nevertheless, the functional and anatomical conservations suggest that larval and adult brains are more similar than previously thought and render larvae a valuable system to describe the functional principles of insect brains.

Architecture of the larval OA/TA system with respect to reward signaling

The larval MBs, containing mostly third-order olfactory neurons, were shown to be centers for olfactory learning (Michels et al., 2005; Pauls et al., 2010b). Their intrinsic neurons, the Kenyon cells, get olfactory information via projection neurons as well as

aversive and appetitive sugar-dependent stimuli via dopaminergic neurons (Gerber and Stocker, 2007; Gerber et al., 2009; Selcho et al., 2009; Thum et al., 2011). Honjo et al. (2009) suggested that OA/TA neurons, potentially signaling sugar reward only innervate the larval MB calyx. However, we identified one type of neuron in the sVMmd and sVMmx clusters that innervates the MB calyces, called sVUM1 (Fig. 3 and 6), and two additional neurons, sVPMmx (Fig. 2 and 6), that innervate the base of the MB vertical lobe in the contralateral hemisphere. The arborizations are also visible in the complete expression pattern of *Tdc2-GAL4* (Fig. 1H). This labeling in the base of the MB vertical lobe is also visible in Honjo et al (2009). Thus, individual OA/TA neurons have the anatomical properties to potentially signal sugar reinforcement directly onto the calyces *and* vertical lobes of the larval MBs and other types of neurons located in the same brain regions.

OA/TA signaling is sufficient and necessary in insects to establish appetitive memories

Pharmacological, genetic, optogenetic or electrophysiological investigations strongly suggest that in bees and flies OA/TA is involved in appetitive reinforcement signaling (Burke et al., 2012; Hammer, 1993; Hammer and Menzel, 1998; Honjo and Furukubo-Tokunaga, 2009; Kim et al., 2013; Schroll et al., 2006; Schwaerzel et al., 2003; Unoki et al., 2005; 2006). In particular, Hammer (1993) reported for the honeybee that the activation of a single VUM_{mx1} neuron or OA injection into the antennal lobe or MB can substitute for the internal rewarding function during associative olfactory conditioning of the proboscis extension reflex (Hammer, 1993; Hammer and Menzel, 1998).

The finding that activation of OA/TA neurons is sufficient to mediate the internal

reward signaling were supported in *Drosophila* larvae and adults, as pairing an odor presentation with optogenetical or thermogenetical activation of nearly all OA/TA neurons in the CNS induces appetitive olfactory associative learning (Burke et al., 2012; Schroll et al., 2006).

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Additionally, in various model organisms insect OA/TA signaling was shown to be *necessary* for appetitive olfactory learning but seems to be dispensable for odor perception or general learning ability (Burke et al., 2012; Honjo and Furukubo-Tokunaga, 2009; Schwaerzel et al., 2003; Unoki et al., 2005; 2006). In adult *Drosophila, TβH* mutant flies with elevated TA and reduced OA levels have a specific impairment for odor-sucrose learning (Schwaerzel et al., 2003) and the alpha1-like octopamine receptor OAMB is required in α/ß and γ mushroom body neurons for appetitive short term memory (STM)(Kim et al., 2013) and also in dopaminergic neurons for odor-arabinose learning (Burke et al., 2012). In *Drosophila* larvae, Honjo and Furukubo-Tokunaga (Honjo and Furukubo-Tokunaga, 2009) showed that the output of OA/TA neurons during training is necessary to form appetitive memory but is dispensable during test. Unfortunately, in larvae it is not possible to use *TβH* mutants or other ways of genetic interference with the overall OA/TA system as this strongly affects larval locomotion and therefore makes it impossible to test for olfactory learning as it requires proper locomotion (Saraswati et al., 2004; Selcho et al., 2012; Selcho et al., 2009). Nevertheless, it is believed that OA/TA signaling contains aspects of the internal rewarding function that are *sufficient and necessary* for appetitive learning throughout different insect species.

Perturbing side effects hamper the interpretation on OA/TA function for reward

learning

However, blocking OA/TA neurons using specifically the driver line *Tdc2-GAL4* does not always impair reward learning (Fig. 5). It can not be excluded that some of the described phenotypes might not be confirmed as learning effects *per se*, as OA/TA are generally known to have modulatory functions on various behaviors. Most critically, for *Drosophila* larvae OA/TA was shown to be necessary for locomotion. *TβH* mutant larvae with elevated TA and reduced OA levels spent more time in pausing episodes than wildtype larvae and displayed a reduction in speed and linear translocation (Saraswati et al., 2004; Selcho et al., 2012). In addition, ablation of OA/TA neurons within the VNC impaires larval locomotion suggesting these neurons to be essential in the modulation of crawling (Selcho et al., 2012). Thus, manipulation of the OA/TA system alters larval locomotion that likely affects the behavioral performance of the larva during and after appetitive olfactory learning, independent of the internal reward processing.

OA/TA function in larval reward learning

In line with published data we also found that the OA/TA system is involved in larval sugar reward learning (Fig.5)(Honjo and Furukubo-Tokunaga, 2009; Schroll et al., 2006). Interesting Honjo et al. published that OA is necessary for sugar reward learning when exposing larvae to an odor and a concomitant sucrose reinforcer for 30 min in a one-trial non-reciprocal training regime (Honjo and Furukubo-Tokunaga, 2005; 2009). As sucrose offers a nutritional benefit this result seems to partially contradict our results. However, there are several possible explanations: i) The effects described by Honjo et al. (2009) are extremely small. The response indices for the related experiment show values of about 0.1 (c.f. Figure 4, Honjo and Furukubo-

Tokunaga, 2009). This means that the described behavioral phenotype for 50 tested animals was only based on 5 larvae more on the odor/reward side compared to the no-odor/no-reward side. In addition, in their study the experimental animals tested had a significantly altered sucrose perception (c.f. supplement table 1). Thus, the relative small odor-sucrose phenotype observed might be due to altered sucrose reward processing during training. ii) Both, the observed phenotype in odor-sugar learning and sugar perception may rely on altered locomotion, which was not controlled in their behavioral data set. Notably, *Tdc2-GAL4*/UAS-*Hid,rpr* larvae showed reduced preference indices towards fructose in our assay. This phenotype could be rescued by the expression of *tshGAL80* indicating that this reduction was based on altered locomotion (data not shown). iii) There are multiple differences in the used behavioral protocols that induce different memory phases. Indeed, odor-fructose training using three training cycles leads to an immediate memory that is independent of *rutabaga* and *dunce* (A. Widmann, manuscript in preparation). As the odor-sucrose memory by Honjo et al. is completely absent in these two classical STM mutants the discrepancy about the requirement of OA/TA neurons might be based on the analysis of cAMP-dependent STM (Honjo and Furukubo-Tokunaga, 2005) versus a cAMP-independent memory phase (Fig.5). This is in line with recent data in adult flies as odor stimulation and concurrent OA network activation establishes only a short lasting memory (Burke et al., 2012).

Thus, OA/TA signaling in the brain and SOG is likely necessary for larval appetitive learning by encoding the reinforcing function for certain aspects of sugar reward that potentially induce a short lasting memory. Based on our single cell description it is now possible to test if this effect is triggered by single neurons of the OA/TA system and how these neurons get input from recently described gustatory sensory neurons

that respond to fructose (Mishra et al., 2013).

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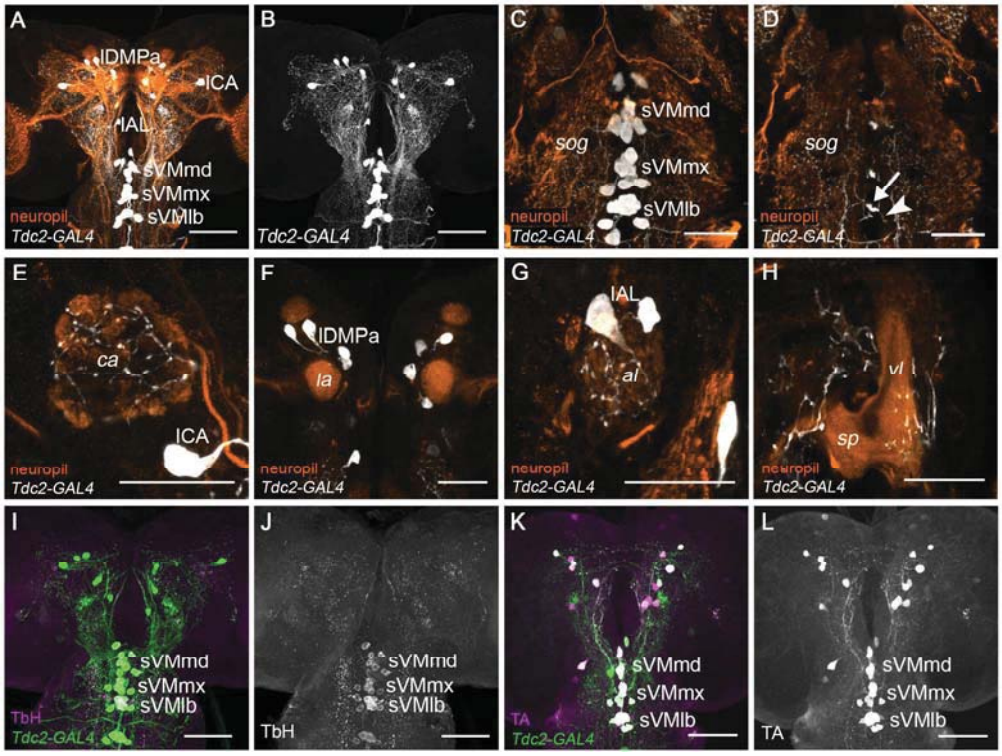

Fig. 1: Octopaminergic/tyraminergic neurons of the larval brain and SOG. A-H: Confocal z-projections showing the expression pattern of *Tdc2-GAL4*;UAS-*Cam2.1* (white) in combination with axonal tract and neuropil markers (orange: FasII/ChAT staining). I-L: Double staining of the *Tdc2-GAL4* expressing (green) and tyramine-β-hydroxylase (TβH) or tyramine (TA) immunolabeled (magenta and white) neurons. A-B: Whole mount projection of the brain and SOG showing the cell clusters and their arborizations. C-D: Selected regions of the SOG. C: The three sVM cell clusters. D: The sVUM neurons of each sVM cluster send their primary neurite along the midline (arrow) while the primary neurites of the sVPM cells run in parallel to the midline (arrowhead). E-H: Higher magnifications of different brain neuropils, visualizing the cell cluster locations in the brain. OA/TA neurons innervate some prominent brain neuropils like the calyces (ca; E), antennal lobes (al; G) and vertical lobes (vl; H), I-J: The *Tdc2-GAL4*-positive cells of the sVM clusters overlap with TβH antibody labeling, indicating that they contain OA. K-L: The *Tdc2-GAL4*-positive cells of both the brain and SOG overlap with TA antibody labeling. Scale bars: A-B, I-L 50µm; C-H 25µm.
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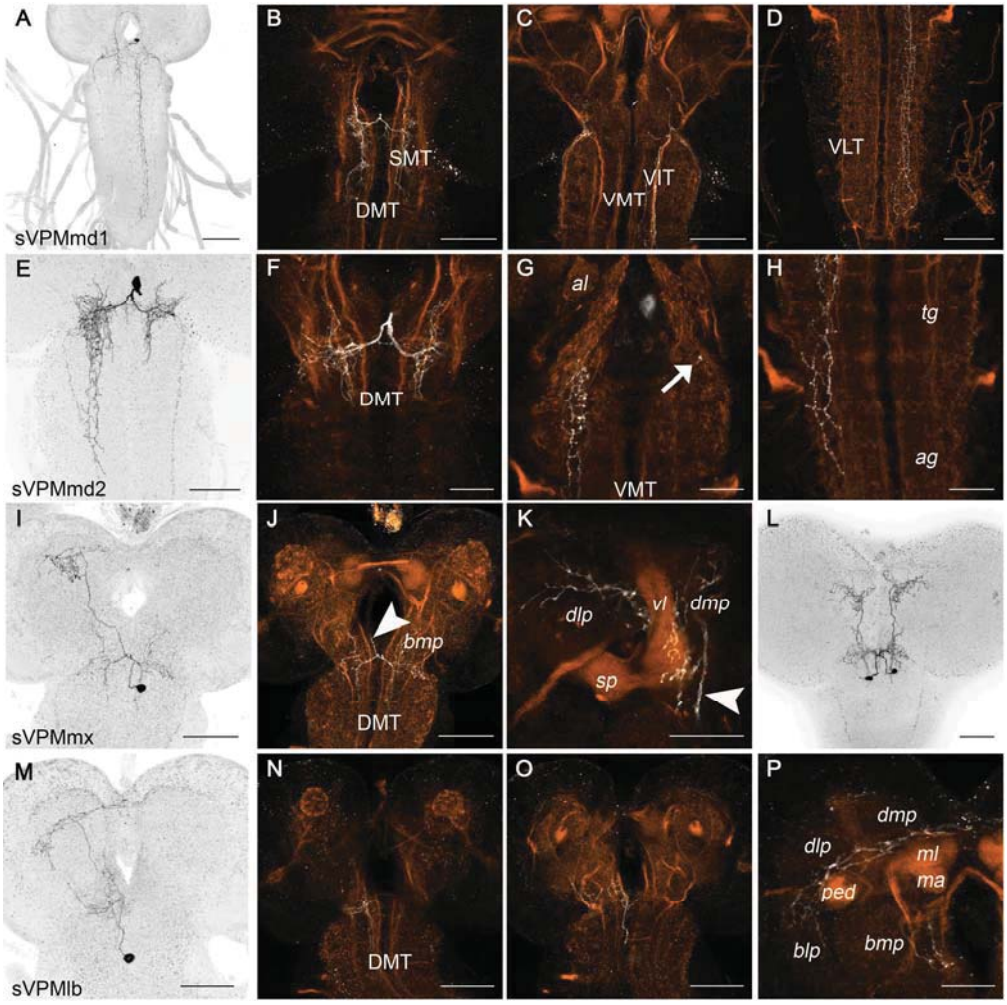


Fig. 2: Octopaminergic/tyraminergic larval sVPM neurons. The left column shows the arborizations of each single cell in the CNS. The characteristic branching pattern of the primary neurite of each VM cluster neuron in the dorsalmost SOG/tritocerebrum is visible in the second column. The rest of each row illustrates higher magnifications of characteristic neuropil regions innervated by each cell type (white: Tdc2-GAL4;UAS-mCD8::GFP; orange: FasII/ChAT staining). A-D: sVPMmd1 innervates the SOG and VNC and sends a ramification around the oesophagus. C: The ventral SOG shows on both sides arborizations at the far lateral edges. D: Ipsilaterally, a descending fiber innervates the ventral thoracic (tg) and abdominal ganglion (ag). E-H: The sVPMmd2 cell type innervates the SOG, bmp and contralateral VNC. G: Contralateral arborizations in the medioventral SOG. The ipsilateral side shows a few dot-like terminals. H: Contralaterally, the innervations reaches the fourth abdominal neuromere. I-L: The sVPMmx cell type innervates the SOG, bmp and the contralateral vertical lobe (vl), dlp and dmp. K: A secondary neurite projects from the branching point of the primary neurite lateral to the oesophagus and ramifies in the basal vl and the adjacent dorsal protocerebrum. L: The sister neurons of the sVPMmx cell type. M-P: The sVPMlb cell type innervates the contralateral SOG, bmp, blp, dlp and dmp. N, O: In contrast to the other neurons of the sVM clusters, the primary neurite of sVMlb sends ramifications into the contralateral SOG before reaching its dorsal part. In the most anterior SOG region, branches innervate the most medial part of the ipsilateral SOG. P: Neurites ramify dorsal to the medial lobe (ml) and peduncle (ped), innervating the dorsal blp. The medial bmp is also innervated. DMT dorsomedial, SMT superficial medial, VIT ventrointermediate, VMT ventromedial, VLT ventrolateral tract (after Nassif et al., 2003). bmp basomedial, blp basolateral, dmp dorsomedial, dlp dorsolateral protocerebrum (after (Selcho et al., 2009)). Scale bars: A-E, I-J, L-O 50µm; F-H, K, P 25µm. 172x171mm (300 x 300 DPI)

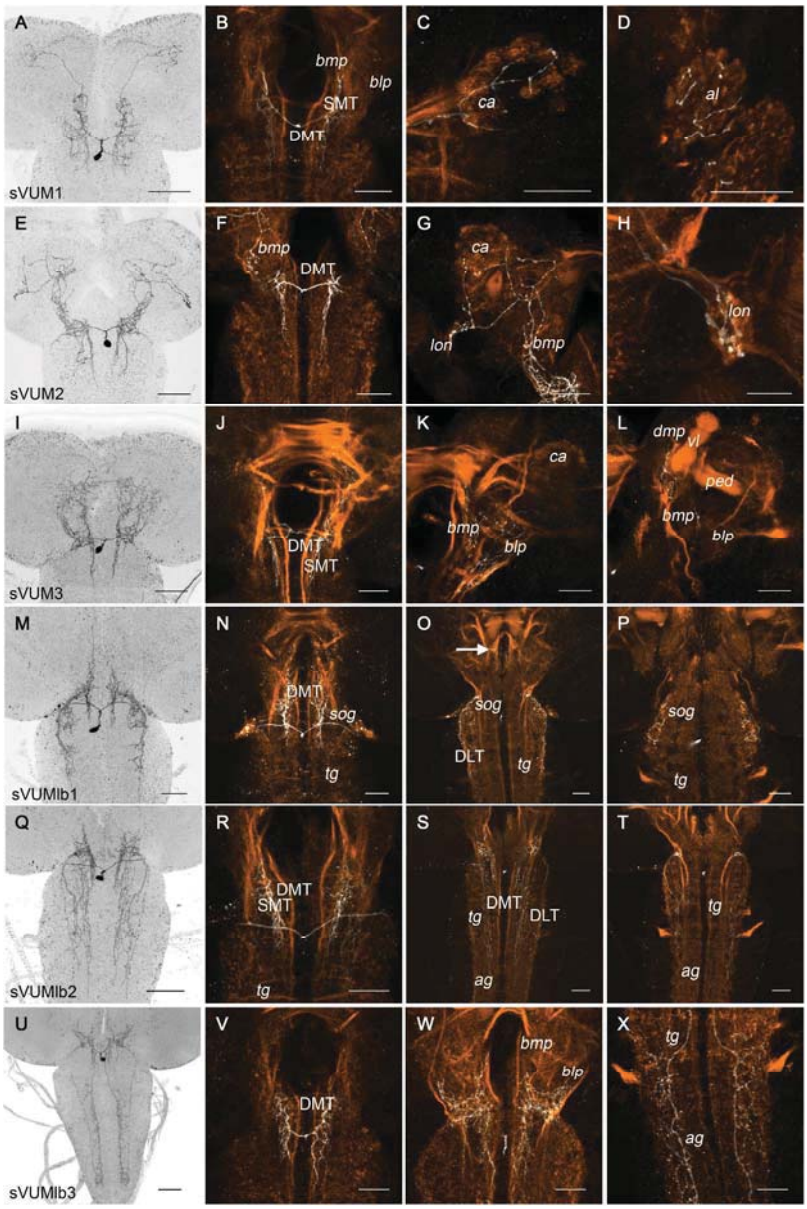


Fig. 3: Octopaminergic/tyraminergic larval sVUM neurons. The left column shows the arborizations of each single cell. The characteristic branching of the primary neurite of each VM cluster neuron in the most dorsal part of the SOG/tritocerebrum is visible in the second column. The rest of each row illustrates higher magnifications of characteristic neuropil innervations of each cell type (white: Tdc2-GAL4;UAS-mCD8::GFP; orange: FasII/ChAT staining). A-D: The sVUM1 cell type was found in the first (sVUMmd1) and second (sVUMmx1) VM cluster. It innervates the SOG, al, bmp, blp and the calyces (ca). C: Via the inner antennocerebral tracts, dot-like terminals reach the ca of the mushroom bodies. D: The al is also innervated. E-H: The sVUM2 cell type was found in the first (sVUMmd2) and second (sVUMmx2) VM cluster. It innervates the SOG, bmp, larval optic neuropil (lon), dmp and dlp. G: Arborizations in the bmp and dmp. Those in the dlp are in the vicinity of the ca. G, H: The LON is innervated by dot-like endings. I-L: The sVUM3 cell type was found in the first (sVUMmd3) and second (sVUMmx3) VM cluster. It innervates the SOG, bmp, blp and dmp. K: The cell branches in the posterior bmp and blp. Arborizations posterior to the medial lobe and medial appendix are visible. L: The ramifications posterior to the medial lobe reach the dmp

medial to the vl. M-P: The sVUMlb1 cell type innervates the SOG and thoracic ganglion (tg). O: Lateral arborizations in the SOG and tg. A neurite runs lateral to the oesophagus (arrow). P: Innervation of the anterior lateral SOG. Q-T: The sVUMlb2 cell type innervates the SOG, blp, tg and abdominal ganglion (ag). S: Arborizations in the dorsomedial SOG, tg and ag. T: The lateral tg and ag are innervated. U-X: The sVUMlb3 cell type innervates the SOG, blp, bmp, tg and ag. W: Arborizations in the posterior bmp and blp. The dorsoanterior SOG shows dense ramifications, extending in dorsoposterior direction along the midline. X: One axon per side runs posterior next to the midline. Reaching the third thoracic neuromere, it turns to the VIT and ramifies in the third thoracic and all abdominal neuromeres. DMT dorsomedial, SMT superficial medial, VIT ventrointermediate tract (after (Nassif et al., 2003)). bmp basomedial, blp basolateral, dmp dorsomedial, dlp dorsolateral protocerebrum (after (Schwaerzel et al., 2003)). Scale bars: A, E, I, M, Q, U 50µm; B-D, F-G, J-L, N-P, R-T, V-X 25µm; H 10µm.
257x386mm (300 x 300 DPI)

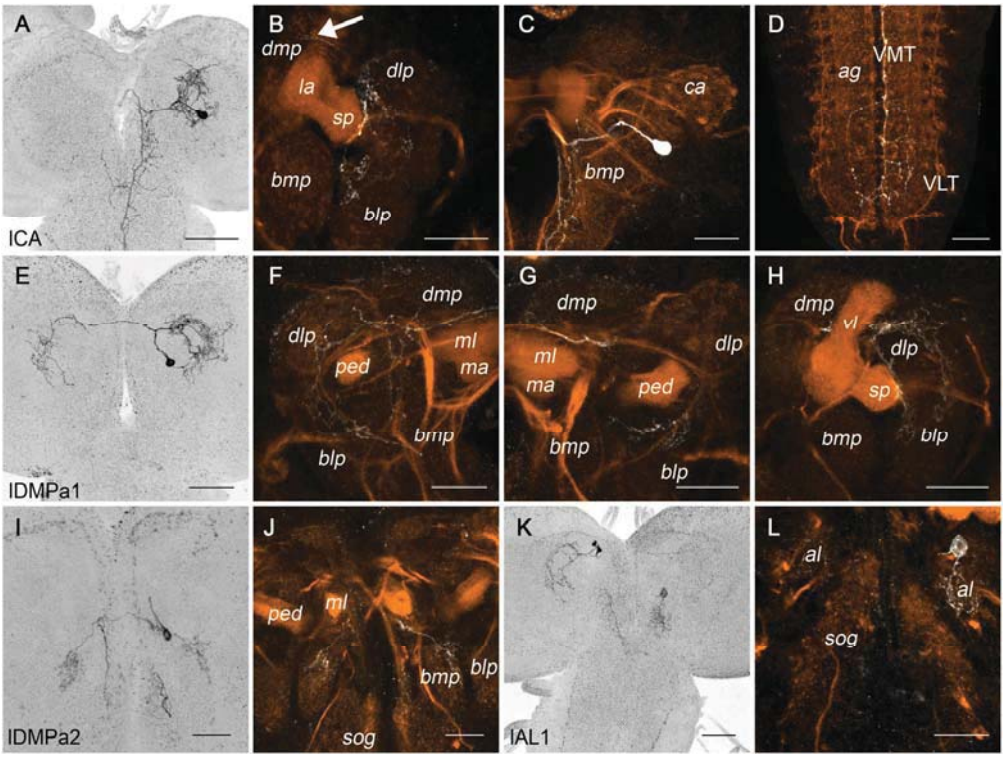


Fig. 4: Potential tyraminergic neurons of the larval brain. The projection pattern of each single cell is shown in the left column. The other pictures illustrate higher magnifications of characteristic arborization patterns of each cell type (white: Tdc2-GAL4;UAS-mCD8::GFP; orange: FasII/ChAT staining). A-D: The lCA cell type innervates the dlp, blp, bmp, SOG, tg and ag. B: Arborizations in the dlp and blp; a neurite runs anterior to the vl and ends in the most lateral dmp (arrow). C: A neurite runs toward the midline dorsal to the oesophagus, turns into the contralateral dorsal bmp and projects to the VNC thereby arborizing in the posteriomedial bmp. D: The neurite projects along the VMT to the ninth abdominal neuromere, sending arborizations into the ipsi- and contralateral ventromedial ag. E-H: The lDMPa1 cell type innervates the dmp, dlp, blp and bmp. F: Innervation of the dmp, dlp, blp and bmp of the contralateral side. G: Arborizations in the posterior dmp and blp. H: At an anterior level, the dlp, blp and most lateral dmp are innervated. I-J: The lDMPa2 cell type innervates the bmp. J: Ramifications in the anterior bmp of both sides of the brain. K-L: The lAL1 cell type innervates the SOG and both al. J: Arborizations in both al. VMT ventromedial, VLT ventrolateral tract (after Nassif et al., 2003). bmp basomedial, blp basolateral, dmp dorsomedial, dlp dorsolateral protocerebrum (Selcho et al., 2009). Scale bars: A, E, K 50µm; B-D, F-J, L 25µm.
129x96mm (300 x 300 DPI)

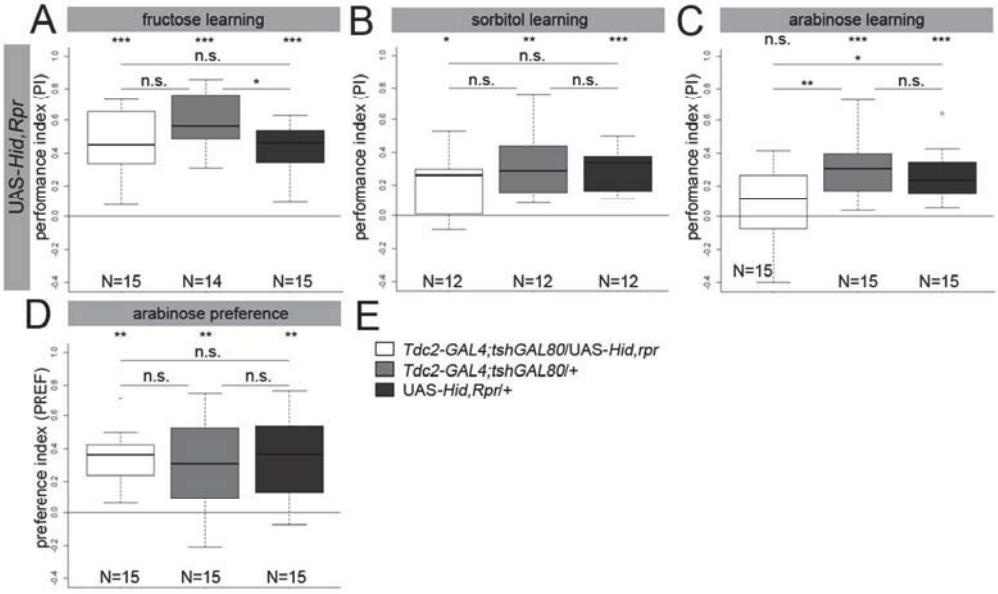


Fig. 5: Ablation of OA/TA neurons in the brain and SOG specifically affects odor-arabinose learning. A,B: Restricted OA/TA cell ablation within the brain and SOG, leaving locomotor circuits intact, did not lead to significant impairment in odor-fructose (A) or odor-sorbitol (B) learning (all $p>0.05$) indicating that these neurons are dispensable to mediate sugar reward as these sugars both offer a nutritional value. C: In contrast, ablation of the same OA/TA neurons affected odor-arabinose learning ($p<0.05$) that was shown to not offer any nutritional benefit to the larva. D: Genetic ablation of these neurons did not specifically alter naïve preferences to arabinose. The sample size for each experiment is given under each box-plot. Significance values between groups are indicated above the respective box-plots. Significance against chance level that indicates if a group showed appetitive olfactory learning (in A-C) or gustatory preference (in D) are presented on the very top for each group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.s. not significant.
107x64mm (300 x 300 DPI)

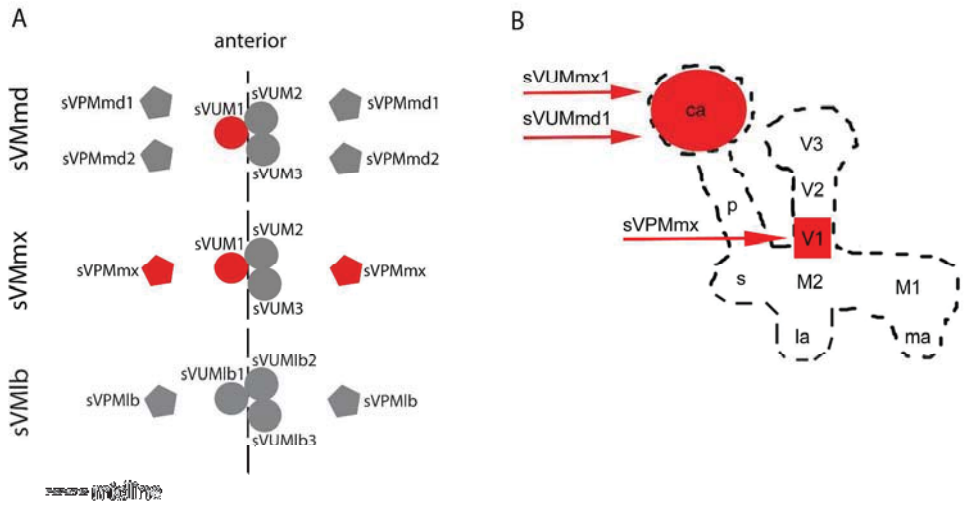


Fig. 6: Overview of the anatomical organization of the OA/TA system. A: A schematic view on the organization of the SOG clusters based on our single cell approach: three different types of ventral unpaired median (VUM) neurons are present in the mandibular as well as the maxillary cluster (sVUM1, sVUM2 and sVUM3). The three sVUM neurons of the labial cluster are of a different type. In addition each cluster gave rise to a second group of neurons that are mirror symmetrically organized with respect to the midline and therefore organized in pairs (sVPM). Two pairs of VPM cells were present in the mandibular cluster (sVPMmd1 and sVPMmd2), one pair in the maxillary cluster (sVPMmx) and one pair in the labial cluster (sVPMlb). B: Based on the single cell labeling of individual OA/TA neurons three cells were identified that innervate the mushroom body. sVUMmx1 and sVUMmd1 innervate the calyces of the mushroom bodies and sVPMmx projects onto the base of the vertical lobe of the contralateral mushroom body. Thus a single mushroom body gets potential input by three different OA/TA neurons at two localy distinct subregions (shown in red in A and B).

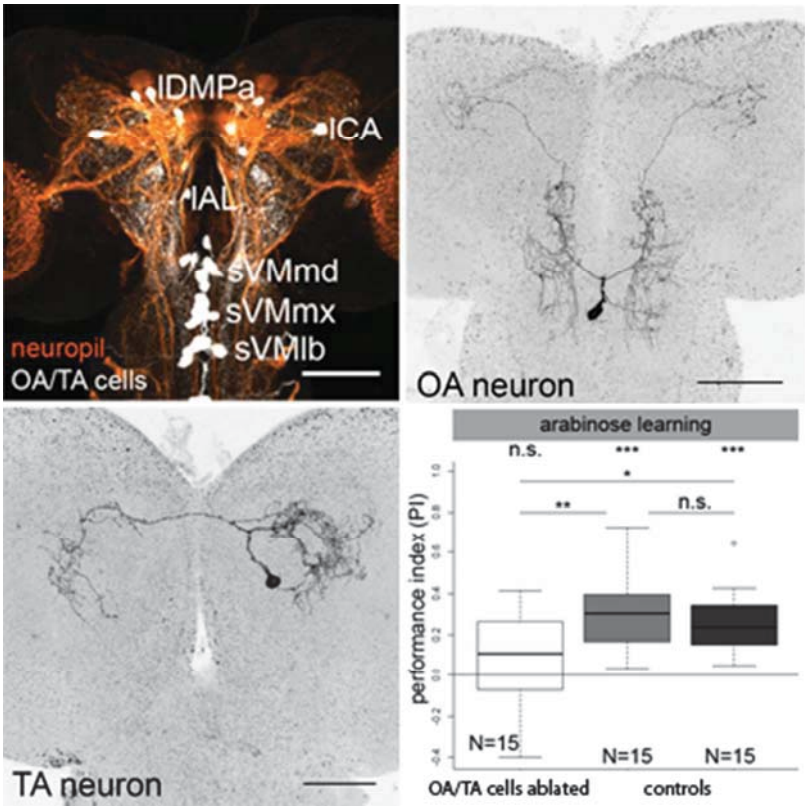
Table 1: Primary antibodies.

Antibody	Host	Immunogen	Manufacturer, [RRIDs]	Working dilution
Anti-GFP	rabbit, polyclonal serum	Purified green fluorescent protein (GFP), a 27-kDa protein derived from the jellyfish *Aequorea Victoria*	A6455, Molecular Probes (Eugene, OR) [AB_221570]	1:1000
Chicken anti-GFP	chicken, polyclonal	Recombinant GFP containing a 6-his tag	AB16901, Chemicon (Temecula, CA) [AB_90890]	1:150, 1:170
ChAT4B1	mouse, monoclonal	80-kDa *Drosophila* Choline acetyltransferase protein	ChAT4B1, DSHB (Iowa City, IA) [AB_528122]	1:100
1D4 anti-Fasciclin II	mouse, monoclonal	Bacterially expressed fusion peptide containing the intracellular C-terminal 103 amino acids of the PEST transmembrane form of FasII	1D4, DSHB (Iowa City, IA) [AB_528235]	1:55
TβH	rat, polyclonal	A bacterially expressed purified internal part of the protein (*Sal–Xho* fragment)	Monastirioti et al., 1996	1:75
Anti-p-Tyramine	rabbit, polyclonal	p-Tyramine-glutaraldehyde-N-alpha-acetyl-L-lysine-N-methylamide	AB124, Chemicon, (Temecula, CA) [AB_90432]	1:200

Table 2.

cell type	central brain regions				mushroom bodies						other neuropil regions					hits per cell type
	dmp	dlp	bmp	blp	ma	vl	la	sp	ped	ca	al	lon	sog	tg	ag	
sVPMmd1			bs										bs	il	il	19
sVPMmd2			bs										bs; cl	cl	cl	6
sVPMmx	cl	cl	bs			cl							bs			14
sVPMlb	cl	cl	cl	cl									cl; (il)			2
sVUMmd1		bs	bs							sm	sm		bs			6
sVUMmx1																11
sVUMmd2	sm	sm	sm									bs	sm			9
sVUMmx2																10
sVUMmd3			sm	sm									bs			4
sVUMmx3																3
sVUMlb1													sm	sm		7
sVUMlb2													sm	sm	sm	6
sVUMlb3			sm	sm									sm	sm	sm	3
sVUMlb4																
ICA		il	il; (cl)	il									il; bs	il; (cl)	il; (cl)	7
IDMPa1	bs	bs		bs												17
IDMPa2			bs													3
IAL1											bs		bs			9

central brain regions: dmp- dorsomedial, dlp- dorsolateral, bmp- basomedial, blp- basolateral protocerebrum; mushroom bodies: ma- medial appendix, la- lateral appendix, vl- vertical lobe, sp- spur, ped- pedunculus, ca- calyx; al- antennal lobes; lon- larval optic neuropil; sog- suboesophageal ganglion; ventral nerve cord: tg- thoracic ganglion, ag- abdominal ganglion; symmetrical (sm), unsymmetrical: both sides (bs), ipsilateral (il), contralateral (cl).



141x141mm (72 x 72 DPI)

In this study we characterized a subset of about 39 octopaminergic/tyraminergic cells situated within the *Drosophila* larval central brain and SOG on the single cell level. These neurons are likely necessary for larval appetitive learning by encoding the reinforcing function for certain aspect of sugar reward.