Biogenic amines in the *Drosophila melanogaster* larval central nervous system – an anatomical and behavioural function description

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1 Summary

1.1 German version


Für diese Art von Untersuchungen eignet sich die Taufliege Drosophila melanogaster hervorragend. Dieser Modellorganismus weist, besonders auch in der larvalen Form, viele Vorzüge auf. Genetische Werkzeuge ermöglichen beispielsweise eine spezifische Manipulation von definierten Zelltypen, welche es unter anderem erlauben, auf eine einfache Weise Verhaltensdefizite oder die Lokalität von einzelnen Neuronen nachzuweisen.

Eine solche Charakterisierung ist im Kapitel 3 für das Lern- und Gedächtniszentrums, dem sogenannten Pilzkörper, dargestellt. Bei der Beschreibung der beteiligten Neuronen, bestätigen wir, was auch schon früher gezeigt wurde, dass biogene Amine bei diesen neuronalen Verschaltungen eine wichtige Rolle spielen. Darum analysiere ich in den folgenden Kapiteln (4-7) unter anderem die unterschiedlichen Effekte der biogenen Amine auf das larvale Lernen.

Nachdem ich in einer früheren Studie die Anatomie der 5-HT-Neurone im larvalen Gehirn charakterisierte und zeigen konnte, dass das genetische Ausschalten von 5-HT per se keinen Effekt auf unterschiedliche Konditionierungen sowie das naive Präferenzverhalten in der Larve hat, untersuche ich im Kapitel 4 die Anatomie der 5-HT Rezeptorenzellen und teste, ob sie ebenfalls entbehrlich für die verschiedenen Verhaltensformen sind.

Biogenic amines are metabolic derivates of amino acids. Serotonin (5-HT), dopamine (DA), histamine (HA), octopamine (OA) and tyramine (TA) are some of the best investigated biogenic amines. They can act as neurotransmitters, neuromodulators, and/or neurohormones, subsequently playing an essential role in a wide range of processes and behaviours in vertebrates. This includes for example the activation of blood platelets after an injury, the coordination of locomotion, or sleep. In addition, biogenic amines control feelings like happiness, aggression or reward. Interestingly, they influence also many of these behaviours and processes in addition to several others in invertebrates. In insects for example, biogenic amines control flying, aggressive behaviour, reward, sleep but also egg laying or visual...
processes. This work follows a bidirectional approach, focusing on the behavioural effect in insects, mainly in fruit flies, on the one hand, and on the anatomical distribution in the central nervous system of biogenic amines and their corresponding receptors on the other. From the behavioural point of view, simple innate behaviours, mainly naïve olfactory and gustatory preferences, and complex classical conditioning based learning and memory will be analysed.

The fruit fly *Drosophila melanogaster* acts as an ideal model organism for this kind of research. Genetic tools allow to specifically manipulate a defined set of cells, which facilitate the investigation of behavioural phenotypes or anatomical analysis. The *Drosophila melanogaster* larva has been well researched as it consists of a relative small number of cells. In particular, neuronal circuits involved in olfactory and gustatory perception and learning and memory have been the subject of intensive research. Moreover, and in comparison to other organisms, the central nervous system is less complex, thus enabling us to examine brain structures from neuronal up to synaptic levels.

A characterisation of such a brain structure termed mushroom body – the learning and memory centre – is shown in chapter 3. Thereby we confirm, what also other studies have revealed, that biogenic amines play an important role in these neuronal circuits. Thus, the following chapters (4-7) consider inter alia the effect of biogenic amines on larval learning and memory.

In an earlier study, I described the anatomical distribution of 5-HT in the larval CNS and showed, that genetic inference of 5-HT expressing cells *per se* did not affect conditioning and innate behaviours. In chapter 4, I expand experiments on 5-HT receptors expressing neurons. Thereby I test if they are also dispensable for different kinds of behaviours and display the location of these neurons in the CNS.
The main interest in chapter 5 is on the dopaminergic system in larvae. Therefore, we focus on a defined set of cells termed PAM cluster, which are essential for learning and memory in adults. Like in the chapter before, we characterize the anatomy of this dopaminergic cluster (only made up by eight neurons) and test the effect on learning and naïve preference behaviours in larvae.

In chapter 6, neurons expressing OA and TA are analysed in the larval brain. Additionally, we test if genetic impairment of these neurons affects odour conditioning applying various sugars.

Considering the HA system, I analyse in chapter 7 mutated larvae that are unable to synthesise HA or one out of two known HA receptors. These animals are tested for naïve preference as well as for learning and memory.

In summary, the results of this thesis and other studies revealed that all analysed biogenic amines and their corresponding receptors are expressed in the larval CNS. Nevertheless, genetic manipulations have mostly no or only a weak observable effect on naïve olfactory and gustatory preference behaviour. In contrast, I show that all investigated systems are somehow involved in different learning and memory processes.
2 General Introduction

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2.1 Biogenic amines

Biogenic amines are metabolic derivates of single amino acids containing at least one amino group. Tryptophan, tyrosine, and histidine act as precursors of some of the most extensively studied biogenic amines: serotonin (5-HT), dopamine (DA), histamine (HA), tyramine (TA) and octopamine (OA) (Figure 1) [1].

![Synthesis of biogenic amines in invertebrates](image)

Biogenic amines can act as neurotransmitters, neurohormones and/or neuromodulators [1, 2].

As a result, many diverse processes including sleep, locomotion, chemosensation, and learning and memory are mediated by the different systems of biogenic amines and their appropriate receptors (in general G-protein coupled receptors (GPCRs)) [1, 3]. Some systems share common functions; others might act in opposing ways. Comparing vertebrates and invertebrates, there are some parallels and some differences in biogenic amine functions and organisation (Table 1).
Table 1: Overview of biogenic amine function examples and receptor types

<table>
<thead>
<tr>
<th>5-HT system</th>
<th>vertebrates</th>
<th>invertebrates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>function (examples)</strong></td>
<td>cardiovascular system, gastro-intestinal tract, sleep, sexuality and aggression, intraocular pressure, appetite, mood</td>
<td>cardiovascular system, gastro-intestinal tract, sleep, sexuality and aggression, locomotion, lifespan, sensitivity to heat</td>
</tr>
<tr>
<td><strong>receptor types</strong></td>
<td>15 5-HT receptors assigned to 7 classes: 5-HT₁, 5-HT₇ (Humans)*</td>
<td>5-HT₁₆, 5-HT₁₈, 5-HT₇, 5-HT₂₆, and 5-HT₂₈ (Fruit fly)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DA system</th>
<th>vertebrates</th>
<th>invertebrates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>function (examples)</strong></td>
<td>locomotion, reward, addiction, motivation</td>
<td>learning and memory, reward, addiction, locomotion, circadian rhythm, arousal, sleep</td>
</tr>
<tr>
<td><strong>receptor types</strong></td>
<td>D1-D5 (Humans)</td>
<td>dumb, damb, D2R and DopEcR (Fruit fly)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OA system</th>
<th>vertebrates</th>
<th>invertebrates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>function (examples)</strong></td>
<td>trace amine, fewer functions are identified</td>
<td>egg laying, sleep, reward, appetitive learning and memory, aggression</td>
</tr>
<tr>
<td><strong>receptor types</strong></td>
<td>trace amine associated receptors (TAAR) (Humans)</td>
<td>Oamb, Octβ1R, Octβ2R, and Octβ3R (Fruit fly)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA system</th>
<th>vertebrates</th>
<th>invertebrates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>function (examples)</strong></td>
<td>trace amine, less functions are identified</td>
<td>appetite regulation, olfaction, foraging labour</td>
</tr>
<tr>
<td><strong>receptor types</strong></td>
<td>trace amine associated receptors 1 and 2 (TAAR1 and TAAR2) (Humans)</td>
<td>TyrR, TyrRII and TyrRIII (Fruit fly)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HA system</th>
<th>vertebrates</th>
<th>invertebrates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>function (examples)</strong></td>
<td>gastro-intestinal tract, sleep-wake cycles, appetite</td>
<td>venom (inducing inflammatory responses in victims), vision, olfaction</td>
</tr>
<tr>
<td><strong>receptor types</strong></td>
<td>H1-H4 (Humans)</td>
<td>HisC11 and ort (Fruit fly)*</td>
</tr>
</tbody>
</table>

Note: In general receptors are GPCRs. Ligand-gated ion channels (indicated by *) are 5-HT₃ in humans and HisC11 as well as ort in fruit flies.
To get a rough idea of each mentioned biogenic amines system a short description including some key functions and the receptor organisation of vertebrates and invertebrates is displayed. Be aware, these are just examples of key functions, since biogenic amines are involved in countless more processes.

2.1.1 The serotonergic system

In both vertebrates as well as in invertebrates a tremendous variety of diverse body functions are mediated by the 5-HT system. For example, 5-HT plays a key role in the regulation of the cardiovascular system [4-9], the gastro-intestinal tract [10-12], sleep-wake cycle [13, 14], or sexual and aggressive behaviour [15-21]. In humans, 5-HT contributes to the regulation of intraocular pressure, appetite, pain, or mood [20, 22]. Additionally, diseases such as migraine [23] and probably also schizophrenia and depression are reported to be a result of impaired 5-HT signalling [24-26]. In different invertebrate model systems, it was shown that 5-HT signalling regulates locomotion or learning and memory processes [27-33]. Furthermore, studies in the fruit fly *Drosophila melanogaster* have revealed that 5-HT regulates growth, lifespan, sensitivity to heat, or resistance to starvation [34-37].

5-HT acts as natural ligand of different 5-HT receptors. The 5-HT receptor type variation differs among species. In honeybees four different exclusively GPCR types, were identified: 5-HT\textsubscript{1A}, 5-HT\textsubscript{7}, 5-HT\textsubscript{2A}, and 5-HT\textsubscript{2B} [38-40]. In fruit flies an additional receptor was found: 5-HT\textsubscript{1B} [41-44]. In worms, beside three GPCRs (SER-1, SER-4, and SER-7), one ligand-gated ion channel type (MOD-1) was described [45-48]. In humans, 15 distinct genes encoding 5-HT receptors have been identified in total. These proteins have been assigned to seven receptor classes (5-HT\textsubscript{1} to 5-HT\textsubscript{7}) [49]. Comparable to the 5-HT receptors in invertebrates these receptors are also GPCRs, except members of the 5-HT\textsubscript{3} receptor family, which are ligand-gated ion channels [50, 51].
2.1.2 The dopaminergic system

The DA systems of invertebrates and vertebrates share several common functions like locomotion, motivation, addiction or reward [52, 53]. In humans, many disorders like schizophrenia, Parkinson’s disease, or hyperactivity disorder are elicited by disturbed DA signalling [23, 53-55]. In invertebrates including worms and different insects, DA is also important for learning and memory processes [56-58]. In fruit flies for example, DA signalling is necessary for acquisition of aversive and appetitive memory [59-63]. Additionally, it was shown that DA regulates circadian rhythm, arousal, sleep, and courtship [52, 64-70].

In the fruit flies, DA signalling is mediated by three different GPCRs [52]. These receptors are, based on their biochemical and pharmacological properties divided in two subfamilies, termed D1-like and D2-like. The D1-like receptors include the DopR1 (also known as DA1 or dumb) and the Dop1R2 (also known as damb) [71, 72]. The D2-like receptor is called Dop2R (or D2R) [73]. An additional, fourth receptor was identified, the DopEcR [74]. It is activated by both dopamine and ecdysteroid. A similar organisation was described in mammals. Here, five GPCRs are divided in D1-like (D1 and D5) and D2-like (D2-D4) receptors [75]. An orthologue of the DopEcR in mammals is not known.

2.1.3 The octopaminergic system

OA was originally discovered in the salivary glands of the octopus and functions as neurotransmitter, neuromodulator, and hormone in many different invertebrate species [2, 76]. In mammals, the concentration of OA is rather low (nanomolar), indicating that OA is functioning as trace amine [77, 78]. However, the knowledge of exact function of trace amines in the human body is far from complete. Nonetheless, it was observed that these molecules are involved in the occurrence of some human disorders like hypertension and depression [79]. Contrary to the situation in mammals the function of OA in invertebrates is
postulated as an important neuroactive substance involved in sleep, egg laying, and aggression behaviour [80-82]. It is also suggested that OA does act as one of the main neurotransmitter/modulator in insect reward signalling. Fruit flies or crickets lacking OA or OA receptors show for example impaired appetitive learning and memory [59, 83]. However, in fruit flies an increasing amount of studies show that also DA is involved in signalling reward [61, 84-87].

Four different OA receptors (all GPCRs) are present in the fruit fly: Oamb, Octß1R, Octß2R, and Octß3R [88-91]. In locusts also four different OA receptors are found and termed: OA1, OA2A, OA2B, and OA3 [92]. In humans, a family of distinct from but related to classical biogenic amine receptors have been reported, the trace amine associated receptors (TAAR) [93, 94]. These GPCRs are activated by trace amines including octopamine [93].

2.1.4 The tyraminergic system

TA acts primarily as precursor of OA in invertebrates (see Figure 1) and vertebrates [1, 95]. However, it was postulated that TA not only acts as a precursor, but might be used as a neuroactive amine as well. Receptors with high affinity for TA were found in vertebrates and invertebrates [93, 96]. In mammals, the concentration of TA is at similar rather low levels like OA, indicating that TA is also functioning as trace amine [77, 95]. It is known that the trace amine associated receptors TAAR1 and TAAR2 have high affinity for tyramine [97]. If and which body functions are affected by this signalling needs further investigation. Functional descriptions of the TA system are rare and challenging in insects as well since many neurons express both OA and TA. A study using blowflies showed that TA is involved in appetite regulation by experiences of food flavours [98]. Other investigations address the function of TA receptors. In fruit flies signalling via TyrR might be involved in olfactory behaviour [99]. TyrR is one of at least three different TA GPCRs (TyrR, TyrRII, TyrRIII) [88, 96, 100]. In
bees TA signals via two different GPCR (AmTAR1, AmTAR2) and can increase gustatory responsiveness or regulate division of foraging labour [101-103].

2.1.5 The histaminergic system
HA is a pharmacological component of venom from different species like spider, bees, locusts, snakes, wasps, or mosquitos and induces inflammatory responses including itching and vasodilation (resulting in skin reddening) in human [104-107]. Beside the function in the gastrointestinal system, where HA is essential for gastric acid secretion, the molecule also acts as a neuroactive molecule in the central nervous system (CNS) and regulates sleep-wake cycles or appetite [107]. In insects HA seems to be the major neurotransmitter in the visual system [108, 109]. Additionally, HA might be involved in olfactory processes by inhibition of the honeybee, bumblebee, and spiny lobster antennal lobes and olfactory lobes, respectively [110-112].

Interestingly, whereas in fruit flies and other insects HA binds on ligand-gated ion channels (HisCl1 and HisCl2 or also called “ort”), in humans exclusively GPCRs were described (H1-H4) [113-117]. The existence of a histamine-gated ion channel in mammals is still under debate [118].

2.2 What is the effect of biogenic amine systems on naïve preference and associative olfactory learning and memory?

The main goal of this work is to investigate the effect of biogenic amine systems on behaviour functions with focus on naïve preference and associative olfactory learning and memory in *Drosophila melanogaster* larvae. The fruit fly, in particular the larva, is an ideal model organism to investigate functions of biogenic amines. Genetic manipulation allows to examine the role of the 5-HT, DA, OA/TA and HA system from simple to more complex behaviour. To understand behavioural functions, knowledge about the anatomical
organisation is very helpful. For example, to study molecular processes that induce behaviour, anatomical analysis (e.g. the expression patterns of receptors initiating second messenger cascades) is essential. Therefore, this work focuses additionally on the distribution of the different aminergic systems in the larval central nervous system. In the following you will find arguments why *Drosophila* larvae are used for this kind of research (chapter 2.3), how the larval CNS is organized in general (chapter 2.4), and information about innate (chapter 2.6) and learned larval behaviour (chapter 2.8). Additionally, I will show how biogenic amines are distributed in the larval CNS (chapter 2.5) and the current state of knowledge of the effect of biogenic amines on larval naïve preference as well as on learning and memory behaviours (chapters 2.7 and 2.9). Finally, an overview of the different chapters of the main part will be given (chapter 2.10).

### 2.3 *Drosophila melanogaster* larva - a powerful model system

There are many convincing arguments why *Drosophila melanogaster* larvae can be used to investigate the effect of biogenic amines *in vivo*.

First, the genetic accessibility provides powerful tools. Mutations in genes of interest can be induced by chemicals, radiation, or by transposable elements (e.g. P-elements) [119-121]. P-element insertions can be used for a binary manipulation tool commonly known as the GAL4/UAS-system [122]. The GAL4-line defines the site of expression. P-elements of these driver lines consist of endogenous promotor fragments followed by the transgene GAL4. The protein GAL4 is a transcriptional activator of the yeast *Saccharomyces cerevisiae* which is originally not present in the fruit fly [123]. The UAS-line defines what will be expressed. P-elements of this effector lines consist of the upstream activation sequence (UAS) followed by one or more effector genes. After crossing the two parental lines, (the GAL4 driver and the UAS effector line), offspring express, under the control of a specified promotor, GAL4.
GAL4 then binds to the UAS and induces transcription of the defined effector gene (Figure 2). This design allows to specifically manipulate a distinct set of cells or tissue in the F1 generation [124]. There are a lot of different effectors lines available [125]. They can be used for example for triggered programmed cell death (e.g. UAS-hid, rpr [126, 127]) or inactivation of neurons (e.g. UAS-tnt [128]). Moreover, light- or thermosensitive forms of effector genes allow expression at a defined time point by applying light (e.g. via channelrhodopsin, [129]) or increased temperature (e.g. shibire [130]). Such effector lines are handy for investigating behavioural functions including learning and memory *in vivo*. Furthermore, UAS sequences can be followed by genes coding for fluorescence proteins like GFP (green fluorescence protein) that are helpful to visualize cells in anatomical studies [62, 131].

![Figure 2: Schematic description of the GAL4/UAS-System. The GAL4-line with a specific promoter upstream of the GAL4 gene is crossed to the UAS-line containing GAL4 binding sites (= upstream activation site, UAS) and an effector gene (P generation). Larvae with both transgenic constructs express GAL4, which bind to the UAS and induces expression of an effector protein (F1 generation) (adapted from Brand and Perrimon 1993 [122]).](image-url)
For manipulating the different biogenic systems, various specific GAL4-lines are obtainable. To generate them, promotor fragments of enzymes, that are essential for the synthesis of biogenic amines (see Figure 1), were used (e.g. Tdc-GAL4\cite{132}). GAL4- and UAS-lines that were utilized in the studies of this thesis are listed in the appendix (chapter 12).

Second, for each aminergic system specific antibodies are available targeting directly biogenic amines (e.g. anti-TA) or enzymes involved in the synthesis (e.g. anti-TH). Moreover, combining genetic tools and specific antibodies allows an analysis up to single cell levels (e.g. used in chapter 5 or in \cite{133}). Thereby single cells or a small set of cells can be stained via a thermo-sensitive activatable flipase that induces expression of GFP under the control of a GAL4 driver line (e.g. TRH-GAL4 \cite{134}). By an additional antibody staining in a different colour (e.g. anti-5-HT) the specificity of the GAL4/UAS system can be tested.

Third, using Drosophila as a model organism has further advantages like short generation times, high amounts of offspring per female, and low rearing costs. These (as well as all mentioned advantages above and below) are reasons why the fruit fly has become one of the most studied model organisms over the last decades. Thus, a high amount of comparable research articles is available including numerous articles on aminergic systems also in the context of behavioural functions and anatomy.

Finally, fruit flies have a relatively “simple” brain. Compared to human brains comprising approximately 86 billion of neurons, the fly brain consists only of about 135,000 of neurons with estimations predicting that there are about 10 times less neurons in the larval central brain \cite{135-137}.

In summary, Drosophila melanogaster, and especially the larva is an ideal model organisms to study biogenic amines also in the context of complex behaviours like learning and memory.
2.4 Anatomical organisation of the *Drosophila* larval central brain

The central brain, located in the anterior part of the larva consists of two hemispheres and the ventral nerve cord (VNC) [138]. The suboesophageal ganglion (SOG), also known as suboesophageal zone, links the hemispheres and the VNC. One central brain hemisphere of an early *Drosophila* larval contains of about 1000-1400 neurons with somata located in the cortex [139-141]. From there axons project into different compartments, arborize and form synapses with dendrites of other neurons [139]. The different neuropils are made up by these compartments and can act as functional units. During larval development, most of the compartments persists and grow isometrically, but some compartments mature anisometrically, divide or develop newly [140].

Nomenclature of compartments is often based on the position in the brain, like the basoanterior compartment (BC) or the basoposterior lateral compartment (BPM). In contrast, the neuropils that are often anatomically and functionally well described, are termed with trivial names, like the mushroom body (MB) or the antennal lobe (AL) (Figure 3). To visualize neuropil structures an antibody against Fascicline II (FasII) is frequently used. The membrane bound protein Fas II, a homologue of the vertebrate N-CAM, is expressed in differentiating neurons during embryogenesis and persists throughout larval development [139, 142]. Additionally, a second neuropil marker anti-ChAT (choline acetyltransferase) can be applied. ChAT activity strongly correlates with acetylcholine levels [143]. Most brain neurons are cholinergic and can therefore be stained by anti-ChAT [140]. In the following neuropil structures of the *Drosophila* larval CNS are described.
Figure 3: Anatomical organisation of the third instar Drosophila larval CNS. The background of the larval CNS regions is stained by a combination of anti-FasII (for axon tracts) and anti-ChAT (for neuropiles). Preparations were flattened during mounting and thus eliminate the typical 90° rotation of the central nervous system (CNS) at the intersection between the suboesophageal ganglion (sog) and ventral nerve cord (vnc) (dashed line). (A) The orientation refers to the body axis (A: dorsal; l: lateral; p: posterior). (A) and (D) show the brain at a middle anteroposterior level; (B) and (C) represent more anterior and more posterior levels, respectively. Separated by the mushroom body region (mb), each hemisphere is divided in four subregions: dorsomedial protocerebrum (dmp), dorsolateral protocerebrum (dlp), basomedial protocerebrum (bmp) and basolateral protocerebrum (blp). bmp and blp are separated by a lack of the anti-ChAT staining (A arrow). The mb nomenclature is depicted in the right hemisphere (A–C): vertical lobe (vl), medial lobe (ml), spur (sp), pedunculus (ped), calyx (ca) medial appendix (ma) and lateral appendix (la). Antennal lobes (al) in the anterior part of the brain (B) and the mb in the posterior part (C) were taken as additional subregions. (D) The vnc is divided into the thorathic ganglion (tg) and the abdominal ganglion (ag). Scale bars: 50 µm. (adapted from Selcho et al. 2009 [62])

2.4.1 Suboesophageal ganglion and antennal lobes

The larval brain has one AL per hemisphere located between the MB and the SOG with a metameric structure and a diameter of about 20-30 µm [144, 145]. One AL consists of 21 glomeruli that are connected to 21 olfactory receptor neurons (ORN). Each neuron targets one glomerulus, resulting in a 1:1 connectivity [146].

Compared to the MB and AL, the anatomical architecture of the SOG is less well characterized. The SOG is innervated by gustatory receptor neurons (GRN) [147]. Depending on the segmental and sensory organ origin of the GRN, two major and two small sub regions of each of the bilaterally organized SOG sides were described [148].

SOG and AL function as primary chemosensory input brain centres [147, 149, 150].
2.4.2 Calyces and mushroom bodies

The two bisymmetrical mushroom body calyces (CAs) are located dorsally in each hemisphere. The structures comprise of a small number of relatively large glomeruli and are innervated by projection neurons (PNs) [151]. The neurons building the CAs and MBs are called Kenyon cells (KCs). Their somata are located more peripherally in the cortex. Axons of KCs form the lobes after passing the CAs and the peduncle. The larval MBs consist of two pairs of lobes, the vertical and the medial lobes that are separated in different compartments [152, 153]. KCs of the different compartments have synapses with MB input and output neurons. In chapter 3 we describe the whole connectome of the first instar larval MB. Therefore about 150,000 transmission electron microscopy images were generated and fused to a 3D volume from which 387 MB related neurons and their synaptic connections were reconstructed manually.

CAs and MBs act as main integration centre of the larval brain – especially for learning and memory [154].

2.4.3 Larval optic neuropils

The larval optic neuropils (LONs) function as first visual information processing brain centre. The location of these paired small compartments is close to the optic anlagen in each lateral hemisphere [155]. Photoreceptor neurons, originating from larval eyes, enter via the optic nerve the brain laterally and terminate in the LONs [156]. Three groups of neurons (lateral neurons, optic lobe pioneer neurons, and serotonergic neurons) form synapses with the photoreceptor neurons in the LONs and project to different brain regions [155, 157, 158].

2.4.4 Ventral nerve cord

The VNC is functionally equivalent to the mammalian spinal cord [159]. The VNC is segmentally organized into three thoracic and eight abdominal segments, and it contains
approximately 3000 cells (in the early larval state) [141]. The neuropil is divided into three mirrored domains (medial, intermediate and lateral) containing sets of Fas II-positive descending or ascending axon tracts that can act as landmarks [141, 160]. Mechanosensory and nociceptive axons terminate in the ventral region of the VNC, whereas the motor domain is located more dorsally [161].

2.5 Expression pattern of different biogenic amines systems in *Drosophila* larvae CNS

The 5-HT expression pattern of about 84-96 neurons in the larval CNS was described in different studies [1, 133, 162]. By using the flip-out-technique I could characterize 5-HTergic neurons up to single cell level in an earlier study [133]. My findings suggest innervation of 5-HT positive neurons in various CNS regions including the SOG, AL, LON, and VNC but not in the CA or MB. A comprehensive description of the 5-HT receptor system is lacking. Thus, I characterized the expression pattern of four specific 5-HT receptor GAL4 lines (chapter 4).

DAergic neurons were described in the eighties by immunoreactivity [163]. Later, Selcho et al. characterized the system by single cell staining using TH-GAL4 [62]. Somata and innervation of about 90 DA neurons are distributed over the whole CNS. In contrast to the 5-HT system, DAergic neurons innervate also different regions of the MB and the CA. In chapter 3 we identify which MB input neurons are DAergic. We additionally describe an unknown DA cluster innervating specifically the medial lobe of the MB (chapter 5).

Monastirioti et al. described OAergic neurons with somatas mainly in the VNC and the SOG projecting also to the hemispheres by immunohistochemistry technics [164]. Later Selcho et al. investigated the expression pattern of OA and/or TA expressing neurons in the VNC with the help of of specific antibodies and GAL4-lines up to single cell level [165]. By applying
the same technics, we expanded the characterization of about 39 OA/TA expressing neurons located in the SOG and the hemispheres (chapter 6).

Larval CNS HA immunostaining was shown by Phyton and Stocker in perspective of chemosensory neurons [166]. HAergic cells innervate CNS regions like the VNC, SOG and different regions of the protocerebrum. But a combinatorial staining with neuropil specific antibodies for landmarks is absent.

2.6 *Drosophila* larval behaviour – naïve preference

Contrary to the rich behaviour repertoire of adult *Drosophila*, larval behaviour is limited in terms of not exhibiting any flying, mating, egg laying, and grooming behaviour. Furthermore, it is not clear if larvae are able to express aggression or show sleeping behaviour. One of the main, possibly the most important goal of larvae is eating. To survive in the food source environment, where they typically hatch, it is important to sense and valuate chemical cues [167]. Thus, innate preference behaviour is essential. In general preference refers per definition to a biased choice between two or more conditions in the same sensory modality but of different quantity or quality [168]. In *Drosophila* larvae, different naïve preference behaviours are explored including visual, olfactory, gustatory, thermal and food preference [168-173]. My focus in this work is on naïve olfactory and gustatory preference and how they are affected when different biogenic systems are impaired.

For decades, the larval olfactory and gustatory systems have been studied. Each chemosensory system is bilaterally organized (Figure 4). Odours are perceived by dendrites arborisations of ORNs located in the dorsal organs (DO), whereas dendritic arborisations of GRN located in the terminal organs (TO), the ventral organs (VO) and the DO perceive gustatory input. In addition to these three external organ pairs, four internal organ pairs located close to the oesophagus are important for gustatory perception: the dorsal (DPS), the
ventral (VPS), the posterior (PPS) and the dorsal pharyngeal organs (DPO) [145, 150, 174]. GRNs connect the sensory organs with the brain via four different nerve pairs: the antennal nerves (AN), the labral nerves (LN), the maxillary nerves (MNs) and the labial nerves (LBNs). All nerves terminate at the primary gustatory brain centre the SOG. In contrast ORNs are exclusively part of the ANs that bifurcate and innervate the ALs too. Thus, the ALs function as primary olfactory brain centres. More downstream, the lateral horn is thought to organize naïve olfactory behaviour [147]. But, how the chemosensory information is further processed, and a behavioural output is generated is still not fully investigated in the larva. However, a recently published screening approach revealed a neural-behaviour map where more than 1000 neural lines that evoke 29 different behaviours (behaviotypes) are mapped [175].

Figure 4: Schematic overview of the chemosensory system of Drosophila larvae. From the three external organs, the dorsal (DO), terminal (TO) and ventral (VO), neurons project via the dorsal, terminal and ventral organ ganglion (DOG, TOG and VOG) along the antennal nerve (AN) and the maxillary nerve (MN) to the larval brain. Olfactory processes of the DO innervate the larval antennal lobe (LAL). Local interneurons (LN) connect the glomeruli of the LAL and projection neurons (PN) innervate Kenyon cells (KC) in the Calyx region and the lateral horn (LH). Gustatory processes of the DO, TO and VO innervate the suboesophageal ganglion (SOG) via AN or MN. Four pharyngeal organs are located along the pharynx: the dorsal, ventral, posterior sense organ (DPS, VPS, PPS) and the dorsal pharyngeal organ (DPO). The DPS, the DPO and the PPS send projections along the labral nerve (LN), the VPS along the labial nerve (LBN) to the SOG. (adapted from Gerber and Stocker 2007 [149] and Apostolopoulou et al. 2015 [176])

Drosophila larvae are attracted by different gustatory stimuli. These include sugars offering (e.g. fructose, sucrose, glucose, maltodextrin, trehalose, and sorbitol) or lacking nutritional
(e.g. xylose and arabinose) benefits [169, 177] and sodium chloride at low concentration [178]. In contrast, high salt concentrations [178] and different bitter components like quinine [179, 180] and caffeine [181] induce aversive responses.

*Drosophila* larvae are able to perceive more than 50 different odours [182]. Testing larvae for their naïve preference for these odours, they are attracted by nearly all of the tested odours (e.g. acetone). A small set of further odours cause no (e.g. 1.8 cineole) or aversive responses (e.g. undiluted xylene) [182, 183].

There exist different approaches that allow measuring chemotaxis choice behaviour. Two of them, mainly used in this work, are shown below (Figure 5).

![Figure 5: Gustatory and Olfactory preference assays](image)

- **Figure 5:** Gustatory and Olfactory preference assays. (A) Gustatory preference is measured on Petri dishes containing a layer that is divided into two halves: one containing pure agarose, the other containing a test-t agarose solution (e.g. sugar or salt solution). (C) Olfactory preference is measured on Petri dishes containing agarose layer and two containers: one is filled with one odour; the other is empty. Preference indexes are calculated according to the formulas in (B) and (D). (E) provides a legend for (A) and (C).

### 2.7 The effect of different biogenic amines systems in *Drosophila* larval naïve preference

*Drosophila* larvae lacking 5-HT show naïve preference for different sugars and avoidance of salt. Additionally, it was shown that 5-HT seems to be dispensable for naïve odour preference testing amyl acetate, benzaldehyde and heptanol [133, 184]. But nonanol avoidance of 5-HT
mutant larvae was increased [184]. I expanded these experiments and tested larvae lacking 5-HT receptors for naïve olfactory and gustatory preference using four different odours, three sugars, and salt at high concentration (chapter 4).

DA might be required for perceiving benzaldehyde and possibly also fructose and amyl acetate, but not octanol or salt [62]. In contrast, larvae with down regulated or lacking DA receptor expression perceived salt, quinine hemisulfate, sucrose, amyl acetate, and benzaldehyde comparable to controls [185, 186]. We examined the effect of blocked neuronal output of a subset of DA cells on naïve preference applying different sugars and odours (chapter 5).

The effect of OA/TA signalling on larval naïve preference is not yet studied (up to my knowledge). In chapter 6 our focus was mainly on the expression pattern of OA/TA larval CNS neurons but also on behaviour effects of OA/TA signalling including gustatory preference.

Investigations of the HA systems with focus on behaviour in fruit flies are rare. Information about the effect of HA signalling on naïve gustatory and olfactory preference is missing. In chapter 7 I show behaviour data (fructose, salt, amyl acetate and benzaldehyde preference performance) of larvae lacking HA or the HA receptor ort.

2.8  Drosophila larval behaviour - associative olfactory learning and memory

Although, larvae have rather simple brains with respect to neuron numbers, they are able to show learning and memory behaviour. In the last decades, mostly classical (also known as Pavlovian) conditioning was in focus of Drosophila larval research [187]. In this behavioural assay an unconditioned stimulus (US) is paired with a subsequent conditioned stimulus (CS) for several times. Afterwards, a presentation of the CS alone elicits an animal response. An
often-investigated form of classical conditioning is the associative olfactory learning and memory. Here, an odour A (CS\textsubscript{A}) is paired with a US and an odour B (CS\textsubscript{B}) is unpaired. After a training phase, where animals explored both situations for several times, they are tested for odour preference (A vs. B). This assay can be modified by using different rewarding (e.g. different sugars) and punishing (e.g. high concentrated salt, electric shock, light) stimuli (US), different odours, omitting one odour, or by varying training and test durations [188]. The paradigm mainly used in this work is shown below (Figure 6).

**Figure 6: Drosophila larvae associative olfactory learning and memory assay.** (A) provides a scheme how associative olfactory learning can be applied. Larvae are placed on a Petri dish containing an agarose layer and odour A (upper line, left). 5 minutes later larvae are transferred to a Petri dish containing an unconditioned stimulus (US) solution (e.g. sugar solution) paired with odour B. After 5 minutes, this procedure is repeated two more times. After this training phase of 30 minutes, larvae are tested on a Petri dish covered by an agarose layer (in case of sugar learning) and odour A on one site and odour B on the other site. Larvae are counted on each odour region and preference index 1 (PR1) is calculated (B). This experiment is done reciprocally (A, lower line) and therefore PR2 can be calculated. The performance index (PI) is calculated by the formula shown in (B). (C) shows a legend for (A). Note, aversive olfactory learning and memory shares the same protocol with exception of the test plate, which is filled with an agar unconditioned stimulus solution (e.g. salt solution).

The coincidence detection between US and CS neurons allows to associate an odour with a gustatory stimulus. This process takes place in the MB [188, 189]. Odour information (CS) is transmitted from the AL via projection neurons to the glomeruli of the CA [146]. On the other hand, it is still not fully investigated how the transduction of US information occurs. Yet, it is
known, that MB input neurons, expressing mostly DA (called DAN) or OA (called OAN), innervate different MB regions (see also chapters 3 and 5). Convening US and CS information in the intrinsic KC induces learning and memory specific signal cascades including the PKA (protein kinase A) and/or PKC (protein kinase C) containing pathway and results in altered postsynaptic activity of MB output neurons (MBONs) [185]. Like in naïve preference behaviour processes, it is unclear how information is transferred further downstream and how a specific locomotion output is generated.

2.9 The effect of different biogenic amines systems in Drosophila larval associative olfactory learning and memory

5-HT seems to be dispensable for larval appetitive learning and memory behaviour [133]. This is independent of the nutrition value of the sugar used as US (see appendix chapter 12). And also aversive electric shock learning and memory is not affected in larvae lacking 5-HT [133]. To confirm if this is also true for aversive salt learning and memory, I examine the effect of 5-HT and four 5-HT receptors (chapter 4). Additionally, I test larvae lacking 5-HT receptors using fructose as US (chapter 4).

Like in other insects, the DA system is necessary for aversive olfactory learning and memory in Drosophila larvae [62, 129, 185, 186, 190]. It was even shown that DA signalling is sufficient for aversive learning and memory [129]. Appetitive learning and memory behaviour is also affected in larvae lacking DA or DA receptors [62, 186]. Accordingly, DA neurons signal punishment and reward. In the adult system it was shown that a cluster of DA positive neurons (termed protocerebral anterior medial - PAM) is specifically required for signalling reward [61]. In chapter 5 we investigate the effect of this DA cluster (termed primary PAM - pPAM) in larval learning and memory behaviour.
It was shown that temperature induced inhibition of OA/TA signalling results in reduced appetitive sucrose learning and memory whereas light induced activation of the larval OA/TA neurons paired with an odour induces appetitive learning and memory [129]. This indicates, that OA/TA signalling is necessary and sufficient for this behaviour. We further test, if OA/TA signalling is also required for associating an odour with additional sugars containing different nutrition values (chapter 6).

HA was in the focus of entomologists investigating mostly the visual system. Another function that is attributed to HA in fruit flies are mechanosensory cues resulting e.g. in grooming [191]. To examine if HA is also involved in larval learning and memory behaviour, I investigate the effect of HA signalling by using HA and ort mutated larvae (chapter 7).

2.10 Objectives of this work


We reconstruct all MB intrinsic and MB in- and output neurons of one first instar larva and describe this circuit at synaptic resolution. We also define neurotransmitter identity of MB related neurons.

Chapter 4: Anatomy and Behavioral Function of Serotonin Receptors in Drosophila melanogaster Larvae

(Huser et al. 2017, published in PLOS ONE, doi: 10.1371/journal.pone.0181865)

Focusing on the receptor level of the 5-HT system, I describe the distribution of individual 5-HT receptor specific GAL4 expression in the larval CNS. Additionally, I show the effect of disrupted 5-HT receptor expression on naïve preference and appetitive and aversive olfactory learning and memory.
Chapter 5: Four Individually Identified Paired Dopamine Neurons Signal Reward in Larval Drosophila.


We describe the larval anatomy and the behavioural function of a paired DAergic cluster termed pPAM. Our focus is mainly on appetitive learning and memory but we also illustrate results of tested aversive learning and memory as well as naïve preference.

Chapter 6: Characterization of the Octopaminergic and Tyraminergic Neurons in the Central Brain of Drosophila Larvae


We analyze the anatomy of the larval OA/TA system in the brain and SOG up to a single-cell level. In addition, we show the effect of the OA/TA system on appetitive olfactory learning and memory.

Chapter 7: Behavioral function of the histaminergic system in Drosophila melanogaster larvae

(Huser and Thum 2018, in preparation)

Using mutated animals with impaired HA signalling or the loss of HA receptor expression, I examine the effect of the HA system on larval naïve preference as well as aversive and appetitive olfactory learning and memory.
2.11 References


BIOGENIC AMINES IN THE CENTRAL NERVOUS SYSTEM OF DROSOPHILA MELANOGASTER LARVAE - AN ANATOMICAL AND BEHAVIOURAL FUNCTION DESCRIPTION

GENERAL INTRODUCTION

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3 The complete connectome of a learning and memory center in an insect brain

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CHAPTER 3

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3.1 Abstract

Associating stimuli with positive or negative reinforcement is essential for survival, but a complete wiring diagram of a higher-order circuit supporting associative memory has not been previously available. Here we reconstruct one such circuit at synaptic resolution, the *Drosophila* larval mushroom body. We find that most Kenyon cells integrate random combinations of inputs but that a subset receives stereotyped inputs from single projection neurons. This organization maximizes performance of a model output neuron on a stimulus discrimination task. We also report a novel canonical circuit in each mushroom body compartment with previously unidentified connections: reciprocal Kenyon cell to modulatory neuron connections, modulatory neuron to output neuron connections, and a surprisingly high number of recurrent connections between Kenyon cells. Stereotyped connections found between output neurons could enhance the selection of learned behaviours. The complete circuit map of the mushroom body should guide future functional studies of this learning and memory centre.

3.2 Main

Massively parallel, higher-order neuronal circuits such as the cerebellum and insect mushroom body (MB) serve to form and retain associations between stimuli and reinforcement in vertebrates and evolutionarily complex invertebrates\(^1,2,3,4,5,6\). Although these systems provide a biological substrate for adaptive behaviour, no complete synapse-resolution wiring diagram of their connectivity has been available to guide analysis and aid understanding. The MB is a higher-order parallel fibre system in many invertebrate brains, including hemimetabolous as well as holometabolous insects and their larval stages\(^6\). MB function is essential for associative learning in adult insects\(^1,3,4,5\) and in *Drosophila* larvae\(^1,7,8\), from the earliest larval stages onwards\(^9\). Indeed, the basic organization of the adult and the
larval MB and their afferent circuits is very similar; however, larvae have about an order of magnitude fewer neurons. Thus, to systematically investigate the organizational logic of the MB, we used serial section electron microscopy to map with synaptic resolution the complete MB connectome in a first-instar Drosophila larva (L1; Fig. 1a). L1 are foraging animals capable of all behaviours previously described in later larval stages, including adaptive behaviours dependent on associative learning (Fig. 1b). Their smaller neurons enable fast electron microscopy imaging of the entire nervous system and reconstruction of complete circuits.

Models of sensory processing in many neural circuits feature neurons that fire in response to combinations of sensory inputs, generating a high-dimensional representation of the sensory environment. The intrinsic MB neurons, the Kenyon cells (KCs), integrate in their dendrites inputs from combinations of projection neurons (PNs) that encode various stimuli, predominantly olfactory in both adult and larva, but also thermal, gustatory, and visual in adult and larva (reported here for the first time, to our knowledge). Previous analyses in adults and larvae suggest that the connectivity between olfactory PNs and KCs is random, but they do not eliminate the possibility of some degree of bilateral symmetry, which requires access to the full PN-to-KC wiring diagram in both hemispheres.

The MB contains circuitry capable of associating reward or punishment with the representation of the sensory environment formed by KCs. KCs have long parallel axons that first run together, forming the peduncle, and then bifurcate, forming the so-called lobes, in both larvae and adults. KCs receive localized inputs along their axonal fibres from dopaminergic as well as octopaminergic modulatory neurons (DANs and OANs, respectively) that define separate compartments. DANs and OANs have been shown to convey reinforcement signals in adult insects and larval Drosophila.
Figure 1 Mushroom bodies of a first-instar Drosophila larva. a, KCs from an electron microscopy volume of the whole central nervous system. b, Associative learning in first-instar larvae: in a petri dish, we presented an odour (cloud) and red light, either paired (left) or unpaired (right), and computed the performance index. Control larvae (attP2;UAS-CsChrimson) receiving paired stimuli did not learn, whereas larvae in which optogenetic activation of dopaminergic (PAM cluster) neurons (GMR58E02-GAL4;UAS-CsCrimson) was paired with odour showed robust appetitive learning (P < 0.0001). c, Diagram of the literature’s MB circuitry model. PN relay sensory stimuli to KC dendrites. MBON dendrites and MBIN (DAN, OAN, and other) axons tile the parallel KC axons, defining compartments (coloured boxes). MBINs signal reward or punishment, and KCs synapse onto output neurons (MBONs). d, PN–to–KC connectivity, colour-coded by percentage of inputs on KC dendrites. Uniglomerular olfactory PNs (Olfactory PNs) and other PNs synapse onto single-claw or multi-claw KCs. Stars indicate KCs with identical input patterns on the left and right hemispheres. For the PNs on the right of the black vertical lines, the first 18 columns are left–right homologous PNs, the last column (left) and last five columns (right) are hemisphere-specific PNs. e, Number of KCs integrating inputs from uniglomerular olfactory PNs, multiglomerular olfactory PNs, non-olfactory PNs, or a mixture of these PN types. L, left; R, right. f, Earlier-born KCs join the lineage bundle closer to the neuropil surface than later-born ones, meaning older KCs present fewer claws than younger ones. Distances span from the point where the KC joins the bundle to the joining point of the KC nearest to the neuropil. Differences between all groups are significant (***P < 0.0001; single-claw and multi-claw KC comparison P = 0.0237).
The dendrites of the mushroom body output neurons (MBONs) respect the DAN compartments in adults and larvae. It has been shown in adult *Drosophila* that co-activation of KCs and DANs can associatively modulate the KC–MBON synapse. Thus, the compartments represent anatomical and functional MB units where sensory input (KCs) is integrated with internal reinforcement signals (DANs/OANs) to modulate instructive output for behavioural control (MBONs). However, the synaptic connectivity of KCs, DAN/OANs, and MBONs at this crucial point of integration was previously unknown.

Furthermore, studies in adult *Drosophila* have shown that, despite the compartmental organization of the MB, many MBONs interact with MBONs from other compartments, suggesting that the MBON network functions combinatorially during memory formation and retrieval. However, a comprehensive account of all MB neuron connections is lacking. Thus, to provide a basis for understanding how the MB, a prototypical parallel fibre learning and memory circuit, functions as an integrated whole, we provide a full, synapse-resolution connectome of all MB neurons of an L1 *Drosophila* larva.

### 3.3 Results

We reconstructed all the KCs in both brain hemispheres of an L1 *Drosophila* larva and identified all of their pre- and postsynaptic partners. We found 223 KCs (110 on the left, 113 on the right), of which 145 were mature (73 on the left, 72 on the right). Immature KCs either lack or have tiny dendrites, and their axons terminate early with filopodia typical of axon growth cones. Every mature KC presents a well-developed dendrite, and its axon innervates all of the MB compartments. Although the number of KCs was different between the two sides (Fig. 1a), we found exactly 24 MBONs, 7 DANs, 2 paired and 2 unpaired OANs, and 5 additional modulatory input neurons (which we call MBINs or mushroom body input
neurons, a term we also use to refer to all the modulatory neurons collectively) of unknown neurotransmitter identity (Supplementary Fig. 1a and Supplementary Table 3). An additional pair of GABAergic neurons, homologous to the anterior paired lateral (APL) neurons in the adult fly\textsuperscript{29,30}, synapses reciprocally with all mature ipsilateral KCs (Extended Data Fig. 1).

### 3.3.1 PN inputs to the KCs

We identified all the sensory PNs and their connections onto KCs. KC dendrites have claw-like structures that wrap around PN axon boutons in the MB calyx\textsuperscript{6} (Extended Data Fig. 2b). We identified input from 21 uniglomerular olfactory PNs on each side\textsuperscript{12}, 5 and 7 multiglomerular PNs\textsuperscript{12}, and 14 and 16 non-olfactory PNs on the left and right sides, respectively. Non-olfactory PNs include thermal, visual, gustatory, mixed olfactory, and possibly other modalities (Fig. 1d, e and Supplementary Table 1). A subset of mature KCs receive input from only one PN (single-claw KCs), while the remaining KCs (multi-claw KCs, as in the adult fly\textsuperscript{17}) receive input from two to six PNs\textsuperscript{14} (Fig. 1d and Extended Data Fig. 2c). Notably, single-claw KCs are born earlier than multi-claw KCs (Fig. 1f). KCs with different numbers of claws receive roughly the same number of synapses summed across claws (Extended Data Fig. 2a). This suggests that multi-claw KCs may require input from multiple PNs to respond, assuring combinatorial selectivity\textsuperscript{31,32}.

Two features of the PN-to-KC connectivity are immediately visible (Fig. 1d): the contrast between the ordered and apparently disordered connections onto single-claw KCs and multi-claw KCs, respectively, and the existence of KCs that do not receive any uni-glomerular PN input. Most KCs (77\%) receive exclusively olfactory input from uni- or multi-glomerular PNs, while the others receive non-olfactory input (Fig. 1d, e). Prominent among these are two KCs per side that exclusively receive thermal information. No structure is apparent in the olfactory PN input to multi-claw KCs (Fig. 1d; see Extended Data Fig. 3b–e for further analysis), consistent with previous analyses in adults\textsuperscript{16,17} and larvae\textsuperscript{14}. Structure was found,
however, when all the PNs were included in the analysis, reflecting the presence of specialized non-olfactory KCs (Extended Data Fig. 3a). We also determined that PN connections onto multi-claw KCs in the left and right hemispheres are statistically independent. Only one bilateral pair of multi-claw KCs receives input from the same set of homologous PNs (marked by asterisks in Fig. 1d), no more than predicted by a random model ($P = 0.52$; Extended Data Fig. 3e). Such asymmetry has not been observed previously in the L1, where strongly connected presynaptic partners of identified neurons have always been seen to have left and right homologues $^{11,12}$.

In contrast to the randomness of multi-claw KCs, the number of single-claw KCs is significantly greater than predicted by random models (Fig. 2a), and Fig. 1d shows clear structure in their wiring. If single-claw KCs sampled random PNs, we would expect to find about five pairs of single-claw KCs innervated by the same PN per hemisphere (‘duplicates’). The data indicate zero left-hemisphere and one right-hemisphere duplicate ($P < 0.002$ and $P < 0.005$ in the random model, respectively). By contrast, the random model predicts that duplicates should be rare for multi-claw KCs, and the data reveal only two ($P = 0.93$). The fact that single-claw KCs appear earliest in development suggests that a top priority, initially, is to assure that a complete set of signals is relayed to the MBONs, which is not guaranteed with random wiring. Indeed, we calculated that 27 (left) and 44 (right) randomly wired multi-claw KCs, on average, would be needed to achieve the same level of coverage of PN inputs as the 17 (left) and 19 (right) single-claw KCs (Methods).
Figure 2 KC connectivity reduces redundancy and optimizes stimulus discrimination. a, Distribution of KC claw numbers compared with random models. Random models have significantly fewer single-claw KCs ($P < 10^{-5}$). Grey circles and lines denote mean and 95% confidence intervals. b, Classification error rate of a readout of the KC representation trained on a stimulus discrimination task. Observed connectivity (blue and orange) is compared with random models (grey) in which KCs have different distributions of average claw numbers. In a and b, grey circles and lines denote mean and 95% confidence intervals for random models. c, Average performance of models with purely random connectivity (grey) or random multi-claw plus non-random single-claw KCs (white). Standard error of the mean is smaller than the marks. d, Number of APL-to-KC synapses, which is correlated with claw number. e, Number of presynaptic KC-to-KC connections, which is inversely related to postsynaptic claw number. Dendro-dendritic (filled circles) or all synapses (open circles) are shown. In d and e, coloured circles and lines denote mean ± s.e.m. from the reconstructed data. f, Dimension of KC representation in models with only feedforward PN-to-KC connections (FF), with APL-mediated inhibition (FF + APL), or with inhibition and excitatory KC-to-KC connections (FF + APL + KC). Dimension is slightly reduced by dendritic KC-to-KC connections (right, filled bars) but strongly reduced by axonic KC-to-KC connections (open bars). Facilitatory axonic KC-to-KC connections (hatched bars) yield an intermediate reduction.

3.3.2 PN-to-KC connectivity optimizes associative learning

The lack of duplication in PN-to-KC connections suggests that MB wiring is well suited to promote KC diversity. We hypothesized that this diversity produces a high-dimensional odour representation that supports stimulus classification. To test this idea, we developed a model in which KCs produce sparse responses to random combinations of odour-evoked PN activity (Methods). We compared the performance on a stimulus classification task of a model MBON
in networks with the observed PN-to-KC connectivity and completely randomly connected models with varying degrees of connectivity (see Methods). Fully random networks have few of the single-claw KCs observed in the reconstruction (Fig. 2a).

The observed connectivity leads to performance superior to the average of purely random model networks (Fig. 2b). Motivated by the observation that unique single-claw KCs are born early in MB development (Fig. 1f), we hypothesized that their presence may be particularly important when the number of KCs is small. We therefore compared the performance of networks with only randomly connected KCs to that of networks with the same total number of KCs but containing a subpopulation of unique single-claw KCs. In networks with few KCs, the presence of single-claw KCs substantially improves performance (Fig. 2c). As additional KCs are added, the advantage of single-claw KCs diminishes. For networks constructed using estimates of adult Drosophila KC connectivity, where single-claw KCs have not been identified, unique single-claw KCs provide only a small benefit.

### 3.3.3 Inhibitory KC interactions via the APL neuron

The MB is innervated by the GABAergic (γ-aminobutyric-acid-releasing) APL neuron in the adult fly and in the larva. APL synapses reciprocally with all mature KCs (Extended Data Fig. 1a). Although APL receives most of its input from multi-claw KCs, individual single-claw KCs typically have more synapses onto APL dendrites than multi-claw KCs (Extended Data Fig. 1b, c). Conversely, APL connects more strongly to KCs with more claws (Fig. 2d and Extended Data Fig. 1c). We extended our model to include APL-mediated feedback inhibition and assessed its effect on the dimension of the KC representation. The dimension quantifies the level of decorrelation of KC responses and is related to their ability to support stimulus classification (Fig. 2b, c; see Methods). The addition of recurrent APL inhibition increases the dimension by approximately 30% (Fig. 2f), consistent with the proposed role of APL in maintaining sparse, decorrelated KC responses. The increased inhibition
received by multi- compared with single-claw KCs might reflect a greater need for decorrelation of their responses given the larger overlap of their PN inputs.

### 3.3.4 Recurrent KC interactions

We next examined whether KCs directly interact with each other, a possibility suggested by previous studies in *Drosophila* and other species. We found that, on average, 60% of the synapses received by KCs come from other KCs and 45% of KC output synapses are onto other KCs (Fig. 2e and Extended Data Fig. 2d–g). Most KC-to-KC synapses are axo-axonic and located in the peduncle and MB lobes; a much smaller fraction are dendro-dendritic and located in the calyx (Extended Data Fig. 2h). The largest number of KC-to-KC connections occurs between the two thermosensory KCs (Supplementary Table 1), but these KCs also make large numbers of connections to olfactory and visual KCs. Single-claw KCs make more recurrent connections, on average, than multi-claw KCs, and both single- and multi-claw KCs tend to connect reciprocally to KCs of the same type (Extended Data Fig. 4a–g). However, we found no relationship between the similarity of the PN inputs to KC pairs and the number of KC-to-KC synapses formed between them (correlation coefficients 0.006 and −0.02 for left and right hemispheres; \( P > 0.3 \), compared with shuffled network).

KC s have been shown to be cholinergic in the adult, so it is likely that KC-to-KC connections are depolarizing. When strong dendro-dendritic KC connections are added to our model, dimension only decreases slightly (Fig. 2f). Additional subthreshold facilitatory axo-axonic KC-to-KC connections also have a weak effect (Fig. 2f, hatched bars). However, if the axo-axonic connections we model are stronger, the dimension collapses because of the large correlations introduced by this recurrence (Fig. 2f, open bars). Although our model does not reveal a functional role for KC-to-KC connections, it is possible that processes that we did not model, such as experience-dependent modulation of their synaptic strengths, may modify the
representation to favour either behavioural discrimination or generalization\textsuperscript{37}. Further characterization of these connections is needed to assess this hypothesis.

### 3.3.5 MBINs and MBONs

We next identified the MBONs and modulatory neurons (MBINs) associated with every MB compartment (Fig. 3a). Each compartment is innervated by one to five MBONs and one to three MBINs (most often DANs and OANs), except for the shaft compartment of the medial lobe which lacks MBINs in L1 larvae (but has them in L3 (ref. 8)). Most MBONs and MBINs innervate a single MB compartment (although most MBINs present a bilateral axon, innervating both the left and right homologous compartments), with few exceptions (MBIN-l1 and 5/24 MBONs; Fig. 3d and Extended Data Fig. 5). Most MBINs (over 80\%) are primarily presynaptic to other MB neurons, but some allocate 50\% or more of their outputs to non-MB neurons (Extended Data Fig. 6a and Supplementary Table 2b). We also found that larger MBIN axons make more synapses onto KCs, and likewise for KC synapses onto larger MBON dendrites (Extended Data Fig. 6b, c).

Antibody labelling of green fluorescent protein (GFP)-labelled neurons showed that seven MBINs are dopaminergic (DAN-c1, -d1, -f1, -g1, -i1, -j1, and -k1; Extended Data Fig. 5), four are octopaminergic (OAN-a1, -a2, -e1, and -g1), three others are neither dopaminergic nor octopaminergic (MBIN-e1, -e2, and -l1), and two were not technically accessible (MBIN-b1 and -b2) (Supplementary Fig. 2a). MBONs are glutamatergic, cholinergic, or GABAergic (Supplementary Fig. 2b), the same set of neurotransmitters seen for adult MBONs\textsuperscript{18} (Supplementary Table 3).
Figure 3 A canonical circuit in every MB compartment. a, Electron microscopy-reconstructed DAN/OAN/MBINs (green) and MBONs (magenta). b, Canonical circuit present in every MB compartment, with previously unknown KC synapses onto MBINs (DAN, OAN, and others), and from these onto MBONs. c, Example of an MBIN (green dot) synapse with a KC (white dot) and an MBON (magenta dot). The same KC is also presynaptic to the MBON in close proximity. Dense- and clear-core vesicles are present near the DAN presynaptic site. d, The connectivity matrix between KCs, DAN/OAN/MBINs, and MBONs of the right-hemisphere MB shows specific, compartment-centric synapses among cells types. Each entry represents the number of synapses from a row to a column (values are averaged for KCs). Note the absence of DAN in SHA (develops later in larval life). DAN/OAN/MBINs synapse only onto MBONs innervating their compartment, and axoaxonically onto same-compartment DAN/OAN/MBINs. Note multi-compartment MBONs in the vertical lobe and lateral appendix (LA). CA, calyx; IP, intermediate peduncle; LP, lower peduncle; LT, lower toe; UT, upper toe; IT, intermediate toe.

3.3.6 A canonical circuit motif in each MB compartment

Electron microscopy reconstruction of MBONs and MBINs revealed a canonical circuit motif (Fig. 3b, d and Extended Data Fig. 5) that appears in every compartment, independent of MBIN or MBON neurotransmitters (except in the shaft compartment lacking modulatory input in L1). In this motif, KCs synapse onto MBONs, as previously shown in adult...
Drosophila⁵,⁶,²⁴,³⁶ and bees and locusts³,⁶,³⁸. However, we identified two unexpected connection types. The first are numerous KC-to-MBIN connections (Fig. 3d and Extended Data Fig. 5). Second, a sizeable fraction of MBIN presynaptic sites (which are polyadic) simultaneously contacts both KCs and MBONs, with generally at least one of the postsynaptic KCs synapsing onto one of the postsynaptic MBONs within less than a micrometre of the MBIN–KC synapse (Fig. 3c). Thus, MBINs synapse onto both the pre- and postsynaptic sides of many KC-to-MBON synapses. For comparison, MBONs receive on average 3.44% of their input from individual MBINs and 1.3% from an individual KC. If 5% of the 73 mature KCs are active in response to a given odour, as has been shown in the adult³¹, then the MBONs receive on average about 4.8% of their input from active KCs, very similar to the percentage of MBIN input.

MBINs convey reinforcement signals and are thought to modulate the efficacy of KC-to-MBON connections through volume release in the vicinity of KC presynaptic terminals¹,³,⁴,⁵,⁶,²⁴,²⁵,²⁶,²⁷ and, as expected, we observed MBIN axon boutons containing dense-core vesicles (Extended Data Fig. 7). We also observed dense-core vesicles in addition to clear-core vesicles in one-third of KCs (Extended Data Fig. 7), consistent with findings in the adult that many KCs co-release short neuropeptide F (sNPF) with acetylcholine³⁶.

Our electron microscopy reconstruction also revealed MBIN synapses containing clear vesicles (Extended Data Fig. 7 and Fig. 3c) indistinguishable from those that release classical neurotransmitters¹¹. All MBIN terminals with clear-core vesicles contained dense-core vesicles, mostly within presynaptic boutons (Extended Data Fig. 8). This suggests that MBINs may have two concurrent modes of action onto KCs and MBONs: activation via synaptic release (of dopamine, octopamine, or fast neurotransmitters³⁹) and neuromodulation via volume release. These two modes, coupled with the diverse connection types among KCs, MBINs, and MBONs, may provide a powerful and flexible substrate for associative learning.
3.3.7 Heterogeneous KC-to-MBON/MBIN connections

Previous studies in adult *Drosophila* have shown that different lobes and compartments within the lobes are involved in forming different types of memory\(^1,4,5,19,20,21,22,25,26,28,40,41\). Functional studies in larvae\(^8,42\) also suggest that vertical and medial lobes may be implicated in distinct types of memory formation (aversive and appetitive, respectively). Our electron microscopy study shows that all mature KCs make synaptic connections with MBONs and MBINs (DANs, OANs, and others) in both the vertical and medial MB lobes (Extended Data Figs 9a–c and 10a–c and Supplementary Table 4a, b). Furthermore, individual MBONs are innervated by between 40% and almost all of the KCs, with an average of 70% (Extended Data Fig. 9a). This extensive innervation provides MBONs access to the high-dimensional KC representation and suggests that individual KCs may be involved in the formation and storage of associations involving multiple stimuli and valences, as suggested for the adult\(^1,4,5\) (but see ref. 43).

Our electron microscopy reconstruction reveals that, in the larva, the axon terminals of all MBINs overlap with all KCs within a compartment and could potentially connect to all KCs, unlike in the adult\(^18,43\). Nevertheless, only subsets of KCs synapse onto either the MBIN or the MBON, or both, within a given compartment, with a broad distribution in the number of synaptic contacts (Extended Data Figs 9d, e and 10d, e and Supplementary Table 4a, b). Estimating connection strength using synapse number, distinct subsets of KCs synapse strongly with MBINs and MBONs in distinct compartments (Extended Data Figs 9c and 10c and Supplementary Tables 1 and 4a, b). This could arise from an innately broad distribution of synapse strengths or activity-dependent changes in synapse number. Either way, this heterogeneity implies that distinct MBONs and MBINs respond to heterogeneous combinations of active KCs.
3.3.8 Comprehensive profile of MBON inputs

Electron microscopy reconstruction and synaptic counting provides a comprehensive view of the signals carried by the MBONs (Fig. 4a, b and Supplementary Table 2a). In general, MBONs are among the neurons in the L1 larval brain receiving the largest number of inputs, with a median of 497 and a maximum of about 1,500 postsynaptic sites. The median for other neurons reconstructed so far is around 250 in L1 (refs 11, 12).

We analysed the detailed structure of KC-to-MBON connectivity to determine the nature of the sensory signals relayed to MBONs. About 23% of the KC input to MBONs originates in KCs that integrate inputs from non-olfactory PNs (Fig. 4b, c), significantly more than in networks with shuffled PN modalities ($P < 0.05$). This pattern of non-olfactory input is stereotyped: the fraction of non-olfactory input received by homologous MBONs across hemispheres is more similar than across different MBONs in the same hemisphere ($P < 0.001$, Mann–Whitney $U$-test). These observations, along with the sparse activity of olfactory KCs seen in adult flies$^{31}$, suggest that non-olfactory signals may have a large influence on the activity of certain MBONs despite the small number of non-olfactory KCs. To quantify this influence for one modality, we compared the total number of synapses made by thermosensory KCs onto the different MBONs to 0.05 times the total number of synapses made by non-thermosensory KCs (Extended Data Fig. 9f). This ratio quantifies the relative influence of a stimulus that activates thermosensory KCs to a typical odour stimulus that activates 5% of olfactory KCs$^{31}$. The ratio is high for some MBONs (-d3, -o1, and -b3) and stereotyped for homologous MBONs across hemispheres, suggesting that these MBONs may be wired to respond strongly to non-olfactory cues, such as temperature.
Figure 4 MBON inputs and circuits. a, MBONs are in the compartments innervated by their dendrites. Most connections among MBONs are axo-axonic, and fewer are axo-dendritic. Few MBONs avoid synapsing to others. Most inter-lobe connections are mediated by GABAergic (MBON-g1, -g2, -h1, -h2) and glutamatergic (MBON-i1, -j1, -k1) MBONs, potentially providing a substrate for lateral inhibition between compartments of opposite valence. b, Fraction of MBON inputs by neuron type. Left and right homologous MBONs are shown adjacent. Only some vertical lobe and lateral appendix MBONs get less than 80% of their input from MB neurons and less than 60% from KCs. Almost all multi-compartment MBONs (MBON-m1, -n1, -o1 and -p1) have a higher fraction of input from non-MB neurons than single-compartment MBONs. The fraction of inputs from PNs to MBONs via KCs is shown within the fraction of KC input (different shades of blue), computed by the product of the PN-to-KC and KC-to-MBON connections. Most MBONs receive a high fraction of olfactory input via KCs while few MBONs (-b3, -o1) get nearly half of their inputs via KCs from non-olfactory PNs. c, Percentages of different types of PN input to the KCs. While there are almost equal numbers of olfactory (olf.) and non-olfactory PNs synapsing onto KCs, non-olfactory PNs represent only about 12% of the inputs to KC dendrites.
We also examined whether the olfactory input received by homologous MBONs is stereotyped by computing the correlation between the number of connections they receive from each olfactory PN via KCs. Unlike for non-olfactory input, homologous MBONs across hemispheres receive a less similar pattern of olfactory PN input than different MBONs in the same hemisphere ($P > 0.99$, Mann–Whitney $U$-test), arguing against stereotypy. Therefore the lack of stereotypy in the olfactory PN-to-KC connectivity and the greater degree of stereotypy in the non-olfactory PN-to-KC connectivity are inherited by the MBONs.

Notably, MBONs do not receive their inputs exclusively from KCs, DANs, and other MBONs. We found that some compartments (Fig. 4b) have two kinds of MBONs that differ in the amount of input they receive from non-MB sources. Some MBONs are postsynaptic almost exclusively to MB neurons (over 90%), while some receive 50% or more of their inputs from non-MB neurons on dendritic branches outside the MB compartments (Fig. 4b and Supplementary Table 2a). MBONs with significant input from outside the MB typically receive input from other MBONs as well. The convergence of modulatory neurons, of olfactory, thermal, and visual KCs, and of non-MB inputs onto some MBONs suggests they may be flexible sites for learning and integration of multisensory and internal state information (via DANs, as suggested by functional studies in the adult$^{19,28,44,45}$, and possibly via the other new non-MB inputs identified here).

### 3.3.9 The MBON output network

In adult *Drosophila*, MBONs form a multi-layered feedforward network$^{18,28}$. Consistent with this, we found synaptic connections between MBONs in different compartments, on both the ipsi- and contralateral sides, that create a bilaterally symmetric structured feedforward circuit (Fig. 4a and Supplementary Table 5). Most inter-lobe connections are mediated by GABAergic (MBON-g1, -g2, -h1, -h2) and glutamatergic (MBON-i1, -j1, -k1) MBONs, potentially providing a substrate for lateral inhibition between the lobes. Glutamate has
previously been found to be inhibitory in the fly\textsuperscript{46}, but an excitatory effect on some neurons cannot be excluded. Some vertical lobe MBONs could also disinhibit (via inhibition of inhibitory medial lobe MBONs), or directly excite other vertical lobe MBONs, potentially providing within-region facilitation.

In addition, there is a hierarchy of interactions across regions of the MB. MBONs of the peduncle and calyx are exclusively at the bottom of the inhibitory hierarchy, receiving inputs from GABAergic MBONs from both the vertical lobe and the medial lobe, but not synapsing onto any other MBONs (Fig. 4a). Furthermore, the medial lobe may also disinhibit the peduncle MBON-c1 that the vertical lobe inhibits (Fig. 4a).

3.3.10 MBON connections to MBINs

The MB connectome further reveals several feedback connections from MBONs onto MBINs of the same compartment (Fig. 5a, b). MBON-e2 from the tip compartment of the vertical lobe (UVL) synapses onto the dendrites of OAN-e1 outside the MB (bilateral; Fig. 5a and Supplementary Fig. 1b). MBON-q1 from the intermediate (IVL) and lower vertical lobe (LVL) compartments is presynaptic to the axons of the DANs from these two compartments in addition to the MBIN-l1 from the lateral appendix compartment (Fig. 5b and Supplementary Fig. 1b). An MBON-to-DAN feedback connection was found in the α1 compartment of the adult vertical lobe and is implicated in the formation of long-term memory\textsuperscript{23}.

Notably, we also found hetero-compartment feed-across connections where MBONs innervating one region of the MB synapse onto MBINs innervating other regions (Fig. 5c, d and Supplementary Fig. 1b). The feed-across motifs could play a role during conflicting memory formation\textsuperscript{20,41} or during reversal learning, and more generally they could enhance the flexibility of modulatory input to the MB.
3.4 Discussion

We provide the first complete wiring diagram of a parallel fibre circuit for adaptive behavioural control. Such circuits exist in various forms, for example the cerebellum in vertebrates and the MB in insects. They contribute to multiple aspects of behavioural control including stimulus classification, the formation and retrieval of Pavlovian associations, and memory-based action selection. A comprehensive wiring diagram of such a multipurpose structure is an essential starting point for functionally testing the observed structural connections and for elucidating the circuit implementation of these fundamental brain functions.
Even though individual neurons may change through metamorphosis, many of the basic aspects of the MB architecture are shared between larval and adult *Drosophila* stages and with other insects (see Supplementary Tables 6 and 7). We therefore expect that the circuit motifs identified here are not unique to the L1 developmental stage, but instead represent a general feature of *Drosophila* and insect MBs.

### 3.4.1 A canonical circuit in each MB compartment

Our electron microscopy reconstruction revealed a canonical circuit in each MB compartment featuring two unexpected motifs in addition to the previously known MBIN-to-KC and KC-to-MBON connections. First, we were surprised to observe that the number of KC-to-MBIN and KC-to-MBON synapses are comparable. As KCs were shown to be cholinergic in adults, KC-to-MBIN connections could be potentially depolarizing. Untrained, novel odours can activate DANs in adult *Drosophila* and OANs in bees. Similar brief short-latency activations of dopamine neurons by novel stimuli are observed in monkeys, too, and are interpreted as salience signals. Learning could potentially modulate the strength of the KC-to-MBIN connection, either weakening it or strengthening it. The latter scenario could explain the increase in DAN activation by reinforcement-predicting odours observed in adult *Drosophila*, bees, and monkeys. In addition, dopamine receptors have been shown to be required in *Drosophila* KCs for memory formation. Another unexpected finding was that MBINs synapse directly onto MBONs, rather than only onto KCs. Such a motif could provide a substrate for neuromodulator-gated Hebbian spike-timing dependent plasticity, which has been observed in the locust MB.

### 3.4.2 Dimensionality of the KC representation

In addition to random and bilaterally asymmetric olfactory and structured non-olfactory PN-to-KC connectivity (Extended Data Fig. 3), our analysis identified single-claw KCs whose
number and lack of redundancy are inconsistent with random wiring (Fig. 2). Random wiring has previously been shown to increase the dimension of sensory representations when the number of neurons participating in the representation is large compared with the number of afferent fibres, as in the cerebellum or adult MB\textsuperscript{13,33}. However, our model shows that when the number of neurons is limited, random wiring alone is inferior to a combination of random and structured connectivity that ensures each input is sampled without redundancy. The presence of single-claw KCs may reflect an implementation of such a strategy. In general, our results are consistent with a developmental program that produces complete and high-dimensional KC sensory representations to support stimulus discrimination at both larval and adult stages.

3.4.3 Mutual inhibition in the MBON–MBON network

This study reveals the complete MBON–MBON network at synaptic resolution (Fig. 4a). Previous studies in the larva have shown that odour paired with activation of medial and vertical lobe DANs leads to learned approach\textsuperscript{8} and avoidance\textsuperscript{7,42}, respectively. Our connectivity analysis reveals that glutamatergic MBONs from the medial lobe laterally connect to MBONs of the vertical lobe. The glutamatergic MBON–MBON connections could be inhibitory\textsuperscript{46}, although further studies are needed to confirm this. Furthermore, inhibitory GABAergic MBONs from the vertical lobe laterally connect to MBONs of the medial lobe. An example is the feedforward inhibition of medial lobe MBON-i1 output neuron by the vertical lobe GABAergic MBON-g1, -g2 output neurons. A similar motif has been observed in the \textit{Drosophila} adult, where aversive learning induces depression of conditioned odour responses in the approach-promoting MBON-MVP2 (MBON-11), which in turn disinhibits conditioned odour responses in the avoidance-promoting MBON-M4/M6 (MBON-03) because of the MBON-MVP2 to MBON-M4/M6 feedforward inhibitory connection\textsuperscript{18,25,26,27,28,40}. 
Combining the present connectivity analysis of the MBON–MBON network in the larva and previous studies in the adult *Drosophila*\(^5\)\(^{18,25,28,40}\), the rule seems to be that MBONs encoding opposite learned valance laterally inhibit each other. Such inhibitory interactions have been proposed as a prototypical circuit motif for memory-based action selection\(^{50}\).

### 3.5 Methods

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

#### 3.5.1 Circuit mapping and electron microscopy

We reconstructed neurons and annotated synapses in a single, complete central nervous system from a 6-h-old [*iso*] *Canton S* GI × *w [iso]* 5905 larva acquired with serial section transmission electron microscopy at a resolution of 3.8 nm × 3.8 nm × 50 nm, which was first published in ref. 11 with the detailed sample preparation protocol. Briefly, the central nervous systems of 6-h-old female larvae were dissected and fixed in 2% gluteraldehyde 0.1 M sodium cacodylate buffer (pH 7.4) to which an equal volume of 2% OsO\(_4\) in the same buffer was added, and microwaved at 350-W, 375-W and 400-W pulses for 30 s each, separated by 60-s pauses, and followed by another round of microwaving but with 1% OsO\(_4\) solution in the same buffer. Then samples were stained *en bloc* with 1% uranyl acetate in water by microwave at 350 W for \(3 \times 3\) 30 s with 60-s pauses. Samples were dehydrated in an ethanol series, then transferred to propylene oxide and infiltrated and embedded with EPON resin. After sectioning the volume with a Leica UC6 ultramicrotome, sections were imaged semi-automatically with Leginon\(^{51}\) driving an FEI T20 transmission electron microscope (Hillsboro), and then assembled with TrakEM2 (ref. 52) using the elastic method\(^{53}\).
To map the wiring diagram, we used the Web-based software CATMAID\textsuperscript{54}, updated with a novel suite of neuron skeletonization and analysis tools\textsuperscript{55}, and applied the iterative reconstruction method\textsuperscript{55}. All annotated synapses in this wiring diagram fulfilled the four following criteria of mature synapses\textsuperscript{11,55}: (1) there was a clearly visible T-bar or ribbon; (2) there were multiple vesicles immediately adjacent to the T-bar or ribbon; (3) there was a cleft between the presynaptic and the postsynaptic neurites, visible as a dark–light–dark parallel line; (4) there were postsynaptic densities, visible as black streaks hanging from the postsynaptic membrane.

In this study, we validated the reconstructions as previously described\textsuperscript{11,55}, a method successfully used in multiple studies\textsuperscript{11,55,56,57,58,59,60}. Briefly, in Drosophila, as in other insects, the gross morphology of many neurons is stereotyped and individual neurons are uniquely identifiable on the basis of morphology\textsuperscript{61,62,63}. Furthermore, the nervous system in insects is largely bilaterally symmetrical and homologous neurons are reproducibly found on the left and the right side of the animal. We therefore validated MBON, DAN, OAN, MBIN, APL, and PN neuron reconstructions by independently reconstructing synaptic partners of homologous neurons on the left and right sides of the nervous system. By randomly re-reviewing annotated synapses and terminal arbors in our dataset, we estimated the false positive rate of synaptic contact detection to be 0.0167 (1 error per 60 synaptic contacts). Assuming the false positives were uncorrelated, for an $n$-synapse connection the probability that all $n$ were wrong (and thus that the entire connection was a false positive) occurred at a rate of $0.0167^n$. Thus, the probability that a connection was false positive reduced dramatically with the number of synaptic connections contributing to that connection. Even for $n = 2$ synapse connections, the probability that the connection was not true was 0.00028 (once in every 3,586 two-synapse connections) and we called connections with two or more connections ‘reliable’ connections. See refs 11, 55 for more details.
3.5.2 Identification of PNs

The olfactory PNs from this same electron microscopy volume were previously traced and identified\(^{12}\), with each olfactory glomerulus being innervated by exactly one single uniglomerular PN in the larva\(^64\). To identify the photosensory and thermosensory PNs connected to KCs, we identified all neurons downstream of 12 photosensory neurons\(^{65}\) in the left and right brain hemispheres and all neurons downstream of three previously characterized cold-sensing neurons, expressed in the 11F02-GAL4 line\(^66\).

3.5.3 Learning in first-instar larvae with a substitution experiment

Learning experiments were performed as described in refs 8, 10, 42, 67. The fly strain \(PGMR58E02\)-GAL4\(_{attP2}\) (Bloomington Stock Center number 41347) and the \(attP2\) control strain were crossed to \(P20XUAS-IVS-CsChrimson.mVenus_{attP18}\) (Bloomington Stock Center number 55134). Flies were reared at 25 °C in darkness on 4% agarose with a yeast and water paste including retinal in a 0.5 mM final concentration. First-instar feeding-stage larvae in groups of 30 individuals were placed on plates filled with 4% agarose and the odour ethyl-acetate (100-times diluted in distilled water) was presented on filter papers located on the lid. Larvae were exposed to constant red light (630 nm, power 350 μW cm\(^{-2}\)) during this odour presentation for 3 min. Subsequently larvae were transferred to a new plate and no odour was presented in the dark for 3 min. This paired training cycle was repeated twice. We also performed the unpaired experiment (odour presentation in the dark and red light without odour) with another group of 30 naive larvae. After a 5-min test with odour presentation on one side of the lid, larvae were counted on the side of the odour, no odour, and a 1 cm area in the middle of the plate. Preference and performance indices were calculated as in refs 8, 67: that is, for both the paired and unpaired, the number of larvae on the no-odour side were subtracted from the number of larvae on the odour side, and the result was divided by the total
number of larvae on the plate (including those in the middle). The performance index was half of the value for the paired minus the unpaired preference scores.

### 3.5.4 Random models of PN-to-KC connectivity

To generate random PN-to-KC models, individual PN connection probabilities were computed as the number of multi-claw KCs contacted divided by the total number of connections onto multi-claw KCs. Probabilities were computed separately for the two hemispheres. To generate the random networks in Fig. 2a, we assumed that PN-to-KC connections were formed independently according to these probabilities. We then iteratively generated KCs until the number of multi-claw KCs matched the number found in the reconstruction. To compare dimension and classification performance for random networks with varying degrees of connectivity (Fig. 2c), we scaled the individual PN connection probabilities so that, on average, each KC received between one and ten claws (Fig. 2b, c). In this case, we fixed the total number of KCs (single- and multi-claw) to be equal to the number found in the reconstruction.

To obtain KCs with a specified number of claws (Fig. 2b), PN connections were determined by weighted random sampling using the individual connection probabilities described above. When comparing detailed connectivity statistics with random models (Extended Data Fig. 3), we used this type of sampling to match the KC claw count distribution for the random models to that of the data.

We also evaluated how many random multi-claw KCs would be required to ensure coverage of the subset of PNs that were connected to single-claw KCs. We restricted our analysis to these PNs and iteratively added random multi-claw KCs, with the claw counts distribution determined by the reconstructed connectivity, until each PN was connected to at least one KC.
For this analysis, PN connection probabilities were computed using the full PN-to-KC connectivity matrix.

### 3.5.5 Model of sparse KC responses

We assumed that the activity of the $i$th KC was given by $s_i = \left[\sum J_{ij} x_j - \theta_i\right]_+$, where $\left[\cdot\right]_+$ denotes rectification, $J$ is the matrix of PN-to-KC connections, $x_j$ is the activity of the $j$th PN, and $\theta_i$ is the KC activity threshold. Simulated odour-evoked PN activity was generated by sampling the value of each $x_j$ independently from a rectified unit Gaussian distribution. The value of $\theta_i$ was adjusted so that each KC fired for a fraction $f = 0.05$ of odours. The entries of $J$ were either the synapse counts from the reconstructed data or, for the case of random connectivity, chosen in the manner of the previous section with a weight sampled randomly from the distribution of PN-to-multi-claw-KC synaptic contact numbers found in the reconstruction.

To assess classification performance (Fig. 2b, c), the KC responses to eight odours were evaluated (ten odours were used when simulating the adult MB). Odour responses again consisted of rectified unit Gaussian random variables, but corrupted by Gaussian noise with standard deviation 0.2. A maximum-margin classifier was trained to separate the odours into two categories to which they were randomly assigned, and the error rate was assessed when classifying odours with different noise realizations. Dimension (Fig. 2f) was assessed by estimating the KC covariance matrix $C_{ij} = \langle (z_i - \langle z_i \rangle)(z_j - \langle z_j \rangle) \rangle$, where $z_i$ is the $z$-scored activity of neuron $i$, using 1,000 random simulated odours. We then computed $\text{dim} = \frac{\sum \hat{\lambda}_i^2}{\sum \hat{\lambda}_i^2}$, where $\{\hat{\lambda}_i\}$ are the eigenvalues of $C$ (ref. 33).

To implement recurrence, we modified the model so that the activity of the $i$th KC was given by $s_i(t) = \Theta(\sum J_{ij} x_j + \sum J^{\text{rec}}_{ik} s_k(t-1) - \theta_i)$, where $J^{\text{rec}}_{ik}$ is a matrix of recurrent KC-to-KC interactions and $t$ is the timestep. $J^{\text{rec}}$ is equal to $\alpha_1 J^{\text{KC\rightarrow KC}} + \alpha_2 J^{\text{APL}}$, where $J^{\text{KC\rightarrow KC}}$ was determined by the KC-to-KC synapse counts (either dendro-dendritic only or both dendro-
dendritic and axo-axonic) and $J_{jk}^{APL} \propto c_i^{APL \rightarrow KC} c_k^{KC \rightarrow APL}$, with $c_i^{APL \rightarrow KC}$ representing the number of APL synapses onto the $i$th KC and $c_k^{KC \rightarrow APL}$ representing the number of synapses onto APL from the $k$th KC. $J^{APL}$ was scaled so that the average of its entries equalled that of $J$. The scalar $\alpha_1 = 0.33$ was chosen so that the strength of KC-to-KC and PN-to-KC connections were comparable, while $\alpha_2 = 0.05$ represents the gain of the APL neuron. A single randomly chosen KC was updated on each timestep, with the number of timesteps equal to five times the number of KCs. We also modelled facilitatory KC-to-KC interactions by assuming KC-to-KC connections only depolarized the postsynaptic neuron when it was already above threshold.

### 3.5.6 Immunostaining

Third-instar larvae were put on ice and dissected in PBS. For all antibodies, brains were fixed in 3.6% formaldehyde (Merck) in PBS for 30 min except anti-dVGlut, which required Bouin’s fixation\textsuperscript{68}. After several rinses in PBT (PBS with 1% or 3% Triton X-100; Sigma-Aldrich), brains were blocked with 5% normal goat serum (Vector Laboratories) in PBT and incubated for at least 24 h with primary antibodies at 4 °C. Before application of the secondary antibodies for at least 24 h at 4 °C or for 2 h at room temperature, brains were washed several times with PBT. After that, brains were again washed with PBT, mounted in Vectashield (Vector Laboratories), and stored at 4 °C in darkness. Images were taken with a Zeiss LSM 710M confocal microscope. The resulting image stacks were projected and analysed with the image processing software Fiji\textsuperscript{69}. Contrast and brightness adjustment, rotation, and arrangement of images were performed in Adobe Photoshop (Adobe Systems).

### 3.5.7 Antibodies

Brains were stained with the following primary antibodies: polyclonal goat anti-GFP fused with FITC (1:1,000, Abcam, ab6662), polyclonal rabbit anti-GFP (1:1,000, Molecular Probes,
A6455), polyclonal chicken anti-GFP (1:1,000, Abcam, ab13970), monoclonal mouse anti-TH (1:500, ImmunoStar, 22941), polyclonal rabbit anti-TDC2 (1:200, CovalAb, pab0822-P), monoclonal mouse anti-ChAT (1:150, Developmental Studies Hybridoma Bank, ChaT4B1), polyclonal rabbit anti-GABA (1:500, Sigma, A2052), and rabbit anti-dVGlut (1:5,000) for identifying GFP-positive, dopaminergic, octopaminergic, cholinergic, GABAergic, and glutamatergic neurons. The following secondary antibodies were used: polyclonal goat anti-chicken Alexa Fluor 488 (1:200, Molecular Probes, A11039), polyclonal goat anti-rabbit Alexa Fluor 488 (1:200, Molecular Probes, A11008), polyclonal goat anti-rabbit Alexa Fluor 568 (1:200, Molecular Probes, A11011), polyclonal goat anti-rabbit Cy5 (1:200, Molecular Probes, A10523), polyclonal goat anti-mouse Alexa Fluor 647 (1:200, Molecular Probes, A21235), and polyclonal goat anti-rabbit Alexa Fluor 647 (1:200, Molecular Probes, A21245).

3.5.8 Identifying GAL4 lines that drive expression in MB-related neurons

To identify GAL4 lines (listed in Supplementary Table 3) that drove expression in specific MB-related neurons, we performed single-cell FLP-out experiments (for FLP-out methodology see refs 11, 71) of many candidate GAL4 lines. We generated high-resolution confocal image stacks of the projection patterns of individual MBONs/DANs/OANs (multiple examples per cell type), which allowed their identification. Most MBONs/DANs/OANs were uniquely identifiable on the basis of the dendritic and axonal projection patterns (which MB compartment they projected to and the shape of input or output arbor outside the MB). These were also compared with previously reported single-cell FLP-outs of dopaminergic and octopaminergic neurons in the larva. Some compartments were innervated by an indistinguishable pair of MBONs or MBINs.
3.5.9 Estimating the birth order of KCs

KCs arise from four neuroblasts per hemisphere that divide continuously from the embryonic to the late pupal stages\textsuperscript{76,77}. The primary neurite of newborn KCs grows through the centre of the MB peduncle, pushing existing KCs to the surface of the peduncle\textsuperscript{78} (Extended Data Fig. 4h). As new cells are added, their somas remain closer to the neuroblast at the surface of the brain and push away existing somas. The point of entry into the lineage bundle of each KC primary neurite remains as a morphological record of the temporal birth order, with some noise. For every KC, we measured the distance from the point where its primary neurite joined the lineage bundle to a reference point consisting of the point where the neurite of the last KC (potentially the oldest of that lineage) joined the bundle, before the bundle entered the neuropil (Fig. 1f) to form the peduncle together with the other three bundles. This measurement revealed that single-claw KCs are born early in MB development, followed by two-claw KCs, three-claw KCs, and so on (Fig. 1f), with the last-born KCs being immature and expected to develop into multi-claw KCs later in larval life\textsuperscript{79}.

3.5.10 Data availability

The electron microscopy volume is available at http://openconnecto.me/catmaid/, entitled ‘acardona_0111_8’. The coordinates for the skeletons modelling neuronal arbors and for the synapses, and the data tables from which all graphs were made, are in the Supplementary Information. Source data for figures and Extended Data figures are available in the Supplementary Information (see below and https://www.nature.com/articles/nature23455#f15). Any other data are available from the corresponding authors upon reasonable request.
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3.7 Author information

3.7.1 Contributions

3.7.2 Competing interests

The authors declare no competing financial interests.

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3.8 Extended Data

Extended Data Figure 1: Connectivity of the larval APL neuron. a, Morphology of the right-hemisphere larval APL neuron. While APL dendrites are postsynaptic to the KCs in LA, LVL, IVL, and all medial lobe compartments, its axon is both post- and presynaptic to the MB calyx. Presynaptic sites in red and postsynaptic sites in blue. b, APL connectivity with KC types. Connections are displayed as fractions of input onto the receiving neurons. APL forms more axo-dendritic connections with multi-claw than with single-claw KCs. All mature KCs connect to APL on its dendrite as well as on its axon. c, Strength of synaptic connections between KCs and the APL neuron for single-claw and multi-claw KCs separately. While single-claw KCs have a higher synapse count connecting to the APL dendrites than multi-claw KCs, both groups of KCs project a similar number of synapses to the APL axon. In the calyx, APL makes more synapses onto multi-claw than onto single-claw KCs. In the lobes, single-claw KCs make more synapses with APL pre- than postsynaptically.
Extended Data Figure 2: Input onto KC dendrites and KC–KC connections. a, Total synaptic input onto KC dendrites from PNs, the APL neuron, and calyx MBINs for single- and multi-claw KCs from both hemispheres. Insert: sum of the KC postsynaptic sites in the calyx as a function of the number of claws. Grey circles show individual KCs and black line shows the mean. Young KCs that have no claws or only form short branches in the calyx have few postsynaptic sites. Mature KCs forming one to six claws present a similar total amount of postsynaptic sites in the calyx. b, A PN bouton (blue) and its associated KC dendritic arborizations. Stars indicate all dendrites of the single-claw KC for this PN. c, Prototypical examples of KCs according to the structure of their dendrites, commonly known as ‘claws’. In first instar, we define each ‘claw’ (indicated by arrowheads) as a connection from a PN providing at least 10% of the postsynaptic sites of the KC dendritic arbor. This connectivity-based definition generally agrees with the count of physically separate claw-like dendritic branches that wrap around the axon terminals of the PNs. Single-claw KCs have not yet been described in the adult fly or other insects. d–g, Frequency of different numbers of postsynaptic KC partners of KCs with at least two-synapse connections, plotted separately for three different types of KC. d, Postsynaptic partners of single-claw KCs. e, Postsynaptic partners of multi-claw KCs. f, Postsynaptic partners of young KCs. g, KC input onto KCs as a percentage of total KC input. For most KCs, more than 50% of their presynaptic partners are other KCs. h, Morphology of an example reconstructed KC (left) and example electron micrographs showing KC–KC synapses (right). Dendro-dendritic connections are in the calyx compartment (1 and 2) and axo-axonic connections are located in the peduncle (3), vertical lobe (4), and medial lobe (5).
Extended Data Figure 3: Test of structure in PN-to-KC connectivity. a, We performed principal component analysis on the PN-to-KC connectivity matrices for the left and right hemispheres. The analysis was restricted to multi-claw KCs. The variance explained by each principal component in descending order is compared with that obtained from random models in which KCs sample PNs according to the individual PN connection probabilities (see Methods). Grey circles and bars denote mean and 95% confidence intervals for the variance explained by each principal component of the random connectivity matrices, while the black circles indicate the values obtained from data. Note a small deviation from the random model, probably because of the non-olfactory PNs. b, Same as a but restricted to connections between olfactory PNs and KCs. c, Same as b but combining the PN-to-KC connectivity for both hemispheres. The data are compared with a random model in which KCs in each hemisphere sample PNs randomly and independently (grey), and with a bilaterally symmetrical model in which the connectivity is random but duplicated across the two hemispheres (pink). d, To assess the ability of our method to identify structured connectivity, we also generated connectivity matrices in which a weak bias was added (blue). Networks were generated in which PNs were randomly assigned to one of two groups. For each KC, the probabilities of connecting to PNs belonging to one randomly chosen group were increased by 1%, while the probabilities for the other group were decreased by 1% (the baseline probabilities were on average approximately 5%). This procedure was performed independently for each KC and led to networks in which KCs preferentially sampled certain PNs. The data are inconsistent with this model, illustrating that biases of ∼1% connection probability can be identified using our methods. e, As an independent method to identify structure in the PN-to-KC wiring, we considered the distribution of olfactory PN overlaps for all KC pairs, defined as the number of olfactory PNs from which both KCs received input. This quantity specifically identified biases in the likelihood of KCs to sample similar inputs. As in b, no such structure was identified.
Extended Data Figure 4: KC–KC clustering. Synaptic connectivity between KCs reveals two communities within the MB. a, Heat map representation of the KC–KC network adjacency matrix, sorted by community structure as discovered by the Louvain method, which identifies groups of KCs with more within-group connections than expected by chance. We denote the denser community on each side as ‘Group 1’ and the other community as ‘Group 2’. Number of cells in each group is shown in the column labels. b, The number of observed 2+ synapse connections between pairs of KCs within and between groups in the same side of the body, normalized by the total number of all possible such connections (L, left; R, right). c, Distribution of number of synapses per edge for connections within and between each group. Boxes indicate interquartile interval, whiskers the 95th percentile, cross indicates median, outliers shown. d, Claw distribution by group (both sides aggregated). Note that all single-claw KCs are in Group 1. e, Total number of anatomical input synapses onto Group 1 and Group 2 KCs, including from non-KC sources. Group 1 cells have significantly more inputs than Group 2 cells on each side (P < 10^−10, t-test with Bonferroni correction). f, Total number of anatomical output synapses from Group 1 and Group 2 KCs, including synapses onto non-KC targets. Group 1 cells have significantly more outputs than Group 2 cells on each side (P < 10^−10, t-test with Bonferroni correction). g, KC anatomy labelled by group for the right MB. Note that both groups of KCs come from each lineage cluster. Labelled spots indicate locations of cross-section views in h. h, Cross-sections of the two principal axon branches of KCs in the left (above) and right (below) MBs at the locations indicated in g. Orientations of each cross-section are arbitrary.
Extended Data Figure 5: Neurons in the canonical circuit in every MB compartment. Neuronal morphology and connectivity of MBINs and MBONs participating in the canonical circuit motif of each MB compartment (MBINs in green and MBONs in magenta). Neurons connecting to the left-hemisphere MB are displayed in anterior view (right hemisphere has the same morphology, data not shown). Locations of MBINs synapsing on MBONs in a given compartment are shown depending on their location (inside the MB neuropil, black; outside the MB, orange). MBIN axons and MBON dendrites tile the MB into 11 distinct compartments. For each compartment, the MBIN and MBON neurons and their connections as a fraction of total input to the receiving neuron are shown on the right. MBONs are diverse in the neurotransmitter that they release (see legend). All compartments present the canonical circuit motif except for SHA, which does not develop its DAN until later in larval life.
Extended Data Figure 6: Fractions of postsynaptic inputs by cell type. a, Fractions of synaptic output from MBINs onto KCs, other MBINs, MBONs, and other neurons. Some MBINs show a high percentage of connections to MB neurons while others connect with less than 50% of their synapses to MB neurons. b, Number of MBIN–KC synapses in relation to the number of total synaptic outputs from MBIN axons, showing a positive correlation between presynaptic sites on the MBIN axon and synapses dedicated to KC population. c, Number of KC–MBON synapses in relation to the number of total synaptic inputs to MBON dendrites, showing a positive correlation between postsynaptic sites on the MBON dendrite and fraction of input from KCs.
Extended Data Figure 7: MBIN presynaptic vesicle types. Examples of electron micrographs of MBIN presynaptic sites and vesicles. We found three types of vesicle: large dense-core, small dense-core, and small clear vesicles. Octopaminergic and dopaminergic neurons contain small clear vesicles in addition to other vesicle types. While OANs have all the same type of large dense-core vesicles, DANs show a variety of small dense-core vesicles. We found small dense-core vesicles in one-third of KCs. Some of these were single-claw, others were multi-claw, some received olfactory input, and others non-olfactory PN input. The largest number of dense-core vesicles was observed in the two thermosensory KCs. Scale bar, 500 nm in all panels.
Extended Data Figure 8: Dense-core vesicles in OANs and DANs. a, Morphology of OAN-a1 and -a2 innervating the calyx of both MBs. b, Location of presynaptic sites (red) and dense-core vesicles (DCVs/black) along the axon of OAN-a1 and -a2. c, DCVs colour-coded by their distance to the closest presynaptic site on the axon of OAN-a1 and -a2. d, Distance (in micrometres) of DCVs to the closest presynaptic site sorted by the value for OAN-a1 and -a2. Most DCVs are within 2 μm from a presynaptic site, just a few are further away and appear to be in transit. e, Presynaptic sites colour-coded by their distance to the closest DCV on the axon of OAN-a1 and -a2. f, Distance (in micrometres) of presynaptic sites to the closest DCV sorted by the value for OAN-a1 and -a2. Half of the presynaptic sites have a DCV associated within 20 μm. Some presynaptic sites have no close DCV associated and are located in the dendrites of OAN-a1 and -a2 (data not shown). g, Morphology of DAN-i1 right innervating the upper toe of the MB medial lobe in both hemispheres with the location of presynaptic sites (red) and DCVs (black). h, Zoom-in onto the medial lobes from g. i, DCV colour-coded by their distance to the closest presynaptic site on the axon of DAN-i1 right. j, Presynaptic sites colour-coded by their distance to the closest DCV on the axon of DAN-i1 right. k, Distance (in micrometres) of DCVs to the closest presynaptic site sorted by the value for DAN-i1 right and left together. Some DCVs are further away from presynaptic sites than 10 μm; these DCVs are in the dendrites of the DAN (shown in g). And distance (in micrometres) of presynaptic sites to the closest DCV sorted by the value for DAN-i1 right and left together. Most of the presynaptic sites have a DCV associated within 1 μm.
Extended Data Figure 9: KC-to-MBON synaptic connections. a, Percentage of mature KCs that are presynaptic to a given MBON. b, Frequency of the percentage of KCs presynaptic to MBONs (bin width is 10%). c, Frequency of the percentage of MBONs each KC connects to for single-claw and multi-claw KCs separately. All single-claw KCs connect with at least 75% of all MBONs present in their own hemisphere. d, Percentage of dendritic MBON inputs from individual KCs in the left brain hemisphere. KCs are ranked by their number of synapses to the MBON for each MBON separately (each line represents an MBON). Note the rank order of KCs is different for every MBON. Note also that a few MBONs receive very strong synaptic input from approximately 20 KCs and less than 3% from the remaining KCs, while most MBONs receive less than 4% of dendritic input from all KCs. e, Same as d, but for the right brain hemisphere. f, Effective strength of thermosensory input to MBONs. The thermosensory fraction is defined as the number of synapses received by an MBON from thermosensory KCs divided by 0.05 times the number of synapses received from all other KCs. The fraction thus represents the relative influence of an input that activates the thermosensory KCs compared with that of a typical stimulus that activates 5% of KCs.
Extended Data Figure 10: MBIN-to-KC synaptic connections. a, Percentage of mature KCs that are postsynaptic to a given MBIN. On average, 68% of KCs in a compartment are postsynaptic to at least one MBIN of that compartment. b, Frequency of the percentage of KCs postsynaptic to at least one MBIN of that compartment. c, Frequency of the percentage of KCs postsynaptic to MBINs for single-claw and multi-claw KCs separately. All single-claw KCs receive synaptic input from at least 50% of all MBINs present in their own hemisphere. d, Percentage of axonic outputs from MBINs connecting to individual KCs in the left brain hemisphere. KCs are ranked by their number of synapses they receive from an MBIN for each MBIN separately (each line represents an MBIN). Note the rank order of KCs is different for every MBIN. Note also that a few MBINs connect very strongly to approximately ten KCs and less than 2% to the remaining KCs, while most MBINs dedicate less than 2% of their axonic output to all KCs. e, Same as d, but for the right brain hemisphere.
3.9 Supplementary information

Supplementary Table 1: Connectivity matrix of the entire MB network. Number of synapses for each edge in the MB network. Neurons in rows are presynaptic to neurons in columns. PN-PN connections are almost all dendro-dendritic and occur within the antennal lobe. (See attached zip file in the online version of the paper).

Supplementary Table 2: Presynaptic partners of MBONs and postsynaptic partners of MBINs. a Number of input synapses from different types of neurons to MBONs. b Number of output synapses from MBINs to different types of neurons. Square brackets indicate the number of neurons in that group, e.g. “KCs [#223]” means there are 223 neurons of the KC type contributing to the amount of synapses in the corresponding table cells.

Supplementary Table 3: MBINs and MBONs are listed. The neurotransmitter for each cell is shown for this study and formerly published studies. Known synonyms of cells and their reference publication are indicated.
Supplementary Table 4: MBIN-to-KC and KC-to-MBON connectivity matrices. 

a Connectivity matrix from MBINs to KCs. Color-coded by the number of postsynaptic sites on the KC axonic arbor. KCs are ranked by their number of claws. 

b Connectivity matrix from KCs to MBONs. Color-coded by the number of postsynaptic sites on the MBON dendritic arbor. KC ranking is the same as in a.
Supplementary Table 5: MBON-MBON connectivity matrix. Connectivity matrix of MBON interactions color-coded by the type and strength of connections. Axo-dendritic connections are displayed in magenta, axo-axonic in dark grey and dendro-dendritic in light grey. The matrix contains ipsi- and contralateral connections of MBONs. Homologous neurons from the left and right brain hemispheres are fused. Connection strength is shown as percent of inputs received by the postsynaptic neuron from a particular presynaptic neuron.
Supplementary Table 6: Common features of larval and adult MB.

Common function
The MB is a cerebellum-like structure (Farris, 2011) and essential for olfactory learning and memory in adult (Kaun et al., 2007; Heisenberg et al., 1985; Gerber et al., 2004; Margulies et al., 2005; Davis, 2011) and larva (Honjo and Furukubo-Tokunaga, 2009; Pauls et al., 2010; Michels et al., 2011; Diederich et al., 2013; Aceves-Pina and Quinn, 1979) as well as in the honey bee (Menzel, 2001, 2014), comparison of adult and larval MB reviewed in (Gerber and Stocker, 2007; Stocker, 2009).

Common basic neuronal organization:
The MB KCs are derived from the same 4 neuroblast lineages in adult and larva (Ito and Hotta, 1992; Truman and Bate, 1988; Kunz et al., 2012; Thomas et al., 1984; Lee et al., 1999). KCs have long parallel axons in a bundle forming the peduncle and later bifurcate into the vertical and medial (horizontal) lobes (Armstrong et al., 1996; Kunyasu et al., 2002).

The MB is a convergence point for PNs, MBINs, KCs and MBONs in adult and larval reviewed (Heisenberg, 2003; Gerber et al., 2009; Straussfeld et al., 2009; Davis, 2011; Oswald and Waddell, 2015).
The MB is subdivided in a relatively small number of compartments in adult (Tanaka et al., 2008; Aso et al., 2014) and larva (Pauls et al., 2010) and this study.

MB compartments are defined by overlapping innervation of individual MBONs and MBINs in adult and larva (Tanaka et al., 2008; Aso et al., 2014) and larva (Pauls et al., 2010) and this study.

A single GABAergic neuron (the APL neuron) innervates most MB compartments in adult (Liu and Davis, 2009) and larva (Masuda-Nakagawa et al., 2014) and this study.

Common calyx organization:
In calyx, many KCs get olfactory input in adult (Turner et al., 2008; Honegger et al., 2011; Caron et al., 2013) and larva (Python and Stocker, 2002; Ramseyers et al., 2005; Masudo-Nakagawa et al., 2005; Da et al., 2013; Berck et al., 2016) and some KCs get input from other modalities (including temperature and light) in adult (Bang et al., 2011; Kirkhart and Scott, 2015; Vogt et al., 2016) and larva (revealed by the connectome in this study). KC dendrites wrap around the PN axon boutons by forming “claw”-like structures (Yasuyama et al., 2002; Leis et al., 2009).

Multi-claw KCs randomly sample PN inputs in adult (Murthy et al., 2008; Caron et al., 2013) and larva (Masuda-Nakagawa et al., 2005) and this study.

Calyx receives input of two octopaminergic neurons in adult (Schwaerzel et al., 2003; Busch et al., 2009) and larva (Selcho et al., 2009, 2014 and this study).

Common global compartment organization:
MBINs are either dopaminergic or octopaminergic in adult (Schwaerzel et al., 2003; Mao and Davis, 2009; Waddell, 2013; Aso et al., 2014) and larva (Selcho et al., 2009, 2014 and this study) and have been shown to convey reinforcement signals in bees and locust (Hammer, 1995; Verger et al., 2007; Cassenaer and Laurent, 2007) as well as in the adult fly (Aso et al., 2010; Liu et al., 2012; Burke et al., 2012; Plaças et al., 2012; Yamagata et al., 2015). This requires the expression of specific amine receptors in insects (Kim et al., 2007; Qin et al., 2012; Selcho et al., 2009; McQuillan et al., 2012).

There are 21 MBON types in adult (Aso et al., 2014; Oswald and Waddell, 2015) and larva (this study).

MBONs are mainly glutamatergic, cholinergic and GABAergic in adult (Aso et al., 2014) and larva (shown in this study). Co-activation of KCs and MBINs has been shown to modulate the KC-MBON synapses in flies (Sejourné et al., 2011; Plaças et al., 2013; Pat et al., 2013) and in bees (Menzel and Marz, 2005).

Common synaptic compartment organization:
The EM connectome in the larva revealed a compartment circuit motif: KCs not only receive input from DANs but also synapse back onto DANs DANs synapse onto KCs but also directly onto MBONs KCs synapse onto MBONs (previously shown in adult Drosophila (Ito et al., 1998) and bees (Maulieshagen, 1993)).

Ongoing EM reconstruction of one adult compartment (s-3) confirms the same canonical circuit motif exists in the adult (Gerry Rubin, personal communication).

KC-KC connections
The EM connectome in the larva revealed a high degree of KC-KC interconnectivity: an individual KC receives 59% of its total synaptic input from other KCs. Individual EM sections from peduncle of bees and crickets suggest KC-KC connections (Schürmann, 1971, 1974) and a light-microscopy study in Drosophila suggests presynaptic KC sites in calyx (Christiansen et al., 2011). While total fraction of synaptic input onto individual KCs from other KCs cannot be evaluated in the adult in the absence of a comprehensive EM volume spanning all MB related neurons and a comprehensive reconstruction of the MB, ongoing EM reconstruction of an entire adult a-3 adult compartment confirms the same degree of compartmental a-axo-axonic KC-KC connectivity (Gerry Rubin, personal communication): an individual KC makes 48% of its total synaptic output in a compartment onto other KCs in both larva and adult.

Common feedforward and feedback organization:
A light microscopy study in the adult suggests cross-compartment MBON-MBON interactions (Aso et al., 2014), and a recent functional study shows MBON-MV2 inhibits MBON-M4/M6 (Perisse et al., 2016). The EM connectome and antibody labelling in the larva revealed extensive MBON-MBON synaptic connections between different compartments that can implement lateral inhibition.

Light microscopy and functional studies in the adult reveal MBON-DAN feedback connections in some compartments (Aso et al., 2014; Ichinose et al., 2015). The EM connectome in the larva revealed MBON-DAN feedback connections in some compartments.
Supplementary Table 7: Differences between larval and adult MB.

1. The number of KCs is larger (2000) in the adult enabling an even greater dimensionality expansion (Ito and Hotta, 1992; Technau and Heisenberg, 1982).

2. There are multiple parallel lobe systems in the adult composed of different KC types (Yang et al., 1995; Tanaka et al., 2008; Perisse et al., 2013; Aso et al., 2014).

3. Larvae present single-claw KCs (Ramaekers et al., 2005), and this study, so far not found in the adult.

4. We found no DPM neuron in the larva – a key neuron of the adult circuit (Waddell et al., 2000; Keene et al., 2004, 2006; Yu et al., 2005; Krashes and Waddell, 2008; Haynes et al., 2015).

5. Both larval and adult MB are clearly structured in vertical and horizontal (medial) lobe systems, but the larva has one horizontal (medial) and one vertical lobe, and the adult has three horizontal and two vertical lobes (Lee et al., 1999).

6. In the adult a few compartments are innervated by a much larger number of DANs from the PAM cluster than is the case for any of the larval compartments (Aso et al., 2014).
BIOGENIC AMINES IN THE CENTRAL NERVOUS SYSTEM OF DROSOPHILA MELANOGASTER LARVAE
- AN ANATOMICAL AND BEHAVIOURAL FUNCTION DESCRIPTION

CHAPTER 3
CONNECTOME

a Morphology of MBINs and MBONs

left MBIN right

MBON

left MBIN right

MBON

left MBIN right MBON

SVA MBON-i2 MBON-g1 MBON-g2

IT DAN-\( g1 \) MBON-f1

UT DAN-\( e1 \) MBON-e2

LT DAN-\( k1 \) MBON-e2 MBON-i1

CA OAN-\( a1 \) MBON-a1 MBON-a2

LA/LV/LVL MBON-i1 MBON-c1

LP DAN-\( c1 \) MBON-c1 MBON-b1 MBON-b2

LA/LVL/lVL MBON-r1 MBON-c1

P MBON-k3 MBON-\( d1 \) MBON-c1

MULTIPLE APL

LA MBON-d2 MBON-k2
Atlas Figure 1: Morphology of MBINs and MBONs and of their connections in the feedback motifs. a Neuronal morphology of MBINs and MBONs per MB compartment in anterior view (MBINs in green and MBONs in magenta). Left and right brain hemisphere neurons are displayed separately. The transparent mesh represents the volume of the KC arbors; the legend of compartments is at the bottom right. b Neuronal morphology of MBINs and MBONs participating in feedback and feed-across motifs (green, MBINs; magenta MBONs). Neurons connecting to the left and right MB are displayed separately in anterior view. Locations of the feedback and feed-across connections as shown in Figure 5 are shown depending on their location (inside the MB neuropil, black; outside the MB, orange). Feedback and feed-across connections from compartments of different lobes are located outside of the MB neuropil. Feed-across connections of compartments of the same lobe are located inside the MB neuropil.
### Antibody light microscopy analysis for MBINs

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<tr>
<th>Neuron</th>
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Atlas Figure 2: Antibody light microscopy analysis for mushroom body input and output neurons. a) Neurotransmitter properties of mushroom body input neuron somata as revealed by selected GAL4 lines. Each row shows from left to right: the name of the individual neuron, a combined anti-GFP (green) and anti-TH (TH: tyrosine hydroxylase; magenta) staining, both antibody stainings separately in black and white, the combined anti-GFP (green) and anti-TDC (TDC: tyrosine decarboxylase) staining, both antibody stainings separately in black and white. Whether a cell is dopaminergic or octopaminergic is listed at the end of each row. For OAN-e1 (OAN: octopaminergic input neuron) we found an overlap of GFP expression and anti-TDC in its soma. In addition, Selcho and coworkers (2014) anatomically described three additional octopaminergic neurons that we cite here and include them in our analysis, renaming them. For DAN-c1, d1, f1, g1, i1, j1 and k1 (DAN: dopaminergic input neuron) we found overlap of GFP expression and anti-TH in their somata. No overlap was detected for MBIN-e1, e2 and I. Scale bars: 5µm. b) Neurotransmitter properties of mushroom body output neuron somata and the anterior paired lateral (APL) neuron soma as revealed by selected GAL4 lines. Each row shows from left to right: the name of the individual neuron, combined anti-GFP (green) and anti-ChAT (ChAT: choline acetyltransferase; magenta) stainings, both antibody stainings separately in black and white, the combined anti-GFP (green) and anti-GABA (GABA: gamma aminobutyric acid) staining, both antibody stainings separately in black and white, the combined anti-GFP (green) and anti-GLUT (GLUT: vesicular glutamate transporter) staining, both antibody stainings separately in black and white.
Whether a cell is cholinergic, GABAergic or glutamatergic is listed at the end of each row. In four cases MBONs show up as pairs of the same type. In all cases the antibody profile of both cells is identical. Thus, we name them individually although we cannot distinguish them; indistinguishable cells have identical connectivity within the MB. For MBON-a1, a2, c1 and e1 (MBON: mushroom body output neuron) we found an overlap of GFP expression and anti-ChAT in their somata. For MBON-b1, b2, d1, g1, h1, h2, m1 and APL we found overlap of GFP expression and anti-GABA in their somata. For MBON-e2, h1, h2, i1, j1 and k1 we found overlap of GFP expression and anti-GLUT in their somata. Scale bars: 5µm.

### 3.9.1 Supplementary references


3.10 References


4 Anatomy and Behavioral Function of Serotonin Receptors in *Drosophila melanogaster* Larvae


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4.1 Abstract

The biogenic amine serotonin (5-HT) is an important neuroactive molecule in the central nervous system of the majority of animal phyla. 5-HT binds to specific G protein-coupled and ligand-gated ion receptors to regulate particular aspects of animal behavior. In Drosophila, as in many other insects this includes the regulation of locomotion and feeding.

Due to its genetic amenability and neuronal simplicity the Drosophila larva has turned into a useful model for studying the anatomical and molecular basis of chemosensory behaviors. This is particularly true for the olfactory system, which is mostly described down to the synaptic level over the first three orders of neuronal information processing.

Here we focus on the 5-HT receptor system of the Drosophila larva. In a bipartite approach consisting of anatomical and behavioral experiments we describe the distribution and the implications of individual 5-HT receptors on naïve and acquired chemosensory behaviors. Our data suggest that 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{7}$ are dispensable for larval naïve olfactory and gustatory choice behaviors as well as for appetitive and aversive associative olfactory learning and memory. In contrast, we show that 5-HT/5-HT$_{2A}$ signaling throughout development, but not as an acute neuronal function, affects associative olfactory learning and memory using high salt concentration as a negative unconditioned stimulus.

These findings describe for the first time an involvement of 5-HT signaling in learning and memory in Drosophila larvae. In the longer run these results may uncover developmental, 5-HT dependent principles related to reinforcement processing possibly shared with adult Drosophila and other insects.

4.2 Introduction

The biogenic amine serotonin (5-HT) exerts an essential role in a wide range of insect behaviors by its action as neurotransmitter, neuromodulator, and/or neurohormone (reviewed
by [1]). Accordingly, for the adult fruit fly *Drosophila melanogaster* it was shown that 5-HT signaling is involved in chemosensation [2, 3], aggression [4, 5], mating [6], feeding [7, 8], and locomotion [7, 9].

As in vertebrates, 5-HT acts as natural ligand for a group of G protein-coupled receptors and ligand-gated ion channels found in the central and peripheral nervous systems [10-12]. 5-HT receptors mediate both excitatory and inhibitory function. In the *Drosophila* genome five different 5-HT G protein-coupled receptors have been previously identified, called 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_7$ [13-18]. 5-HT receptor expression was found in distinct adult fly brain regions including the mushroom bodies (MB), central complex, optic lobes and antennal lobes (AL) [19-24]. Consequently, differential 5-HT receptor requirement was reported for aggression (5-HT$_{1A}$ and 5-HT$_{2A}$), sleep (5-HT$_{1A}$), feeding (5-HT$_{1B}$ and 5-HT$_{2A}$), circadian entrainment and behavior (5-HT$_{1B}$ and 5-HT$_{2A}$), and courtship and mating (5-HT$_7$) [8, 19-21, 23, 25, 26]. Moreover, 5-HT/5-HT receptor signaling was shown to be required for learning and memory as 5-HT$_{1A}$, 5-HT$_{2A}$, and 5-HT$_7$ were reported to be involved in short and long term association [27]. Additionally, anesthesia-resistant memory formation was supposed to be mediated through 5-HT$_{1A}$ receptors expressed in the mushroom body [28].

At the larval stage only a few studies have addressed the anatomical organization and functional relevance of 5-HT receptors. It was described that putative 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2A}$, and 5-HT$_7$ positive cells can be found in the larval central nervous system (CNS) [19, 21, 23, 29]. This allows 5-HT to specifically regulate certain aspects of larval physiology and behavior. In detail, distinct larval 5-HT receptor function was reported for locomotion (5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_7$), reduced light avoidance (5-HT$_{1A}$), juvenile-to-adult transition (5-HT$_7$), feeding (5-HT$_{2A}$), and modulation of heart rate frequency (5-HT$_{2A}$ and 5-HT$_{2B}$) [13, 30-34].
However, these studies often focus on a single type of 5-HT receptor and thus miss a comprehensive side-by-side investigation of each receptor on the anatomical and behavioral level. Accordingly, one emphasis of this study was to investigate the organization of several different 5-HT receptors within the larval brain in parallel. To this end we used the Gal4-UAS system that allows for reproducible expression of effector genes in defined subsets of potential 5-HT receptor cells [35-37]. Based on the limitation of available Gal4 lines we focused our analysis on four of the five 5-HT receptors: 5-HT$_{1A}$-Gal4, 5-HT$_{1B}$-Gal4, 5-HT$_{2A}$-Gal4 (also often called 5-HT$_{2Dro}$-Gal4 [21]), and 5-HT$_{7}$-Gal4. Please note that 5-HT$_{2B}$ was only recently identified [13] and it is not part of this study. Thereby, we provide an initial analysis of larval 5-HT receptor system based on genetic tools that are state of the art and widely used in the field. We want to emphasize that it is not clear for all 5-HT receptor Gal4 lines if and how reliable the Gal4 expression reflects the endogenous receptor gene expression patterns. Due to technical limitations antibody and in-situ information is almost completely unavailable. Consequently, in the longer run, more sophisticated genetic tools have to be established and co-localization studies have to be performed.

Here, we applied a two-part approach by analyzing the expression patterns and the behavioral function of potential 5-HT receptor cells in Drosophila larvae. The behavioral assays include measurements for naïve olfactory and gustatory preferences performed via simple choice tests on agarose filled test plates [38]. In addition, a more advanced design allows to study associative olfactory learning and memory [39-47]. Presenting an odor (the conditioned stimulus (CS)) simultaneously with an aversive unconditioned stimulus (US) induces experience dependent avoidance of the CS. Conversely, if the same CS is paired with an appetitive US, animals can be trained to develop a preference for the CS. Thus, depending on previous experience, the same odor can trigger either avoidance or attraction [41]. Taken together, a comprehensive set of behavioral assays to analyze larval chemosensory behavior is
at hand that allows investigating simple choice behavior and associative olfactory learning and memory in *Drosophila* larvae.

In an earlier study, we could show that 5-HT positive cells are *per se* neither necessary for naïve gustatory, olfactory and light preferences nor for associative olfactory learning and memory (using fructose and electric shock as positive and negative reinforcers, respectively) [48]. However, this does not exclude a specific contribution of distinct 5-HT receptors for chemosensory behaviors. Different 5-HT receptors act antagonistically. 5-HT$_7$ was shown to activate adenylyl cyclases and increases cAMP levels, whereas 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors inhibit cAMP production [14, 15, 18]. Thus, ablation of the entire 5-HT system can affect both assisting and inhibitory functions of antagonistic 5-HT receptors at the same time. We have therefore expanded our analysis on the role of 5-HT signaling on larval chemosensory behavior by focusing on distinct 5-HT receptor functions.

### 4.3 Materials and methods

#### 4.3.1 Flies

Flies were maintained on standard *Drosophila* medium at 25°C or 19°C under 12h light /dark conditions. All 5-HT receptor specific lines, 5-HT$_{1A}$-Gal4 [29], 5-HT$_{1B}$-Gal4 [49] (Bloomington Stock Center no. 24240), 5-HT$_{2A}$-Gal4 [21, 50] (Bloomington Stock Center no. 19367), 5-HT$_7$-Gal4 [23], were kindly provided by Charles Nichols. Note that the 5-HT$_{2A}$-Gal4 construct is an enhancer trap piggyBac construct in the 5-HT$_{2A}$ locus that reduces its expression by nearly 90% [21]. TRH-GAL4 [78] was kindly provided by Serge Birman. For behavioral experiments, wild type *Canton-S* (WT CS) flies and the effector lines UAS-hid,rpr [51, 52] and UAS-shi$^{ts}$ [53] (Bloomington Stock Center no. 44222) were used. w$^{1118}$ flies (kindly provided by Martin Heisenberg) were crossed with UAS- and Gal4 lines to obtain heterozygous controls. To visualize the Gal4 expression pattern, we used UAS-mCD8::GFP
[54] (kindly provided by Hiromu Tanimoto) and UAS-myr::tomato [55, 56] (Bloomington Stock Center no. 32221). In all cases five to six day old feeding third instar larvae were used.

4.3.2 Immunostaining

Experiments were performed as described before [57, 58]. Third instar larvae were put on ice and dissected in phosphate buffered saline (PBS). Brains were fixed in 3.6% formaldehyde (Merck, Darmstadt) in PBS for 30 min. After washing with PBT (PBS with 3% Triton-X 100, Sigma Aldrich, St. Louis, MO), brains were blocked with 5% normal goat serum (Vector Laboratories, Burlingame, CA) in PBT for one to two hours and then incubated for two days with first antibodies at 4°C. Before applying the secondary antibodies for one or two days at 4°C, brains were washed with PBT. Finally, brains were again washed, mounted in Vectashield (Vector Laboratories) between two cover slips and stored at 4°C in darkness. This protocol was used for data presented in Fig 1A, 1A, 1AI, 1AII, 1B, 1BI, 1BVI, 1C, 1CIV, and Fig 2.

In addition we used a second protocol developed at the HHMI Janelia research campus (https://www.janelia.org/project-team/flylight/protocols). Larval tissues were dissected, fixed and washed as described above. After blocking with 3% normal goat serum (Vector Laboratories, Burlingame, CA) in PBT for 1 hr, tissues were incubated for two days with first antibodies at 4°C. After multiple rinses in PBT, tissues were incubated 2 days at 4°C in the cocktail of secondary antibodies. Nervous systems were then washed two to three times in PBT, mounted on poly-L-lysine (Sigma-Aldrich) coated coverslips and then transferred to a coverslip staining jar (Electron Microscopy Sciences) to dehydrate through a graded ethanol series. Afterwards tissues were cleared in xylene, and mounted in DPX (Sigma). This protocol was used for data presented in Fig 1AIII - 1AVII, 1BII - 1BV, 1BVI, 1C1 - 1CIII, 1CV - 1DVI.

Images were taken with Zeiss confocal laser microscopes LSM 550 and LSM 780. The resulting image stacks were projected and analyzed with Image-J (NIH;
https://imagej.nih.gov/ij/) software. Contrast and brightness adjustment as well as rotation and organization of images were performed in Photoshop (Adobe Systems Inc., San Jose, CA).

### 4.3.3 Immunofluorescence Antibodies

Anti-GFP (polyclonal/rabbit/A6455/Molecular Probes/1:1000), anti-GFP-FITC (polyclonal/goat/ab6662/abcam/1:1000) and anti-DsRed (polyclonal/rabbit/632496/Clonetech/1:200) were used for visualizing Gal4 lines expression patterns. Two different mouse antibodies for staining the neuropil (anti-ChAT (monoclonal/mouse/ChAT4B1/DSHB/1:100)) and the axonal tracts (anti-Fasciclin II (monoclonal/1d4 anti-Fas/DSHB/1:50)) were applied to provide landmarks within the larval CNS. 5-HT cells were visualized with anti-5-HT (polyclonal/rabbit/S5545/Sigma Aldrich/1:1000), dopaminergic cells with anti-TH (monoclonal/mouse/22941/Immuno-Star/1:500), respectively.

As secondary antibodies goat anti-rabbit IgG Alexa Fluor 488 (polyclonal/A11008/Molecular Probes/1:200), goat anti-mouse IgG Alexa Fluor 647 (polyclonal/A21235/Molecular Probes/1:200), goat anti-mouse IgG Alexa Fluor 405 (polyclonal/A31553/Molecular Probes/1:200), goat anti-mouse IgG Cy3 (polyclonal/A10521/Molecular Probes/1:200), and goat anti-rabbit IgG Cy5 (polyclonal/A10523/Molecular Probes/1:200) were used.

### 4.3.4 Behavioral experiments

Five to six day old feeding third instar larvae were used for all behavioral experiments. The assays were performed either at 22°C or at 35°C using UAS-*hid*,rpr and UAS-*shi*<sub>P</sub> effector lines, respectively. In case of UAS-*shi*<sub>P</sub>, larvae were additionally incubated for 2 min in a water bath at 37°C prior to behavioral experiments. For further details on experimental protocols we added the following description and refer to earlier studies [38-40, 43, 57, 59-62].
4.3.5 Associative olfactory learning and memory

Experiments were conducted on test plates filled with a thin layer of 2.5% agarose containing either pure agarose or agarose plus reinforcer. We used 1.5 M sodium chloride (SALT) and 2.0 M D-fructose (FRU). As olfactory stimuli, we used 10 μl amyl acetate (AM, diluted 1:250 in paraffin oil) and benzaldehyde (BA, undiluted). Odorants were loaded into custom-made Teflon containers with perforated lids. Learning and memory were tested by exposing a first group of 30 animals to BA, while crawling on agarose medium containing sugar as a positive reinforcer or high salt concentration as a negative reinforcer. After 5 min, larvae were transferred to a fresh test plate 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. Please note that for aversive olfactory learning and memory the test plate included the same high salt concentration as respective training plates. After 5 min, individuals were counted on the AM side (#AM), the BA side (#BA), and in a neutral zone. 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:

\[(1a) \text{PREF}_{\text{AM+}/\text{BA}} = (\# \text{AM} - \# \text{BA}) / \# \text{TOTAL}\]

\[(1b) \text{PREF}_{\text{AM/BA+}} = (\# \text{AM} - \# \text{BA}) / \# \text{TOTAL}\]

To measure specifically the effect of associative learning and memory we calculated the associative performance index (PI) as the difference in preference between the reciprocally trained larvae:

\[(2) \text{PI} = (\text{PREF}_{\text{AM+}/\text{BA}} - \text{PREF}_{\text{AM/BA+}}) / 2\]
Negative PIs thus represent aversive olfactory learning and memory, whereas positive PIs indicate appetitive olfactory learning and memory. Division by 2 ensures scores are bound within (-1; 1).

### 4.3.6 Chemosensory preference

For gustatory preference tests, 2.5% agarose (Sigma Aldrich Cat. No.: A5093, CAS No.: 9012-36-6) solution was boiled in a microwave oven and filled as a thin layer into test plates (85 mm diameter, Cat. No.: 82.1472, Sarstedt, Nümbrecht). After cooling, the agarose was removed from half of the plate. The empty half was filled with 2.5% agarose solution containing sodium chloride (SALT, Sigma Aldrich Cat. No.: S7653, CAS No.: 7647-14-5; 2.0 M and 1.5 M) and D-fructose (FRU, Sigma Aldrich Cat. No.: 47740, CAS No.: 57-48-7; 2.0 M). Assay plates were used at the same day shortly after preparation to avoid diffusion of the stimuli from one side to the other. Groups of 30 larvae were placed in the middle of the plate, allowed to crawl for 5 min, and then counted on the stimulus containing side, the agarose only side, and a neutral zone. The neutral zone covers 1 cm from top to bottom of the Petri dish between the left and right sides. It thereby separates both halves and covers the transition from pure agarose to agarose plus gustatory stimulus. By subtracting the number of larvae on the pure agarose side (#nS) from the number of larvae on the stimulus side (#S) divided by the total number of counted larvae (#TOTAL), a preference index for the respective chemosensory stimulus was calculated:

\[
\text{PREF} = \frac{(#S - #nS)}{#TOTAL}
\]

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

For olfactory preference tests, a similar assay was used except that olfactory stimuli were presented in custom-made Teflon containers with perforated lids presented on only pure
agarose containing test plates. As olfactory stimuli amyl acetate (AM, Fluka Cat. No.: 46022; CAS No.: 628-63-7; diluted 1:250 in paraffin oil, Fluka Cat. No.: 76235, CAS No.: 8012-95-1) and benzaldehyde (BA, Fluka Cat. No.: 12010, CAS No.: 100-52-7; undiluted) were used.

4.3.7 Acutely blocking synaptic output with shibire

To acutely block synaptic output of defined sets of cells we used UAS-*shi* [53]. Immediately before the experiment, larvae were incubated for 2 min in a water-bath at 37°C. The behavioral experiments were then performed as described before, at a restrictive temperature of about 35°C in a custom made chamber.

4.3.8 Statistical methods

Statistical analysis and visualizations were done with R (version 2.15.2), R studio (version 0.98.1028) and Adobe Photoshop (version CC 2015.5). Behavioral data are visualized as box plots with medians (middle lines), 25% / 75% percentiles (box boundaries), and 10% / 90% percentiles (whiskers). Sample size in each case is n= 10 - 20. Kruskal-Wallis tests (KWT) were performed and, in case of significance, followed by Wilcoxon rank-sum tests (WRT); Bonferroni corrections were used for multiple comparisons as applicable (indicated with BWRT). Likewise, Wilcoxon signed-ranked tests (WST) were used to compare values against chance level. Visualization of statistical evaluations: if only n.s. is shown the initial KWT did not suggest for a difference between the three groups (p > 0.05). When differences between each group are shown this provides the results of the BWRT as the initial KWT suggested for singnificance (p < 0.05). P values were rounded to three decimal places except for cases that would have resulted in zero. Here additional decimal places are given. Further details including raw data can be found in supplement table 1.
4.4 Results

4.4.1 Expression Patterns of Four Specific 5-HT Receptor Gal4 Lines in the Larval Brain

To analyze the cellular anatomy of different 5-HT receptor Gal4 lines in the CNS of third instar larvae of Drosophila, we crossed 5-HT1A-Gal4, 5-HT1B-Gal4, and 5-HT7-Gal4 with UAS-mCD8::GFP. We used an anti-GFP antibody to identify details of the cellular innervation and morphology and anti-Fasciclin II (FasII) / anti-Choline Acetyltransferase (ChAT) background staining (Fig 1), which label axonal tracts [63] and neuropils [64], respectively. The double labeling approach thus allows us to map the different 5-HT receptor Gal4 expression patterns into a common neuropil reference. This approach was hitherto not applied to all of these lines at the larval stage. Please note, to clearly disentangle 5HT2A-Gal4 dependent expression from marker expression we crossed 5-HT2A-Gal4 with UAS-myr::tomato and labelled specifically Gal4 positive cells via an anti-DsRed antibody [65]. This is necessary as the enhancer trap piggyBac construct carries a “3xP3-EYFP” marker that is also recognized by the anti-GFP antibody [50, 66, 67]. The “3xP3-EYFP” marker is expressed under the control of the endogenous Pax6 gene and thus on its own drives expression in the larval visual system and brain [68, 69].

4.4.1.1 5-HT1A-Gal4

5-HT1A-Gal4 expression was found throughout the larval CNS including both brain hemispheres and the ventral nerve cord (VNC) (Fig 1A). A closer inspection revealed innervation of the protocerebrum (arrow in Fig 1AII and 1AIV), the AL (arrow in Fig 1AIV), to a lesser degree of the suboesophageal ganglion (SOG; Fig AIII), and mushroom body calyx (CA; arrow in Fig 1AV). In contrast, the MB lobes and larval optic neuropil (LON) were not labeled (Fig 1AVI and 1AVII). Characteristic for the 5-HT1A-Gal4 expression pattern was a row of cell bodies localized close to the midline in each thoracic and abdominal segment, (Fig 1A
and 1A1) as well as a set of about 15 cell bodies dorsolateral of the CA innervating different protocerebral regions (Fig 1A). In addition, several neurons were labeled per thoracic and abdominal segment that leave the brain and potentially target the peripheral system of the larvae (arrows in Fig 1A). The obtained expression pattern is comparable to the ones described before [15, 29].

Figure 1 Gal4 expression patterns of four potential 5-HT receptor lines. 5-HT1A-, 5-HT1B-, 5-HT7-, and 5-HT2A-Gal4 positive cells are shown in A, B, C, and D, respectively. (A-C) Gal4 lines were crossed with UAS-mCD8::GFP to analyze...
their expression patterns (green; anti-GFP staining) in addition to reference labeling of the central nervous system (CNS) (magenta; anti-ChAT/anti-FasII double-staining). In (D) 5-HT2A-Gal4 was crossed with UAS-myr::tomato to visualize its expression pattern (green; anti-dsRed staining) within the larval CNS (magenta; anti-ChAT/anti-FasII double-staining). For all four lines the first column shows a frontal view onto a z-projection of the entire CNS. In addition, for each line representative z-projections of close-ups of the ventral nerve cord (VNC), one hemisphere (HEMI), the suboesophageal ganglion (SOG), one antennal lobe (AL), the calyx (CA) of the mushroom body (MB), the lobes of the MB, and the larval optic neuropil (LON) are shown from left to right. Below each close-up only the GFP channel is shown as an inverted black and white image to visualize innervation patterns with higher contrast and no neuropil background. White arrows highlight aspects of the expression patterns that are further described in the results. Additional abbreviations: VL vertical lobe, ML medial lobe, PED peduncle; Scale bars: 50 μm (in A, B, C, D) and 20 μm (in all other panels).

4.4.1.2 5-HT1B-GAL4

5-HT1B-Gal4 also showed expression in extended regions throughout the whole larval CNS, including both hemispheres and the VNC (Fig 1B). A closer inspection revealed innervation in cells at the tip and midline of the SOG (arrows in 1BIII). A small number of only about 20, likely embryonic-born Kenyon cells (KC) [70], innervate the entire MB at the CA and at the surface of the MB peduncle, vertical and medial lobe (arrows in Fig 1Bv and 1Bvi). No expression was found in the AL and LON (Fig 1Biv and 1Bvii). Besides the pronounced staining along the midline, a set of cells with somata in the lateral abdominal segments project to the terminal plexus of the VNC (arrows in Fig 1Bi). These 5-HT1B positive cells were reported to express the peptide hormone leucokinin and play a role in larval turning behavior [71]. An additional characteristic of the 5-HT1B-Gal4 line is its massive expression laterally in both hemispheres that give rise to the adult optic lobes after metamorphosis (arrows in Fig 1Bii and 1Bvii) [72]. Our results are in line with earlier findings of the expression patterns of 5-HT1B [19, 71].

4.4.1.3 5-HT7-Gal4

5-HT7-Gal4 expression was detected throughout the whole larval CNS including both hemispheres and the VNC (Fig 1C). We could not find innervation within the MB lobes and the LON by potential 5-HT7 cells (Fig 1Cvi, Cvii). Yet, innervation was detected in the VNC, SOG, AL and CA (arrows in Fig 1Ci – 1Cv). Seven cells with somata in close proximity to the AL densely innervate the AL (Fig 1Civ). These are likely projection neurons as they
follow the antennocerebral tract an innervate the CA (arrows in Fig 1C\textsubscript{V}). The peduncle of the MB was weakly innervated by a MB extrinsic neuron called BL neuron (right arrow in Fig 1C\textsubscript{VI}) \cite{70}. In addition, 5-HT\textsubscript{7}-Gal\textsubscript{4} shows dense innervation within the SOG potentially co-localizing with the 5-HT positive SE0 clusters \cite{34, 48, 73}. These cells were reported to be involved in feeding and also in linking the external environment with the internal endocrine system \cite{34, 73}. Overall the observed expression is consistent with the one reported before \cite{23}.

4.4.1.4 5-HT\textsubscript{2A}-Gal\textsubscript{4}

5-HT\textsubscript{2A}-Gal\textsubscript{4} showed an expression pattern that is remarkably different when compared to the other three driver lines (Fig 1D). There are nearly no somata detectable within the larval CNS (Fig 1D\textsubscript{I}-D\textsubscript{VII}). Yet, there is strong labelling within the SOG, likely by axonal terminals from neurons of the peripheral nervous system that enter the brain via different nerves (arrows in Fig 1D\textsubscript{III}). However, due to technical limitations we were not able to clearly localize these cells outside of the CNS. We are nonetheless convinced that these cells are 5-HT\textsubscript{2A}-Gal\textsubscript{4} positive due to their initial description by Nichols \cite{21} using the same Gal\textsubscript{4} line. In addition, we found a second type of brain innervation from the periphery, this time into the LON (arrow in Fig 1D\textsubscript{VII}). Please note that the larval MB was not innervated. Detectable staining was limited to adjacent fiber bundles (lower arrow in Fig 1D\textsubscript{VI}) and weak innervation of the dorsal protocerebrum (upper arrow in Fig 1D\textsubscript{VI}).

In summary, the four analyzed Gal\textsubscript{4} expression patterns suggest that 5-HT receptors may be expressed broadly throughout the brain in partially overlapping patterns including primary olfactory and gustatory brain centers (AL and SOG). Distinct receptors may be limited to particular types of cells like peripheral sensory neurons that project to the SOG (5-HT\textsubscript{2A}-Gal\textsubscript{4}) and MB Kenyon cells that are known to be involved in learning and memory (5-HT\textsubscript{1B}-Gal\textsubscript{4}).
Yet, these results are based on Gal4 expression patterns that may not represent the endogenous gene expression patterns and therefore have to be handled with care.

### 4.4.2 Co-localization of 5-HT Receptor Gal4 lines with Serotonin and Tyrosine-Hydroxylase

To examine if the larval 5-HT system is controlled in an auto-regulatory manner [74-76], we crossed 5-HT1A-, 5-HT1B-, and 5-HT7-Gal4 with UAS-mCD8::GPF, used the same primary antibody mixture as before (anti-ChAT/anti-FasII and anti-GPF), but added anti-5-HT (Fig 2A, 2C and 2E). We did not analyze 5-HT2A-Gal4 due to the low number of stained somata within the larval brain (Fig 1D), but rather afferent projections of peripheral neurons that have their somata outside the CNS. All three driver lines exhibited no co-labeling of Gal4 positive and anti-5-HT staining (Fig 2A, 2C, 2E). This indicates that 5-HT receptors may localize mostly postsynaptically in the larva. This interpretation matches with earlier results that have analyzed 5-HT/5-HT receptor co-localization [23, 29]. Yet, we also found one exception: consistent with previous work [29] a single pair of 5-HT1A stained cells in the VNC co-localized with 5-HT immunolabeling (Fig 2AII, arrowheads).

In addition, we also analyzed if potential 5-HT receptor cells are dopaminergic. It was shown that different aminergic systems directly connect on each other. Octopaminergic neurons for example signal a specific aspect of sugar reinforcement directly onto dopaminergic neurons that express an α-adrenergic-like octopamine receptor called OAMB [77]. To address if the larval serotonergic system can directly act on dopaminergic neurons we applied a triple staining approach. We used anti-TH for the visualization of dopaminergic neurons, anti-GFP to label 5-HT receptor positive cells and anti-ChAT/anti-FasII for a reference staining of the larval CNS (Fig 2B, 2D and 2F). For the tested lines, 5-HT1A-, 5-HT1B-, and 5-HT7-Gal4, we did not detect co-localization with dopamine synthesizing cells. Therefore, it is rather unlikely that the 5-HT system directly signals as a neurotransmitter on dopaminergic neurons.
Figure 2 Co-expression of 5-HT1A-, 5-HT1B-, and 5-HT7-Gal4 with anti-5-HT and anti-TH. Expression patterns of 5-HT1A-, 5-HT1B-, and 5-HT7-Gal4 crossed with UAS-mCD8::GFP are visualized using a triple staining protocol. Anti-GFP (green) was used to label Gal4 positive cells, anti-ChAT/anti-FasII (blue) was used as a reference labeling for the CNS, and anti-5-HT (red, in A, C, E) or anti-TH (red, in B, D, F) was used to identify 5-HT or dopaminergic cells, respectively. 5-HT2A-Gal4 was not included in this experiment due to the low number of cell bodies inside the CNS (Fig 1D). There was no co-staining detected between 5-HT receptor Gal4 expression patterns and anti-TH (B, D, F). The same is true for the co-expression analysis with anti-5-HT (A, C, E); the only exception was a single pair of neurons in the terminal segment of the VNC labeled by 5-HT1A-Gal4 (arrowhead in A). (A-F) show frontal views onto z-projections of the entire CNS (left) and representative z-projections of close-ups of one or both hemispheres and SOG (right top) and abdominal ganglion (right bottom). Scale bars: 50 μm (left) and 20 μm (right top and bottom).

4.4.3 Ablation of 5-HT2A-Gal4 positive cells during development impairs aversive associative olfactory learning and memory

To examine if larvae lacking potential 5-HT receptor cells are able to associate an odor with a positive or negative gustatory stimulus, we utilized a well-established standard paradigm (reviewed in [38]). As olfactory stimuli we used benzaldehyde (BA) and amyl acetate (AM).
As appetitive and aversive gustatory unconditioned stimuli we used fructose (FRU, 2.0 M) and high sodium chloride concentration (SALT, 1.5 M), respectively. This is possible, because ablation of potential 5-HT receptor cells did neither change naïve olfactory responses towards amyl acetate (diluted 1:250 in paraffin oil), benzaldehyde (undiluted), heptanol (undiluted), and nonanol (undiluted) (Fig S1); nor gustatory responses to sodium chloride (2M and 1.5M for 5-HT2A-Gal4), fructose (2M), arabinose (2M), and sorbitol (2M) (Fig S2; Fig S3).

Ablation of 5-HT1A-Gal4, 5-HT1B-Gal4, and 5-HT7-Gal4 positive cells throughout development did not change appetitive olfactory learning and memory (Fig 3B, KWT p = 0.061; Fig 3C, KWT p = 0.007; BWRT p = 0.189 compared to 5-HT1B-Gal4/+ and p = 0.052 compared to UAS-hid,rpr/+; Fig 3D, KWT p = 0.061) as well as aversive olfactory learning and memory (Fig 3F, KWT p = 0.233; Fig 3G, KWT p = 0.006; BWRT p = 1.000 compared to 5-HT1B-Gal4/+ and p = 0.016 compared to UAS-hid,rpr/+; Fig 3H, KWT p = 0.303).

Yet, 5-HT2A-Gal4/UAS-hid,rpr experimental larvae showed impaired aversive olfactory learning and memory (Fig 3I; WST p = 0.131), which was significantly different compared to both genetic controls (Fig 3I; KWT p = 0.010; BWRT p = 0.021 compared to 5-HT2A-Gal4/+, p = 0.034 compared to UAS-hid,rpr/+). Appetitive olfactory learning and memory did not differ among the three tested genotypes (Fig 3E; KWT p = 0.606). Repetition of the experiments with increased sample size gave rise to the same results (Fig S4).

In summary, we thus conclude that 5-HT1A-, 5-HT1B-, and 5-HT7-Gal4 positive cells were not necessary for appetitive and aversive olfactory learning and memory. Yet, for 5-HT2A-Gal4 we got a different result; unlike appetitive olfactory learning and memory, aversive olfactory learning and memory was impaired.
Figure 3 Ablation of potential 5-HT2A receptor cells throughout development impairs aversive olfactory learning and memory. 5-HT1A-, 5-HT1B-, 5-HT7-, and 5-HT2A-Gal4 lines were crossed with UAS-hid,rpr to genetically induce apoptosis in potential 5-HT receptor cells. In addition, Gal4 lines and UAS-hid,rpr were crossed with w1118 to obtain heterozygous genetic control larvae. (A) provides a color scheme for the three different groups used in each experiment. Appetitive olfactory learning and memory using fructose reinforcement is shown at the top (B-E). Aversive olfactory learning and memory is shown at the bottom (F-I). In most cases, experimental larvae and genetic control groups behaved similar. However, ablation of 5-HT2A-Gal4 positive cells specifically impaired aversive olfactory learning and memory (I), while leaving appetitive olfactory learning and memory intact (E). Sample size (n = 10–14) is indicated at the bottom of each box plot. Differences against zero are given at the top of each box plot. Differences between all three groups or individual groups are shown at the bottom of the panel. Visualization of statistical evaluations: if only n.s. is shown the initial Kruskal-Wallis test (KWT) did not suggest for a difference between the three groups (p > 0.05). When differences between each group are shown this provides the results of the Wilcoxon rank-sum tests with Bonferroni corrections (BWRT) as the initial KWT suggested for significance (p < 0.05). *** (p < 0.001), ** (p < 0.01), * (p < 0.05), n.s. (not significant p ≥ 0.05).

4.4.4 5-HT2A receptor function throughout development is necessary for aversive olfactory learning and memory

To investigate if the memory impairment seen for 5-HT2A-Gal4/UAS-hid,rpr experimental larvae was due to changes in the 5-HT2A receptor function, we next tested a homozygous
hypomorphic $5-HT_{2A}$ receptor mutant. In detail, Nichols [21] showed via quantitative real-time PCR analysis for this line that $5-HT_{2A}$ expression is reduced by about 90% compared to wild type (Oregon-R) and $w^{1118}$ flies.

Figure 4 Impaired $5-HT_{2A}$ receptor function throughout development impairs aversive olfactory learning and memory. Homozygous $5-HT_{2A}$ receptor gene mutants and wild-type control larvae (WT CS) were used to analyze aversive (B) and appetitive (C) olfactory learning and memory, olfactory amyl acetate (AM, in D) and benzaldehyde (BA in E) preferences and gustatory sodium chloride (SALT, in F) and fructose (FRU, in G) preferences. (A) provides a color scheme for the two different groups used in each experiment. Whereas mutant larvae showed olfactory and gustatory preferences as well as appetitive olfactory learning and memory comparable to WT CS larvae, aversive olfactory learning and memory was significantly reduced. Sample size (n = 11–17) is indicated at the bottom of each box plot. Differences against zero are given at the top of each box plot. Differences between mutant and wild type larvae are shown at the bottom of the panel. *** (p < 0.001), ** (p < 0.01), * (p < 0.05), n.s. (not significant p ≥ 0.05).
Homoyzgous $5-HT_{2A}$ mutant larvae showed a similar reduction for aversive olfactory learning and memory when compared to wild-type control ($WT\ CS$) larvae (Fig 4B; WRT $p= 0.001$). Again, the behavioral phenotype was specific for aversive olfactory learning and memory and did not affect appetitive olfactory learning and memory (Fig 4C; WRT $p= 1.000$ compared to $WT\ CS$). In addition, we performed control experiments with $5-HT_{2A}$ mutant and $WT\ CS$ larvae to test for proper olfactory and gustatory chemotaxis. This was necessary to exclude perturbing defects in task-relevant sensory-motor abilities. $5-HT_{2A}$ receptor mutants displayed olfactory AM and BA preferences as well as gustatory SALT and FRU preferences that did not significantly differ from wild type controls (Fig 4D, 4E, 4F, 4G; WRT $p= 0.173$, $p= 0.154$, $p= 0.148$, $p= 0.516$, respectively).

Based on these results we conclude that $5-HT_{2A}$ receptor function throughout development is necessary for aversive olfactory learning and memory using high salt concentration as a unconditioned stimulus.

4.4.5 Ablation of the $5-HT/5-HT_{2A}$ receptor system throughout development specifically impairs aversive odor-salt learning and memory

In an earlier study we had shown that larvae lacking $5-HT$ cells are able to establish an association between an odor and a punishing stimulus [48]. At first sight the behavioral phenotypes shown in Fig 3I and 4B, therefore, appear to be contradictory. Yet, in our initial experiments we used electric shock instead of high salt concentration as unconditioned stimulus. Thus, it is possible that manipulation of the $5-HT$ system does not affect aversive olfactory learning and memory in general but is rather restricted to gustatory high salt concentration. To investigate whether this is the case we utilized the same approach as used in Huser et al. (2012). We crossed the $TRH\ Gal4$ line [78] that expresses in most of the larval $5-HT$ cells (Fig 5A; [48]) as the $tryptophan\ hydroxylase\ (TRH)$ gene was reported to catalyse the rate-limiting step of $5-HT$ synthesis from tryptophan to 5-hydroxy-tryptophan [1].
Figure 5. 5-HT/5-HT2A signaling throughout development specifically impairs odor-salt learning and memory. (A) The TRH-Gal4 line was crossed with UAS-mCD8::GFP to visualize its expression pattern (green; anti-GFP staining) in addition to a reference labeling of the CNS (magenta; anti-ChAT/anti-FasII double-staining). (B) The TRH-Gal4 line was crossed with UAS-hid,rpr to genetically induce apoptosis in 5-HT cells. In addition, the Gal4 line and UAS-hid,rpr were crossed with w1118 to obtain heterozygous genetic control larvae. Above the panel a color scheme describes the three different groups used in the experiment. Ablation of 5-HT cells completely abolished aversive olfactory learning and memory reinforced by high salt concentration. (C) shows an overview on the experimental procedure that was used in larvae to test for odor-electric shock learning and memory. (D) Ablation of 5-HT2A-Gal4 positive cells via UAS-hid,rpr did not impair odor-electric shock learning and memory. Sample size (n = 15) is indicated at the bottom of each box plot. Differences against zero are given at the top of each box plot. Differences between the groups are shown at the bottom of the panel. Visualization of statistical evaluations: if only n.s. is shown the initial KWT did not suggest for a difference between the three groups (p > 0.05). When
differences between each group are shown this provides the results of the BWRT as the initial KWT suggested for significance (p < 0.05). *** (p < 0.001), ** (p < 0.01), * (p < 0.05), n.s. (not significant p ≥ 0.05). Scale bar: 50 μm.

Experimental TRH-Gal4/UAS-hid,rpr larvae having most of their 5-HT cells ablated throughout development [48] did not show aversive odor-salt learning and memory (Fig 5B; WST p= 0.762). The behavior was different from both genetic controls (Fig 5B; KWT p= 0.002; BWRT p= 0.002 compared to TRH-Gal4/+, p= 0.038 compared to UAS-hid,rpr/+). The specificity for the unconditioned stimulus is further supported by a second experiment. Also 5-HT2A-Gal4/UAS-hid,rpr experimental larvae that received odor-electric shock training performed similar as genetic controls and were not impaired in learning and memory (Fig 5C and 5D; KWT p= 0.470). We thus conclude that 5-HT/5-HT2A receptor signaling throught development is necessary for aversive olfactory learning and memory reinforced by high salt concentration.

4.4.6 Acute blockage of neuronal output of 5-HT and 5-HT2A receptor cells does not impair odor-salt learning and memory

To address if 5-HT function is acutely required for odor-salt learning and memory we used the temperature-sensitive dominant negative form of dynamin UAS-shibirets (shi^{ts}) [53]. Thereby, we specifically disrupted synaptic vesicle recycling during training and testing but not during development.

An acute block of neurotransmission of 5-HT2A-Gal4 and TRH-Gal4 positive cells did not affect odor-salt learning and memory (Fig 6C, 6D; WST p= 0.001 and p= 0.020). For both experiments we did not observe a difference between the three particular genotypes (Fig 6C, 6D; KWT p= 0.414 and p= 0.655).
Figure 6 Acute blockage of synaptic output of 5-HT cells and potential 5-HT2A receptor cells does not affect chemosensory behavior. The 5-HT2A-Gal4 and TRH-Gal4 lines were crossed with UAS-shi/ts to genetically interfere with synaptic transmission only during training and testing but not during development. In addition, the Gal4 lines and UAS-hid,rpr were crossed with w1118 to obtain heterozygous genetic control larvae. (A) provides a color scheme for the different groups used in each experiment. (B) shows the temperature regime that was applied to block synaptic output at the restrictive temperature of 35°C specifically during training (30 min) and testing (5 min). Immediately before the experiment, larvae were incubated (2 min) in a water-bath at 37°C. Aversive olfactory learning and memory reinforced by high salt concentration (C and D), olfactory preferences for AM and BA (E–H) and gustatory preferences for SALT (I and J) were analyzed. In none of the cases experimental larvae behaved significantly different compared to both genetic control groups. We thus reason that blockage of synaptic output of 5-HT2A-positive cells does not impair all tested chemosensory behaviors. Sample size (n = 12–18) is indicated at the bottom of each box plot. Differences against zero are given at the top of each box plot. Differences between all three groups or individual groups are shown at the bottom of the panel, except for SALT, where it is placed above the box plots. Visualization of statistical evaluations: if only n.s. is shown the initial KWT did not suggest for a difference between the three groups (p > 0.05). When differences between each group are shown this provides the results of the BWRT as the initial KWT suggested for significance (p < 0.05). *** (p < 0.001), ** (p < 0.01), * (p < 0.05), n.s. (not significant p ≥ 0.05).

In addition, experimental larvae were tested for olfactory AM and BA preferences and gustatory SALT preference. 5-HT2A-Gal4/UAS-shi/ts were attracted by both odors (Fig 6E, 6G;
WST p= 0.001 and p= 0.048) and avoided SALT (Fig 6I; WST p= 0.033). For all experiments there was no difference between experimental and control larvae (Fig 6E, 6G, 6I; KWT p= 0.560, KWT p= 0.660, KWT p= 0.127, respectively). The same results were observed with TRH-Gal4 (Fig 6F, 6H, 6J; KWT p= 0.017; BWRT p= 0.029 compared to TRH-Gal4/+ p= 0.622 compared to UAS-shi⁴⁹/+; KWT p= 0.597, KWT p= 0.251, respectively). We thus conclude that acute blockage of neurotransmission of the 5-HT signaling system did not affect odor-salt learning and memory reinforced by high salt concentration as well as tested naïve chemosensory preferences.

4.5 Discussion

4.5.1 Ablation of 5-HT/5-HT2A receptor signaling during development impairs aversive olfactory learning and memory reinforced by high salt concentration

Serotonin is a biogenic amine, an important neuroactive molecule within the CNS of several insect species. Serotonin, dopamine, histamine, octopamine, and tyramine are amines which have been extensively studied in Drosophila (reviewed in [1, 79, 80]). Each one of the five aminergic neuronal systems – including the serotonergic system - exhibits a stereotypic pattern of a small number of neurons that are widely distributed in the larval and adult CNS [1]. Aminergic neurons have attracted much attention in recent years. Thus, the detailed anatomy and various behavioral functions for many aminergic neurons were identified - in numerous cases even at single cell resolution (only focusing on the Drosophila larvae: [48, 57, 58, 62, 81]).

In contrast, much less is known on the receptor side. Due to the lack of specific antibodies and genetic tools to anatomically describe and functionally manipulate aminergic receptor cells only limited access and insight is given. Further, similar to vertebrates, for each amine different receptor genes were identified that in turn often couple to different signaling
pathways and thereby provide a substrate for complex multi-dimensional functions (reviewed in [1, 82]). This complexity is additionally complicated by differences in the nomenclature (5-HT7 or 5-HT7Dro was also called 5-HT-drol; 5-HT1A or 5-HT1Adro was also called 5-HT-dro2A [10]) and the identification of new receptor genes (5-HT2B was only recently described [13]).

In this study we used state of the art Gal4 lines to anatomically describe and functionally evaluate the role of 5-HT1A, 5-HT1B, 5-HT2A, and 5-HT7 receptor cells with respect to larval chemosensory behaviors [21-23, 26, 27, 29-32, 71]. Due to the lack of specific antibodies this approach is limited as it is not possible to validate the correctness of each Gal4 expression pattern. We found that putative 5-HT/5-HT2A receptor signaling during development is necessary for odor-salt learning and memory. The finding is based on three mutually supportive results. First, ablation of potential 5-HT2A receptor cells throughout development specifically impairs odor-salt learning and memory (Fig 3). Second, 5-HT2A receptor mutant larvae show the same impairment specifically for odor-salt learning and memory (Fig 4). Third, ablation of 5-HT cells throughout development abolishes specifically odor-salt learning and memory (Fig 5), while leaving appetitive odor-sugar and aversive odor-electric-shock learning and memory intact [48]. Thereby, we describe for the first time a potential involvement of the 5-HT system in larval learning and memory. The mode of action, however, of the putative 5-HT/5-HT2A receptor signaling is related to the development of the animal and independent of an acute neuronal function (Fig 6).

4.5.2 5-HT and 5-HT2A receptor cells are dispensable for most of the chemosensory behaviors tested

In addition, we found that potential 5-HT1A, 5-HT1B, and 5-HT7 receptor cells are not necessary for any of the tested chemosensory behaviors (Fig S1, S2, 3). However, given the missing verification for each of the three Gal4 lines expression patterns this result has to be interpreted carefully. Moreover, 5-HT2A receptor cells were not required for olfactory and
gustatory preferences and appetitive olfactory learning and memory (Fig S1, S2, S3, S4, 3). Overall, it is remarkable that the obtained behavioral phenotypes are limited, particularly as several studies revealed that 5-HT receptors are essential for different aspects of larval behavior. For locomotion, for instance, 5-HT1B function was required within a small set of abdominal leucokinin positive neurons to suppress rearing [71]. RNAi-dependent knock-down of 5-HT1B, 5-HT2A, and 5-HT7 receptors within the MB Kenyon cells increased the distance larvae crawled within 140 seconds [32]. Furthermore, 5-HT2A and 5-HT7 receptor mutant larvae showed a reduced number of body-wall contractions [31]. Thus, it seems that larval locomotion requires the entire set of 5-HT receptors within the CNS to organize different aspects of the motor program. Nevertheless, 5-HT receptor function is not limited to locomotion. Pan-neuronal overexpression of 5-HT1A, but not 5-HT1B, 5-HT2A, and 5-HT7, increased the time larvae spend in the light [33]. In addition, 5-HT2A mutants and pan-neuronal knock-down of 5-HT2A reduces larval feeding [13]. This means that individual 5-HT receptors are also important for different kinds of larval behavior. For 5-HT2A this at least includes locomotion, feeding, and odor-salt learning and memory. Future work needs to address whether different sets of cells regulate distinct behavioral outputs or if a single set of cells has multiple functions. Interestingly, in our hands genetic interference with 5-HT receptor function did not affect olfactory and gustatory preferences nor appetitive olfactory learning and memory (Fig S1, S2, S3, S4, 3). Thus, defects in certain aspects of locomotion did not prevent larvae from making chemosensory choices, at least within the test interval of 5 minutes that we have applied. Loss or knock-down of 5-HT receptor function does not completely compromise larval crawling and therefore still allows larvae to orientate within their chemosensory environment over longer time periods. In the future, recently established automated locomotion tracking techniques can be implemented that allow to reconstruct and evaluate larval runs and turns with high spatial and temporal resolution [83-87]. This will
allow to evaluate if 5-HT receptor function is necessary for immediate chemosensory responses or if particular aspects of the chemosensory behavioral output are changed (for example turn rate and run distance).

Interestingly, although 5-HT2A function is required for feeding [13], larvae with missing 5-HT2A cells or reduced 5-HT2A expression are able to associate an odor with a food reward (Fig 3, 4, S4). This might suggest that at least two different systems process gustatory information in the larval CNS: one pathway modulates feeding and depends on 5-HT function, a second pathway is important for appetitive learning and memory and independent of 5-HT and its receptors. Indeed, it was shown that 5-HT function affects all feeding related motor patterns, including head tilting, mouth hook movement, and pharyngeal and esophagus movements [73, 88]. Whether the processing of the sweetness and/or the nutritional value of sugars contributes differently to these proposed pathways is not clear [45]. A similar dual system was recently also found for the processing of bitter quinine [89].

4.5.3 The developmental effect of 5-HT/5-HT2A receptor signaling is specific for odor-salt learning and memory

We have shown that 5-HT cells and 5-HT2A receptor function is necessary for odor-salt learning and memory during development. During learning olfactory stimuli are sensed by only 21 olfactory receptor neurons, which are housed in a single sensillum at the head of the larva, the dorsal organ [90-95]. The olfactory information from a given olfactory receptor neuron is further conveyed by 21 uniglomerular and 14 multiglomerular projection neurons from the AL to the lateral horn and the CA region of the MB [93, 96-99]. Here, intrinsic MB Kenyon cells provide a substrate for synaptic plasticity as olfactory information converges with gustatory reward and punishment signals from different sets of dopaminergic neurons [57, 62, 100, 101]. Further downstream only a limited number of about two dozen MB output neurons [70] transfer the information onto premotor centers to trigger learned behavior.
Given that 5-HT cells and 5-HT$_{2A}$ receptor function is dispensable for appetitive olfactory learning and memory (Fig 3, 4, S4) and given that 5-HT cells [48] as well as 5-HT$_{2A}$-Gal4 positive cells (Fig 5) are not necessary for aversive odor-electric shock learning and memory, we assume that 5-HT/5-HT$_{2A}$ receptor signaling is specifically necessary for salt reinforcement processing. Otherwise, due to the overlapping neuronal circuits (including the olfactory circuit, the mushroom body, and the premotor and motor centers), more general impairments in learning and memory would have occurred.

Where in the reinforcing pathway does 5-HT/5-HT$_{2A}$ receptor signaling become effective? Unfortunately, very little is known about the neuronal pathways signaling aversive salt reinforcement. In *Drosophila* larvae there is no evidence for direct 5-HT input onto the MB [48]. In adults, however, 5-HT positive neurons innervate the MB lobes and CA, termed DPM (dorsal paired medial) and CSD (contralaterally projecting serotonin-immunoreactive deutocerebral) neuron [102, 103]. Yet, the DPM neuron is not present at the larval stage [70] and the CSD neuron only innervates both AL and the lateral protocerebrum but misses the adult specific innervation of the CA and lateral horn [103].

Instead, 5-HT$_{2A}$-Gal4 line expression nearly exclusively innervates the SOG (Fig 1). Most of the innervation comes from cells having their somata outside the CNS. Unfortunately, due to technical limitations we were not able to clearly localize them. However, TRH-Gal4 has a pronounced expression within the SOG (Fig 5 [48]). Thus, although we favor the hypothesis that 5-HT/5-HT$_{2A}$ receptor signaling, which is necessary for salt reinforcement can be attributed to the SOG, future work is needed for validation. In addition, we cannot exclude that the 5-HT$_{2A}$ receptor Gal4 expression pattern may be misleading, given that we were able to show that acute blockage of 5-HT/5-HT$_{2A}$ receptor neurotransmission does not impair odor-salt learning and memory (Fig 6). Thus, neuromodulatory or developmental processes may underlie 5-HT/5-HT$_{2A}$ receptor function. Indeed, 5-HT$_{2A}$ receptor gene expression pattern
markedly changes over larval development [21]. Therefore, it is possible that the impairment of odor-salt learning and memory is based on cellular functions, which are no longer included in the expression pattern of third instar larvae that we used for our analysis. This renders a localization of the cellular effects rather difficult.

4.5.4 5-HT and 5-HT2A receptor signaling regulate developmental processes

Before adopting their roles as neurotransmitters in the mature CNS, neuroactive substances function in the establishment of neural networks [104]. This is also true for 5-HT that can serve as a neurotransmitter and a neuromodulator in all animal phyla studied (reviewed in [105]). In vertebrates it was shown that 5-HT modulates different developmental events, including neuronal migration, cell differentiation, and synaptogenesis (reviewed in [106]). In invertebrates 5-HT regulates - among other processes - cell division in mollusca and the development of the AL during metamorphosis in moths [107, 108]. For *Drosophila* larvae it was shown that dopa decarboxylase mutants that are devoid of 5-HT and dopamine increase the extent of branching of 5-HT projections to the proventriculus and midgut, similar to larvae in which neuronal 5-HT synthesis was constitutively knocked-down [76, 109]. In addition, constitutively knocked-down 5-HT synthesis showed an increased number and size of varicosities in 5-HT fiber projections to the proventriculus [76]. This kind of autoregulation for the organization of 5-HT varicosities was also described in the larval CNS in 5-HT neurons of the A7 segment of the abdominal ganglion [76, 110].

Developmental functions of 5-HT signaling are not limited to larval stages but also include embryonic development. High levels of 5-HT2A receptor expression occur already at 3 hours of embryonic development and match with the seven-stripe pattern of the pair-rule gene *Fushi tarazu* [16]. 5-HT signaling through the 5-HT2A receptor triggers changes in cell adhesiveness that are necessary for normal germband extension during gastrulation [111, 112].
In summary, there is good evidence that 5-HT/5-HT_{2A} receptor signaling serves several developmental functions, including the regulation of neuronal connectivity in addition to their classic role in synaptic transmission. Future work needs to address how this might affect unconditioned stimulus processing using high salt concentration. In the longer run this may uncover 5-HT dependent organizational principles of reinforcement processing shared with adult *Drosophila* and other insects.

### 4.6 Acknowledgements

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### 4.7 Author Contributions

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4.8 Supporting information

4.8.1 Materials and Methods

4.8.1.1 Flies

Flies were maintained on standard Drosophila medium at 25°C or 19°C under 12h light/dark conditions. All 5-HT receptor specific lines, 5-HT1A-Gal4 [29], 5-HT1B-Gal4 [49] (Bloomington Stock Center no. 24240), 5-HT2A-Gal4 [21, 50] (Bloomington Stock Center no. 19367), 5-HT7-Gal4 [23], were kindly provided by Charles Nichols. Note that the 5-HT2A-Gal4 construct is an enhancer trap piggyBac construct in the 5-HT2A locus that reduces its expression by nearly 90% [21]. For behavioral experiments, the effector line UAS-hid,rpr [51, 52] was used. w1118 flies (kindly provided by Martin Heisenberg) were crossed with UAS- and Gal4 lines to obtain heterozygous controls. In all cases third instar larvae were analyzed.

4.8.1.2 Chemosensory preference

For gustatory preference tests, 2.5% agarose (Sigma Aldrich Cat. No.: A5093, CAS No.: 9012-36-6) solution was boiled in a microwave oven and filled as a thin layer into test plates (85 mm diameter, Cat. No.: 82.1472, Sarstedt, Nümbrecht). After cooling, the agarose was removed from half of the plate. The empty half was filled with 2.5% agarose solution containing sodium chloride (SALT, Sigma Aldrich Cat. No.: S7653, CAS No.: 7647-14-5; 2.0 M and 1.5 M), D-fructose (FRU, Sigma Aldrich Cat. No.: 47740, CAS No.: 57-48-7; 2.0 M),
D-sorbitol (SORB, Sigma Aldrich Cat. No.: W302902, CAS No.: 50-70-4; 2.0 M), or D-arabinose (ARA, Sigma Aldrich Cat. No.: 10850, CAS No.: 10323-20-3; 2.0 M). Assay plates were used at the same day shortly after preparation to avoid diffusion of the stimuli from one side to the other. Groups of 30 larvae were placed in the middle of the plate, allowed to crawl for 5 min, and then counted on the stimulus containing side, the agarose only side, and a neutral zone. The neutral zone covers 1 cm from top to bottom of the Petri dish between the left and right sides. It thereby separates both halves and covers the transition from pure agarose to agarose plus gustatory stimulus. By subtracting the number of larvae on the pure agarose side (#nS) from the number of larvae on the stimulus side (#S) divided by the total number of counted larvae (#TOTAL), a preference index for the respective chemosensory stimulus was calculated:

\[
\text{PREF} = \frac{(#S - #nS)}{#\text{TOTAL}}
\]

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

For olfactory preference tests, a similar assay was used except that olfactory stimuli were presented in custom-made Teflon containers with perforated lids presented on only pure agarose containing test plates. As olfactory stimuli amyl acetate (AM, Fluka Cat. No.: 46022; CAS No.: 628-63-7; diluted 1:250 in paraffin oil, Fluka Cat. No.: 76235, CAS No.: 8012-95-1), benzaldehyde (BA, Fluka Cat. No.: 12010, CAS No.: 100-52-7; undiluted), 1-heptanol (HEP, Sigma Aldrich Cat. No.: H2805, CAS No.: 111-70-6; undiluted) and 1-nonanol (NON, Sigma Aldrich Cat. No.: 131210, CAS No.: 143-08-8; undiluted) were used.
4.8.2 Figures

Figure S1 Ablation of potential 5-HT receptor cells does not alter olfactory preferences towards four different odors. 5-HT$_{1A}$-, 5-HT$_{1B}$-, 5-HT$_{7}$-, and 5-HT$_{2A}$-Gal4 lines were crossed with UAS-hid,rpr to genetically induce apoptosis in potential...
5-HT receptor cells. In addition, Gal4 lines and UAS-hid.rpr were crossed with w^1118 to obtain heterozygous genetic control larvae. (A) provides a color scheme for the three different groups used in each experiment. Naïve olfactory preferences for amyl acetate (AM, in B, C, D, E), benzaldehyde (BA, in F, G, H, I), heptanol (HEP, in J, K, L, M), and nonanol (NON, in N, O, P, Q) were analyzed. In none of the cases experimental larvae behaved significantly different from both genetic control groups. We thus reason that ablation of potential 5-HT receptor cells does not impair the ability of the larvae to detect olfactory cues. The sample size (n = 11–16) is indicated under each box plot. Differences against random distribution are given at the top of each box plot. Differences between all three groups or individual groups are shown at the bottom of the panel. *** (p < 0.001), ** (p < 0.01), * (p < 0.05), n.s. (not significant p ≥ 0.05).
Figure S2 Ablation of potential 5-HT receptor cells does not alter gustatory preferences towards four different tastants. 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>7</sub>, and 5-HT<sub>2A</sub>-Gal4 lines were crossed with UAS-hid,rpr to genetically induce apoptosis in potential 5-HT receptor cells. In addition, Gal4 lines and UAS-hid,rpr were crossed with w<sup>1118</sup> to obtain heterozygous genetic control larvae. (A) provides a color scheme for the three different groups used in each experiment. Naïve gustatory preferences for sodium chloride (SALT, in B, C, D, E), fructose (FRU, in F, G, H, I), arabinose (ARA, in J, K, L, M), and sorbitol (SOR, in N, O, P, Q) were analyzed. In none of the cases experimental larvae behaved significantly different to both genetic control groups. We thus reason that ablation of potential 5-HT receptor cells does not impair the ability of the larvae to detect gustatory stimuli. Sample size (n = 13–20) is indicated under each box plot. Differences against random distribution
are given at the top of each panel. Differences between all three groups or individual groups are shown at the bottom of the panel, except for SALT, where it is placed above the box plots. *** (p < 0.001), ** (p < 0.01), * (p < 0.05), n.s. (not significant p ≥ 0.05).

Figure S3 Ablation of potential 5-HT<sub>2A</sub> receptor cells does not alter gustatory preferences towards 1.5 M sodium chloride. 5-HT<sub>2A</sub>-Gal4 was crossed with UAS-hid,rpr to genetically induce apoptosis in potential 5-HT<sub>2A</sub> receptor cells. In addition, the Gal4 line and UAS-hid,rpr were crossed with w<sup>1118</sup> to obtain heterozygous genetic control larvae. Naïve gustatory preferences for 1.5 M sodium chloride (SALT) was analyzed. Experimental larvae behaved at the same level as both genetic control groups. We thus reason that ablation of potential 5-HT<sub>2A</sub> receptor cells does not impair the ability of the larvae to detect 1.5 M sodium chloride. Sample size (n = 15) is indicated under each box plot. Differences against random distribution are given at the top of each panel. Differences between all three groups or individual groups are shown above the box plots. n.s. indicates that the initial KWT did not suggest for a difference between the three groups (p > 0.05). *** (p < 0.001), ** (p < 0.01), n.s. (not significant p ≥ 0.05).
Ablation of potential 5-HT2A receptor cells throughout development impairs aversive olfactory learning and memory. 5-HT2A-Gal4 was crossed with UAS-hid,rpr to genetically induce apoptosis in potential 5-HT2A receptor cells. In addition, the Gal4 line and UAS-hid,rpr were crossed with w^1118 to obtain heterozygous genetic control larvae. (A) provides a color scheme for the three different groups used in each experiment. (B) Appetitive olfactory learning and memory using fructose reinforcement is shown at the top. (C) Aversive olfactory learning and memory is shown at the bottom. (B) For appetitive olfactory learning experimental larvae and genetic control groups behaved similar. Yet, ablation of 5-HT2A-Gal4 positive cells throughout development specifically impaired aversive olfactory learning and memory (C). Sample size (n = 17–20) is indicated at the bottom of each box plot. Differences against zero are given at the top of each box plot. Differences between all three groups or individual groups are shown at the bottom of the panel. Visualization of statistical evaluations: if only n.s. is shown the initial KWT did not suggest for a difference between the three groups (p ≥ 0.05). When differences between each group are shown this provides the results of the BWRT as the initial KWT suggested for significance (p > 0.05). *** (p < 0.001), ** (p < 0.01), * (p < 0.05), n.s. (not significant p ≥ 0.05).

4.8.3 Additional data

Additional data including raw behavior data and anatomical movies of GAL4 expression can be found online (http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0181865#pone-0181865-g002).
4.9 References


5 Four Individually Identified Paired Dopamine Neurons Signal Reward in Larval *Drosophila*.


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5.1 Summary

Dopaminergic neurons serve multiple functions, including reinforcement processing during associative learning [1–12]. It is thus warranted to understand which dopaminergic neurons mediate which function. We study larval Drosophila, in which only approximately 120 of a total of 10,000 neurons are dopaminergic, as judged by the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme of dopamine biosynthesis [5, 13]. Dopaminergic neurons mediating reinforcement in insect olfactory learning target the mushroom bodies, a higher-order “cortical” brain region [1–5, 11, 12, 14, 15]. We discover four previously undescribed paired neurons, the primary protocerebral anterior medial (pPAM) neurons. These neurons are TH positive and subdivide the medial lobe of the mushroom body into four distinct subunits. These pPAM neurons are acutely necessary for odor-sugar reward learning and require intact TH function in this process. However, they are dispensable for aversive learning and innate behavior toward the odors and sugars employed. Optogenetical activation of pPAM neurons is sufficient as a reward. Thus, the pPAM neurons convey a likely dopaminergic reward signal. In contrast, DL1 cluster neurons convey a corresponding punishment signal [5], suggesting a cellular division of labor to convey dopaminergic reward and punishment signals. On the level of individually identified neurons, this uncovers an organizational principle shared with adult Drosophila and mammals [1–4, 7, 9, 10] (but see [6]). The numerical simplicity and connectomic tractability of the larval nervous system [16–19] now offers a prospect for studying circuit principles of dopamine function at unprecedented resolution.

5.2 Results and Discussion

5.2.1 Four Paired Tyrosine-Hydroxylase-Positive Neurons of the Previously Undescribed pPAM Cluster Innervate the Larval Mushroom Body

Judged by the defects of dopamine receptor mutants, the dopaminergic system is necessary for aversive and appetitive olfactory learning [5]. However, although it was revealed that
aversive learning can come about by dopaminergic cells covered by the TH-Gal4 driver, including those of the DL1 cluster [5], the cellular identity of neurons involved in appetitive learning of the larva remained clouded. We aimed to reveal the nature of these cells.

We use an antibody that specifically recognizes the enzyme tyrosine hydroxylase (TH) to identify neurons as likely to be dopaminergic [5, 20], as the TH enzyme specifically catalyzes the rate-limiting step of dopamine biosynthesis. We confirm the three previously reported cell clusters (DL1, DL2, and DM; Figure 1A) [5, 20–22]. We additionally uncover a cluster located anteriorly and medially, consisting of four pairs of TH-positive neurons (Figures 1B–1I). We termed this cluster the primary-lineage protocerebral anterior medial (pPAM) cluster and the respective neurons pPAM1–4. This cluster is not evident in the larval TH-Gal4 expression pattern and had therefore previously escaped attention (in flp-out experiments from TH-Gal4, only a faint expression in pPAM2 was rarely observed [5]).

We then screened the larval expression patterns of the Janelia collection of Gal4 driver strains [23] for coverage of pPAM neurons and identified the strains R30G08, R58E02, and R64H06 (Figures 2 and S1). These driver strains show specific expression in two, three, and four pPAM neurons, respectively, per brain hemisphere (Figures 2A–2I and S1). Analyses of flp-out expression patterns [24] for each strain show that R30G08 covers the pPAM1,3 neurons, whereas R58E02 expresses in pPAM1,3,4 and R64H06 in all four pairs of pPAM cluster neurons (Figure S1). These pPAM neurons innervate the mushroom body medial lobe at four distinct tiles (Figures 1E–1I and S1). The flp-out experiments also showed rare expression in the pPAM2 neuron in the R30G08 and R58E02 driver strains (4 out of 97 brains; boxed in gray in Figure S1). Across the large number of animals involved in behavioral testing, though, such low-probability expression would be without measurable consequence [25].
Figure 1 Four Paired Neurons, pPAM1–4, Subdivide the Mushroom Body Medial Lobes into Four Distinct Tiles (A–D) Anti-TH labeling shows likely dopaminergic neurons (green), and anti-ChAT/FasII staining reveals the neuropil/axonal tracts (magenta) of partial brain projections from TH-Gal4;UAS-mCD8::GFP larvae. (A) In the posterior half of the brain, the three previously identified clusters of TH-positive neurons called DL1, DL2, and DM can be discerned. (B) In the anterior half of the brain, a paired cluster of four TH-positive neurons is here identified and named primary-lineage protocerebral anteriormedial cluster (pPAM1–4, arrows). (C and D) TH-positive neurons densely innervate the mushroom body, including its vertical lobe (vl), lateral appendix (la), and medial lobe (ml) (only the right brain hemisphere is shown; the insert in D shows the four cell bodies of the mushroom body-projecting pPAM neurons). (E–H) Flp-out clones of Gal4 strains (see Figure 2) that cover the pPAM cluster reveal distinct innervation in the tiles of the mushroom body medial lobe, symmetrically for both hemispheres. Green labeling shows anti-GFP staining; magenta labeling is as above. For a more detailed anatomical description, see Figure S1. (I) Schematic of the tiled organization of the medial lobe of the right brain hemisphere and the innervation by the pPAM1–4 neurons. Scale bars, 50 μm (A and B) and 25 μm (C–H). See also Figure S1.

Using the dendritic marker DenMark to reveal postsynaptic regions [26] and Synaptobrevin-GFP to label presynaptic regions [27], we found that presynaptic staining from pPAM cluster neurons was detectable in the medial lobe and postsynaptic labeling across the lateral and medial protocerebrum and weakly in the medial lobe (Figures 2J–2O).

Taken together, these data suggest that the four paired pPAM neurons deliver a likely dopaminergic signal to the medial lobe of the mushroom body and do so individually for separate mush-room body lobe tiles.
Figure 2 Ga4 Strains Covering pPAM Cluster Neurons (A, D, and G) The R30G08 driver strain covers two likely dopaminergic TH-positive neurons, namely pPAM1,3. (A and D) The R30G08 driver strain was crossed to the UAS-mCD8::GFP effector strain. A z projection of an anterior view of a larval whole-mount brain is shown using anti-GFP (green) and, as neuropil/axonal tract markers, anti-ChAT/FasII (magenta). The pPAM1,3 neurons are identified based on expression in their respective tiles of the medial lobe and by flp-out clones (Figure S1 shows one of the rare flp-out clones of pPAM2). Asterisks in (D) highlight the four different subunits of the medial lobe (same in E and F). (G) Same genotype as above. Co-labeling of anti-GFP and anti-TH suggests a likely dopaminergic nature of the pPAM1,3 neurons covered by the R30G08 driver strain. (B, E, and H) The R58E02 driver strain covers three likely dopaminergic TH-positive neurons, namely pPAM1,3,4. Other details are as above. Supporting flp-out clones are shown in Figure S1 (Figure S1 shows one of the rare flp-out clones of pPAM2). (C, F, and I) The R64H06 driver strain covers all four likely dopaminergic TH-positive pPAM1–4 neurons. Note the innervation of the complete medial lobe. Other details are as above. Supporting flp-out clones are
displayed in Figure S1. (J–O) Polarity of the pPAM cluster neurons. (J and M) With R58E02 (J) and R64H06 (M) used as
driver strains, UAS-DenMark was expressed to mark postsynaptic, input regions (red) and UAS-nsyb::GFP to mark
presynaptic, output regions (green). Anti-ChAT/FasII neuropil staining is shown in blue. (K and N) The postsynaptic, input
regions (shown in green; neuropil and axonal tracts are shown in magenta) of the pPAM neurons are mainly located in the
medial and lateral protocerebrum, with additional sparse signals from the medial lobe. (L and O) The presynaptic, output
regions (shown in green; neuropil and axonal tracts are shown in magenta) are limited to the medial lobe of the mushroom
body. Scale bars, 50 μm (A–C) and 25 μm (D–O). See also Figure S1.

5.2.2 pPAM Neurons Acutely Function for Appetitive, but Not Aversive,
Learning

We crossed the driver strains R30G08, R58E02, and R64H06 for the expression of the
apoptosis proteins Hid and Reaper to ablate [28, 29] the respective sets of pPAM neurons
(Figures S4A and S4B). These animals were then tested in an odor-sugar associative memory
paradigm [30]. As sugars we used fructose, arabinose, and sorbitol [31, 32], as they differ in
nutritional value and thus conceivably in the set of sensory neurons that they activate.

Ablation of only the pPAM1,3 neurons in the R30G08 strain left the rewarding effect of all
three sugars largely unaffected (Figures 3A–3D); ablating the pPAM1,3,4 neurons in the
R58E02 strain reduced only fructose and sorbitol reward learning (Figures 3E-3H); ablating
pPAM1-4 — that is, all neurons of the cluster — in the R64H06 strain reduced learning for all
three sugars (Figures 3I-3L). By a combinatorial argument, the pPAM4 neuron thus appears
to be required for the full rewarding effects of fructose and sorbitol, whereas the pPAM2
neuron appears to be required for the full rewarding effect of arabinose (Figure 3R; “labeled
line hypothesis’’). However, acute silencing of synaptic output from pPAM2 neurons does not
impair the rewarding effect of arabinose (Figures 4C–4H). It therefore seems possible that
ablating progressively more pPAM neurons leads to more severe reductions in sugar reward
learning (Figures 4A and 4B; ‘‘mass action hypothesis’’). In any event, in none of the cases of
defective odor-reward learning did we find gross defects in task-relevant sensory-motor
abilities (Figures S3A–S3E).

We next examined the requirement of pPAM neurons for aversive learning [33–36]. Removal
of the pPAM1,3,4 neurons using the driver strain R58E02 had no effect on odor-quinine
memory scores (Figure 3M). Use of the driver strain R64H06 that covers all pPAM neurons confirms this result (Figure 3P). Thus, the pPAM neurons appear dispensable for odor-quinine learning.

We further examined the effects of acutely blocking synaptic output from the pPAM neurons. We expressed a temperature-sensitive dynamin (Shibire<sup>ts</sup>) in the pPAM1,3,4 neurons to block their synaptic output only during the experiment; this is sufficient to reveal their acute requirement for appetitive learning using fructose as sugar reward (Figure 3N). This manipulation left task-relevant sensory-motor function intact (Figures S3F–S3H).

To test whether the impairment in fructose reward learning upon disabling the pPAM neurons is related to dopamine function, we knocked down the dopamine-synthetizing TH enzyme by RNAi, using the driver R64H06 covering all pPAM neurons. This manipulation led to a reduction in fructose reward learning (Figure 3Q), whereas task-relevant sensory-motor function remained intact (Figures S3I–S3K). This result also makes it un-likely that non-pPAM neurons covered in R64H06 contribute to the phenotype, as these do not express TH.

Based on the results so far, we suspected that optogenetic activation of pPAM neurons by transgenic Channelrhodopsin2 expression might substitute for reward stimulation [8]. Activation of the pPAM1,3,4 neurons as covered by the R58E02 driver was sufficient for such reward substitution (Figure 3O, left), provided that retinal was fed to the larvae to enable Channelrhodopsin2 function (Figure 3O, right). Notably, this rewarding effect was strong enough to overcome the otherwise slightly punitive effect of the light needed to activate Channelrhodopsin2 (see genetic controls in Figure 3O).

We conclude that the pPAM neurons mediate a likely dopaminergic appetitive reinforcement signal toward the mushroom body.
Figure 3 pPAM Neurons Mediate Reward Signals In all panels (except D, H, L, and R), associative performance indices are shown for tests immediately after associative, classical conditioning. The three pPAM-specific driver strains R30G08, R58E02, and R64H06 were crossed to the effector UAS-hid,pr to induce apoptosis (A–M and P), to UAS-shi to acutely block synaptic output (N), to UAS-ChR2 to artificially activate them (O), or to UAS-TH-RNAi to knock down TH function (Q). Box plots represent the median as the middle line and 25%/75% and 10%/90% as box boundaries and whiskers, respectively. Sample size in each case is n = 16. Differences between groups are depicted below the respective box plots. Small circles indicate outliers. n.s., p > 0.05; *p < 0.05. (A–C) With R30G08 used as driver strain to ablate the pPAM1,3 neurons, associative performance indices are not robustly decreased for any of the three sugar rewards. That is, in no case were associative performance indices upon pPAM1,3 ablation lower than in both genetic controls. (D) Schematic of medial lobe innervation by the pPAM1,3 neurons. Solid fill indicates the ablation, and light fill indicates the presence of the cell.
innervating the respective tile. (E–G) Using R58E02 as driver strain to ablate the pPAM1,3,4 neurons leads to an impairment in odor-fructose (both p < 0.05) and odor-sorbitol (both p < 0.05) learning, but not for arabinose as reward (both p > 0.05). (H) Schematic of medial lobe innervation by the pPAM1,3,4 neurons. (I–K) Ablation of all four pPAM neurons using R64H06 as a driver strain leads to an impairment for all three sugar rewards (all p < 0.05). (L) Schematic of medial lobe innervation by the pPAM1–4 neurons. (M) Aversive olfactory learning using quinine as punishment is not decreased upon ablation of pPAM1,3,4 using R58E02 as driver (all p > 0.05). (N) To test for the acute function of the pPAM1,3,4 neurons, we expressed a temperature-sensitive dynamin using UAS-shi^ts1 from the R58E02 driver. An acute block of synaptic output from these neurons, at restrictive temperature, strongly reduces odor-fructose associative function (both p < 0.05). At a permissive temperature, synaptic output remains intact in the experimental and the control genotypes, and no difference in associative function is detectable between strains (both p > 0.05). (O) To test whether optogenetic activation of the pPAM1,3,4 neurons is sufficient to substitute for a reward, we used the R58E02 driver in combination with UAS-ChR2 to express Channelrhodopsin2. The behavioral experiment then is the same as above, except that the sugar reward is replaced by light-activation of the pPAM1,3,4 neurons. That is, one odor is presented together with light stimulation and thus with activation of pPAM1,3,4, whereas the second odor is presented in darkness. Only larvae of the experimental genotype, but not of the genetic controls, show an associative difference in preference between these groups (left; @ retinal; both p < 0.05). This shows that activation of the pPAM1,3,4 neurons is sufficient to mediate a reward signal. Without feeding retinal, which is required for Channelrhodopsin2 function, no such appetitive learning is observed in any genotype (right; @ no retinal; both p > 0.05). (P) Aversive olfactory learning using quinine as punishment is not decreased even upon ablation of the entire pPAM cluster using R64H06 as driver (both p > 0.05). (Q) Knockdown of TH function in all four pPAM neurons using R64H06 as driver strain leads to impaired learning using fructose as a reward (both p < 0.05). (R) Labeled line hypothesis. The defects in associative function upon ablating subsets of pPAM neurons as shown in Figure 3 could be explained by a combinatorial argument suggesting that pPAM4 is essential for a fructose/sorbitol reward signal, whereas pPAM2 is essential for an arabinose reward signal. See also Figures S2, S3, and S4.

5.2.3 Reinforcement Signaling in Larval Drosophila

Our discovery of the four paired pPAM neurons as mediators of an appetitive reinforcement signal in larval Drosophila complements earlier work showing that a distinct set of likely dopaminergic neurons, included in the TH-Gal4 expression pattern, is sufficient as an aversive reinforcement signal in these animals [8]. Such division of labor uncovers an organizational principle shared with adult Drosophila, though at massively reduced cell numbers, a principle that may hold true in mammals, as well [1–4, 7, 9, 10, 37] (but see [6]).

Given that all four pPAM neurons innervate the medial lobes of the mushroom bodies, our study points to the medial lobe as site of odor-reward memory trace formation. Regarding aversive learning, the likely dopaminergic inputs to other regions could provide this function [5]. This situation, again at much reduced cell numbers, uncovers a principle shared with adult Drosophila (Figure S4) [1–4, 10, 15, 38, 39].

Likewise similar to the situation in adults [2], activation of a set of likely octopaminergic/tyraminergic neurons is sufficient to mediate an appetitive reinforcement effect, too [8]. In honey-bees, activation of a single, unpaired and likely octopaminergic neuron, the VUMmx1 neuron, is sufficient to signal appetitive reinforcement [40].
Figure 4 Alternative Hypothesis of Reward Processing in the pPAM Neurons (A) Mass action hypothesis. The number, rather than the identity, of pPAM neurons may matter for reward function, such that defects in associative function are the more likely to be observed the more pPAM neurons are affected. The figure illustrates that all pPAM neurons may thus be involved in mediating the reward function of all three sugars. (B) Semi-schematic presentation of the decrease in associative function upon ablating two pPAM neurons (in R30G08), three pPAM neurons (in R58E02), or all four pPAM neurons (in R64H06); each data point refers to the median performance index (PI) for either one of the three sugars. This plot suggests that defects get stronger the more pPAM neurons are ablated. (C–H) Within the mushroom bodies, the driver strains NP7139 and NP7231 cover only the pPAM2 neuron. (C, D, F, and G) Driver strains were crossed to the UAS-mCD8::GFP effector strain. z projections are shown, either of the entire brain (C and F) or of the mushroom body (D and G), with an anterior view using anti-GFP (green) and, as neuropil/axonal tract markers, anti-ChAT/FasII (magenta). Use of NP7139 (C–E) or NP7231 (F–H) as driver strains to block synaptic output from the pPAM2 neuron does not impair learning with arabinose as reward (all p > 0.05); this does not support the labeled line hypothesis (Figure 3R). Asterisks highlight the four different subunits of the medial lobe in (D) and (G). Box plots represent the median as the middle line and 25%/75% and 10%/90% as box boundaries and whiskers, respectively. Scale bars, 25 μm (C, D, F, and G).
Within the mushroom body, this neuron innervates the olfactory input regions in the calyx. A similar type of neuron exists in adult and larval *Drosophila* [41, 42]. The way in which the dopaminergic and the octopaminergic/tyraminergic systems jointly organize appetitive reinforcement signaling is a fascinating issue. These systems may differentially convey nutritional and non-nutritional aspects of reward and/or different kinds of reward [2, 11, 31, 41, 43].

Thus, reinforcement processing in the larval and the adult *Drosophila* brain follows similar principles of circuit organization—however, with strikingly reduced cell numbers in the larval case. The larval pPAM cluster features only four neurons, whereas in the adult there are about 30 times more of these neurons [1, 2, 15]. Although this may allow for the representation of more kinds of ‘‘valuables’’ in the adult (Figure S4) [11, 44, 45], the numerical simplicity of the larval nervous system, together with the ongoing efforts toward its complete connectome [19], might bring a full-brain, single-cell, and single-synapse understanding of memory into reach for the larva.

### 5.3 Experimental Procedures

#### 5.3.1 Fly Strains

Flies were reared under standard conditions unless mentioned otherwise. UAS-*mCD8::GFP* (*w*.;*P[20XUAS-IVS-mCD8::GFP]attP2*; Bloomington Stock Center no. 32194) and *y, w, hsp70-flp; Sp/CyO; UAS-CD2y+ > mCD8::GFP/TM6b* [24] were used to analyze the morphology of the pPAM cluster. UAS-*nsy::GFP*; UAS-*DenMark* (*w1118; L1/CyO; P[UAS-DenMark]3, P[UAS-syt.eGFP]3*; Bloomington Stock Center no. 33065) was used to label pre- and postsynaptic terminals [26]. The Gal4 strains R30G08/TM6b, R58E02, and R64H06/TM6b (*w1118; P[GMR30G08-GAL4]attP2/TM6; *w1118; P[GMR58E02-GAL4]attP2; *w1118; P[GMR64H06-GAL4]attP2/TM6*; Bloomington Stock Center nos.
48101, 41347, 49608) were identified by screening the database of [23]. The Gal4 strains NP7139 and NP7231 (\textit{w}[*] \textit{P}[^{+mW.hs}] = \textit{GawB}); Kyoto Stock Center nos. 114098 and 114162) were identified by screening the NP collection. \textit{UAS-hid,rpr} (\textit{y, w}1118, \textit{P}\{\textit{UAS-hid}\}, \textit{P}\{\textit{UAS-rpr}\}) was used to ablate neurons [28, 29, 46]; \textit{UAS-shits} (\textit{w}1118; \textit{P}\{\textit{UAS-shits1}\}; Bloomington Stock Center no. 44222) [47] was used to acutely block synaptic output; and \textit{UAS-ChR2} (\textit{w}[*];\textit{P}\{\textit{UAS-ChR2.S}\};3; Bloomington Stock Center no. 9681) allows activation of neurons by blue light [8]. \textit{UAS-TH-RNAi} was used to interfere specifically with \textit{TH} gene function (TriP JF01813; Bloomington Stock Center no. 25796) [48]. The strains were not isogenized before the experiments.

5.3.2 Immunostaining

Third-instar larvae were put on ice and dissected in PBS [5, 41]. Brains were fixed in 3.6% formaldehyde (Merck) in PBS for 30 min. After eight rinses in PBT (PBS with 3% Triton X-100; Sigma-Aldrich), brains were blocked with 5% normal goat serum (Vector Laboratories) in PBT for 2 hr and incubated for 2 days with primary antibodies at 4° C. Before application of the secondary antibodies for 2 days at 4° C, brains were washed eight times with PBT. After secondary antibody incubation, brains were washed eight times with PBT, mounted in Vectashield (Vector Laboratories) and stored at 4° C in darkness. Images were taken with a Zeiss LSM 510M confocal microscope with 25x or 40x glycerol objectives. The resulting image stacks were projected and analyzed with Image J (NIH; http://imagej.nih.gov/ij). Contrast and brightness adjustment, rotation, and arrangement of images were performed in Photoshop (Adobe Systems).

For the single-cell staining \textit{y, w, hsp70-flp; Sp/CyO; UAS>CD2y+::mCD8::GFP/TM6b} virgins were crossed to R30G08, R58E02, or R64H06 males. A single heat shock of 37° C was applied for 18 min by placement of the vials in a water bath. For the onset of heat shock, we chose different times from 0 to 200 hr after egg laying.
5.3.3 Antibodies

For analysis of Gal4 expression patterns and individual neurons a rabbit anti-GFP antibody (A6455; Molecular Probes; 1:1000) and two different mouse antibodies for staining the cholinergic neuropil (ChAT4B1; DSHB; 1:150) and axonal tracts (1d4 anti-FasciclinII; DSHB; 1:50) were applied [5, 41]. DA neurons were visualized with a polyclonal antibody against TH (1:800) [5]. Pre- and postsynaptic structures were identified using the conjugated goat GFP FITC antibody (ab 6662; Abcam; 1:1000) to label the UAS-nsyb::GFP effector and rabbit anti-DsRed (632496; Clonetech; 1:200) to visualize the UAS-DenMark effector.

As secondary antibodies, goat anti-rabbit IgG Alexa Fluor 488 (A11008; Molecular Probes; 1:200), goat anti-mouse IgG Alexa Fluor 647 (A21235; Molecular Probes; 1:200), goat anti-mouse IgG Cy3 (A10521; Molecular Probes; 1:200) and goat anti-rabbit IgG Cy5 (A10523; Molecular Probes; 1:200) were used.

5.3.4 Odor-Sugar Learning

Experiments were conducted on assay plates filled with a thin layer of 2.5% agarose containing either pure agarose (Sigma Aldrich cat. no. A5093; CAS no. 9012-36-6) or agarose plus D-fructose (Sigma Aldrich cat. no. 47740; CAS no. 57-48-7), D-arabinose (Sigma Aldrich cat. no. A3131; CAS no. 10323-20-3), or D-sorbitol (Sigma Aldrich cat. no. W302902; CAS no. 50-70-4) at a concentration of 2 M [30, 31]. As olfactory stimuli, we used 10 μl amyl acetate (AM; Fluka 46022; CAS no. 628-63-7; diluted 1:250 in paraffin oil, Fluka 76235; CAS no. 8012-95-1) and benzaldehyde (BA; undiluted; Fluka 12010; CAS no. 100-52-7). Odorants were loaded into custom-made Teflon containers (4.5 mm diameter) with perforated lids [30]. A first group of 30 animals was exposed to AM while crawling on agarose medium containing in addition sugar as a positive reinforcer. After 5 min, larvae were transferred to a fresh, pure-agarose Petri dish and exposed to BA (AM+/BA). This cycle of training trials was repeated two more times. A second group of larvae received reciprocal
training (AM/BA+). Then larvae were transferred onto test plates containing pure agarose on which AM and BA were presented on opposite sides. After 5 min, individuals were counted as located on the AM side (# AM), the BA side (# BA), or in a 10 mm neutral zone. We determined a preference index for each training group as follows (these preference indices are documented in Figure S2):

\[
\text{Pref}_{\text{AM+/BA}} = \frac{(\# \text{AM} - \# \text{BA})}{\# \text{Total}} \quad \text{(Equation 1A)}
\]

\[
\text{Pref}_{\text{AM+/BA}} = \frac{(\# \text{AM} - \# \text{BA})}{\# \text{Total}} \quad \text{(Equation 1B)}
\]

To measure specifically the effect of associative learning, we then calculated the associative performance index (PI) as the difference in preference between the reciprocally trained larvae:

\[
\text{PI} = \frac{\text{Pref}_{\text{AM+/BA}} - \text{Pref}_{\text{AM+/BA}}}{2} \quad \text{(Equation 2)}
\]

Negative PIs thus represent aversive associative learning, whereas positive PIs indicate appetitive associative learning. Division by 2 ensures scores are bound within (-1; 1). The sequence of training trials (i.e., AM+/BA or BA/AM+) was alternated across repetitions of the experiment.

5.3.5 Odor-Quinine Learning

Odor-quinine learning was performed as described above for odor-sugar learning [33], except that instead of sugar 6 mM quinine (quinine-hemisulfate; Sigma Aldrich cat. no. Q1250; CAS no. 207671-44-1) was used with 1% agarose [49]. Given that learned aversive behavior is a form of learned escape, the testing situation needs to actually warrant escape; therefore, quinine needs to be added to the test plate [33].

5.3.6 Substitution Experiment

To substitute an actual sugar reward by remotely activating neurons, we used UAS-ChR2 [8]. Fly strains were reared on standard Drosophila medium that included retinal (100 mM final
concentration; Sigma Aldrich cat. no. R2500; CAS no. 116-31-4) at 25°C in darkness. A group of 30 feeding-stage third-instar larvae were placed onto plates containing 2.5% agarose and exposed to either AM or BA. During the presentation of the first odor, the larvae were exposed to blue light (470 nm; ~ 20 000 lux) for 5 min. The second odor was then presented in darkness. As described for odor-sugar learning, training was performed reciprocally and the sequence of training trials was alternated across repetitions of the experiment. Data were then scored as above.

5.3.7 Acutely Blocking Synaptic Output with shibire
to
To acutely block synaptic output, we used UAS-shi[47]. The larvae were incubated for 2 min in a water bath at 37°C. The behavioral experiments were then performed as described before, at a restrictive temperature of about 35°C. Control experiments were performed with incubation at room temperature and at a permissive temperature of about 23°C.

5.3.8 Statistical Methods
Kruskal-Wallis tests were performed and, in case of significance, followed by Wilcoxon rank-sum tests; Holm-Bonferroni corrections were used for multiple comparisons as applicable. Likewise, Wilcoxon signed-ranked tests were used to compare values against chance level.

All statistical analyses were performed with R version 2.14.0 and Windows Excel 2010. Figure alignments were done with Adobe Photoshop. The behavioral data are presented as boxplots (middle line, median; box boundaries, 25%/75% quantiles; whiskers, 10%/90% quantiles; circles, outliers). Asterisks and “n.s.” indicate p > 0.05 and p < 0.05, respectively.
5.4 Supplemental Information

Figure S1 Additional data related to Figure 1 and 2. Anatomy of the four paired pPAM neurons and coverage of pPAM neurons revealed by clonal analysis from the driver strains R30G08, R58E02 and R64H06. The pPAM cluster...
consists of four paired neurons, named pPAM1-4. Shown are frontal views of Z-projections on the mushroom body using anti-GFP (green) and anti-ChAT/FasII (magenta) antibodies. From left to right, the first column shows a projection of the mushroom body region; the second column shows the respective green channel only; the third and fourth columns present detailed views of the ipsi- and contralateral medial lobe, respectively; the rightmost column depicts the ipsilateral innervation in the protocerebrum. Preparations were obtained using the flp-out technique from the driver strains R30G08 (for pPAM1 and pPAM3) and R64H06 (for pPAM2 and pPAM4). Similar morphology was found for all clones of a given neuron for the three Gal4 lines. All four neurons have their cell body located in the anterior medial part of the brain. The primary neurite projects posterior up to an area medial of the base of the vertical lobe. Here, the primary neurite splits into two secondary branches. The first branch projects dorsally to form postsynaptic structures in the dorsal protocerebrum. The second branch runs basomedial to innervate the contralateral medial lobe in the same tile. Based on the gross anatomy of the medial lobe innervation, presynaptic structures (and some postsynaptic as well) are very likely. Irrespective of these general features, each pPAM neuron is individually identifiable: (A-E) The pPAM1 neuron innervates the medial lobe most laterally compared to all other pPAM neurons, and is largely presynaptic in this region. Its postsynaptic innervation is not only restricted to an area medial to the vertical lobe of the mushroom body but also includes areas of the dorsolateral protocerebrum, further lateral of the vertical lobe. The shown clone is derived from the R30G08 driver strain. (F-J) The pPAM2 neuron innervates the basal tip of the medial lobe, and is largely presynaptic in this region. Its postsynaptic innervation is restricted to the medial part of the brain next to the vertical lobe. In several cases pPAM2 clones showed a second or even a third presynaptic branch that crossed the midline more dorsally than the one innervating the medial lobes; these ended in the dorsomedial part of the contralateral brain by forming a small number of synaptic boutons. The shown clone is derived from the R64H06 driver strain. (K-O) The pPAM3 neuron innervates the dorsal tip of the medial lobe, and is largely presynaptic in this region. Its postsynaptic arborization includes the medial part of the ipsilateral brain in a more widespread fashion, and more dorsally compared to pPAM2. The shown clone is derived from the R30G08 driver strain. (P-T) The pPAM4 neuron innervates the very medial part of the medial lobe, and is largely presynaptic in this region. Its postsynaptic innervation is restricted to the most dorsomedial and basomedial protocerebrum. The shown clone is derived from the R64H06 driver strain. Scale bars: 25 µm. 

(U-AF) All images show Z-projections of frontal views onto the larval brain. Clones of pPAM neurons are labeled by anti-GFP (green); anti-ChAT/FasII antibodies are used to visualize neuropil structures/axonal tracts (magenta). Typically, clones are obtained at frequencies of around 10% (solid green lines). In contrast, clones for the pPAM2 neuron in the driver strains R30G08 and R58E02 were yielded at much lower frequencies (solid grey line). In these two driver strains the pPAM2 neuron therefore does not show in the full expression pattern (note that in Figure 2D and E the pPAM2 tile is spared). Across the large number of animals involved in behavioral testing such low-probability expression is without measurable consequence, as the larvae in our assay behave independently of each other [S1]. (U, V, W, X) Single cell clones from R30G08 were regularly found for the pPAM1 and pPAM3 neuron (solid green lines), but only rarely for pPAM2 (solid grey line). (Y, Z, AA, AB) Clones from R58E02 regularly yield the pPAM1, pPAM3, and pPAM4 neuron (solid green line), but only rarely pPAM2 (solid grey line). Note that (Z) shows a double-cell clone of the pPAM2 neuron in both hemispheres. Asterisks highlight the location of the four different subunits of the medial lobe in V and Z. (AC, AD, AE, AF) Clones from R64H06 are regularly obtained for all four pPAM neurons (solid green line). Note that (AE) shows a double-cell clone of the pPAM3 neuron in both hemispheres. Scale bars: 50 µm.
Figure S2: Additional data related to Figure 3. Preference indices. Preference Indices of the reciprocally trained groups underlying the associative Performance Indices shown in Figure 3. Preference Indices are measured either after the odor AM.
was paired with the respective reinforcer (e.g. AM+, left boxes for each genotype) or after the odor BA was paired with the reinforcer (e.g. BA+, right boxes). Positive values indicate approach towards AM, negative values indicate approach towards BA. Differences in preference between reciprocally trained groups indicate associative memory.

### Cell ablation experiments

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### Blockage of neuronal output (measured at restrictive temperature)

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### TH knock-down via UAS-TH-RNAi using the driver line R64H06

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Figure S3: Additional data related to Figure 3. Manipulation of pPAM neurons leaves task-relevant sensory-motor faculties intact. Gustatory preference of experimentally naïve animals towards all three sugar rewards (A: fructose, B: arabinose, and C: sorbitol, respectively) is compared for those experimental groups that did show defects in the respective learning experiment upon ablation of pPAM neurons (see Figure 3A-K) and their respective genetic controls. Likewise, in D and E the olfactory preference of experimentally naïve animals towards the to-be-associated odors AM (D) and BA (E) is shown. In F-H the preference of experimentally naïve animals for the to-be-associated stimuli (fructose, AM, BA) upon blocking synaptic output from pPAM1,3,4 in the R58E02 driver strain (see Figure 3N) is shown. In I-K the preference of experimentally naïve animals for the to-be-associated stimuli (fructose, AM, BA) upon expression of UAS-TH-RNAi in the entire pPAM cluster via the R64H06 driver strain (see Figure 3Q) is shown. In none of the cases do differences in preference reveal (p > 0.05 in Kruskal-Wallis tests), arguing that the defects in learning shown in Figure 3 are not secondary to defects in task-relevant sensory motor faculties.
Figure S4: Additional data related to Figure 3. Verification of genetically induced cell ablation, identification of potential synaptic connections of pPAM neurons as judged by GFP reconstitution across synaptic partners (GRASP), and comparison of adult and larval expression patterns (A and B) R64H06/UAS-hid,rpr labeled with anti-TH (to visualize potential dopaminergic neurons, green) and anti-ChAT/FasII antibodies (to reveal neuropil structures/axonal tracts, magenta). (A) Likely dopaminergic neurons in the posterior half of the brain are not altered and the three previously known
clusters DL1, DL2, and DM are visible. (B) In the anterior half of the brain all four pairs of the pPAM cluster neurons are specifically ablated (arrow). Scale bars: 25 µm. (C–F) Partial Z-projection of frontal views for one hemisphere of larval brains of R58E02-LexA, LexAop-mCD4::GFP11; UAS-mCD4::GFP1-10 crossed with dNPF-Gal4 (C), OK107-Gal4 (D), GR43a-Gal4 (E) or TDC2-Gal4 (F), respectively. Reconstituted GFP is visualized in green; the neuropil/axonal tracts are visualized by anti-ChAT/FasII staining (magenta). The pPAM1,3,4 neurons as covered by the R58E02 LexA driver are in close proximity to neurons included in neuropeptide F positive neurons of the dNPF-Gal4 driver (C, arrow), mushroom body Kenyon cells included in the OK107-Gal4 expression pattern (D, arrow), and tyraminergic/octopaminergic neurons included in the TDC2-Gal4 expression pattern (F, arrows). No GRASP signal was detectable when using GR43a-Gal4 together with R58E02-LexA (E). Scale bars: 50 µm. (G–I) Comparison of the adult (left) and larval (right) innervation of the mushroom body lobes by the driver strains R64H06, R58E02 and R30G08. R64H06 and R58E02 label nearly the entire larval medial lobe and show a similarly broad innervation at the adult stage. R30G08 innervation is more limited at both stages. Also note the higher number of medial-lobe mushroom body subunits and related PAM neurons in adults (12 subunits and about 120 PAM neurons, rather than 4 subunits and 4 pPAM neurons in the case of larvae). This prompts the tempting speculation that adults can perceive, classify and distinguish rewarding stimuli in more different types than larvae.

5.4.1 Supplemental Experimental Procedures

5.4.1.1 Fly strains

Fly strains were reared on standard Drosophila medium at 25°C with a 14/10 h light/dark cycle [S2,S3]. For the GRASP experiments the following strains were used: R58E02-LexA/CyO, LexAop-mCD4::GFP11, UAS-mCD4::GFP1-10/TM6B virgins were crossed to dNPF-Gal4, OK107-Gal4, GR43a-Gal4 or TDC2-Gal4, respectively (Bloomington Drosophila Stock Center No.: 854, 9313, 25682 and 57637) [S4].

5.4.1.2 GRASP

In the F1 generation of these crosses, larval brains were dissected and stained using the described standard protocol (for details see section “Immunostaining”) [S2].

5.4.1.3 Antibodies

To analyze the larval expression patterns in the different GRASP experiments two different mouse antibodies for staining the cholinergic neuropil (ChAT4B1; DSHB, Iowa City, IA, 1:150) and axonal tracts (1d4 anti-Fasciclin II; DSHB, Iowa City, IA; 1:50) were applied [S2]. As secondary antibody a goat anti-mouse IgG Alexa Fluor 647 (A21235, Molecular Probes, 1:200) was used. The reconstituted GFP expression was analyzed based on its strong endogenous signal (shown in Figure S3). There was no difference detectable between the two kinds of GFP signal.
5.4.1.4 Sensory acuity tests

Sensory tests were performed using standard methods [S3, S5, S6]. For olfactory acuity tests, 2.5% agarose solution (Sigma Aldrich Cat. No.: A5093; CAS No.: 9012-36-6) was boiled in a microwave and filled as a thin layer into Petri dishes (85 mm diameter, Cat. No.: 82.1472, Sarstedt, Nümbrecht). After cooling, closed Petri dishes were kept at room temperature and were used on the same day. For gustatory acuity tests, the agarose dishes were prepared in the same way, except that after cooling the agarose was removed from half of the plate. The empty half was filled by 2.5% agarose solution in addition containing 2 M fructose, 2 M arabinose, or 2 M sorbitol.

For olfactory acuity assays, 10 µl of either pure benzaldehyde or diluted amyl acetate (1:250 in paraffin oil) were loaded into a custom-made Teflon container. Olfactory preferences were tested by placing 30 larvae in the middle of the Petri dish with an odor-filled Teflon container on one side and an empty container on the other side (AIR). Larvae were then counted after 5 minutes as being located either on the odor side, the no-odor side, or a middle neutral zone (a stripe of about 10 mm width running vertically in the middle of the plate). For gustatory acuity tests, 30 larvae were put in the middle of a Petri dish that contained pure agarose on one side and agarose plus a gustatory stimulus (fructose, arabinose or sorbitol) on the other side. Larvae were counted after 5 minutes as being located on either the sugar side, the no-sugar side, or a middle neutral side (an area of about 10 mm width running vertically in the middle of the plate).

The indices for sensory acuity were then calculated as follows:

\[
\text{Preference Index} = \frac{\# \text{ ODOR} - \# \text{ AIR}}{\# \text{ TOTAL}}
\]

Positive Preference Indices therefore indicate attraction of the particular odor (amyl acetate or benzaldehyde, as indicated above each panel of Figure S4).
Preference Index = (# SUGAR - # PURE AGAROSE) / # TOTAL

Positive Preference Indices therefore indicate attraction of the particular sugar (fructose, arabinose, or sorbitol, as indicated above each panel of Figure S4).

5.4.2 Supplemental References


5.5 Author contributions


5.6 Acknowledgments

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5.7 References


6 Characterization of the Octopaminergic and Tyraminergic Neurons in the Central Brain of Drosophila Larvae


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6.1 Abstract

*Drosophila* larvae are able to evaluate sensory information based on prior experience, similarly 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 the dopaminergic modulatory systems in reinforcement signaling. Here we use genetics to analyse the anatomy, up to the single-cell level, of the octopaminergic/tyraminergic system in the larval brain and subesophageal 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 signalling 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 larval locomotion, whereas the others in the subesophageal ganglion and brain hemispheres mediate reward signaling.

6.2 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 behavioural response by assessing the consequences of their own actions 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) showed that in honeybees a single OA/TA neuron called \textit{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 \textit{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 insects is widely accepted.

Several groups have recently reported that sugar reinforcement includes at least two different components in \textit{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 nonnutritious sugars, such as arabinose and xylose, form a less robust memory reinforced by OA (Burke and Waddell, 2011). Thus, OA seems to signal specifically the sweet component of the multifactory sugar reward signal. Therefore, it was suggested that OA/TA neurons involved in processing of sweet taste during conditioning signal onto DA neurons that target the mushroom bodies (Burke et al., 2012). However, this was recently questioned; the \textit{alpha1}-like octopamine receptor OAMB was shown to be sufficient for reward learning in \textit{alpha/beta} and \textit{gamma} 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 \textit{Drosophila} depend on combined processing of the DA and OA/TA systems.

Immunohistochemical work showed that OA is synthesized mainly in unpaired median (UM) neurons of insects whose cell bodies are located either ventrally (VUM neurons) or dorsally (DUM neurons) in the subesophageal 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, 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 and SOG, 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 larval 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 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 involved neither 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 nonnutritious sugar reward processing necessary for establishing appetitive memories.
Based on multiple roles of OA/TA proved in distinct insect systems, such as locomotion, learning and memory, stress-induced behaviors, and 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 in understanding how specific behavioural functions are executed within distinct subcircuits of a complex neuronal network.

6.3 Materials and Methods

6.3.1 Fly strains

Flies were cultured according to standard methods (for details see Selcho et al., 2009, 2012). For the behavioral experiments, UAS-Hid,rpr (Kurada and White, 1998; White et al., 1996) were crossed with the 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 with w1118. 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 with 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.

6.3.2 Immunofluorescence

Immunostaining

The staining protocol of third-instar larval CNS has been described in detail by Selcho et al.
To detect TA we used a modified staining protocol of Sinakevitch and Strausfeld (2006; see also Busch et al., 2009), also described in detail by 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; 1:1,000; RRID: AB_221570; Table 1) in combination with two different mouse antibodies labeling the neuropil (anti-ChAT, ChAT4B1, anticholineacetyltransferase; DSHB, Iowa City, IA; 1:100; RRID: AB_528122; Table 1) and axonal tracts (anti-FasII, 1d4, antifasciclin II; DSHB; 1:55, RRID: AB_528235; Table 1), respectively. Anti-GFP with an antibody against tyramine β-hydroxylase produced in rats (anti-TβH; Monastirioti et al., 1996; kindly provided by M. Monastirioti, IMBB, FORTH, Greece; 1:75; Table 1) was used to analyse whether all Tdc2-GAL4 positive neurons are octopaminergic. TA was labeled via a polyclonal antibody against glutaraldehyde coupled p-TA (anti-TA, AB124; Chemicon, 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 Immunoresearch; 1:250), Cy3 goat anti-rabbit IgG (111–165-003; Jackson Immunoresearch; 1:100), and Cy3 goat anti-mouse IgG (A10521; Molecular Probes; 1:100; RRID:AB_1500665; or 115–166 003; Jackson Immunoresearch; 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 with a molecular weight of about 30 kDa in lysates prepared from Escherichia 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).

TβH

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

**Anti-p-tyramine**

The anti-TA antibody was used to characterize tyraminergic neurons 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 labelled ligand and different unlabeled catecholamine conjugates (including TA conjugate) was determined. The best displacement was observed with the TA conjugate, whereas the OA conjugate was 42 times less immunoreactive (Geffard et al., 1984).

**Table 1 Primary Antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Immunogen</th>
<th>Manufacturer [RRIDs]</th>
<th>Working dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-GFP</td>
<td>Rabbit, polyclonal</td>
<td>Purified green fluorescent protein (GFP), a 27-kDa protein derived from the jellyfish <em>Aequorea victoria</em></td>
<td>A6455, Molecular Probes [Eugene, OR] [AB_221570]</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Chicken anti-GFP</td>
<td>Chicken, polyclonal</td>
<td>Recombinant GFP containing a 6-his tag</td>
<td>AB16901, Chemicon [Temecula, CA] [AB_90890]</td>
<td>1:150, 1:170</td>
</tr>
<tr>
<td>ChAT4B1</td>
<td>Mouse, monoclonal</td>
<td>80-kDa <em>Drosophila</em> choline acetyltransferase protein</td>
<td>ChAT4B1, DSHB (Iowa City, IA) [AB_528122]</td>
<td>1:100</td>
</tr>
<tr>
<td>1D4 anti-Fasciclin II</td>
<td>Mouse, monoclonal</td>
<td>Bacterially expressed fusion peptide containing the intracellular C-terminal 103 amino acids of the PEST transmembrane form of Fasβ</td>
<td>1D4, DSHB (Iowa City, IA) [AB_528235]</td>
<td>1:55</td>
</tr>
<tr>
<td>TβH</td>
<td>Rat, polyclonal</td>
<td>A bacterially expressed purified internal part of the protein (Sal-Xho fragment)</td>
<td>Monastirioti et al., 1996</td>
<td>1:75</td>
</tr>
<tr>
<td>Anti-p-tyramine</td>
<td>Rabbit, polyclonal</td>
<td>p-Tyramine-glutaraldehyde-N-alpha-acetyl-L-lysine-N-methylanilide</td>
<td>AB124, Chemicon, [Temecula, CA] [AB_90432]</td>
<td>1:200</td>
</tr>
</tbody>
</table>

**6.3.3 Microscopy and figure production**

CNS preparations were scanned using a confocal light scanning microscope (Leica TCS SP5; Leica Microsystems, Wetzlar, Germany). The images scanned with a step size of 1 µm or 0.8 µm thickness were analysed in the software program ImageJ (NIH, Bethesda, MD; RRID: nif-
0000–30467). Contrast, brightness, and coloring were adjusted in Photoshop (Adobe Systems, San Jose, CA).

### 6.3.4 Behavioral experiments

Appetitive olfactory learning and naive preferences to tastants applied were tested by 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 with a thin layer of agarose solution containing either pure 2.5% agarose or 2.5% agarose plus fructose, sorbitol, or arabinose at concentration of 2 M. As olfactory stimuli, we used 10 µl amyl acetate (AM Fluka catalog No. 46022; diluted 1:250 in paraffin oil, Fluka catalog No. 76235) and benzaldehyde (BA, undiluted, Fluka catalog No. 12010). Odorants were loaded into custom-made Teflon containers (4.5-mm diameter) with perforated lids as described by 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 minutes, larvae were transferred to a fresh Petri dish in which they were allowed to crawl on pure agarose medium for 5 minutes 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 minutes, individuals were counted on the AM side (No. AM), the BA side (No. BA), and in a neutral zone (for further details a video is available in 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 (No. total), we calculated a preference index for each training group.

$$\text{PREF}_{\text{AM+/BA}} = \frac{\text{No. AM} - \text{No. BA}}{\text{No. total}}$$
We then compiled a performance index (PI).

\[
PREF_{AM/BA+} = \frac{(No. AM - No. BA)}{No. total}
\]

\[
PI = \frac{(PREF_{AM+/BA} - PREF_{AM/BA+})}{2}
\]

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

### 6.3.5 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 minutes, 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 (No. nS) from the number of larvae on the sugar side (No. S) divided by the total number of counted larvae (No. total), a PI for the sugar was calculated.

\[
PREF = \frac{(No. S - No.nS)}{No. total}
\]

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

### 6.3.6 Statistical analysis

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 performed with R version 2.13.2 (RRID: nif-0000-10474). Figure alignments were adjusted in 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 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.

### 6.4 Results

#### 6.4.1 Anatomy of the OA/TA neurons in the larval brain and SOG

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 describe the OA/TA neurons precisely, we labeled the GAL4-expressing cells with Cameleon2.1 (Diegelmann et al., 2002) and double stained them with antibodies directed toward $\text{T\beta H}$ and TA, to examine the neurotransmitter/modulator of the cells (Fig. 1). Cameleon2.1 was used because it provides a stronger and more stable expression compared with UAS-GFP, similar to the results published from a prior study on the thoracic and abdominal OA/TA system (Selcho et al., 2012).

$Tdc2$-GAL4/UAS-Cameleon2.1 shows GAL4 expression in about 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), whereas ~ 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), and 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 two 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). As 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).

By 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). From the neurons described above, only those in the SOG were recognized by the antibody, and in none of our stainings were the neurons in the hemispheres labeled (Fig. 1I,J; n = 27). Thus, our data suggest 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 labelled 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 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 did the sVM cells. 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, whereas 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 suggest significant difference in TβH levels between sVM cells and brain hemisphere neurons.

Figure 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) and 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 such as 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 the SOG overlap with TA antibody labeling. Scale bars = 50 μm in A,B,I–L; 25 μm in C–H.

6.4.2 Anatomy of single OA/TA neurons in the larval SOG

To understand better the potential neuronal network underlying sugar reward reinforcement, 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 14 different OA/TA or TA cell types (summarized in Table 2 and Fig. 6).

The three sVM clusters consist of paired and unpaired neurons, 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, 3, second row).

Table 2 Characteristics of 14 Different OA/TA or TA Cell Types

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Central brain regions</th>
<th>Mushroom bodies</th>
<th>Other neuropil regions</th>
<th>Hits per cell type</th>
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</table>

1Central brain regions: dmp, dorsomedial protocerebrum; dlp, dorsolateral protocerebrum; bmp, basomedial protocerebrum; 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, subesophageal ganglion; ventral nerve cord; tg, thoracic ganglion; ag, abdominal ganglion; arborizations visible at: bs, both sides; il, ipsilateral; cl, contralateral.

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 sVPMmd neurons are descending neurons, whereas those of the sVPMmx and sVPMmb clusters are of the ascending type. As 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, whereas descending VUM cells are located in the lb cluster. 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.

### 6.4.3 VPM neurons of the SOG

sVPM<sub>md1</sub> innervates the SOG and VNC (Fig. 2A–D) and sends a process around the esophagus (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<sub>md2</sub> 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<sub>md2</sub> innervates the contralateral VNC as far as the fourth abdominal neuromere (Fig. 2A,H). In contrast to the sVPM<sub>md</sub> cells, the sVPM<sub>mx</sub> 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 esophagus and ramifies in the basal vertical lobe and adjacent dorsal protocerebrum (Fig. 2K). The sVPM<sub>lb</sub> 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<sub>lb</sub> 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 (Fig. 2O).
**Figure 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 esophagus. 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 (arrow). H: Contralaterally, the innervations reach the fourth abdominal neuromere. I–L: The sVPMmx cell type innervates the SOG, bmp, and contralateral vertical lobe (vl), dlp, and dmp. K: A secondary neurite projects from the branching point of the primary neurite lateral to the esophagus 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 sVVM clusters, the primary neurite of sVPMb 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, dorso medial tract; SMT, superficial medial tract; VIT, ventrointermediate tract; VMT, ventromedial tract; VLT ventrolateral tract (after Nassif et al., 2003). bmp, basomedial protocerebrum; blp, basolateral protocerebrum; dmp, dorsomedial protocerebrum; dlp, dorsolateral protocerebrum (after Selcho et al.,). Scale bars = 50 μm in A–E, I–J, L–O; 25 μm in F–H, K, P.
6.4.4 VUM neurons of the SOG

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\textsubscript{md1}) and maxillar (sVUM\textsubscript{mx1}) 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\textsubscript{md2}) and maxillar (sVUM\textsubscript{mx2}) VM clusters (Fig. 3E–H), innervates the SOG; the basomedial, dorsomedial (Fig. 3G), and dorsolateral protocerebrum, close to the MB calyx; and the larval optic neuropil (Fig. 3G,H). The sVUM3 cell type, found again in mandibular (sVUM\textsubscript{md3}) and maxillar (sVUM\textsubscript{mx3}) 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\textsubscript{lb1} 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 esophagus (3O). The second cell type of the labial VM cluster, sVUM\textsubscript{lb2} (Fig. 3Q–T), innervates the SOG (Fig. 3R–T), the basomedial protocerebrum, and medial and lateral areas of thoracic and abdominal neuromeres (Fig. 3S,T). A third labial VM cell type, sVUM\textsubscript{lb3} (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).
Figure 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 (sVUM_{md1}) and second (sVUM_{mx1}) VM cluster. It innervates the SOG, al, bmp, blp, and 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 (sVUM_{md2}) and second (sVUM_{mx2}) VM clusters. It
innervates the SOG, bmp, larval optic neuropil (lom), dmp, and dlp. G: Arborizations in the bmp and dmp. Those in the dlp are in the vicinity of the ca. H: The LON is innervated by dot-like endings. I–L: The sVUM3 cell type was found in the first (sVUMmd3) and second (sVUMmd2) VM clusters. 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 sVUM3 cell type innervates the SOG and thoracic ganglion (tg). Q: Lateral arborizations in the SOG and tg. A neurite runs lateral to the esophagus (arrow). P: Innervation of the anterior lateral SOG. Q–T: The sVUMmd2 cell type innervates the SOG, bmp, 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 sVUMmd3 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 dorso-posterior 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 tract; SMT, superficial medial tract; VIT, ventrointermediate tract (after Nassif et al., 2003).

bmp, basomedial protocerebrum; blp, basolateral protocerebrum; dmp, dorsomedial protocerebrum; dlp, dorsolateral protocerebrum (after Selcho et al., 2009). Scale bars = 50 μm in A, E, I, M, Q, U; 25 μm in B–D, F, G, J, L, N, P, R–T, V–X; 10 μm in H.

6.4.5 Anatomy of single OA/TA neurons in the larval brain hemispheres

For 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, two cell types were identified, both of which innervate the two brain hemispheres. At least two cells belong to the lDMPa1 type and one to the lDMPa2 type, but 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 in Figure 4G, H, 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).
Figure 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 ICA 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 esophagus, turns in the contralateral dorsal bmp, and projects to the VNC, thereby arborizing in the ipsilateral posterior medial 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 IDMPa1 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 IDMPa2 cell type innervates the bmp, basomedial protocerebrum, blp, basolateral protocerebrum, dmp, dorsomedial protocerebrum, dllp, dorsolateral protocerebrum (after Selcho et al., ). Scale bars = 50 μm in A,E,K; 25 μm in B–D,F–J,L.

6.4.6 The role of OA/TA neurons in reward processing in larval associative learning

A recent study in adult Drosophila indicated OA/TA neurons to be important in mediating sweet taste onto dopaminergic neurons, to reinforce sugar odor learning (Burke et al., 2012). In our previous work we were able to show that dopaminergic neurons are required both for reward and for punishment learning in larvae (Selcho et al., 2009). This suggests 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 unconditioned stimulus (US; Rohwedder et al., 2012). Ectopic expression of both UAS-\textit{Hid} (head involution defective) and UAS-\textit{rpr} (reaper) leads via DNA-fragmentation and chromatin condensation to apoptosis (Kurada and White, 1998; White et al., 1996). As shown in 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 \textit{Tdc2-GAL4;tsh-GAL80. 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 with fructose as a reward (Fig. 5A). \textit{Tdc2-GAL4;tshGAL80/UAS-Hid,rpr} experimental larvae showed significant learning scores ($P = 6.104 \times 10^{-5}$) and performed on control level ($P = 0.1456$ compared with \textit{Tdc2-GAL4;tshGAL80/+} and $P = 0.5125$ compared with \textit{UAS-Hid,rpr/+}). Additionally, \textit{Tdc2-GAL4;tshGAL80/UAS-Hid,rpr} larvae exhibited robust appetitive memory in sorbitol learning, comparable to that of genetic controls (Fig. 5B; $P = 0.1186$ for \textit{Tdc2-GAL4;tshGAL80/+} and $P = 0.0566$ for \textit{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, because it is present for both fructose and sorbitol. In contrast, \textit{Tdc2-GAL4;tshGAL80/UAS-Hid,rpr} larvae failed to show significant learning scores in arabinose odor learning (Fig. 5C; $P = 0.285$). Experimental larvae performance was significantly reduced compared with \textit{Tdc2-GAL4;tshGAL80/+} ($P = 0.0070$) and \textit{UAS-Hid, rpr/+} ($P = 0.0278$) controls. Thus, OA/TA
neurons in the brain and SOG seem to be required for 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; learning scores were indistinguishable from controls using fructose and sorbitol as rewarding US (Fig. 5A,B). Tdc2-GAL4;tsh-GAL80/UAS-Hid,rpr showed normal attraction behaviour toward arabinose ($P = 0.0089$) and performed indistinguishably from genetic controls (Fig. 5D; $P = 0.8034$ for Tdc2-GAL4; tshGAL80/+ and $P = 0.7088$ for UAS-Hid,rpr/+).

**Figure 5** Ablation of OA/TA neurons in the brain and SOG specifically affects arabinose odor learning. A-B: Restricted OA/TA cell ablation within the brain and SOG, leaving locomotor circuits intact, did not lead to significant impairment in fructose odor (A) or sorbitol odor (B) learning (all $P > 0.05$), indicating that these neurons are dispensable in mediating sugar reward in that these sugars both offer nutritional value. C: In contrast, ablation of the same OA/TA neurons affected arabinose odor learning ($P < 0.05$) that was shown not to 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 whether 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.

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 from adult flies (Burke et al., 2012; Burke and Waddell, 2011).

### 6.5 Discussion

#### 6.5.1 Architecture of the larval OA/TA system on the single-cell level in comparison with other insects

In invertebrates and vertebrates, the activity of distinct neuronal networks within the CNS and consequently the final behavioral output are modulated by biogenic amines such as 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 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. Although most of the neurons situated in the brain seem to have normal TA levels but lower OA levels (or even no OA), all remaining neurons (about 25) within the three subesophageal clusters are tyraminergic and octopaminergic. The basic organization within the SOG shows remarkable similarities to 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; and 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 in either the mandibular or the 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 antennal lobe, for instance, which 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 larvalborn MB neurons innervated by sVPMmx neurons are pruned during early puparium formation and retain their main processes only 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, no data are available on the development of these OA/TA neurons over metamorphosis. Thus, from the similarities 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 for describing the functional principles of insect brains.

6.5.2 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 information via dopaminergic neurons (Gerber and Stocker, 2007; Gerber et al., 2009; Selcho et al., 2009; Thum et al., 2011). Honjo and Furukubo-Tokunaga (2009) suggested that OA/TA neurons, potentially signaling sugar
reward, innervate only the larval MB calyx. However, we identified one type of neuron in the sVMmd and sVMmx clusters that innervates the MB calyces, called sVUM1 (Figs. 3, 6), and two additional neurons, sVPMmx (Figs. 2, 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 an article by Honjo and Furukubo-Tokunaga (2009). Thus, individual OA/TA neurons have the anatomical properties potentially to 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.

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**Figure 6 Overview of the anatomical organization of the OA/TA system.** A: Schematic view of 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 receives potential input by at least three different OA/TA neurons at two locally distinct subregions (shown in red in A,B).
6.5.3 OA/TA signaling is sufficient and necessary in insects to establish appetitive memories

Pharmacological, genetic, optogenetic, and 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 was supported in Drosophila larvae and adults; pairing an odor presentation with optogenetic or thermogenetic activation of nearly all OA/TA neurons in the CNS induces appetitive olfactory associative learning (Burke et al., 2012; Schroll et al., 2006).

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 sucrose odor learning (Schwaerzel et al., 2003) and the alpha1-like octopamine receptor OAMB is required in α/β and γ mushroom body neurons for appetitive short-term memory (STM; Kim et al., 2013) and also in dopaminergic neurons for arabinose odor learning (Burke et al., 2012). For Drosophila larvae, 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 tests. Unfortunately, in larvae, it is not possible to use TβH mutants or other methods of genetic interference with
the overall OA/TA system, because this strongly affects larval locomotion and therefore makes it impossible to test for olfactory learning, which requires proper locomotion (Saraswati et al., 2004; Selcho et al., 2012). Nevertheless, it is believed that OA/TA signaling includes aspects of the internal rewarding function that are sufficient and necessary for appetitive learning throughout different insect species.

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

However, blocking OA/TA neurons using specifically the driver line \textit{Tdc2-GAL4} does not always impair reward learning (Fig. 5). It cannot be excluded that some of the described phenotypes might not be confirmed as learning effects per se, because OA/TA are generally known to have modulatory functions on various behaviors. Most critically, for \textit{Drosophila} larvae, OA/TA was shown to be necessary for locomotion. \textit{TβH} mutant larvae with elevated TA and reduced OA levels spent more time in pausing episodes than wild-type 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 impairs 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 behavioural performance of the larva during and after appetitive olfactory learning, independent of the internal reward processing.

6.5.5 OA/TA function in larval reward learning

We also found, in line with published data, that the OA/TA system is involved in larval sugar reward learning (Fig. 5; Honjo and Furukubo-Tokunaga, 2009; Schroll et al., 2006). Honjo and Furukubo Tokunaga (2005, 2009) found that OA is necessary for sugar reward learning when exposing larvae to an odor and a concomitant sucrose reinforcer for 30 minutes in a one-trial nonreciprocal training regime. Sucrose offers a nutritional benefit, so this result
seems partially to contradict our results. However, there are several possible explanations. 1) The effects described by Honjo and Furukubo-Tokunaga (2009) are extremely small. The response indices for the related experiment show values of about 0.1 (cf. Fig. 4, Honjo and Furukubo-Tokunaga, 2009). This means that the described behavioural phenotype for 50 tested animals was based only on five larvae more on the odor/reward side compared with the no-odor/no-reward side. In addition, in their study, the experimental animals tested had a significantly altered sucrose perception (cf. Supp. Info. Table 1). Thus, the relative small sucrose odor phenotype observed might be due to altered sucrose reward processing during training. 2) Both the observed phenotype in sugar odor learning and sugar perception may rely on altered locomotion, which was not controlled in their behavioural data set. Notably, Tdc2-GAL4/UAS-Hid,rpr larvae showed reduced preference indices toward 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). 3) There are multiple differences in the behavioral protocols that induce different memory phases. Indeed, fructose odor training using three training cycles leads to an immediate memory that is independent of rutabaga and dunce (A. Widmann, manuscript in preparation). The sucrose odor memory by Honjo and Furukubo-Tokunaga (2005) is completely absent in these two classical STM mutants, so the discrepancy about the requirement of OA/TA neurons might be based on the analysis of cAMP-dependent STM vs. a cAMP-independent memory phase (Fig. 5). This is in line with recent data for adult flies; odor stimulation and concurrent OA network activation establish 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 whether this effect is triggered by single neurons of the OA/TA system and how these neurons
receive input from recently described gustatory sensory neurons that respond to fructose (Mishra et al., 2013).

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6.7 Conflict of interest statement

The authors declare no competing interests.

6.8 Role of Authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: MS, DP, RFS, AST. Acquisition of data: MS, DP, AH. Analysis and interpretation of data: MS, DP, AH, RFS, AST. Drafting of the manuscript: MS, DP, AH, RFS, AST. Critical revision of the manuscript for important intellectual content: MS, DP, RFS, AST. Statistical analysis: MS, DP. Obtained funding: MS, DP, RFS, AST. Administrative, technical, and material support: MS, DP, AH, RFS, AST. Study supervision: AST.

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7 Behavioral function of the histaminergic system in *Drosophila melanogaster* larvae

*In preparation*

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7.1 Abstract

The biogenic amine histamine (HA) is an important neuroactive molecule in the visual system of insects. In *Drosophila melanogaster* HA binds to two different ligand-gated ion receptors *hisCl1* (also referred to as *hclB*) and *hisCl2* (also referred to as *ort* or *hclA*) and regulates in addition to the visual system also mechanosensory and temperature induced behavior.

The model organism *Drosophila melanogaster* has a lot of advantages to study the effect of biogenic amines on different kinds of behavior. Due to its genetic amenability and neuronal simplicity *Drosophila*, especially larvae, are well suited to investigate chemosensory behavior. Neuronal networks involved in larval olfaction, gustation, and learning and memory have been described, often up to the single-cell level.

Here we focus on the histaminergic system of the *Drosophila* larva. In detail, we investigate the effect of impaired HA signaling on naïve and acquired chemosensory behavior. Our data suggest that reduced HA synthesis does neither affect larval naïve olfactory and gustatory preference nor appetitive and aversive associative olfactory learning and memory. In contrast, we show that a mutation in the HA receptor gene *ort* results in reduced appetitive and aversive olfactory learning and memory but leaves olfactory and gustatory choice behavior intact.

These findings provide a first evidence that the histaminergic system is involved in larval behavior processes. In the long run, these results may uncover further involvements of the HA signaling in larval behavior processes possibly shared with adult *Drosophila* and other insects.
7.2 Introduction

Biogenic amines play an essential role in a wide range of insect behaviors [1-3]. Among the best studied aminergic molecules are dopamine, tyrosine, histamine (HA), octopamine and serotonin. HA seems to be the major neurotransmitter in the visual system in insects. In many different insects including various flies, cockroaches and bees it was shown that HA acts as a neurotransmitter in photoreceptors [4-9]. In Drosophila melanogaster adults HA-like immunoreactivity was detected in the retina, the lamina and the medulla [10] and genetic depletion of histamine synthesis results in reduced optomotor responses [11]. In contrast, larval photoreceptor cells of Drosophila that project to the brain are not HA-like immunoreactive [10] and mutant larvae showed normal photokinetic responses [11]. In flies it was reported that HA signaling additionally regulates mechanosensory behavior [11] and temperature preference [12]. Beside the findings, that HA does not affect photokinesis, a specification of the HA system related to larval behavior is to our knowledge completely lacking. Accordingly, the emphasis of this study is to investigate the role of the HA system in larval behavior. We focus on the effect of genetic depletion of HA signaling on chemosensory behavior including appetitive and aversive olfactory learning and memory as well as olfactory and gustatory naïve preference.

To this end, we used two different mutant lines. First, to investigate the effect of HA we took advantage of a mutant deficient in the histidine decarboxylase (hdc) gene. HDC acts as an essential enzyme in histamine synthesis. The allele hdcJK910 is a hypomorphic mutation, as histamine synthesis is 10-fold less than in wild type [13, 14]. Second, to analyze the effect of manipulating HA receptor neurons we used a mutant deficient in the ora transientless (ort) gene, coding for the HA receptor ort (also known as hclA or hisCl2). The null mutant ort1 has a deletion that leads to the loss of the sequence encoding a substantial portion of the N-terminal extracellular domain [15].
The used behavioral assays include measurements for naïve olfactory and gustatory preferences performed as simple choice tests on agarose filled test plates [16]. Variations in the design allow to study associative olfactory learning and memory [17] in addition. Presenting an odor (the conditioned stimulus [CS]) simultaneously with an aversive unconditioned stimulus (US) may induce experience dependent avoidance of the CS. Conversely, if the same CS is paired with an appetitive US, animals can be trained to develop a preference for the CS [18]. Thus, depending on previous experience, the same odor can trigger either avoidance or attraction [19, 20]. Accordingly, a comprehensive set of behavioral assays to analyze larval chemosensory behavior exists that allows investigating simple choice behavior and also associative tasks in Drosophila larvae.

7.3 Materials and methods

7.3.1 Flies

Flies were maintained on standard Drosophila medium at 25°C or 19°C under 12h light /dark conditions. HdcJK910 (Bloomington Stock Center no. 64203) and ort1 (Bloomington Stock Center no. 1133) were ordered from Bloomington. For behavioral experiments, wild type Canton-S (WT CS) was used.

7.3.2 Behavioral experiments

Five to six-day old feeding third instar larvae were used for all behavioral experiments. The assays were performed at 22°C. For further details on experimental protocols we referred to earlier studies [16, 18, 21-24]). In addition, we provide a short description in the following.

7.3.3 Chemosensory preference

For gustatory preference tests, a 2.5% agarose (Sigma Aldrich Cat. No.: A5093, CAS No.: 9012-36-6) solution was boiled in a microwave oven and filled as a thin layer into assay plates. After cooling, the agarose was removed from half of the plate. The empty half was
filled with 2.5% agarose solution containing sodium chloride (SALT, Sigma Aldrich Cat. No.: S7653, CAS No.: 7647-14-5; 1.5M), and D-fructose (FRU, Sigma Aldrich Cat. No.: 47740, CAS No.: 57-48-7; 2.0M). Immediately after preparation of the plates, groups of 30 larvae were placed in the middle of the plate, allowed to crawl for 5 min, and then counted on the stimulus containing side, the agarose only side, and the neutral zone (about 1 cm between both sides). By subtracting the number of larvae on the pure agarose side (#nS) from the number of larvae on the stimulus side (#S) divided by the total number of counted larvae (#TOTAL), a preference index for the respective chemosensory stimulus was calculated:

\[
PREF = \frac{(S - nS)}{TOTAL}
\]

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

For olfactory preference test, a similar assay was used except that olfactory stimuli were presented in custom-made Teflon containers with perforated lids presented on only pure agarose containing assay plates. As olfactory stimuli amyl acetate (AM, Fluka Cat. No.: 46022; CAS No.: 628-63-7; diluted 1:250 in paraffin oil, Fluka Cat. No.: 76235, CAS No.: 8012-95-1) and benzaldehyde (BA, Fluka Cat. No.: 12010, CAS No.: 100-52-7; undiluted) were used.

### 7.3.4 Classical conditioning

Experiments were conducted on assay plates (85mm diameter, Cat. No.: 82.1472, Sarstedt, Nümbrecht) filled with a thin layer of 2.5% agarose (Sigma Aldrich Cat. No.: A5093, CAS No.: 9012-36-6) containing either pure agarose or agarose plus reinforcer. We used 1.5 M sodium chloride (Sigma Aldrich Cat. No.: S7653, CAS No.: 7647-14-5) and 2.0 M D-fructose (Sigma Aldrich Cat. No.: 47740, CAS No.: 57-48-7). As olfactory stimuli, we used 10 μl amyl acetate (Fluka Cat. No.: 46022, CAS No.: 628-63-7; diluted 1:250 in paraffin oil, Fluka
Odorants were loaded into custom-made Teflon containers with perforated lids. 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 or salt as a negative reinforcer. After 5 min, larvae were transferred to a fresh test plate 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. Please note that for aversive learning the test plate included salt. After 5 min, individuals were counted on the AM side (#AM), the BA side (#BA), and in a neutral zone. 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:

\[
(1a) \text{PREF}_{AM+BA} = \frac{\text{# AM} - \text{# BA}}{\text{# TOTAL}}
\]

\[
(1b) \text{PREF}_{AM/BA+} = \frac{\text{# AM} - \text{# BA}}{\text{# TOTAL}}
\]

To measure specifically the effect of associative learning that is of the odor-reinforcement contingency, we then calculated the associative performance index (PI) as the difference in preference between the reciprocally trained larvae:

\[
(2) \text{PI} = \frac{(\text{PREF}_{AM+/BA} - \text{PREF}_{AM/BA+})}{2}
\]

Negative PIs thus represent aversive associative learning, whereas positive PIs indicate appetitive associative learning. Division by 2 ensures scores are bound within (-1; 1).
7.3.5 Statistical methods

Statistical analysis and visualizations were done with R (version 2.15.2), R studio (version 0.98.1028) and Adobe Photoshop (version CC 2015.5). Behavioral data are visualized as box plots with medians indicated by lines in the middle and 25% / 75% and 10% / 90% as box boundaries and whiskers, respectively. Sample size in each case is $n = 11 - 21$. Wilcoxon rank-sum tests were performed for multiple comparisons. Likewise, Wilcoxon signed-ranked tests were used to compare values against chance level. P values were rounded to three decimal places except for cases that would have resulted in zero. Here additional decimal places are given.

7.4 Results

7.4.1 Mutation in the hdc\textsuperscript{JK910} gene does neither affect olfactory learning and memory nor chemosensory chemotaxis

To examine if larvae lacking histamine are able to associate an odor with a positive or negative gustatory stimulus, we applied a well-established two-group, reciprocal mass training design (reviewed in [16]). As olfactory stimuli (CS) we utilized BA and AM. As appetitive and aversive gustatory stimuli (US) we used FRU (2.0 M) and SALT (1.5 M), respectively.

Homozygous hdc\textsuperscript{JK910} experimental larva showed appetitive olfactory learning and memory (Figure 1B; Wilcoxon signed-ranked test $p=0.0002$) that did not differ from WT CS (Figure 1B; Wilcoxon rank-sum test $p=0.724$ compared to WT CS). Hdc\textsuperscript{JK910} larvae also learnt to avoid an odor that was paired with a punishing gustatory stimulus (Figure 1C; Wilcoxon signed-ranked test $p=0.001$). Learning and memory was similar as for experimental and control larvae (Figure 1C; Wilcoxon rank-sum test $p=0.840$ compared to WT CS).
To test if larval olfactory responses to different odors depend on histamine signaling, 30 third instar larvae were placed on a test plate filled with agarose together with a Teflon container that contained an odor on one side and a second container without odor on the other side. Olfactory preferences for two different odors were analyzed: amyl acetate (AM, diluted 1:250 in paraffin oil) and benzaldehyde (BA, undiluted).

Mutation of \textit{hdc}^\text{JK910} did not affect larval olfactory preferences towards AM (Figure 1D; Wilcoxon signed-ranked test \( p = 0.001 \)). Similar results were obtained testing \textit{hdc}^\text{JK910} mutant larvae for BA preference (Figure 1E; Wilcoxon signed-ranked test \( p = 0.001 \)). In both tests, experimental and control larvae were on the same level (Figure 1D, 1E; Wilcoxon rank-sum tests \( p = 0.206 \) and \( p = 0.481 \)).

For naïve gustatory preference, we prepared test plates filled with an agarose layer on one side and a gustatory stimulus (1.5 M sodium chloride SALT or fructose FRU 2.0 M) diluted in agarose layer on the other side.

Homozygous \textit{hdc}^\text{JK910} mutation did not prevent larvae from avoiding SALT (Figure 1F; Wilcoxon signed-ranked test \( p = 0.001 \)) and did not reveal a difference compared to WT CS (Figure 1F; Wilcoxon rank-sum tests \( p = 0.221 \)). Experimental larvae showed also a preference towards FRU (Figure 1G; Wilcoxon signed-ranked test \( p = 0.002 \)), that did not differ compared to control larvae (Figure 1G; Wilcoxon rank-sum tests \( p = 0.518 \)).

In summary, hdc function seems to be unnecessary as \textit{hdc}^\text{JK910} mutants behave on wild type level. We thus conclude that HA might be dispensable for aversive and appetitive olfactory learning and memory as well as for olfactory and gustatory naïve preference.
Figure 1 Impaired HA synthesis does not affect appetitive and aversive olfactory learning and memory and naïve olfactory and gustatory preference 

Homozygous *hdc JK910* gene mutants and wild-type control larvae (WT CS) were used to analyze aversive (B) and appetitive (C) olfactory learning and memory, olfactory AM (diluted 1:250 in paraffin oil) (D) and BA (undiluted) preferences and (E) gustatory FRU (F) and SALT (G) preferences. (A) provides a color scheme for the two different groups used in each experiment. Mutant larvae showed appetitive and aversive olfactory learning and memory, as well as olfactory and gustatory naïve preferences comparable to wild-type larvae. Sample size (n = 13 - 21) is indicated at the bottom of each box plot. Differences against zero are given at the top of each box plot. Differences between mutant and wild type larvae are shown at the bottom of the panel. *** (p < 0.001), ** (p < 0.01), * (p < 0.05), n.s. (not significant, p ≥ 0.05).
7.4.2 Mutation in the ort\textsuperscript{1} gene elicited impaired aversive and appetitive olfactory learning and memory but no impairment of gustatory and olfactory chemotaxis.

Homozygote ort\textsuperscript{1} mutant larvae showed appetitive olfactory learning and memory (Figure 2B; Wilcoxon signed-ranked test \(p=0.036\)), which was significantly reduced compared to wild type control larvae (Figure 2B; Wilcoxon rank-sum tests \(p=0.0001\)). Similar results were obtained when testing aversive olfactory learning and memory. Experimental larvae were able to establish an aversive olfactory memory (Figure 2C; Wilcoxon signed-ranked test \(p=0.021\)), but scores were significantly reduced compared to WT CS (Figure 2C; Wilcoxon rank-sum tests \(p=0.044\)). In contrast, when ort\textsuperscript{1} receptor mutants were tested for olfactory naïve preference, they showed attraction to both odors AM and BA (Figure 2D, 2E; Wilcoxon signed-ranked tests \(p=0.002\) and \(p=0.006\)) that did not significantly differ compared to wild type controls (Figure 2D, 2E; Wilcoxon rank-sum tests \(p=0.249\) and \(p=0.115\)). Testing gustatory preference, mutants showed preference for FRU and avoidance of SALT (Figure 2F, 2G; Wilcoxon signed-ranked tests \(p=0.002\) and \(p=0.010\)). FRU and SALT performance was similar to wild type control larvae (Figure 2F, 2G; Wilcoxon rank-sum tests \(p=0.183\) and \(p=0.139\)).

In conclusion, mutation in the HA receptor gene ort\textsuperscript{1} affects olfactory learning and memory and but not olfactory and gustatory naïve preference.
Figure 2 Impaired ort1 receptor function results in reduced appetitive and aversive olfactory learning and memory but normal AM, BA, FRU, and SALT preference. Homozygous ort1 gene mutants and wild-type control larvae (WT CS) were used to analyze aversive (B) and appetitive (C) olfactory learning and memory, olfactory AM (diluted 1:250 in paraffin oil) (D) and BA (undiluted) preferences and (E) gustatory FRU (F) and SALT (G) preferences. (A) provides a color scheme for the two different groups used in each experiment. Mutants showed compared to wild-type larvae impaired appetitive and aversive olfactory learning and memory. Preference for AM, BA, FRU, and SALT was not affected. Sample size (n = 11 - 19) is indicated at the bottom of each box plot. Differences against zero are given at the top of each box plot. Differences between mutant and wild type larvae are shown at the bottom of the panel. *** (p < 0.001), ** (p < 0.01), * (p < 0.05), n.s. (not significant, p ≥ 0.05).
7.5 Discussion

7.5.1 HA signaling is neither necessary for naïve gustatory and olfactory preference nor appetitive and aversive olfactory learning and memory

With our experimental approach, we could show that a mutation in the \( hdc^{JK910} \) allele does not affect naïve gustatory preference testing FRU and SALT or naïve olfactory preference testing AM and BA (Figure 1). These findings are in line with a prior study analyzing the anatomical description of the histaminergic system [25]. Python and Stocker did not observe any HA staining in neurons of the dorsal organ and the terminal organ, both primary chemosensory organs of the larval head [16, 25]. Additionally, they found no HA positive cells innervating the antennal lob, the primary olfactory brain center [16, 25]. The primary gustatory brain center, the subesophageal ganglion, shows some histaminergic arborizations. However, putative gustatory terminals seem not to be HA positive [16, 25].

\( Hdc^{JK910} \) mutant larvae showed appetitive and aversive olfactory learning and memory behavior on wild type level (Figure 1), indicating that HA signaling is not involved in this kind of processes. This is squaring with the lack of HA staining in projection neurons [25], which connect the antennal lobe with the mushroom body, a higher brain center crucial for learning and memory [26, 27]. Projection neurons are mostly acetyl choline positive [25]. Additional mushroom body input neurons express other biogenic amines like dopamine and octopamine [24, 28, 29]. If this kind of neurons are also HA positive would be part of further studies. However, this was not reported to be the case in adult \( Drosophila \).

7.5.2 Ort\( ^l \) mutant larvae showed olfactory and gustatory naïve preference on wild type level but impaired olfactory learning and memory

The mutation in the \( ort^l \) allele of the HA receptor leads to reduced appetitive and aversive olfactory learning and memory (Figure 2). There are no comparable results in the \( Drosophila \) field (fly and larva) available. In addition, an anatomical analysis of \( ort \)-positive cells in...
lupae is missing. In flies ort expression was analyzed with a fluorescein-labeled antisense of ort riboprobe [30]. Expression was detected in different CNS regions including the ventral nerve cord, the subesophageal ganglia as well as not further defined lateral und central brain regions. Further investigations taking advantage of the UAS/GAL4-system [31] revealed ort expression in the first visual neuropil, the lamina as well as in the medulla [30, 32]. Direct evidence of ort expression in the mushroom body or its input and output neurons was not found. However, depending on the gene, expression patterns can change dramatically through development and we cannot exclude larval ort expression in neurons necessary for olfactory learning and memory processes. To examine where and how the HA system is involved, further anatomical and molecular investigations are necessary.

7.5.3 Mutation in the receptor gene, but not in hdc, affects learning and memory

Our results suggest a behavioral effect in the HA receptor mutant but no effect when HA synthesis is reduced. At first sight this appears to be contradictory. In the following probable explanations for this phenomenon are pointed out. First, ortI is a null mutation without expression detectable that leads to reduced learning and memory performance (Figure 2). HdcJK910 is a hypomorphic mutation and histamine synthesis is 10-fold less compared to wild type expression [13, 14]. HdcJK910 mutant larvae behave on wild type level (Figure 1). Thus, we cannot exclude that 10% of HA expression is sufficient for intact learning and memory behavior at wild type level. Second, the mutation in the ortI gene might result in a deletion of another unknown gene or regulatory sequence necessary for learning and memory processes. Third, HA might not exclusively bind to the ort receptor. Other potential unknown ligands, independent of HA synthesis, could activate the chloride channel by binding to the ort receptor and elicit learning and memory processes. Fourth, in addition to ort (also referred to as hclA or hisCl2) HA can target a second HA receptor type called hisCl1 (also referred to as hclB). [33-35]. Even though both belong to the Cys-loop ligand-gated ion channel
superfamily [34], they might be expressed and change membrane potential in different sets of neurons (e. g. GABAergic and cholinergic neurons). The expression of an HA receptor in an inhibitory GABAergic neuron might result in a different response compared to the response when HA targeting another HA receptor expressed in an excitatory cholinergic neuron. However, as mentioned before, the anatomical distribution of the two HA receptors is unknown and must be examined in the future. In addition, to test this hypothesis with a behavior approach further experiments including the use of genetic manipulations of the hisCl1 receptor are essential. Regrettably, we had no access to such genetic tools and were not able to investigate the effect of the second receptor type on larval behavior.

### 7.6 Outlook

This study gives a first overview of the effect of HA on *Drosophila* larval behavior. Our focus is on chemosensory choice and association behavior. The effect of the HA system on further larval behavior processes should be considered in future studies. Additionally, an anatomical analysis up to a single cell level similar to other biogenic amines [24, 28, 36] is missing and would help to understand behavioral processes. To confirm the evidence that the ort1 mutation affects larval learning and memory processes, investigations using other genetic tools are necessary. To test if the effect of the ort1 mutation on learning and memory is limited to the larval stage, research should be expanded to the adult system.

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7.9 References


8 General Discussion

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The biogenic amines serotonin (5-HT), dopamine (DA), octopamine (OA), tyramine (TA) and histamine (HA) are involved in a large number of various behaviours in *Drosophila* like locomotion, sleep and aggression [1-6]. The focus of this work is to uncover the involvement of biogenic amines in innate behaviour, mainly naïve olfactory and gustatory preference, as well as in associative olfactory learning and memory in *Drosophila* larvae. In addition, I analyse the distribution of biogenic amine systems in the CNS.

In detail, this work provides the description of a whole connectome of the learning and memory brain centre – the mushroom body (chapter 3). By the analysis of this neuronal circuit, we see that biogenic amines are part of it. In larvae, mostly DA and OA expressing neurons innervate the MB and are known to play an important role in learning and memory [7-9]. Accordingly, in chapter 5 the CNS anatomy of the DAergic pPAM cluster is illustrated and we discover the necessity and sufficiency of this cells in appetitive olfactory learning and memory for the first time in larvae. Additionally, the expression of OA/TA neurons on single cell resolution and the effect of these neurons in appetitive learning and memory is given in chapter 6. But not only these biogenic amines are expressed in the larval CNS and are involved in learning and memory processes. I expand the experiments and analyse the 5-HT as well as the HA systems. After investigating in an earlier study, the expression pattern of 5-HTergic neurons up to single cell level [10], I show in chapter 4 a comprehensive overview of specific 5-HT receptor GAL4-lines expression and the effect of genetic interference of these lines on naïve preference and learning and memory. I reveal that specifically 5-HT/5-HT\(_{2A}\) signalling seems important for aversive salt conditioning. In chapter 7 the effect of the HA system on naïve preference and learning and memory is tested – possibly for the first time – in larvae. I show a preliminary hint that the HA receptor *ort* could be involved in mediating learning and memory in *Drosophila* larvae.
Table 1: Overview of Larval CNS Expression and Innervation of Aminergic Systems. Third instar *Drosophila* larvae were stained with specific antibodies or via anti-GFP labelling of specific GAL4-lines. Note: The use of TDC-GAL4 or anti-TDC indicates the presence of TA/or/and OA. Anti-TA, -OA or -TbH is not often utilized due to the weak or unspecific staining pattern. Thus, a clear separation of the OA and TA system is difficult and an additional column referring to both systems OA/TA is added.

### Larval CNS Anatomy: Innervation of Aminergic Systems

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**Explanation:** X = expression, O = no expression, ~ = unclear/weak expression if at all. NA = not analysed. 1-20 = reference, A = data in appendix, bold = chapter in this thesis.

8.1 Biogenic amines and the corresponding receptors are found in the CNS but are not per se involved in inducing an innate behavioural response

The larval CNS is subdivided into different brain regions essential for the receiving of various sensory information and inducing innate behavioural responses. For example, the larval optic neuropil and the ventral nerve cord act as visual and mechanosensory primary brain centres, respectively [24, 25]. Table 1 gives an overview of the innervation in different larval brain regions by neurons expressing respective biogenic amines or their receptors. This table (as well as tables 2 and 3) provides a comprehensive overview of the current knowledge in larval research by summarizing the results of this thesis in combination with results of other studies. Note, that although the complex collection of corresponding articles was done carefully, a lack of some important results cannot be excluded. In perspective of innate behaviour, I am particularly interested in naïve olfactory and gustatory preference. Olfactory and gustatory stimuli are mainly sensed in sensilla located in the larval head [26-28]. From there olfactory and gustatory receptor neurons innervate primary brain centres – the antennal lobes (ALs) and suboesophageal ganglion (SOG) [29]. Neurons of all analysed biogenic amine systems innervate the SOG and neurons of many systems (5-HT, OA/TA and maybe DA and HA) innervate also the AL (table 1, chapter 4 and [7, 9, 10, 14, 19]). Therefore, we assumed that biogenic amines may affect naïve gustatory and olfactory preference. Surprisingly, they seem not to be required for these kinds of innate behaviours. In chapter 4-7 we show that genetic interferences of the 5-HT receptors (5-HT$_{1A-7}$), the DA expressing pPAM cluster, the OA/TA expressing neurons, and the HA (HA and ort) system do not impair naïve preference for different odours, sugars, and salt at high concentration (table 2). Similar results were shown in earlier studies for 5-HT and the DA receptors DopR1 and Dop1R2 (table 2 and [7, 10, 30, 31]). In contrast, impairment of DA expressing cells via TH-GAL4 seems to affect naïve preference behaviour induced by benzaldehyde and possibly also by amyl acetate and fructose.
Our results indicate that biogenic amines might be in general dispensable for naïve olfactory and gustatory preference. Though, we cannot exclude that some systems are necessary for non-tested odours or gustatory naïve preference. But why do biogenic amines expressing neurons innervate primary brain regions like the SOG and the AL? Biogenic amines function as neurotransmitters and/or neuromodulators. To generate naïve preference behaviour the molecules may act as modulators and fine tune signals rather than act as classical neurotransmitter. This is in line with the findings of Dacks and co-workers [34]. They show that at least the Drosophila adult 5-HT system modulates the sensory circuit of the AL. 5-HT increases the responsiveness of inhibitory GABAergic local interneurons as well as the sensitivity of PNs in an odour-dependent manner. The 5-HT system may modulate and fine-tune responsiveness also in the larvae but this effect is possibly not observable in the applied preference assays (chapter 4). Thus, biogenic amine systems might be involved in odour and possibly also gustatory information processing, but not per se necessary for observable naïve preference behaviour. In a further run, the description of the whole brain connectome on the resolution level of synapses – analogue to the connectome done in chapter 3 – will be a first step to understand the whole neuronal circuitry involved in mediating innate behaviours. Additionally, calcium imaging and electrophysiological analysis of potentially involved neurons might reveal a clearer picture of the modulatory effect of biogenic amines.
Table 2: Overview of the Effects of Aminergic Systems on Larval Naïve Preference Behaviour. Various aminergic systems were inactivated via mutations or different UAS-effector lines. Note: A clear separation of the OA and TA system is difficult, thus one column refers to both systems OA/TA.

### Larval Naïve Preference Behaviour after Inactivation of Aminergic Systems

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<th>5-HT 1B</th>
<th>5-HT 2A</th>
<th>5-HT 7</th>
<th>DA</th>
<th>pPAM</th>
<th>Dop R1</th>
<th>Dop 1R2</th>
<th>Dop EcR</th>
<th>Dop 2R</th>
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<th>Oamb</th>
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<th>Oct β2R</th>
<th>Oct β3R</th>
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Explanation: \(X\) = impaired choice behaviour, \(O\) = not impaired choice behaviour, \(~\) = unclear/partial impaired choice behaviour, NA = not analysed, \(^1\)-\(^{20}\) = reference, \(^*\) = data in appendix, bold = chapter in this thesis

### Reference

8.2 Biogenic amine systems are expressed in different brain regions and are involved in associative olfactory learning and memory

The mushroom body (MB) integrates various sensory information and acts as learning and memory brain centre in insects. A model of the MB function in classical conditioning based learning and memory in Drosophila is described by Heisenberg [35]. In summary, his model shows that MB Kenyon cells (KCs) of the MBs receive olfactory information (conditioned stimulus) via projection neurons (PNs) and gustatory information (unconditioned stimulus) via OA or DA positive MB input neurons (MBINs). More downstream KCs synapse to MB output neurons (MBONs) that are connected to further neurons and finally induce muscle contractions to generate learning and memory based behaviour. In chapter 3, we provide for the first time a complete connectome up to synapsis level of a whole MB circuit validate and expand the model of Heisenberg. In the L1 larva, about 110-113 paired intrinsic KCs are synaptically connected to 14 paired and two unpaired MBINs and 24 paired MBONs. In addition to this linear signalling MBIN > KC > MBON, feedback loops KC > MBIN or direct connections between MBIN > MBON are found (chapter 3). In the calyx (CA) regions of the MBs, KCs are connected to 40-45 paired (PNs). Half of them (21 pairs) project from the ALs to the CAs and are olfactory PNs (for more details on synaptic level see [37]). Phyton and Stocker revealed that PNs are cholinergic [14]. For the description of the MB connectome, I was in particular interested in the documentation of the neurotransmitter identity (inclusive biogenic amines) of the MBINs and MBONs (see below and chapter 3).
Table 3: Overview of the Effects of Aminergic Systems on Olfactory Larval Learning and Memory. Various aminergic systems were inactivated via mutations or different UAS-effector lines. Note: A clear separation of the OA and TA system is difficult, thus one column refers to both systems OA/TA.

Larval Olfactory Learning and Memory Behaviour after Inactivation of Aminergic Systems

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<th>5-HT 7</th>
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<th>pPAM</th>
<th>Dop R1</th>
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Explanation: X = impaired learning and memory behaviour, O = not impaired learning and memory behaviour, ~ = unclear/partial impaired learning and memory behaviour, NA = not analysed. ¹-²⁰ = reference, ^ = data in appendix, bold = chapter in this thesis

8.2.1 The role of the 5-HT system in associative olfactory learning and memory is not consistent and fully understood

5-HT expressing neurons innervate the MB in *Drosophila* adults but likely not in larvae [10, 38, 39]. Coincidentally, the presence of 5-HT receptors in KCs was reported in adults but my analysis of specific 5-HT receptor GAL4 expression suggests a lack of all analysed 5-HT receptors in larval KCs (chapter 4 and [40, 41]). These differences between the adult and larval stage are not only found on anatomical level. There is also evidence that the effect of 5-HT signalling on learning and memory behaviour might differ between the adult and the larval stage. First, in adults specific 5-HTergic neurons (termed DPM neurons) synapse to the α/β lobes of the MBs, which express 5-HT1A receptors. This 5-HT/5-HT1A signalling is required for an intermediate-term memory formation named anaesthesia-resistant memory (ARM) [42]. Widmann et al. showed that ARM formation also take place in larvae [31]. However, by using the same standard training protocol I do not see an impaired aversive learning and memory of larvae lacking also 5-HT1A receptor expressing neurons (chapter 4). Thus, this circuit seems adult specific and develops presumably during or after metamorphosis as larvae do not have DPM neurons at all and I show in chapter 4 that 5-HT1A receptors are likely not expressed in the MBs (table 1 and [43]). Second, pharmacological and genetic manipulation of 5-HT1A, 5-HT1B, 5-HT2, and 5-HT7 receptors revealed impaired adult short term (STM) and long-term memory (LTM) formation testing aversive olfactory electric shock conditioning [44]. In contrast, I do not observe impaired electric shock learning and memory in larvae with ablated 5-HT or 5-HT2A (also termed 5-HT2) expressing neurons. However, further investigations must confirm this effect as adult specific. I tested only 5-HT2A for aversive learning and memory induced by an electric shock US. Thus, experiments should be expanded using other 5-HT receptors. In addition, it is still unclear which kind of memory form is tested by the use of the electric shock protocol. However, it was shown that STM is present in larvae but relatively short-lived and possibly no longer observable after the
training period I use [31]. If *Drosophila* larvae can establish LTM at all and how it can be tested is still under debate and needs further investigations. There is a third hint that there might be some differences between adults and larvae. Sitaraman et al. showed that performance in appetitive olfactory learning of flies with genetically impaired 5-HT expression was reduced [45]. In contrast, I observe appetitive olfactory learning scores on genetic control level testing larvae with a nearly complete lack of 5-HT or 5-HT receptors expression (chapter 4 and [10]). Considering all these results, the question arises if the effect of the 5-HT system on learning and memory is limited to the adult system? In chapter 4 I give a first hint for the necessity of the 5-HT system in larval learning and memory by showing that 5-HT/5-HT$_{2A}$ signalling specifically impairs larval odour-salt learning and memory. This might be a developmental effect, as acute blockage of synaptic output of 5-HT cells and potential 5-HT$_{2A}$ receptor cells does not affect aversive conditioning. How and when in development 5-HT/5-HT$_{2A}$ signalling affects specifically the establishment of this kind of larval learning and memory might be part of further investigations. In a first run, one could test larvae at various larval stages for aversive olfactory salt learning and memory and analyse expression pattern at the corresponding time points. Based on negative results one could subsequently impair 5-HT/5-HT$_{2A}$ signalling at different time points in embryonic development via a thermosensitive genetic interference of the GAL4 expression (e.g. via GAL80). However, it still might be challenging to investigate how 5-HT/5-HT$_{2A}$ signalling affects aversive salt reinforcement, because in general very little is known about the underlying neuronal pathways. But, it would be interesting to test, if this effect is specific for salt or if 5-HT/5-HT$_{2A}$ signalling is also required for aversive learning and memory induced by other negative gustatory US. To test this, different gustatory tastants like quinine or caffeine could be used [46, 47].
The number of studies describing the 5-HT system involved in learning and memory in Drosophila larvae and adults is rather small compared to other systems like DA. This matter of fact might be incidental. But probably the role of 5-HT in behavioural processes is more complex. As already mentioned above, 5-HT appears to be an important modulatory player, which also affects locomotion and developmental processes [2, 48-51]. That makes the investigation of observable changes in condition based behaviours caused by impaired 5-HT signalling difficult. Therefore, a clear, generally valid statement on the effect of 5-HT related to specific forms of olfactory learning and memory in Drosophila is not yet feasible, whereas in other invertebrates, mainly in Aplysia californica 5-HT acts as an important player in learning and memory [52].

8.2.2 The DA system is necessary and sufficient for aversive and appetitive associative olfactory learning and memory

DAergic neurons innervate the mushroom body of Drosophila in the larval (chapter 3 and 5 and [7]) and the adult stage (e.g. the comprehensive descriptions of [53] and [54]). In chapter 5 we show that larval DAergic neurons innervate parts of the MB peduncles and vertical lobes as well as all regions of the medial lobes. Whether DAergic neurons also innervate the CA is under debate. Selcho et al. [7] detected at least weak staining in these regions, whereas we (chapter 3) as well as Honjo and Furukubo-Tokunaga [9] could not observe any CA innervation.

DA seems to be the main biogenic amine that is necessary and sufficient for both appetitive and aversive olfactory learning and memory. This was demonstrated in numerous adult (e.g. [55-58] and in some larval studies (chapter 3 and 5 and [7, 8]). Depending on the kind of signalling – aversive or appetitive US – distinct sets of DAergic neurons innervate specific regions of the MB (chapters 3 and 5, [54, 55, 59]). Similar to the adult system a subset of larval DAergic neurons called pPAM innervate the medial lobes of the MBs (chapter 5). We
show that this cluster consisting of only four pairs of cells in larvae is necessary and sufficient for appetitive olfactory learning and memory. As mentioned above, DA is important for aversive learning and memory. It is likely that not the whole set of DAergic cells are essential for this behaviour. In Drosophila adults for example it was shown that the PPL cluster, innervating the vertical MB lobes, is essential for aversive olfactory learning and memory [57]. In larvae, the DAergic neurons DAN-f1 and DAN-g1 neurons of the DL1 cluster innervate the vertical MB lobes and are good candidates for investigating aversive olfactory learning and memory in larvae (chapter 3 and [7]). Thus, one could, similar to the approach applying in chapter 5, use specific GAL4-lines covering DL1 neurons to investigate the DA subset or cluster necessary for aversive olfactory conditioning.

Some recent anatomical and behavioural studies considered the DA system also on receptor level. D1-like receptors DopR1 or Dop1R2 are expressed in the larval MBs but not in the CAs [7, 15]. Mutations in both receptor genes lead to impaired larval aversive salt conditioning [7, 31]. Appetitive fructose conditioning is only affected in case of mutated DopR1 but not Dop1R2 [7]. The expression patterns of the D2-like DopR2 receptor and the ecdysone and dopamine dual receptor DopEcR is not yet analysed in the larval brain. It was however shown that Dop2R receptors are involved in appetitive and aversive learning and memory processes in larvae and in aversive STM and ARM in adults [36, 60]. It might be worth to test the effect of DopEcR in larval learning and memory as two recent studies showed modulatory effects of mushroom body neuronal activity by DopEcR [61] and an involvement of this receptor in courtship memory in adults [62].

In summary, the DA system is the best investigated biogenic amine in Drosophila conditioning. Numerous publications validate the contribution of DAergic neurons and DA receptors in aversive and appetitive olfactory learning and memory.
The OA/TA system is involved in appetitive and possibly also aversive associative olfactory learning and memory processes

OA and TA are expressed in the larval brain and innervate different regions (table 1, chapter 3 and 6 and [9]). Note that anatomical and behavioural functions are often analysed via genetic manipulation or immunostaining of TDC, the enzyme important for TA synthesis. TA acts as neuroactive molecule but also as precursor of OA. Thus, in this case a clear separation between the OA and TA system is not possible and consequently often termed as OA/TA (or imprecisely simple as OA) system. To specifically distinguish between the anatomy and behavioural functions of the TA and OA system further investigations are indispensable. In larvae, OA/TA neurons innervate the CAs and the MB vertical lobes (chapter 3 and 6). Interestingly, OA neurons not only synapse to KCs but also to MB output neurons (chapter 3).

Reward is mediated by OA in different insect species [63-66]. In Drosophila larvae and adults OA neurons signalling is necessary and sufficient for appetitive olfactory learning and memory [8, 9, 58, 59]. We show in chapter 6 that OA/TA signalling is specifically necessary for appetitive arabinose but not fructose olfactory learning and memory in larvae. If the larval OA/TA system is explicitly important for signalling sugar lacking nutrition value (like arabinose) is under debate as Honjo and Furukubo-Tokunaga showed impaired larval performance applying sucrose that is a nutritious sugar [9]. However, this difference might be due to technical reasons or due to the lack of controlling locomotion side effects (see chapter 6 and [1]). In the future, it might be of interest to investigate the subset of OA/TA neurons that are essential for arabinose learning and memory by the use of GAL4 lines covering only subsets of OA/TA neurons.

Two recently published articles suggesting an effect of the OA system on aversive learning and memory in adults [67, 68]. It was shown that impaired OA synthesis (via TβH-RNAi knocked down or TβH mutants) showed reduced olfactory electric shock learning and
memory (STM and ARM). This is in high contrast to former studies postulating that OA exclusively signals positive US.

The anatomical organisation of OA and TA receptors in larvae was part of a recent publication [13]. Unfortunately, this study does not use an additional landmark staining (e.g. for the neuropil) and therefore the precise brain region of the expression pattern is difficult to determine. The effect of signalling via different OA and TA receptors on larval naïve preference or conditioning is still unknown. The effect of OAMB on larval appetitive learning and memory appears particularly promising for investigations in the future, as signalling via this receptor is necessary for appetitive learning and memory in adult Drosophila [59, 69].

In conclusion, it is widely accepted that the OA or OA/TA system is essential for signalling appetitive US in Drosophila and many other insects, while a possible effect in aversive olfactory learning and memory must be confirmed in further studies.

8.2.4 The HA system might be involved in associative olfactory learning and memory processes

HA expressing neurons are present in the larval brain and innervate the VNC and the SOG (table 1 and [14]). Due to the lack of studies analysing HA expression in combination with neuropil staining, it is difficult to identify innervation of other brain regions like the MB. In adults HA receptors are mainly expressed in the visual CNS but also in regions like the thoracic ganglia [70, 71]. If and where in the larval brain the HA receptors ort and His Cl1 are present is not known. To analyse the HA system on an anatomical level, specific antibodies and GAL4-lines are required.

In chapter 7 I show that specifically impaired HA receptors ort but not HA expression affects larval olfactory learning and memory. Putting this result in the context of the current state of research, there is no evidence that the HA system is involved in learning and memory
processes in Drosophila. However, up to my knowledge there is also no study showing that HA is dispensable for this behaviour. In vertebrates, the role of HA is studied intensively because of its important role in allergic and inflammation processes [72, 73]. A relative small number of studies show an involvement of HA in cognition [74]. But it is still unclear whether HA directly affects learning processes rather than controlling several homeostatic functions that can interfere indirectly with the learning process [74]. Interestingly, the vertebrate HA receptor H$_2$ is coupled to Gs, that mediates the activation of the adenylylcyclase and protein kinase A, which phosphorylates proteins and activates the transcription factor cyclic-AMP-response element (CRE)-binding protein (CREB) [75]. This pathway is known to be essential for memory formation in Drosophila [31, 76, 77]. It will be of interest to investigate if such second messenger signalling cascade induced by HA is also existing in Drosophila. However, up to now, only ionotropic HA receptors are reported in Drosophila, while in vertebrates, G protein-coupled HA receptors were described.

Whether and how the HA system is involved in learning and memory processes in insects, including Drosophila, has to be verified in further studies.

8.3 References


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54. Mao Z, Davis RL. Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity. Frontiers


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Für eine hohe Produktivität und ein angenehmes Arbeitsklima mit viel Humor, offenen Ohren für Freudiges und Suboptimales und unterhaltsamen Konversationen hat das tolle A-TEAM gesorgt. Ich bin immer gerne ins Labor gekommen. Vielen Dank Anne, Anna, Astrid, Anthi, Andi und TilmAn (und viele weitere, «A»-enthaltende Namen…). Ich werde euch und die
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10 Author Contribution

Chapter 3: The complete connectome of a learning and memory center in an insect brain (Eichler et al. 2017)
>> Investigation (anatomy experiments - immunostainings)

Chapter 4: Anatomy and Behavioral Function of Serotonin Receptors in Drosophila melanogaster Larvae (Huser et al. 2017)
>> Conceptualization, Data curation, Formal analysis, Investigation (anatomy and behavior experiments), Methodology, Visualization, Writing – original draft, Writing – review & editing

Chapter 5: Four Individually Identified Paired Dopamine Neurons Signal Reward in Larval Drosophila. (Rohwedder et al. 2016)
>> Investigation (Anatomy experiments)

Chapter 6: Characterization of the Octopaminergic and Tyraminergic Neurons in the Central Brain of Drosophila Larvae (Selcho et al. 2014)
>> Acquisition of data (behavior experiments), Analysis and interpretation of data, Drafting of the manuscript, Administrative, technical, and material support

Chapter 7: Behavioral function of the histaminergic system in Drosophila melanogaster larvae (Huser and Thum 2018, in preparation)
>> Conceptualization, Data curation, Formal analysis, Investigation (behavior experiments), Visualization, Writing
11 Bibliography

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BIOGENIC AMINES IN THE CENTRAL NERVOUS SYSTEM OF DROSOPHILA MELANOGASTER LARVAE

- AN ANATOMICAL AND BEHAVIOURAL FUNCTION DESCRIPTION

**BIBLIOGRAPHY**

Please be advised that we experienced an unexpected issue that occurred on Saturday and Sunday January 20th and 21st that caused the site to be down for an extended period of time and affected the ability of users to access content on Wiley Online Library. This issue has now been fully resolved. We apologize for any inconvenience this may have caused and are working to ensure that we can alert you immediately of any unplanned periods of downtime or disruption in the future.


Waddell, S. "Reinforcement Signalling in Drosophila; Dopamine Does It All after All." Curr. Opin. Neurobiol. 23 (// 2013): 324-29.


12 Appendix

12.1 Appendix 1: Genetic lines used in this thesis

Table 1 GAL4- and UAS-lines that were used in this work.

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## BIOGENIC AMINES IN THE CENTRAL NERVOUS SYSTEM OF DROSOPHILA MELANOGASTER LARVAE

- AN ANATOMICAL AND BEHAVIOURAL FUNCTION DESCRIPTION

### APPENDIX

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#### UAS-line: Specific for: Chapter:

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<td>induces cell apoptosis</td>
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<td>dominant negative form of thermo-sensitive dynamin – disrupted synaptic vesicle recycling</td>
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12.2 Appendix 2: Energy dependant sugar learning of larvae with impaired 5-HT synthesis

Figure 1 The Serotonergic Neurons of the CNS are not Necessary for Appetitive low and high Energy Sugar induces Olfactory Learning and Memory. For testing appetitive olfactory learning, we utilized a two-group, reciprocal training design consisting of two half trials that give rise to a final performance index. Third instar larvae with impaired serotonergic neurons preferred an odor that was paired with 2-M arabinose and sorbitol. In none of the learning assays significant differences between experimental and control larvae were found. Under each boxplot of the figure for each genotype the sample size is shown; n = 10-15. Asterisks above each boxplot indicate, if the data is significantly different from zero. *< 0.05; **< 0.01; ***< 0.001.