Paracrine and synaptic serotonin have opposite effects on olfactory processing in *Drosophila* via distinct signaling pathways

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Abbreviated title: Paracrine signaling in the *Drosophila* antennal lobe

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Abstract
Virtually all nervous systems are subject to neuromodulation that alters how signals are processed within and between neurons. Deciphering how modulatory neurons integrate within circuits is complicated because their transmitters can be released either synaptically or in a paracrine fashion to activate a multitude of receptors. Moreover, the functional consequences that different sources of a neuromodulator can have on a neuronal circuit is difficult to establish. We used whole-cell recordings, pharmacology, and genetic manipulations in female *Drosophila melanogaster* to show that serotonin (5-HT) can reach the antennal lobe (AL), an olfactory neuropil, in a paracrine fashion. The DA1 glomerulus in flies is nearly void of direct serotonergic innervation yet is highly sensitive to 5-HT pharmacology. This sensitivity remains even when the only 5-HT immunoreactive neurons in the AL are genetically ablated, suggesting that 5-HT must enter the AL from outside sources. This paracrine 5-HT establishes a basal tone of the modulator that suppresses olfactory responses and is detected by local interneurons (LN) expressing only the excitatory 5-HT7R. This population of LNs postsynaptically inhibits the output neurons of the AL. Conversely, activation of serotonergic neurons directly innervating the AL boosts olfactory output. This modulation is not sensitive to 5-HT7R manipulation. Our results demonstrate that 5-HT can influence distant circuits and have opposing effects on the same network depending on the source and postsynaptic neurons that it targets.

Significance Statement
Neuromodulation is an essential component of sensory processing and imparts flexibility in how stimuli are represented in the brain. However, the manner in which modulatory systems interact with sensory systems is complicated due to their ability to signal via traditional synaptic mechanisms and via volume, or paracrine, transmission. Here we demonstrate that low concentrations of paracrine-released 5-HT serve to diminish olfactory responses in the *Drosophila* olfactory system while high concentrations of the same modulator released synthetically boost responses. Serotonin’s unique effects on a neuronal circuit depends on the source of release and the distinct receptors that it activates.

Introduction
Neuromodulation imparts flexibility in neural circuits by altering the intrinsic properties of neurons and the synaptic strength between them [1,2]. Thus, neuromodulation can optimize the computations of neural circuits depending on the animal's behavioral state or sensory environment. Serotonin (5-HT) represents a quintessential neuromodulator, having been identified in nearly all animal species examined [3], and it has been implicated in cognition [4], sensory [5], and motor function [6]. Despite their prevalence and rich history in neuroscience, the function and organization of serotonergic systems has remained elusive and enigmatic. Complexities in the study of 5-HT arise in part from the manner in which the transmitter is released as well as the large variety of postsynaptic 5-HT receptors (5-HTRs) expressed throughout the brain.
Traditional neurotransmitters are emitted from clear vesicles at well-defined synapses. In such cases a one-to-one wiring diagram, or connectome, can be constructed to elucidate how a circuit functions and how information flows between a group of neurons. Serotonergic systems are more complicated as the modulator can be exocytosed from both small clear vesicles at distinct synapses or extrasynaptically from dense core vesicles to influence distant targets [7]. This latter form of communication is termed paracrine signaling, or volume transmission [8], and depends on both diffusion and active processes. Paracrine signaling inherently implies a weak correlation between the precise anatomical innervation of a serotonergic neuron and the range of targets that it can influence within a circuit, thus making it difficult to attribute activity in an individual serotonergic neuron and modulation of downstream cells. Once released into the synapse or extracellular space (ECS) via paracrine signaling, 5-HT can bind a plethora of receptors each with their own binding affinities, kinetics, and downstream second messenger systems. Currently, 14 5-HTRs from 7 receptor families in humans have been described [9]. Most circuits in the brain express multiple 5-HTR types across their neurons and a given neuron can often express multiple 5-HTRs, further complicating analyses of neuromodulation. Whether synaptic and paracrine-released 5-HT target unique receptors and originate from different sources is critical to formulating conceptual models of how this modulator works in the brain.

In this study, we use the first olfactory relay of Drosophila, the antennal lobe (AL), to compare the properties of paracrine and synaptic 5-HT in sensory processing. Specifically, we examine the source, function, and mechanism of detection of synaptic and paracrine derived 5-HT. The AL is an ideal system to study serotonergic transmission because only one pair of 5-HT neurons termed the contralaterally-projecting serotonin-immunoreactive deuterocerebral interneurons (CSDns) innervate the AL (Figure 1A). The CSDNs innervate most glomeruli in the AL, but also selectively exclude others such as the pheromone sensitive glomerulus, DA1. Importantly, the odor responses of DA1 projection neurons (PNs, an analog of mammalian mitral cells (MCs)) are sensitive to serotonergic pharmacology [10,11], suggesting that 5-HT enters the glomerulus in a paracrine fashion. By using a combination of pharmacology, whole-cell electrophysiology, RNAi knock down, and 2-photon imaging, we identify the receptor and cell class responsible for detecting paracrine 5-HT at the DA1 glomerulus and propose that synaptic and paracrine 5-HT serve distinct functions in olfactory processing.

Results

Low concentrations of serotonin suppress olfactory signaling

We used pharmaceutical reagents to manipulate the basal or resting concentration of serotonin within a physiological range in the AL. Methysergide is a broad 5-HTR antagonist and should remove most tonic 5-HTR activation at rest [12]. Methysergide boosted DA1 PN odor responses to their preferred ligand, cis-
vaccenyl acetate (cVA), suggesting 5-HT has a suppressive effect at low concentrations (Figures 1B and 1C). Next, we used fluoxetine, a selective blocker of the serotonin transporter, to lightly elevate the concentration of 5-HT. Consistent with a suppressive role for 5-HT, fluoxetine diminished DA1 responses (Figures 1D-1F). High concentrations of exogenous 5-HT boost odor responses in *Drosophila* [10,11], but such effects have never been reported with endogenous release. We used the channelrhodopsin Chrimson [13] to stimulate the CSDns in an attempt to evoke the highest amount of endogenous 5-HT release directly into the AL. Importantly, the DA1 glomerulus receives little to no direct serotonergic innervation and thus does not respond to CSDn stimulation [11]. We thus sampled random PNs instead and found that direct release of 5-HT from the CSDns enhanced their odor evoked activity (Figure 1G). These results show that 5-HT can have opposing effects on PN responses based on concentration and that lower concentrations reduce PN odor-evoked activity.

The inability of the CSDns to modulate DA1 responses suggests that 5-HT from outside sources may permeate into the AL in a paracrine fashion [14]. To demonstrate this point directly, we expressed temperature sensitive diphtheria toxin in the CSDns to acutely kill these cells (Figure 2A and B) and then monitored the effects of methysergide on DA1 PN odor responses. Methysergide application boosted odor responses suggesting that 5-HTR activation occurs in the AL despite the absence of viable CSDns (Figures 2B and D). In fact, the amplitude of odor response enhancement was not statistically significant between flies with or without intact CSDns (Figure 2E, Student’s t-test, p = 0.135, n = 8 control and n = CSDn ablated). Boosting was not observed when we targeted all serotonergic neurons for acute ablation (Figure 2D), suggesting that methysergide’s effects at this glomerulus are 5-HT specific and that 5-HTRs do not signal in the absence of 5-HT (Figures 2F - H). These results demonstrate that serotonin from outside the AL circulates in the extracellular space in a paracrine fashion to establish a low basal concentration of 5-HT within the DA1 glomerulus that sets a baseline inhibitory tone during olfactory processing.

**Paracrine 5-HT is detected by LNs expressing 5-HT7R**

We next performed a pharmaceutical screen to determine which receptors are responsible for detecting and signaling low paracrine levels of 5-HT at the DA1 glomerulus. Antagonists for each of the five *Drosophila* serotonin receptors [15-18] were tested for their ability to both mimic and occlude methysergide’s ability to boost of DA1 PN responses (Figures 3A - E). Most antagonists had the opposite effect and reduced odor responses. Only the 5-HT7R antagonist SB258719 both boosted odor activity and prevented methysergide from further elevating DA1 PN responses (Figure 3E). Because the effects of SB258719 were more transient, we utilized methysergide in subsequent experiments after verifying that the *Drosophila* 5-HT7 receptor was effectively blocked by methysergide *in vivo* (Figure S1). The antagonist screen thus implicates 5-HT7Rs in mediating paracrine signaling in the AL.

DA1 odor responses could be influenced by 5-HT7R activity in either ORNs, PNs, or LNs. Currently there are no validated antibodies available for any
of the serotonin receptors in flies to identify which neurons express the receptor. We thus used previously described 5-HTR MiMIC T2A-GAL4 protein-trap and gene-trap transgenic Gal4 lines [19-21] and determined their overlap with the DA1 PNs (Figures 4A - E) [22,23]. We found no overlap between DA1 PNs and each of the 5 MiMIC 5-HTR lines, suggesting that DA1 PNs themselves are not the target of paracrine 5-HT’s effects in the AL. Consistent with this genetic approach, photo-uncaging serotonin had no direct effect on DA1 PNs (Figure S1). Previous studies have shown that ORNs as a population do not express 5-HT7R and only express 5-HT2BR [21]. We confirmed that the cognate ORNs to the DA1 glomerulus do express 5-HT2BR (Figure S2). However, because neither PNs nor ORNs express 5-HT7R specifically, LNs are the most likely cell class within the AL to employ the 5-HT7R receptor to detect paracrine signaling within the DA1 glomerulus.

We thus expressed an RNAi against the 5-HT7R in various promoter lines to identify the population of LNs that mediates the boosting of DA1 PN odor responses in methysergide (Figures 5A - C). The GH298-Gal4 and NP3056-Gal4 promoter lines each express broadly within the AL but have virtually no overlap in their expression patterns [24]. We also screened the Janelia FlyLight collection [25] for Gal4 lines generated from fragments of the 5-HT7R promoter and identified R70A09-Gal4 as expressing exclusively in 12 LNs per hemisphere (Figure 5D). Expression of the 5-HT7R-RNAi in either NP3056 or R70A09 blocks the effects of methysergide. Interestingly, NP3056 and R70A09 have little overlap in their expression patterns (Figure S3), suggesting that both populations of LNs must express functional 5-HT7Rs to block the effects of the antagonist and paracrine 5-HT. We converted R70A09-Gal4 into a Q-system promoter line [26] using the HACK method [27] to test for the overlap between the R70A09 and MiMIC 5-HT7R lines. Most R70A09 LNs are labeled in the MiMIC 5-HT7R protein trap line, suggesting they indeed express the receptor (Figure 5E). Interestingly, we also examined the overlap between the 5-HT7R-expressing R70A09 LNs and the MiMIC 5-HTR lines for the additional four 5-HTRs and found little to no overlap between these populations (Figure S4). These data reveal that there exists a population of LNs in the AL that exclusively expresses the 5-HT7 receptor and is partly responsible for the suppressive effects of low 5-HT concentrations.

If R70A09 LNs use 5-HT7Rs to detect low levels serotonin, then expressing the 5-HT7R-RNAi in these cells should not only prevent the modulation of DA1 PNs odor responses to drops in 5-HT concentration, but also to increases in 5-HT. We thus repeated our previous experiments and exposed the preparation to fluoxetine to slightly elevate 5-HT concentrations. Fluoxetine typically suppresses odor responses (Figure 1D) but failed to do so when 5-HT7Rs where knocked down in R70A09 LNs (Figures 5F - H). Because serotonin can have opposing effects at high and low concentrations in the AL (Figure 1 and ref. [11]), we next applied a high concentration of 5-HT mimicking synaptic release to the same preparations to determine if 5-HT7Rs in R70A09 LNs also signal higher serotonin concentrations. DA1 odor responses boosted to
exogenous 5-HT suggesting that R70A09 LNs do not utilize 5-HT7Rs to signal higher concentrations of serotonin (Figures 5F - H).

5-HTR7-expressing LNs suppress odor responses via postsynaptic inhibition

Paracrine 5-HT onto LNs could suppress odor responses indirectly by either presynaptically inhibiting ORNs or postsynaptically targeting PNs. The best described role for GABAergic LNs in the AL is to mediate gain control by presynaptically targeting ORNs [28-30]. Interestingly, we found that methysergide had no effect on ORN output as measured via GCaMP6s [31] (Figures 6A - C). Methysergide did however boost DA1 PN odor responses (Figures 6D - F), suggesting that R70A09 LNs likely target postsynaptic PNs directly.

To demonstrate a connection between R70A09 LNs and DA1 PNs, we expressed Chrimson in the LNs while recording whole-cell from DA1 PNs. Stimulation of R70A09 LNs reliably inhibited the PNs (Figure 6G). However, as LNs in the Drosophila AL can be highly interconnected, we used our recently developed TERPS technique [11,32] to establish if this connection is direct and monosynaptic. We co-expressed Chrimson along with the TTX-insensitive sodium channel NaChBac [33,34] in the LNs and recorded from DA1 PNs (Figure 6H). TTX was then applied to eliminate all polysynaptic connections. The inhibitory connection between R70A09 LNs and DA1 PNs is monosynaptic and GABAergic as it remains even after the application of TTX and is sensitive to GABA pharmacology (Figures 6I - J).

Discussion

Determining the precise connectome of a neuronal circuit is essential to probing its function and ultimately determining the computations that it performs [35]. However, it is also appreciated that additional forms of extra-connectomic signaling, such as ephaptic coupling [36] and neuromodulation complicate the interpretation of simple cell-to-cell wiring diagrams [37]. Neuromodulation can be particularly tricky as the link between anatomy and physiology can be obscured by the great distances that modulatory compounds travel and the variety of receptors with which they can interact. Here, we exploited the well-described anatomy and genetic tools of Drosophila to show that in addition to synaptic release from the CSDn, 5-HT infiltrates the AL in a paracrine fashion. This paracrine 5-HT establishes a tonic modulatory tone and is detected in part by 5-HT7Rs on a distinct subset of GABAergic local interneurons that postsynaptically inhibit PNs. These data show a specialized function for paracrine 5-HT in the AL that is mediated by a specific receptor and opposite the role of synaptic 5-HT.

The sources of 5-HT in the AL

Neuromodulators, including serotonin, can be secreted at conventional synapses and also directly into the ECS where they can impact distant targets. The CSDn is the likely source of synaptic 5-HT in the AL as it is the only serotonergic neuron to directly innervate this neuropil. Activation of the CSDn is sufficient to
release high concentrations of 5-HT sufficient to boost PN odor responses, thus mimicking exogenous application. The source of paracrine 5-HT that permeates into the AL is currently unknown. We previously proposed that the concerted activity of the entire population of serotonergic neurons sets the modulatory tone in the AL as stimulation of these cells is sufficient to modulate DA1 odor responses in a 5-HT dependent manner [11]. In this case, robust and coordinated activation of 5-HT neurons could overwhelm transport mechanisms and allow the modulator to remain in the ECS longer to diffuse further. Indeed optogenetic activation of larval serotonergic neurons can cause 5-HT spikes lasting tens of seconds to minutes [38].

Alternatively, a select few serotonergic neurons could contribute disproportionately to paracrine 5-HT. How might some neurons have a greater ability to transmit 5-HT to the AL over others? While distance to the AL neuropil seems like an obvious criterion, other factors including the path tortuosity, volume fraction of the ECS, and the relative abundance of clearance mechanisms along the way can also influence the efficiency of extracellular communication [8,39,40]. Additionally, 5-HT may be secreted directly into the haemolymph of the animal to circulate long distances. Serotonin is known to enter both the vertebrate CSF and invertebrate haemolymph [41,42]. In both leeches [43] and Aplysia [44], stimulation of few serotonergic neurons is sufficient to raise 5-HT levels in the haemolymph. Finally, glia may act as intermediaries for long-distance transmission by sequestering 5-HT and releasing it elsewhere [45,46]. Such factors make it difficult to predict a priori which neurons may contribute most to paracrine release of 5-HT into the AL. Manipulations of subsets of 5-HT neurons will be needed to resolve these issues.

**Cellular detection of paracrine 5-HT signaling**

Our data suggest that PN odor responses can be differentially modulated depending on the source of 5-HT (Figure 7A). Serotonin from the CSDn boosts odor responses while the resting tone of 5-HT is set by the population of serotonergic neurons and suppresses such responses. These two processes must be governed by distinct receptors as the knock down of 5-HT7R via RNAi blocks paracrine 5-HT’s effects in the AL but still permits boosting to higher concentrations of exogenous modulator. Thus, the same neuromodulator can have diverse effects within a small circuit depending on the receptor to which it binds [1]. One of the main factors differentiating synaptic versus paracrine 5-HT is likely concentration. The 5-HT7R is well suited for detecting paracrine 5-HT because it is the highest affinity serotonin receptor in *Drosophila* [18]. This allows the receptors to signal small changes in 5-HT levels at low concentrations. Most extrasynaptic receptors are high affinity G-coupled metabotropic receptors, and the few ionotropic examples tend to be higher affinity than their synaptic counterparts [47,48].

Interestingly, we found a specific subset of GABAergic LNs to be responsible for detecting paracrine 5-HT within the DA1 glomerulus. We propose that paracrine 5-HT binds to excitatory 5-HT7Rs on these cells to boost their activity and suppress PN output (Figure 7B). LNs are an ideal target for
modulation as they can influence several aspects of olfactory coding in both flies [49,50] and mice [51,52]. Serotonin is also a potent modulator of GABAergic transmission in vertebrates [53] and 5-HT targets local interneurons in the olfactory bulb (OB) as well [54-56]. It is critical to note however that the DA1 glomerulus may not be representative of all glomeruli. We demonstrated that DA1 PNs do not express the 5-HT7R endogenously, but PNs innervating other glomeruli likely do. In fact, multiple cell classes in the AL express the receptor [21] but may do so in a glomerulus specific manner. Thus, it is likely that the specific effect of paracrine 5-HT differs slightly across glomeruli and is dependent on the unique expression pattern of 5-HT7R within that glomerulus. Importantly, data regarding expression patterns of 5-HT7R comes primarily from genetic approaches that label neurons according to the activation of the promoters for these receptors [19,20]. Thus, while we can discern which cell classes express specific 5-HTRs, there is currently no data to verify if individual LNs can traffic these receptors to dendrites innervating specific glomeruli. Future studies employing physiology or novel molecular tools will be vital for determining how 5-HTRs are dispersed across the AL to modulate the odor responses of PN innervating different glomeruli.

The role of paracrine 5-HT in the AL
Inhibition in the AL [28,29,57] and OB [58,59] targets presynaptic ORN terminals mediating a form of divisive gain control. Divisive gain control is important as it can prevent the saturation of downstream PN and MC responses allowing olfactory circuits to encode over a wider range of odor concentrations [30,49,60]. This form of gain control also allows the postsynaptic cell to utilize its entire dynamic range as ORN activity increases. Finally, targeting ORNs leaves the window of temporal integration in PNs unaltered by inhibitory conductances. Interestingly, our results suggest that one function of paracrine 5-HT may be to specifically shift the balance between pre- and postsynaptic inhibition. The properties of postsynaptic inhibition are distinct of those described above and are subtractive rather than divisive [60,61]. Postsynaptic inhibition may be beneficial for suppressing PN firing from a broader distribution of inputs rather than just its cognate ORNs. Subtractive gain control can also reduce the maximum firing rate of a neuron thus potentially diminishing its influence on perception regardless of stimulus strength. This may be advantageous for suppressing behavior when sensory stimuli are presented in the wrong context. The utilization of divisive and subtractive gain control within the same circuit is not unique to olfaction, as it has also been described in the attentional regulation of vision [62].

An alternative function for the suppressive nature of paracrine 5-HT in the AL may be to provide stability to olfactory processing under modulation. Data from the lobster stomatogastric ganglion (STG) indicate that dopamine has opposing actions on the same neurons depending on whether the concentration is in the low tonic range (nM) or higher concentrations mimicking synaptic release (µM) [63]. This mechanism has a homeostatic effect and stabilizes the rhythmic pattern of STG motor output [64]. Similarly, we found that low and high concentrations of serotonin at the DA1 glomerulus also have opposing effects.
Bidirectional modulation by the same transmitter has been reported in numerous other systems [65-67], suggesting it may be a common feature of the central nervous system across diverse phylogenetic groups. Our results argue that the set point of such bidirectional modulation in the AL could be dynamically altered as it arises from different sources and influences distinct olfactory neurons.

Material and Methods

Fly stocks
Flies were reared on Nutri-Fly Bloomington Formulation (Flystuff.com, San Diego, CA) at 25°C. All experiments were performed on female flies 1–3 days post-eclosion. All fly stocks containing the optogenetic transgene Chrimson [13] were raised on rehydrated potato flakes (Carolina Biological, Burlington, NC) mixed with all-trans-retinal. A list of all genotypes and their sources for each figure is listed in Table 1.

Odor and odor delivery
An odor was presented as previously described [11]. In brief, a carrier stream of carbon-filtered house air was presented at 2.2 L/min to the fly continuously. A solenoid was used to redirect 200 ml/min of this air stream into an odor vial before rejoining the carrier stream, thus diluting the odor a further 10-fold prior to reaching the animal. cVA (Pherobank, Wageningen, Netherlands) was delivered as a pure odorant. For all experiments, the odor was presented every 40 s.

Whole-cell Electrophysiology
In vivo whole-cell recordings were performed as previously described [11]. Data were low-pass filtered at 5 kHz using an AM Systems model 2400 amplifier (AM Systems, Carlsberg, Washington) and digitized at 10 kHz. Pipettes were pulled from thin-walled borosilicate glass (World Precision Instruments, Sarasota, FL; 1.5 mm outer diameter, 1.12 mm inner diameter) to a resistance of 8–12 MΩ. To visualize neurons, we used oblique illumination from an infrared LED guide through a fiber optic (Thorlabs, Newton, New Jersey). The external recording solution contained, (in mM) 103 NaCl, 3 KCl, 5 N-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid, 8 trehalose, 10 glucose, 26 NaHCO3, 1 NaH2PO4, 1.5 CaCl2, and 4 MgCl2 (adjusted to 270–275 mOsm). The saline was bubbled with 95% O2/5% CO2 and reached a pH of 7.3. Our internal solution contained, (in mM) 140 potassium aspartate, 10 HEPES, 4 MgATP, 0.5 Na3GTP, 1 EGTA, and 1 KCl. For whole cell recordings, a small hyperpolarizing current was applied to offset the depolarization caused by the pipette seal conductance. The cells’ resting potentials were adjusted slightly to match the firing rate of similar neurons obtained in cell-attached recordings. Neurons which did not fire spontaneously or that had depolarized membrane potentials upon break-in were excluded from the study. DA1 PNs were labeled using the Q/QUAS system [26] with the MZ19-QF promoter. These cells were identified based on their soma location in the lateral cluster of the antennal lobe and responsiveness to cVA.
TERPS
TERPS was performed as previously described [32]. We expressed NaChBac and Chrimson in LNs with the GMR70A09-Gal4 line. This promoter line labels LNs and only a small set of additional neurons outside the AL. TTX (1 μM) was used to block all spiking in the AL. We confirmed this by depolarizing each neuron and observing that they could no longer fire action potentials. We used a high-powered red LED (Red XP-E, 620-630 nm wavelength) and Buckpuck driver (RapidLED, Randolph, Vermont) to stimulate Chrimson expressing neurons. The LED was mounted directly underneath the preparation and light was presented at 0.238 mW/mm² as measured by a Thorlabs light meter PM100A with light sensor S130C. Flies expressing Chrimson were raised on food containing 0.2 mM all-trans retinal. All-trans retinal was prepared as a stock solution in ethanol (35 mM), and 28 μl of this stock was mixed into approximately 5 ml of rehydrated potato flakes and added to the top of a vial of conventional food.

Photoactivation of caged serotonin We used a high-power UV LED (M365LP1, 365 nm wavelength, Thorlabs) to elicit photolysis of caged-serotonin. The light was presented at 0.2 mW with 20 msec pulse. The external saline containing caged-serotonin was recirculated at the beginning of experiments, and we replaced it every experiment. To prevent undesirable photolysis, we shielded the preparation from ambient light.

Two-photon calcium imaging
We used female flies aged 3 days post-eclosion and reared at room temperature. Imaging experiments were performed at room temperature. Flies were dissected in the same manner as those used in whole cell recordings and perfused with the same extracellular solution. The saline was bubbled with 95% O₂ / 5% CO₂ and reached a pH of 7.3. 920 nm wavelength light was used to excite GCaMP6s under two-photon microscopy. The microscope and data acquisition were controlled by ThorImage 3.0 (Thorlabs, Inc.). An image of sample was scanned at a speed of 60 frames/second and averaged every 10 frames. Thus, the sample was recorded at 6 frames/second. Odors were delivered for 1 second after the first 2 seconds of each trial. An inter trial interval of 80 s was applied between each trial. An ROI was set manually for each experiment that surrounded the DA1 glomerulus. Calcium transients (ΔF/F) were measured as changes in fluorescence, in which ΔF/F was calculated by normalizing the fluorescence brightness changes over the baseline period (the first 2 seconds of each trial before the odor delivery).

Pharmacology
The following chemicals were used in this study at the concentrations indicated: methysergide maleate (50 μM, Tocris, CAS 129-49-7), fluoxetine (10 μM, Tocris/Sigma, CAS 56296-78-7), WAY100635 (20 μM, Tocris, CAS 634908-75-1), SB216641 (20 μM, Tocris, CAS 193611-67-5), ketanserin (20 μM, Tocris, CAS 83846-83-7), metaclopamide (20 μM, Sigma, CAS 364-62-5), SB258719
(20 μM, Tocris, CAS 1217674-10-6), CGP54626 (50 μM, Tocris, CAS 149184-21-4), mecamylamine (100 μM, Sigma, CAS 826-39-1), tetrodotoxin (TTX) (1 μM, Tocris, CAS 18660-81-6), picrotoxin (5 μM, Sigma, CAS 124-87-8), NPEC-caged-serotonin (50 μM, Tocris, CAS 1257326-22-9), and serotonin (100 μM, Sigma, CAS 153-98-0). Serotonin solutions were made fresh from powder immediately prior to each experiment and wrapped tightly in aluminum foil to prevent oxidation by light. We used a peristaltic pump to recirculate the external recording solution in all experiments using pharmaceuticals. Drugs were added sequentially to the same recirculating solution.

Cell ablation

Cell ablation by diphtheria toxin was performed as previously described [11]. In brief, a temperature-sensitive variant of diphtheria toxin was expressed in the CSDn or Trh neurons by the GAL4/UAS system. One day post eclosion, adult flies were transferred to 31°C for three days. The efficiency of the diphtheria toxin was assessed by immunohistochemistry with the serotonin antibody for each preparation post hoc.

Immunohistochemistry

Dissected brains were fixed with 4% paraformaldehyde for 15 min at room temperature. Fixed tissue was blocked with PBS containing 2% Triton X-100 and 10% normal goat serum (NGS) for 30 min and then incubated for 1 day each (with washing in between) at room temperature in PBS containing 1% Triton X-100, 0.25% NGS, and a primary antibody or secondary antibody solution. The brain was mounted in 20 μl Vectashield medium. We used the following primary antibodies at the indicated dilutions: 1:50 mouse anti-bruchpilot (nc82) (Developmental Studies Hybridoma Bank, Iowa), 1:1000 rabbit anti-5HT (Sigma, S5545). Secondary antibodies from Invitrogen were used at dilutions of 1:250, which were Alexa Fluor 633 goat anti-mouse IgG (Life Technologies, A21050), Alexa Fluor 568 goat anti-mouse IgG (Life Technologies, A11004), Alexa Fluor 633 goat anti-rabbit (Invitrogen, A21071). Confocal images were acquired with Zeiss LMS710 confocal laser scanning microscope under 40x or 63x magnification.

Statistical analysis

Values are given as means ± SEM. Wilcoxon signed rank test were performed for all paired comparisons between one treatment and the control within the same group.

Figure Legends

Figure 1. Serotonin modulates olfactory responses in a concentration dependent manner.
(A) A schematic of the Drosophila antennal lobe showing relevant neurons and connections. Olfactory receptor neurons (ORNs) make feed forward connections onto the dendrites of projection neurons (PNs) in olfactory glomeruli (light colored circles in AL). ORNs expressing the same olfactory receptor are colored identically and project to the same individual glomerulus. GABAergic local interneurons (dark blue) allow communication between glomeruli and participate in various forms of lateral and intra-glomerular inhibition. The AL receives all of its serotonergic innervation from a pair of neurons termed the CSDns (red) that innervate nearly all glomeruli excluding pheromone-sensitive glomeruli, such as DA1.

(B) A sample recording of a DA1 PN odor response to cVA. All recordings in this manuscript were from DA1 PNs. PN responses refers specifically to DA1 PN responses hereafter unless otherwise noted. Top, raw voltage trace. Middle, ticks represent the timing of individual action potentials from the recording above. Bottom, a peristimulus time histogram (PSTH) of the firing rate of the trial above.

(C) The mean and SEM of PSTH's in saline and after the application of 50 µM methysergide (used throughout the study). Odor stimulus is 500ms and is used throughout subsequent figures. The vertical bar indicates 50 spikes per second.

(D) The mean and SEM of PSTH's in saline and after the application of 10 µM fluoxetine.

(E) A time series showing the effects of both methysergide and fluoxetine. Data are normalized to the mean of the response without drugs.

(F) Summary statistics of methysergide and fluoxetine experiments (C - E). Each gray circle represents one preparation. Each horizontal and vertical lines are the mean and SEM across preparations respectively. Wilcoxon signed rank test, n = 8 methysergide (p = 0.007) and n = 6 fluoxetine (p = 0.031). ** = p < 0.01, * = p < 0.05. Symbols used throughout subsequent figures.

(G) A unity plot showing boosting of odor responses of randomly sampled non-DA1 PNs after CSDn stimulation. Responses were significantly elevated, paired Student's t-test performed on the each of the 11 cells preferred stimulus before and after stimulation, p = 0.042.

Figure 2. Paracrine released serotonin reaches the DA1 glomerulus from outside the AL.

(A) Image of the *Drosophila* brain with CSDns intact and labeled with a 5-HT antibody. CSDns are labeled with red arrowheads and white arrowheads point to other serotonergic neurons. Scale bar = 50 µm for panels A, B and F. Background staining with the nc82 antibody reveals the brain's general anatomy.
(B) Staining, imaging, and labeling performed as in panel A. The CSDn was ablated with a temperature-sensitive variant of diphtheria toxin while other serotonergic neurons remain intact. White dashed circles indicate the location where the CSDns reside in control animals.

(C) PSTHs showing odor responses in saline and following methysergide application.

(D) Quantification of previous experiment. n = 10, paired Student’s t-test (p = 0.002).

(F) An image showing the brain after acute ablation of all 5-HT neurons by expressing diphtheria toxin with the trh-Gal4 promoter line. Some non-specific 5-HT labeling is observed, but it does not localize to cell bodies suggesting all 5-HT neurons were successfully ablated.

(G) PSTHs showing odor responses in saline and following methysergide application when all 5-HT neurons are ablated.

(H) Quantification of previous experiment. n = 11, paired Student’s t-test, N.S. = not significant (p = 0.205).

**Figure 3.** The Drosophila 5-HT7R likely mediates the suppression of odor responses at low concentration of serotonin.

(A) *Left,* a PSTH of DA1 odor responses recorded in saline and after the application of the 5-HT1AR antagonist WAY100635. *Middle,* a time series of the effects of the antagonist and with subsequent application of methysergide. *Right,* quantification of the preceding experiments. n = 7, saline vs. drug p = 0.047, drug vs. methysergide p = 0.15. All comparisons including subsequent panels where performed with a Wilcoxon signed-rank test.

(B) As in A but for the 5-HT1BR antagonist SB216641. n = 8, saline vs. drug p = 0.23, drug vs. methysergide p = 0.016

(C) As in A but for the 5-HT2AR antagonist ketanserin. n = 7, saline vs. drug p = 0.11, drug vs. methysergide p = 0.94

(D) As in A but for the 5-HT2BR antagonist metaclopamide. n = 6, saline vs. drug p = 0.031, drug vs. methysergide p = 0.68

(E) As in A but for the 5-HT7R antagonist SB258719. n = 8, saline vs. drug early p = 0.05 (†), saline vs. drug late p = 0.84, drug vs. methysergide p = 0.74.

**Figure 4.** DA1 PNs do not express 5-HTRs.
(A) The QMz-19 promoter line was used to examine the overlap between DA1 PNs and 3 MiMIC-5-HT1AR gene trap promoter lines. Red labeled cells are DA1 PNs, green cells are express in the MiMIC lines, and yellow neurons indicate overlapping populations. This nomenclature carries through all subsequent panels. No yellow neurons are observed. All combinations were tested at least 2 times.

(B) As in A but testing for overlap between Mz-19 DA1 PNs and a MiMIC-5-HT1B gene trap line.

(C) As in A examining the overlap with MiMIC-5-HT2A gene trap lines.

(D) As in A but examining the overlap with MiMIC-5-HT2B gene trap lines.

(E) As in A but examining the overlap with MiMIC-5-HT7 protein trap line.

Figure 5. R70A09-Gal4 LNs use 5-HT7Rs to detect and signal low but not high concentrations of 5-HT.

(A) An RNAi against the 5-HT7R was expressed in 3 LN lines and tested for its ability to block the effects of methysergide. The expression of the construct in the GH298 LN line did not block the effects of methysergide. Expression in both NP3056 and R70A09 did occlude the effects of methysergide. From left to right, n = 7, 8, 7, Wilcoxon sign-ranked test (p = 0.015, p = 0.64, p = 0.812).

(B) A PSTH of DA1 responses when the 5-HT7R-RNAi is expressed in R70A09 LNs.

(C) A time series of DA1 responses from the previous experiment.

(D) The R70A09-Gal4 line expresses exclusively in approximately 12 LNs per hemisphere.

(E) The MiMIC-5-HT7 protein trap and the R70A09-Q promoter lines have extensive overlap. 9.3 ± 1.0 R70A09 cells overlap with the 11.25 ± 0.69 LNs of the MiMIC line [21]. n = 6.

(F) PSTH's of DA1 odor responses in fluoxetine and exogenous 5-HT. The 5-HT7R-RNAi was expressed in R70A09LNs.

(G) A time series of the odor responses in fluoxetine and subsequently 5-HT.
Figure 6. R70A09 LNs utilize GABA to suppress DA1 odor responses via postsynaptically.

(A) Olfactory responses were assessed pre-synaptically by expressing GCaMP6s in the DA1 cognate ORNs, OR67d.

(B-C) Blocking basal levels of 5-HT with methysergide had no effect on OR67d odor responses. n = 9, Wilcoxon signed-rank test (p = 0.426).

(D) PN odor responses were assessed by expressing GCaMP6s in DA1 PNs.

(E-F) Methysergide boosted PN responses. n = 8, Wilcoxon signed-rank test (p = 0.008).

(G) Whole-cell recordings from DA1 PN with chrimson expression in R70A09 LNs. DA1 PNs show hyperpolarization when R70A09 LNs were stimulated. Each trace indicates different trials.

(H) TERPS analysis was used to determine if the connection is monosynaptic. Chrimson and NaChBac were expressed in R70A09 and TTX was applied to block all polysynaptic connections. LNs were activated with red light while responses were measured in DA1 PNs via whole-cell recording.

(I) Activating R70A09 LNs results in a PN hyperpolarization that is blocked by GABAergic pharmacology.

(J) Quantification of the previous experiments. n = 5, Wilcoxon signed-rank test (p = 0.031).

Figure 7. A model for paracrine and concentration dependent 5-HT signaling in the *Drosophila* AL.

(A) A schematic of the *Drosophila* brain showing the AL and serotonergic neurons in red. Only the CSDn directly innervates the AL and its activation boosts PN responses through unknown receptors. This is denoted with the + next to the CSDn. Low concentrations of 5-HT suppress PN output and persist even in the absence of the CSDn, suggesting they permeate into the AL via the extracellular space and haemolymph in the fly. Position of 5-HT based on previous reports [68,69].
(D) Within the glomerulus low levels of 5-HT suppress odor response by activating GABAergic LNs that target PNs postsynaptically. Methysergide blocks the 5-HT7R which should hyperpolarize these LNs and boosts DA1 PN odor responses.

**Supplementary Figure 1.** Methysergide antagonizes the *Drosophila* 5-HT7R.

(A) A control experiment in 5-HT is photo-uncaged using UV light while recording whole-cell from a DA1 PN in TTX saline. Uncaging 5-HT has no detectable effect on the PNs suggesting they do not express 5-HTRs. This result is consistent with immune staining in Figure 4.

(B) The 5-HT7R was expressed in DA1 PNs by Gal4/UAS resulting in their depolarization upon UV-photo uncaging of 5-HT. The depolarization was effectively blocked by methysergide.

(C) SB258719 also effectively blocked the effects of 5-HT at the 5-HT7R.

(D) Quantification of the results in B and C. We calculated mean membrane potentials from 1 and 10 sec after UV stimulus over 1.5 sec as peak and plateau responses respectively. From Left to right, n = 5, 7, 6, 6, 6, Wilcoxon signed-rank test (p = 0.071, p = 0.031, p = 0.218, p = 0.031).

**Supplementary Figure 2.** DA1 ORNs express 5-HT2BR.

(A) The 5208 MiMIC line expresses broadly in the brain and displays a characteristic crossing in the ORN commissure labeled with a white arrow head.

(B) ORNs where isolated in the MIMIC line but expressing flippase in the ORNS using the OR83b-Q promoter line and labeling neurons using a FRT>STOP>FRT-GFP transgene. GFP is only expressed ORNs where the stop cassette has been removed.

(C) A higher zoom image of ORNs in the AL.

(D) An nc82 stain showing glomeruli in the AL.

(E) DA1 is outlined showing expression of the 5-HT2BR.

(F-J) As A – E but for the a different 5-HT2BR promoter line, 6500.

**Supplementary Figure 3.** R70A09, NP3056, and GH298 promoter lines label 3 distinct populations of LNs with minimal overlap.
(A) GFP is expressed in the HACKed R70A09 Q system line and RFP is expressed in the Gal4 parent line. There is perfect overlap between the HACKed promoter line and the GAL4 line.

(B) The HACKed R70A09 line is used to test overlap with the broadly expressing LN line, NP3056. Little overlap is observed between these lines.

(C) The same strategy is used to reveal little overlap between R70A09 and the GH298-Gal4 LN line.

Supplementary Figure 4. 5-HT7R expressing LNs do not express other 5-HTRs

(A) (Left) The R70A09-Q promotor line expresses mCherry labeling LNs on the lateral aspect of the AL. (Middle) The MiMIC-5-HT1A-GAL4 promoter line is labeled with mcd8-GFP in the same preparation. (Right) A merge of the two previous panels showing no overlap in expression. White outlines demarcate R70A09 LNs.

(B) As in A above but showing the lack of overlap between R70A09 LNs and MiMIC 5-HT1B neurons.

(C) As in A above but showing the lack of overlap between R70A09 LNs and MiMIC 5-HT2A neurons.

(D) As in A above but showing the lack of overlap between R70A09 LNs and MiMIC 5-HT2B neurons.

References


Ito K, Suzuki K, Estes P, Ramaswami M, Yamamoto D, Strausfeld NJ. The organization of extrinsic neurons and their implications in the


Nai Q, Dong H-W, Hayar A, Linster C, Ennis M. Noradrenergic Regulation of GABAergic Inhibition of Main Olfactory Bulb Mitral Cells Varies as a


Figure 3

A  Odor (cVA)
Saline
WAY 100635
5-HT1AR
25 spikes/s

B  Saline
SB 216641
5-HT1BR

C  Saline
Ketanserin
5-HT2AR
25 spikes/s

D  Saline
Metaclopramide
5-HT2BR
40 spikes/s

E  Saline
SB 258719
5-HT7R
25 spikes/s

WAY 100635 + Methy
SB 216641 + Methy
Ketanserin + Methy
Metaclopramide + Methy
SB 258719 + Methy

N.S., *
Figure 6

(A) Schematic of ORNs (Odor Receptor Nerves) and PNs (Projecting Nerves) from Or67d-Gal4 > UAS-GCaMP6s, showing fluorescence changes in DA1 neurons with Saline and Methysergide.

(B) Time courses of ΔF/F (%), showing a comparison between Saline and Methysergide.

(C) Graph showing ΔF/F (ΔF/F ∫dt) for Saline and Methysergide.

(D) Similar to (A) for Mz19-Gal4 > UAS-GCaMP6s.

(E) ΔF/F (%) time courses for Saline and Methysergide.

(F) ∫ΔF/F dt graph for Saline and Methysergide.

(G) Recorded spike activity from DA1 PNs and LNs with Saline.

(H) Diagram showing ORNs, DA1 PNs, LNs, and the expression of R70A09-G4 > Chrismion + NaChBac.

(I) Recorded responses from LNs with Saline + TTX + Picrotoxin + CGP + Mecamylamin.

(J) ∆Mv graph showing saline and the combined effect of TTX, Picrotoxin, CGP, and Mecamylamin.
Table 1. Drosophila genotypes used in this study.

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<th>Figure</th>
<th>Genotypes (transgene with Bloomington number)</th>
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<td>1B-F</td>
<td>QF-Mz19 (41573), QUAS-mCD8-GFP (30002); +/-</td>
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<td>Gal4-R60F02 (48228)/+</td>
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<td>QF-Mz19, QUAS-mCD8-GFP/UAS-Dipth^ts; Gal4-trh</td>
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<td></td>
<td>(38389)/+</td>
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<td>3</td>
<td>QF-Mz19, QUAS-mCD8-GFP; +/-</td>
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<td>4A</td>
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<td>HT1A^t{MI01140}; 20xUAS-mCD8-GFP (32194)/+</td>
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<td>QF-Mz19, QUAS-mCherry/Mi{MIC}5-HT1A^t{MI01468};</td>
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<td>5-HT7 RNAi (32471)</td>
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<td>Figure 5D</td>
<td>+/+; 20×UAS-mCD8-GFP/Gal4-GMR70A09</td>
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<td>Figure 5E</td>
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<td>Figure 6A-C</td>
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<td>Figure 6D-F</td>
<td>Gal4-Mz19 (34407); 20×UAS-GCamp6s</td>
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<td>Figure 6G-J</td>
<td>QF-Mz19, QUAS-mCD8-GFP/UAS-chrimson (55135); Gal4-GMR70A09/UAS-NachBac (9466)</td>
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<td>Supplementary Figure 1A</td>
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<td>Supplementary Figure 2G-J</td>
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<td>Supplementary Figure 3A</td>
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Gal4-NP3056 was from Kyoto Stock Center #113080
MiMIC 5-HTR Gal4 lines were a gift from Herman Dierick
Supplementary Figure 2

Or83b-QF2$^{G4H}$, 10xUAS-FRT-stop-FRT-GFP; QUAS-FLP

A

5208-T2A-G4

50 μm

B

C

D

E

DA1

F

6500-T2A-G4

G

H

I

J

DA1
Supplementary Figure 3

A

R70A09-QF2-G4H > GFP

R70A09-Gal4 > RFP

Merge

100% overlap

B

R70A09-QF2-G4H > GFP

NP3056-Gal4 > RFP

Merge

C

R70A09-QF2-G4H > GFP

GH298-Gal4 > RFP

Merge

R70A09-QF2

NP3056-Gal4

R70A09-QF2

GH298-Gal4

G4H

n = 6

12.0 ± 1.1

40.2 ± 3.7

0.5 ± 0.8

G4H

n = 4

10.5 ± 1.9

1.0 ± 1.6