

# Serotonergic amplification of odor-evoked neural responses maps onto flexible behavioral outcomes

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Yelyzaveta Bessonova, Barani Raman 

Department of Biomedical Engineering, Washington University in St. Louis

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## Abstract

Behavioral responses to many odorants are not fixed but are flexible, varying based on organismal needs. How such variations arise and the role of various neuromodulators in achieving flexible neural-to-behavioral mapping is not fully understood. In this study, we examined how serotonin modulates the neural and behavioral responses to odorants in locusts (*Schistocerca americana*). Our results indicated that serotonin can increase or decrease appetitive behavior in an odor-specific manner. On the other hand, in the antennal lobe, serotonergic modulation enhanced odor-evoked response strength but left the temporal features or the combinatorial response profiles unperturbed. This result suggests that serotonin allows for sensitive and robust recognition of odorants. Nevertheless, the uniform neural response amplification appeared to be at odds with the observed stimulus-specific behavioral modulation. We show that a simple linear model with neural ensembles segregated based on behavioral relevance is sufficient to explain the serotonin-mediated flexible mapping between neural and behavioral responses.

### eLife assessment

This **useful** work shows that the experimental application of serotonin to locust antennal lobes induces an increased feeding-related response to some odorants (even in food-satiated animals). To explain how the odorant-specific effects are seen despite similar consequences of 5-HT modulation on all projection neuronal types, the authors propose a simple quantitative model built around projection with different downstream connections. While they are consistent with the authors' conclusions, the current panel of experiments is **incomplete** and additional future work will be required to fully support the conclusions the authors currently draw from their observations.

## Introduction

Often the same sensory stimulus can trigger different behavioral responses within a single organism. For example, appetitive odorants that are attractive while hungry may not drive the same behavioral response after feeding to satiety.<sup>1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46,47,48,49,50,51,52,53,54,55,56,57,58,59,60,61,62,63,64,65,66,67,68,69,70,71,72,73,74,75,76,77,78,79,80,81,82,83,84,85,86,87,88,89,90,91,92,93,94,95,96,97,98,99,100</sup> How neural circuits process sensory stimuli

to flexibly drive varying outcomes is not fully understood. It is hypothesized that different neuromodulators should be involved in mediating state-dependent changes in neural and behavioral responses.<sup>2,3</sup> However, whether neuromodulation globally changes responses to sensory cues (i.e. non-specific increases or decreases), or mediates selective alterations in sensory input-driven behavior, and how this is achieved, is not clear.<sup>4</sup>

In insects, serotonin (5HT) is a key neuromodulator that is linked with the regulation of many behaviors, including feeding<sup>5</sup>, socialization,<sup>6-8</sup> aggression,<sup>9,10</sup> and mating.<sup>11,12</sup> Food intake is known to be highly dependent on the serotonin levels in the brain of an organism. Elevated levels of serotonin have been shown to decrease the time spent feeding and the amount of consumed food in blowflies and flesh flies.<sup>13,14</sup> In *Drosophila* larvae and adults, inhibition of neural serotonin synthesis or global increase of serotonin levels, increases or suppresses food intake, respectively.<sup>15-17</sup> In locusts, serotonin is linked to triggering phenotypical plasticity. A spike in serotonin levels in the locust brain is highly correlated with the solitary stage,<sup>18</sup> whereas high levels of serotonin in the locust thoracic ganglion are known to trigger the transformation of solitary animals into gregarious ones.<sup>7,8</sup> Serotonin modulation has also been linked with increased activity and aggression in crickets and flies,<sup>10,19</sup> as well as a reduction of courtship behaviors, like male wing extension towards females and mating in flies.<sup>12</sup>

How serotonin alters sensory processing to drive these behavioral changes is yet to be completely resolved. In olfaction, serotonin is known to modify the processing of odor-driven neural signals right from the periphery. In both vertebrates and invertebrates, the serotonergic release is known to reduce olfactory sensory neuron output to the following circuit through pre-synaptic GABAergic inhibition.<sup>20-22</sup> In contrast, exogenous serotonin is known to increase the odor-evoked responses of second-order neurons in the insect antennal lobe and vertebrate olfactory bulb.<sup>20,23</sup> The serotonin-mediated increase in odor-evoked responses has been reported to be odor-specific and hypothesized to enhance the sensitivity to odorants in a state-dependent manner.<sup>24</sup>

How are odor-evoked neural responses modulated to produce flexible, odor-specific changes in behavioral outcomes? In this study, we examined this question in the locust olfactory system. We show that serotonin can increase or decrease innate appetitive behavioral responses in an odor-specific manner. In contrast, exogenous serotonin increased the strength of odor-evoked neural responses for all odorants without altering the temporal processing features or the ensemble response fidelity. We present a simple model to map the serotonin-mediated amplification of neural responses onto odor-specific changes in behavioral outcomes. Finally, we examine the relevance of these findings for modulating hunger-state-dependent modulation of appetitive responses in locusts. In sum, our results provide a more systems-level view of how a specific neuromodulator (serotonin) alters neural circuits to produce flexible behavioral outcomes.

## Results

### Serotonin modulates appetitive behavior in an odor-specific manner

We began by examining how serotonin modulates odor-driven innate behavioral responses in locusts (*S. americana*). In this assay, starved locusts opened their sensory appendages close to the mouth, called maxillary palps, when encountering certain food-related odorants.<sup>25-28</sup> We examined the palp-opening responses (POR) for an odor panel including four odorants: hexanol (HEX), benzaldehyde (BZA), linalool (LOOL), and ammonium (AMN). Note that hexanol (green) is a green-leaf volatile<sup>29</sup>, and benzaldehyde (blue) is a putative locust aggregation pheromone.<sup>30</sup>

Whereas linalool (red) is used in many pesticides.<sup>31</sup> Hence, this panel included odorants that have diverse behavioral preferences.<sup>28</sup> Each odorant was delivered at 1% v/v concentration and presented for ten trials or repetitions in a pseudorandomized order (see Methods).

We used a binary metric to categorize the presence or absence of PORs (**Figure 1A**). The response matrix across locusts (rows) and trials (columns) is summarized in **Figure 1B**. It is worth noting that HEX evoked supra-median(0.62) POR responses, while BZA and LOOL have a response close to the median(0.4) POR response level (mean POR = 0.43 across odorants and locusts). In contrast, AMN elicited sub-median(0.29) POR responses across locusts. We also examined the PORs before and after serotonin (5HT) injection for each locust (see Methods). Notably, we found that the probability of PORs (**Figure 1C**) changed after 5HT injection but only for a subset of odorants (HEX and BZA). Intriguingly, the PORs to LOOL decreased after 5HT injection. Injection of saline or merely repeating the same set of odorants after a three-hour time window (similar time frame as before and after 5HT injection) did not produce any significant change in the PORs (**Supplementary Figs. 1 and 2**; **Supplementary Figure 4** shows POR responses to paraffin oil before and after 5HT application). These results suggest that 5HT altered appetitive behavioral responses in an odor-specific manner.

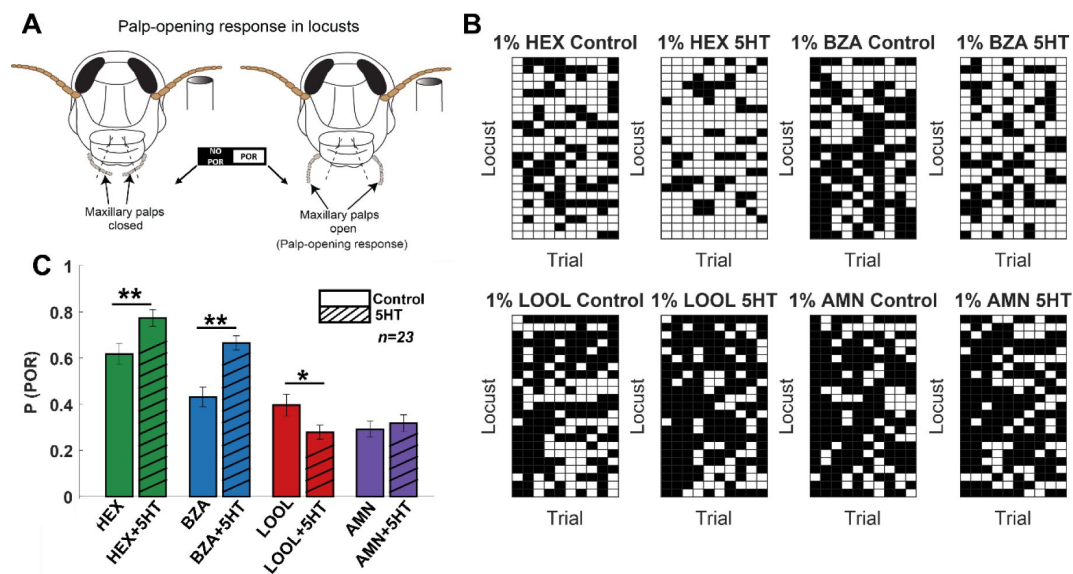
## Serotonin enhances olfactory arousal to odorants

Next, we wondered whether serotonin introduction altered the behavioral dose-response relationship. To examine this, we recorded PORs to the same four odorants at widely different concentrations (**Figure 2**; spanning four log units of concentration). First, even without serotonin application, it can be noted that PORs to the odorants tended to increase as a function of odor intensity. Further, the increase was more significant for HEX and BZA, two odorants that generally elicited more PORs. However, note that there was a detectable decrease in PORs at the highest concentration of HEX and BZA.

Injection of serotonin increased the probability of generating POR for all concentrations of HEX and BZA. In contrast, changes in PORs elicited by LOOL and AMN were only modestly modified after 5HT introduction. Hence, these findings imply that serotonin enhanced appetitive behavioral responses over a wide range of concentrations but only for a subset of odorants (HEX and BZA). Furthermore, the reductions in behavioral responses at the highest intensity of HEX and BZA were no longer detectable or significant after 5HT treatment. In sum, these results indicate that the overall arousal to appetitive odorants is enhanced after serotonin application, and increases in behavioral response are maintained over a wide range of concentrations.

## Serotonin alters the spontaneous activity of projection neurons in the antennal lobe

Next, we investigated the neural basis of the observed serotonergic modulation of behavioral outcomes. To assess this, we intracellularly monitored the spiking activity of projection neurons (PNs) in the antennal lobe. We first compared the spontaneous spiking activities of the same neuron before and after (5HT) serotonin treatment (**Figure 3A vs. 3B**). Note that the baseline spontaneous firing patterns in individual projection neurons changed after bath application of serotonin. The PNs fired spontaneous bursts of spikes after serotonin exposure (**Figure 3A** (before 5HT) vs. **3B** (after 5HT)). To quantify these differences, we computed the inter-spike interval (ISI) distributions across all PNs (**Figure 3 C-E**;  $n = 82$  PNs). The ISI distributions after 5HT application showed a left shift indicating that pairs of consecutive spikes occurred in quick succession (**Figure 3C**). Consistent with these results, the median values for the ISI distribution reduced significantly for each PN recorded (**Figure 3D**). Finally, we plotted the median ISI before 5HT (control; along the x-axis) against median ISI values after 5HT application (y-axis) for each PN (**Figure 3E**). As can be noted, each point represents a single PN. Clustering closer to the X-axis indicates that serotonin altered the excitability of PNs and induced bursts of spikes.



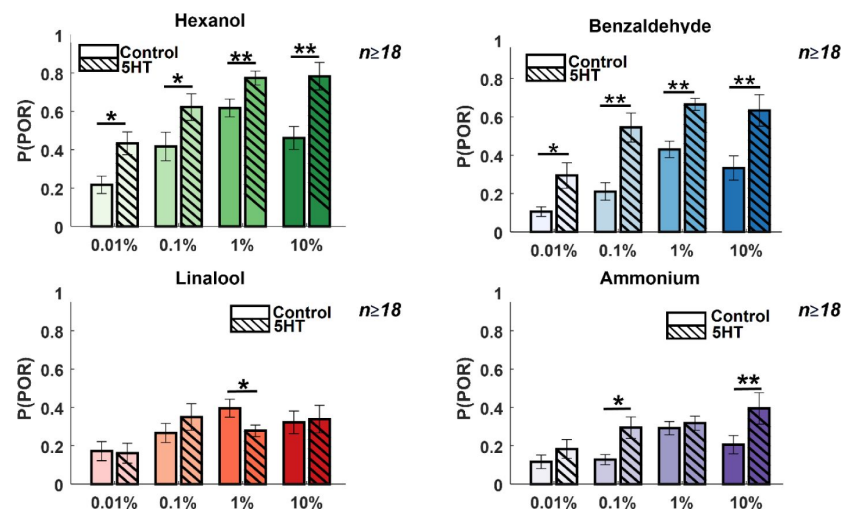
**Figure 1.**

### Serotonin modulates innate appetitive behavioral responses in an odor-specific manner.

**(A)** A schematic of the locust palp opening response (POR) is shown. Starved locusts (> 24 hours) were presented with a panel of four odorants (hexanol, (HEX); benzaldehyde, (BZA); linalool, (LOOL); ammonium, (AMN)) at 1% v/v dilution. Each locust was presented with ten trials of each odorant. The odor pulse duration was 4 seconds and the time between two consecutive odor exposures (inter-trial interval; ITI) was 56 seconds. Movement of the palps during the odor presentation was identified as a positive POR. The presence or absence of a POR was noted for each trial.

**(B)** A summary of trial-by-trial PORs for each locust is shown. Each trial was categorized by the presence (white box) or absence (black box) of a POR. Each row represents the PORs recorded from a single locust, and each column indicates a specific trial. PORs of twenty-three locusts were recorded and summarized as a response matrix. The POR response matrix for the same set of locusts before and after 5HT injection is shown to allow comparison.

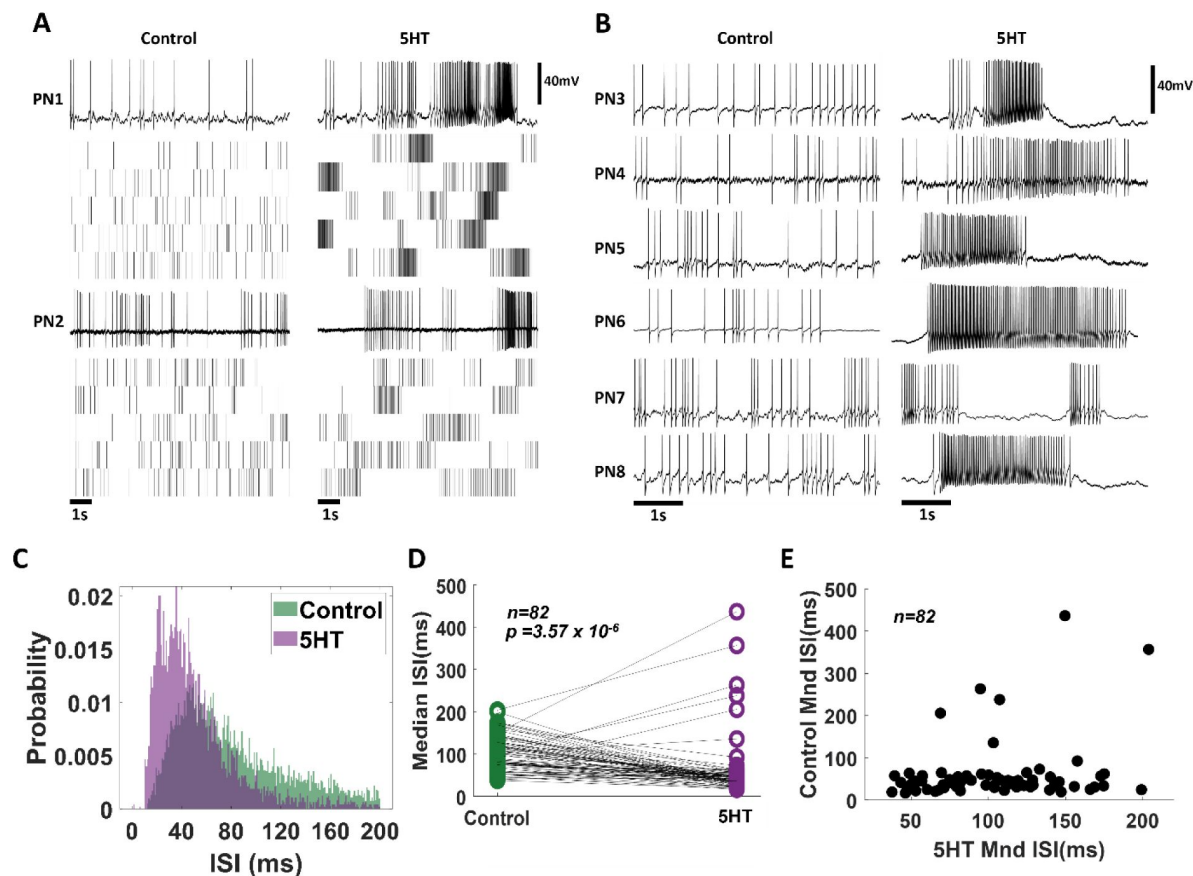
**(C)** The PORs before and after (5HT) serotonin injection are summarized and shown as a bar plot for all four odorants in the panel. Striped bars signify the data collected after the 5HT injection. Significant differences are identified in the plot (one-tailed paired-sample t-test; (\*p<0.05; \*\*p<0.01; standard paired sample t-test).



**Figure 2.**

**Serotonin alters the dose-response relationship for select odorants.**

**The left** solid bar shows the p(POR) for each odorant as a function of concentration before serotonin injection. **The right** striped bar summarizes p(POR) after serotonin injection for the same set of locusts (\* $p < 0.05$ ; \*\*  $p < 0.01$  standard one-tailed paired sample t-test).



**Figure 3.**

### Exogenous serotonin induces bursting behavior in antennal lobe projection neurons.

(A) Representative intracellular recordings showing membrane potential fluctuations as a function of time for two separate projection neurons (PNs) in the locust antennal lobe. A ten-second window when no odorant was presented is shown. Raw voltage traces are shown during the first trial, and spiking rasters are shown for the subsequent trials. Firing patterns before (left) and after (right) serotonin application are shown for comparison. Note, that the spiking activity becomes more bursty after the 5HT application.

(B) Firing patterns for a larger set of PNs are compared before (control) and after (5HT) serotonin. Changes in PN excitability are observed in all recorded PNs.

(C) The distribution of Inter-spike intervals (ISI) across PNs is shown before serotonin application (control; in green), and after serotonin application (purple). Note, that the purple histogram is taller and shifted to the left indicating shorter gaps between consecutive action potentials.

(D) Comparison of the median ISIs for each individual PN before (control) and after 5HT application. The black line connects median ISI values for a single PN in control and 5HT conditions. Note, the majority of the black lines are tilted downwards indicating a reduction in the gap between spikes.

(E) Median ISI values before and after the 5HT application are plotted for each PN.

In addition, we found that the total spiking activity in individual PNs monotonically increased with the magnitude of the current injection (**Supplementary Figure 3**). However, after serotonin injection we found that the spiking activity remained relatively stable and did not systematically vary with the magnitude of the current injection. While the changes in odor-evoked responses may incorporate both excitability changes in individual PNs and recurrent feedback inhibition through GABAergic LNs, these results from our current injection experiments unambiguously indicate that there are changes in excitability at the level of individual PNs.

## Serotonin modulates odor-evoked response intensity but not timing

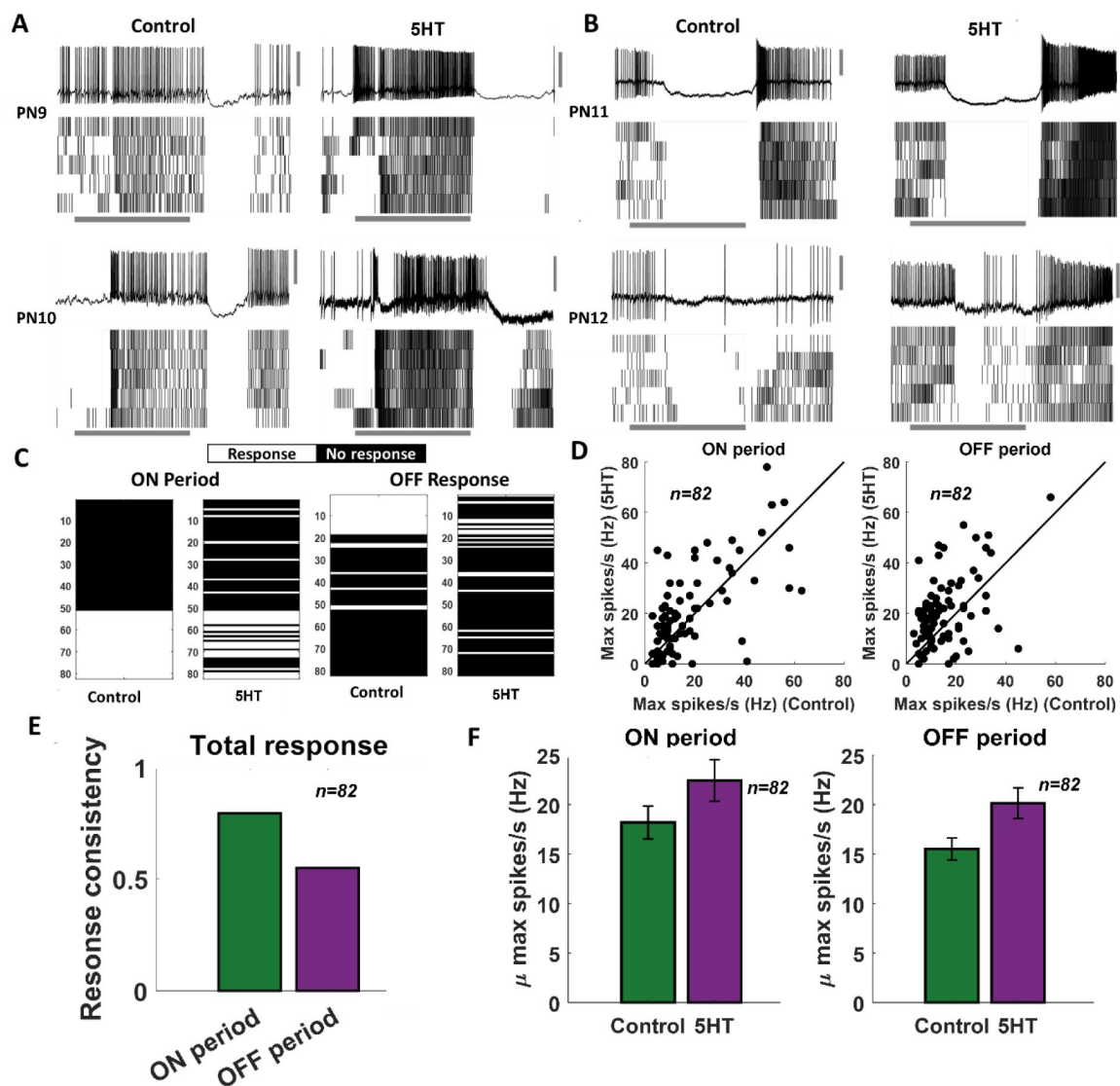
As noted, serotonin modulated the excitability of all individual PNs we recorded. How are the odor-evoked responses modulated, and does serotonergic modulation confound the information about odor identity? To examine this, we analyzed the odor-evoked responses of eighty-two PN-odor combinations. In addition to the four odorants examined in behavioral experiments, the odor panel used included three additional odorants (4-vinyl anisole, 1-nonanol, and octanoic acid). Consistent with previous observations, projection neurons responded to odorants by increasing their spiking activity either during odor presentation (ON response) or after stimulus termination (OFF response) (**Figure 4A**). These odor-evoked response patterns were consistent across trials.

We examined whether the odor-evoked response timing (ON vs OFF responses) was preserved after serotonin application (**Figure 4A**). As can be noted, the stimulus-evoked ON and OFF responses in these four representative PNs remained intact after 5HT application. We found that for a majority of the PNs in our dataset that exhibited either an ON or OFF response, the response timing was maintained after 5HT application (**Figure 4A-C**). Notably, ON responses tended to be more robustly maintained after the 5HT application compared to the OFF responses (**Figure 4C, F**). Furthermore, most of the non-responsive PNs continued to remain inactive during the odorant presentation after serotonin introduction.

What variations in spiking patterns were observed after the 5HT application? Our results indicate that the total number of spikes (i.e. response magnitude) evoked by an odorant increased after 5HT application during both ON and OFF time periods (**Figure 4D, F**). However, mere introduction of saline or the solvent in which serotonin was diluted (HCl) did not alter the PN responses (**Supplementary Figure 2**). In sum, these results indicate that serotonin modulates the excitability of individual PNs, but only those PNs that were activated by an odorant tended to increase their response magnitude. Further, the response timing (ON vs OFF periods) was robustly maintained across PNs.

## Robust encoding of odor identity

Is the identity of the odorant robustly encoded by the ensemble-level odor-evoked responses? To understand this, we first visualized the overall PN responses across all neuron-odor combinations in our dataset (**Figure 5A**). Consistent with the individual PN analyses, the changes in spontaneous activities and stability of spiking responses during the odor presentation period can be readily observed. To qualitatively analyze this, we visualized the neural activity using principal component analysis dimensionality reduction. The 82-dimensional PN spike counts in 50ms time-bins were projected onto the top two eigenvectors of the response covariance matrix and connected in the order of occurrence to generate closed-loop trajectories (**Figure 5B**). Neural response trajectories during (ON) and after (OFF) odor presentation periods activated different subsets of PNs and therefore did not have much overlap during these time periods. We also plotted the neural response trajectories before and after 5HT application to allow comparison. Our results indicate that the ON response trajectories before and after 5HT application overlapped with each other indicating that the ensemble activity across the eighty-two PNs was similar before and after serotonin application. Similar results were also observed during the OFF-response window.



**Figure 4.**

### Serotonin alters the magnitude of odor-evoked spiking activity but not its timing.

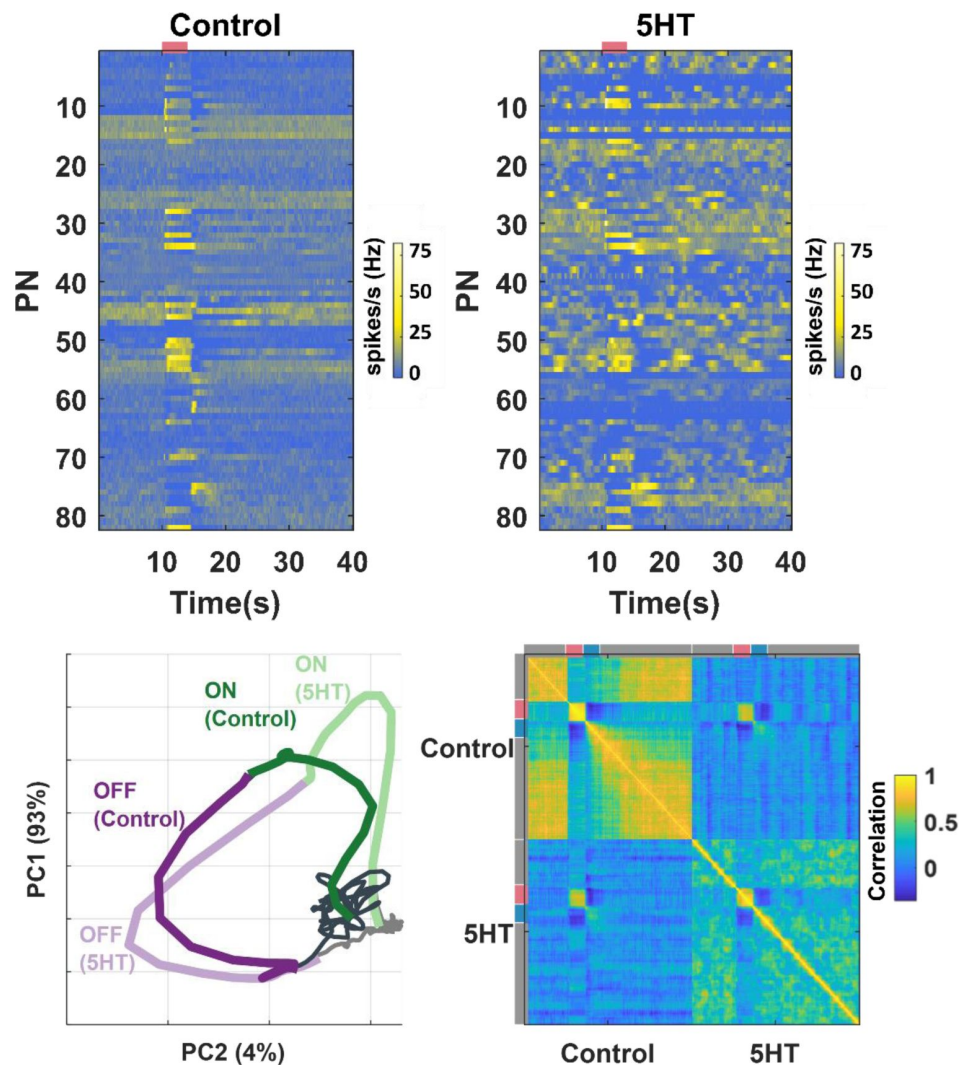
(A-B) Representative plots of the odor-evoked ON responses (PN9-10) and OFF responses (PN11-12) for five trials are shown. The first trial is shown as a raw voltage trace. Spiking activities during all five trials are shown as a raster plot. The horizontal grey bar indicates the four-second odor delivery period. The vertical grey scale bar identifies 40mV.

(C) A binary plot categorizing PN activity as responsive or non-responsive during odor presentation (ON responses) or after odor termination (OFF responses). Response categorization before and after 5HT application is shown for each PN to examine response robustness.

(D) Left panel: Peak spiking activity for each PN during odor presentation in the control condition and after 5HT application. Right panel: Comparing peak spiking activity observed during the OFF period.

(E) Fraction of PNs that maintain their response or lack of response to an odorant before and after 5HT application are quantified during ON and OFF periods.

(F) Left panel: Mean odor-evoked spiking activity across PNs during odor presentation in the control condition and after 5HT application is compared. Error bars indicate SEM. Right panel: Similar plot comparing mean spiking activity across cells during the OFF period.



**Figure 5.**

**Ensemble-level odor-evoked response patterns robustly maintain odor identity after 5HT treatment.**

(A) Trial-averaged spiking activity as a function of time is shown for eight-two neurons. The hotter color identifies the higher average firing rates per bin (200 ms). Each row represents one PN, each column represents one-time bin. The red bar identifies the odor presentation time period. The heatmap on the left shows the PN activity matrix before 5HT application, and the heatmap on the right shows the PN activity matrix (neurons sorted in the same order) after(5HT) exposure.

(B) PN odor-evoked responses (n=82 projection neuron-odor combinations) are visualized after dimensionality reduction using Principal Component Analysis (PCA). The neural responses were binned in 50 ms windows and projected onto the top two eigenvectors of the response covariance matrix and connected in the order of occurrence to generate the response trajectory shown. Neural response trajectories evoked during the OFF period are shown in purple, and ON response trajectories are shown in green. Darker colors indicate response trajectories before 5HT treatment, and lighter shades show neural trajectories after 5HT treatment.

(C) A correlation matrix summarizing the similarity between each 82-dimensional PN activity vector with all other response vectors is shown. Different time segments (spontaneous (grey), odor ON (red), and odor OFF (blue)) are indicated along the X and Y axes. The hotter color indicates a higher correlation.

To further support the qualitative dimensionality reduction analysis, we performed a quantitative correlation analysis using high-dimensional PN activity vectors (**Figure 5C**). As can be noted, the structure of spontaneous activity before and after serotonin changed, and as a result, the correlation between them became negative. Consistent with the results from the PCA analysis, we found that the odor-evoked responses before and after 5HT application were highly correlated.

Taken together, these results indicate that the identity of the odorant is robustly maintained by ensemble neural activity. Both ON and OFF responses continue to preserve the information they carry about the odorant.

## A simple linear model explains serotonergic modulation of neural-behavioral mapping

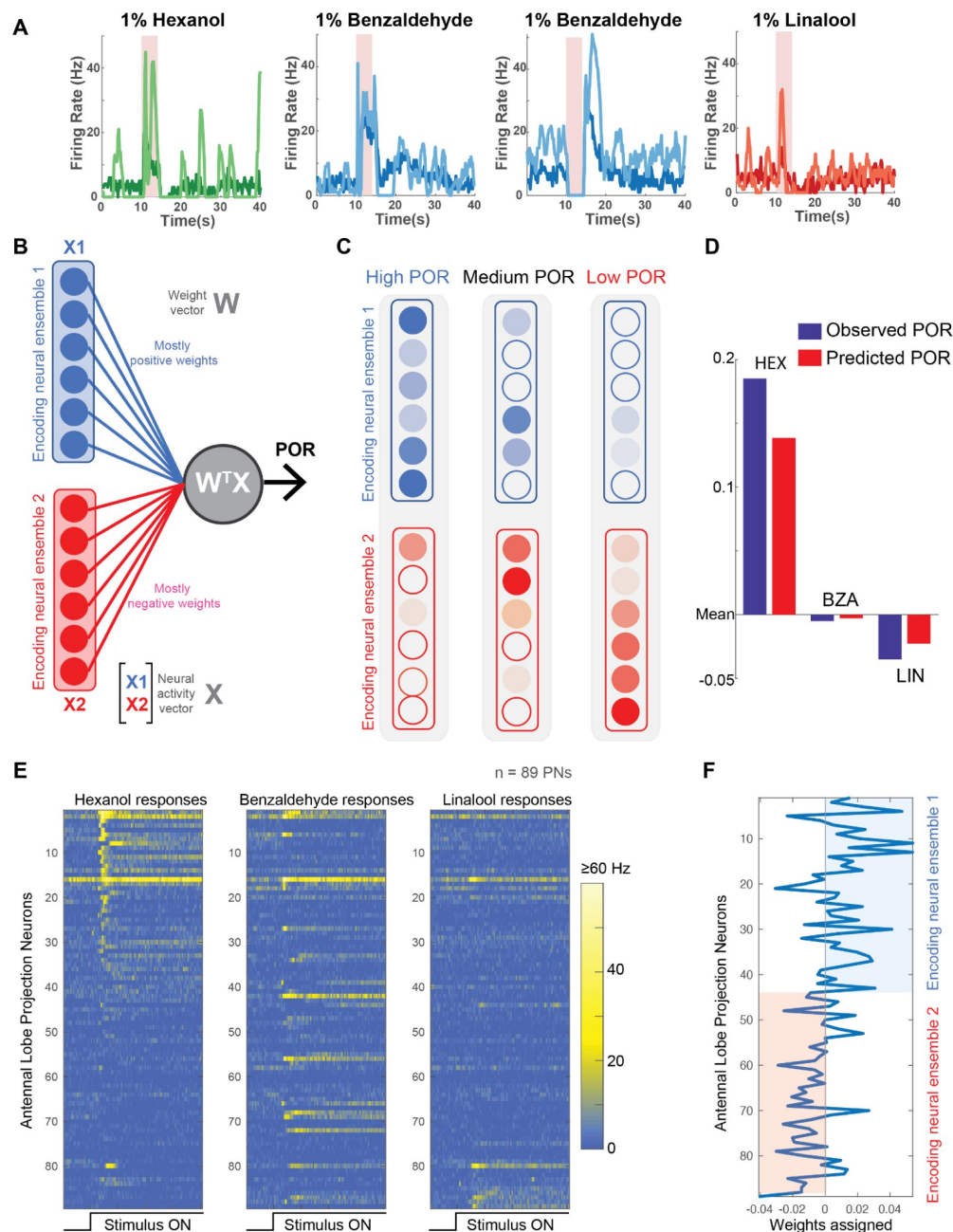
Our results indicate that serotonin modulated behavioral responses in an odor-specific manner. However, neural responses elicited by all odorants increased (**Figure 6A**). These results seem to be at odds with each other. To gain mechanistic insights regarding how 5HT uniformly amplifies neural responses while also generating odor-specific changes in behavioral outcomes, we performed a simple linear regression (**Figure 6B**). We used a previously published extracellular recording dataset<sup>28</sup> consisting of odor-evoked responses of 89 PNs to HEX, BZA, and LOOL to build the model. The output of the model was the amount of increase or decrease in PORs compared to the overall mean response levels across all odorants used in our behavioral experiments (see Methods). Note that this model is equivalent to assigning a weight to each projection neuron and using the weighted sum of projection neuron responses to generate the observed POR output.

We hypothesized that the antennal lobe projection neurons can be divided into two non-overlapping ensembles: Encoding Ensemble 1 is assigned mostly positive weights and Encoding Ensemble 2 are assigned negative weights (**Figure 6C**). Odorants that evoke supra-median PORs should activate more neurons in Encoding Ensemble 1, and those that produce sub-median-level PORs are expected to activate neurons in Encoding Ensemble 2 more. It is worth noting that this architecture is similar to having ‘neuron – anti-neuron’ pairs where one decoding neuron weighs the positive contribution to generate PORs, and the second decoding neuron collects the negative contributions and suppresses the same behavioral output. Such ‘neuron – anti-neuron’ pairs have been utilized for predicting overall motor outputs<sup>32–34</sup>, and are highly consistent with the emerging view from other insect models that have shown mushroom body output neurons form segregated channels to drive opposing behaviors<sup>35,36</sup>.

Our results indicate that this simple linear regression-based model was sufficient to robustly predict the observed PORs (**Figure 6D**). Furthermore, as expected, HEX and LOOL activated highly distinct neural ensembles. HEX-activated projection neurons received mostly positive weights and LOOL-activated neurons received negative weights (**Figure 6E**). Any increase in positively weighted PN responses (HEX response after 5HT) should increase the overall POR, whereas the increase in negatively weighted PN responses (LOOL after 5HT) should similarly decrease the behavioral output. Segregating odor encoding into behavior-specific channels in the antennal lobe would allow serotonin to amplify neural responses to all odorants, while still generating odor-specific increases or decreases in behavior.

## Hunger-state vs. serotonergic modulation of appetitive behavioral responses

Serotonin is regarded as one of the neuromodulators associated with feeding behaviors<sup>5</sup>. Therefore, we wondered whether serotonin modulates the behavioral appetitive responses of locusts in a hunger-state-dependent manner. To understand this, we first examined the PORs in locusts that were fed grass blades before the experiments (satiated). (**Figure 7**; **Supplementary**



**Figure 6**

### Neural data maps onto the behavioral results.

(A) PSTHs of four representative PNs are shown before (darker shade color) and after 5HT (lighter shade color) application. Note that 5HT increased overall response amplitudes to all odors in the panel.

(B) A schematic of the linear model used for predicting PORs given neural data. Each neuron is assigned a weight. The weighted sum of PN activity is fit to PORs values for HEX, BZA, and LOOL (see Methods). The neurons were split into two ensembles based on their assigned weights.

(C) Odorants that evoke stronger PORs are expected to activate more PNs that receive positive weights. In contrast, odorants that reduce POR output compared to the mean response are expected to activate more PNs that received negative weights.

(D) Comparison of observed versus predicted POR values across locusts for the three odors used in the model.

(E) Odor-evoked responses of 89 PNs to HEX, BZA, and LOOL are shown. The PNs are ordered based on the difference in peak responses to HEX and LOOL (i.e. HEX activated PNs are at the top and LOOL-activated neurons are at the bottom).

(F) Weights assigned to each PN are shown. PNs are ordered the same as in **panel E**.

**Figure 6** ) Compared to the hungry locusts (**Figure 1** ), the fed locusts responded less to HEX and BZA. However, consistent with results from the hungry locusts, the introduction of serotonin increased the appetitive POR responses to HEX and BZA. Intriguingly, the appetitive responses of fed locusts treated with 5HT were comparable or slightly higher than the responses of hungry locusts to the same set of odorants. It is worth noting that responses to LOOL and AMN, non-food related odorants with weaker PORs, remained unchanged in fed locusts treated with 5HT. Therefore, we conclude that, like in many species, serotonin influence food-driven behaviors in locusts. However, since 5HT increased behavioral responses in both fed and hungry locusts, the precise role of 5HT modulation and whether it underlies hunger-state dependent modulation of appetitive behavior still remains to be determined.

In sum, our results reveal a clear mapping between serotonergic modulation of odor-driven neural responses and how it regulates innate appetitive behavioral outcomes.

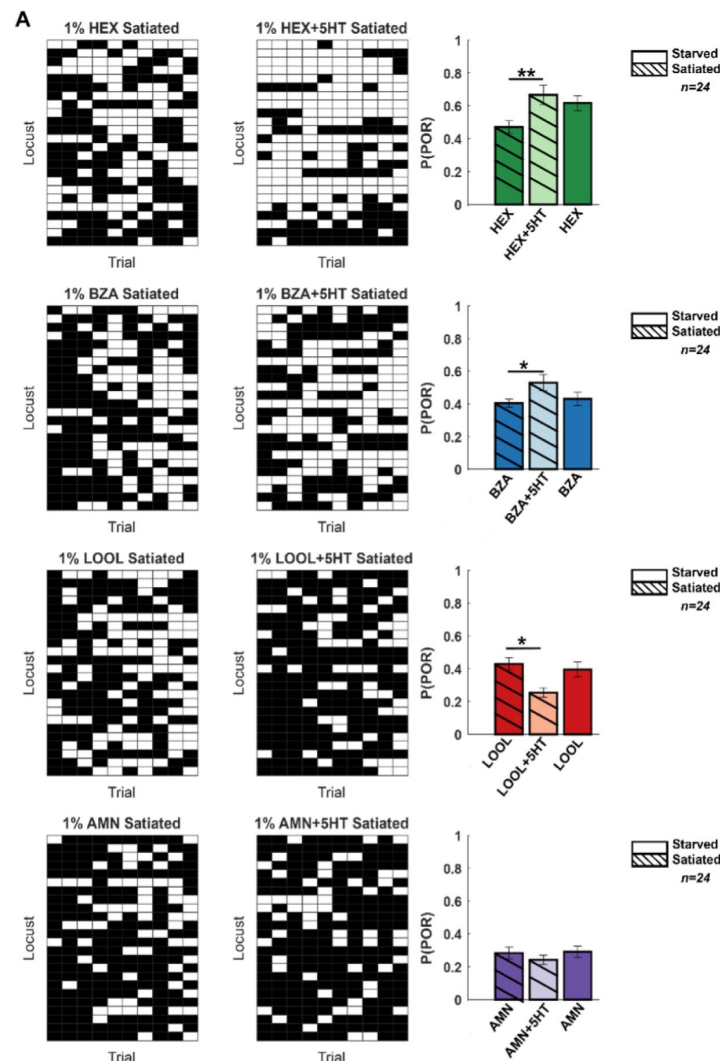
## Discussion

We examined how serotonin modulates odor-evoked neural and behavioral responses in locusts to a small but diverse panel of odorants. Our behavioral results revealed that serotonin increased innate appetitive responses to a subset of odorants (HEX and BZA). In contrast, responses to aversive or neutral odorants like LOOL<sup>31</sup> or AMN<sup>37</sup> decreased or had no significant change in their response levels. Consistent with the behavioral result, we found that the strength of the stimulus-driven responses increased in several PNs in the antennal lobe. However, the overall combination of neurons activated, and their temporal patterns of activation (ON vs. OFF responses), remained consistent. As a direct consequence, the identity of the odorant could be robustly maintained after exogenous serotonin introduction. Finally, fed locusts injected with serotonin generated similar appetitive responses to food-related odorants as starved locusts indicating the role of serotonin in hunger state-dependent modulation of odor-evoked responses.

Prior results from a number of invertebrate and vertebrate models have shown similar changes in odor-evoked neural responses in the antennal lobe<sup>20,24,38–40</sup>. The increase in the spiking activity of second-order neurons seems inconsistent with the serotonergic gating of sensory input through pre-synaptic inhibition<sup>21,22</sup>. Our results indicate that serotonin also modified the overall excitability of individual PNs and made them fire action potentials in bursts. Hence, it is possible that the increased neural sensitivity could compensate for the decreased input from sensory neurons. The change in input-output mapping as a result of serotonin introduction resulted in an increase in the behavioral response to different concentrations of HEX and BZA.

In contrast to the changes in the response strength, our results indicate that the timing of odor-evoked spiking activity was robustly maintained. As a result, the combination of neurons activated during and immediately after the presentation of the odorant remained similar before and after the introduction of serotonin. Hence, serotonergic modulation altered sensitivity to some odorants without altering the identity of the odorant. These results are consistent with prior imaging studies in flies that reported odor-specific changes in glomerular activity<sup>24</sup>. Further, this interpretation of our physiological results is consistent with the behavioral observation that serotonin altered response levels in an odor-specific manner.

However, behavioral responses could both increase or decrease depending on the odor identity. In contrast, our neural data indicated an enhancement for all odorants examined. This apparent mismatch between neural and behavioral responses could be resolved using a very simple linear regression model. In the model, a subset of neurons that were activated strongly by odorants with stronger PORs received positive weights. In contrast, the subset of neurons that responded to odorants that evoked fewer PORs received negative weights. Notably, the segregation of neural



**Figure 7.**

### Hunger-state dependent serotonergic modulation of appetitive behavioral responses.

**Left:** A summary of trial-by-trial PORS to the same four odorants used in [Figure 1](#). The same convention was used in [Figure 1](#) (POR – white; no POR – black). Each row represents PORS recorded across a single locust in ten trials. Twenty-four locusts were used. Each column represents a trial. Each odorant was presented at 1% v/v concentration. The POR matrix for the same set of locusts before and after 5HT injection is shown to allow comparison. **Right:** PORS are summarized and shown as a bar plot for all four odors for satiated locust (highlighted with lines), before (dark shade), and after 5HT injection (lighter shade). To allow comparison with POR in starved locust, results from [Figure 1](#) are re-plotted in solid bars without stripes. Significant differences are identified in the plot (one-tailed paired-sample t-test; (\* $p < 0.05$ ; \*\* $p < 0.01$ ; standard paired sample t-test).

subsets based on the behavioral relevance and the opposing weights assigned to them was sufficient to produce odor-specific increase or decrease in behavioral PORs as observed during serotonergic modulation.

The model used for mapping neural responses onto behavior only required that odorants that evoke or suppress PORs activate distinct sets of neurons. It is worth noting that such segregation of neural responses could happen at any neural circuit along the olfactory pathway. Our extracellular recording data (**Fig. 6E**) indicate that hexanol (high PORs) and linalool (low PORs) do indeed activate highly non-overlapping sets of PNs in the antennal lobe. Hence, our results suggest that the segregation of neural activity based on behavioral relevance begins very early along the olfactory pathway.

While serotonin is implicated in a range of behaviors, the modulation of feeding behavior is widely conserved across invertebrates and vertebrates. Hence, we finally explored whether this role is conserved in locusts as well. We found that the appetitive response evoked by a food-related odorant (HEX; green leaf volatile<sup>29</sup>) was reduced in animals that were fed. Notably, we found that serotonin application in locusts that were fed recovered back their appetitive response to HEX. Earlier studies have shown that serotonergic neurons could mediate a hunger-state dependent switch in behavioral response preference (attraction vs. repulsion) to the same food-related odorant in fruit fly larvae<sup>1</sup>. Our studies complement these findings and show how serotonin could modulate behavioral responses to different odorants in a stimulus-specific manner.

## Data and code availability

All data and the custom code used to generate figures in this paper are available in Figshare: [https://figshare.com/articles/dataset/Serotonergic\\_amplification\\_of\\_odor-evoked\\_neural\\_responses\\_maps\\_flexibly\\_onto\\_behavioral\\_outcomes/25365442](https://figshare.com/articles/dataset/Serotonergic_amplification_of_odor-evoked_neural_responses_maps_flexibly_onto_behavioral_outcomes/25365442)

## Acknowledgements

We thank members of the Raman Lab (Washington University in St. Louis) and members of the Behavioral Plasticity Research Institute for their feedback on the manuscript and earlier presentation. We thank Pearl Olsen for insect care. We thank Ryan Sumida for writing the code to truncate and grab the behavioral video files. We thank Jacob Kelley for validating our FigShare data and code. This research was supported by NSF Grant # 2021795 to B.R.

## Author contributions

YB and BR conceived the study and designed the experiments/analyses. YB performed all the behavioral and electrophysiology experiments and analyzed the data. BR developed the model. YB and BR wrote the paper. BR obtained the funds and supervised all aspects of the work.

## Competing Interest Statement

The authors declare no competing interests.

## Methods

### Animals

We used adult *Schistocerca americana* of both sexes from a crowded colony for our electrophysiology and behavioral experiments. Sixth-instar locusts were identified by the developed wings and soft cuticle in the neck area.

### Odor Stimulation

The odor stimulus was delivered using a standard procedure<sup>26,28,41–43</sup>. Briefly, all odorants were diluted to their 0.01-10% concentration by volume (v/v) in mineral oil. Ammonium alone was diluted in distilled water. 20 ml of diluted odor solution was kept in 60 ml sealed bottles. During stimulus presentations, a constant volume (0.1 L per minute) from the odor bottle headspace was displaced and injected into the carrier stream using a pneumatic pico pump (WPI Inc., PV-820). A vacuum funnel placed right behind the locust antenna ensured the removal of odor vapors.

### Odor Panel

The odorants were selected based on chemical structure and ecological relevance. Therefore, we chose a very diverse odor panel that consisted of a food odorant – hexanol<sup>29</sup>, a sexual maturation pheromone – 4-vinyl anisole<sup>44</sup>, and a putative aggregation pheromone – benzaldehyde<sup>30</sup>. In addition, we included an acid – octanoic acid, a base – ammonium, and an alcohol – 1-nonanol in our experiments. These odors were specifically selected based on our previous neural and behavioral data<sup>26,28,45</sup>. For some experiments, we chose to focus on four odorants that had a diverse range of palp opening response rates: supra-median (hexanol), median (benzaldehyde), and sub-median (linalool) POR response levels.

### Behavior experiments

**Sixth instar** locusts of either sex were starved for 24-48 hours before the experiment or taken straight from the colony and fed blades of grass for the satiated condition. Locusts were immobilized by placing them in the plastic tube and securing their body with black electric tape (**Supplementary Figure 7**). The head of the locusts along with the antenna and maxillary palps protruded out of this immobilization tube so they could be freely moved. Note that the maxillary palps are sensory organs close to the mouth parts that are used to grab food and help with the feeding process. Locusts were given 20 – 30 minutes to acclimatize after placement in the immobilization tube.

Each locust was presented with one concentration of four odorants (hexanol, benzaldehyde, linalool, and ammonium) in a pseudorandomized order. The odor pulse was 4 s in duration and the inter-pulse interval was set to 60 s. The experiments were recorded using a web camera (Microsoft). The camera was fully automated with the custom MATLAB script to start recording 2 seconds before the odor pulse and end recording at odor termination. An LED was used to track the stimulus onset/offset. The POR responses were scored offline. Responses to each odorant were scored a 0 or 1 depending on whether the palps remain closed or opened (**Figure 1A**). A positive POR was defined as a movement of the maxillary palps during the odor presentation time window as shown on the locust schematic (**Figure 1A**).

## Serotonin treatment

After the initial set of POR experiments, a 0.1 M serotonin solution was injected directly into the locust's head. The needle of a U-100 syringe was inserted slightly under the cuticle of the locust head, ~ 1mm above the median ocellus. 1ul of the solution was injected and the opening was sealed with a small amount of melted dental wax. The locust was left to stabilize for 3 hours after the injection and before the second set of behavioral experiments.

We did electrophysiology experiments 5-10 minutes after bath application of 5HT. A longer delay (3 hrs) was required for our behavioral experiments as the locusts tended to be a bit more agitated with larger spontaneous movements of palps as well as exhibited unprompted vomiting.

## Electrophysiological Experiments Surgery

Sixth instar locusts of either sex were used for these experiments. The legs and wings were removed and the locust was immobilized on a platform. The head was fixed with wax and a cup was built around the head to hold saline solution. The locust antennae were held in place using clear tubing and allowed to pass through the wax cup. The cuticle between the antenna was removed and the air sacs/trachea was removed to expose the brain. Additionally, the gut was removed and a metal wire platform was placed underneath the brain to lift and stabilize it. Finally, the transparent sheath was removed by carefully using sharp forceps. Locust's brains were super-fused with artificial saline buffer. A visual demonstration of this entire protocol is available online<sup>43</sup> [\[link\]](#).

## Electrophysiology

Intracellular recordings were performed using glass electrodes (resistance 8-15 MΩ) filled with intracellular saline (130mM L-aspartic acid potassium salt, 2mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 10mM HEPES, 10mM EGTA, 2mM Na<sub>2</sub>ATP, 3mM D-glucose, 0.1mM cAMP; osmolarity ~315mmol/kg; pH 7.0). Glass electrodes were pulled using micropipette puller (Sutter Instrument, Novato, CA). Spontaneous firing, as well as real-time odor-evoked responses, were recorded in the current clamp configuration. Each set of experiments consisted of 5 consecutive 40-second trials, with 20-second intervals in between for each odorant/odor concentration. Odor stimulation was performed at the 10<sup>th</sup> second of the trial for 4 seconds. Voltage signals were amplified (Axoclamp-2B, Molecular Devices) and saved using a custom MATLAB script.

After monitoring the responses to the odor panel, a serotonin solution was applied directly into the bath using a thin nozzle pipette. The serotonin solution (0.01M serotonin hydrochloride in locust saline buffer) was made fresh before every experiment due to its light sensitivity. The same set of recordings and odor panel was repeated 5-10 minutes following serotonin application.

## Analysis

### Probability of POR calculation

POR responses were scored in a binary fashion. POR responses of locusts were summed across ten trials and across all locusts. The probability for each odorant was calculated as follows:

$$p(POR) = \frac{Total\ Score_{odor}}{Total\ \#\ of\ locusts \times 10} \quad (1)$$

Significant differences between the POR responses observed before and after serotonin application were calculated using a single-tailed paired-sample *t*-test ('*ttest*' built-in function in MATLAB).

## Electrophysiology data

In total, electrophysiological data from 82 odor-neuron combinations was obtained intracellularly and used for analyses. Each recording was preprocessed and converted into a response matrix. MATLAB built-in function “findpeaks” was used for identifying action potentials. In total, our dataset includes recordings from 19 PNs. Seven PNs were tested on a panel of seven odorants (4-vinyl anisole, 1-nonanol, octanoic acid, Hex, Bza, Lool, and Amn) and the remaining twelve were tested with the four main odorants used in the study (Hex, Bza, Lool, and Amn). Note that in the locust antennal lobe only PNs fire full-blown sodium spikes. GABAergic local neurons only fire calcium spikelets and can be easily distinguished from PNs (**Supplementary Figure 5** [↗](#)).

## PN response classification

We defined 4 s of odor presentation as an ON period, and the 4 s immediately following odor termination as an OFF period. For each PN, we used the mean baseline response during the pre-stimulus time period + 6.5 standard deviations of baseline activity as the threshold that needs to be exceeded to be classified as a response.

## Dimensionality reduction analysis

We used Principal Component Analysis (PCA) to visualize ensemble PN activity. The spiking activity for each PN during 4 s of odor presentation was averaged across all 5 trials and binned in 200ms non-overlapping time bins. In this manner, we obtained a 164 PN x 200 time-bin matrix for all odorants. The first 82 rows included PN responses before serotonin introduction and the other 82 rows included the responses of the same set of neurons after exogenous serotonin application. The response covariance matrix was calculated, and the data was projected onto the top two eigenvectors corresponding to the largest eigenvalues.

## Correlation analysis

Similar to the PCA analysis, the data matrix used for this analysis was 164 PN x 200 time bins (40 s; 200 ms time bins). This included the 82-dimensional PN spike count vectors before, during, and after odor presentations. Spike counts were averaged across five trials.

The correlation analysis was done time-bin-by-time-bin. Each pixel or matrix element in time-bin-by-time-bin correlation plots (**Figure 5C** [↗](#)) indicates the correlation value between neural activity vectors observed in the  $i^{\text{th}}$  and  $j^{\text{th}}$  time bins. All time-bin-by-time-bin correlation analyses were computed using high-dimensional response vectors. Correlations were calculated as:

$$C_{ij} = \frac{\text{cov}(X_i, X_j)}{\sigma_i \cdot \sigma_j} \quad (2)$$

Here,  $i$  and  $j$  represent time bins,  $X_i$ ,  $X_j$  represents the population activity vector in the  $i^{\text{th}}$  and  $j^{\text{th}}$  time bin respectively,  $\sigma_i$ ,  $\sigma_j$  are standard deviations of spiking activities during the  $i^{\text{th}}$  and  $j^{\text{th}}$  time bins respectively.

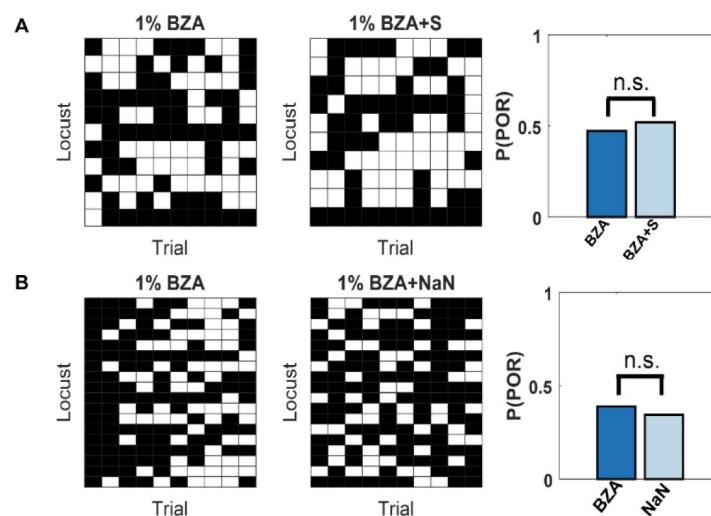
## Linear Regression Model

We used a recently published dataset<sup>28</sup> [↗](#) of extracellular PN responses to HEX, BZA, and LOOL to build our linear regression model. The input to the model was the spiking activity across the eighty-nine neurons (89 PNs x 40 s; 89 x 800 response matrix for each odorant; each time bin was 50 ms in duration). The response matrix for the three odorants was concatenated ( $\mathbf{X}$ ; 89 x 2400). The PN weights were determined as follows:

$$\mathbf{W} = (\mathbf{X}\mathbf{X}^T)^{-1}\mathbf{X}\mathbf{Y} \quad (3)$$

$\mathbf{X}$  denotes the concatenated matrix of neural activity. Each column of  $\mathbf{X}$  represents trial-averaged firing activity across 89 PNs in a 50 ms time bin. Neural responses before, during, and after the termination of all three odorants (hex, bza, and lool) were included.  $\mathbf{Y}$  is a row vector with values set to mean subtracted p(POR) only during those time bins when HEX, BZA, and LOOL were presented (zeros otherwise).

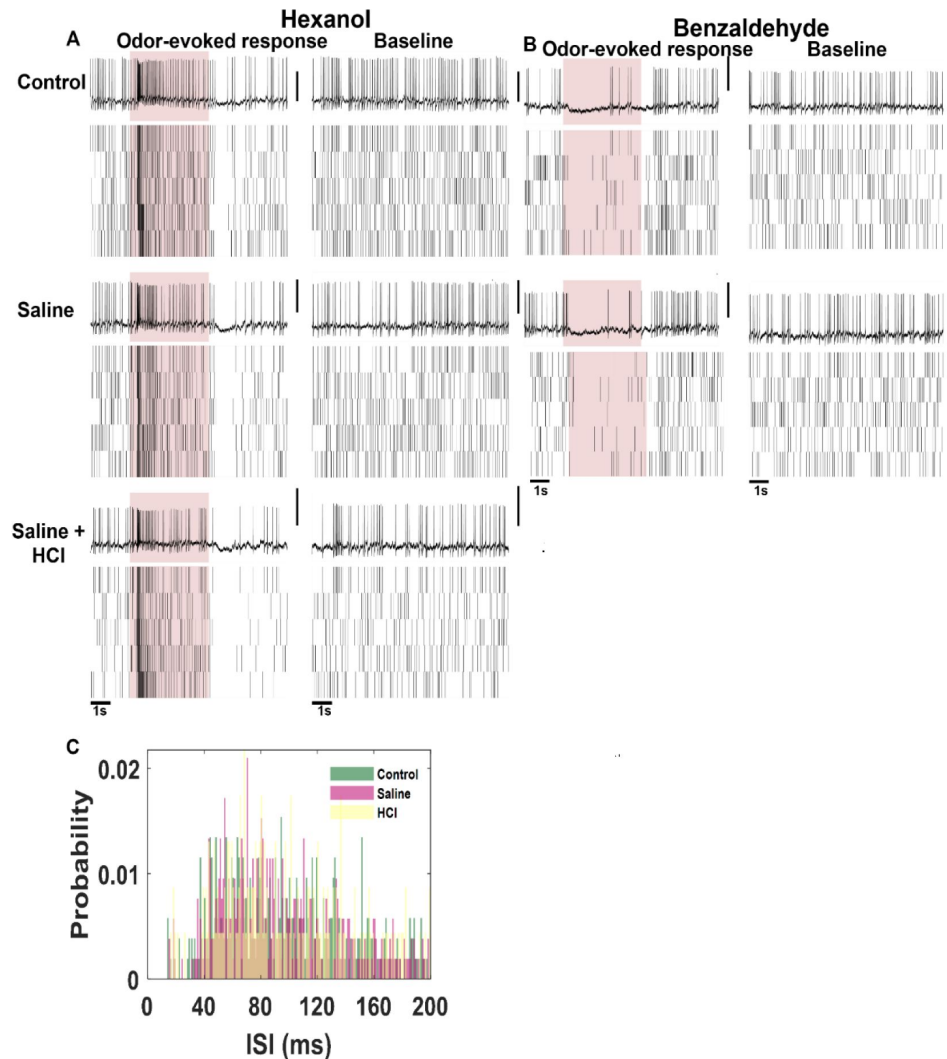
## Supplementary figures



**Supplementary Figure 1.**

### Saline injection control experiments.

Benzaldehyde at 1% concentration by volume (v/v) was used in these experiments. On the left, the raw POR response matrix is shown. Same color convention as [Figure 1](#). Each row represents PORs recorded from a single locust ( $n=11$  for saline injection (top panel) and  $n = 18$  locusts when the injection was absent (bottom panel)) over 10 consecutive trials (columns). The probability of POR is shown as a bar plot on the right side. The results before and after the injection of locust saline are presented in **panel A**. **Panel B** shows the results from a second control experiment. We compared POR responses to BZA before and after 3-hour wait (without any injection). This time period is comparable to the time lag between before and after 5HT injections. Significance in POR differences tested using standard paired-sample t-test. (\*\*indicates  $p<0.05$ , n.s. – not significant)

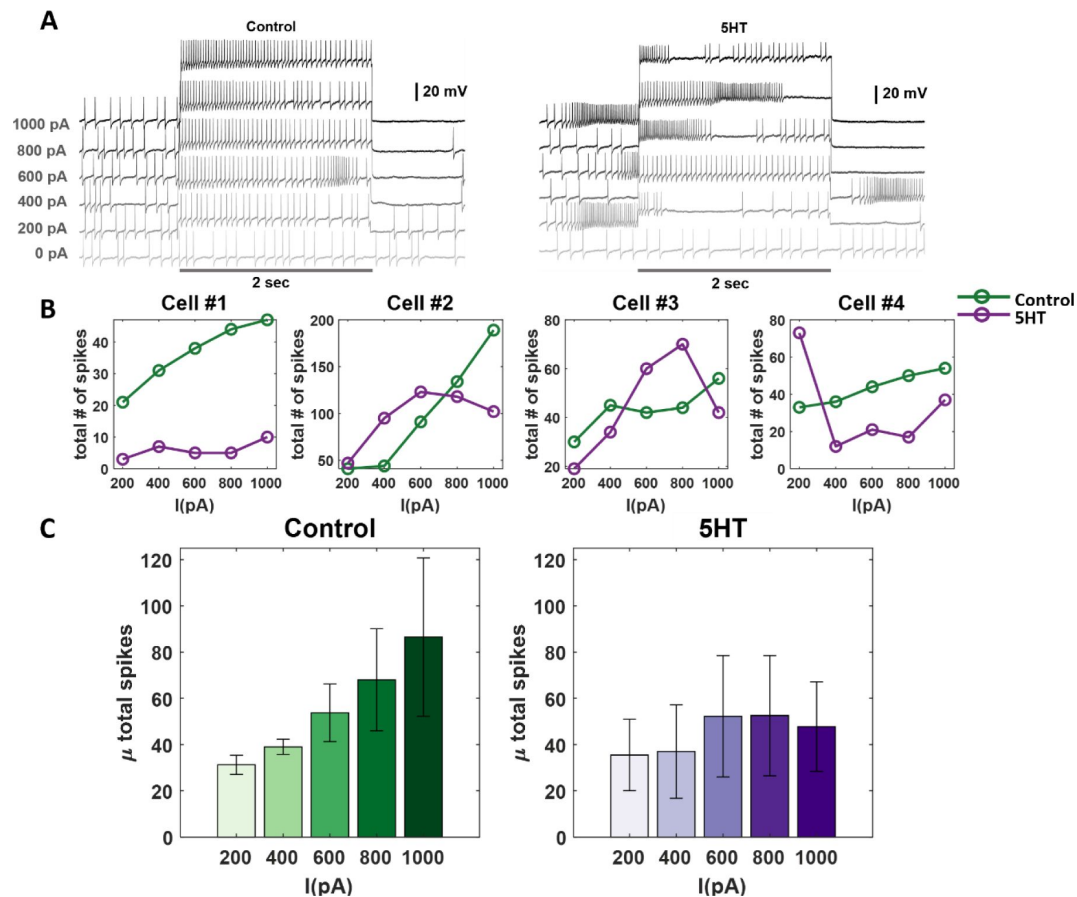


**Supplementary Figure 2.**

### Electrophysiology control experiments.

(A, B) Representative voltage signals and rasters showing spontaneous and odor-evoked responses before and after saline injection. Saline with the addition of HCl (Saline+HCl) was used as an additional control because HCl is the solvent used to dilute serotonin.

(C) Interspike interval (ISI) distribution is shown for the unmanipulated and the two control cases shown in panels A, and B.



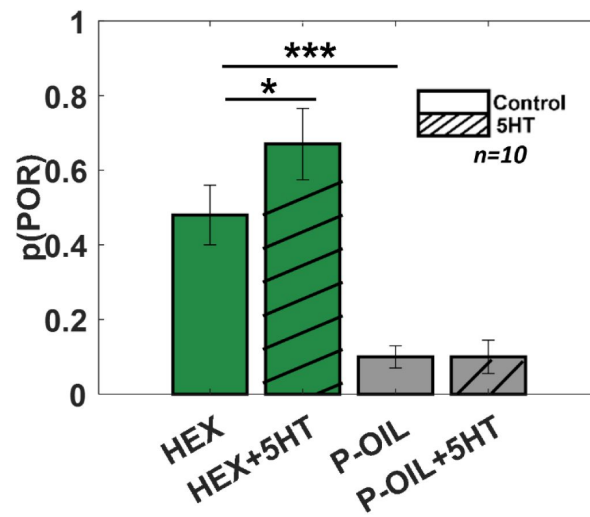
**Supplementary Figure 3.**

### Current-injection induced spiking activity in individual PNs is altered after serotonin application.

**(A)** Representative intracellular recordings showing membrane potential fluctuations as a function of time for one projection neuron (PNs) in the locust antennal lobe. A two-second window when a positive current pulse (200-1000pA range; 2 s duration) was applied is shown. Firing patterns before (left) and after (right) serotonin application are shown for comparison. Note, the spiking activity induced by current injection changed after the 5HT application. The black bar represents the 20mV scale.

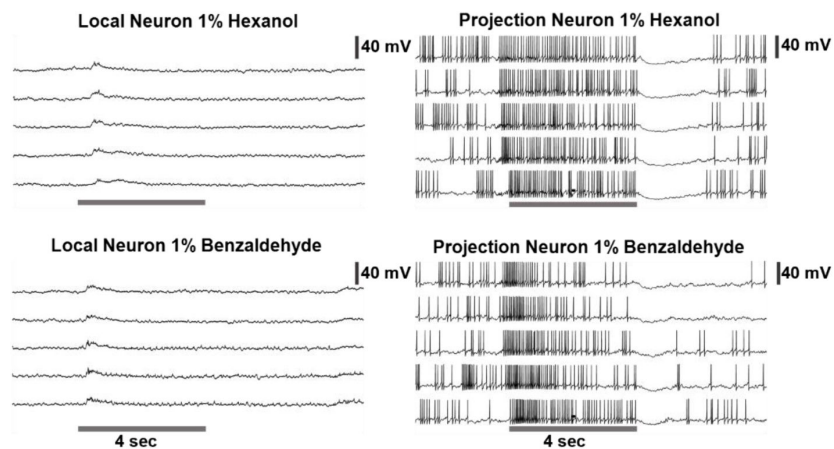
**(B)** Dose-response curves showing the total number of action potentials for each recorded PN during the 2-second current pulse before (green) and after (purple) serotonin application. Note that the current intensity was systematically increased from 200 pA to 1000 pA.

**(C)** The mean number of spikes across the four recorded cells during current injection is shown. The color progression represents the intensity of applied current ranging 200pA (leftmost bar) to 1000pA (rightmost bar). The dose-response trends before (green) and after (purple) 5HT application are shown for comparison. The error bars represent SEM across the four cells.



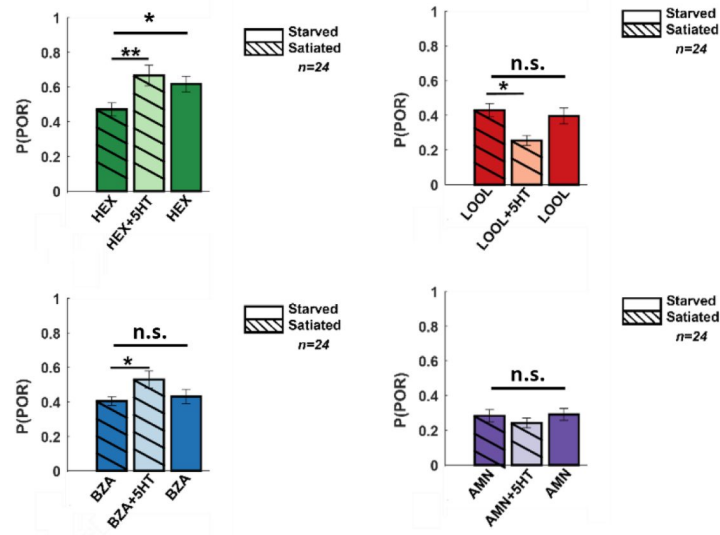
**Supplementary Figure 4.**

Serotonin does not alter the palp-opening responses evoked by paraffin oil (i.e. the solvent used to dilute odorants). The PORs before and after (5HT) serotonin injection are summarized and shown as a bar plot for hexanol and paraffin oil. Striped bars signify the data collected after 5HT injection. Significant differences are identified in the plot (one-tailed paired-sample t-test; (\* $p < 0.05$ ).



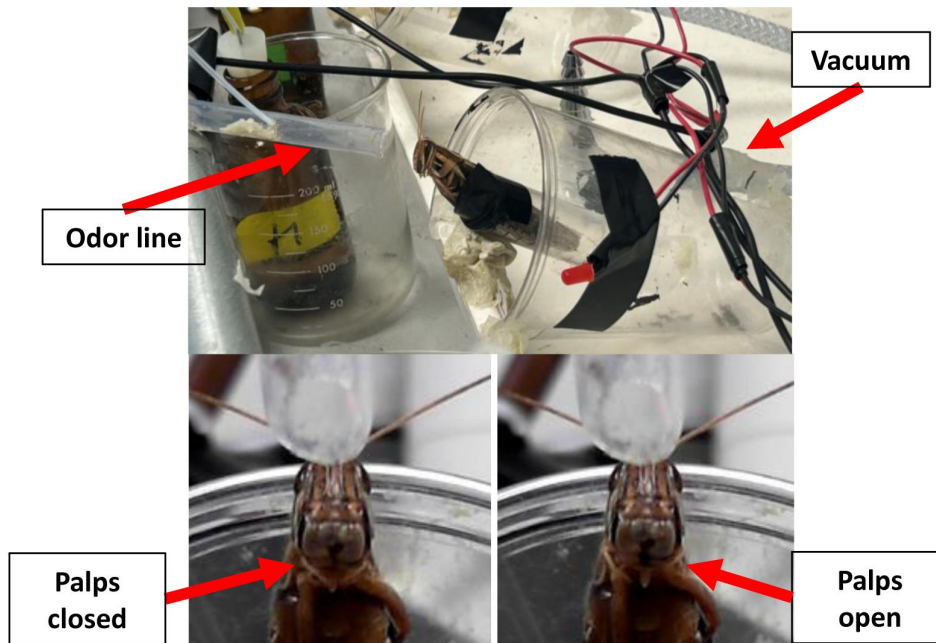
**Supplementary Figure 5.**

**Left:** Representative raw voltage traces recorded from a local neuron before, during and after a 4-second odor pulse are shown. Note that the local neurons in the locust antennal lobe do not fire full-blown sodium spikes but only fire small calcium spikelets. **On the right:** Representative raw voltage trace recorded from a representative projection neuron is shown for comparison. Clear sodium spikes are clearly visible during spontaneous and odor-evoked periods. The gray bar represents 4 seconds of odor pulse. The vertical black bar represents the 40mV.



**Supplementary Figure 6.**

Summarized PORs and shown as a bar plot for all four odors for satiated locust (highlighted with lines), before (dark shade), and after 5HT injection (lighter shade). To allow comparison before 5HT injection for starved locust plotted as well (dark shade plain).



**Supplementary Figure 7.**

Pictures showing the behavior experiment setup and representative palp-opening responses in a locust.

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## Article and author information

### Yelyzaveta Bessonova

Department of Biomedical Engineering, Washington University in St. Louis

### Barani Raman

Department of Biomedical Engineering, Washington University in St. Louis

**For correspondence:** [barani@wustl.edu](mailto:barani@wustl.edu)

ORCID iD: [0000-0002-7866-155X](https://orcid.org/0000-0002-7866-155X)

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## Editors

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## Reviewer #1 (Public Review):

Summary:

This manuscript explores the impact of serotonin on olfactory coding in the antennal lobe of locusts and odor-evoked behavior. The authors use serotonin injections paired with an odor-evoked palp-opening response assay and bath application of serotonin with intracellular recordings of odor-evoked responses from projection neurons (PNs).

Strengths:

The authors make several interesting observations, including that serotonin enhances behavioral responses to appetitive odors in starved and fed animals, induces spontaneous bursting in PNs, directly impacts PN excitability, and uniformly enhances PN responses to odors.

Weakness:

The one remaining issue to be resolved is the theoretical discrepancy between the physiology and the behavior. The authors provide a computational model that could explain this discrepancy and provide the caveat that while the physiological data was collected from the antennal lobe, but there could be other olfactory processing stages involved. Indeed other processing stages could be the sites for the computational functions proposed by the model. There is an additional caveat which is that the physiological data were collected 5-10 minutes after serotonin application whereas the behavioral data were collected 3 hours after serotonin application. It is difficult to link physiological processes induced 5 minutes into serotonin application to behavioral consequences 3 hours subsequent to serotonin application. The discrepancy between physiology and behavior could easily reflect the timing of action of serotonin (i.e. differences between immediate and longer-term impact).

Overall, the study demonstrates the impact of serotonin on odor-evoked responses of PNs and odor guided behavior in locust. Serotonin appears to have non-linear effects including changing the firing patterns of PNs from monotonic to bursting and altering behavioral responses in an odor-specific manner, rather than uniformly across all stimuli presented.

<https://doi.org/10.7554/eLife.91890.2.sa1>

**Reviewer #2 (Public Review):****Summary:**

The authors investigate the influence of serotonin on feeding behavior and electrophysiological responses in the antennal lobe of locusts. They find that serotonin injection changes behavior in an odor-specific way. In physiology experiments, they can show that projection neurons in the antennal lobe generally increase their baseline firing and odor responses upon serotonin injection. Using a modeling approach the authors propose a framework on how a general increase in antennal lobe output can lead to odor-specific changes in behavior.

**Strengths:**

This study shows that serotonin affects feeding behavior and odor processing in the antennal lobe of locusts, as serotonin injection increases activity levels of projection neurons. This study provides another piece of evidence that serotonin is a general neuromodulator within the early olfactory processing system across insects and even phyla.

**Weaknesses:**

I still have several concerns regarding the generalizability of the model and interpretation of results. The authors cannot provide evidence that serotonin modulation of projection neurons impacts behavior.

The authors show that odor identity is maintained after 5-HT injection, however, the authors do not show if PN responses to different odors were differently affected after serotonin exposure.

Regarding the model, the authors show that the model works for odors with non-overlapping PN activation. However, only one appetitive, one neutral, and one aversive odor has been tested and modeled here. Can the fixed-weight model also hold for other appetitive and aversive odors that might share more overlap between active PNs? How could the model generate BZA attraction in 5-HT exposed animals (as seen in behavior data in Figure 1) if the same PNs just get activated more?

The authors should still not exclude the possibility that serotonin injections could affect behavior via modulation of other cell types than projection neurons. This should still be discussed, serotonin might rather shut down baseline activation of local inhibitory neurons - and thus lead to the interesting bursting phenotypes, which can also be seen in the baseline response, due to local PN-to-LN feedback.

The authors did not fully tone down their claims regarding causality between serotonin and starved state behavioral responses.

There is no proof that serotonin injection mimics starved behavioral responses.

<https://doi.org/10.7554/eLife.91890.2.sa0>

**Author response:**

The following is the authors' response to the original reviews.

**Reviewer #1 (Public Review):****Summary:**

*This manuscript explores the impact of serotonin on olfactory coding in the antennal lobe of locusts and odor-evoked behavior. The authors use serotonin injections paired with an odor-evoked palp-opening response assay and bath application of serotonin with intracellular recordings of odor-evoked responses from projection neurons (PNs).*

*Strengths:*

*The authors make several interesting observations, including that serotonin enhances behavioral responses to appetitive odors in starved and fed animals, induces spontaneous bursting in PNs, and uniformly enhances PN responses to odors. Overall, I had no technical concerns. Weaknesses:*

*While there are several interesting observations, the conclusions that serotonin enhanced sensitivity specifically and that serotonin had feeding-state-specific effects, were not supported by the evidence provided. Furthermore, there were other instances in which much more clarification was needed for me to follow the assumptions being made and inadequate statistical testing was reported.*

*Major concerns.*

- To enhance olfactory sensitivity, the expected results would be that serotonin causes locusts to perceive each odor as being at a relatively higher concentration. The authors recapitulate a classic olfactory behavioral phenomenon where higher odor concentrations evoke weaker responses which is indicative of the odors becoming aversive. If serotonin enhanced the sensitivity to odors, then the dose-response curve should have shifted to the left, resulting in a more pronounced aversion to high odor concentrations. However, the authors show an increase in response magnitude across all odor concentrations. I don't think the authors can claim that serotonin enhances the behavioral sensitivity to odors because the locusts no longer show concentration-dependent aversion. Instead, I think the authors can claim that serotonin induces increased olfactory arousal.*

The reviewer makes a valid point. Bath application of serotonin increased POR behavioral responses across all odor concentrations, and concentration-dependent aversion was also not observed. Furthermore, the monotonic relationship between projection neuron responses and the intensity of current injection is altered when serotonin is exogenously introduced (see Author response image 1; see below for more explanation). Hence, our data suggests that serotonin alters the dose-response relationship between neural/behavioral responses and odor intensity. As recommended, we have followed what the reviewer has suggested and revised our claim to serotonin inducing increase in olfactory arousal. The new physiology data has been added as Supplementary Figure 3 to the revised manuscript.

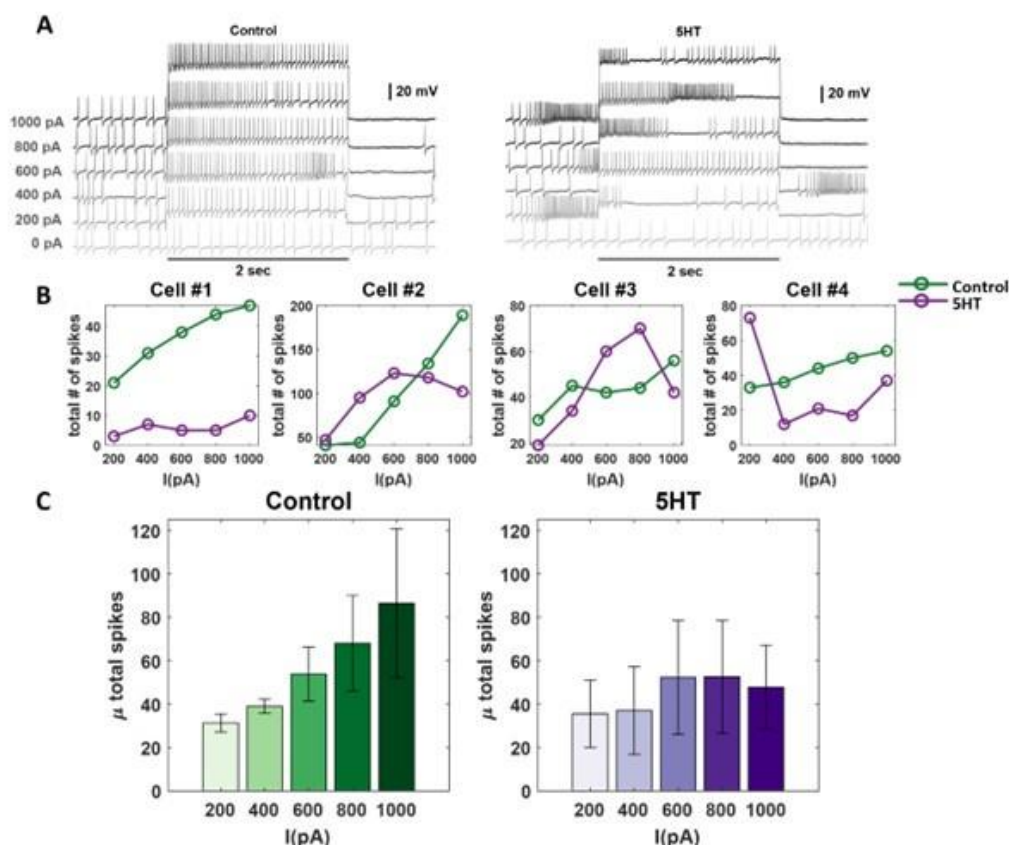
- The authors report that 5-HT causes PNs to change from tonic to bursting and conclude that this stems from a change in excitability. However, excitability tests (such as I/V plots) were not included, so it's difficult to disambiguate excitability changes from changes in synaptic input from other network components.*

To confirm that the PN excitability did indeed change after serotonin application, we performed a new set of current-clamp recordings. In these experiments, we monitored the spiking activities in individual PNs as we injected different levels of current injections (200 – 1000 pico Amperes). Note that locust LNs that provide recurrent inhibition arborize and integrate inputs from a large number of sensory neurons and projection neurons. Therefore, activating a single PN should not activate the local neurons and therefore the antennal lobe network.

We found that the total spiking activity monotonically increased with the magnitude of the current injection in all four PNs recorded (Author response image 1). However, after serotonin injection, we found that the spiking activity remained relatively stable and did not systematically vary with the magnitude of the current injection. While the changes in odor-evoked responses may incorporate both excitability changes in individual PNs and recurrent feedback inhibition through GABAergic LNs, these results from our current injection experiments unambiguously indicate that there are changes in excitability at the level of individual PNs. We have added this result to the revised manuscript.

# **Author response image 1.**

Current-injection induced spiking activity in individual PNs is altered after serotonin application. (A) Representative intracellular recordings showing membrane potential fluctuations as a function of time for one projection neuron (PNs) in the locust antennal lobe. A two-second window when a positive 200-1000pA current was applied is shown. Firing patterns before (left) and after (right) serotonin application are shown for comparison. Note, the spiking activity changes after the 5HT application. The black bar represents the 20mV scale. (B) Dose-response curves showing the average number of action potentials (across 5 trials) during the 2second current pulse before (green) and after (purple) serotonin for each recorded PN. Note that the current intensity was systematically increased from 200 pA to 1000 pA. The (C) The mean number of spikes across the four recorded cells during current injection is shown. The color progression represents the intensity of applied current ranging 200pA (leftmost bar) to 1000pA (rightmost bar). The dose-response trends before (green) and after (purple) 5HT application are shown for comparison.. The error bars represent SEM across the four cells.



- *There is another explanation for the theoretical discrepancy between physiology and behavior, which is that odor coding is further processing in higher brain regions (ie. Other than the antennal lobe) not studied in the physiological component of this study. This should at least be discussed.*

This is a valid argument. For our model of neural mapping onto behavior to work, we only need the odorant that evokes or suppresses PORs to activate a distinct set of neurons. Having said that, our extracellular recording results (Fig. 6E) indicate that hexanol (high POR) and linalool (low POR) do activate highly non-overlapping sets of PNs in the antennal lobe. Hence, our results suggest that the segregation of neural activity based on behavioral relevance already begins in the antennal lobe. We have added this clarification to the discussion section.

- *The authors cannot claim that serotonin underlies a hunger state-dependent modulation, only that serotonin impacts responses to appetitive odors. Serotonin enhanced PORs for starved and fed locusts, so the conclusion would be that serotonin enhances responses regardless of the hunger state. If the authors had antagonized 5-HT receptors and shown that feeding no longer impacts POR, then they could make the claim that serotonin underlies this effect. As it stands, these appear to be two independent phenomena.*

This is also a valid point. We have clarified this in the revised manuscript.

#### **Reviewer #2 (Public Review):**

##### *Summary:*

*The authors investigate the influence of serotonin on feeding behavior and electrophysiological responses in the antennal lobe of locusts. They find that serotonin injection changes behavior in an odorspecific way. In physiology experiments, they can show that antennal lobe neurons generally increase their baseline firing and odor responses upon serotonin injection. Using a modeling approach the authors propose a framework on how a general increase in antennal lobe output can lead to odorspecific changes in behavior. The authors finally suggest that serotonin injection can mimic a change in a hunger state.*

##### *Strengths:*

*This study shows that serotonin affects feeding behavior and odor processing in the antennal lobe of locusts, as serotonin injection increases activity levels of antennal lobe neurons. This study provides another piece of evidence that serotonin is a general neuromodulator within the early olfactory processing system across insects and even phyla.* *Weaknesses:*

*I have several concerns regarding missing control experiments, unclear data analysis, and interpretation of results.*

*A detailed description of the behavioral experiments is lacking. Did the authors also provide a mineral oil control and did they analyze the baseline POR response? Is there an increase in baseline response after serotonin exposure already at the behavioral output level? It is generally unclear how naturalistic the chosen odor concentrations are. This is especially important as behavioral responses to different concentrations of odors are differently modulated after serotonin injection (Figure 2: Linalool and Ammonium).*

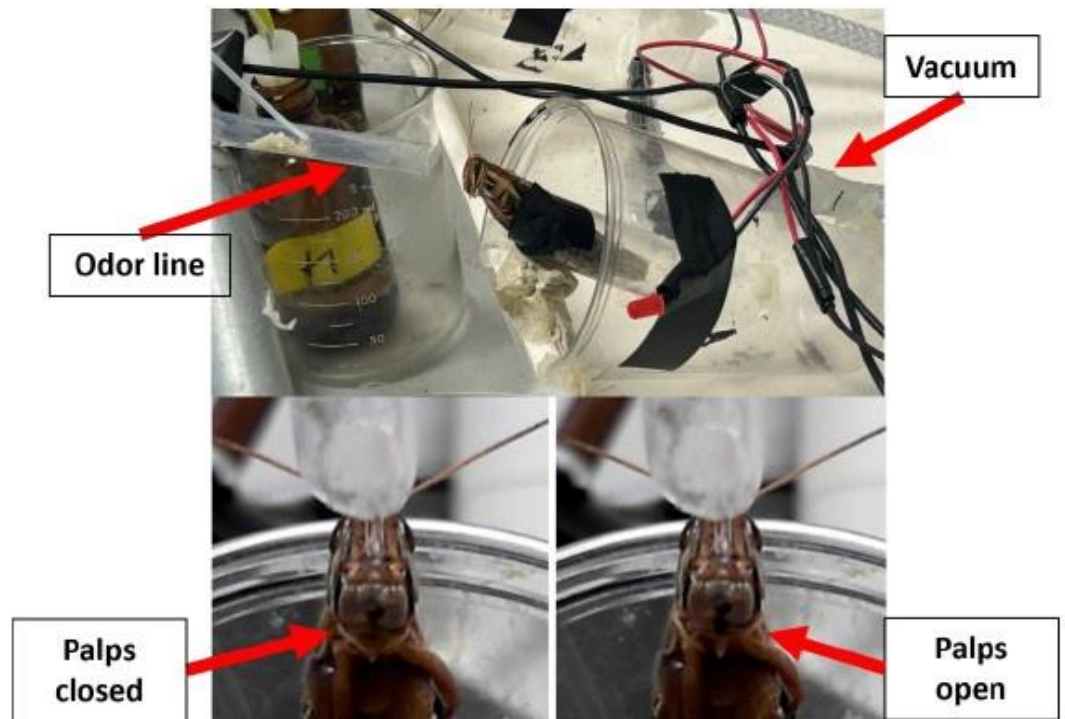
POR protocol: Sixth instar locusts (*Schistocera americana*) of either sex were starved for 24-48 hours before the experiment or taken straight from the colony and fed blades of grass for the satiated condition. Locusts were immobilized by placing them in the plastic tube and securing their body with black electric tape (see Author response image 2). Locusts were given 20 - 30 minutes to acclimatize after placement in the immobilization tube. As can be noted, the head of the locusts along with the antenna and maxillary palps protruded out of this immobilization tube so they can be freely moved by the locusts. Note that the maxillary palps are sensory organs close to the mouth parts that are used to grab food and help with the feeding process.

It is worth noting that our earlier studies had shown that the presentation of ‘appetitive odorants’ triggers the locust to open their maxillary palps even when no food is presented (Saha et al., 2017; Nizampatnam et al., 2018; Nizampatnam et al., 2022; Chandak and Raman, 2023.) Furthermore, our earlier results indicate that the probability of palp opening varies across different odorants (Chandak and Raman, 2023). We chose four odorants that had a diverse range of palp-opening: supra-median (hexanol), median (benzaldehyde), and sub-median (linalool). Therefore, each locust in our experiments was presented with one concentration of four odorants (hexanol, benzaldehyde, linalool, and ammonium) in a pseudorandomized order. The odorants were chosen based on our physiology results such that they evoked different levels of spiking activities.

The odor pulse was 4 s in duration and the inter-pulse interval was set to 60 s. The experiments were recorded using a web camera (Microsoft) placed right in front of the locusts. The camera was fully automated with the custom MATLAB script to start recording 2 seconds before the odor pulse and end recording at odor termination. An LED was used to track the stimulus onset/offset. The POR responses were manually scored offline. Responses to each odorant were scored a 0 or 1 depending on if the palps remained closed or opened. A positive POR was defined as a movement of the maxillary palps during the odor presentation time window as shown on the locust schematic (Main Paper Figure 1).

#### **Author response image 2.**

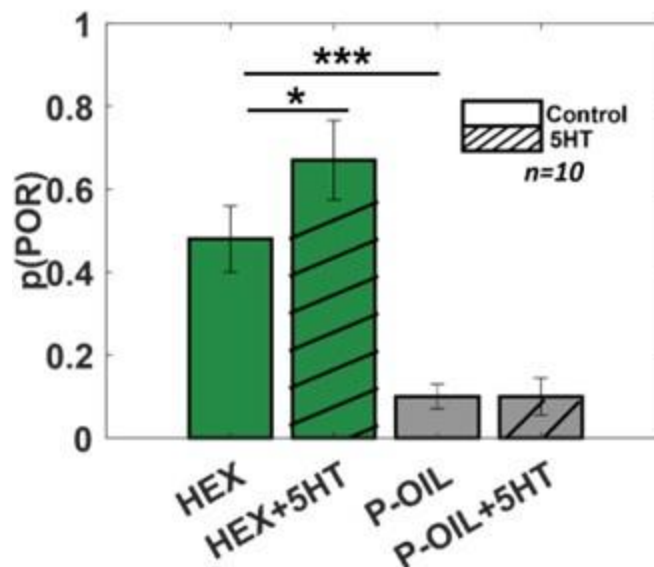
Pictures showing the behavior experiment setup and representative palp-opening responses in a locust.



As the reviewer inquired, we performed a new series of POR experiments, where we explored POR responses to mineral oil and hexanol, before and after serotonin injection. For this study, we used 10 locusts that were starved 24-48 hours before the experiment. Note that hexanol was diluted at 1% (v/v) concentration in mineral oil. Our results reveal that locusts PORs to hexanol (~ 50% PORs) were significantly higher than those triggered by mineral oil (~10% PORs). Injection of serotonin increased the POR response rate to hexanol but did not alter the PORs evoked by mineral oil (Author response image 3).

### Author response image 3.

Serotonin does not alter the palp-opening responses evoked by paraffin oil. The PORs before and after (5HT) serotonin injection are summarized and shown as a bar plot for hexanol and paraffin oil. Striped bars signify the data collected after 5HT injection. Significant differences are identified in the plot (one-tailed paired-sample t-test; (\* $p < 0.05$ )).

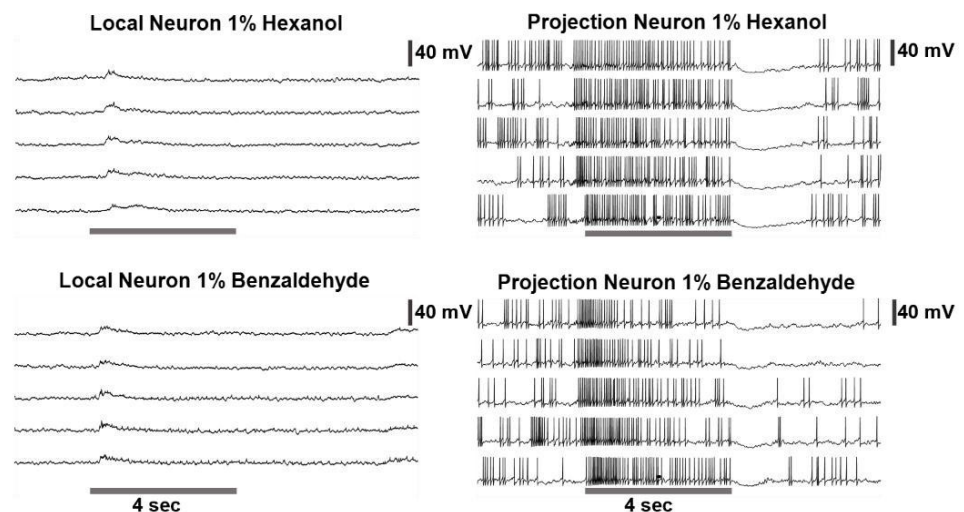


Regarding recordings of potential PNs - the authors do not provide evidence that they did record from projection neurons and not other types of antennal lobe neurons. Thus, these claims should be phrased more carefully.

In the locust antennal lobe, only the cholinergic projection neurons fire full-blown sodium spikes. The GABAergic local neurons only fire calcium 'spikelets' (Laurent, TINS, 1996; Stopfer et al., 2003; see Author response image 4 for an example). Hence, we are pretty confident that we are only recording from PNs. Furthermore, due to the physiological properties of the LNs, their signals being too small, they are also not detected in the extracellular recordings from the locust antennal lobe. Hence, we are confident with our claims and conclusion.

#### Author response image 4.

PN vs LN physiological differences: Left: A representative raw voltage traces recorded from a local neuron before, during, and after a 4-second odor pulse are shown. Note that the local neurons in the locust antennal lobe do not fire full-blown sodium spikes but only fire small calcium spikelets. On the right: A representative raw voltage trace recorded from a representative projection neuron is shown for comparison. Clear sodium spikes are clearly visible during spontaneous and odor-evoked periods. The gray bar represents 4 seconds of odor pulse. The vertical black bar represents the 40mV.



*The presented model suggests labeled lines in the antennal lobe output of locusts. Could the presented model also explain a shift in behavior from aversion to attraction - such as seen in locusts when they switch from a solitary to a gregarious state? The authors might want to discuss other possible scenarios, such as that odor evaluation and decision-making take place in higher brain regions, or that other neuromodulators might affect behavioral output. Serotonin injections could affect behavior via modulation of other cell types than antennal lobe neurons. This should also be discussed - the same is true for potential PNs - serotonin might not directly affect this cell type, but might rather shut down local inhibitory neurons.*

There are multiple questions here. First, regarding solitary vs. gregarious states, we are currently repeating these experiments on solitary locusts. Our preliminary results (not included in the manuscript) indicate that the solitary animals have increased olfactory arousal and respond with a higher POR but are less selective and respond similarly to multiple odorants. We are examining the physiology to determine whether the model for mapping neural responses onto behavior could also explain observations in solitary animals.

Second, this reviewer makes the point raised by Reviewer 1. We agree that odor evaluation and decisionmaking might take place in higher brain regions. All we could conclude based on our data is that a segregation of neural activity based on behavioral relevance might provide the simplest approach to map non-specific increase in stimulus-evoked neural responses onto odor-specific changes in behavioral outcome. Furthermore, our results indicate that hexanol and linalool, two odorants that had an increase and decrease in PORs after serotonin injection, had only minimal neural response overlap in the antennal lobe. These results suggest that the formatting of neural activity to support varying behavioral outcomes might already begin in the antennal lobe. We have added this to our discussion.

Third, regarding serotonin impacting PNs, we performed a new set of current-clamp experiments to examine this issue (Author response image 1). Our results clearly show that projection neuron activity in response to current injections (that should not incorporate feedback inhibition through local neurons) was altered after serotonin injection. Therefore, the observed changes in the odor-evoked neural ensemble activity should incorporate modulation at both individual PN level and at the network level. We have added this to our discussion as well.

*Finally, the authors claim that serotonin injection can mimic the starved state behavioral response. However, this is only shown for one of the four odors that are tested for*

behavior (HEX), thus the data does not support this claim.

We note that Hex is the only appetitive odorant in the panel. But, as reviewer 1 has also brought up a similar point, we have toned down our claims and will investigate this carefully in a future study.

**Recommendations for the authors:**

**Reviewer #1 (Recommendations For The Authors):**

- *Was the POR of the locusts towards linalool and ammonium higher than towards a blank odor cartridge? I ask because the locusts appear to be less likely to respond to these odors and so I am concerned that this assay is not relevant to the ecological context of these odors. In other words, perhaps serotonin did not enhance the responses to these odors in this assay, because this is not a context in which locusts would normally respond to these odors.*

The POR response to linalool and ammonium is lower and comparable to that of paraffin oil. Serotonin does not increase POR responses to paraffin oil but does increase response to hexanol (an appetitive odorant). We have clarified this using new data (Author response image 5).

- *It seems to me that Figure 5C is the crux for understanding the potential impact of 5-HT on odor coding, but it is somewhat confusing and underutilized. Is the implication that 5-HT decorrelates spontaneous activity such that when an odor stimulus arrives, the odor-evoked activity deviates to a greater degree? The authors make claims about this figure that require the reader to guess as to the aspect of the figure to which they are referring.*

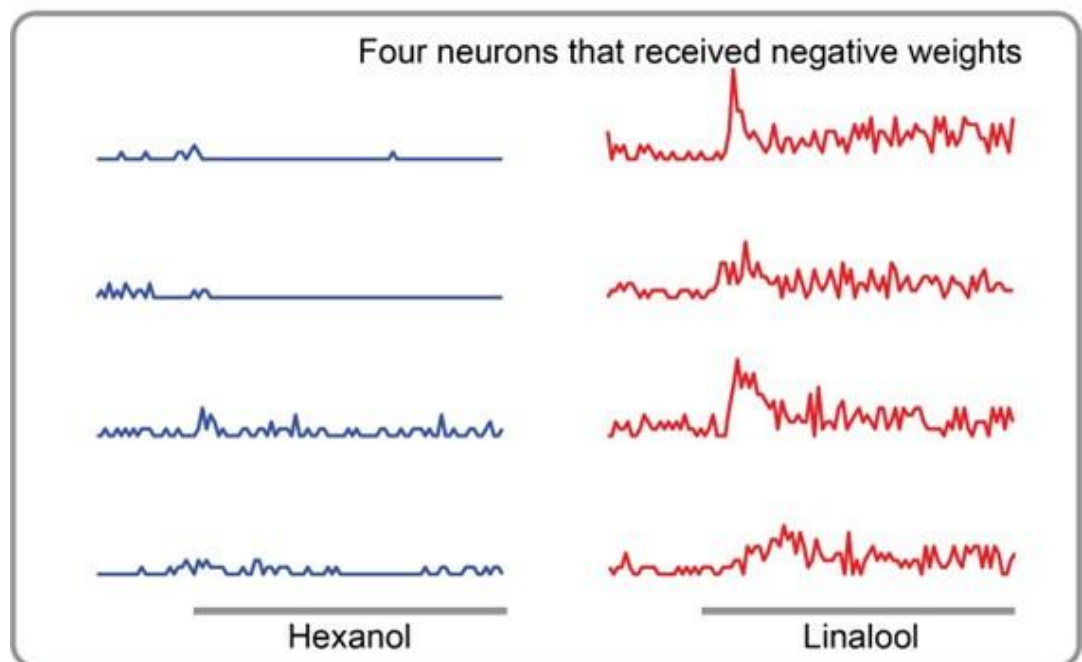
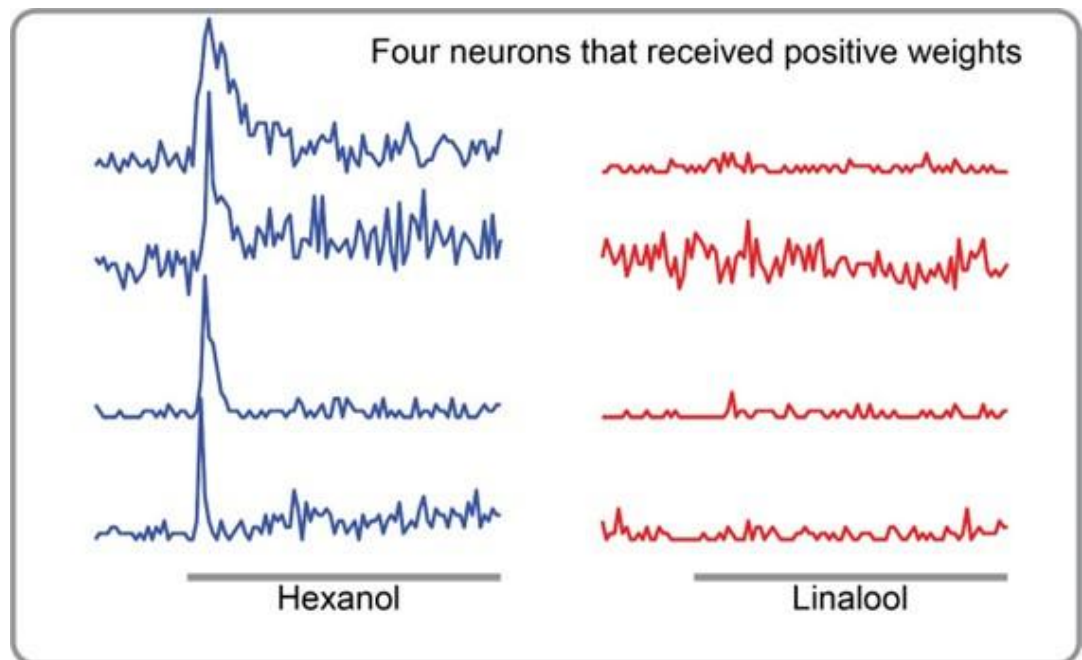
The reviewer makes an astute observation. Yes, the spontaneous activity in the antennal lobe network before serotonin introduction is not correlated with the ensemble spontaneous activity after serotonin bath application. Remarkably, the odor-evoked responses were highly similar, both in the reduced PCA space and when assayed using high-dimensional ensemble neural activity vectors. Whether the changes in network spontaneous activity have a function in odor detection and recognition is not fully understood and cannot be convincingly answered using our data. But this is something that we had pondered.

- *The modeling component summarized in Figure 6 needs clarification and more detail. Perhaps example traces associated with positive weighting within neural ensemble 1 relative to neural ensemble 2? I struggled to understand conceptually how the model resolved the theoretical discrepancy between physiology and behavior.*

As recommended, here is a plot showing the responses of four PNs that had positive weights to hexanol and linalool. As can be expected, each PN in this group had higher responses to hexanol and no response to linalool. Further, the four PNs that received negative weights had response only to linalool.

**Author response image 5.**

Odor-evoked responses of four PNs that received positive weights in the model (top panel), and four PNs that were assigned negative weights in the model (bottom).

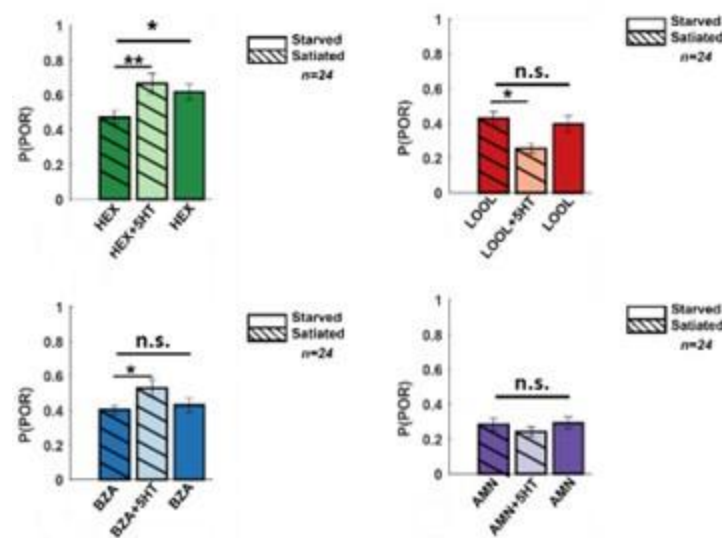


- Was there a significant difference between the PORs of hungry vs. fed locusts? The authors state that they differ and provide statistics for the comparisons to locusts injected with 5-HT, but then don't provide any statistical analyses of hungry vs. fed animals.

The POR responses to HEX (an appetitive odorant) were significantly different between the hungry and starved locusts.

# Author response image 6.

A bar plot summarizing PORs to all four odors for satiated locust (highlighted with stripes), before (dark shade), and after 5HT injection (lighter shade). To allow comparison before 5HT injection for starved locust plotted as well (without stripes). The significance was determined using a one-tailed paired-sample ttest(\*p<0.05).

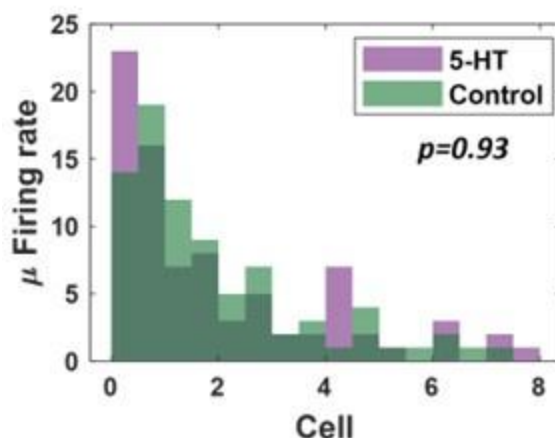


- Were any of the effects of 5-HT on odor-evoked PN responses significant? No statistics are provided.

We examined the distribution of odor-evoked responses in PNs before and after 5HT introduction. We found that the overall distribution was not significantly different between the two (one-tailed pairedsample t-test; p = 0.93).

# Author response image 7.

Comparison of the distribution of odor-evoked PN responses before (green) and after (purple) 5HT introduction. One-tailed paired sample t-test was used to compare the two distributions.



- *The authors interchangeably use "serotonin", "5HT" and "5-HT" throughout the manuscript, but this should be consistent.*

This has been fixed in the revised manuscript.

- *On page 2 the authors provide an ecological relevance for linalool as being an additive in pesticides, however, linalool is a common floral volatile chemical. Is the implication that locusts have learned to associate linalool with pesticides?*

Linalool is a terpenoid alcohol that has a floral odor but has also been used as a pesticide and insect repellent [Beier et al., 2014]. As shown in Author response image 2, it evoked the least POR responses amongst a diverse panel of 22 odorants that were tested. We have clarified how we chose odorants based on the prior dataset in the Methods section.

- *In Figure 1, there should be a legend in the figure itself indicating that the black box indicates the absence of POR and the white box indicates presence, rather than just having it in the legend text.*

Done.

- *In Figure 2, the raw data from each animal can be moved to the supplements. The way it is presented is overwhelming and the order of comparisons is difficult to follow.*

Done.

- *For the induction of bursting in PNs by the application of 5-HT, were there any other metrics observed such as period, duration of bursts, or peak burst frequency? The authors rely on ISI, but there are other bursting metrics that could also be included to understand the nature of this observation. In particular, whether the bursts are likely due to changes in intrinsic biophysical properties of the PNs or polysynaptic effects.*

We could use other metrics as the reviewer suggests. Our main point is that the spontaneous activity of individual PNs changed. We have added a new current-injection experiments to show that the PNs output to square pulses of current becomes different after serotonin application (Author response image 1)

- *Were 4-vinyl anisole, 1-nonanol, and octanoic acid selected as additional odors because they had particular ecological relevance, or was it for the diversity of chemical structure?*

These odorants were selected based on both, chemical structure and ecological relevance. The logic behind this was to have a very diverse odor panel that consisted of food odorant – Hexanol, aggregation pheromone – 4-vinyl anisole, sex pheromone – benzaldehyde, acid – octanoic acid, base – ammonium, and alcohol – 1-nonanol. Additionally, we selected these odors based on previous neural and behavioral data on these odorants (Chandak and Raman, 2023, Traner and Raman, 2023, Nizampatnam et al, 2022 & 2018; Saha et al., 2017 & 2013).

**Reviewer #2 (Recommendations For The Authors):**

*The electrophysiology dataset combines all performed experiments across all tested different PN-odor pairs. How many odors have been tested in a single PN and how many PNs have been tested for a single odor? This information is not present in the current manuscript. Can the authors exclude that there are odor-specific modulations?*

In total, our dataset includes recordings from 19 PNs. Seven PNs were tested on a panel of seven odorants (4-vinyl anisole, 1-nonanol, octanoic acid, Hex, Bza, Lool, and Amn), and the remaining twelve were tested with the four main odorants used in the study (Hex, Bza, Lool, and Amn). This information has been added to the Methods section

*How did the authors choose the concentrations of serotonin injections and bath applications - is this a naturalistic amount?*

The serotonin concentration for ephys experiments was chosen based on trial-error experiments:

0.01mM was the highest concentration that did not cause cell death. For the behavioral experiments, we increased the concentration (0.1 M) due to the presence of anatomical structures in the locust's head such as air sacks, sheath as well as hemolymph which causes some degree of dilution that we cannot control.

*Behavior experiments were performed 3 hours after injection - ephys experiments 5-10 minutes following bath application. Can the authors exclude that serotonin affects neural processing differently on these different timescales?*

We cannot exclude this possibility. We did ePhys experiments 5-10 minutes after bath application as it would be extremely hard to hold cells for that long.

A longer delay was required for our behavioral experiments as the locusts tended to be a bit more agitated with larger spontaneous movements of palps as well as exhibited unprompted vomiting. A 3hour period allowed the locust to regain its baseline level movements after 5HT introduction. [This information has been added to the methods section of the revised manuscript]

*Concerning the analysis of electrophysiological data. The authors should correct for changes in the baseline before performing PCA analysis. And how much of the variance is explained by PC1 and PC2?*

We did not correct for baseline changes or subtract baseline as we wanted to show that the odor-evoked neural responses still robustly encoded information about the identity of the odorant.

*The authors should perform dye injections after recordings to visualize the cell type they recorded from. Serotonin might affect also other cell types in the antennal lobe.*

As mentioned above, in the locust antennal lobe only PNs fire full-blown sodium spikes, and LNs only fire calcium spikelets (Author response image 4). Since these signals are small, they will be buried under the noise floor when using extracellular recording electrodes for monitoring responses in the AL antennal lobe.

Hence we are pretty certain what type of cells we are recording from.

*There were several typos in the manuscript, please check again.*

We have fixed many of the grammatical errors and typos in the revised version.