nature portfolio

Peer Review File

Olfactory sensory neuron population expansions influence projection neuron adaptation and enhance odour tracking

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Following their previous work establishing the link between OSN number expansion and D. sechellia's nori seeking behavior, here the authors try to address the sensory processing and underlying neural mechanism. To understand the developmental mechanism of ab3 neuron expansion, they nicely used quantitative trait locus and identified two regions on 3rd chromosome and X chromosome, respectively, are highly correlated to this neuron number expansion phenotype. This is very elegant and not a simple task. They then built many sophisticated genetic tools to address questions on neural mechanisms. They concluded that the OSN expansion are not accompanied by increases in the numbers of cognate PNs; instead, the dendrite arborization and overall synaptic connections between PNs and ORNs increased.

In the end, they concluded that the system achieves nori preference through increasing OSN numbers and in the end functions to local interneurons to weaken lateral inhibition, which decreases PN adaption and leads to persistence response. This is an elegant story with well conducted experiments. Yet, my overall though toward this manuscript is that the story goes too deep in the neural activities and physiological properties of Or85b OSNs/VM5d PNs and Or22a OSNs/DM2 PNs in the second half of the main figures and extended Figures. This almost derailed the main theme because Or85b OSNs/VM5d PNs are not involved in the specialized noni response of D. sechellia.

(Major comments)

1. (Extended data Fig 4C) The mean trajectory ground speed of Or22a[RFP] D. sec is faster than wild D. sec and D. mel. Based on this result, the author claimed that Or22a mutant D. sec do not have obvious impairment in flight performance. However, it seems that these flies flew much faster than controls. Could this contribute to their different behaviors than controls in the downwind half and upwind half regions (Fig 2g)? In other words, whether the results shown in Fig. 2g is due to "abnormal" sensation (e.g., over sensitive) to nori odor plume or lacking the persistence of sensing nori odor plume?

2. (Fig 3a) The author nicely tuned the light intensity to have Or22 OSNs' neural spiking rate similar to that upon noni stimulation. However, when they used the light to stimulate one side of Or22a OSNs, the ΔWBA did not show up-and-down patterns upon light on and off, which was seen when stimulated with noni odor (Fig. 2b). Why? Does the flow per se contribute to the ΔWBA pattern in Fig 2b? Albeit this question, the authors convincingly demonstrated that the number of Or22a OSNs in D. sechellia strongly correlate to their tracing behavior toward the odor from nori juice (Fig. 3d).

3. (Fig 4g) Based on the result showed in Fig. 4d, the overall GFP intensity in D. sechellia DM2 is weaker than that in D. melanogaster DM2. That may partly explain why the difference of post-synaptic puncta numbers in DM2 of these two species become smaller albeit statistically significant. If the authors compare the density of puncta (puncta numbers/glomerular volume), the density of which in D. sechellia DM2 may be smaller than that in D. melanogaster DM2. VM5d may also be the case. If this is true, I am curious whether the authors have any thought to this.

4. (Fig. 5) Two types of stimulation were applied in this study. When applied different odor concentrations, D. sechellia VM5d PNs showed lower spiking frequencies than that of D. melanogaster but the normalized GCaMP activities between OSNs and PNs correlate well in both species. However, when pulse odor was applied, OSNs activities decreased and PN activity did not change in 10th stimulation in D sechellia, which is not the case in D. melanogaster. Why?

5. (Fig. 5) To understand the PN mechanism in such OSN expansion, they focused on VM5d PNs and use 2-hepanone to examine PN properties. The authors explained this is because they have GAL4 driver to sparsely label VM5d PNs. I do have a concern that also VM5d OSNs have neural expansion and VM5d PNs have similar trends of structural changes to DM2 PNs, VM5d PNs are not specialized toward noni but response to a general odor 2-hepanone. Can the VM5d PN properties reflect the properties of DM2 PNs and explain the persistent response to noni?

6. Extended data Fig 8e nicely showed that D. Sechellia Or22a receptors per se also lead to the stronger PN activities than D. melanogaster Or22a receptors. How such Or receptor properties may contribute to the results shown in Fig. 5h?

7. (Fig 5, Fig6, Extended data Figures 7-12) In these figures, authors investigated tremendous efforts to characterize the electrophysiological properties and neural activities of Or85b/VM5d and Or22a/DM2 channels between two species. Yet, in addition to some similarity, there are significant differences between these two sensory channels upon odor stimulation (e.g., Fig 5g, Fig 5h). Most important, Or22a/DM2 but not Or85b/VM5d channel is responsible for the specialized noni tracking behavior of D. Sechellia, which is the main theme of this story. From this aspect, Or85b/VM5d data would derail the attention and even confuse readers. However, I have to say, these experiments are carefully performed and not tedious and; data are solid and good.

8. Is it possible that the effect of Or22a neuronal expansion to PN persistence is through following mechanism: OSNs → excitatory LNs → inhibitory LNs → OSNs? In this case, excitatory LNs are additionally recruited in D. sechellia due to stronger odor input.

(Minor comments)

1. Fig 1c: the number of ab4 neurons (OR56a and Or7a) also increased in D. sechellia, why did not mention this?

2. Through the tethered fly assay, Or22a mutant D. sechellia showed defect in the persistence to nori (Fig. 2c). In the wind tunnel assay, Or22a mutants stayed less than control both in the upwind area and downwind area (Fig 2g). In the first behavior assay, Or22a mutant D. sechellia behave similar to control D. melanogaster, while in the second behavior assay, D. sechellia behave differently than control D. melanogaster in the downwind half. I am not sure why the authors would conduct these two different behavior assays. Is either one of them good enough?

3. (Fig 4c, figure legend) "Left, representative image of VM5d PNs ....." → DM2 and VM5d PNs?

4. (Fig. 4) The VM5d glomerulus of D. sechellia VM5d glomerulus was nicely identified through the PN responses to particular odor and side-by-side compared to that of D. melanogaster VM5d PNs (Extended Fig 7). Turns out the position of VM5d glomerulus shows medial shift in the AL of D. sechellia. I am curious whether the authors have any thought to such glomerular shift. This may have some consequence to the local interneuron network in the AL.

5. (Fig. 4f) Since Or 85b OSNs did not contribute to the persistent sensing of nori (Fig. 2c), I am curious why the authors compared the dendrite surface of VM5d PNs in two species and not that of DM2 PNs.

6. (line 1553) "...subject to imaging analysis in shown in a dark colour." → ".....is shown...."?

7. (line 439-441) "We next pharmacologically impaired cholinergic neurotransmission to diminish excitatory connections of OSNs, which include OSN-PN and likely also OSN-LN synapses." → This also includes LN-LN synapses because some LNs are cholingeric. Although excitatory LNs form elctrical synapses with PNs, ORNs and LNs, it does not exclude these identified eLNs or other cholingeric LNs form synapses to other LNs.

Reviewer #2 (Remarks to the Author):

In this study Takagi et al. explore the genomic, behavioral and functional consequences of species specific expansion of sensory neuron types for olfactory processing. This is an important topic because ecological changes in stimulus representation have been reported across taxa and sensory modalities, but there is little work to link anatomical changes with physiological and behavioral changes, or the genomic underpinnings that enable these expansions to occur.

They demonstrate that changes at multiple genomic loci result in a change in the proportion of olfactory sensory neurons (OSNs) that express certain Or proteins and that this change in OSN population demography results in more robust odor tracking and synaptic connections on a per PN basis without causing an increase in sensitivity. There is an increase in DM2 PN sensitivity due to changes in the relative sensitivity of the Or22a protein itself. Instead there is less decay over time in response to pulsed odor delivery, implying that the OSN expansion results in an improved fidelity with which odor dynamics are represented. This is impacted by pharmacological manipulations of GABA signaling implying that lateral interaction at least partially contribute.

Overall, this is a tour de force effort combining many levels of analyses to provide a very nice holistic study. It is a well written study with clear figures that communicate the points well. I have only one major concern (for which I provide two approaches to resolve that do not require new data collection) and a few minor concerns. I commend the authors on this exciting body of work.

Major Concern.

-The authors interpret the impact of Or22a and Or85b expansion as being on the ability of PNs to track odor dynamics by demonstrating a lack of response decay to pulsed stimuli. This is one time scale upon which we can consider encoding of stimulus dynamics, but another would be the ability of PNs to track the onset of each individual odor pulse. One could imaging that if PNs are strongly activated by the first pulse, they cannot increase their firing rate further in response to the next odor pulse. This would result in the PNs being WORSE at tracking odor dynamics. One solution could be to run an analysis of the PN recordings that have already been made to determine how well they track pulses (power spectral density analysis could work) to demonstrate that they are truly better at tracking the odor dynamics. As it stands this study measures adaptation (change from first to tenth pulse) rather than fidelity of odor tracking. The second solution would simply be to change the language to say that this expansion results in combating sensory adaptation.

Minor Concerns.

-I personally think the title of the paper sells this work short. The authors have found a really exciting effect on sensory adaptation and the current title focuses on what the sensory expansion doesn't do, which feels like a bit of a wet blanket. Something along the lines of "Sensory neuron population expansion enhances odour tracking by preventing projection neuron adaptation". The authors may feel that this oversells the case, but I think the title should reflect how the expansion impacts PN activity, rather than what it does not do to PN activity.

-In the discussion section the authors propose that there are likely changes in connectivity of DM2 PNs in the lateral horn or mushroom body. This is a very interesting point and definitely worthy of future study. It would be worth citing Seeholzer et al 2018 who showed that connectivity changes in higher order brain centers can result in changes in odor preference across Drosophila species.

-The final summary panel would be more informative with a cartoon schematic comparing the connectivity changes between melanogaster and sechellia.

NCOMMS-24-19278-T: RESPONSE TO REVIEWERS

We thank the reviewers for their careful reading and constructive criticisms of our manuscript. Below, we provide responses to each of the raised issues.

Reviewer #1

Following their previous work establishing the link between OSN number expansion and D. sechellia's nori seeking behavior, here the authors try to address the sensory processing and underlying neural mechanism. To understand the developmental mechanism of ab3 neuron expansion, they nicely used quantitative trait locus and identified two regions on 3rd chromosome and X chromosome, respectively, are highly correlated to this neuron number expansion phenotype. This is very elegant and not a simple task. They then built many sophisticated genetic tools to address questions on neural mechanisms. They concluded that the OSN expansion are not accompanied by increases in the numbers of cognate PNs; instead, the dendrite arborization and overall synaptic connections between PNs and ORNs increased.

In the end, they concluded that the system achieves nori preference through increasing OSN numbers and in the end functions to local interneurons to weaken lateral inhibition, which decreases PN adaption and leads to persistence response. This is an elegant story with well conducted experiments. Yet, my overall though toward this manuscript is that the story goes too deep in the neural activities and physiological properties of Or85b OSNs/VM5d PNs and Or22a OSNs/DM2 PNs in the second half of the main figures and extended Figures. This almost derailed the main theme because Or85b OSNs/VM5d PNs are not involved in the specialized noni response of D. sechellia.

RESPONSE: Please see below a detailed response regarding the depth of analyses on different olfactory pathways.

(Major comments)

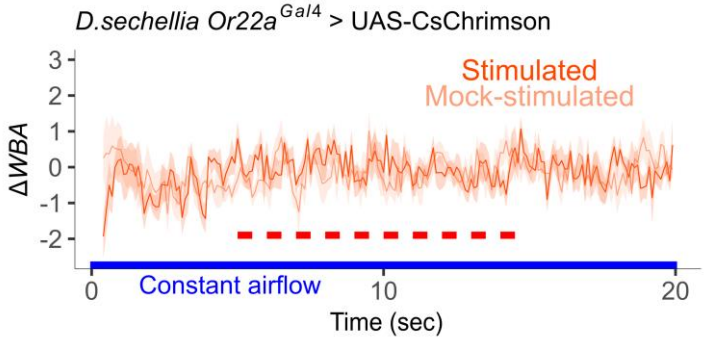
1. (Extended data Fig 4C) The mean trajectory ground speed of Or22a[RFP] D. sec is faster than wild D. sec and D. mel. Based on this result, the author claimed that Or22a mutant D. sec do not have obvious impairment in flight performance. However, it seems that these flies flew much faster than controls. Could this contribute to their different behaviors than controls in the downwind half and upwind half regions (Fig 2g)? In other words, whether the results shown in Fig. 2g is due to "abnormal" sensation (e.g., over sensitive) to nori odor plume or lacking the persistence of sensing nori odor plume?

RESPONSE: We suspect that the higher ground speed of *D. sechellia Or22a* mutants reflects, in part, defects in noni odour plume detection of these animals, as studies in *D. melanogaster* demonstrate that odour encounter leads to

deceleration of animals during flight (e.g., doi:10.1242/jeb.172023, doi:10.1101/2023.11.30.569086v3). The observed phenotype in the wind tunnel is therefore likely a combination of defective sensation leading to lack of persistence and indirect effects on flight speed. We added a comment, and cited these references, in the legend to Supplementary Fig. 4c.

2. (Fig 3a) The author nicely tuned the light intensity to have Or22 OSNs' neural spiking rate similar to that upon noni stimulation. However, when they used the light to stimulate one side of Or22a OSNs, the ΔWBA did not show up-and-down patterns upon light on and off, which was seen when stimulated with noni odor (Fig. 2b). Why? Does the flow per se contribute to the ΔWBA pattern in Fig 2b? Albeit this question, the authors convincingly demonstrated that the number of Or22a OSNs in D. sechellia strongly correlate to their tracing behavior toward the odor from nori juice (Fig. 3d).

RESPONSE: Like the reviewer, our first hypothesis on why the optogenetic activation did not cause up-and-down patterns in the *ΔWBA* time course was the absence of airflow. To test this, we performed combined optogenetic activation with humidified airflow, but these conditions did not induce the obvious up-and-down patterns observed in noni odour responses *(Reviewer Figure 1)*, suggesting some other factor(s) explain the difference. We note that the unilateral, optogenetic activation of a single olfactory channel is likely to produce a different olfactory percept compared to noni odour activation of the entire, bilateral olfactory system, so it is not surprising that behavioural dynamics between these two experiments is different.



Reviewer Figure 1. Optogenetic activation of Or22a OSNs in *D. sechellia* under constant airflow.
Time course of *ΔWBA* (mean ± SEM) where red bars indicate the timing of light application (ten 500 ms pulses with 500 ms intervals). The blue bar indicates the presence of airflow. *n* = 8.

We modified the text to highlight this observation (lines 240-244):

"Pulsed optogenetic activation of D. sechellia Or22a OSNs induced attractive behaviour with a similar magnitude as pulsed odour stimuli – though not evoking the same time-locking of responses to individual pulses as for odours – demonstrating the sufficiency of this single olfactory pathway for evoking behaviour."

3. (Fig 4g) Based on the result showed in Fig. 4d, the overall GFP intensity in D. sechellia DM2 is weaker than that in D. melanogaster DM2. That may partly explain why the difference of post-synaptic puncta numbers in DM2 of these two species become smaller albeit statistically significant. If the authors compare the density of puncta (puncta numbers/glomerular volume), the density of which in D. sechellia DM2 may be smaller than that in D. melanogaster DM2. VM5d may also be the case. If this is true, I am curious whether the authors have any thought to this.

RESPONSE: The reviewer is correct in noting that the staining intensity in *D. sechellia* DM2 is lower compared to *D. melanogaster* DM2 (most likely due to a positional effect on transgene insertion site), which might lead to the underestimation of synaptic puncta in this species. As suggested, we compared the density of puncta in this glomerulus but found there is no difference between species (*D. sechellia* 0.320 $\pm$ 0.014 puncta/μm^3, *D. melanogaster* 0.327 $\pm$ 0.005 puncta/μm^3; $P > 0.5$, unpaired t-test). We also analysed VM5d, where we found that the synaptic puncta density is lower in *D. sechellia* (0.26 $\pm$ 0.009 puncta/μm^3), compared to *D. melanogaster* (0.39 $\pm$ 0.009 puncta/μm^3) ($P < 0.001$, unpaired t-test), but it is unclear if this represents a real species difference and/or reflects transgene expression differences. If the latter scenario is true, this would presumably only lead to an underestimation of the higher number of synaptic puncta in *D. sechellia* VM5d compared to *D. melanogaster* VM5d (which is the main conclusion we draw from these experiments).

As the analysis with the Dα7:GFP reporter probably vastly underestimates the number of synapses – explaining why we chose to refer to “synaptic puncta” instead of “synapses” – we deliberately avoided making deeper interpretations of relative differences between DM2 and VM5d in the two species. Electron microscopic level connectomic analysis would be the best way to compare synapse numbers across species, which is beyond the scope of this study. Nevertheless, our data support the claim that there are more synaptic connections between OSNs and PNs in both DM2 and VM5d in *D. sechellia*, which has not been previously demonstrated.

4. (Fig. 5) Two types of stimulation were applied in this study. When applied different odor concentrations, D. sechellia VM5d PNs showed lower spiking frequencies than that of D. melanogaster but the normalized GCaMP activities between OSNs and PNs correlate well in both species. However, when pulse odor was applied, OSNs activities decreased and PN activity did not change in 10th stimulation in D sechellia, which is not the case in D. melanogaster. Why?

RESPONSE: There are several points in this comment that we address individually in the hope to fully answer this question.

When applied different odor concentrations, D. sechellia VM5d PNs showed lower spiking frequencies than that of D. melanogaster but the normalized GCaMP activities between OSNs and PNs correlate well in both species.

The lower odour-evoked spiking of *D. sechellia* VM5d PNs is only statistically significant at two intermediate concentrations, so we remarked upon this more in counterpoint to our original expectation that PN sensitivity would be higher in *D. sechellia*, because of the larger number of OSNs. GCaMP imaging provides a distinct measure of neuronal activity (in a different cellular compartment i.e. dendrites for calcium imaging versus soma for patch-clamping, the latter better reflecting axonal spike rates), so it is not so entirely surprising that the quantitative differences between species do not fully correspond. However, the global conclusion - i.e. that more OSNs do not lead to increased sensitisation of PNs – is supported by both approaches.

However, when pulse odor was applied, OSNs activities decreased and PN activity did not change in 10th stimulation in D sechellia, which is not the case in D. melanogaster. Why?

Here the reviewer is referring to the data in Fig. 5i (OSNs) and Fig. 5j (PNs), and we also refer to similar analyses with long-odour pulses in Supp. Fig. 10. For *D. melanogaster*, the persistent OSN calcium response throughout the 10 pulses (or long odour stimulus) is consistent with previous work describing sustained activity in OSN axon termini upon long odour stimulation (doi:10.7554/eLife.43735). Why *D. sechellia* display a decrease is unclear, but we note this is relatively small: the overall shapes of the curves in Supp. Fig. 10a (*D. melanogaster*) and Supp. Fig. 10c (*D. sechellia*) are very similar, and for the pulsed odours (Fig. 5i), there is a somewhat variable strength of response throughout the 10 pulses, for which we do not have a good explanation. However, despite this decrease in OSN activity in *D. sechellia* OSNs, the PN calcium responses are maintained across odour pulses (or the long single stimulus) in this species, but not in *D. melanogaster*. These differences further highlight the distinction that must exist in OSN→PN transformations in these species, which is the main focus of our study.

5. (Fig. 5) To understand the PN mechanism in such OSN expansion, they focused on VM5d PNs and use 2-hepanone to examine PN properties. The authors explained this is because they have GAL4 driver to sparsely label VM5d PNs. I do have a concern that also VM5d OSNs have neural expansion and VM5d PNs have similar trends of structural changes to DM2 PNs, VM5d PNs are not specialized toward noni but response to a general odor 2-hepanone. Can the VM5d PN properties reflect the properties of DM2 PNs and explain the persistent response to noni?

RESPONSE: The reviewer raises the reasonable point of why we focussed on the Or85c/b-VM5d pathway in some experiments and the Or22a-DM2 pathway in others. In part, we were constrained by the availability of genetic tools in *D.*

sechellia (where currently we only have transgenic reagents to analyse VM5d PNs), and in part we were guided by the biology. In the latter context, although Or22a appears to have a more important behavioural role in the tethered fly assay, we note that *D. sechellia Or85c/b* mutants do have a phenotype, with reduced persistence of odour tracking (Fig. 2c). Moreover, in our previous study (doi:10.1038/s41586-020-2073-7), loss of either the Or85c/b or Or22a pathways have similar defects in a long-range noni attraction assay. Finally, while the best-known Or85c/b ligand, *2*-heptanone, is not noni specific, the same is also true for the best-known ligand of *D. sechellia* Or22a, methyl hexanoate, which is found in many fruits. Thus the evidence doesn't point to Or22a being a "specialised" noni odour sensor and Or85c/b a general odour detector.

Our focus on the Or85b/c pathway for many of the physiological experiments was also motivated by the similarity in receptor tuning between species, which enabled analysis of the specific contribution of a higher OSN number to PN response properties. Nevertheless, we show in our calcium imaging experiments, that DM2 PNs display a similar lack of adaptation in *D. sechellia* compared to *D. melanogaster* (Fig. 5k) as we observed for VM5d PNs (Fig. 5j), supporting the idea that the physiological consequences are generalisable across olfactory pathways with increased OSN number.

We appreciate that it does not render the manuscript a straightforward read to describe analysis of multiple olfactory pathways; in the revised version, we have endeavoured to clarify the above points as far as possible (e.g. lines 197-202; 323-326):

*"Loss of Or22a abolished attraction of flies towards noni. Or85c/b and Ir75b mutants show less persistent attraction but retained some, albeit transient, turning towards this stimulus. Flies lacking Or35a behaved comparably to wild-type strains (**Fig. 2c**). These results point to Or22a as an important olfactory receptor required for D. sechellia to respond behaviourally to noni odour, with additional contributions of Or85c/b and Ir75b."*

"Moreover, the partner Or85b OSNs' sensitivities to the best-known agonist, 2-heptanone, are indistinguishable between species. This enabled us to assess the specific impact of OSN population expansion on PN responses (in contrast to the Or22a pathway, which also exhibits receptor tuning differences)."

6. Extended data Fig 8e nicely showed that D. Sechellia Or22a receptors per se also lead to the stronger PN activities than D. melanogaster Or22a receptors. How such Or receptor properties may contribute to the results shown in Fig. 5h?

RESPONSE: If we understand the reviewer's comment correctly, they are asking whether the difference in receptor tuning explains the differences in OSN and PN dose-response curves shown in Fig. 5h. For the OSNs, we previously showed that replacing the *D. sechellia Or22a* allele with the *D. melanogaster Or22a* allele confers a *D. melanogaster*-like odour response profile on these neurons, as measured by peripheral electrophysiological recordings (doi:10.1038/s41586-020-

2073-7). Unfortunately, we are unable to perform calcium imaging (in OSNs or PNs) in such "receptor-swap" flies for technical reasons, as these animals express fluorescent markers that interfere with the measurement of GCaMP signals.

7. (Fig 5, Fig6, Extended data Figures 7-12) In these figures, authors investigated tremendous efforts to characterize the electrophysiological properties and neural activities of Or85b/VM5d and Or22a/DM2 channels between two species. Yet, in addition to some similarity, there are significant differences between these two sensory channels upon odor stimulation (e.g., Fig 5g, Fig 5h). Most important, Or22a/DM2 but not Or85b/VM5d channel is responsible for the specialized noni tracking behavior of D. Sechellia, which is the main theme of this story. From this aspect, Or85b/VM5d data would derail the attention and even confuse readers. However, I have to say, these experiments are carefully performed and not tedious and; data are solid and good.

RESPONSE: Please see our response to point 5 above.

8. Is it possible that the effect of Or22a neuronal expansion to PN persistence is through following mechanism: OSNs → excitatory LNs → inhibitory LNs → OSNs? In this case, excitatory LNs are additionally recruited in D. sechellia due to stronger odor input.

RESPONSE: This is indeed a possibility, as excitatory LNs are known to excite inhibitory LNs through chemical and electrical synapses in *D. melanogaster* (doi:10.1016/j.neuron.2010.08.041). As LNs are incredibly diverse in their physiological and neuroanatomical properties (doi:10.1016/j.cub.2023.10.041), the detailed examination of these hypotheses in *D. sechellia* would require a number of additional neurogenetic tools, which we feel is beyond the scope of the current manuscript. However, we added the following sentence to the Discussion to incorporate this suggestion (lines 487-488):

"Excitatory cholinergic LNs might also contribute to such lateral inhibition by activating GABAergic LNs in response to OSN inputs."

(Minor comments)

1. Fig 1c: the number of ab4 neurons (OR56a and Or7a) also increased in D. sechellia, why did not mention this?

RESPONSE: We did not specifically highlight the increase in ab4 Or56a neurons both because the fold-change is substantially lower than that for the ab3 (Or85c/b and Or22a) and ac3I (Ir75b) neurons, and for narrative simplicity in this manuscript. *D. melanogaster* Or56a is involved in sensing harmful microbes (doi:10.1016/j.cell.2012.09.046) so seems unlikely to play a role in attraction to noni. There are several other (minor) species differences in OSN number that we do not specific mention in the text, but we hope that our broad survey of the

antennal populations, as presented in Fig. 1c might stimulate investigations by other researchers.

2. Through the tethered fly assay, Or22a mutant D. sechellia showed defect in the persistence to nori (Fig. 2c). In the wind tunnel assay, Or22a mutants stayed less than control both in the upwind area and downwind area (Fig 2g). In the first behavior assay, Or22a mutant D. sechellia behave similar to control D. melanogaster, while in the second behavior assay, D. sechellia behave differently than control D. melanogaster in the downwind half. I am not sure why the authors would conduct these two different behavior assays. Is either one of them good enough?

RESPONSE: These assays are complementary, allowing us to investigate different aspects of attractive behaviour (long-range and short-range; long- and short-timescale). The wind tunnel assay (Fig. 2g) added detail and resolution to our previous study (doi:10.1038/s41586-020-2073-7) where we only quantified the ability of flies to locate an odour source; here, by tracking individual flies in the new dataset, we gained insights into flight metrics. The tethered fly assay gave us the possibility to study individual animals and combine behaviour with optogenetic stimulation of selected olfactory pathways, allowing us to establish the role of individual pathways in attraction. While we appreciate that there are differences in the observed phenotypes in these assays, this is unsurprising given the substantial difference in spatial scale and temporal dynamics by which stimuli are presented (which likely has a very impact on the perception of the stimulus), as well as how behaviour is quantified. While we do not claim to fully understand the specific contribution of all olfactory pathways to all aspect of odour-guided behaviour, collectively they emphasise the importance of these pathways, and we feel it is valuable to present data from both assays.

3. (Fig 4c, figure legend) “Left, representative image of VM5d PNs …….” → DM2 and VM5d PNs?

RESPONSE: We have corrected the figure legend.

4. (Fig. 4) The VM5d glomerulus of D. sechellia VM5d glomerulus was nicely identified through the PN responses to particular odor and side-by-side compared to that of D. melanogaster VM5d PNs (Extended Fig 7). Turns out the position of VM5d glomerulus shows medial shift in the AL of D. sechellia. I am curious whether the authors have any thought to such glomerular shift. This may have some consequence to the local interneuron network in the AL.

RESPONSE: It is certainly possible that changes in glomerular position (which might be driven by increase/decreases in glomerular size) influence connectivity patterns with other antennal lobe neurons. We do not current have the tools to assess such a possibility, but we have made brief mention of this interesting point in the Discussion (lines 488-492):

"We cannot exclude that LNs display species-specific innervations or connectivity – potentially influenced by shifts in glomerular position due to their volume changes – but testing this idea will require genetic drivers to visualise and manipulate subsets of this highly diverse neuron type."

5. (Fig. 4f) Since Or 85b OSNs did not contribute to the persistent sensing of nori (Fig. 2c), I am curious why the authors compared the dendrite surface of VM5d PNs in two species and not that of DM2 PNs.

RESPONSE: Please see our response to Major comment 5, above. For measurement of dendrite surface area, we were limited to analysis of VM5d PNs simply because of current lack of genetic tools to target/label individual DM2 PNs in *D. sechellia*.

6. (line 1553) "…subject to imaging analysis in shown in a dark colour." → "…..is shown…."?

RESPONSE: Corrected.

7. (line 439-441) "We next pharmacologically impaired cholinergic neurotransmission to diminish excitatory connections of OSNs, which include OSN-PN and likely also OSN-LN synapses." → This also includes LN-LN synapses because some LNs are cholingeric. Although excitatory LNs form elctrical synapses with PNs, ORNs and LNs, it does not exclude these identified eLNs or other cholingeric LNs form synapses to other LNs.

RESPONSE: We have adapted the phrasing accordingly (lines 432-434):

"We next pharmacologically impaired cholinergic neurotransmission to diminish excitatory connections of OSNs, which include OSN-PN, as well as OSN-LN and some LN-LN synapses"

Reviewer #2

In this study Takagi et al. explore the genomic, behavioral and functional consequences of species specific expansion of sensory neuron types for olfactory processing. This is an important topic because ecological changes in stimulus representation have been reported across taxa and sensory modalities, but there is little work to link anatomical changes with physiological and behavioral changes, or the genomic underpinnings that enable these expansions to occur.

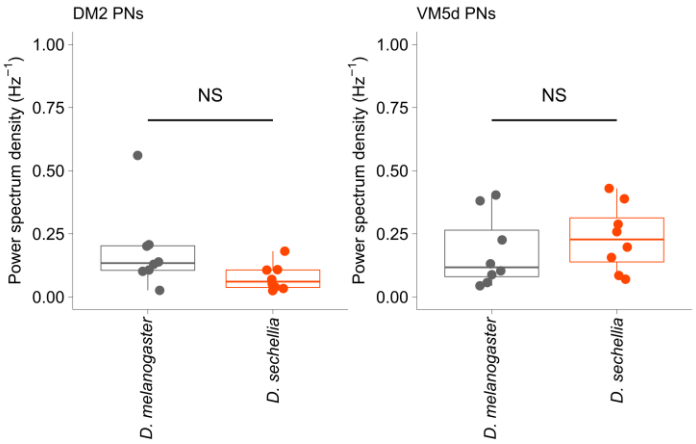
They demonstrate that changes at multiple genomic loci result in a change in the proportion of olfactory sensory neurons (OSNs) that express certain Or proteins and that this change in OSN population demography results in more robust odor tracking and synaptic connections on a per PN basis without causing an increase in sensitivity. There is an increase in DM2 PN sensitivity due to changes in the relative sensitivity of the Or22a protein itself. Instead there is less decay over time in response to pulsed odor delivery, implying that the OSN expansion results in an improved fidelity with which odor dynamics are represented. This is impacted by pharmacological manipulations of GABA signaling implying that lateral interaction at least partially contribute.

Overall, this is a tour de force effort combining many levels of analyses to provide a very nice holistic study. It is a well written study with clear figures that communicate the points well. I have only one major concern (for which I provide two approaches to resolve that do not require new data collection) and a few minor concerns. I commend the authors on this exciting body of work.

Major Concern.

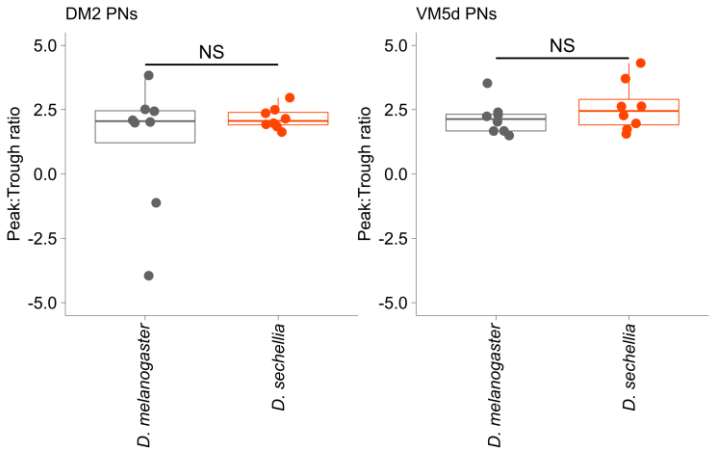
-The authors interpret the impact of Or22a and Or85b expansion as being on the ability of PNs to track odor dynamics by demonstrating a lack of response decay to pulsed stimuli. This is one time scale upon which we can consider encoding of stimulus dynamics, but another would be the ability of PNs to track the onset of each individual odor pulse. One could imaging that if PNs are strongly activated by the first pulse, they cannot increase their firing rate further in response to the next odor pulse. This would result in the PNs being WORSE at tracking odor dynamics. One solution could be to run an analysis of the PN recordings that have already been made to determine how well they track pulses (power spectral density analysis could work) to demonstrate that they are truly better at tracking the odor dynamics. As it stands this study measures adaptation (change from first to tenth pulse) rather than fidelity of odor tracking. The second solution would simply be to change the language to say that this expansion results in combating sensory adaptation.

RESPONSE: We thank the reviewer for bringing up this interesting idea. To quantify how well the PNs track odour pulses, we first performed power spectral density analysis as the reviewer suggested. We extracted the timeframe where odour pulses were applied, calculated the power spectrum density (PSD), and compared the 2.5 Hz component (which corresponds to the 200/200 ms ON/OFF odour cycle) in DM2 and VM5d PNs. We found that in both DM2 and VM5d PNs, there are no significant difference in PSD between species (*Reviewer Figure 2*).



Reviewer Figure 2. Power spectral density analysis of DM2 and VM5d PNs in response to odour pulse trains.

To further examine this possibility, we performed a parallel analysis to compare the peak-to-trough (P:T) ratio. We quantified the ratio between peak and trough GCaMP signals in response to individual odour pulses, averaged across 10 pulses, and compared the P:T ratio across species. Again, there were no significant differences in the P:T ratio across species in both DM2 and VM5d PNs (*Reviewer Figure 3*).



Reviewer Figure 3. Peak-to-trough analysis of DM2 and VM5d PNs in response to odour pulse trains.

These additional analyses do not support the notion that *D. sechellia* PNs are better at tracking noni odour dynamics. However, at the behavioural level, *D. sechellia* is clearly superior to *D. melanogaster* in noni-plume tracking, and we have ensured our use of the word "tracking" is only in the context of describing animal behaviour, and not PN responses.

Minor Concerns.

- I personally think the title of the paper sells this work short. The authors have found a really exciting effect on sensory adaptation and the current title focuses on what the sensory expansion doesn't do, which feels like a bit of a wet blanket. Something along the lines of "Sensory neuron population expansion enhances odour tracking by preventing projection neuron adaptation". The authors may feel that this oversells the case, but I think the title should reflect how the expansion impacts PN activity, rather than what it does not do to PN activity.

RESPONSE: We have reflected carefully on the title, striving to avoid over-claims about the causal effects of sensory population expansion on PN adaptation, and the differences in PN adaptation on odour tracking behaviour, which we have not directly demonstrated. However, we tried to find a compromise between the previous title and the reviewer's suggestion and modified the title to: *"Olfactory sensory neuron population expansions influence projection neuron adaptation and enhance odour tracking".*

- In the discussion section the authors propose that there are likely changes in connectivity of DM2 PNs in the lateral horn or mushroom body. This is a very interesting point and definitely worthy of future study. It would be worth citing Seeholzer et al 2018 who showed that connectivity changes in higher order brain centers can result in changes in odor preference across Drosophila species.

RESPONSE: We are great admirers of the Seeholzer 2018 study as one of the first to demonstrate how changes in central neural pathways underlies species-specific behaviours. However, this paper describes differences in contact pheromone behavioural responses, not odour preferences, and therefore we did not find a good place to cite it in our Discussion (we are additionally already somewhat over the recommended reference limit).

- The final summary panel would be more informative with a cartoon schematic comparing the connectivity changes between melanogaster and sechellia.

RESPONSE: This is an excellent idea and we have added a simple schematic in Fig. 6d to accompany our discussion about known and speculated differences in circuit structure and function based upon the data in our work.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have fully addressed my points. I would support the publication of this manuscript.

Reviewer #2 (Remarks to the Author):

The authors have addressed all of my concerns and I congratulate them on an excellent study.

NCOMMS-24-19278A: RESPONSE TO REVIEWERS

We thank the reviewers for their positive comments on the revised version of the manuscript.