

Opposing chemosensory functions of closely related gustatory receptors

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Abstract

Most animals possess functionally distinct population of taste cells, expressing receptors that are tuned to compounds of different valence. This organizational feature allows for discrimination between chemicals associated with different taste modalities and facilitates sensing of foods contaminated with toxic chemicals. In the fruit fly *D. melanogaster*, primary sensory neurons express taste receptors that are tuned to distinct chemicals, thereby activating neural ensembles that elicit either feeding or avoidance behavior. Members of a family of ligand gated receptor channels, the Gustatory receptors (Gr), play a central role in these behaviors. In general, closely related, evolutionarily conserved Gr proteins are co-expressed in the same type of taste neurons, tuned to chemically related compounds and therefore triggering the same behavioral response. Here, we report that members of the Gr28 subfamily are expressed in largely non-overlapping sets of taste neurons in *Drosophila* larvae, detect chemicals of different valence and trigger opposing feeding behaviors. We determined the intrinsic properties of Gr28 neurons by expressing the mammalian Vanilloid Receptor (VR1), which is activated by capsaicin, a chemical to which wild type *Drosophila* larvae do not respond. When VR1 is expressed in Gr28a neurons, larvae become attracted to capsaicin, whereas expression of VR1 in Gr28bc neurons triggers avoidance to capsaicin. Thus, the Gr28 proteins, which have been evolutionarily conserved over the last 65 million years in insects, represent the first taste receptor subfamily in which specific members mediate behavior with opposite valence. We also identified denatonium benzoate, a synthetic bitter compound that shares structural similarities with natural bitter chemicals, as a ligand for a receptor complex containing a Gr28bc or Gr28ba receptor subunit. AlphaFold structure prediction, combined with the limited sequence conservation in the putative binding pockets of various Gr28 proteins, creates a theoretical framework for targeted *in vivo* structure function studies to precisely map residues critical for ligand recognition.

eLife assessment

This **valuable** study focuses on the role of the Gr28 family of insect chemoreceptors. Using the *Drosophila* larva, the authors show that taste neurons expressing different members of this family of bitter taste receptors trigger opposite behavior - attraction and repulsion. They establish the minimal bitter taste receptor subunit composition needed in these neurons to mediate the repulsion of bitter tastants. The evidence presented is **convincing**, using well-validated and controlled tools and experiments.

Introduction

Meaningful animal behavior is established through cooperative engagement of multiple sensory systems. In many insects, the chemosensory system plays a central role in such integration processes. The fruit fly *Drosophila melanogaster* has served as the primary insect model system for elucidating the molecular basis and neural circuitry of both olfaction and taste, by virtue of the vast genetic resources, amenability to both neurophysiological recording and live imaging, and simple yet powerful behavioral assays, allowing investigators to link genes to chemosensory behavior and neural activity (Montell, 2021 [↗](#)).

The gustatory system in *Drosophila* is characterized by several insect specific features with regard to anatomy and the molecular nature of taste receptors. For example, taste cells are primary sensory neurons, with dendritic projections that express taste receptors, while long axons project that convey taste information directly to the brain. In adult flies, these neurons, referred to as Gustatory Receptor Neurons (GRNs) are distributed across several appendages, such as labial palps, legs and presumably the antennae. Additionally, some GRNs reside internally, arranged in cell clusters along the pharynx (Joseph and Carlson, 2015 [↗](#))(Amrein, 2016 [↗](#))(Scott, 2018 [↗](#)). Similarly, *Drosophila* larvae have numerous structures located both on the head surface as well as internally along the larval pharynx, that assess chemical compounds prior and during ingestion, respectively (Kwon et al., 2011 [↗](#))(Apostolopoulou et al., 2015 [↗](#))(Rist and Thum, 2017 [↗](#)). Like most insects, *Drosophila* employs taste receptors encoded by two major gene families, the *Gustatory receptor* (*Gr*) and the *Ionotropic Receptor* (*IR*) genes, to sense all chemicals, such as appetitive food compounds, noxious and toxic chemicals as well as pheromones. Both *Gr* and *IR* based receptors are thought of form complexes composed of several different subunits, partial composition of which is known for only a few *Gr*-based taste receptors for sugars and bitter compounds (Kwon et al., 2007 [↗](#))(Jiao et al., 2008 [↗](#))(Fujii et al., 2015 [↗](#))(Shim et al., 2015 [↗](#))(Gonzales et al., unpublished), as well as gaseous carbon dioxide (Jones et al., 2007 [↗](#)) and even fewer *IR*-based fatty acid and carboxylic acid receptors (Ahn et al., 2017 [↗](#))(Tauber et al., 2017 [↗](#))(Chen and Amrein, 2017 [↗](#)). Interestingly, at least some GRNs express members of both families, although no evidence for receptor complexes consisting of both *IR* and *Gr* subunits has been reported to date.

The *Gr* genes represent the largest taste receptor gene family in insects. In *D. melanogaster*, it comprises 60 genes predicted to encode 68 proteins, expression of which has been characterized (Kwon et al., 2011 [↗](#))(Weiss et al., 2011 [↗](#))(Ling et al., 2014 [↗](#))(Choi et al., 2016 [↗](#))(Rist and Thum, 2017 [↗](#)). Several *Gr* genes have been functionally characterized in adult flies using genetic mutations, combined with either electrophysiological recordings, Ca^{2+} imaging studies and behavioral analyses (reviewed by Montell, 2021 [↗](#)) but only a few have been studied in larvae (Mishra et al., 2013 [↗](#))(Apostolopoulou et al., 2016 [↗](#))(Choi et al., 2016 [↗](#))(Mishra et al., 2018 [↗](#))(Choi et al., 2020 [↗](#)). The *Gr28* gene subfamily is of particular interest for a number of reasons: First, it is one of the most conserved *Gr* subfamily, homologs of which can be found across all insect families and even more distant arthropods (Eyun et al., 2017 [↗](#))(Suzuki et al., 2018 [↗](#))(Fujii et al., 2023 [↗](#)). Second, the *Gr28* genes are expressed not only in the gustatory system, but in many other organs, especially the central nervous system (CNS) and non-chemosensory neurons of the peripheral nervous system (PNS), suggesting that they have functions beyond gustation and are important to sense chemical signals unrelated to food (Thorne and Amrein, 2008 [↗](#)). Notably, evidence for such roles has been reported before any direct link to gustatory perception was discovered. Ni and colleagues showed that *Gr28bd* is essential for high temperature avoidance in flies (Ni et al., 2013 [↗](#)), while Xiang and collaborators found that *Gr28* mutant larvae were deficient in UV light avoidance (Xiang et al., 2010 [↗](#)). Third, the only gustatory function for any

Gr28 gene in larvae thus far is sensing of ribonucleosides and RNA in larvae (Mishra et al., 2018 [DOI](#)), which was recently established as a novel appetitive taste quality common to a broad range of dipteran insects (Fujii et al., 2023 [DOI](#)).

Here, we present a functional characterization of the *Gr28* genes and the respective GRNs in *Drosophila* larvae. Four of the six *Gr28* genes (*Gr28a*, *Gr28ba*, *Gr28bc* and *Gr28be*) are expressed in the larval taste system, subdivided into two, functionally distinct neuronal ensembles. The first such ensemble is defined by expression of *Gr28bc* and comprises four neurons, two of which also express *Gr28ba*, *Gr28bc*, *Gr28be* and the bitter core receptor gene *Gr66a*, while the other two neurons express only *Gr28bc*, *Gr28a* and *Gr66a*. The second ensemble is characterized by 12 GRNs that express *Gr28a*, including the pair of GRNs expressing the *Gr28b* genes. When the mammalian Vanilloid Receptor 1 (VR1) was expressed under the control of specific GAL4 drivers, we found that *Gr28a* and *Gr28bc* neurons mediate opposing taste behavior in the presence of capsaicin, the ligand for VR1. Specifically, *Gr28a:VR1* larvae show strong attraction for capsaicin, while *Gr28bc:VR1* larvae show strong avoidance of capsaicin. Furthermore, inactivation experiments reveal that the *Gr28bc* GRNs are necessary to sense bitter compounds, specifically denatonium, quinine, lobeline, and caffeine. However, *Gr28* mutant larvae fail to avoid only denatonium, but none of the other bitter compounds, a phenotype rescued by expression of *Gr28bc* or *Gr28ba*. Thus, to our knowledge, the related *Gr28* genes represent the first subfamily where members mediate functionally opposing taste behaviors.

Results

Expression of the *Gr28* genes in the larval taste organs

The peripheral chemosensory system of the larvae is subdivided into bilaterally symmetrical, ‘external’ and ‘internal’ taste organs (Stocker, 2008). The three external organs reside near the tip of the head and organized as paired ganglia, the Dorsal, Terminal and Ventral Organ Ganglia (DOGs, TOGs and VOGs) that house the GRN cell bodies, each neuron projecting a dendrite to the sensory organ structures (DO, TO and VO) at the head surface, while carrying information via their axons to the subesophageal zone (SEZ) in the brain (**Figure 1A** [DOI](#)). The DOG harbors 21 olfactory neurons and 18 presumptive taste neurons. For clarity, numbers below refer to neurons on one side of the bilaterally symmetrical structures of all organs, thus the total neuron number in a larva is double that number. The presumptive GRNs located in the DOG fall into two distinct groups, based on their dendritic projections: 11 presumptive GRNs, four of which were shown to express *Gr* genes (Apostolopoulou et al., 2015 [DOI](#)), extend dendritic projections to the base of the DO, while 7 neurons project dendrites to the TO (the dorsolateral group, **Figure 1A** [DOI](#)) (Kwon et al., 2011 [DOI](#)). The TOG contains 30 neurons, all sending dendrites to the TO (the distal group). The internal taste structures, referred to as the dorsal/ventral pharyngeal sense organ (DPS/VPS, 33 neurons), and the posterior pharyngeal sense organs (PPS, 6 neurons) are located along the pharynx and sense chemicals as they pass towards the digestive system. We note that not all these neurons are confirmed GRNs, either by function or expression of *Gr* or *IR* genes, albeit based on their location and anatomy, most are thought to be GRNs (Stewart et al., 2015 [DOI](#)) (Rist and Thum, 2017 [DOI](#)) (Sánchez-Alcañiz et al., 2018 [DOI](#)).

Our initial expression analysis revealed that four of the six *Gr28* genes (*Gr28a*, *Gr28ba*, *Gr28bc* and *Gr28be*) are expressed in larval taste organs, in addition to cells in the gut, the brain and non-chemosensory cells of the larvae (Mishra et al., 2018 [DOI](#)). To further delineate the putative chemosensory roles of these genes, we performed a more detailed co-expression analysis between *Gr28* genes and with respect to the bitter receptor gene *Gr66a*, using the bimodal expression systems *GAL4/UAS* to label *Gr28* expressing neurons and *LexA/lexAop* to mark *Gr66a* neurons (**Figure 1B** [DOI](#) and **1C** [DOI](#); note that the neuron numbers below refer to the number present in one of each paired ganglion). In the TOG, we found expression of all four *Gr28* genes, along with that

Gr66a, which was expressed in 3 or 4 neurons (one neuron exhibits often stronger expression of *Gr66a* than the two or three remaining neurons). This number slightly smaller than the 6 neurons reported using a distinct *Gr66a-GAL4* driver (Kwon et al., 2011). *Gr28ba*, *Gr28bc* and *Gr28be* are co-expressed in a single neuron with *Gr66a* (Figure 1B). In contrast, *Gr28a* is expressed in a distinct TOG neuron than *Gr66a* (Figure 1B), and by extension, in a different neuron than the co-expressed *Gr28b* genes, which we independently confirmed using a *Gr28bc-QF* driver (Figure 1-figure supplement 1). In the DOG, we find only a single *Gr28a* neuron, while none of the *Gr28b* genes, or *Gr66a*, is expressed there (Figure 1B). Expression in the internal sensory organs was similarly complex. *Gr66a* was found in one neuron in the DPS/VPS, co-expressed with *Gr28bc* as well as *Gr28a* (Figure 1C). None of the *Gr28b* genes is expressed in the PPS, but two neurons express *Gr28a*, one of which also expresses *Gr66a* (Figure 1C). In summary, the internal sensory organs can be subdivided in three distinct groups: *Gr66a/Gr28b/Gr28a* positive neurons, *Gr66a/Gr28a* positive neurons and *Gr28a^{only}* neurons, which contrasts expression in the external sensory organs where *Gr28a* and the *Gr28b* genes are mutually exclusive.

Subsets of *Gr28* neurons mediate opposing feeding behaviors

We previously showed that at least one of the six *Gr28* genes is necessary for feeding attraction to RNA, ribonucleosides and ribose using a well-established two choice feeding assay (Mishra et al., 2018). Specifically, larvae homozygous for a *Gr28* null allele, a deletion of the entire *Gr28a* gene and most of the coding region of all *Gr28b* genes ($\Delta Gr28$), lose their ability to sense these compounds, a phenotype that is restored when single *UAS-Gr28* genes are expressed in *Gr28a* neurons using *Gr28a-GAL4*. This observation and the largely non-overlapping expression of *Gr28a* and the *Gr28b* genes suggests that respective neurons represent functionally distinct entities. To investigate this possibility, we took advantage of the mammalian Vanilloid Receptor 1 (VR1), a TRP channel that is activated by capsaicin (Caterina et al., 1997). *Drosophila* have no *VR1* like-gene in their genome and do not respond to capsaicin behaviorally, but flies are attracted to this chemical when a modified *VR1* gene (*VR1E600K*) is expressed in sweet taste neurons (Marella et al., 2006). Thus, we expressed *VR1E600K* (henceforth referred to *UAS-VR1*) under the control of the four *Gr28-GAL4* drivers (*Gr28a-GAL4*, *Gr28ba-GAL4*, *Gr28bc-GAL4* and *Gr28be-GAL4*) in larvae and tested their response to capsaicin using the two-choice preference assay. Additionally, we expressed *UAS-VR1* in bitter neurons as well as sweet neurons using respective *GAL4* drivers (*Gr66a-GAL4* and *Gr43a^{GAL4}*) (Scott et al., 2001) (Miyamoto et al., 2012). Consistent with the observation in adults (Marella et al., 2006), *w¹¹¹⁸* control larvae and larvae carrying *UAS-VR1* or *Gr-GAL4* genes alone were unresponsive to 0.1 mM or 0.5 mM capsaicin (Figure 2A). However, larvae expressing *UAS-VR1* under the control of *Gr28a-GAL4* or *Gr43a-GAL4* showed strong appetitive responses to both capsaicin concentrations (Figure 2A). In contrast, larvae expressing *UAS-VR1* under the control of *Gr28bc-GAL4* or *Gr66a-GAL4* showed robust avoidance behavior to both concentrations of capsaicin. Lastly, expression of *UAS-VR1* with the *Gr28ba-GAL4* or *Gr28be-GAL4* driver, which is co-expressed in a single TO neuron with *Gr28bc* (Figure 1B), did cause neither attraction to nor avoidance of capsaicin.

Expression difference between *Gr28bc* and *Gr28ba/be* is a single neuron in the DPS/VPS (Figure 1C), a neuron required to mediate avoidance of capsaicin in *Gr28bc-GAL4*, *UAS-VR1* larvae. Even though this GRN also expresses *Gr28a*, it appears unlikely that it is necessary to mediate appetitive behavior as well. Indeed, we could further restrict the necessary set of appetitive neurons to four *Gr28a* positive neurons (*Gr28a^{only}* GRNs), as suppression of *VR1* expression in *Gr66a* GRNs (one being the *Gr28bc* expressing GRN in the DPS/VPS) did not affect capsaicin preference (Figure 2B). We further note that activation of the two *Gr28bc* neurons by capsaicin drives repulsion to the same degree as when all seven *Gr66a* expressing neurons are activated. Taken together, this analysis indicates that *Gr28a-GAL4* is expressed in appetitive inducing neurons, while *Gr28bc-GAL4* neurons mediate avoidance behavior.

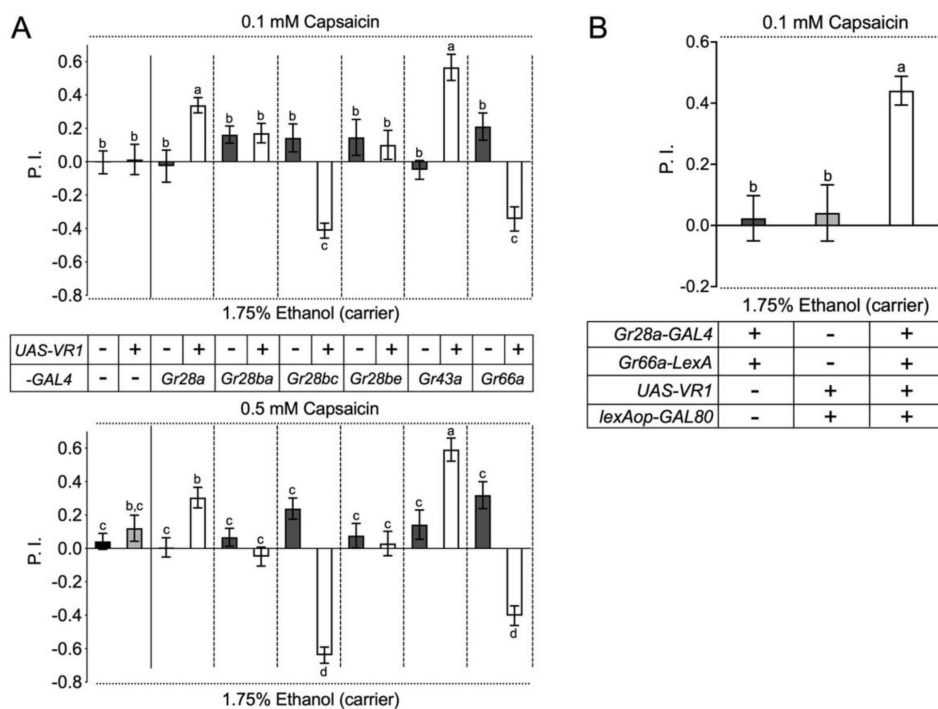


Figure 2.

Taste behavior of larvae mediated by different *Gr28* GRNs

A) *Gr28a* GRNs mediate capsaicin preference, while *Gr28bc* GRNs elicit capsaicin avoidance in larvae. Control larvae (w^{1118} , *UAS-VR1*/+ and *Gr* (indicated *Gr* genes)-*GAL4*/+) show no preference for or avoidance to 0.1 mM (top) or 0.5 mM (bottom) capsaicin. When expressing *UAS-VR1* in *Gr28a* GRNs (*Gr28a-GAL4/UAS-VR1*), larvae display robust taste preference for capsaicin for both 0.1 and 0.5 mM capsaicin. In contrast, when expressed in *Gr28bc* GRNs (*Gr28bc-GAL4/UAS-VR1*), larvae strongly avoid both 0.1 and 0.5 mM capsaicin. Neither avoidance nor preference was observed when VR1 was expressed in the *Gr28ba* or *Gr28be* GRNs. For comparison, expression of VR1 in sweet taste neurons (*Gr43a^{GAL4}/UAS-VR1*) or bitter taste neurons (*Gr66a-GAL4/UAS-VR1*) led to strong preference for or strong avoidance of capsaicin. Each bar represents the mean $\pm$ SEM of P.I. ($n = 11-42$ assays). The taste behavior of *Gr-GAL4 > UAS-VR1E600K* larvae is compared with 3 controls (w^{1118} , *UAS-VR1E600K*/+ & *Gr-GAL4*/+) by using one-way ANOVA with Bonferroni's multiple comparison tests ($p < 0.05$), whereby different letters indicate statistically significant difference. Dashed lines delineate groups for ANOVA. A short solid line indicates control groups.

B) *Gr28a^{only}* GRNs are necessary and sufficient for inducing capsaicin preference of larvae. When VR1 expression is restricted to GRNs not co-expressing *Gr66a* or *Gr28bc* (*Gr28a^{only}* GRNs) by means of *lexAop-GAL80* under control of *Gr66a-LexA*, larvae show strong preference for capsaicin. Each bar represents the mean $\pm$ SEM of P.I. ($n = 13-18$ assays). Bars with different letters are significantly different (one-way ANOVA with Bonferroni's multiple comparison tests ($p < 0.05$)). Fly genotypes: wild-type: w^{1118} (black), $w^{1118};UAS-VR1E600K$ /+ (light gray), $w^{1118};Gr28a-GAL4$ /+ (dark grey), $w^{1118};Gr28a-GAL4/UAS-VR1E600K$ (white), $w^{1118};Gr28ba-GAL4$ /+ (dark grey), $w^{1118};UAS-VR1E600K/+;Gr28ba-GAL4$ /+ (white), $w^{1118};Gr28bc-GAL4$ /+ (dark grey), $w^{1118};Gr28bc-GAL4/UAS-VR1E600K$ (white), $w^{1118};Gr28be-GAL4$ /+ (dark grey), $w^{1118};Gr28be-GAL4/UAS-VR1E600K$ (white), $w^{1118};Gr43a^{GAL4}/+ (dark grey), $w^{1118};Gr43a^{GAL4}/UAS-VR1E600K$ (white), $w^{1118};Gr66a-GAL4$ /+ (dark grey), $w^{1118};Gr66a-GAL4/UAS-VR1E600K$ (white), $w^{1118};UAS-VR1E600K/+;lexAop-GAL80$ /+ (light gray), $w^{1118};Gr66a-LexA/+;Gr28a-GAL4$ /+ (dark grey) and $w^{1118};Gr66a-LexA/UAS-VR1E600K;Gr28a-GAL4/lexAop-GAL80$ (white).$

Wild type larvae, like adult flies, avoid bitter compounds (Apostolopoulou et al., 2015 [↗](#)) (Apostolopoulou et al., 2016 [↗](#)) (Choi et al., 2016 [↗](#)) (van Giesen et al., 2016 [↗](#)) (Choi et al., 2020 [↗](#)). If *Gr28bc* neurons are indeed central to the avoidance of these chemicals in larvae, we expect that eliminating their function would result in loss of larval avoidance behavior to bitter chemicals. Thus, we inactivated neuronal subsets using the potassium inward rectifying channel Kir2.1 (Baines et al., 2001 [↗](#)) (Paradis et al., 2001 [↗](#)). In contrast to larvae with an intact taste neuron repertoire, larvae in which two *Gr28bc* neurons were inactivated no longer avoided any of these bitter compounds (**Figure 3** [↗](#)). A more subtle phenotype was observed when the single *Gr28bc* neurons were inactivated, eliminating lobeline and caffeine avoidance, but leaving denatonium and quinine repulsion intact, a finding consistent with previous studies (Choi et al., 2020 [↗](#)). These data suggest that two pairs of neurons, one in the TO and one in the D/VPS are necessary and sufficient for avoidance of all four bitter tasting chemicals.

Gr28bc and Gr28ba are subunits of a taste receptor complex for denatonium

We examined whether any of the Gr28 proteins is part of a taste receptor complex detecting these bitter chemicals (**Figure 4** [↗](#)). Surprisingly, only avoidance of denatonium was affected in larvae without any functional *Gr28* gene ($\Delta Gr28$), while responses to quinine, lobeline or caffeine was not significantly affected. Moreover, avoidance to caffeine increased significantly (**Figure 4A** [↗](#)). We next tested which Gr28 protein is necessary for denatonium avoidance and expressed individual *Gr28* genes under the control of the *Gr28bc-GAL4* driver. Only when either *Gr28ba* or *Gr28bc* was expressed in these neurons, denatonium avoidance was fully recovered, whereas expression of *Gr28bb*, *Gr28bd*, *Gr28be* or *Gr28a* failed to do so (**Figure 4B** [↗](#)). This observation indicates that despite the high level of similarity between these receptors, recognition of denatonium is dependent on specific Gr28b subunits, features that are present in Gr28ba and Gr28bc, but not any of the other Gr28 proteins.

Previous work had established that *Gr66a* is required for caffeine avoidance, in both larvae and adult flies (Moon et al., 2006 [↗](#)) (Lee et al., 2009 [↗](#)) (Apostolopoulou et al., 2016 [↗](#)). We confirmed this, as *Gr66a* mutant larvae show little if any avoidance of caffeine, while avoidance of lobeline was not affected (**Figure 4-figure supplement 1** [↗](#)). Somewhat surprisingly, avoidance of denatonium and quinine increased significantly. This is likely a result of the heteromeric composition of bitter taste receptor complexes, whereby absence of Gr66a allows Grs that tuned to denatonium (such as Gr28bc or Gr28ba) and quinine be incorporated into multimeric receptor complexes.

Finally, we investigated neuronal responses in larvae expressing the Ca^{2+} indicator GCaMP6m in *Gr28bc-GAL4* GRNs (**Figure 5** [↗](#)). We developed a whole larvae imaging preparation, whereby larvae were placed in an “imaging chamber” to minimize head movements (**Figure 5A** [↗](#)), and visualized neural activity in real time (Chen et al., 2013 [↗](#)) upon exposure to the four bitter compounds, as well as sucrose, ribose and fructose (**Figure 5B** [↗](#) to **5D** [↗](#)). All bitter compounds elicited rapid Ca^{2+} increases in *Gr28bc* GRNs in the TO, while none of the sugars did (**Figure 5C** [↗](#) and **5D** [↗](#)). When neural activity was recorded in $\Delta Gr28$ homozygous mutant larvae (**Figure 5E** [↗](#)), cellular responses to denatonium and quinine were severely reduced, while responses to both caffeine and lobeline were not affected. Consistent with our behavioral analysis (**Figure 4B** [↗](#)), re-expression of either *Gr28ba* or *Gr28bc*, but not *Gr28bb*, *Gr28bd*, *Gr28be* or *Gr28a* rescued cellular response to denatonium, but not to quinine (**Figure 5E** [↗](#)). Together, these experiments identified *Gr28bc* and *Gr28ba* as redundant subunit of a denatonium receptor complex, a complex that does not require *Gr66a* or any of the other *Gr28b* subunits.

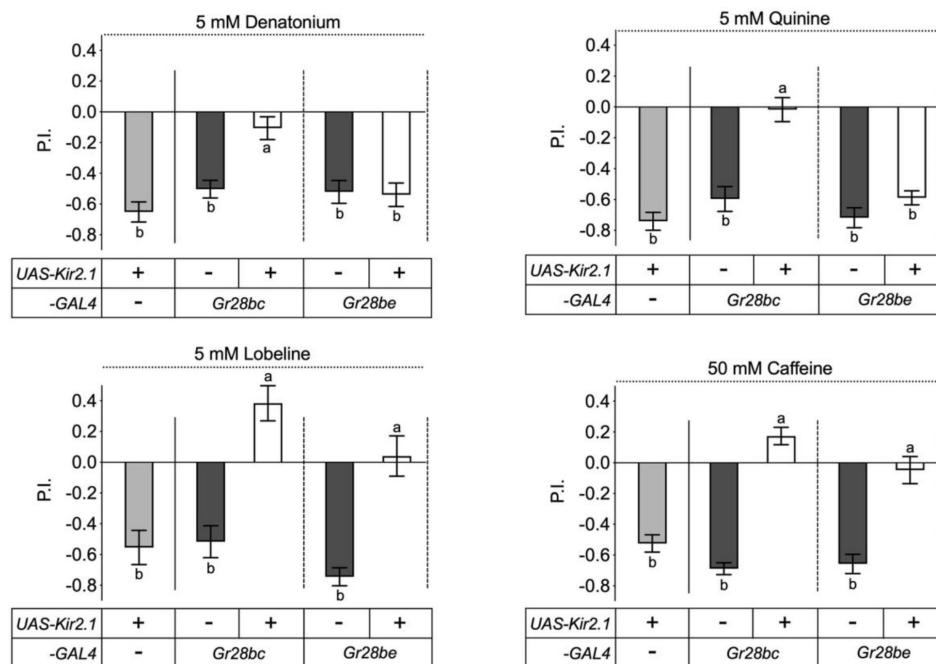


Figure 3.

The *Gr28b* neurons mediate avoidance behavior to bitter compounds

Inactivation of *Gr28bc* GRNs (*Gr28bc-GAL4/UAS-Kir2.1*) elicits significantly reduced avoidance of larvae to all four bitter compounds tested: denatonium, quinine, lobeline and caffeine, while control larvae carrying either the driver or the reporter only showed strong avoidance of these compounds. In contrast, flies with inactivated *Gr28be* GRNs (*Gr28be-GAL4/UAS-Kir2.1*) still avoid denatonium and quinine (top), but no longer avoid lobeline and caffeine (bottom). Each bar represents the mean $\pm$ SEM of P.I. ($n = 11-20$ assays). The taste behavior of *Gr-GAL4 > UAS-Kir2.1* larvae is compared with 2 controls (*UAS-Kir2.1/+* & *Gr28b-GAL4/+*) by using Kruskal-Wallis test by ranks with Dunn's multiple comparison tests ($p < 0.05$). Bars with different letters are significantly different. Dashed lines delineate groups for ANOVA. A short solid line indicates a control. Fly genotypes: $w^{1118}; UAS-Kir2.1/+$ (light grey), $w^{1118}; Gr28bc-GAL4/+$ (dark grey), $w^{1118}; Gr28bc-GAL4/UAS-Kir2.1$ (white), $w^{1118}; Gr28be-GAL4/+$ (dark grey), $w^{1118}; Gr28be-GAL4/UAS-Kir2.1$ (white).

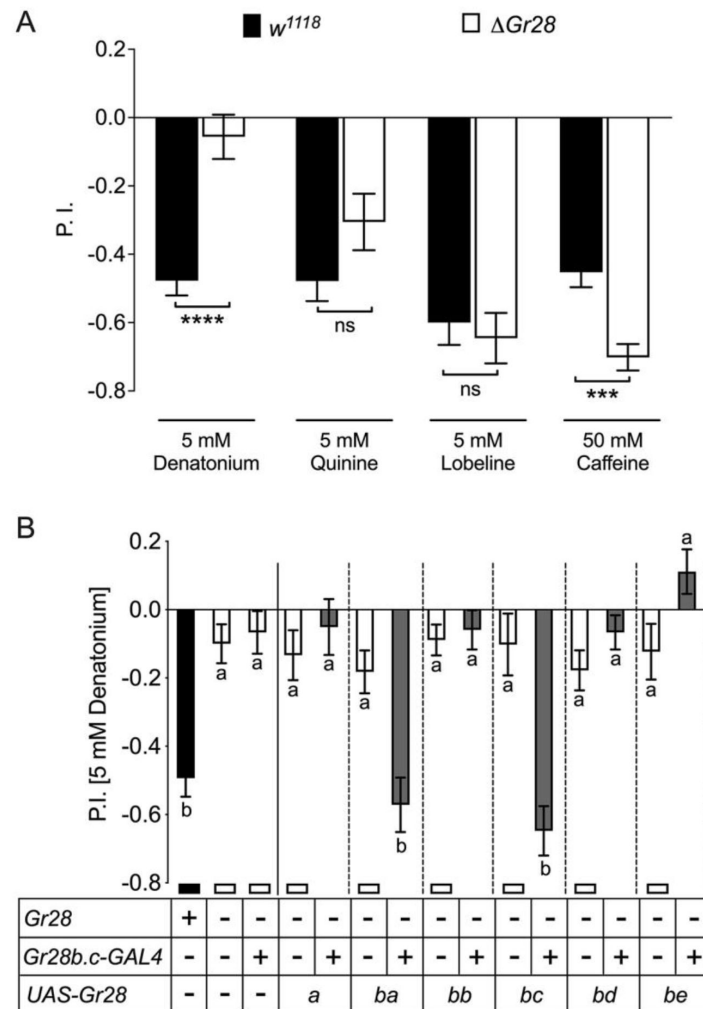


Figure 4.

Role of individual *Gr28* genes in bitter taste avoidance

A) Some of the *Gr28* genes are strictly required for sensing denatonium. Wild-type (w^{1118}) larvae, but not *Gr28* mutant larvae, strongly avoid denatonium. In contrast, *Gr28* mutant ($w^{1118};\Delta Gr28/\Delta Gr28$) larvae do not show significantly reduced avoidance of quinine, lobeline and caffeine. Each bar represents the mean $\pm$ SEM of P.I. ($n = 12-22$ assays). Asterisks indicate a significant difference between the *Gr28* mutant and wild-type larvae (Two-tailed, Mann-Whitney U test, **** $p < 0.0001$, *** $p < 0.001$, ns: not significant).

B) Single *Gr28b* genes can rescue avoidance response to denatonium when expressed in *Gr28bc* neurons of *Gr28* mutant larvae. Each bar represents the mean $\pm$ SEM of P.I. ($n = 11-22$ assay). The behavior of *Gr28* mutant larvae expressing *UAS-Gr28* transgenes under control of the *Gr28bc-GAL4* driver was compared to *Gr28^{+/+}* control (w^{1118} , filled bar), and three *Gr28* mutant controls ($\Delta Gr28$, $\Delta Gr28$ plus driver, and $\Delta Gr28$ plus respective *UAS-Gr28* reporter, plain bar) using Kruskal-Wallis test by ranks with Dunn's multiple comparison tests ($p < 0.05$). Bars with different letters are significantly different. Fly genotypes: wild-type: w^{1118} (black), mutants: $w^{1118};\Delta Gr28/\Delta Gr28$, $w^{1118};\Delta Gr28/\Delta Gr28$ *Gr28bc-GAL4*, $w^{1118};\Delta Gr28/\Delta Gr28;UAS-Gr28$ (indicated *Gr28* genes)/+ and $w^{1118};\Delta Gr28/\Delta Gr28$ *UAS-GCaMP6m;UAS-Gr28* (for *Gr28bb* or *Gr28bc* genes)/+ (white), rescues: $w^{1118};\Delta Gr28/\Delta Gr28$ *Gr28bc-GAL4;UAS-Gr28* (indicated *Gr28* genes)/+ and $w^{1118};\Delta Gr28$ *UAS-GCaMP6m/\Delta Gr28* *Gr28bc-GAL4;UAS-Gr28* (for *Gr28bb* or *Gr28bc* genes)/+ (grey).

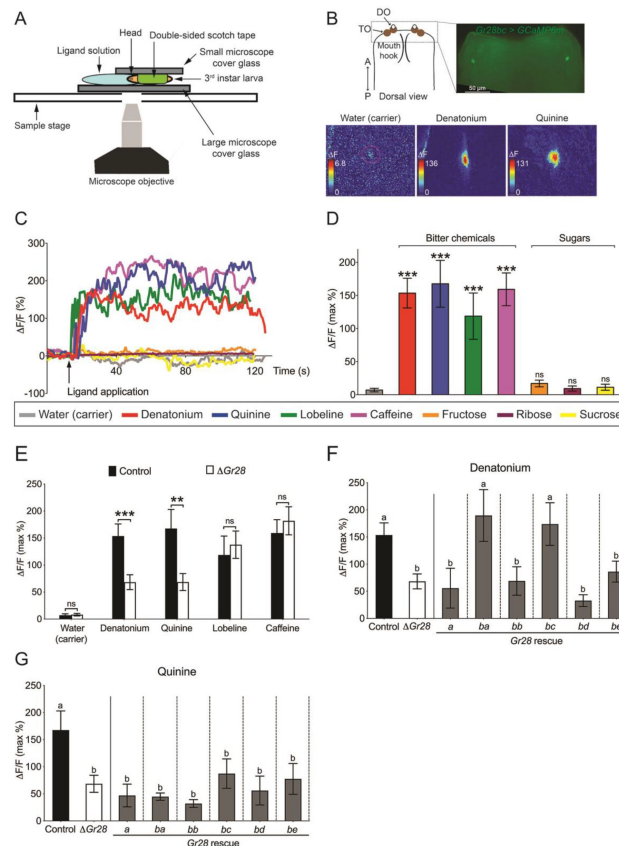


Figure 5.

Cellular activation of *Gr28bc* GRNs by bitter compounds that require *Gr28ba* or *Gr28bc* function.

A) Diagram of Ca^{2+} imaging experimental set up.

B) Representative still images of Ca^{2+} response in the *Gr28bc* expressing TO neuron. Ca^{2+} responses of the *Gr28bc* GRNs upon stimulation with indicated ligands. ΔF indicates the changes in fluorescence light intensity of the cell body before/after ligand application.

C & D) Representative traces (C) and quantified Ca^{2+} responses (D) of the *Gr28bc* GRNs after stimulation with indicated ligands. Fly genotype: *w¹¹¹⁸; Gr28bc-GAL4/UAS-GCaMP6m*. Each bar represents the mean $\pm$ SEM of Ca^{2+} imaging with 12 – 16 larvae. Asterisks indicate a significant difference between carrier (water) and indicated ligands (Two-tailed, Mann-Whitney U test, *** $p < 0.001$, ns: not significant).

(E) Neurons of larvae lacking the *Gr28* genes exhibit significantly reduced responses to denatonium and quinine. The *Gr28bc* expressing TO GRN of *Gr28* mutant larvae ($\Delta Gr28$) display significantly reduced Ca^{2+} responses to denatonium and quinine but not to lobeline or caffeine when compared to *Gr28bc* expressing TO of wild type controls. Responses to denatonium, but not quinine, is restored by expression of single *Gr28b* (*Gr28ba* or *Gr28bc*) genes (grey). Larvae genotypes: *Gr28⁺* control (black bar): *w¹¹¹⁸; Gr28bc-GAL4/UAS-GCaMP6m*. *Gr28⁻* control (white bar): *w¹¹¹⁸; $\Delta Gr28$ Gr28bc-GAL4/ $\Delta Gr28$ UAS-GCaMP6m*. Each bar represents the mean $\pm$ SEM with 13 – 16 larvae. Asterisks indicate a significant difference between *Gr28⁺* control and *Gr28* mutant ($\Delta Gr28$) larvae (Two-tailed, Mann-Whitney U test, *** $p < 0.001$, ** $p < 0.01$ ns: not significant).

(F and G) *Gr28bc* or *Gr28ba* rescues denatonium responses in GRNs of $\Delta Gr28$ larvae. Expression of *Gr28bc* or *Gr28ba* in under control of *Gr28bc-GAL4* restores responses to denatonium, but not quinine in GRNs of $\Delta Gr28$ larvae. Each bar represents the mean $\pm$ SEM of Ca^{2+} imaging with 12 – 17 larvae. The Ca^{2+} responses of *Gr28* mutant larvae expressing *UAS-Gr28* transgenes under *Gr28bc-GAL4* driver is compared to *Gr28⁺* (black) and *Gr28⁻* (white) controls by using Kruskal-Wallis test by ranks with Dunn's multiple comparison tests ($p < 0.05$). Bars with different letters are significantly different. Dashed lines delineate groups for ANOVA. A short solid line indicates control groups. Fly genotypes: *Gr28⁺* control (black bar): *w¹¹¹⁸; Gr28bc-GAL4/UAS-GCaMP6m*. *Gr28⁻* control (white bar): *w¹¹¹⁸; $\Delta Gr28$ Gr28bc-GAL4/ $\Delta Gr28$ UAS-GCaMP6m*. *Gr28* rescues (grey bar): *w¹¹¹⁸; $\Delta Gr28$ Gr28bc-GAL4/ $\Delta Gr28$ UAS-GCaMP6m; UAS-Gr28/+*. Concentrations of ligands were 100 mM for sugars, 50 mM for caffeine and 5 mM for denatonium, quinine and lobeline.

The Gr28 receptors comprise one of the few Gr subfamilies conserved across diverse insect species (Engsontia and Satasook, 2021 [↗](#))(Agnihotri et al., 2016 [↗](#))(Yu et al., 2023 [↗](#)). Yet, they were the least characterized when compared to other conserved subfamilies, such as the sugar receptors (Gr5a, Gr61a and Gr64a-f), the carbon dioxide receptors Gr21a and Gr63a or the bitter taste receptors. The only ligands associated with the Gr28 proteins were ribonucleosides and RNA, which are appetitive nutrients for larvae and are mediated by *Gr28a* neurons (Mishra et al., 2018 [↗](#)). Indeed, RNA has been found to be an appetitive taste ligand across many dipteran insects, including mosquitoes, and we showed that Gr28 homologs of both *A. aegypti* and *A. gambiae* can rescue the preference for RNA and ribose when expressed in *Gr28a* neurons of Δ Gr28 mutant larvae (Fujii et al., 2023 [↗](#)). Here, we established a precedent for members of the same subfamily for mediating both appetitive and repulsive behavior and being expressed in largely non-overlapping sets of GRNs. In flies, members of other conserved Gr subfamilies are either precisely (*Gr21a* and *Gr63a*)(Jones et al., 2007 [↗](#))(Kwon et al., 2007 [↗](#)), or largely (sugar *Gr* genes) (Fujii et al., 2015 [↗](#)) co-expressed in one type of GRNs, forming multimeric receptors that mediate a single type of behavior.

Our expression analysis in larvae is consistent with earlier reports, despite some variation in number of neurons expressing *Gr66a* and *Gr28b* (Kwon et al., 2011 [↗](#))(Choi et al., 2016 [↗](#)), which is likely due to the use of different *GAL4* drivers and/or variability in expression. Importantly, all *Gr28b* genes are co-expressed with the bitter marker *Gr66a* (Figure 1 [↗](#)), while *Gr28a* is found in a largely, but not entirely distinct set of GRNs. Based on our VR1-capsaicin experiments, the GRNs sharing expression of both *Gr28a* and *Gr66a* (and by extension some *Gr28b* genes) are critical for avoidance, and not required for appetitive behavior (Figure 2 [↗](#)).

Distinct functions of small set of GRNs expressing specific Gr28 subunits

A key finding presented of this study is the observation that *Gr28a^{only}* and *Gr28bc* neurons dictate distinct behavioral programs, the former representing an ensemble of neurons that instruct a larvae to “go towards” chemical source and consume it, while the latter do the opposite (Figure 2 [↗](#)). The set of “go-away” GRNs co-expressing *Gr28bc* and *Gr66a* is remarkably small, consisting of only two pairs, one in the TO and the other in the DPS/VPS. It seems likely that this is the smallest, minimal subset of neurons sufficient for avoidance, as expression of V1R in only the TO pair (under the control of either *Gr28ba-GAL4* or *Gr28be-GAL4*) has no behavioral effect when challenged with capsaicin. The “go-to” neurons are characterized by expression of *Gr28a* and represent a slightly larger set of four GRN pairs (*Gr28a^{only}* only GRNs). Thus, we propose that the minimal requirement to induce “go-to” and “go-away” behavior is defined by distinct sets of GRNs, and that each appears to be composed of neurons located in both external as well as the internal taste organs (Figure 6 [↗](#)).

Co-expression of a subunit for the bitter taste receptors Gr28bc/Gr28ba and Gr28a, a receptor for RNA, a nutrient essential for larval growth (Mishra et al., 2018 [↗](#)) in bitter taste neurons raises interesting questions about the function of Gr28a. As these neurons are necessary for repulsion, we propose that Gr28a might serve as a subunit along with other Grs (possibly Gr28bc and/or Gr66a) in heteromeric bitter taste receptor complexes, detecting yet other bitter compounds. In “go-to”, *Gr28a^{only}* GRNs, Gr28a is likely to form a homomultimeric receptor, since its expression in fructose sensing GRNs confers RNA recognition to these neurons, both in cellular and behavioral assays (Mishra et al., 2018 [↗](#)). We note that the sweet taste receptor Gr5a, a subunit of a multimeric receptor in sweet taste GRNs in the labial palps of flies is also expressed in non-sweet neurons of unknown function (Fujii et al., 2015 [↗](#)). Finally, while Gr28bc was shown to be required

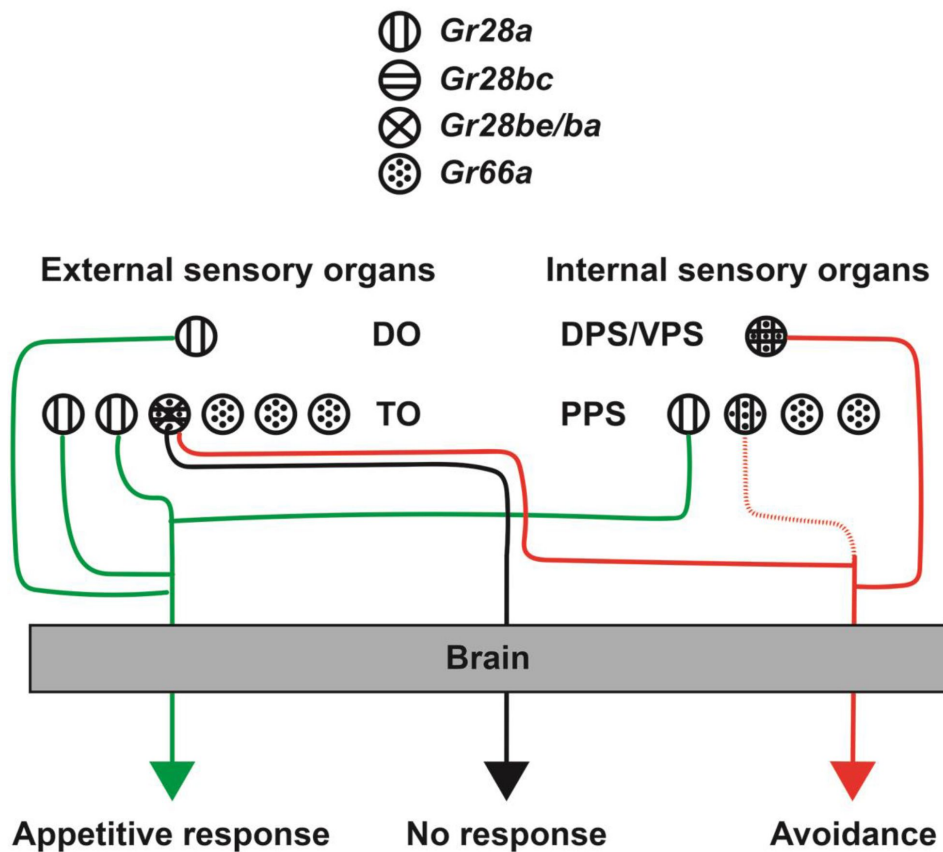


Figure 6

Role of different GRN subsets in taste behavior of larvae

GRNs mediating avoidance behavior express *Gr28bc*, while GRNs necessary for feeding preference express *Gr28a* alone (*Gr28a^{only}* GRNs). Note that each ensemble is composed of at least a pair of neurons located in the external taste organs and a pair of neurons in the internal taste organs.

for detecting and avoiding 5% saponin in flies (Sang et al., 2019 [↗](#)), larvae showed no behavioral response to 0.1% of saponin (Choi et al., 2016 [↗](#))(Choi et al., 2020 [↗](#)), a finding we independently confirmed using both concentration of saponin (data not shown).

Mapping ligand specificity for denatonium

Only Gr28ba and Gr28bc can rescue responses to denatonium when expressed in *Gr28bc* GRNs of Δ Gr28 mutant larvae. Thus, sequence comparison between the unique N-terminal halves of the Gr28 proteins might provide insights as to possible residues important for denatonium recognition/binding, especially residues located in extracellular loops (EL) and transmembrane (TM) regions. When interrogating TM domains 1 to 4 and EL1 and EL2, only seven residues are identical between Gr28ba and Gr28bc (Table 1 [↗](#), Figure 7 [↗](#)). Reducing the stringency requirement by allowing one of the remaining receptors to share the same residue, nine additional sites are identified. Interestingly, five of these sixteen amino acid residues map to four of the nine most probable binding pocket as predicted by Prankweb (<https://prankweb.cz/> [↗](#)), with three residues part of the pocket with the highest probability score (Table 2 [↗](#), Figure 8 [↗](#)). Thus, targeted substitutions of single amino acids residing in predicted ligand binding pockets in other Gr28 proteins and testing such “chimera” provides a theoretical framework for practical experiments using either behavior analysis or Ca²⁺ imaging to precisely define the specific requirement for denatonium specificity. Additional complementary approaches can be taken to identify the critical residues in the predicted binding pockets of other members of this family once their ligands have been identified. Thus, the Gr28 proteins might serve as model to experimentally validate the predictions created by these powerful algorithms.

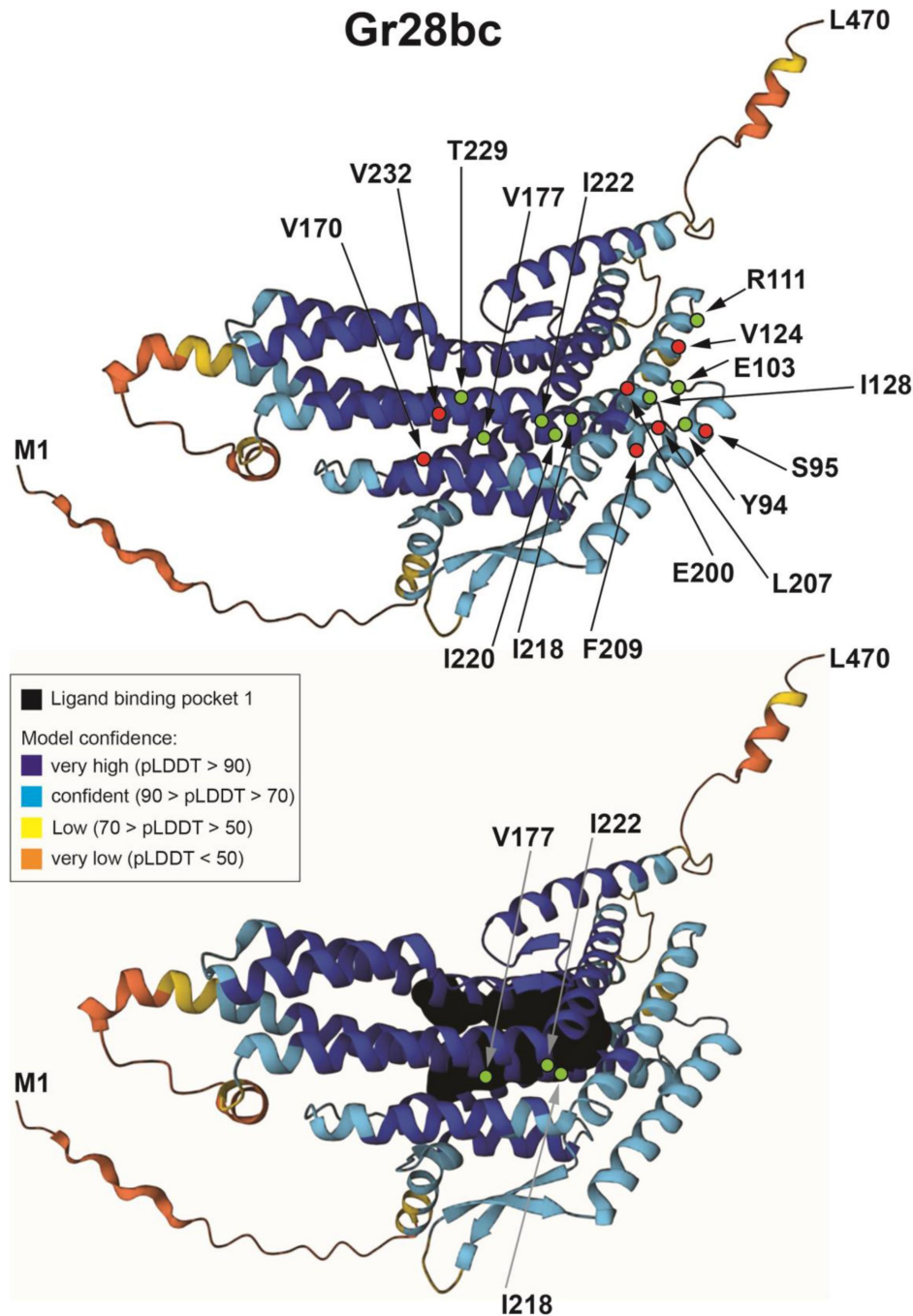


Figure 8.

Identification of candidate amino acids for ligand binding pockets of Gr28bc.

Top: Sixteen amino acids conserved in both Gr28ba and Gr28bc are mapped on 3D structure of Gr28bc using Alphafold (<https://www.alphafold.ebi.ac.uk/>). Red circles indicate amino acid locations of residues identical in Gr28ba and Gr28bc. Green circles indicate amino acid locations of residues identical in one additional Gr28 protein (Gr28bb or Gr28a). Bottom: Prediction of putative ligand binding pocket in of Gr28bc with highest probability using Prankweb (<https://prankweb.cz/>). The three residues part of this pocket and identical between Gr28ba and Gr28bc are indicated in green (V177, I218 & I222). The color displayed in the protein structure indicates a per-residue confidence score (pLDDT) between 0 and 100 as predicted by Alphafold.

Location	Residue (Gr28bc)	Other (if applicable)
EL1	E103	Gr28bb
	R111	Gr28bb
EL2	E200	none
	Y94	Gr28bb
	S95	none
TM2	V124	none
	I128	Gr28bb
TM3	V170	none
	V177	Gr28bb
TM4	L207	none
	F209	none
	I218	Gr28a
	I220	Gr28bb
	I222	Gr28bb
	T229	Gr28a
	V232	none

The 7 amino acid residues identical in the amino-terminal region of Gr28bc and Gr28ba are shown in bold. The nine additional amino acids also identical in one additional Gr28 proteins are also listed. These residues are considered potentially critical for recognition of denatonium, since only Gr28ba and Gr28bc can rescue response to denatonium when expressed in *Gr28bc* neurons of $\Delta Gr28/\Delta Gr28$ mutant larvae.

Table 1.

Conserved amino acids in the amino termini of Gr28ba and Gr28bc

Name	Score	Probability	Residue_ids
Pocket 1	10.35	0.533	F173, V177 , S180, M181, V184, N185, F188, H215, I218 , A219, I222 , S226, L357, Y358, I360, A361, S364, G368, R371
Pocket 2	4.54	0.172	F117, M121, F318, L319, V322, F323, Y436, I439, L440
Pocket 3	4.44	0.166	S104, V105, A106, Q122, I123, L207 , T210, F211, Q214, F350, Q354
Pocket 4	3.47	0.11	I314, S315, F318, F419, L425, T428, I429, A432, T436
Pocket 5	3.17	0.093	L178, M181, F182, N185, T216, I220 , V151, H55
Pocket 6	2.36	0.05	V105, T192, L196, L207, H208, F211, F350
Pocket 7	2.01	0.037	L359, I362, I363, Y426, I429, S430, L433
Pocket 8	1.45	0.017	I213, T216, H55, H86
Pocket 9	1.07	0.007	R46, F49, H50, Y53, Y61, I62, F78

Probability and expansion of predicted ligand binding sites in Gr28bc as predicted by Prankweb (<https://prankweb.cz/>). Amino acid residues identical in Gr28ba and Gr28bc alone are in ***bold & italic***, or in **bold** only if a residue is conserved in one additional Gr28 protein.

Table 2.

Prediction of ligand binding sites from protein structure of Gr28bc

Key resources table

Reagent type	Designation	Source or Reference	Identifiers
Antibodies			
Rabbit polyclonal anti-GFP	Anti-GFP	Thermo Fisher Scientific	A6455; RRID: AB_221570
Rat monoclonal anti-mCD8	Anti-mCD8	Thermo Fisher Scientific	MCD0800; RRID: AB_10392843
Alexa 488 conjugated goat anti-rabbit	Green	Thermo Fisher Scientific	A11070; RRID: AB_2534114
Cy3-conjugated goat anti-rat	Magenta	Jackson ImmunoResearch Laboratories Inc.	112-165-072; RRID: AB_2338248
Normal goat serum	Blocking of non-specific antibody binding in tissue staining	SouthernBiotech	0060-01
Chemical compounds			
Caffeine	Caffeine	Millipore-Sigma	C0750
Capsaicin	Capsaicin	Millipore-Sigma	M2028
Denatonium benzoate	Denatonium benzoate	Millipore-Sigma	D5765
Lobeline hydrochloride	Lobeline hydrochloride	Millipore-Sigma	141879
D-(-)-ribose	D-(-)-ribose	Millipore-Sigma	R7500
Quinine hydrochloride dihydrate	Quinine hydrochloride dihydrate	Millipore-Sigma	Q1125
Fructose	Fructose	Spectrum chemical	F1092
Agarose	Agarose	Apexbio	20-102
Sucrose	Sucrose	Macron fine chemicals	8360-06
Charcoal	Charcoal	J.T. Baker	1560-01
Experimental Models: Organisms/Strains			
<i>D. melanogaster</i> : <i>w</i> ¹¹¹⁸	Wild-type control	Bloomington <i>Drosophila</i> Stock Center	BDSC: 3605; FlyBase: FBst0003605
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> { <i>Gr28a-GAL4</i> . <i>T</i> } <i>SF36S</i>	<i>Gr28a-GAL4</i>	Thorne and Amrein, 2008	FlyBase: FBtp0056017
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> { <i>Gr28a-GAL4</i> . <i>T</i> } <i>SF36B1</i>	<i>Gr28a-GAL4</i>	Thorne and Amrein, 2008	FlyBase: FBtp0056017
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> { <i>Gr28b.a-GAL4</i> } <i>NT42aC51a</i>	<i>Gr28ba-GAL4</i>	Thorne and Amrein, 2008	FlyBase: FBtp0054526
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> { <i>Gr28b.c-GAL4</i> } <i>NT21B1</i>	<i>Gr28bc-GAL4</i>	Thorne and Amrein, 2008	FlyBase: FBtp0054528
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> { <i>Gr28b.e-GAL4</i> .4.245} <i>Gr28a3All</i>	<i>Gr28be-GAL4</i>	Scott et al., 2001	FlyBase: FBtp0014672
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>Df</i> (2L) <i>ΔGr28</i>	<i>ΔGr28/ΔGr28</i>	Mishra et al., 2018	FlyBase: FBab0049019

<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> {Gr66C1-GAL4.3.153}	Gr66a-GAL4	Scott et al., 2001	FlyBase: FBtp0014661
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> {UAS-Gr28a.G}attP2	UAS-Gr28a	Ni et al., 2013	Flybase: FBal0344045
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> {UAS-Gr28b.A}attP2	UAS-Gr28ba	Ni et al., 2013	Flybase: FBal0291410
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> {UAS-Gr28b.B}attP2	UAS-Gr28bb	Ni et al., 2013	Flybase: FBal0291412
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> {UAS-Gr28b.C}attP2	UAS-Gr28bc	Ni et al., 2013	Flybase: FBal0291411
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> {UAS-Gr28b.D}attP2	UAS-Gr28bd	Ni et al., 2013	Flybase: FBal0291409
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> {UAS-Gr28b.E}attP2	UAS-Gr28be	Ni et al., 2013	Flybase: FBal0291408
<i>D. melanogaster</i> : <i>w</i> ¹¹¹⁸ ; <i>Ti</i> {GAL4}Gr43a ^{GAL4}	Gr43a ^{GAL4}	Miyamoto et al., 2012	BDSC:93447; Flybase: FBst0093447
<i>D. melanogaster</i> : <i>w</i> ¹¹¹⁸ ; <i>P</i> {UAS-VR1E600K}	UAS-VR1E600K	Marella et al., 2006	Flybase: FBal0215202
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> {lexAop-rCD2-GFP}	lexAop- rCD2:GFP	Lai and Lee, 2006	Flybase: FBst0066687
<i>D. melanogaster</i> : <i>y</i> ¹ <i>w</i> [*] ; <i>P</i> {10XUAS-IVS- mCD8::RFP}su(Hw)attP8	UAS-mCD8:RFP	Bloomington <i>Drosophila</i> Stock Center	BDSC: 32220; Flybase: FBti0131987
<i>D. melanogaster</i> : <i>w</i> ¹¹¹⁸ ; <i>P</i> {20XUAS-IVS- GCaMP6m}attP40	UAS-GCaMP6m	Bloomington <i>Drosophila</i> Stock Center	BDSC: 42748; Flybase: FBti0151346
<i>D. melanogaster</i> : <i>w</i> [*] ; <i>P</i> {UAS- Hsap KCNJ2.EGFP}1	UAS-Kir2.1-GFP	Baines et al., 2001; Paradis et al., 2001	Flybase: FBst0006596
<i>D. melanogaster</i> : <i>w</i> ¹¹¹⁸ ; <i>P</i> {Gr66a- lexA.S}2; TM2/TM6B	Gr66a-LexA	Thistle et al., 2012	BDSC: 93024 Flybase: FBst0093024
Sequence-based reagents			
Gr28bc_F	5'- AATCTAGGTAC CCCGGCTGCT CGTCTCCCTG GATGT-3'	This paper	PCR primers
Gr28bc_R	5'- CGTCAAACCTAG TGACCGCTTCG TTTGAGCTTCA ACC-3'	This paper	PCR primers
Recombinant DNA reagents			
LexA vector CMC105	Insect expression vector	This paper Larsson et al., 2004	Plasmids
Software and Algorithms			
NIS-Elements	N/A	Nikon	N/A
Prism software 9.5.1	Prism software 9.5.1	GraphPad Software	N/A
Adobe photoshop 2022	Adobe photoshop 2022	Adobe	N/A
Other			
Nikon Eclipse Ti inverted microscope	Nikon Eclipse Ti inverted microscope	Nikon	N/A
Nikon A1 confocal microscope	Nikon A1R confocal microscope system	Nikon	N/A
Pertri dish, 60 x 15 mm	Pertri culture dish	Falcon	REF353004
Microscope cover glass, 24 x 50 mm	Microscope cover glass	VWR	16004-098
Microscope cover glass, 12CIR-1	Microscope cover glass	Thermo Fisher Scientific	1254580

Drosophila Stocks

Flies were maintained on standard corn meal food in plastic vials under a 12 h light/dark cycle at 25°C. The *w¹¹¹⁸* strain (Bloomington Drosophila Stock Center, number 3605) was used as a wild-type control. Fly strains used: *Gr28a-GAL4(SF36S)* for immunostaining and **Figure 2B**, and *SF36B1* for all other experiments), *Gr28ba-GAL4(NT42aC51a)*, *Gr28bc-GAL4(NT21B1)* (Thorne and Amrein, 2008); *Gr28be-GAL4(Gr28a3AI)* and *Gr66a-GAL4* (Scott et al., 2001); *ΔGr28(54B3)* (Mishra et al., 2018); *UAS-Gr28a*, *UAS-Gr28ba*, *UAS-Gr28bb*, *UAS-Gr28bc*, *UAS-Gr28bd* and *UAS-Gr28be* (Ni et al., 2013); *Gr43a^{GAL4}* (Miyamoto et al., 2012); *Gr66a-LexA* (Thistle et al., 2012); *UAS-VR1E600K* (Marella et al., 2006); *UAS-Kir2.1-GFP* (Baines et al., 2001)(Paradis et al., 2001); *lexAop-rCD2:GFP* (Lai and Lee, 2006), *UAS-GCaMP6m*, *UAS-mCD8:RFP* and *Gr66a^{ex83}* (Bloomington Drosophila Stock Center, numbers 42748, 32220 and 35528)

Chemicals

Caffeine (Cat No. C0750), capsaicin (Cat No. M2028), denatonium benzoate (Cat No. D5765) and lobeline hydrochloride (Cat No. 141879), D(-)-ribose (Cat No. R7500) and quinine hydrochloride dihydrate (Cat No. Q1125) were purchased from Millipore-Sigma, with a purity of > 95%. Fructose (Cat No. F1092) and agarose (Cat. No. 20-102) were purchased from Spectrum chemical and Apexbio, respectively. Sucrose (Mfr. No. 8360-06) and charcoal (Cat No. 1560-01) were purchased from Macron fine chemicals and J.T. Baker, respectively. A stock solution for capsaicin (20 mM) was prepared in 70% ethanol and stored at 4° C protected from light for up to one year. Stock solutions for bitter chemicals were prepared in Millipore Q water and stored at -20° C. Stock solutions for sugars were prepared in Millipore Q water and stored at 4° C for up to one month. A stock solution for ribose was treated with charcoal (10% of the weight of ribose used for stock solution) overnight at 4° C and sterile-filtrated (0.45 μm) to remove unrelated odor. Stock solutions were diluted to the final concentration using Millipore Q water prior to each experiment.

Immunofluorescence

Immunofluorescence of larval heads was performed based on the protocol described in Croset and colleagues (Croset et al., 2016) with minor modification. Heads of third instar larvae were dissected using microscissors in phosphate buffered saline (PBS) and immediately fixed in PBS with 4% paraformaldehyde for 1 hour at 4° C. Heads were washed six times in washing buffer (PBS with 0.1% Triton X-100) for 20 min and blocked for 1 h in washing buffer containing 5% heat-inactivated goat serum (SouthernBiotech, Cat. No. 0060-01), followed by incubation with the primary antibodies (rabbit anti-GFP, 1:1,000 dilution; rat anti-mCD8, 1:200 dilution, ThermoFisher Scientific) at 4°C overnight. Heads were washed six times for 20 min in washing buffer and blocked in washing buffer containing 5% heat-inactivated goat serum for 1 hour. Heads were incubated with the secondary antibodies (goat anti rabbit ALEXA 488, 1:500 dilution, ThermoFisher Scientific; goat anti-rat Cy3, 1:300 dilution, Jackson ImmunoResearch Laboratories Inc.) at 4°C overnight. Heads were washed six times in washing buffer for 20 min. Washes were carried out at room temperature under gentle agitation. Heads were mounted with VectaShield (Vector Lab, Cat No. H-1200) on a microscope slide and images were obtained using a Nikon A1R confocal microscope system. Adobe photoshop 2022 was used further to process images.

Larval two-choice preference assay

Two-choice preference assay of larvae was conducted as described in Mishra et al. (Mishra et al., 2013) with minor modification. Flies were placed on standard corn meal food in plastic vials and allowed to lay eggs for 24 h under a 12 h light/dark cycle at 25°C. Flies were removed from food vials and feeding-stage third-instar larvae were collected. All dishes for two-choice preference assay were prepared just prior each experiment. Petri dishes (60 x 15 mm, Falcon, Cat. No. REF353004) with two halves marked on the bottom were filled with melted plain 1% agarose

or 1% agarose containing 1.75% ethanol (for capsaicin preference). After the agarose solidified, one half was removed and replaced with 1% agarose solution containing taste ligands (capsaicin or bitter compound). For each experiment, 15 larvae from food vials were briefly rinsed twice with Millipore Q water and placed along the middle separating pure and ligand containing agarose. After 16 min, photos were taken for record keeping and used to calculate larval preference indices. Larvae that crawled onto the wall of a dish or dug in the agarose were excluded. The preference index (P.I.) was calculated as follow: $PI = (N_{\text{tastant}} - N_{\text{plain}}) / N_{\text{Total}}$, whereby N is the number of larvae in the tastant sector, the plain agarose sector and the total number on the plate. Positive values indicate a preference for capsaicin or bitter compound while negative values indicate repulsion (avoidance).

Calcium imaging

Calcium imaging was performed in *Gr28bc* GRNs expressed in the terminal organ of feeding-stage, third instar larvae, reared as described for the larval two-choice preference assay. For each experiment, larvae from food vials were briefly rinsed twice with Millipore Q water and were mounted dorsally on a large microscope cover glass (24 x 50 mm, VWR, Cat. No. 16004-098) using double-sided scotch tape and covered with a small microscope cover glass (12CIR-1, Thermo Fisher Scientific, Cat. No. 1254580). Millipore Q water (40 μ l) was applied to the tip of the larval head, and the preparation was placed on the stage of a Nikon eclipse Ti inverted microscope. Images were obtained every 500 ms, starting 15 s before application and ending 105 s after ligand application. Each recording was initiated by applying water (40 μ l) to set a baseline. The first ligand solution (40 μ l of bitter chemical or sugar) was applied thereafter, followed by 5 washes with carrier (100 μ l of water). After a 3-minute pause to allow the preparation to recalibrate, a second ligand solution (40 μ l bitter chemical or sugar) was applied. To assure validity in experiments with *Gr28* mutants and rescues, each recording was concluded with application of caffeine, and recordings were included only if caffeine generated a positive response. Baseline fluorescence, which was determined from the average of five frame measurements from a region next to the cell immediately before ligand application, was subtracted from the actual measurements. $\Delta F/F$ (%) = (fluorescence light intensity of the cell body – baseline/baseline) x 100. $\Delta F/F$ (max %) is the maximum value within 40 seconds after ligand application.

Generation of transgenic *Gr28bc-LexA* flies

To generate the *Gr28bc-LexA* driver, a 1.3 kb DNA fragment immediately upstream of the *Gr28bc* start codon was amplified from *w¹¹¹⁸* flies using a forward (5'-AATCTAGGTACCCGCTGCTCGTCTCCCTGGATGT-3') and a reverse (5'-CGTCAAACTAGTGACCGCTTCGTTTGAGCTTCAACC-3') primer. *Acc65I* and *SpeI* sites included in the primer sequence (underlined) were incorporated such that the amplified fragment was amenable to directional cloning into the LexA vector CMC105 (Larsson et al., 2004 [4](#)). The clone chosen was confirmed by DNA sequence analysis. Transgenic flies were generated by standard P-element transformation of *w¹¹¹⁸* embryos (Rainbow Transgenic Flies Inc., Camarillo, CA).

Statistical analysis

Statistical analyses were conducted using Prism software 9.5.1 (GraphPad Software). Larval two-choice preference assay and Ca^{2+} imaging data were analyzed for normal distribution using D'Agostino-Pearson omnibus and Shapiro-Wilk normality tests. When groups did not meet the assumption for normal distribution, nonparametric statistics was used. For comparison between multiple groups, one-way ANOVA or Kruskal-Wallis test by ranks (nonparametric one-way ANOVA) was performed to test for difference of mean or rank distribution. As a post hoc test, Bonferroni's or Dunn's (nonparametric) multiple comparison tests were employed to compare two specific groups. One-way ANOVA with Bonferroni's multiple comparison tests were used in **Figure 2** [4](#). Kruskal-Wallis test by ranks with Dunn's multiple comparison tests were used in **Figures 3** [4](#), **4B** [4](#) and **5F** [4](#) and **5G** [4](#)). For comparison between two groups, Mann-Whitney U test

(nonparametric t test, **Figures 4A**, **5D**, **5E** and **Figure 4-figure supplement 1**) with two-tailed P value was used to compare means of two groups. Sample size for larval two-choice preference assays and Ca^{2+} imaging experiments were based on Mishra et al (Mishra et al., 2018).

Acknowledgements

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Source data files

Figure 2-Source Data 1. (A) Taste preference assay of larvae expressing VR1 in different *Gr28* GRNs for 0.1 mM (top) or 0.5 mM (bottom) capsaicin. (B) Taste preference assay of larvae with expression of VR1 in *Gr28a^{only}* GRNs using *lexAop-GAL80* under control of *Gr66a-LexA*.

Figure 3-Source Data 1. Taste preference assay for bitter compounds of larvae with inactivated *Gr28bc* or *Gr28be* GRNs using expression of *UAS-Kir2.1*.

Figure 4-Source Data 1. (A) Taste preference assay of wild type and *Gr28* mutant larvae for bitter compounds. (B) Taste preference assay of *Gr28* mutant larvae for denatonium expressing single *Gr28* genes in *Gr28bc* GRNs.

Figure 5-Source Data 1. (D) Ca^{2+} responses of *Gr28bc* GRNs in the TO to bitter compounds and sugars. (E) Ca^{2+} responses of *Gr28* mutant larvae to bitter compounds. (F&G) Ca^{2+} responses of *Gr28* mutant larvae to denatonium (F) or quinine (G) expressing single *Gr28* genes in *Gr28bc* GRNs.

Figure 4-figure supplement 1-Source Data 1. Taste preference assay of *Gr66a* mutant larvae for bitter compounds.

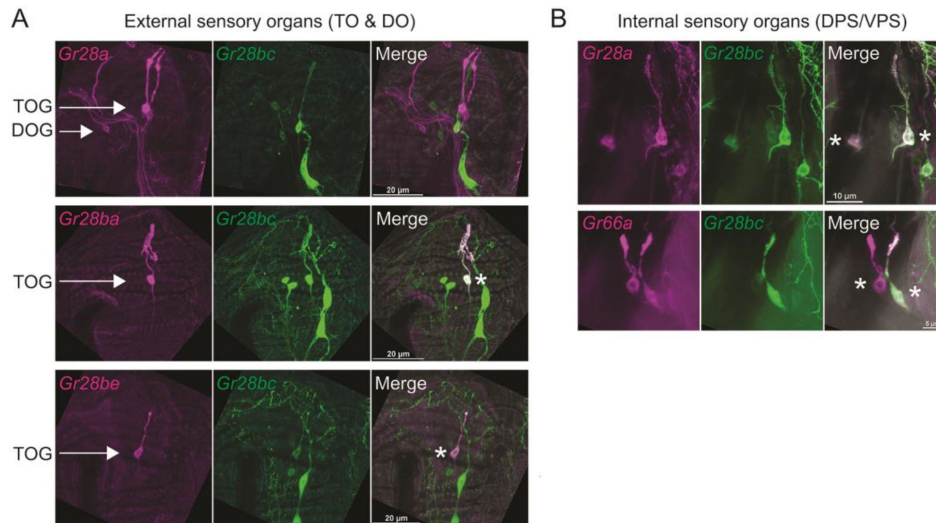


Figure 1-figure supplement 1.

Co-expression analysis between *Gr28* genes in larval sensory organs

Immunostaining with anti-GFP (green) and anti-mCD8 (magenta) antibodies on whole-mount preparations of the heads from larvae of the genotypes: (1) *UAS-mCD8:RFP lexAop-rCD2:GFP;Gr28a-GAL4* or *Gr28be-GAL4* or *Gr66a-GAL4/+;Gr28bc-LexA/+*; (2) *UAS-mCD8:RFP lexAop-rCD2:GFP/+; Gr28ba-GAL4/Gr28bc-LexA*. Asterisks refer to GRN expressing both *Gr28bc* and the indicated *Gr66a* or *Gr28* gene. Note that images show only one of the bilaterally symmetrical external organs in the DOG/TOG (A) and both bilaterally symmetrical organs in the DPS/VPS (B).

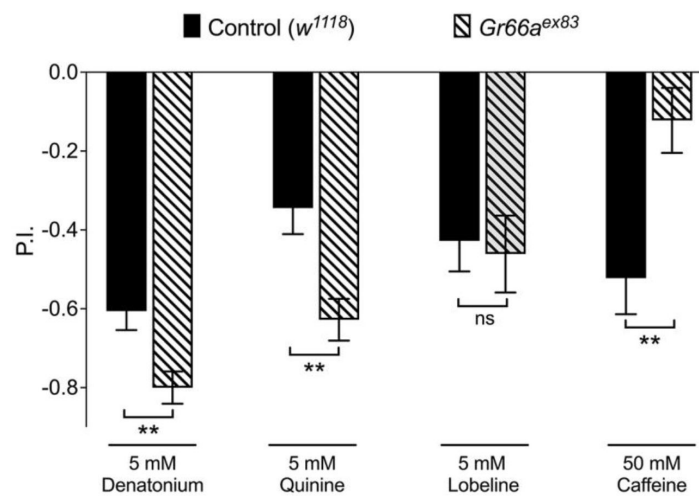


Figure 4-figure supplement 1.

Gr66a gene is necessary for caffeine avoidance of larvae

Gr66a null mutant larvae are unable to avoid of caffeine but none of other bitter compounds. Each bar represents the mean ± SEM of P.I. (n = 11-12 assays). Asterisks indicate a significant difference between the *Gr66a* mutant (*Gr66a^{ex83}*) and control larvae (*w¹¹¹⁸*) (Two-tailed, Mann-Whitney U test, ** p < 0.01, ns: not significant).

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Reviewer #1 (Public Review):

Summary:

Ahn and Amrein characterize the expression of members of the Gr28 family of gustatory receptors in taste neurons in the *Drosophila melanogaster* larva, define the behaviorally-relevant ligands for these receptors, and use chemogenetic experiments to show, strikingly, that different neurons have opposite behavioral responses to the chemogenetic ligand. They go on to show what neurons need to be silenced to lose responses to bitters, and very nicely show what subunits of the Gr28 bitter receptors are necessary and sufficient for responses to bitters. This is a nice piece of work, rigorously carried out, that tackles the neurons and receptors that drive innate responses to tastants in *Drosophila* larvae.

Strengths:

1. The chemogenetic experiments in Figure 2 are cleanly done with very clear results, and the subsetting in Figure 2B further clarifies the cellular requirements for the behavior.
2. The rescue experiments with different Gr28 subunits in the Gr28 mutant are creative and clear.

Weaknesses:

1. The authors should define early and clearly that expression of Gr28 genes studied in this paper relies not on looking at the endogenous gene but at the expression of Gal4 under the control of enhancers from these loci. The Gal4 drivers are useful and important tools, but the possibility exists that their expression is not 100% congruent with the endogenous expression of these receptors. I would not require a comprehensive validation of the lines by RNA in situ hybridization compared to the Gal4 driver lines but would recommend the disclaimer be made and that the authors are more precise in talking about the expression of the marker rather than the expression of the specific receptor gene.

2. The important chemogenetic behavioral data would benefit from a clearer presentation including a cartoon to explain what the behavior is and how it is scored. Figure 2 is the key figure in this paper and it would be helpful if the figure were re-organized to guide the non-expert reader to the key result. I recommend labeling the positive controls Gr43a as "sweet" and Gr66a as "bitter" and perhaps organize the presentation to have the negative control at the left, then Gr28ba that had no effect, then group Gr28a with Gr43a for positive valence and Gr28bc with Gr66a for negative valence. I'm not sure what the value is of showing both 0.1 mM and 0.5 mM capsaicin, the text does not explain. The experiment in Figure 2B is important but non-experts will not understand what is being done here - can the authors please provide a cartoon like those in Figure 1 showing what cells are being subjected to chemogenetics and how this differs from Figure 2A?

3. The AlphaFold ligand docking in Figure 8 is conducted with Gr28bc monomers, which are unlikely to be the in vivo relevant structure, given that the related OR/ORCO ancestor structures are tetramers. I recommend that this component of the paper either be removed entirely or that the authors redo the in silico work using the AlphaFold-Multimer package reported by Hassabis and Jumper in 2022

<https://www.biorxiv.org/content/10.1101/2021.10.04.463034v2>. It will be interesting to see what a tetramer structure looks like with the ligand.

Reviewer #2 (Public Review):

This study investigates how genes in the Gr28 family of gustatory receptors function in the taste system of *Drosophila* larvae. Gr28 genes are intriguing because they have been

implicated in taste as well as other functions, such as sensing temperature and ultraviolet light. This study makes several new findings. First, the authors show that four Gr28 genes are expressed in putative taste neurons, and these neurons can be largely divided into subsets that express Gr28a versus Gr28bc. The authors then demonstrate that these two neuronal subsets drive opposing behaviors (attraction versus avoidance) when activated. The avoidance-promoting neurons respond to bitter compounds and are required for bitter avoidance, and Gr28bc and Gr28ba were specifically implicated in bitter detection in these cells. Together, these findings provide insight into the complexity of taste receptor expression and function in *Drosophila*, even within a single receptor subfamily.

The conclusions are well-supported by the experimental data. Strengths of the paper include the use of precise genetic tools, thorough analyses of expression patterns, carefully validated behavioral assays, and well-controlled functional imaging experiments. The role of Gr28bc neurons is more thoroughly explored than that of Gr28a neurons. However, a previous study from the same lab (Mishra et al., 2018) showed that Gr28a neurons detect RNA and ribose, which are attractive to larvae. Presumably, this is the attractive response that is being recapitulated upon artificial activation of Gr28a neurons.

I only have one technical concern: In Figure 2B, the authors do not show confirmation that using Gr66a-lexA driving lexAop-Gal80 eliminates Gal4-driven gene expression in the desired cells (cells co-expressing Gr66a and Gr28a). This is important for interpreting the behavioral experiment in order to demonstrate that the Gr28a cells mediating attraction are distinct from Gr66a/Gr28bc cells.

Reviewer #3 (Public Review):

a) Important findings

- This study confirms that Gr28 subfamily members are expressed in distinct sets of taste neurons in *Drosophila* larvae, supporting previous findings (e.g., Kwon et al., 2011).
- Neurons expressing different members of the Gr28 family exhibit distinct behavioral responses when chemically activated with capsaicin.
- Silencing experiments reveal that neurons expressing Gr28bc are necessary for larval avoidance of four bitter compounds, whereas neurons expressing Gr28be are necessary for avoiding lobeline and caffeine.
- Inserting either Gr28ba or Gr28bc into the GR28 mutant line restored larval avoidance of denatonium.
- Calcium imaging experiments show that Gr28ba and Gr28bc are involved in sensing denatonium, while none of the GR28 family members are involved in detecting quinine.

b) Caveats

- The authors did not acknowledge that neurons expressing members of the GR28 family also express other Gr family members, which could potentially contribute to the detection and behavioral responses to the tested bitter compounds.
- Gal4 lines from various studies exhibit varying expression patterns, highlighting the necessity for improved reagents. These findings also suggest the importance of employing different Gal4 lines for each receptor to validate the results of the current study.
- Activating or silencing neurons pertains to the function of the neurons rather than the receptors.
- Inconsistency is observed in the use of different reagents across the experiments. Specifically, all six Gal4 lines were utilized in the Chemical Activation experiments, while only two lines were employed in the silencing experiments.
- The AlphaFold structure prediction is exciting but lacks conclusive evidence.