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.

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 bitt er 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), which was recently established as a novel appetitive taste quality common to a broad range of dipteran insects (Fujii et al., 2023).

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). 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), extend dendritic projections to the base of the DO, while 7 neurons project dendrites to the TO (the dorsolateral group, Figure 1A)(Kwon et al., 2011). The TOG contains 30 neurons,

142 all sending dendrites to the TO (the distal group). The internal taste structures, referred
143 to as the dorsal/ventral pharyngeal sense organ (DPS/VPS, 33 neurons), and the
144 posterior pharyngeal sense organs (PPS, 6 neurons) are located along the pharynx and
145 sense chemicals as they pass towards the digestive system. We note that not all these
146 neurons are confirmed GRNs, either by function or expression of *Gr* or *IR* genes, albeit
147 based on their location and anatomy, most are thought to be GRNs (Stewart et al.,
148 2015)(Rist and Thum, 2017)(Sánchez-Alcañiz et al., 2018).

149 Our initial expression analysis revealed that four of the six *Gr28* genes (*Gr28a*,
150 *Gr28ba*, *Gr28bc* and *Gr28be*) are expressed in larval taste organs, in addition to cells in
151 the gut, the brain and non-chemosensory cells of the larvae (Mishra et al., 2018). To
152 further delineate the putative chemosensory roles of these genes, we performed a more
153 detailed co-expression analysis between *Gr28* genes and with respect to the bitter
154 receptor gene *Gr66a*, using the bimodal expression systems *GAL4/UAS* to label *Gr28*
155 expressing neurons and *LexA/lexAop* to mark *Gr66a* neurons (Figure 1B and 1C; note
156 that the neuron numbers below refer to the number present in one of each paired
157 ganglion). In the TOG, we found expression of all four *Gr28* genes, along with that
158 *Gr66a*, which was expressed in 3 or 4 neurons (one neuron exhibits often stronger
159 expression of *Gr66a* than the two or three remaining neurons). This number slightly
160 smaller than the 6 neurons reported using a distinct *Gr66a-GAL4* driver (Kwon et al.,
161 2011). *Gr28ba*, *Gr28bc* and *Gr28be* are co-expressed in a single neuron with *Gr66a*
162 (Figure 1B). In contrast, *Gr28a* is expressed in a distinct TOG neuron than *Gr66a*
163 (Figure 1B), and by extension, in a different neuron than the co-expressed *Gr28b*
164 genes, which we independently confirmed using a *Gr28bc-QF* driver (Figure 1-figure
165 supplement 1). In the DOG, we find only a single *Gr28a* neuron, while none of the
166 *Gr28b* genes, or *Gr66a*, is expressed there (Figure 1B). Expression in the internal
167 sensory organs was similarly complex. *Gr66a* was found in one neuron in the DPS/VPS,
168 co-expressed with *Gr28bc* as well as *Gr28a* (Figure 1C). None of the *Gr28b* genes is
169 expressed in the PPS, but two neurons express *Gr28a*, one of which also expresses
170 *Gr66a* (Figure 1C). In summary, the internal sensory organs can be subdivided in three
171 distinct groups: *Gr66a/Gr28b/Gr28a* positive neurons, *Gr66a/Gr28a* positive neurons

172 and *Gr28a*^{only} neurons, which contrasts expression in the external sensory organs
173 where *Gr28a* and the *Gr28b* genes are mutually exclusive.
174
175 **Subsets of *Gr28* neurons mediate opposing feeding behaviors**
176 We previously showed that at least one of the six Gr28 genes is necessary for
177 feeding attraction to RNA, ribonucleosides and ribose using a well-established two
178 choice feeding assay (Mishra et al., 2018). Specifically, larvae homozygous for a *Gr28*
179 null allele, a deletion of the entire *Gr28a* gene and most of the coding region of all
180 *Gr28b* genes (*ΔGr28*), lose their ability to sense these compounds, a phenotype that is
181 restored when single *UAS-Gr28* genes are expressed in *Gr28a* neurons using *Gr28a-*
182 *GAL4*. This observation and the largely non-overlapping expression of *Gr28a* and the
183 *Gr28b* genes suggests that respective neurons represent functionally distinct entities.
184 To investigate this possibility, we took advantage of the mammalian Vanilloid Receptor
185 1 (VR1), a TRP channel that is activated by capsaicin (Caterina et al., 1997). *Drosophila*
186 have no *VR1* like-gene in their genome and do not respond to capsaicin behaviorally,
187 but flies are attracted to this chemical when a modified *VR1* gene (*VR1E600K*) is
188 expressed in sweet taste neurons (Marella et al., 2006). Thus, we expressed
189 *VR1E600K* (henceforth referred to *UAS-VR1*) under the control of the four *Gr28-GAL4*
190 drivers (*Gr28a-GAL4*, *Gr28ba-GAL4*, *Gr28bc-GAL4* and *Gr28be-GAL4*) in larvae and
191 tested their response to capsaicin using the two-choice preference assay. Additionally,
192 we expressed *UAS-VR1* in bitter neurons as well as sweet neurons using respective
193 *GAL4* drivers (*Gr66a-GAL4* and *Gr43a*^{GAL4}) (Scott et al., 2001) (Miyamoto et al., 2012).
194 Consistent with the observation in adults (Marella et al., 2006), *w*^{1118} control larvae and
195 larvae carrying *UAS-VR1* or *Gr-GAL4* genes alone were unresponsive to 0.1 mM or 0.5
196 mM capsaicin (Figure 2A). However, larvae expressing *UAS-VR1* under the control of
197 *Gr28a-GAL4* or *Gr43a-GAL4* showed strong appetitive responses to both capsaicin
198 concentrations (Figure 2A). In contrast, larvae expressing *UAS-VR1* under the control of
199 *Gr28bc-GAL4* or *Gr66a-GAL4* showed robust avoidance behavior to both
200 concentrations of capsaicin. Lastly, expression of *UAS-VR1* with the *Gr28ba-GAL4* or
201 *Gr28be-GAL4* driver, which is co-expressed in a single TO neuron with *Gr28bc* (Figure
202 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.

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 *Gr28be* 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 (*ΔGr28*), 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 *ΔGr28* 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.

DISCUSSION

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 *ΔGr28* 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* (Figure1), 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 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 *ΔGr28* 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.

MATERIALS AND METHODS

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			
D. melanogaster: w^{1118}	Wild-type control	Bloomington *Drosophila* Stock Center	BDSC: 3605; FlyBase: FBst0003605
D. melanogaster: w; P{Gr28a-GAL4.T}SF36S*	*Gr28a-GAL4*	Thorne and Amrein, 2008	FlyBase: FBtp0056017
D. melanogaster: w; P{Gr28a-GAL4.T}SF36B1*	*Gr28a-GAL4*	Thorne and Amrein, 2008	FlyBase: FBtp0056017
D. melanogaster: w; P{Gr28b.a-GAL4}NT42aC51a*	*Gr28ba-GAL4*	Thorne and Amrein, 2008	FlyBase: FBtp0054526
D. melanogaster: w; P{Gr28b.c-GAL4}NT21B1*	*Gr28bc-GAL4*	Thorne and Amrein, 2008	FlyBase: FBtp0054528
D. melanogaster: w; P{Gr28b.e-GAL4.4.245}Gr28a3AII*	*Gr28be-GAL4*	Scott et al., 2001	FlyBase: FBtp0014672
D. melanogaster: w; Df(2L)ΔGr28*	*ΔGr28/ΔGr28*	Mishra et al., 2018	FlyBase: FBab0049019

D. melanogaster: w; P{Gr66C1-GAL4.3.153}*	*Gr66a-GAL4*	Scott et al., 2001	FlyBase: FBtp0014661
D.melanogaster: w; P{UAS-Gr28a.G}attP2*	*UAS-Gr28a*	Ni et al., 2013	Flybase: FBal0344045
D.melanogaster: w; P{UAS-Gr28b.A}attP2*	*UAS-Gr28ba*	Ni et al., 2013	Flybase: FBal0291410
D.melanogaster: w; P{UAS-Gr28b.B}attP2*	*UAS-Gr28bb*	Ni et al., 2013	Flybase: FBal0291412
D.melanogaster: w; P{UAS-Gr28b.C}attP2*	*UAS-Gr28bc*	Ni et al., 2013	Flybase: FBal0291411
D.melanogaster: w; P{UAS-Gr28b.D}attP2*	*UAS-Gr28bd*	Ni et al., 2013	Flybase: FBal0291409
D.melanogaster: w; P{UAS-Gr28b.E}attP2*	*UAS-Gr28be*	Ni et al., 2013	Flybase: FBal0291408
D.melanogaster: w^{1118};Ti{GAL4}Gr43a^{GAL4}	*Gr43a^{GAL4}*	Miyamoto et al., 2012	BDSC:93447; Flybase: FBst0093447
D.melanogaster: w^{1118};P{UAS-VR1E600K}	*UAS-VR1E600K*	Marella et al., 2006	Flybase: FBal0215202
D. melanogaster: w; P{lexAop-rCD2-GFP}*	*lexAop-rCD2:GFP*	Lai and Lee, 2006	Flybase: FBst0066687
D. melanogaster: y^{1}w;P{10XUAS-IVS-mCD8::RFP}su(Hw)attP8*	*UAS-mCD8:RFP*	Bloomington *Drosophila* Stock Center	BDSC: 32220; Flybase: FBti0131987
D. melanogaster: w^{1118}; P{20XUAS-IVS-GCaMP6m}attP40	*UAS-GCaMP6m*	Bloomington *Drosophila* Stock Center	BDSC: 42748; Flybase: FBti0151346
D. melanogaster: w; P{UAS-Hsap\KCNJ2.EGFP}1*	*UAS-Kir2.1-GFP*	Baines et al., 2001; Paradis et al., 2001	Flybase: FBst0006596
D. melanogaster: w^{1118}; P{Gr66a-lexA.S}2;TM2/TM6B	*Gr66a-LexA*	Thistle et al., 2012	BDSC: 93024 Flybase: FBst0093024
Sequence-based reagents			
Gr28bc_F	5′-AATCTAGGTACCCCGGCTGCTCGTCTCCCTGGATGT-3′	This paper	PCR primers
Gr28bc_R	5′-CGTCAAACTAGTGACCGCTTCGTTTGAGCTTCAACC-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 w^{1118} 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*(*Gr28a3AII*) 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)

365

366 **Chemicals**

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

381

382 **Immunofluorescence**

383 Immunofluorescence of larval heads was performed based on the protocol
384 described in Croset and colleagues (Croset et al., 2016) with minor modification. Heads
385 of third instar larvae were dissected using microscissors in phosphate buffered saline
386 (PBS) and immediately fixed in PBS with 4% paraformaldehyde for 1 hour at 4 °C.
387 Heads were washed six times in washing buffer (PBS with 0.1% Triton X-100) for 20
388 min and blocked for 1 h in washing buffer containing 5% heat-inactivated goat serum
389 (SouthernBiotech, Cat. No. 0060-01), followed by incubation with the primary antibodies
390 (rabbit anti–GFP, 1:1,000 dilution; rat anti–mCD8, 1:200 dilution, ThermoFisher
391 Scientific) at 4°C overnight. Heads were washed six times for 20 min in washing buffer
392 and blocked in washing buffer containing 5% heat-inactivated goat serum for 1 hour.
393 Heads were incubated with the secondary antibodies (goat anti rabbit ALEXA 488,
394 1:500 dilution, ThermoFisher Scientific; goat anti–rat Cy3, 1:300 dilution, Jackson
395 Immunoresearch Laboratories Inc.) at 4°C overnight. Heads were washed six times in

396 washing buffer for 20 min. Washes were carried out at room temperature under gentle
397 agitation. Heads were mounted with VectaShield (Vector Lab, Cat No. H-1200) on a
398 microscope slide and images were obtained using a Nikon A1R confocal microscope
399 system. Adobe photoshop 2022 was used further to process images.
400
401 **Larval two-choice preference assay**
402 Two-choice preference assay of larvae was conducted as described in Mishra et
403 al. (Mishra et al., 2013) with minor modification. Flies were placed on standard corn
404 meal food in plastic vials and allowed to lay eggs for 24 h under a 12 h light/dark cycle
405 at 25°C. Flies were removed from food vials and feeding-stage third-instar larvae were
406 collected. All dishes for two-choice preference assay were prepared just prior each
407 experiment. Petri dishes (60 x 15 mm, Falcon, Cat. No. REF353004) with two halves
408 marked on the bottom were filled with melted plain 1% agarose or 1% agarose
409 containing 1.75% ethanol (for capsaicin preference). After the agarose solidified, one
410 half was removed and replaced with 1% agarose solution containing taste ligands
411 (capsaicin or bitter compound). For each experiment, 15 larvae from food vials were
412 briefly rinsed twice with Millipore Q water and placed along the middle separating pure
413 and ligand containing agarose. After 16 min, photos were taken for record keeping and
414 used to calculate larval preference indices. Larvae that crawled onto the wall of a dish
415 or dug in the agarose were excluded. The preference index (P.I.) was calculated as
416 follow: $PI = (N_{tastant} - N_{plain}) / N_{Total,}$ whereby N is the number of larvae in the tastant
417 sector, the plain agarose sector and the total number on the plate. Positive values
418 indicate a preference for capsaicin or bitter compound while negative values indicate
419 repulsion (avoidance).
420
421 **Calcium imaging**
422 Calcium imaging was performed in *Gr28bc* GRNs expressed in the terminal
423 organ of feeding-stage, third instar larvae, reared as described for the larval two-choice
424 preference assay. For each experiment, larvae from food vials were briefly rinsed twice
425 with Millipore Q water and were mounted dorsally on a large microscope cover glass
426 (24 x 50 mm, VWR, Cat. No. 16004-098) using double-sided scotch tape and covered

427 with a small microscope cover glass (12CIR-1, Thermo Fisher Scientific, Cat. No.
428 1254580). Millipore Q water (40 µl) was applied to the tip of the larval head, and the
429 preparation was placed on the stage of a Nikon eclipse Ti inverted microscope. Images
430 were obtained every 500 ms, starting 15 s before application and ending 105 s after
431 ligand application. Each recording was initiated by applying water (40µl) to set a
432 baseline. The first ligand solution (40 µl of bitter chemical or sugar) was applied
433 thereafter, followed by 5 washes with carrier (100 µl of water). After a 3-minute pause to
434 allow the preparation to recalibrate, a second ligand solution (40 µl bitter chemical or
435 sugar) was applied. To assure validity in experiments with *Gr28* mutants and rescues,
436 each recording was concluded with application of caffeine, and recordings were
437 included only if caffeine generated a positive response. Baseline fluorescence, which
438 was determined from the average of five frame measurements from a region next to the
439 cell immediately before ligand application, was subtracted from the actual
440 measurements. ∆F/F (%) = (fluorescence light intensity of the cell body –
441 baseline/baseline) x 100. ∆F/F (max %) is the maximum value within 40 seconds after
442 ligand application.
443
444 **Generation of transgenic *Gr28bc-LexA* flies**
445 To generate the *Gr28bc-LexA* driver, a 1.3 kb DNA fragment immediately
446 upstream of the *Gr28bc* start codon was amplified from w^{1118} flies using a forward (5′-
447 AATCTAGGTACCCCGGCTGCTCGTCTCCCTGGATGT-3′) and a reverse (5′-
448 CGTCAAACTAGTGACCGCTTCGTTTGAGCTTCAACC-3′) primer. *Acc65I* and *SpeI*
449 sites included in the primer sequence (underlined) were incorporated such that the
450 amplified fragment was amenable to directional cloning into the LexA vector CMC105
451 (Larsson et al., 2004). The clone chosen was confirmed by DNA sequence analysis.
452 Transgenic flies were generated by standard P-element transformation of w^{1118} embryos
453 (Rainbow Transgenic Flies Inc., Camarillo, CA).
454
455 **Statistical analysis**
456 Statistical analyses were conducted using Prism software 9.5.1 (GraphPad
457 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. Kruskal-Wallis test by ranks with Dunn's multiple comparison tests were used in Figures 3, 4B and 5F and 5G). 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).

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644

645

646 **FIGURES**

647

648 **Figure 1. Expression of the *Gr28* genes in the larval sensory organs**

649

650 **A) Schematic representation of larval chemosensory system** (Kwon et al.,
651 2011). Three external sensory organs (Dorsal Organ, DO; Terminal Organ, TO;
652 Ventral Organ, VO) hold collectively the dendritic projections of neuronal cell
653 bodies in the respective ganglia (DOG, TOG and VOG). Three clusters of
654 sensory neurons (Dorsa/Ventral Pharyngeal Sensory organ, D/VPS; and
655 Posterior Pharyngeal Sensory organ, PPS) align the pharynx. The antennal
656 nerve (AN) connects the DO neurons to the subesophageal zone (SEZ). The TO
657 and VO neurons project along the maxillary nerve (MN) to the SEZ. The DPS
658 and PPS neurons innervate to the SEZ via labral nerve (LRN). The VPS neurons
659 project to the SEZ through the labial nerve (LBN). Note that olfactory neurons
660 are omitted in the schematic here and in B (below).

661 **B) Expression of the *Gr28* genes in the external sensory organs.** Note that
662 images show only one of the bilaterally symmetrical organs. Possible co-
663 expression between *Gr66a* and different *Gr28b* genes (in B and C; left panels)
664 was assessed using GAL4 and LexA drivers for Gr28 genes and *Gr66a*,
665 respectively. Expression summary (diagram on the right) shows only one side of
666 the bilaterally symmetrical organs. The *Gr28b* genes and *Gr28a* are expressed
667 in different neurons. Likewise, no co-expression is observed between *Gr66a*
668 (marker for bitter GRNs) and *Gr28a,* whereas the *Gr28b* genes are co-expressed
669 in a subset of *Gr66a* neurons.

670 **C) Expression of the *Gr28* genes in internal taste organs.** Note that images for
671 the DPS/VPS show both side of the bilaterally symmetrical ganglion, while
672 images of the PPS show only one side of the bilaterally symmetrical organ.
673 *Gr28a* and *Gr66a* are partially co-expressed, but each gene is also expressed
674 exclusively in a subset of neurons in the PPS. *Gr28bc* but none of the other
675 *Gr28b* genes is co-expressed with *Gr66a* in a single neuron in the DPS/VPS.
676 Expression summary (diagram on the right) shows only one of the bilaterally

symmetrical organs. Immunostaining with anti-GFP (green) and anti-mCD8 (magenta) antibodies was carried out on whole-mount preparations from larvae heads of the following genotypes: (1) *UAS-mCD8:RFP lexAop-rCD2:GFP;Gr66a-LexA/Gr28a-GAL4* or *Gr28bc-GAL4* or *Gr28be-GAL4* (2) *UAS-mCD8:RFP lexAop-rCD2:GFP;Gr66a-LexA/+; Gr28ba-GAL4/+*. Asterisks refer to a GRN expressing both *Gr66a* and the indicated *Gr28* gene. Scale bars are 5 µm.

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 ± 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 ± 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).

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 ± 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

739

740 **A)** Some of the *Gr28* genes are strictly required for sensing denatonium. Wild-type
741 (w^{1118}) larvae, but not *Gr28* mutant larvae, strongly avoid denatonium. In
742 contrast, *Gr28* mutant (w^{1118};*ΔGr28/ΔGr28*) larvae do not show significantly
743 reduced avoidance of quinine, lobeline and caffeine. Each bar represents the
744 mean ± SEM of P.I. (n = 12- 22 assays). Asterisks indicate a significant
745 difference between the *Gr28* mutant and wild-type larvae (Two-tailed, Mann-
746 Whitney U test, **** $p < 0.0001$, *** $p < 0.001$, ns: not significant).

747 **B)** Single *Gr28b* genes can rescue avoidance response to denatonium when
748 expressed in *Gr28bc* neurons of *Gr28* mutant larvae. Each bar represents the
749 mean ± SEM of P.I. (n = 11- 22 assay). The behavior of *Gr28* mutant larvae
750 expressing *UAS-Gr28* transgenes under control of the *Gr28bc-GAL4* driver was
751 compared to $Gr28^{+/+}$ control (w^{1118} , filled bar), and three *Gr28* mutant controls
752 (*ΔGr28*, *ΔGr28* plus driver, and *ΔGr28* plus respective *UAS-Gr28* reporter, plain
753 bar) using Kruskal-Wallis test by ranks with Dunn's multiple comparison tests (p
754 < 0.05). Bars with different letters are significantly different. Fly genotypes: wild-
755 type: w^{1118} (black), mutants: w^{1118};*ΔGr28/ΔGr28*, w^{1118};*ΔGr28/ΔGr28 Gr28bc-*
756 *GAL4,* w^{1118};*ΔGr28/ΔGr28;UAS-Gr28* (indicated *Gr28* genes)/+ and
757 w^{1118};*ΔGr28/ΔGr28 UAS-GCaMP6m;UAS-Gr28 (*for *Gr28bb or Gr28bc* genes)/+
758 (white), rescues: w^{1118};*ΔGr28/ΔGr28 Gr28bc-GAL4;UAS-Gr28* (indicated *Gr28*
759 genes)/+ and w^{1118};*ΔGr28 UAS-GCaMP6m/ΔGr28 Gr28bc-GAL4;UAS-Gr28*
760 (for *Gr28bb* or *Gr28bc* genes)/+ (grey).

761

762 **Figure 5. Cellular activation of *Gr28bc* GRNs by bitter compounds that require**
763 **Gr28ba or Gr28bc function.**

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

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

769 **C & D) Representative traces (C) and quantified Ca^{2+} responses (D) of the**
770 ***Gr28bc* GRNs after stimulation with indicated ligands.** Fly genotype: *w^{1118};*
771 *Gr28bc-GAL4/UAS-GCaMP6m*. Each bar represents the mean ± SEM of Ca^{2+}
772 imaging with 12 – 16 larvae. Asterisks indicate a significant difference between
773 carrier (water) and indicated ligands (Two-tailed, Mann-Whitney U test, *** $p <$
774 0.001, ns: not significant).
775 **(E) Neurons of larvae lacking the *Gr28* genes exhibit significantly reduced**
776 **responses to denatonium and quinine.** The *Gr28bc* expressing TO GRN of
777 *Gr28* mutant larvae (*ΔGr28*) display significantly reduced Ca^{2+} responses to
778 denatonium and quinine but not to lobeline or caffeine when compared to *Gr28bc*
779 expressing TO of wild type controls. Responses to denatonium, but not quinine,
780 is restored by expression of single *Gr28b (Gr28ba* or *Gr28bc)* genes (grey).
781 Larvae genotypes: *$Gr28^{+}$* control (black bar): *w^{1118}; Gr28bc-GAL4/UAS-*
782 *GCaMP6m*. *$Gr28^{-}$* control (white bar): *w^{1118}; ΔGr28 Gr28bc-GAL4/ΔGr28 UAS-*
783 *GCaMP6m*. Each bar represents the mean ± SEM with 13 – 16 larvae. Asterisks
784 indicate a significant difference between *$Gr28^{+}$* control and *Gr28* mutant (*ΔGr28*)
785 larvae (Two-tailed, Mann-Whitney U test, *** $p < 0.001$, ** $p < 0.01$ ns: not
786 significant).
787 **(F and G) *Gr28bc* or *Gr28ba* rescues denatonium responses in GRNs of *ΔGr28***
788 **larvae.** Expression of *Gr28bc* or *Gr28ba* in under control of *Gr28bc-GAL4*
789 restores responses to denatonium, but not quinine in GRNs of *ΔGr28* larvae.
790 Each bar represents the mean ± SEM of Ca^{2+} imaging with 12 – 17 larvae. The
791 Ca^{2+} responses of *Gr28* mutant larvae expressing *UAS-Gr28* transgenes under
792 *Gr28bc-GAL4* driver is compared to *$Gr28^{+}$* (black) and *$Gr28^{-}$* (white) controls by
793 using Kruskal-Wallis test by ranks with Dunn's multiple comparison tests ($p <$
794 0.05). Bars with different letters are significantly different. Dashed lines delineate
795 groups for ANOVA. A short solid line indicates control groups. Fly genotypes:
796 *$Gr28^{+}$* control (black bar): *w^{1118}; Gr28bc-GAL4/UAS-GCaMP6m*. *$Gr28^{-}$* control
797 (white bar): *w^{1118}; ΔGr28 Gr28bc-GAL4/ΔGr28 UAS-GCaMP6m*. *Gr28* rescues
798 (grey bar): *w^{1118}; ΔGr28 Gr28bc-GAL4/ΔGr28 UAS-GCaMP6m; UAS-Gr28*/+.

799 Concentrations of ligands were 100 mM for sugars, 50 mM for caffeine and 5 mM
800 for denatonium, quinine and lobeline.
801
802 **Figure 6 Role of different GRN subsets in taste behavior of larvae**
803
804 GRNs mediating avoidance behavior express *Gr28bc*, while GRNs necessary for
805 feeding preference express *Gr28a* alone (*Gr28a*^{only} GRNs). Note that each ensemble is
806 composed of at least a pair of neurons located in the external taste organs and a pair of
807 neurons in the internal taste organs.
808
809 **Figure 7 Amino acid alignment of the six Gr28 proteins**
810 The sequence alignment was generated using Clustal Omega tool from ClustalW2
811 (https://www.ebi.ac.uk/Tools/msa/clustalo/). IL and EL indicate intracellular loop and
812 extracellular loop, respectively. Note that the C terminal region starting at the IL3 is
813 identical in the Gr28b proteins. Red highlighted letters indicate amino acids identical
814 only in Gr28ba and Gr28bce. Green highlighted letters indicate amino acids conserved
815 in Gr28ba, Gr28bc and one other Gr28 protein. An asterisk below the sequences
816 indicates residues identical in all Gr28 proteins; a colon a strongly conserved residue
817 (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW); and a period moderately
818 conserved residue (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK,
819 NEQHRK, FVLIM, HFY). TM1-7 indicates helical transmembrane segment predicted by
820 HMMTOP 2.0 software (http://www.enzim.hu/hmmtop).
821
822 **Figure 8. Identification of candidate amino acids for ligand binding pockets of**
823 **Gr28bc.**
824 Top: Sixteen amino acids conserved in both Gr28ba and Gr28bc are mapped on 3D
825 structure of Gr28bc using Alphafold (https://www.alphafold.ebi.ac.uk/). Red circles
826 indicate amino acid locations of residues identical in Gr28ba and Gr28bc. Green circles
827 indicate amino acid locations of residues identical in one additional Gr28 protein
828 (Gr28bb or Gr28a).

Bottom: Prediction of putative ligand binding pocket in of Gr28bc with highest probability using Prankweb (https://prankweb.cz/). The three resides 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.

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 (w^{1118}) (Two-tailed, Mann-Whitney U test, ** $p < 0.01$, ns: not significant).

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*.

860

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

864

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

869

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

872

873

874

875

876 **Table 1. Conserved amino acids in the amino termini of Gr28ba and Gr28bc**

877

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**

878

879 The 7 amino acid residues identical in the amino-terminal region of Gr28bc and Gr28ba
880 are shown in bold. The nine additional amino acids also identical in one additional Gr28
881 proteins are also listed. These residues are considered potentially critical for recognition
882 of denatonium, since only Gr28ba and Gr28bc can rescue response to denatonium
883 when expressed in *Gr28bc* neurons of *ΔGr28/ΔGr28* mutant larvae.

884

885 **Table 2. Prediction of ligand binding sites from protein structure of 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

886

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

891

Figure 1

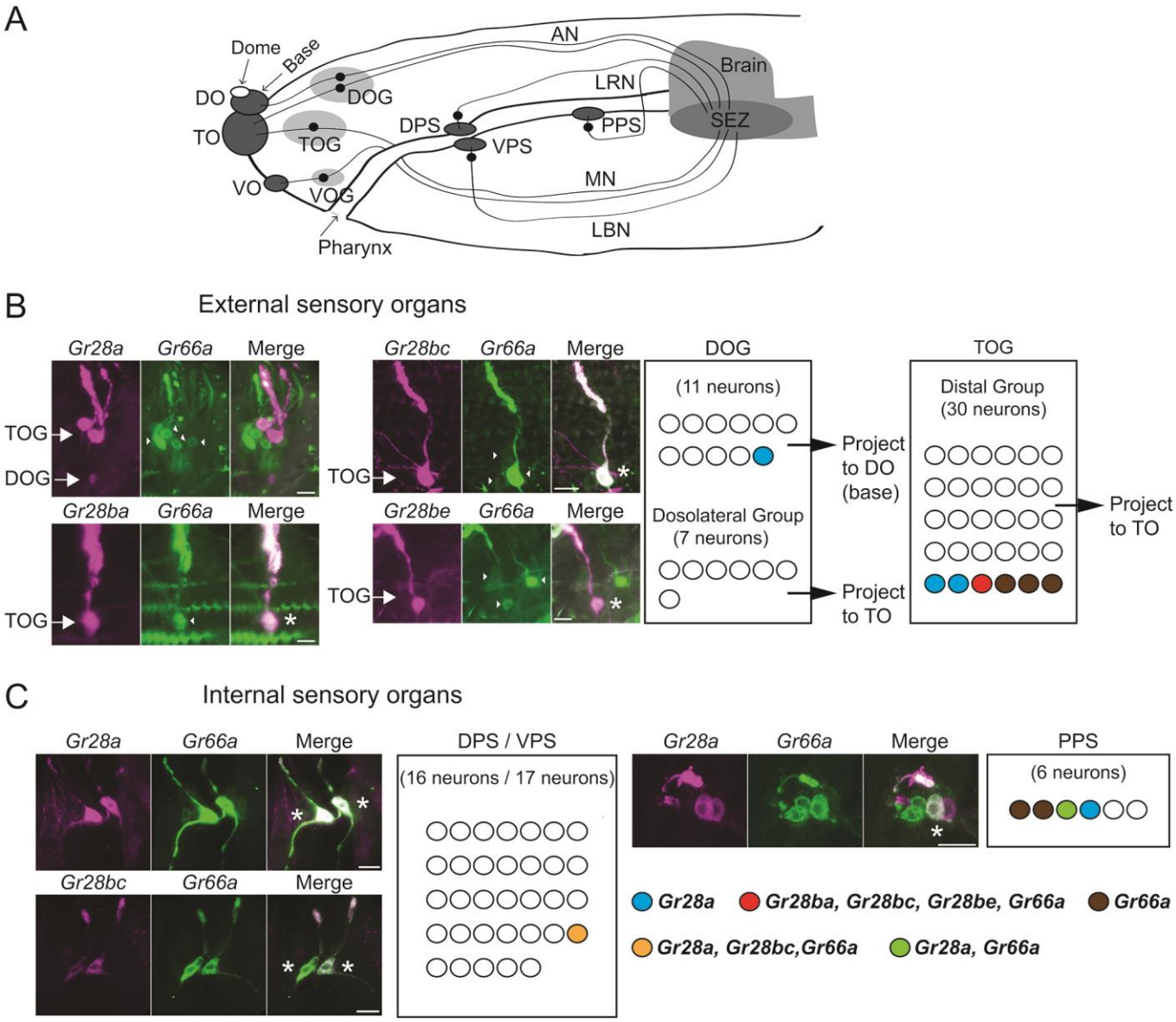
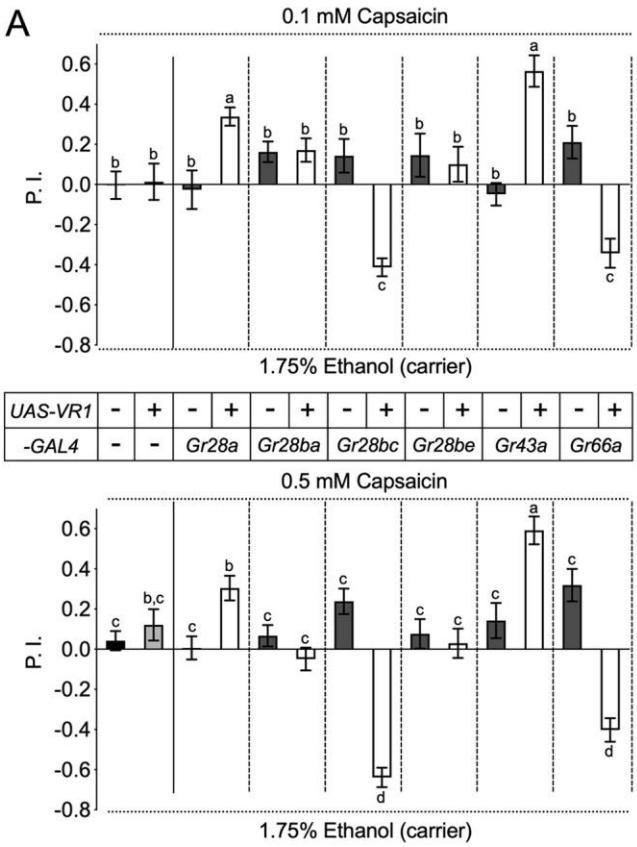

A
Dome
Base
DO
TO
VO
DOG
TOG
VOG
Pharynx
AN
LRN
DPS
VPS
PPS
MN
LBN
Brain
SEZ
B
External sensory organs
Gr28a
Gr66a
Merge
TOG
DOG
Gr28bc
Gr28ba
Gr28be
DOG
(11 neurons)
Project to DO (base)
Dosolateral Group (7 neurons)
Project to TO
TOG
Distal Group (30 neurons)
Project to TO
C
Internal sensory organs
DPS / VPS
(16 neurons / 17 neurons)
PPS
(6 neurons)
Gr28a
Gr28ba, Gr28bc, Gr28be, Gr66a
Gr66a
Gr28a, Gr28bc,Gr66a
Gr28a, Gr66a

Figure 2


A
0.1 mM Capsaicin
P. I.
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
1.75% Ethanol (carrier)
UAS-VR1 - + - + - + - + - + - + - +
-GAL4 - - Gr28a Gr28ba Gr28bc Gr28be Gr43a Gr66a
0.5 mM Capsaicin
P. I.
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
1.75% Ethanol (carrier)

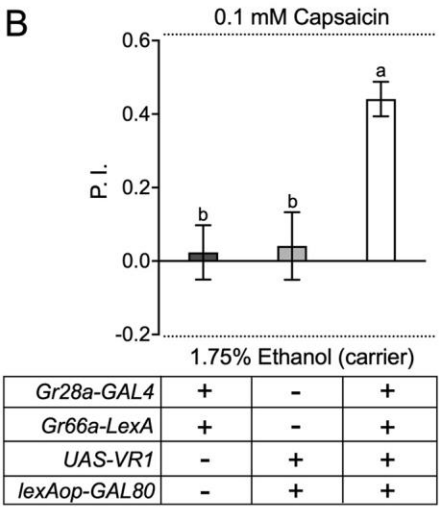

B
0.1 mM Capsaicin
P. I.
0.6
0.4
0.2
0.0
-0.2
1.75% Ethanol (carrier)
Gr28a-GAL4 + - +
Gr66a-LexA + - +
UAS-VR1 - + +
lexAop-GAL80 - + +

Figure 3

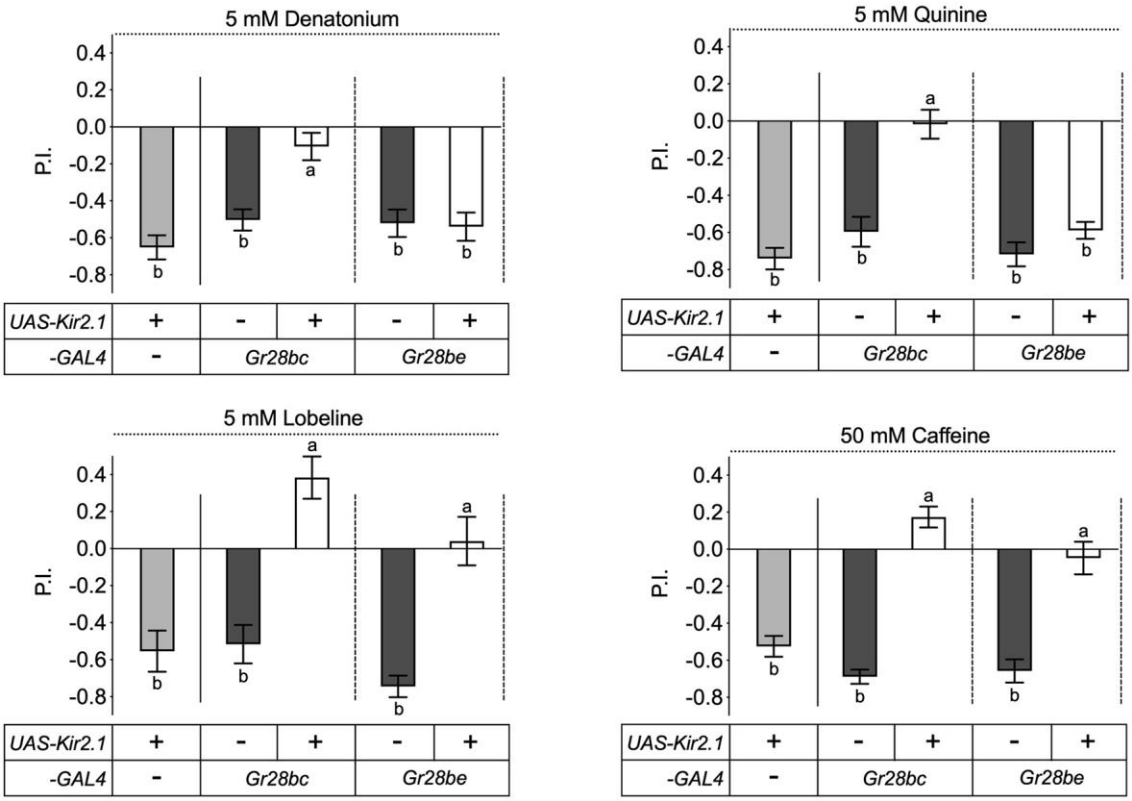

5 mM Denatonium
5 mM Quinine
5 mM Lobeline
50 mM Caffeine
P.I.
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
a
b
UAS-Kir2.1
-GAL4
Gr28bc
Gr28be

Figure 4

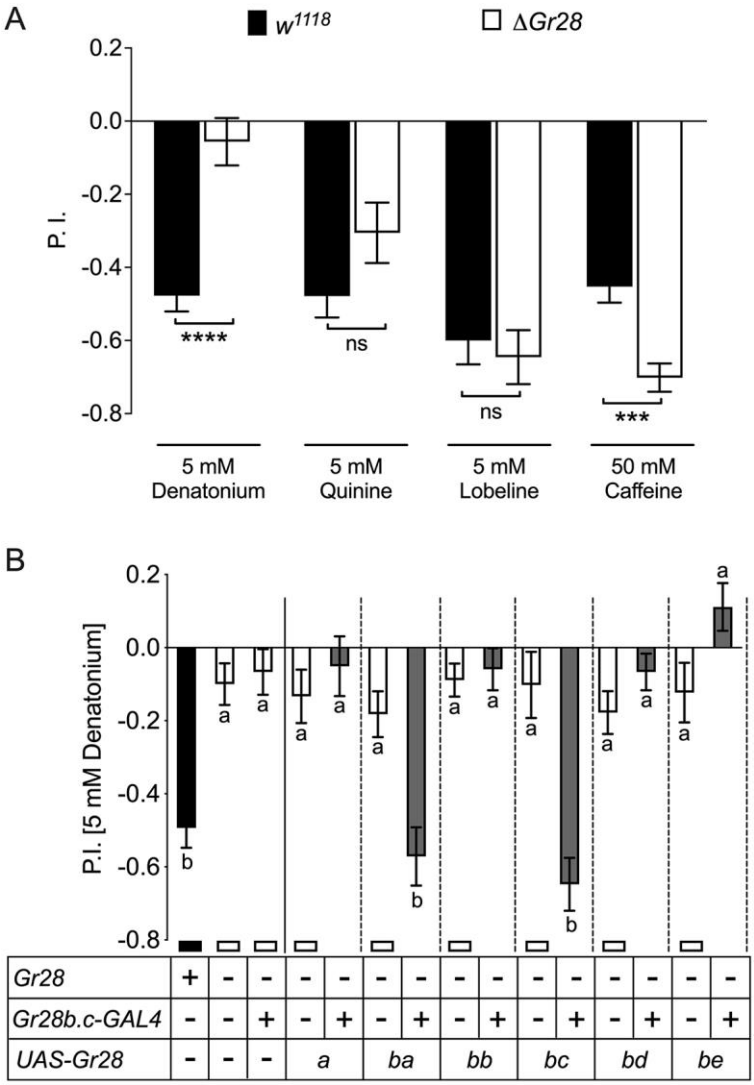

A
w1118
ΔGr28
P. I.
0.2
0.0
-0.2
-0.4
-0.6
-0.8
****
ns
ns
***
5 mM Denatonium
5 mM Quinine
5 mM Lobeline
50 mM Caffeine
B
P.I. [5 mM Denatonium]
Gr28
Gr28b.c-GAL4
UAS-Gr28
a
ba
bb
bc
bd
be

Figure 5

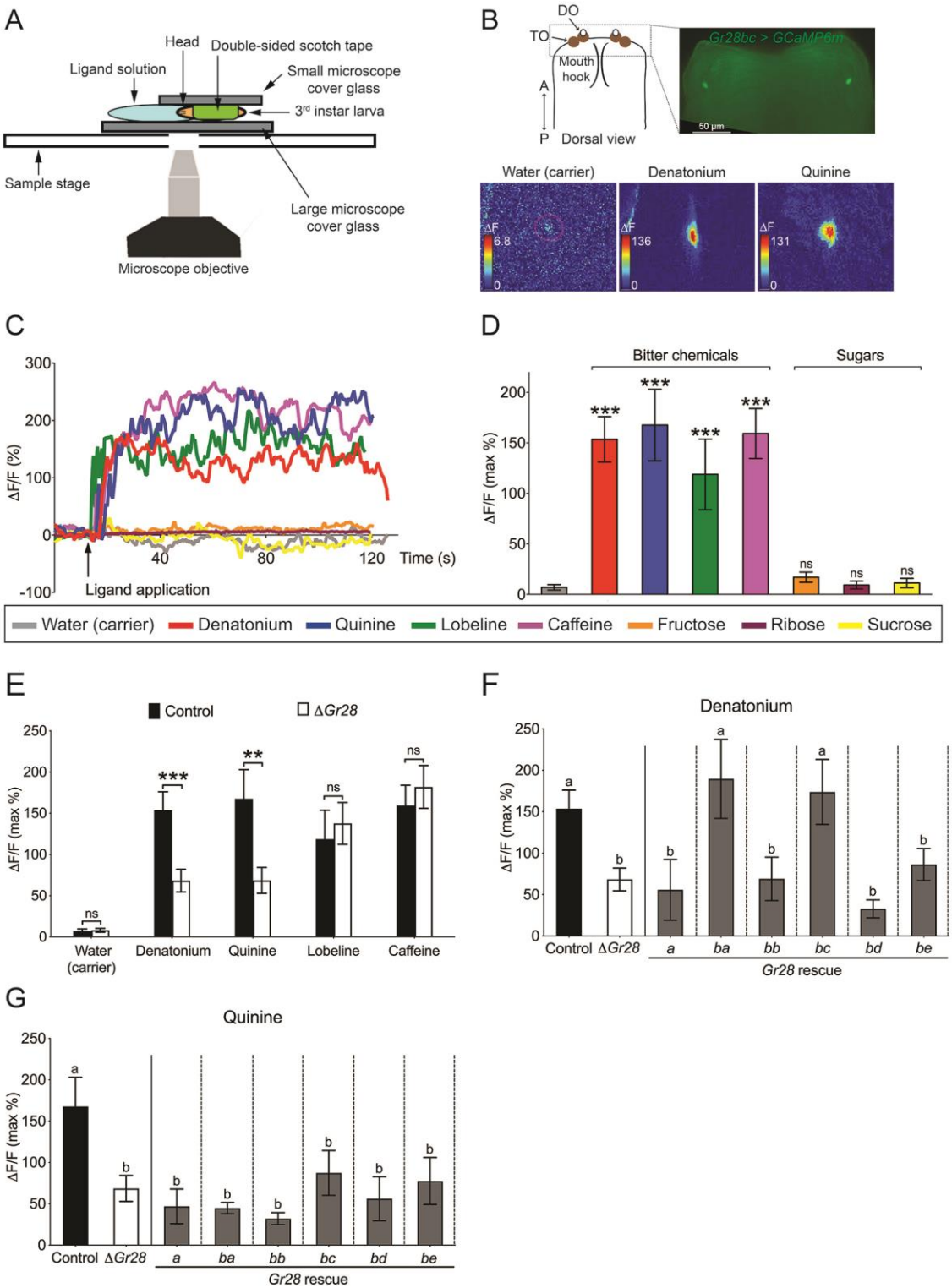

A
Head
Double-sided scotch tape
Ligand solution
Small microscope cover glass
3rd instar larva
Sample stage
Large microscope cover glass
Microscope objective
B
DO
TO
Mouth hook
A
P
Dorsal view
Gr28bc > GCaMP6m
50 µm
Water (carrier)
Denatonium
Quinine
ΔF
6.8
136
131
0
C
ΔF/F (%)
Time (s)
Ligand application
D
Bitter chemicals
Sugars
ΔF/F (max %)
ns
Water (carrier)
Denatonium
Quinine
Lobeline
Caffeine
Fructose
Ribose
Sucrose
E
Control
ΔGr28
ΔF/F (max %)
Water (carrier)
Denatonium
Quinine
Lobeline
Caffeine
F
Denatonium
Control
ΔGr28
a
ba
bb
bc
bd
be
Gr28 rescue
G
Quinine

Figure 6

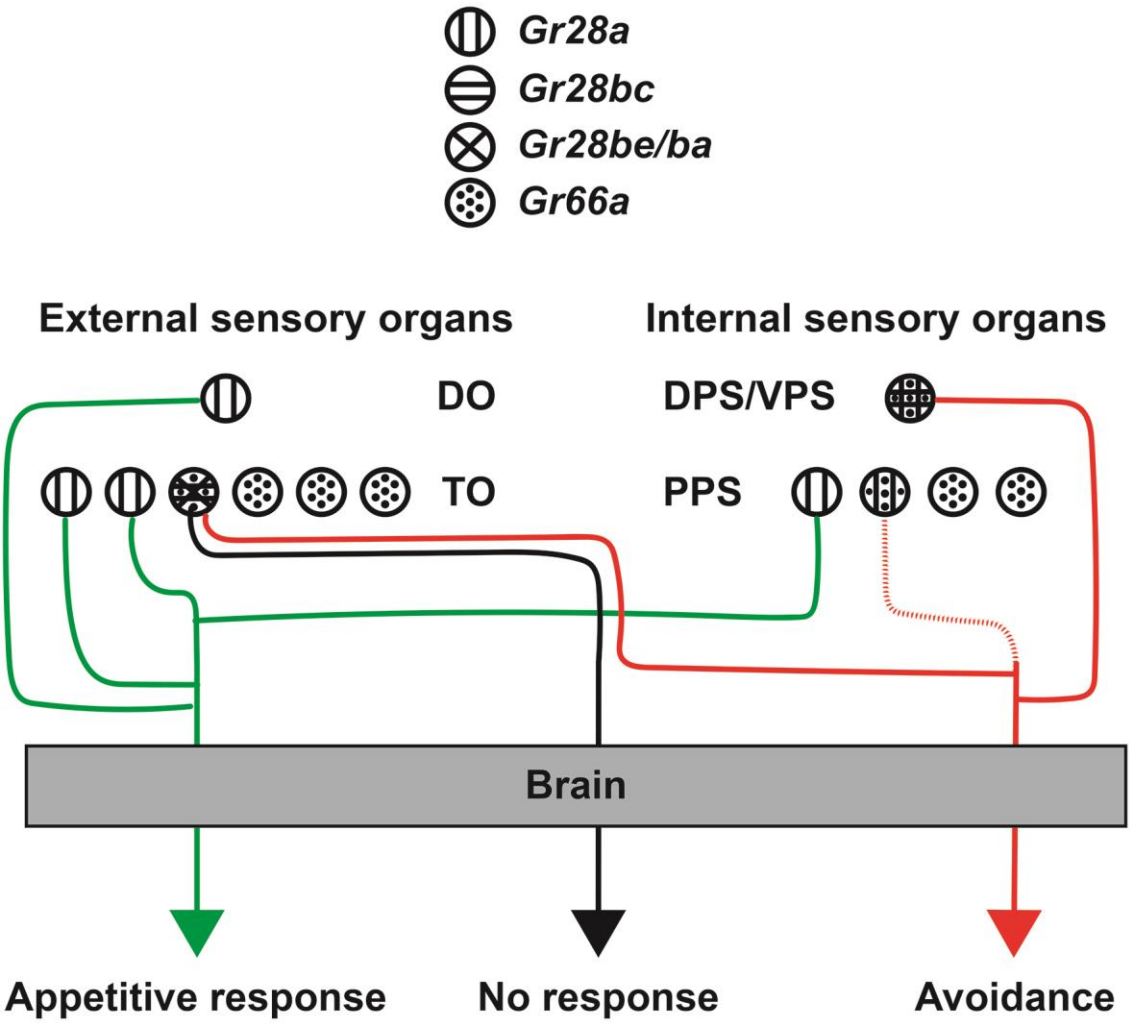

Gr28a
Gr28bc
Gr28be/ba
Gr66a
External sensory organs
Internal sensory organs
DO
DPS/VPS
TO
PPS
Brain
Appetitive response
No response
Avoidance

Figure 7

```
                                                IL1
Gr28ba   ----------------MIRCGLDIFRGCRGRFRYWLSARDCYDSISLMVAIAFALGITPFLVR   47
Gr28bc   MDIEMAKEPVNPTDTPDIEVTPGLCQPLRRRFRRFVTAKQLYECLRPVFHVTYIHGLTSFYIS   63
Gr28bb   -------------------------MSALRRVRKYFISSQVYEALRPLFFLTFLYGLTPFHVV   38
Gr28bd   ----------------------------MSFYFCEIFKPRDAFGAEQTLLLYTYLLGLTPFRL-   35
Gr28be   -----------------------MWLLRRSVGKSGNRPHDVYTCYRLTIFMALCLGIVPYYVS   40
Gr28a    --------------------------MAFKLWERFSQADNVFQALRPLTFIS-LLGLAPFRLN   36
                                                : : .       :   *:. : :
                                TM1                     EL1        TM2
Gr28ba   -RNALGENSLEQSWYGFLNAIFRWLLLAYCYSYI-NLRNESLIGYFMRNHVSQISTRVHDVGG   108
Gr28bc   CDTKTGKKAIKKTIFGYINGIMHIAMFVFAYSLTIYNNCESVASYFFRSRITYFGDLMQIVSG   126
Gr28bb   -RRKMGESYLKMSCFGVFNIFIYICLCGFCYIS-SLRQGESIVGYFFRTEISTIGDRLQIFNG   99
Gr28bd   -RGQAGERQFHLSKIGYLNAFLQLSFFSYCFLAA-LIEQQSIVGYFFKSEISQMGDSLQKFIG   96
Gr28be   -ISSEGRGKLTSSYIGYINIIIRMAIYMVNSFYG-AVNRDTLMSNFFLTDISNVIDALQKING   101
Gr28a    L---NPRKEVQTSKFSFFAGIVHFLFFVLCFGIS-VKEGDSIIGYFFQTNITRFSDGTLRLTG   95
               .  .  :  . :  :.   :           . ::: . *: . :: .      . *
                                IL2                     TM3
Gr28ba   IIAAVFTFILPLLLRKYFLKSVKNMVQVDTQ-LERLRSPVNFNTVVGQVVLVILAVVLLDTVL   170
Gr28bc   FIGVTVIYLTAFVPNHRLERCLQKFHTMDVQ-LQTVGVKIMYSKVLRFSYMVLISMFLVNVLF   188
Gr28bb   LIAGAVIYTSAILKRCKLLGTLTILHSLDTN-FSNIGVRVKYSRIFRYSLLVLIFKLLILGVY   161
Gr28bd   MTGMSILFLCSSIRVRLLIHIWDRISYIDDR-FLNLGVCFNYPAIMRLRLLQIFLINGVQLGY   158
Gr28be   MLGIFAILLISLLNRKELLKLLATFDRLETEAFPRVGVAMHQVAANKKMNRLVIILVGSMVAY   164
Gr28a    ILAMSTIFGFAMFKRQRLVSIIQNNIVVDEI-FVRLGMKLDYRRILLSSFLISLGMLLFNVIY   157
         : .         .    :         ::   :  :   .             :
                EL2                 TM4                         IL3
Gr28ba   LTTGLVCLAKME-VYASWQLTFIFVYELLAISITICMFCLMTRTVQRRITCLHKVLKNLAHQW   232
Gr28bc   TGGTFSVLYSSE-VAPTMALHFTFLIQHTVIAIAIALFSCFTYLVEMRLVMVNK           241
Gr28bb   FVGVFRLLVSLD-VTPSFCVCMTFFLQHSVVSIAICLFCVIAFSFERRLSIINQ           214
Gr28bd   LISSNWMLLGND-VRPIYTAIVAFYVPQIFLLSIVMLFNATLHRLWQHFTVLNQ           211
Gr28be   ITCSFLMISLRDTTTFSISAVISFFSPHFIVCAVSFLAGNVMIKLRIYLSALNE           218
Gr28a    LCVSYSLLVSAT-ISPSFVTFTTFALPHINISLMVFKFLCTTDLARSRFSMLNEILQDILDAH   219
               :               *      :                 :     :*::: .

Gr28ba   DT----R---SLKA-VNQKQRS-------LQCLDSFSMYTIVTKDPAEIIQESMEIHHLICEA   280
Gr28a    IEQLSALELSPMHSVVNHRRYSHRLRNLISTPMKRYSVTSVIRLNPEYAIKQVSNIHNLLCDI   282
                   ::: **::: *          :. :*: :::  :*   *::  :**:*:*:
                        TM5                   EL3          TM6
Gr28ba   AATANKYFTYQLLTIISIAFLIIVFDAYYVLETLLGKSKRESKFKTVEFVTFFSCQMILYLIA   343
Gr28a    CQTIEEYFTYPLLGIIAISFLFILFDDFYILEAILN-PKRLDVFEADEFFAFFLMQLIWYIVI   344
         . * ::**** ** **:*:**:*:** :*:**::*.  ** . *:: **.:**  *:* *::
                                IL4
Gr28ba   IISIVEGSNRAIKKSEKTGGIVHSLLNKTKSAEVKEKLQQFSMQLMHLKINFTAAGLFNIDRT   406
Gr28a    IVLIVEGSSRTILHSSYTAAIVHKILNITDDPELRDRLFRLSLQLSHRKVLFTAAGLFRLDRT   407
         *: *****.*:* :*. *..***.:** *.. *::::* ::*:** * *: *******.:***
                TM7                 EL4
Gr28ba   LYFTISGALTTYLIILLQFTSNSPNNGYGNGSSCCETFNNMTNHTL--                  452
Gr28a    LIFTITGAATCYLIILIQFRFTHHMDDTSSNS-----TNNLHSIHLGD                  450
         * ***:** * *****:**  .   :. ...*      **: .  *
```

Figure 8

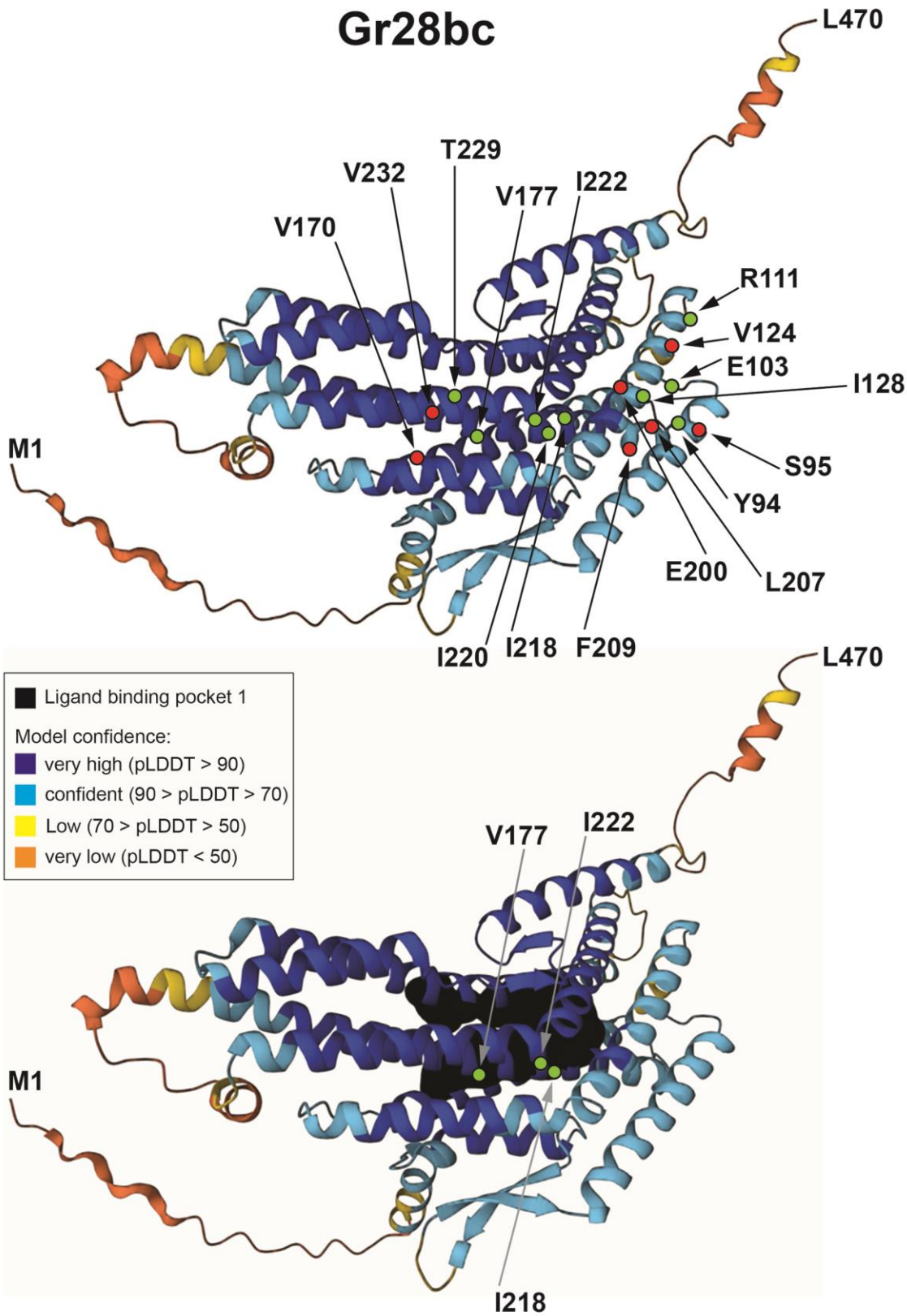

Gr28bc
L470
T229
V232
V177
I222
V170
R111
V124
E103
I128
M1
S95
Y94
E200
L207
I220
I218
F209
Ligand binding pocket 1
Model confidence:
very high (pLDDT > 90)
confident (90 > pLDDT > 70)
Low (70 > pLDDT > 50)
very low (pLDDT < 50)
L470
V177
I222
M1
I218

Figure 1-figure supplement 1

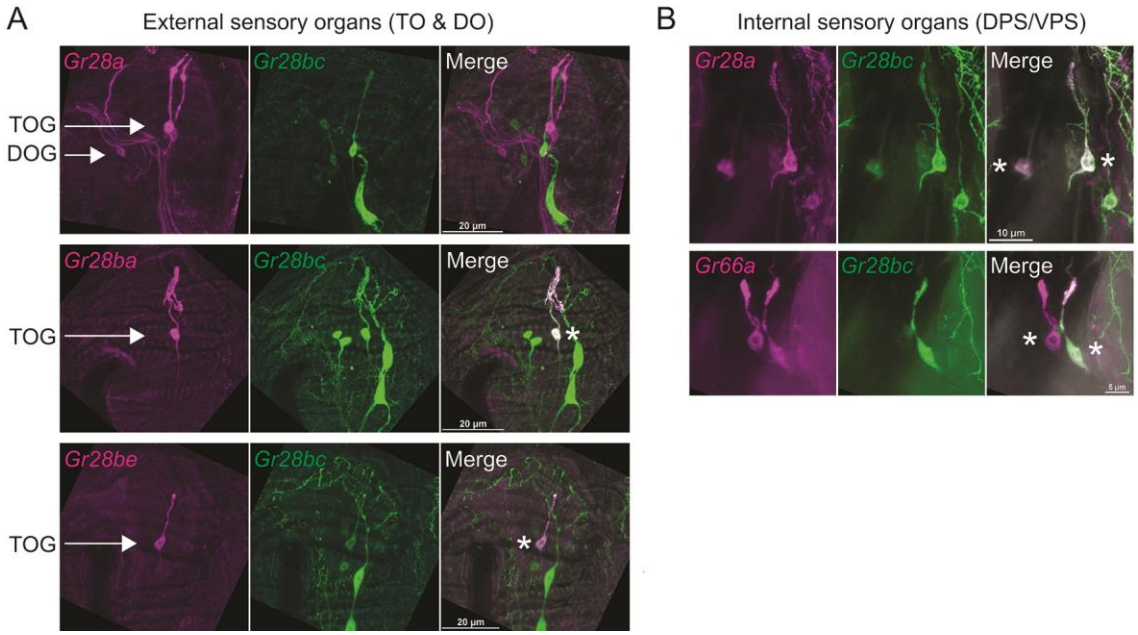
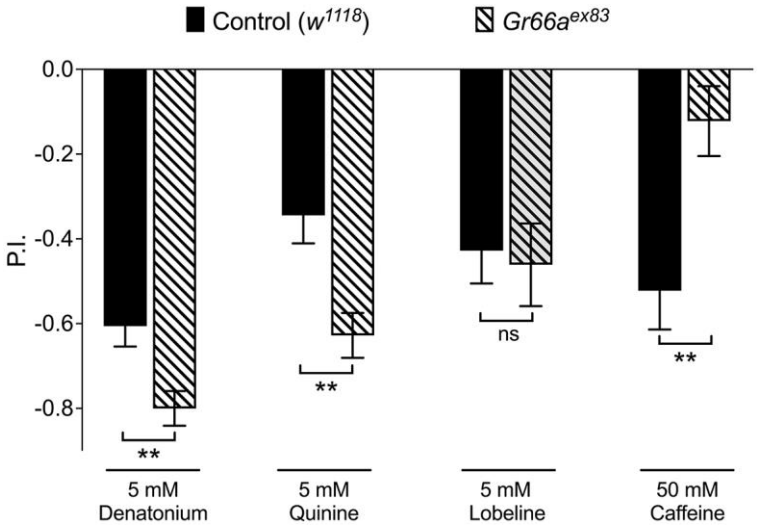



Figure 4-figure supplement 1