Opposing chemosensory functions of closely related gustatory receptors

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ABSTRACT

Most animals have functionally distinct populations of taste cells, expressing receptors that are tuned to compounds of different valence. This organizational feature allows for discrimination between chemicals associated with specific taste modalities and facilitates differentiating between unadulterated foods and foods contaminated with toxic substances. In the fruit fly *D. melanogaster*, primary sensory neurons express taste receptors that are tuned to distinct groups of chemicals, thereby activating neural ensembles that elicit either feeding or avoidance behavior. Members of a family of ligand gated receptor channels, the Gustatory receptors (Grs), 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 wildtype *Drosophila* larvae do not respond. When VR1 is expressed in *Gr28a* neurons, larvae become attracted to capsaicin, consistent with reports showing that *Gr28a* itself encodes a receptor for nutritious RNA. In contrast, expression of VR1 in two pairs of *Gr28b.c* neurons triggers avoidance to capsaicin. Moreover, neuronal inactivation experiments show that the *Gr28b.c* neurons are necessary for avoidance of several bitter compounds. Lastly, behavioral experiments of *Gr28* deficient larvae and live Ca^{2+} imaging studies of *Gr28b.c* neurons revealed that denatonium benzoate, a synthetic bitter compound that shares structural similarities with natural bitter chemicals, is a ligand for a receptor complex containing a Gr28b.c or Gr28b.a subunit. Thus, the *Gr28* proteins, which have been evolutionarily conserved over 260 million years in insects, represent the first taste receptor subfamily in which specific members mediate behavior with opposite valence.

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 *Drosophila* gustatory system is characterized by several insect specific anatomical and molecular features. For example, taste cells are primary sensory neurons, with dendritic processes that express taste receptors, while long axons project and 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 (Amrein, 2016; Joseph and Carlson, 2015; Scott, 2018). Likewise, *Drosophila* larvae have numerous structures located both on the head surface as well as internally along the larval pharynx, where chemical compounds are assessed during their passage towards the digestive system (Figure 1A)(Apostolopoulou et al., 2015; Kwon et al., 2011; Rist and Thum, 2017).

Fruit flies, like most insects, employ taste receptors encoded by two major gene families, the *Gustatory receptor* (*Gr*) and the *Ionotropic Receptor* (*IR*) genes, to sense soluble chemicals, such as appetitive food compounds, noxious and toxic chemicals as well as pheromones. Both Gr- and IR- based receptors are thought to form complexes composed of several, and generally different, subunits. Composition of only a few Gr-based taste receptors for sugars and bitter compounds is known (Jiao et al., 2008; Yavuz et al., 2014; Shim et al., 2015; Sung et al., 2017) (Fujii et al., unpublished), and two Gr proteins, Gr21a and Gr63a, are co-expressed in a small subset of olfactory neurons where they form a receptor complex for gaseous carbon dioxide (Jones et al., 2007; Kwon et al., 2007).

The *Gr* genes represent the largest taste receptor gene family in insects. In *D. melanogaster*, it is comprised of 60 genes predicted to encode 68 proteins, expression of which has been extensively described (Dahanukar et al., 2007; Dunipace et al., 2001; Fujii et al., 2015; Scott et al., 2001; Weiss et al., 2011). 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 (Amrein, 2016; Montell, 2021), but only a few have been studied in larvae (Apostolopoulou et al., 2016a; Choi et al., 2016, 2020; Mishra et al., 2013, 2018).

The *Gr28* gene subfamily is of particular interest for a number of reasons: First, it is one of the most conserved *Gr* subfamilies, homologs of which can be found across all insect families and even more distant arthropods (Eyun et al., 2017; Fujii et al., 2023; Suzuki et al., 2018). The six *Gr28* genes are tightly clustered, with the five *Gr28b* genes transcribed from distinct promotors and unique exons spliced to shared 2nd and 3rd exons, while *Gr28a* is a separate transcription unit (Figure 1B). Overall conservation between the Gr28 proteins is high (equal or more than 50% similarity), characteristic for genes generated through recent gene duplication events. Second, the *Gr28* genes are expressed not only in the gustatory system of larvae and adult flies, but also 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 (Mishra et al., 2018; 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 *Gr28b.d* 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 known gustatory function for any Gr28 protein thus far is sensing of RNA and ribose by Gr28a, mediated by *Gr28a-GAL4* GRNs (Mishra et al., 2018). Remarkably, sensing RNA and ribose is an appetitive taste quality found in other dipteran insects that diverged from *Drosophila* about 260 million years ago, including flesh flies and mosquitoes, and we showed that *Gr28* homologs from *A. aegypti* and *A. gambiae* can restore RNA and ribose preference in

Drosophila Gr28 mutant larvae when expressed in *Gr28a-GAL4* GRNs (Fujii et al., 2023).

Here, we present a detailed expression analysis and functional characterization of the *Gr28* genes and the respective GRNs in *Drosophila* larvae. In addition to *Gr28a,* three of the five *Gr28b* genes (*Gr28b.a, Gr28b.c* and *Gr28b.e*) are also expressed in the larval taste system. Interestingly, GRNs expressing *Gr28a* and the three *Gr28b* genes represent functionally distinct neuronal ensembles, with minimal expression overlap in a single pair of neurons expressing both *Gr28a* and *Gr28b.c*. When the mammalian Vanilloid Receptor 1 (VR1) is expressed under the control of specific GAL4 drivers, we found that *Gr28a-GAL4* and *Gr28b.c-GAL4* neurons mediate opposing taste behavior in the presence of capsaicin, the ligand for VR1. Specifically, *Gr28a-GAL4/UAS-VR1* larvae show strong attraction for capsaicin, while *Gr28b.c-GAL4/UAS-VR1* larvae show strong avoidance of capsaicin. Neuronal inactivation experiments reveal that the *Gr28b.c* GRNs are necessary to sense bitter compounds, such as denatonium, quinine, lobeline, and caffeine. Moreover, Ca^{2+} responses of *Gr28b.c* GRNs to denatonium and quinine are significantly reduced and avoidance behavior of these two chemicals is diminished in *Gr28* mutant larvae, whereas Ca^{2+} responses and avoidance behavior were not affected when challenged with lobeline and caffeine. This implies that Gr28b proteins are subunits of receptor complexes sensing a subgroup of bitter tasting compounds. In summary, the *Gr28* genes encode related Gr proteins mediating both positive and negative valence.

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 are organized as paired ganglia, the Dorsal, Terminal and Ventral Organ Ganglia (DOGs, TOGs and VOGs) that house the GRN cell bodies with dendritic extensions in respective sensory organs (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 GRNs. For clarity, GRN numbers refer to neurons in one of the two, bilaterally symmetrical taste organs. The GRNs located in the DOG fall into two distinct groups, based on their dendritic extensions: 11 presumptive GRNs, four of which were shown to express *Gr* genes (Apostolopoulou et al., 2015), send dendrites to the base of the DO, while 7 neurons extend dendrites to the TO (the dorsolateral group, Figure 1A)(Kwon et al., 2011). The TOG contains 30 neurons, with dendrites located in 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 are moved 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 (Rist and Thum, 2017; Sánchez-Alcañiz et al., 2018; Stewart et al., 2015).

Our initial expression analysis of the *Gr28* genes revealed that four of the six *Gr28* genes (*Gr28a*, *Gr28b.a*, *Gr28b.c* and *Gr28b.e*) were 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; Thorne and Amrein, 2008). This and all previous *Gr* expression studies were performed using bimodal expression systems, mostly *GAL4/UAS*, whereby *Gr* promotors driving *GAL4* are assumed to faithfully reproduce expression of the respective *Gr* genes. Importantly, we analyzed two or four *Gr28-GAL4* insertion lines for each transgene, and at least two generated the same expression pattern (Mishra et al., 2018; Thorne and Amrein, 2008) providing evidence that the drivers reflect a fairly accurate expression profile of respective endogenous genes. To further delineate the putative chemosensory roles of these genes, we performed a more detailed co-expression analysis between the *Gr28* genes and the bitter receptor gene *Gr66a* by combining the *GAL4/UAS* (labeling *Gr28* expressing neurons) with the *LexA/lexAop* (marking *Gr66a* neurons) system (Figure 1C and 1D). In the TOG, we found expression of all four *Gr28* genes, along with that of *Gr66a*, which was expressed in 3 or 4 neurons (this number is slightly smaller than the 6 neurons previously reported

by (Kwon et al., 2011). *GAL4* drivers for *Gr28b.a*, *Gr28b.c* and *Gr28b.e* are co-expressed in a single neuron with *Gr66a-LexA* (Figure 1C). In contrast, *Gr28a-GAL4* is expressed in a distinct TOG neuron than *Gr66a-LexA* (Figure 1C) and the *Gr28b* genes, an observation we independently confirmed using a *Gr28b.c-LexA* driver (Figure 1-figure supplement 1). In the DOG, we find only a single *Gr28a-GAL4* neuron, while none of the *Gr28b* genes, or *Gr66a*, is expressed there (Figure 1C). None of the *Gr28-GAL4* drivers was expressed in the VOG. In the internal sensory organs, *Gr66a-LexA* is found in one GRN in the DPS/VPS, where it is co-expressed with *Gr28b.c-GAL4* as well as *Gr28a-GAL4* (Figure 1D). In the PPS, none of the *Gr28b* genes is expressed, but *Gr28a-GAL4* is found in two neurons, one of which also expresses *Gr66a-LexA* (Figure 1D). In summary, the internal sensory organs can be subdivided in three distinct groups (Figure 1E): *Gr66a/Gr28b.c/Gr28a* positive neurons, *Gr66a/Gr28a* positive neurons and *Gr28a*^{only} neurons, which contrasts the external sensory organs where *Gr28a* and the *Gr28b* genes are expressed in a mutually exclusive fashion.

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)(Figure 2A). Specifically, larvae homozygous mutant for *Gr28* (*ΔGr28*, a deletion of the entire *Gr28a* gene and more than half of the coding region of all *Gr28b* genes, Figure 1B), lose their ability to sense these compounds, a phenotype that is restored when single *UAS-Gr28* reporter transgenes are expressed in *Gr28a-GAL4* neurons. 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*, *Gr28b.a-GAL4*, *Gr28b.c-GAL4* and *Gr28b.e-GAL4*) in

larvae and tested their response to capsaicin using the two-choice preference assay (Figure 2A). Additionally, we expressed *UAS-VR1* in bitter neurons as well as appetitive fructose sensing neurons using respective *GAL4* drivers (*Gr66a-GAL4* and *Gr43a*^{GAL4}) (Mishra et al., 2013; Scott et al., 2001), which served as control larvae (note that fructose sensing *Gr43a*^{GAL4} neurons are distinct from *Gr28a-GAL4* neurons (Mishra et al., 2018)). Just like adult flies, *w*^{1118} control larvae lacking either a *UAS-VR1* reporter, a *Gr-GAL4* driver, or both, were unresponsive to 0.1 mM capsaicin, while the positive control larvae expressing *UAS-VR1* in either fructose sensing GRNs or bitter taste GRNs showed robust attraction to or avoidance of capsaicin (Figure 2B). Consistent with previous findings, which identified *Gr28a* GRNs as appetitive neurons (Mishra et al., 2018), larvae expressing *UAS-VR1* under the control of *Gr28a-GAL4* showed strong appetitive responses to capsaicin (Figure 2B). In contrast, larvae expressing *UAS-VR1* under the control of *Gr28b.c-GAL4* showed robust avoidance behavior to capsaicin, while expression in the single TOG GRN using either *Gr28b.a-GAL4* or *Gr28b.e-GAL4* (also expressing *Gr28b.c*; Figure 1C) caused neither attraction to nor avoidance of capsaicin.

Overlap between *Gr28a* and *Gr66a* in internal GRNs raises the question about their contribution to appetitive and/or avoidance behavior. We deemed it unlikely that these neurons were critical for appetitive behavior, since the one pair located in the DPS/VPS (expressing also *Gr28b.c*) is necessary for capsaicin avoidance (see above). Indeed, when VR1 was suppressed in all *Gr66a/Gr28b.c* neurons by means of the GAL4 suppressor GAL80, these larvae, expressing VR1 in *Gr28a*^{only} GRNs remained strongly attracted to capsaicin (Figure 2B last panel; for effective suppression, see Figure 2- figure supplement 1). Taken together, this analysis indicates that *Gr28a-GAL4* is expressed in appetitive inducing neurons, while *Gr28b.c-GAL4* neurons mediate avoidance behavior.

The capsaicin experiments above suggest that the two larval *Gr28b.c-GAL4* GRNs mediate negative valence. Previous reports have shown that larvae avoid many bitter compounds (Apostolopoulou et al., 2016b, 2015; Choi et al., 2020, 2016; van Giesen et al., 2016), and thus, we expected that eliminating activity of *Gr28b.c-GAL4* GRNs would result in loss of avoidance behavior to at least some bitter chemicals.

Indeed, when *Gr28b.c-GAL4* GRN activity was blocked using the inward rectifying potassium channel Kir2.1 (Baines et al., 2001; Paradis et al., 2001), larvae no longer avoided the four tested bitter compounds denatonium, quinine, lobeline and caffeine (Figure 3). A compound specific avoidance phenotype to lobeline and caffeine was observed when the *Gr28b.e-GAL4* GRNs in the TOG was inactivated. These data, together with the capsaicin experiments, suggest that two pairs of GRNs, one in the TOG and one in the DPS/VPS are necessary and sufficient for avoidance of these four bitter tasting chemicals.

Gr28b.c and Gr28b.a are subunits of a taste receptor complex for denatonium.

We next examined whether any of the Gr28b proteins is part of a taste receptor complex detecting any of these bitter chemicals (Figure 4). Surprisingly, only avoidance of denatonium was affected in larvae lacking the *Gr28* gene cluster (*ΔGr28*; Figure 1B), while avoidance to quinine was somewhat reduced, albeit not significantly, and avoidance to lobeline or caffeine remained robust. In fact, avoidance to caffeine increased modestly, but significantly (Figure 4A). We then examined whether any of the Gr28 proteins was sufficient to restore denatonium avoidance by expressing individual *Gr28* genes under the control of the *Gr28b.c-GAL4* driver. Indeed, either *Gr28b.a* or *Gr28b.c* expression led to a full recovery of denatonium avoidance, while expression of any other *Gr28b* gene, or *Gr28a*, failed to do so (Figure 4B). This observation suggests that despite the high level of similarity between these receptors, recognition of denatonium is dependent on specific structural features present in Gr28b.a and Gr28b.c, but not in any of the other Gr28 proteins.

Since *Gr66a-LexA* is co-expressed in all *Gr28b-GAL4* expressing GRNs, we wondered whether Gr66a is a component of the denatonium receptor. Previous work had established that *Gr66a* is required for caffeine avoidance, in both larvae and adult flies (Apostolopoulou et al., 2016b; Lee et al., 2009; Moon et al., 2006), which we confirmed (Figure 4-figure supplement 1). Surprisingly, avoidance of denatonium and quinine was not diminished, but increased significantly (Figure 4-figure supplement 1). Given the multimeric nature of bitter taste receptors (Sung et al., 2017), one possibility is that the absence of a Gr subunit not required for the detection of denatonium (Gr66a)

could favor formation of multimeric complexes containing Gr subunits that recognize this compound (such as Gr28b.a and/or Gr28b.c).

Finally, we investigated neuronal responses in larvae expressing the Ca^{2+} indicator GCaMP6m in *Gr28b.c-GAL4* GRNs (Figure 5). We developed a whole animal imaging preparation, whereby larvae were placed in an “imaging chamber” to minimize head movements (Figure 5A), and visualized neural activity of the *Gr28b.c-GAL4* GRN in the TOG 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 *Gr28b.c-GAL4* GRNs, while none of the sugars did (Figure 5C and 5D). When neural activity was recorded in *Gr28b.c-GAL4* GRNs of *ΔGr28* homozygous mutant larvae (Figure 5E), Ca^{2+} responses to denatonium and quinine were severely reduced, while responses to both caffeine and lobeline were not affected. Re-expression of either *Gr28b.a* or *Gr28b.c*, but not *Gr28b.b*, *Gr28b.d, Gr28b.e* or *Gr28a* rescued Ca^{2+} response to denatonium, but not to quinine (Figure 5F and 5G). Together, these experiments identified *Gr28b.c* and *Gr28b.a* as redundant subunits 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 (Agnihotri et al., 2016; Engsontia and Satasook, 2021; 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 essential for larvae and detected 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).

Previous studies in adult *Drosophila* have shown that members of conserved Gr protein families such as the carbon dioxide receptors (*Gr21a* and *Gr63a*) (Jones et al., 2007; Kwon et al., 2007) or the receptors for sweet taste encoded by the eight sugar *Gr* genes (Fujii et al., 2015) are largely co-expressed in one type of neuron in the fly's taste organs. For example, with the exception of *Gr5a* (see below), sugar *Gr* genes are only expressed in a single GRN (the "sweet' neuron) of each taste sensilla, and activation of these "sweet" neurons by sugars requires the function of at least two of the 8 sugar *Gr* genes (Dahanukar et al., 2007; Yavuz et al., 2014)(Fujii et al., unpublished). Similarly, the approximately 33 putative bitter taste receptors, which comprise several small conserved subfamilies (Robertson et al., 2003), as well as individual *Gr* genes with little overall similarity to one another, are partially co-expressed in a the bitter GRN of each taste sensilla (Weiss et al., 2011). Molecular genetic studies combined with electrophysiological recordings have shown that at least three different Gr subunits are required to constitute functional receptor complexes that can sense a bitter compound (Shim et al., 2015). We note that two rare exceptions to the heteromeric nature of taste receptor complexes exist, namely the RNA receptor Gr28a and the fructose receptor Gr43, which have been proposed to function as homomultimeric complexes (Mishra et al., 2018, 2013). Cryo-EM structural analysis of the conserved insect Olfactory receptor co-receptor (ORCO) suggests that insect odorant receptors form tetramers (Butterwick et al., 2018), and biochemical characterization and comparative modeling of BmGr9, the *Bombyx* mori homolog of the *Drosophila* Gr43a fructose receptor, supports such structures for Gr proteins as well (Morinaga et al., 2022).

Distinct functions are mediated by small set of GRNs expressing specific Gr28 subunits.

Our expression analysis of the bitter taste receptor gene *Gr66a* and the *Gr28* genes in larvae is consistent with earlier studies, despite some small variation in neuron number (Choi et al., 2016; Kwon et al., 2011), which is likely due to the use of different *GAL4* driver lines and/or variability in expression levels. Importantly, all *Gr28b* genes are co-expressed with the bitter taste receptor *Gr66a* (Figure1) and probably several other putative bitter *Gr* genes (Kwon et al., 2011; Rist and Thum, 2017), while *Gr28a* is

found in a largely, but not entirely, distinct set of GRNs. Whether and what kind of *Gr* genes might be co-expressed with *Gr28* in *Gr28*^{only} GRNs will require more in-depth expression studies and might shed light on other receptors involved in appetitive behaviors of larvae.

A key finding of the work presented here is the observation that *Gr28a*^{only} and *Gr28b.c* neurons dictate distinct behavioral programs, the former representing an ensemble of neurons that instruct larvae to "go towards" a chemical source and consume it, while the latter do the opposite (Figure 2). This observation is reminiscent of a seminal study by Troemel and colleagues in the *C. elegans* chemosensory system, who reported that the valence of a chemical compound is dependent on the identity of a neuron, and not the identity of the molecular receptor the neuron expresses (Troemel et al., 1997). The number of "go-away" GRNs in *Drosophila* larvae co-expressing *Gr28b.c* 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 to induce avoidance behavior, as expression of V1R in only the TO pair (under the control of either *Gr28b.a-GAL4* or *Gr28b.e-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} GRNs). Thus, the minimal requirement to induce "go-to" and "go-away" behavior is defined by distinct sets of GRNs, and each appears to be composed of neurons located in both external as well as the internal taste organs (Figure 7). Co-expression of the RNA receptor Gr28a and in the DPS/VPS GRN essential for bitter taste (Figures 2B and 3) raises interesting questions about additional functions for Gr28a in bitter taste. We note that the sweet taste receptor Gr5a, a subunit of a multimeric trehalose receptor, is also expressed in non-sweet neurons of unknown function (Fujii et al., 2015).

Functional redundancy of taste receptors

Both behavioral analyses as well as Ca^{2+} imaging experiments implicate at least one Gr28b protein as an essential component of a denatonium receptor complex, as *ΔGr28* larvae exhibit total loss of avoidance (Figure 4), and respective GRNs fail to elicit a

response upon exposure to this chemical (Figure 3). What the precise composition of that complex is remains to be determined, but recovery of denatonium responses by expressing either Gr28b.c and/or Gr28b.a indicates that either one of these (or possibly both) is an essential subunit, in addition to other Grs expressed in this GRNs, such as Gr22a and Gr59c (Choi et al., 2020; Rist and Thum, 2017), while Gr66a is unlikely to be part of such a complex based on our behavioral analysis (Figure 4-figure supplement 1). Since only Gr28b.a and Gr28b.c can rescue denatonium responses in *Gr28b.c* GRNs of *ΔGr28* mutant larvae, sequence comparison between the unique N-terminal halves of the Gr28 proteins comprising the first four transmembrane domains and the extracellular loops 1 and 2 might provide insights as to possible residues important for ligand recognition. When interrogating these regions, only seven residues are identical between Gr28b.a and Gr28b.c (Table 1, Figure 6). Reducing the stringency requirement by allowing one of the remaining receptors to share the same residue, nine additional sites are identified. One experimental avenue to validate these residues as important sites contributing to denatonium binding might involve introduction of point mutations that converts respective amino acids of other Gr28b proteins into those found in Gr28b.a/Gr28b.c.

The role of Gr28b proteins in quinine detection is less clear, and the different phenotypes observed in behavioral experiments and Ca^{2+} imaging suggest that at least two molecular types of quinine receptors exist in larvae. Ca^{2+} imaging experiments implicate a role for multiple Gr28b subunits in a quinine receptor complex in the TOG neuron, since single *Gr28b* genes cannot restore the loss of quinine response in *ΔGr28* larvae (Figure 5E and 5G). However, because *ΔGr28* mutant larvae still avoid quinine (Figure 4), at least one Gr28b independent receptor must be exist in one or several other GRNs, one of which is likely the DPS/VPS, since expression of Kir2.1 in that neuron, along with the TOG neuron, totally abolishes quinine avoidance (Figure 3). We note that functional redundancy is also observed in sweet taste receptors, where we found that different combinations of sugar *Gr* genes can restore responses to the same sugar when expressed in tarsal sweet GRNs of sugar blind flies (Fujii et al. unpublished).

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:	*Gr28a-GAL4*	Thorne and	FlyBase:

w; P{Gr28a-GAL4.T}SF36S*		Amrein, 2008	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*	*Gr28b.a-GAL4*	Thorne and Amrein, 2008	FlyBase: FBtp0054526
D. melanogaster: w; P{Gr28b.c-GAL4}NT21B1*	*Gr28b.c-GAL4*	Thorne and Amrein, 2008	FlyBase: FBtp0054528
D. melanogaster: w; P{Gr28b.e-GAL4.4.245}Gr28a3AII*	*Gr28b.e-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-Gr28b.a*	Ni et al., 2013	Flybase: FBal0291410
D.melanogaster: w; P{UAS-Gr28b.B}attP2*	*UAS-Gr28b.b*	Ni et al., 2013	Flybase: FBal0291412
D.melanogaster: w; P{UAS-Gr28b.C}attP2*	*UAS-Gr28b.c*	Ni et al., 2013	Flybase: FBal0291411
D.melanogaster: w; P{UAS-Gr28b.D}attP2*	*UAS-Gr28b.d*	Ni et al., 2013	Flybase: FBal0291409
D.melanogaster: w; P{UAS-Gr28b.E}attP2*	*UAS-Gr28b.e*	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^1w;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:	*Gr66a-LexA*	Thistle et al.,	BDSC: 93024

w^{1118}; P{Gr66a-lexA.S}2;TM2/TM6B		2012	Flybase: FBst0093024
D. melanogaster: w^{1118}; P{lexAop-GAL80. T}	*lexAop-GAL80*	Thistle et al., 2012	Flybase: FBtp0079728
D. melanogaster: w^{1118};P{Gr28b.c-LexA}#8	*Gr28b.c-LexA*	This paper	
Sequence-based reagents			
Gr28b.c_F	5′-AATCTAGGTACCCCGGCTGCTCGTCTCCCTGGATGT-3′	This paper	PCR primers
Gr28b.c_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 Figure 1 and Figure 2B, and *SF36E1* for Figure 2B and Figure 2-figure supplement 1, *Gr28b.a-GAL4*(*NT42aC51a*), *Gr28b.c-GAL4*(*NT21B1*) (Thorne and Amrein, 2008); *Gr28b.e-GAL4*(*Gr28a3AII*) and *Gr66a-GAL4* (Scott et al., 2001); *⊿Gr28*(*54B3*) (Mishra et al., 2018); *UAS-Gr28a*, *UAS-Gr28b.a*, *UAS-Gr28b.b*, *UAS-Gr28b.c*, *UAS-Gr28b.d* and *UAS-Gr28b.e* (Ni et al., 2013); $Gr43a^{GAL4}$ (Miyamoto et al., 2012); *Gr66a-LexA* and *lexAop-GAL80* (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); *Gr28b.c-LexA*(#8)

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. They 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. The next day, 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, followed by incubation 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. Finally, heads were washed six times in washing buffer for 20 min each at room temperature under gentle agitation. Heads were then 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 modifications. 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. Agarose food dishes for two-choice preference assay were prepared just prior each experiment as follows: 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, images 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: $PI = (N_{tastant} - N_{plain}) / N_{Total,}$ whereby N is the number of larvae in the tastant sector, the plain agarose sector and the total number, respectively. Positive values indicate a preference for capsaicin or bitter compound while negative values indicate repulsion (avoidance).

Calcium imaging

Calcium imaging was performed in *Gr28b.c* 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 µl) 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 (40µl) to set a baseline. The first ligand solution (40 µl of bitter chemical or sugar) was applied thereafter, followed by 5 washes with carrier (100 µl of water). After a 3-minute pause to allow the preparation to recalibrate, a second ligand solution (40 µl 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 *Gr28b.c-LexA* flies

To generate the *Gr28b.c-LexA* driver, a 1.3 kb DNA fragment immediately upstream of the *Gr28b.c* start codon was amplified from w^{1118} flies using a forward (5′-AATCTAGGTACCCCGGCTGCTCGTCTCCCTGGATGT-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). The clone chosen was confirmed by DNA sequence analysis. Transgenic flies were generated by standard P-element transformation of w^{1118} 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. 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, Figure 2-figure supplement 1B and Figure 4-figure supplement 1) with two-tailed P value were 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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FIGURES

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

A) **Schematic representation of the larval chemosensory system**. Three external sensory organs (DO, TO and VO) hold collectively the dendritic extensions of neuronal cell bodies in the respective ganglia (DOG, TOG and VOG). Three clusters of sensory neurons (DPS, VPS and PPS) reside along the pharynx. The antennal nerve (AN) connects the DOG neurons to the subesophageal zone (SEZ). The TOG and VOG neurons project along the maxillary nerve (MN) to the SEZ. The DPS and PPS neurons project axons to the SEZ via the labral nerve (LRN). The VPS neurons project to the SEZ through the labial nerve (LBN). Note that olfactory neurons are omitted in the schematic here and in E (below).

B) **Structure of the *Gr28* locus.** The six *Gr28* genes are clustered within 10 kilobases. The five *Gr28b* genes are transcribed from distinct promotors, with first unique exons that are spliced to common second and third exons. *Gr28a* is downstream of the *Gr28b* genes. The *Gr28* mutation (*ΔGr28*) used in this study lacks the shared common exons of the *Gr28b* genes and the entire *Gr28a* gene.

C) **Expression of the *Gr28* genes in the external sensory organs.** Note that images show only one of the bilaterally symmetrical organs. Co-expression between *Gr66a* (a marker for bitter taste GRNs) and different *Gr28* genes (in "Merge" panel) was assessed using *GAL4* and *LexA* drivers for *Gr28* genes and *Gr66a*, respectively. For *Gr28b.a*, *Gr28b.c* and *Gr28b.e* co-expression was observed in each case. However, *Gr66a* and *Gr28a* are expressed in different GRNs. Asterisks refer to a GRN expressing both *Gr66a* and the indicated *Gr28* gene. Scale bars are 5 µm.

D) **Expression of the *Gr28* genes in internal sensory organs.** Note that the bilaterally symmetrical halves of the DPS/VPS are physically close to each other, and the images includes both halves, while the image of the PPS shows only one side of the bilaterally symmetrical organ. *Gr28b.c*, but none of the other *Gr28b* genes, is co-expressed with *Gr66a* in the DPS/VPS. In the PPS, none of

the *Gr28b* genes is found, while *Gr28a* and *Gr66a* are partially co-expressed, but each gene is also expressed exclusively in a subset of GRNs. Asterisks refer to a GRN expressing both *Gr66a* and the indicated *Gr28* gene. Scale bars are 5 µm.

E) **Expression summary:** Only relevant neurons in one of the paired taste organs, are shown with total number of neurons indicated in parenthesis. The cartoon summarizes the immunostainings shown in C) and D). The VOG is not shown as none of the *Gr28-GAL4* drivers is expressed there. Immunostaining was performed on whole-mount preparations from larvae heads of the following genotypes: *UAS-mCD8:RFP lexAop-rCD2:GFP;Gr66a-LexA/Gr28a-GAL4*, *UAS-mCD8:RFP lexAop-rCD2:GFP;Gr66a-LexA/Gr28b.c-GAL4*, *UAS-mCD8:RFP lexAop-rCD2:GFP;Gr66a-LexA/Gr28b.e-GAL4* and *UAS-mCD8:RFP lexAop-rCD2:GFP;Gr66a-LexA/+; Gr28b.a-GAL4/+*. The *Gr43a*^{GAL4} GRNs, which do not overlap with *Gr28a-GAL4* neurons (Mishra et al., 2018) are shown for reference to experiments described in Figures 2 and 7.

Figure 2. Intrinsic valence of different *Gr28* GRNs

A) **Behavioral two-choice preference assay to quantify appetitive and avoidance of larvae for chemical ligands.** 15 feeding stage, 3rd instar larvae are placed along the demarcation of a 1% agarose containing dish (35 mm), with one side plain, and the other side containing ligand. The preference Index (P.I.; see Material and Methods) is calculated after counting location of larvae after 16 minutes.

B) ***Gr28a* GRNs mediate capsaicin preference, while *Gr28b.c* GRNs elicit capsaicin avoidance in larvae.** GRNs expressing the capsaicin receptor VR1 (brown panels) are marked with a red crown in diagrams below. *w*^{1118}, reporter gene only (*UAS-VR1/+*) and respective *GAL4* driver (*Gr-GAL4/+*) larvae serve as negative controls (white panels) and show neither preference for nor avoidance to 0.1 mM capsaicin. Experimental larvae expressing VR1 in *Gr28-GAL4* neurons are shown in blue. Positive control larvae (purple panels) expressing VR1 in

bitter taste GRNs (*Gr66a-Gal4*) or appetitive, sweet GRNs (*Gr43a*^{GAL4}) show expected avoidance to or preference for capsaicin. Experimental larvae (blue panels) expressing VR1 in *Gr28a-GAL4* GRNs display robust preference for capsaicin, which is still observed when expression is further restricted to *Gr28a*^{only} GRNs (*Gr66a-LexA/UAS-VR1; Gr28a-GAL4/lexAop-GAL80*; right panel). In contrast, when VR1 is expressed in *Gr28b.c-GAL4* GRNs, larvae strongly avoid capsaicin. Neither avoidance nor preference was observed when VR1 is expressed in the *Gr28b.a-GAL4* or *Gr28b.e-GAL4* GRNs. Each bar represents the mean ± SEM of P.I. (n = 10- 22 assays). The taste behavior of *Gr-GAL4 > UAS-VR1* larvae is compared to 3 controls (*w*^{1118}, *UAS-VR1/*+ and *Gr-GAL4/*+) using one-way ANOVA with Bonferroni's multiple comparison tests ($p < 0.05$), whereby different letters indicate a statistically significant difference. Dashed lines delineate groups for ANOVA. All control and experimental larvae are in the *w*^{1118} background, carry one copy of the indicated transgene(s) and were generated from crosses of respective strains listed in the Material and Methods.

Figure 3. The *Gr28b.c* neurons mediate avoidance behavior to bitter compounds

Inactivation of *Gr28b.c* GRNs (*Gr28b.c-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, larvae with inactivated *Gr28b.e* GRNs (*Gr28b.e-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 *Gr28b.c-GAL4; UAS-Kir2.1* and *Gr28b.e-GAL4; UAS-Kir2.1* larvae is compared to 2 controls *(UAS-Kir2.1/*+ and *Gr28b.c -GAL4/*+ or *Gr28b.e-GAL4/*+) 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. Fly genotypes: *w*^{1118}*; UAS-Kir2.1/*+ (light gray), *w*^{1118}*; Gr28b.c-GAL4/*+ (dark gray), *w*^{1118}*; Gr28b.c-GAL4/UAS-Kir2.1* (white), *w*^{1118}*; Gr28b.e-GAL4/*+ (dark gray), *w*^{1118}*; Gr28b.e-GAL4/UAS-Kir2.1* (white).

Figure 4. Role of individual *Gr28* genes in bitter taste avoidance

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

B) **Single *Gr28b* genes can rescue avoidance response to denatonium when expressed in *Gr28b.c* neurons of *Gr28* mutant larvae.** The behavior of w^{1118};*ΔGr28/ΔGr28* larvae expressing *UAS-Gr28* transgenes under control of the *Gr28b.c-GAL4* driver was compared to $Gr28^{+/+}$ controls (w^{1118}, black bar), and three *Gr28* mutant controls (*ΔGr28*, *ΔGr28* plus driver, and *ΔGr28* plus respective *UAS-Gr28* reporter, white bar) using Kruskal-Wallis test by ranks with Dunn's multiple comparison tests ($p < 0.05$). Each bar represents the mean ± SEM of P.I. (n = 11- 22 assay). Bars with different letters are significantly different. Dashed lines delineate groups for ANOVA. Fly genotypes: wild-type: w^{1118} (black), mutants: w^{1118};*ΔGr28/ΔGr28*, w^{1118}; *ΔGr28/ΔGr28 Gr28b.c-GAL4,* w^{1118};*ΔGr28/ΔGr28; UAS-Gr28* (indicated *Gr28* genes)/+ and w^{1118}; *ΔGr28/ΔGr28 UAS-GCaMP6m; UAS-Gr28* (for *Gr28b.b or Gr28b.c* genes)/+ (white), rescues: w^{1118}; *ΔGr28/ΔGr28 Gr28b.c-GAL4; UAS-Gr28* (indicated *Gr28* genes)/+ and w^{1118}; *ΔGr28 UAS-GCaMP6m/ΔGr28 Gr28b.c-GAL4; UAS-Gr28* (for *Gr28b.b* or *Gr28b.c* genes)/+ (gray).

Figure 5. Cellular Ca^{2+} responses of *Gr28b.c* GRNs to select bitter compounds requires Gr28b.a or Gr28b.c.

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

B) **Representative still images of Ca^{2+} response in the *Gr28b.c* expressing GRN of the TOG.** Ca^{2+} responses of the *Gr28b.c* GRNs upon stimulation with

indicated ligands. ΔF indicates the changes in fluorescence light intensity of the cell body after ligand application.

C and D) Representative traces (C) and quantified Ca^{2+} responses (D) of the *Gr28b.c* GRNs after stimulation with indicated ligands. Fly genotype: *w^{1118}; Gr28b.c-GAL4/UAS-GCaMP6m*. Each bar represents the mean ± 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. *Gr28b.c* expressing GRNs in the TOG of *Gr28* mutant larvae (*ΔGr28*) have significantly reduced Ca^{2+} responses to denatonium and quinine but not to lobeline or caffeine when compared to *Gr28b.c* expressing GRNs of wild type controls. Larvae genotypes: *$Gr28^{+}$* control (black bar): *w^{1118}; Gr28b.c-GAL4/UAS-GCaMP6m*. *ΔGr28* control (white bar): *w^{1118}; ΔGr28 Gr28b.c-GAL4/ΔGr28 UAS-GCaMP6m*. Each bar represents the mean ± SEM with 13 – 16 larvae. Asterisks indicate a significant difference between *$Gr28^{+}$* control and *ΔGr28* larvae (Two-tailed, Mann-Whitney U test, *** $p < 0.001$, ** $p < 0.01$ ns: not significant).

F and G) *Gr28b.c* or *Gr28b.a* transgenes rescue denatonium responses in GRNs of *ΔGr28* larvae. Expression of *Gr28b.c* or *Gr28b.a* is under control of *Gr28b.c-GAL4* restores responses to denatonium, but not quinine in TOG GRNs of *ΔGr28* larvae. Each bar represents the mean ± SEM of Ca^{2+} imaging with 12 – 17 larvae. The Ca^{2+} responses of *Gr28* mutant larvae expressing *UAS-Gr28* transgenes under *Gr28b.c-GAL4* driver is compared to *$Gr28^{+}$* (black) and *ΔGr28* (white) controls 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. Fly genotypes: *$Gr28^{+}$* control (black bar): *w^{1118}; Gr28b.c-GAL4/UAS-GCaMP6m*. *ΔGr28* control (white bar): *w^{1118}; ΔGr28 Gr28b.c-GAL4/ΔGr28 UAS-GCaMP6m*. *Gr28* rescues (gray bar): *w^{1118}; ΔGr28 Gr28b.c-GAL4/ΔGr28 UAS-GCaMP6m; UAS-Gr28* (indicated Gr28

genes)/+. Concentration of ligands was 100 mM for sugars, 50 mM for caffeine and 5 mM for denatonium, quinine and lobeline.

Figure 6. Amino acid alignment of the six Gr28 proteins.

The sequence alignment was generated using Clustal Omega tool from ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalo/). IL and EL indicate intracellular loop and extracellular loop, respectively. Note that the C terminal region starting at the IL3 is identical in the Gr28b proteins. Red highlighted letters indicate amino acids identical only in Gr28b.a and Gr28b.c. Green highlighted letters indicate amino acids conserved in Gr28b.a, Gr28b.c and one other Gr28 protein. Asterisks below the sequences indicate residues identical in all Gr28 proteins, colons indicate conserved residue (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW), and periods indicate moderately conserved residue (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY). TM1-7 indicate helical transmembrane segments predicted by HMMTOP 2.0 software.

Figure 7. Role of different GRN subsets in taste behavior of larvae

GRNs sufficient for mediating avoidance behavior can be defined by *Gr28b.c-GAL4*, while GRNs sufficient for mediating appetitive behavior are defined by a subset of *Gr28a-GAL4* GRNs (*Gr28a*^{only} GRNs; see also Figure 2B and Figure 2-figure supplement 1). 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. Also, a larger set of avoidance neurons (*Gr66a-GAL4*) might function independently of any *Gr28b.c* neurons, and one set of fructose sensing neurons (*Gr43a*^{GAL4}) distinct from *Gr28a-GAL4* GRNs mediates appetitive behavior.

Table 1. Conserved amino acids in the amino termini of Gr28b.a and Gr28b.c

Location	Conserved in Gr28b.c/Gr28b.a	Other (if applicable)
EL1	E103	Gr28b.b
	R111	Gr28b.b
EL2	**E200**	**none**

TM1	Y94	Gr28b.b
	S95	**none**
TM2	**V124**	**none**
	I128	Gr28b.b
TM3	**V170**	**none**
	V177	Gr28b.b
TM4	**L207**	**none**
	F209	**none**
	I218	Gr28a
	I220	Gr28b.b
	I222	Gr28b.b
	T229	Gr28a
	V232	**none**

The 7 amino acid residues identical in the amino-terminal region of Gr28b.c and Gr28b.a are shown in bold (residue number is taken from Gr28b.c). 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 Gr28b.a and Gr28b.c can rescue response to denatonium when expressed in *Gr28b.c* neurons of *ΔGr28/ΔGr28* mutant larvae.

Figure 1-figure supplement 1. Co-expression analysis between *Gr28* genes in larval sensory organs

Immunostaining with anti-GFP (green) and anti-mCD8 (red) antibodies on whole-mount preparations of the heads from larvae of the genotypes: (1) *UAS-mCD8:RFP lexAop-rCD2:GFP;Gr28a-GAL4* or *Gr28b.e-GAL4* or *Gr66a-GAL4/+;Gr28b.c-LexA/+*: (2) *UAS-mCD8:RFP lexAop-rCD2:GFP;+; Gr28b.a-GAL4/Gr28b.c-LexA*. Asterisks refer to GRN expressing both *Gr28b.c* 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 2-figure supplement 1. Suppression of GAL4 in a subset of *Gr28a-GAL4* neurons

(A) Expression of *GCaMP6m* was restricted to a subset of *Gr28a-GAL4* GRNs by inclusion of *Gr66a-LexA* and *lexAop-GAL80.* Immunostaining with anti-GFP (green) antibody on whole-mount preparations of the heads from larvae. The "control" genotype shown on the left is w^{1118}*; Gr66a-LexA/UAS-GCaMP6m;Gr28a-GAL4/+* The "*lexAop-GAL80*" genotype on the right is w^{1118}*;Gr66a-LexA/UAS-GCaMP6m;Gr28a-GAL4/lexAop-GAL80*. Note the reduced number GFP positive GRNs in the "*lexAop-GAL80*" genotype compared to the "control".

(B) Quantification of GFP positive GRNs in the different sensory organs. Each bar represents the mean ± SEM of GFP expressing cells with 13 – 14 larvae. Asterisks indicate a significant difference between control larvae and larvae expressing *lexAop-GAL80* by under control *Gr66a-LexA* driver (*lexAop-GAL80*) (Two-tailed, Mann-Whitney U test, ** $p < 0.01$, * $p < 0.05$).

Figure 4-figure supplement 1. *Gr66a* 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. Taste preference assay of larvae expressing VR1 in different *Gr* GRNs for 0.1 mM capsaicin. Taste preference assay of larvae with expression of VR1 in $Gr28a^{only}$ GRNs using *lexAop-GAL80* under control of *Gr66a-LexA* for 0.1 mM capsaicin.

Figure 3-Source Data 1. Taste preference assay for bitter compounds of larvae with inactivated *Gr28b.c* or *Gr28b.e* GRNs using expression of *UAS-Kir2.1*.

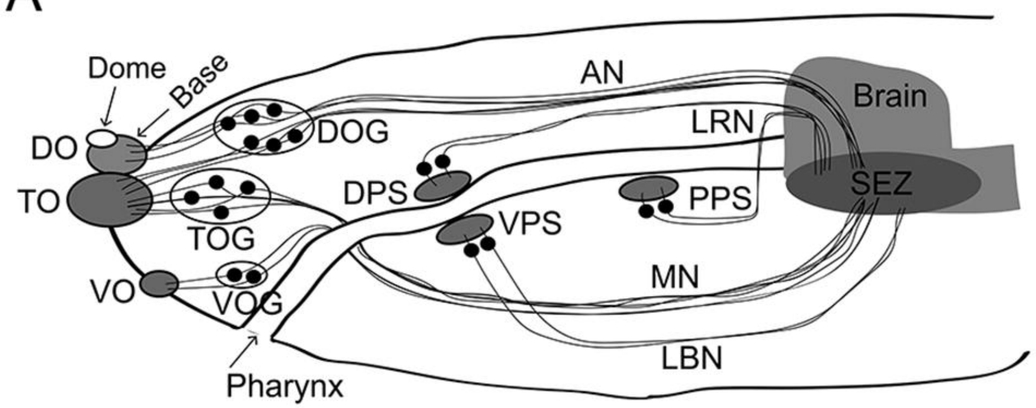
Figure 4-Source Data 1. (A) Taste preference assay of wild type and *Gr28* mutant larvae for bitter compounds. (B) Taste preference assay of *Gr28* mutant larvae for denatonium expressing single *Gr28* genes in *Gr28b.c* GRNs.

Figure 5-Source Data 1. (D) Ca^{2+} responses of *Gr28b.c* GRNs in the TOG 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 *Gr28b.c* GRNs.

Figure 2-figure supplement 1-Source Data 1A & 1B. (B) Quantification of GFP positive GRNs recorded from images of *Gr28a* GRNs expressing *UAS-GCaMP6m* (A).

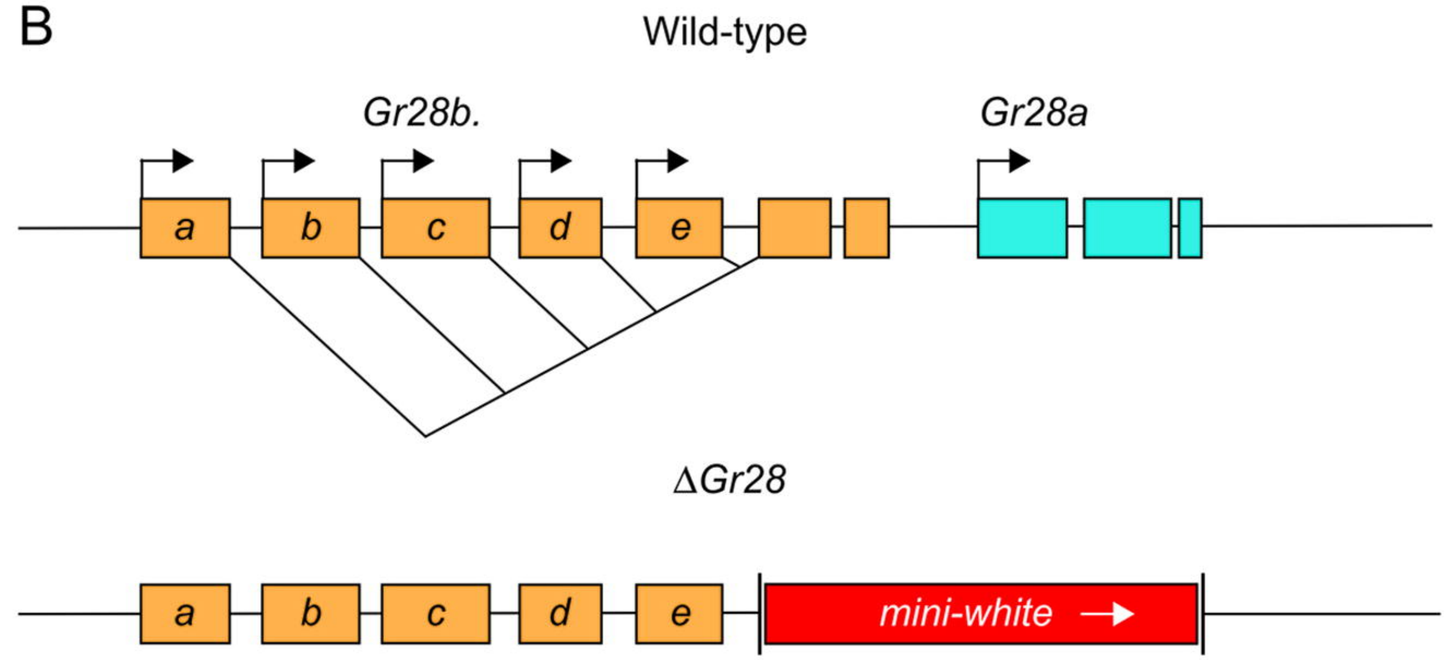
Figure 4-figure supplement 1-Source Data 1. Taste preference assay of *Gr66a* mutant larvae for bitter compounds.

A



DO: Dorsal Organ
TO: Terminal Organ
VO: Ventral Organ
D/T/VOG: Dorsal/Terminal/Ventral Organ Ganglion
D/V/PPS: Dorsal/Ventral/Posterior Pharyngeal Sense Organ
SEZ: subesophageal zone

B



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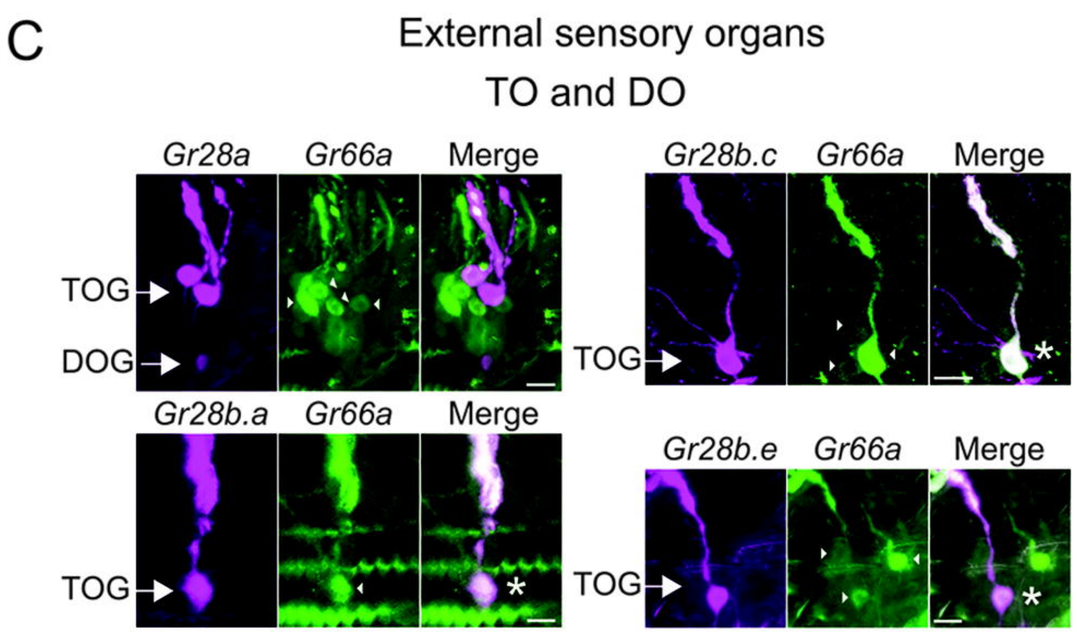

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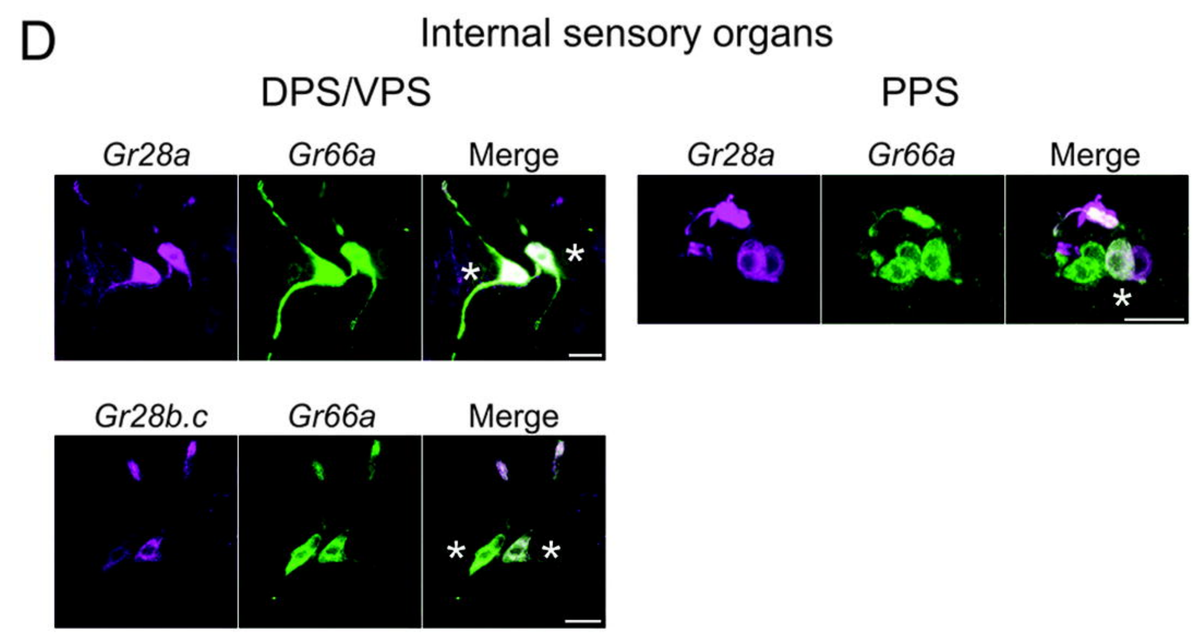

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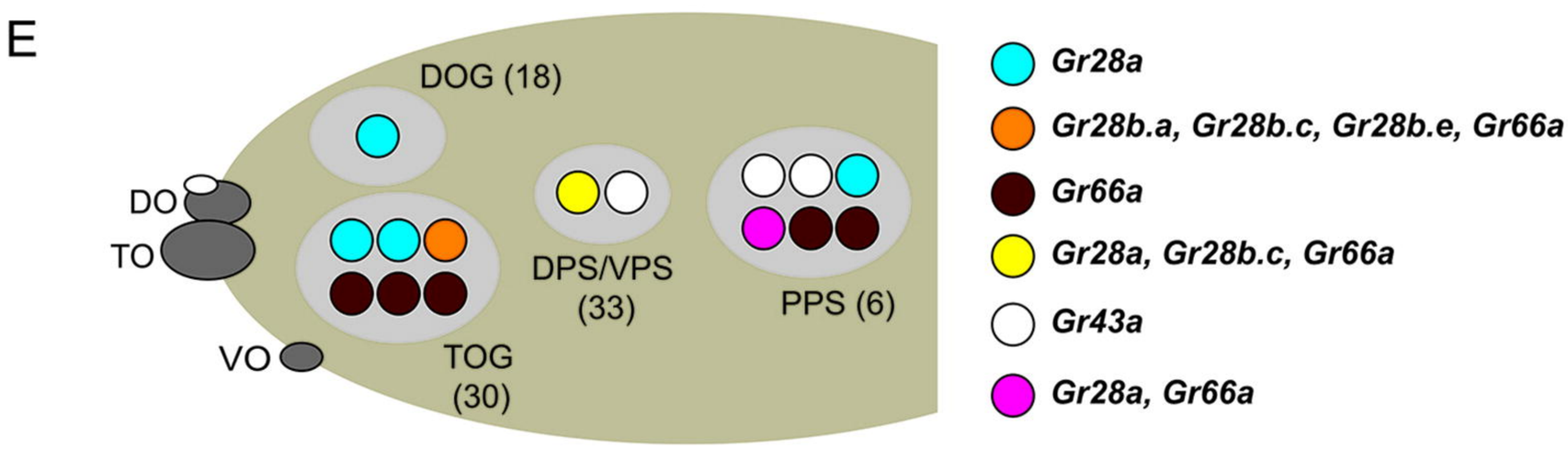

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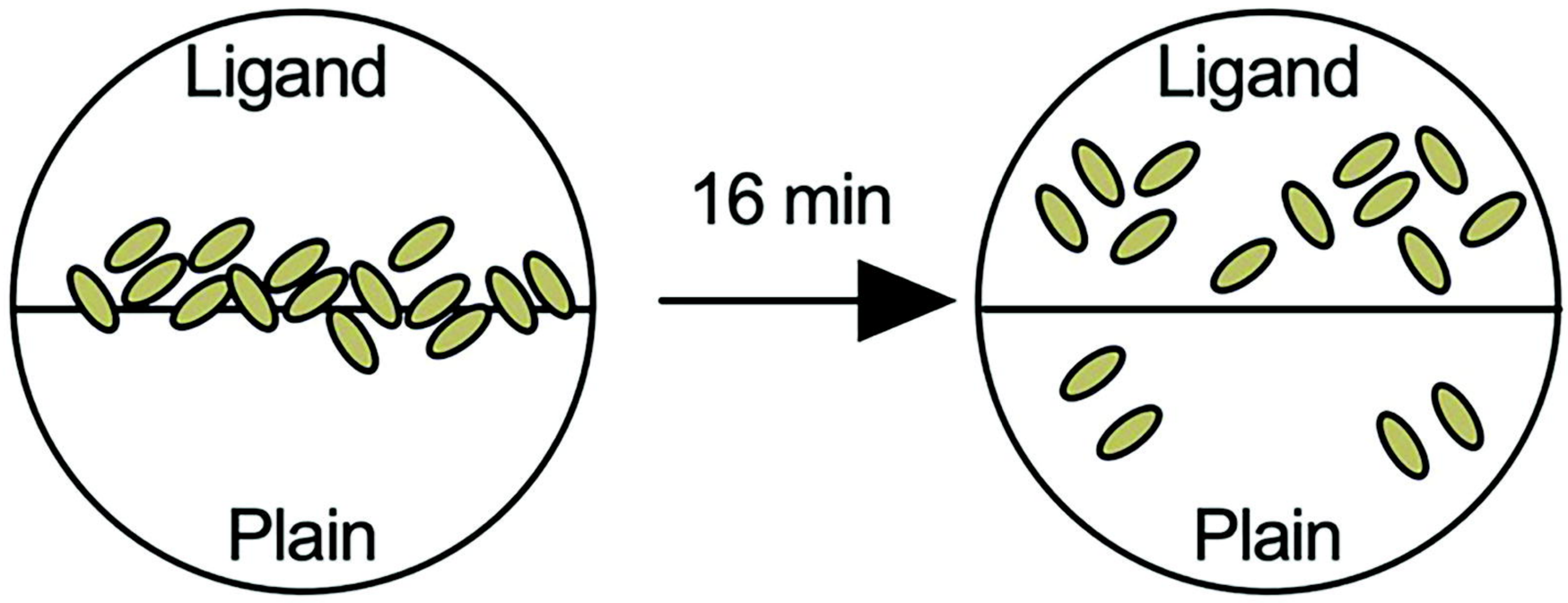


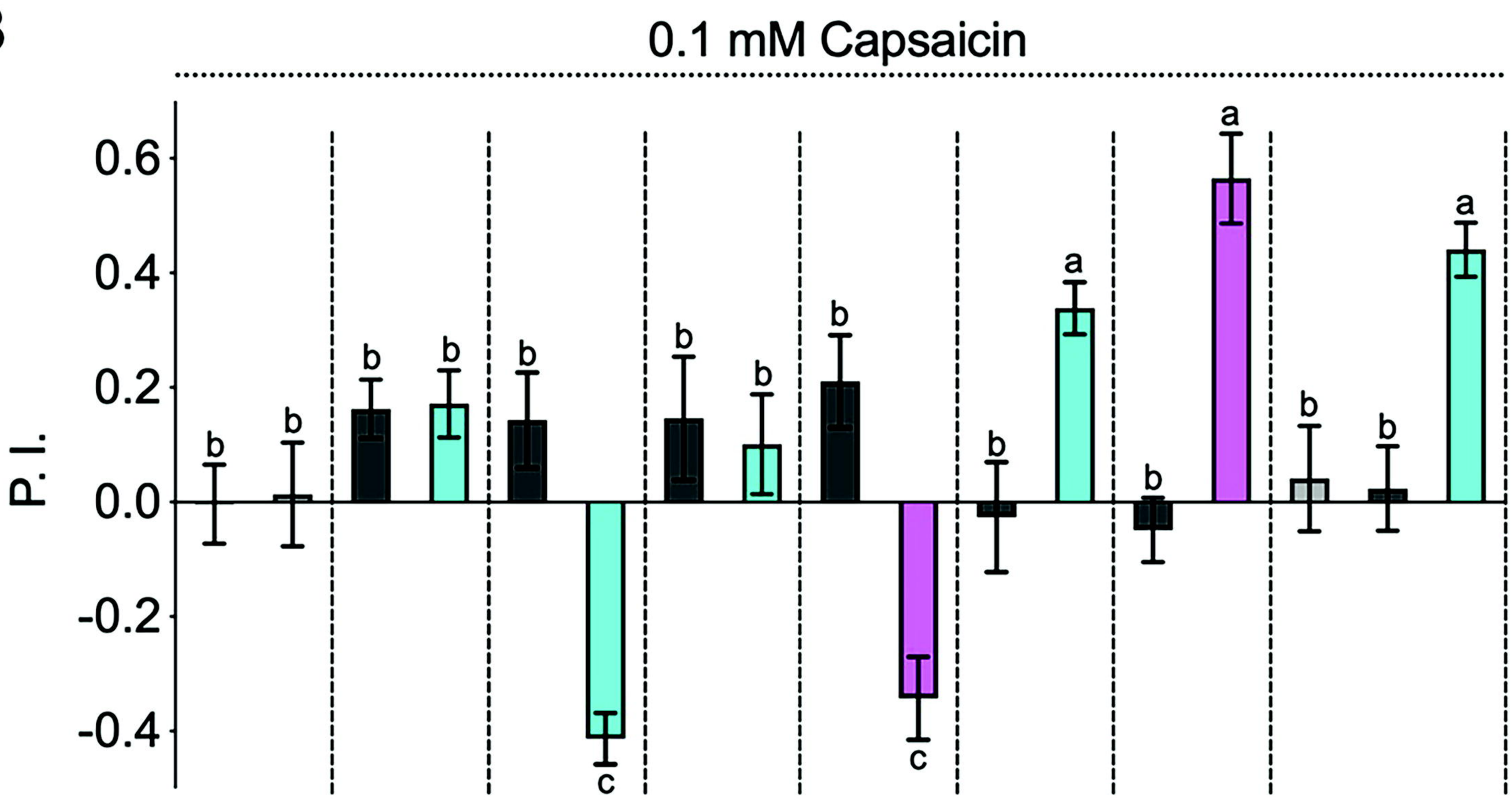
$$P.I. = \frac{N_{Ligand} - N_{Plain}}{N_{Total}}$$

P.I. = 1 : complete preference for ligand

P.I. = 0 : no response to ligand

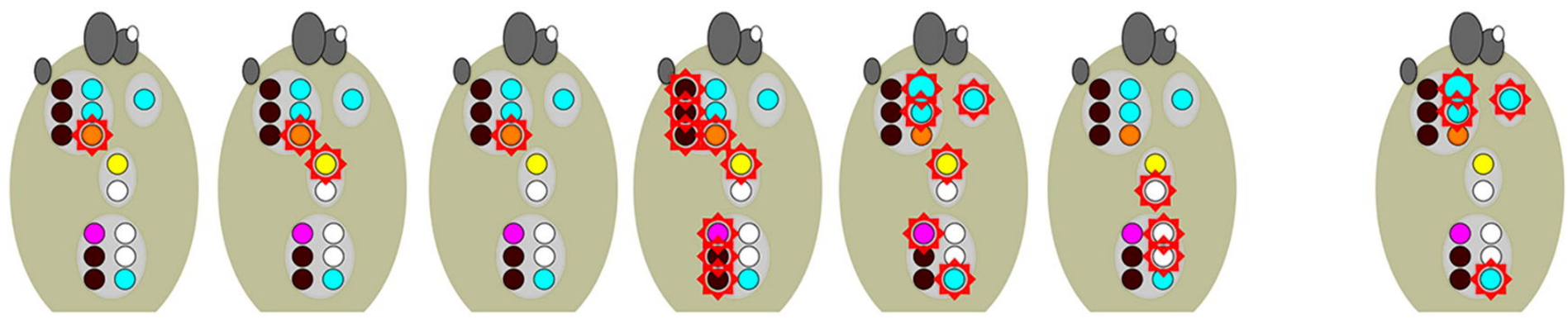
P.I. = -1 : complete avoidance to ligand

B



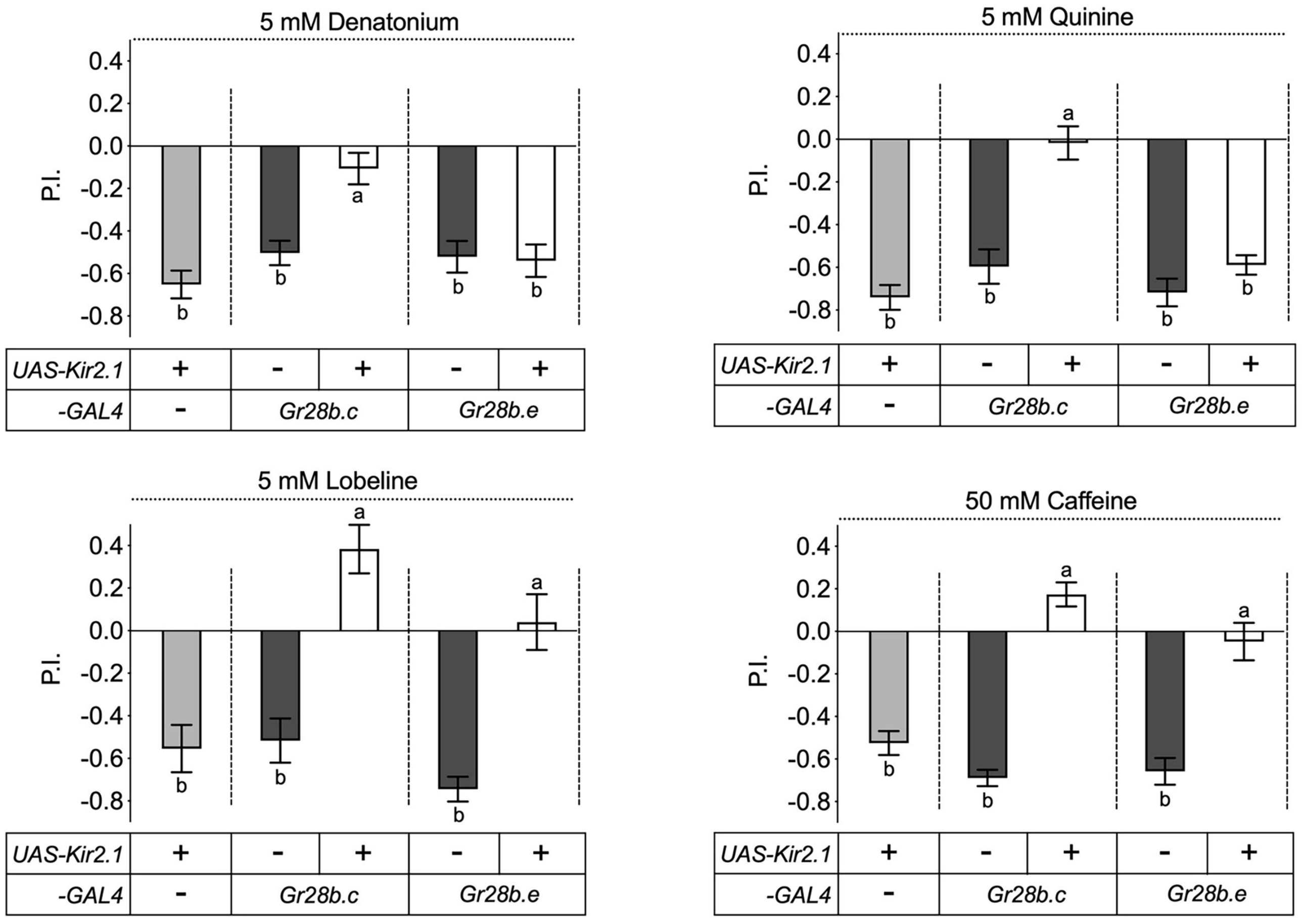
-GAL4	-	-	Gr28b.a		Gr28b.c		Gr28b.e		Gr66a		Gr28a		Gr43a		-	Gr28a	
UAS-VR1	-	+	-	+	-	+	-	+	-	+	-	+	-	+	+	-	+
Gr66a-LexA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
lexAop-GAL80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+

GRNs expressing *UAS-VR1*



Gr28a *Gr28b.a, Gr28b.c, Gr28b.e, Gr66a* *Gr66a*

Gr28a, Gr28b.c, Gr66a *Gr43a* *Gr28a, Gr66a*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0.2
0.0
-0.2
-0.4
-0.6
-0.8
b
b
a
b
b
UAS-Kir2.1
+
-
+
-
+
-GAL4
-
Gr28b.c
Gr28b.e
5 mM Quinine
P.I.
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
b
b
a
b
b
UAS-Kir2.1
+
-
+
-
+
-GAL4
-
Gr28b.c
Gr28b.e
5 mM Lobeline
P.I.
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
b
b
a
b
a
UAS-Kir2.1
+
-
+
-
+
-GAL4
-
Gr28b.c
Gr28b.e
50 mM Caffeine
P.I.
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
b
b
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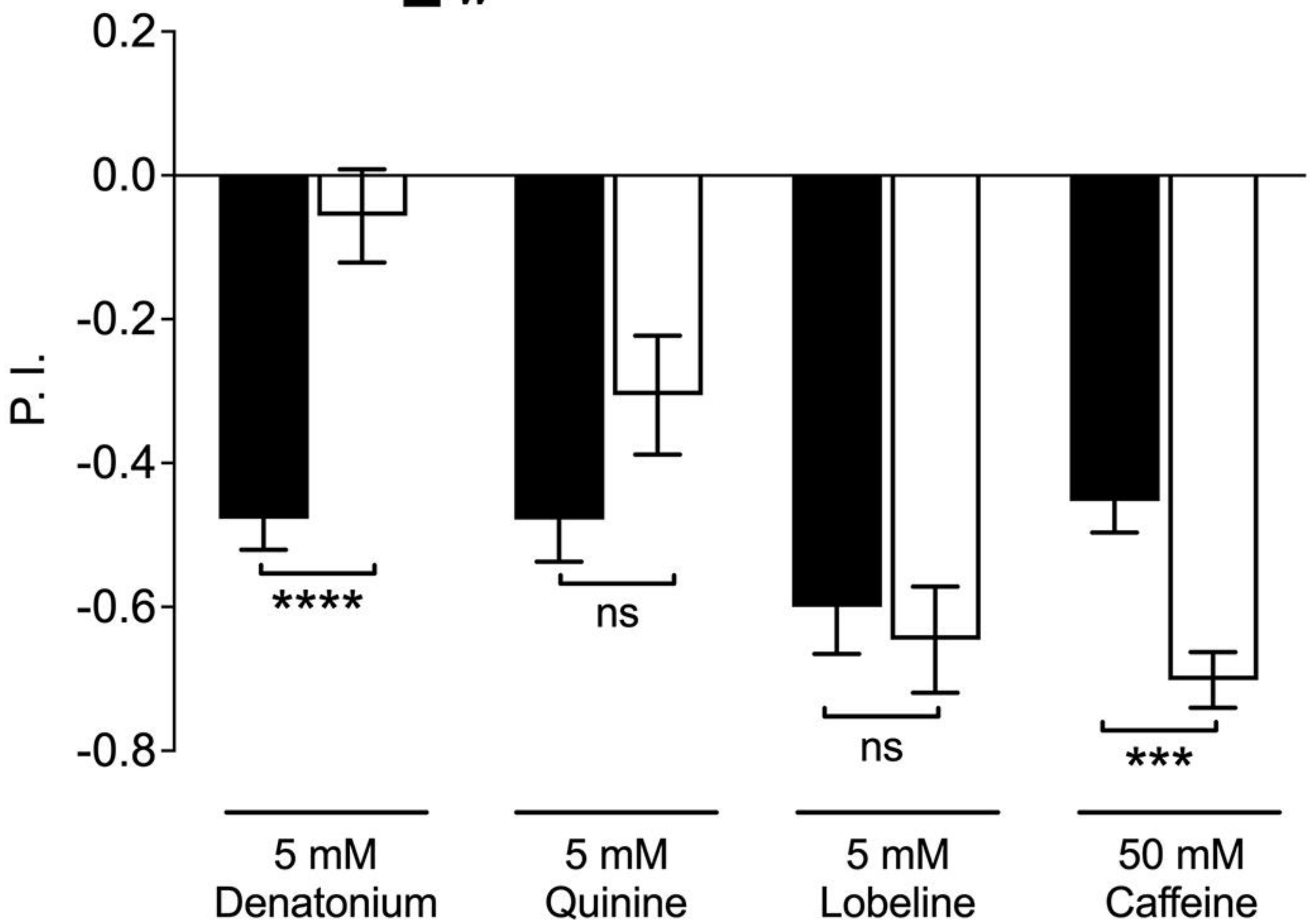
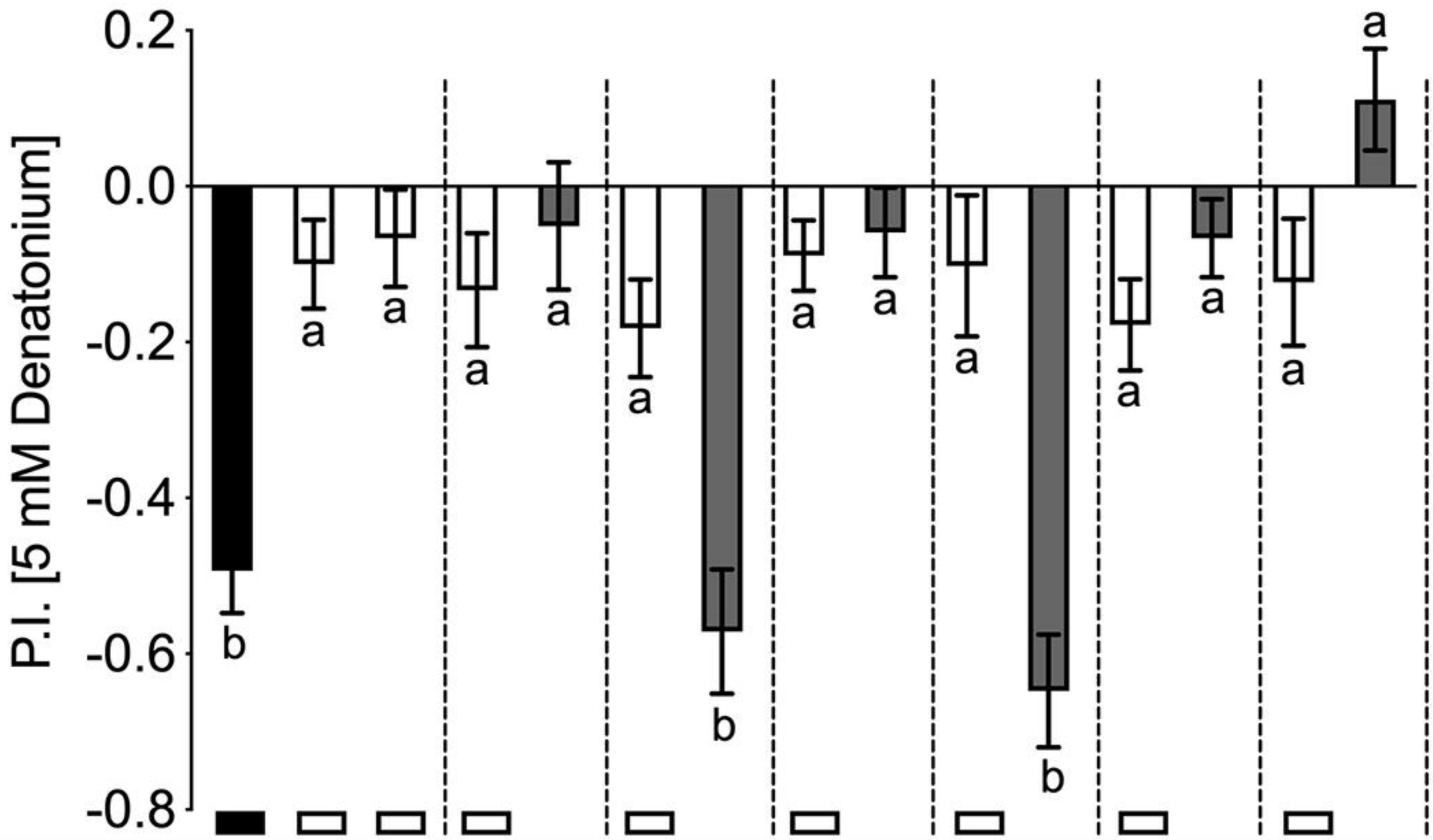
Gr28b.c
Gr28b.e

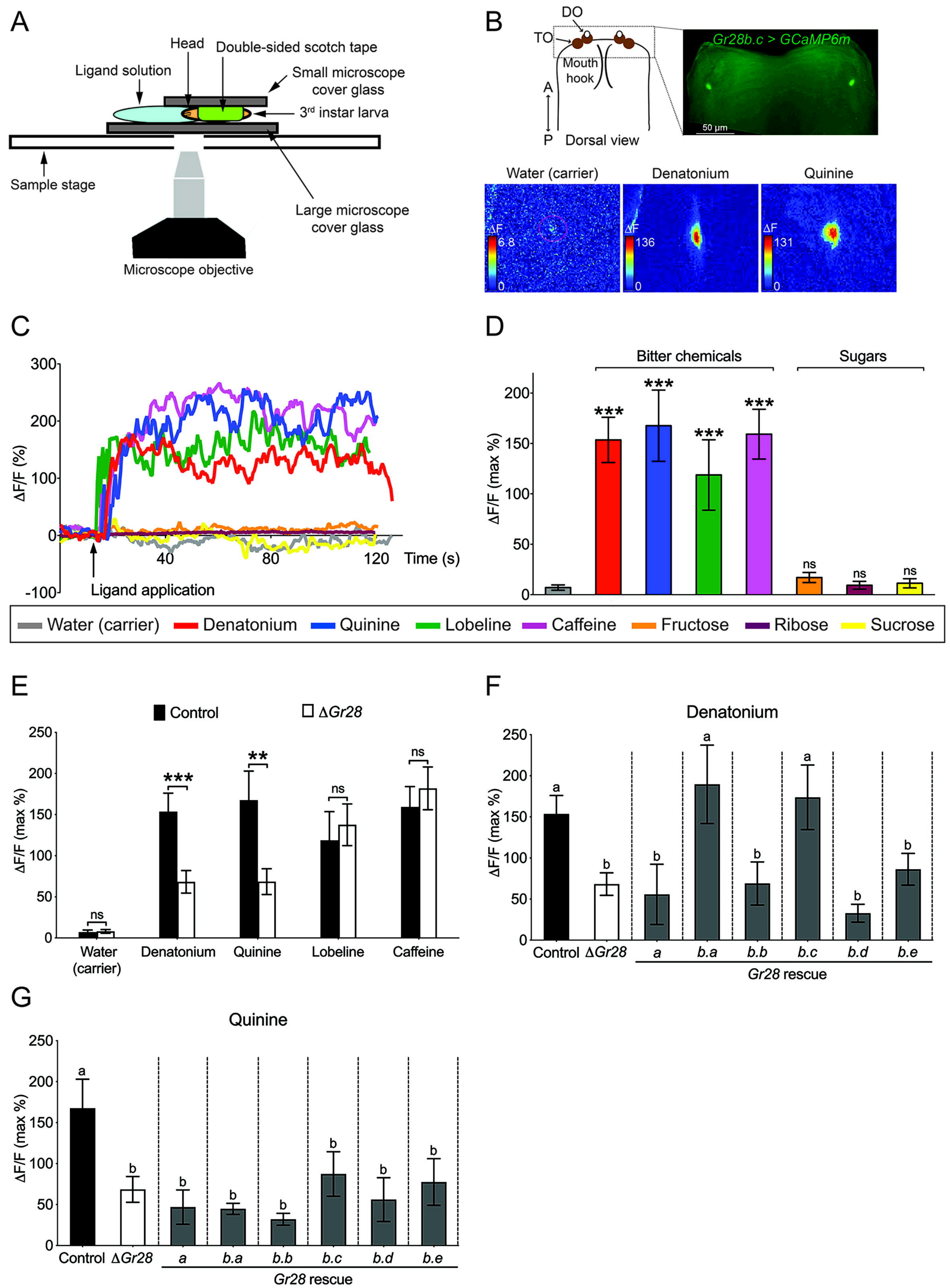
A



B



Gr28	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gr28b.c-GAL4	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+
UAS-Gr	-	-	-	*28a*		*28b.a*		*28b.b*		*28b.c*		*28b.d*		*28b.e*	


A
Head
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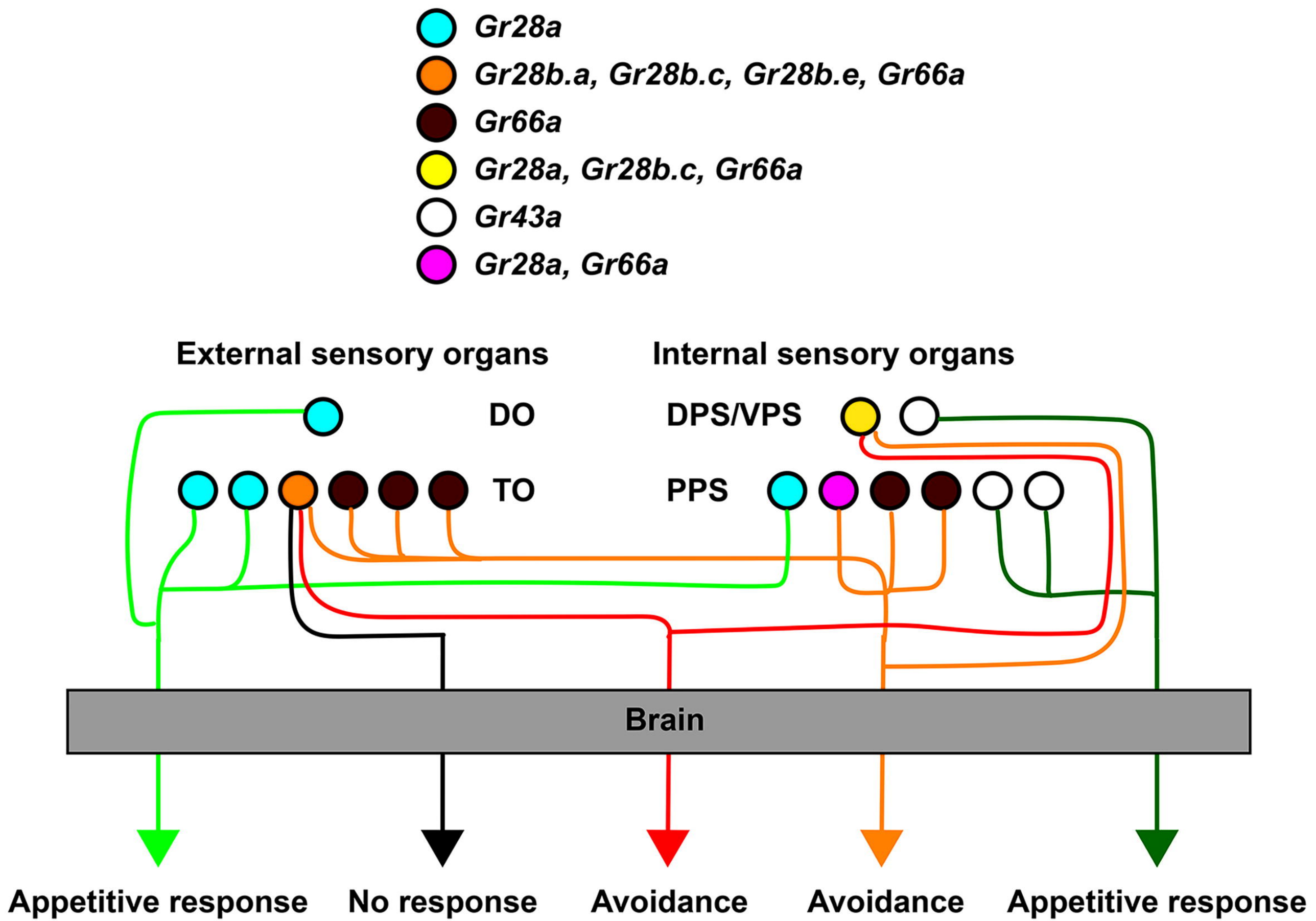
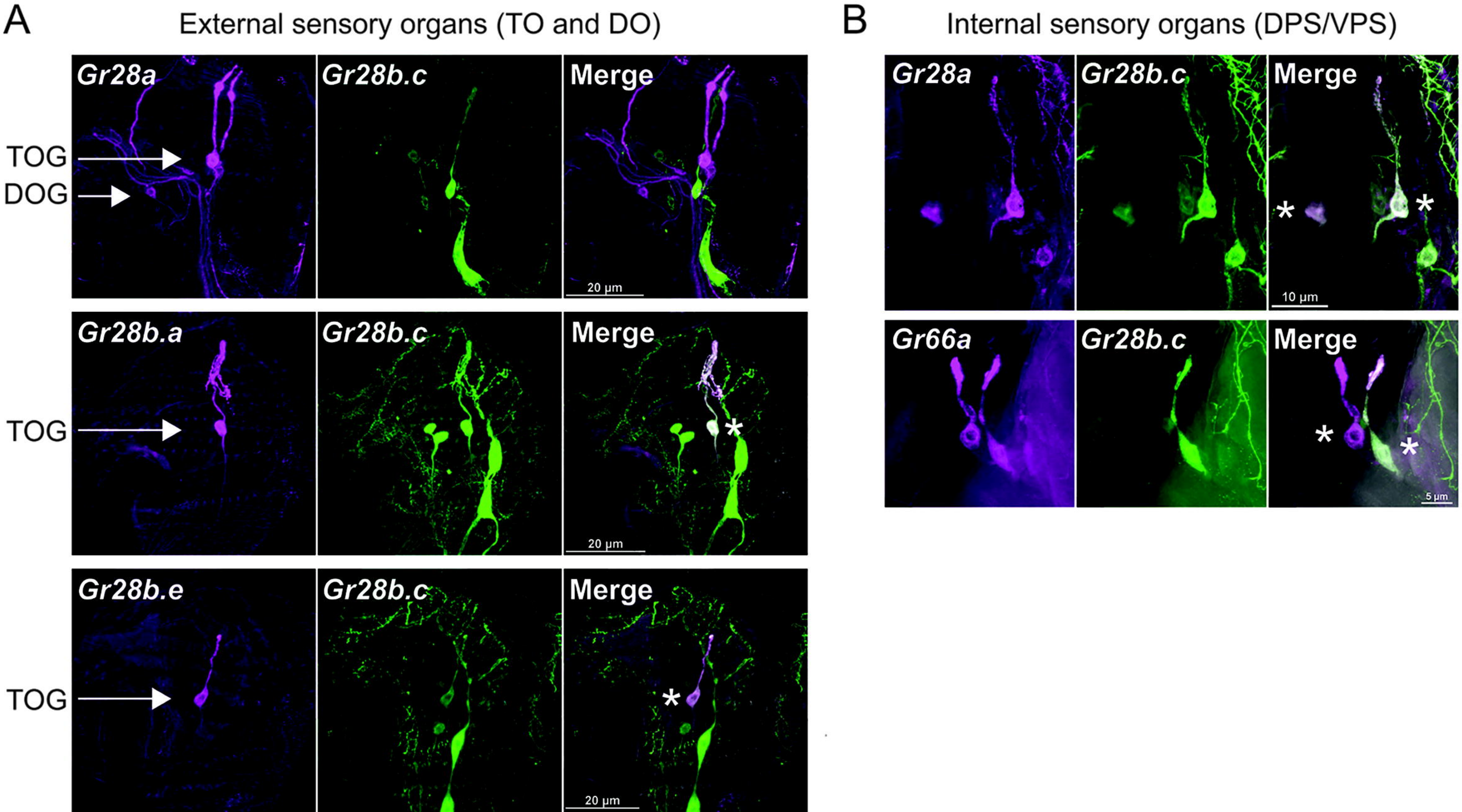
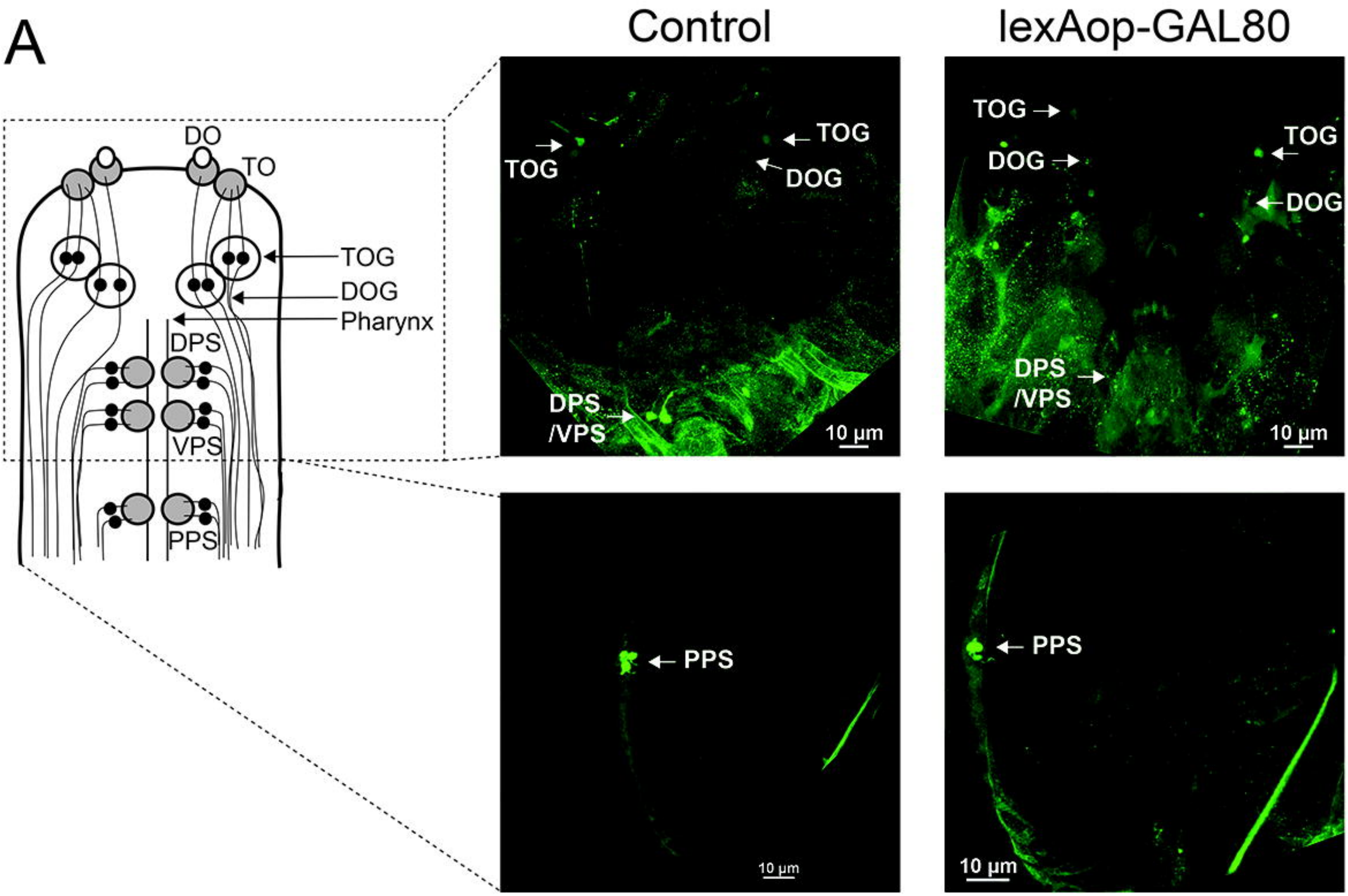
Ligand solution
Small microscope cover glass
3rd instar larva
Sample stage
Large microscope cover glass
Microscope objective
B
DO
TO
Mouth hook
A
P
Dorsal view
Gr28b.c > GCaMP6m
50 µm
Water (carrier)
Denatonium
Quinine
ΔF
6.8
0
136
0
131
0
C
ΔF/F (%)
300
200
100
0
-100
40
80
120
Time (s)
Ligand application
D
Bitter chemicals
Sugars
ΔF/F (max %)
200
150
100
50
0
***
***
***
***
ns
ns
ns
Water (carrier)
Denatonium
Quinine
Lobeline
Caffeine
Fructose
Ribose
Sucrose
E
Control
ΔGr28
ΔF/F (max %)
250
200
150
100
50
0
ns
***
**
ns
ns
Water (carrier)
Denatonium
Quinine
Lobeline
Caffeine
F
Denatonium
ΔF/F (max %)
250
200
150
100
50
0
a
b
b
a
b
a
b
b
Control
ΔGr28
a
b.a
b.b
b.c
b.d
b.e
Gr28 rescue
G
Quinine
ΔF/F (max %)
250
200
150
100
50
0
a
b
b
b
b
b
b
b
Control
ΔGr28
a
b.a
b.b
b.c
b.d
b.e
Gr28 rescue

```
                                                IL1
Gr28b.a   ----------------MIRCGLDIFRGCRGRFRYWLSARDCYDSISLMVAIAFALGITPFLVR   47
Gr28b.c   MDIEMAKEPVNPTDTPDIEVTPGLCQPLRRRFRRFVTAKQLYECLRPVFHVTYIHGLTSFYIS   63
Gr28b.b   -------------------------MSALRRVRKYFISSQVYEALRPLFFLTFLYGLTPFHVV   38
Gr28b.d   ---------------------------MSFYFCEIFKPRDAFGAEQTLLLYTYLLGLTPFRL-   35
Gr28b.e   ------------------------MWLLRRSVGKSGNRPHDVYTCYRLTIFMALCLGIVPYYVS   40
Gr28a     ---------------------------MAFKLWERFSQADNVFQALRPLTFIS-LLGLAPFRLN   36
                                          : : .       :   *:. : :

                      TM1                       EL1          TM2
Gr28b.a   -RNALGENSLEQSWYGFLNAIFRWLLLAYCYSYI-NLRNESLIGYFMRNHVSQISTRVHDVGG   108
Gr28b.c   CDTKTGKKAIKKTIFGYINGIMHIAMFVFAYSLTIYNNCESVASYFFRSRITYFGDLMQIVSG   126
Gr28b.b   -RRKMGESYLKMSCFGVFNIFIYICLCGFCYIS-SLRQGESIVGYFFRTEISTIGDRLQIFNG   99
Gr28b.d   -RGQAGERQFHLSKIGYLNAFLQLSFFSYCFLAA-LIEQQSIVGYFFKSEISQMGDSLQKFIG   96
Gr28b.e   -ISSEGRGKLTSSYIGYINIIIRMAIYMVNSFYG-AVNRDTLMSNFFLTDISNVIDALQKING   101
Gr28a     L---NPRKEVQTSKFSFFAGIVHFLFFVLCFGIS-VKEGDSIIGYFFQTNITRFSDGTLRLTG   95
                . .  :  . :  :.   :            . ::: . *: . :: .      . *

                                IL2                          TM3
Gr28b.a   IIAAVFTFILPLLLRKYFLKSVKNMVQVDTQ-LERLRSPVNFNTVVGQVVLVILAVVLLDTVL   170
Gr28b.c   FIGVTVIYLTAFVPNHRLERCLQKFHTMDVQ-LQTVGVKIMYSKVLRFSYMVLISMFLVNVLF   188
Gr28b.b   LIAGAVIYTSAILKRCKLLGTLTILHSLDTN-FSNIGVRVKYSRIFRYSLLVLIFKLLILGVY   161
Gr28b.d   MTGMSILFLCSSIRVRLLIHIWDRISYIDDR-FLNLGVCFNYPAIMRLRLLQIFLINGVQLGY   158
Gr28b.e   MLGIFAILLISLLNRKELLKLLATFDRLETEAFPRVGVAMHQVAANKKMNRLVIILVGSMVAY   164
Gr28a     ILAMSTIFGFAMFKRQRLVSIIQNNIVVDEI-FVRLGMKLDYRRILLSSFLISLGMLLFNVIY   157
          : .         .   :         ::   :  :   .                :

                EL2                TM4                          IL3
Gr28b.a   LTTGLVCLAKME-VYASWQLTFIFVYELLAISITICMFCLMTRTVQRRITCLHKVLKNLAHQW   232
Gr28b.c   TGGTFSVLYSSE-VAPTMALHFTFLIQHTVIAIAIALFSCFTYLVEMRLVMVNK           241
Gr28b.b   FVGVFRLLVSLD-VTPSFCVCMTFFLQHSVVSIAICLFCVIAFSFERRLSIINQ           214
Gr28b.d   LISSNWMLLGND-VRPIYTAIVAFYVPQIFLLSIVMLFNATLHRLWQHFTVLNQ           211
Gr28b.e   ITCSFLMISLRDTTTFSISAVISFFSPHFIVCAVSFLAGNVMIKLRIYLSALNE           218
Gr28a     LCVSYSLLVSAT-ISPSFVTFTTFALPHINISLMVFKFLCTTDLARSRFSMLNEILQDILDAH   219
                :                 *      :                  :     :*::: .

Gr28b.a   DT----R---SLKA-VNQKQRS-------LQCLDSFSMYTIVTKDPAEIIQESMEIHHLICEA   280
Gr28a     IEQLSALELSPMHSVVNHRRYSHRLRNLISTPMKRYSVTSVIRLNPEYAIKQVSNIHNLLCDI   282
                  ::: **::: *          :. :*: :::  :*   *::  :**:*:*:

                      TM5                     EL3          TM6
Gr28b.a   AATANKYFTYQLLTIISIAFLIIVFDAYYVLETLLGKSKRESKFKTVEFVTFFSCQMILYLIA   343
Gr28a     CQTIEEYFTYPLLGIIAISFLFILFDDFYILEAILN-PKRLDVFEADEFFAFFLMQLIWYIVI   344
          . * ::**** ** **:*:**:*:** :*:**::*.  ** . *:: **.:**  *:* *::

                                IL4
Gr28b.a   IISIVEGSNRAIKKSEKTGGIVHSLLNKTKSAEVKEKLQQFSMQLMHLKINFTAAGLFNIDRT   406
Gr28a     IVLIVEGSSRTILHSSYTAAIVHKILNITDDPELRDRLFRLSLQLSHRKVLFTAAGLFRLDRT   407
          *: *****.*:* :*. *..***.:** *.. *::::* ::*:** * *: *******.:***

          TM7                 EL4
Gr28b.a   LYFTISGALTTYLIILLQFTSNSPNNGYGNGSSCCETFNNMTNHTL--                 452
Gr28a     LIFTITGAATCYLIILIQFRFTHHMDDTSSNS-----TNNLHSIHLGD                 450
          * ***:** * *****:**  .   :. ...*      **: .  *
```

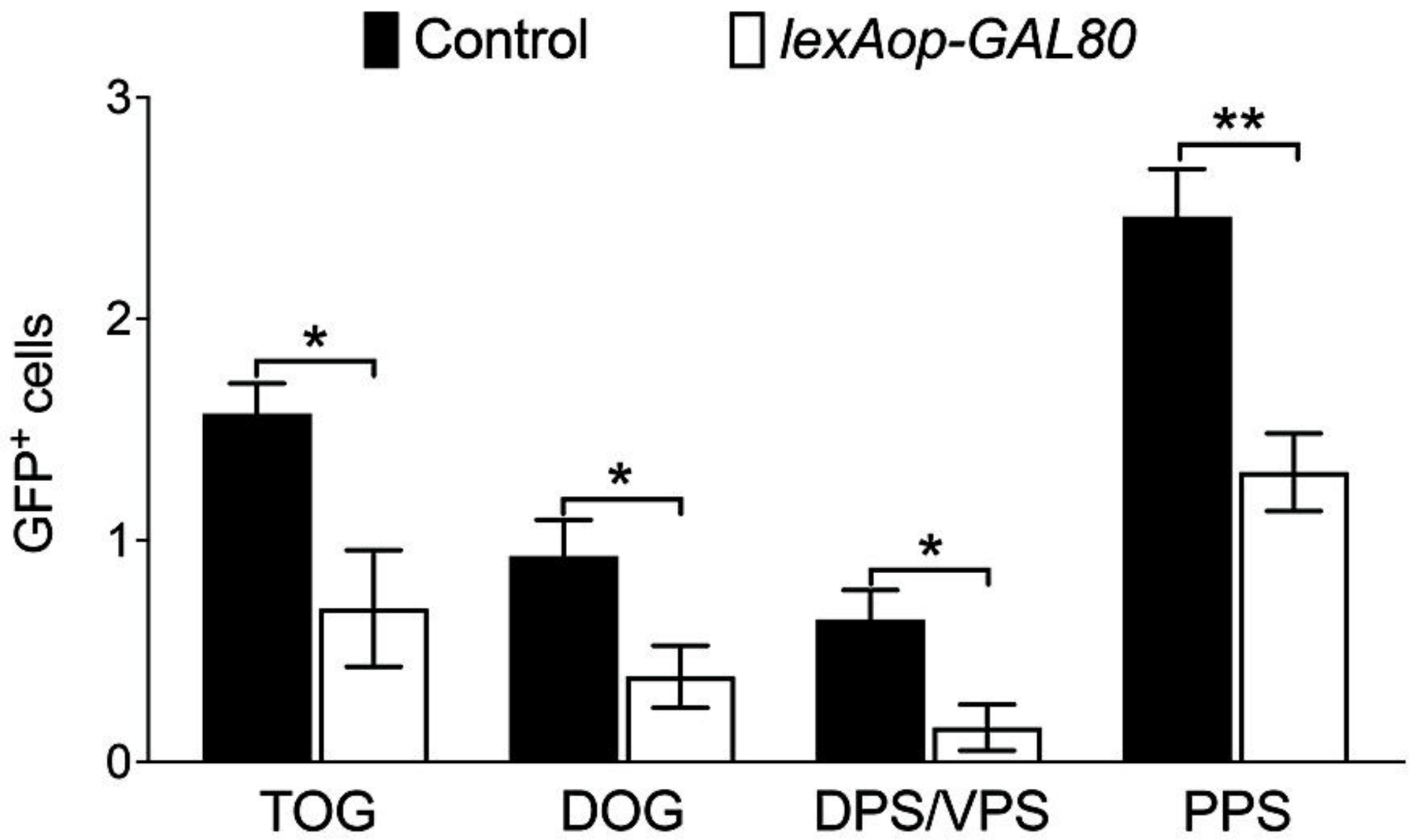

Gr28a
Gr28b.a, Gr28b.c, Gr28b.e, Gr66a
Gr66a
Gr28a, Gr28b.c, Gr66a
Gr43a
Gr28a, Gr66a
External sensory organs
Internal sensory organs
DO
TO
DPS/VPS
PPS
Brain
Appetitive response
No response
Avoidance
Avoidance
Appetitive response

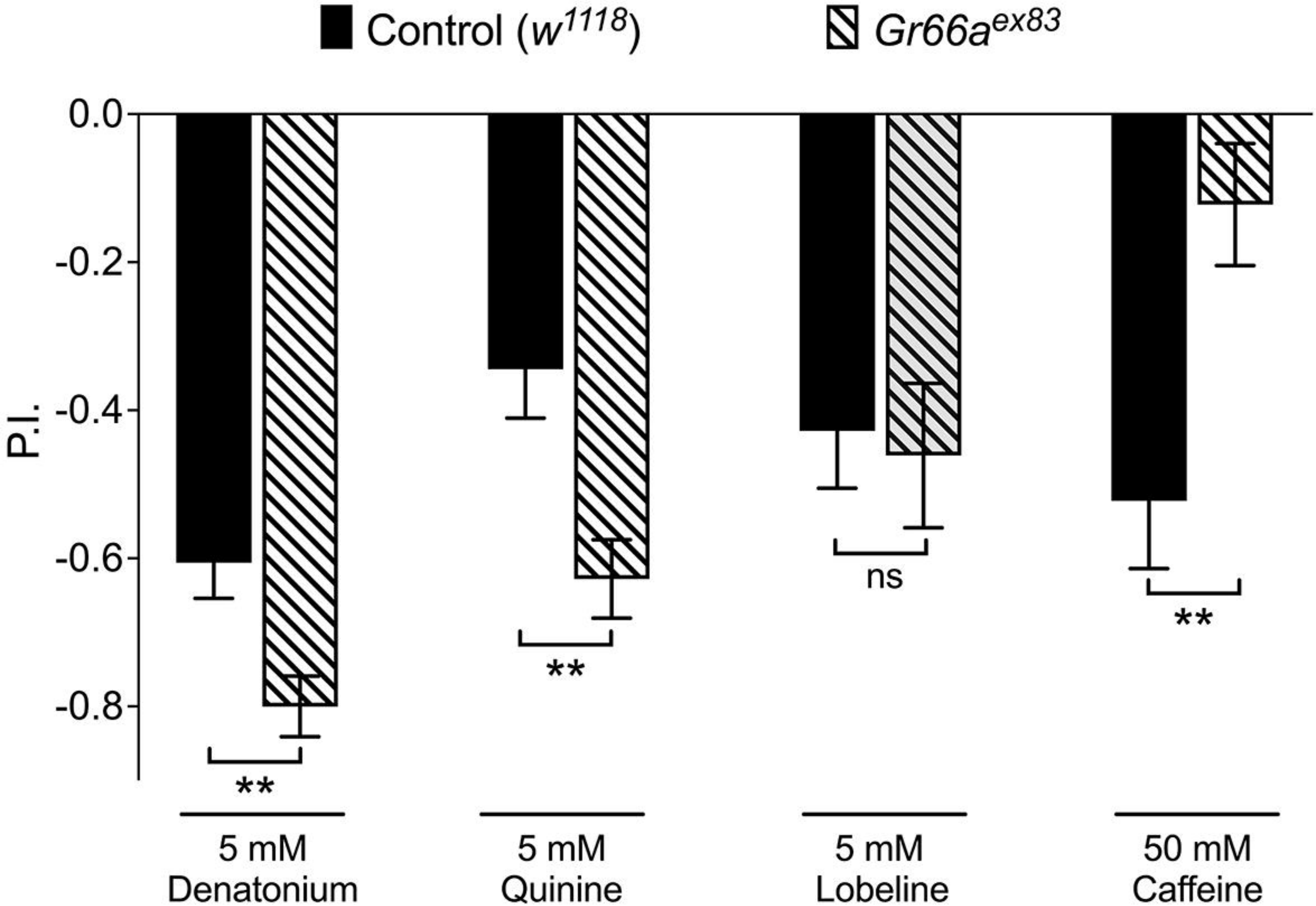

A
External sensory organs (TO and DO)
Gr28a
Gr28b.c
Merge
TOG
DOG
20 µm
Gr28b.a
Gr28b.c
Merge
TOG
20 µm
Gr28b.e
Gr28b.c
Merge
TOG
20 µm
B
Internal sensory organs (DPS/VPS)
Gr28a
Gr28b.c
Merge
10 µm
Gr66a
Gr28b.c
Merge
5 µm

A


Control
lexAop-GAL80
DO
TO
TOG
DOG
Pharynx
DPS
VPS
PPS
TOG
DOG
DPS
/VPS
10 µm
PPS

B


Control
lexAop-GAL80
GFP+ cells
0
1
2
3
*
*
*
**
TOG
DOG
DPS/VPS
PPS


Control (w^{1118})
$Gr66a^{ex83}$
0.0
-0.2
-0.4
-0.6
-0.8
P.I.
**
**
ns
**
5 mM Denatonium
5 mM Quinine
5 mM Lobeline
50 mM Caffeine