Title: Drosophila's sensory responses to bacterial peptidoglycan integrates positive and negative signals

Authors

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Abstract

Interactions between animals, including humans, and surrounding microbes are governed by a delicate balance, crucial for survival. Animals must distinguish and respond adequately to beneficial and harmful microbes to maintain homeostasis. Recent research suggests that bacterial components such as lipopolysaccharide and peptidoglycan (PGN) influence host behavior by modulating neuronal activity. PGN detection by specific neurons can prompt infected female flies to reduce oviposition or trigger avoidance behaviors via gustatory neurons. Using behavioral assays and calcium imaging, we found that PGNs can also act as attractants, activating the sweet taste circuit in a concentration-dependent manner. Our findings demonstrate that flies integrate PGN-derived positive and negative signals to make ad hoc decisions. This dual response underlines the need for *Drosophila* to distinguish between different concentrations of compounds in their environment, integrating sensory data to navigate efficiently in microbe-co-inhabited environments.

Highlights

- Bacterial PGN is attractive to flies at high concentrations
- Gr5a sweet gustatory neurons are activated by PGN in an NF-kB independent manner
- PER to high PGN concentrations remains in axenic flies
- Fly gustatory response to PGN integrates both attractive and aversive signals

46 **Introduction**

47 The interactions between animals, including humans, and the microbes surrounding them are
48 governed by a subtle balance. To understand the mechanisms underlying this equilibrium, the
49 insect *Drosophila melanogaster* serves as a powerful model, as microorganisms are essential
50 for the host's development, survival, nutrient s, or making certain food sources digestible
51 (Storelli et al., 2011). Some beneficial microbes occupy ecological niches that would otherwise
52 be filled by harmful microorganisms if they were absent (Kim et al., 2010). Conversely, certain
53 pathogenic species must be detected, avoided, or even actively combated by animals
54 (Charroux et al., 2020; Chen et al., 2024; Tleiss et al., 2024). The ability of animals to detect,
55 identify, and respond appropriately to the microbial world is therefore essential for their
56 homeostasis and sustainability.

57 Recent studies have begun to explore the hypothesis that essential bacterial cell wall
58 components, such as lipopolysaccharides (LPS) and peptidoglycan (PGN), which are well-
59 characterized ligands for receptors expressed by immune cells, might also modulate host
60 neuronal activity. Such molecular dialogue could allow the host to "sample" its surroundings
61 via its sensory system and adopt behaviors that limit contact with pathogenic microbes or, in
62 case of infection, reduce its impact on the host and its offspring (Kobler et al., 2020; Yanagawa
63 et al., 2017). For instance, PGN detection by a few specific neurons can modulate oviposition
64 behavior in infected females (Kurz et al., 2017; Masuzzo et al., 2022). In this case, the detection
65 of a universal bacterial cell wall component by certain neurons in the brain acts as a molecular
66 alarm, triggering a behavioral change in infected females, reducing their oviposition rate, and
67 potentially conserving energy for the energy-demanding immune response. Other studies
68 demonstrate that bacterial molecular patterns are directly detected by the fly's gustatory
69 system. The detection of LPS by esophageal bitter sensing neurons enables flies to avoid LPS

in feeding and oviposition assays (Soldano et al., 2016). We previously reported that by activating the bitter gustatory network, bacterial PGN can suppress the appetitive effect of a sucrose solution, demonstrating that PGN can be perceived as a bitter substance by flies (Montanari et al., 2024). As microbes are important cues for flies, which feed and reproduce on rotting fruits, we wonder whether they are also detected by the sensory system as positive cues.

In this study, we used the proboscis extension reflex assay (Shiraiwa and Carlson, 2007) and calcium imaging to assess whether a PGN solution could also activate the sweet taste circuit in flies. Our data reveal that PGN can elicits Gr5a+ neuron-dependent activation within the sweet taste network, demonstrating a dual response of the fly's taste system to PGN. This response is repulsive at low concentrations and attractive at higher concentrations, differing from the dual response to salt or hexanoic acid observed in other gustatory neurons (Jaeger et al., 2018; McDowell et al., 2022; Pradhan et al., 2023; Zhang et al., 2013). This dichotomous response suggests that *Drosophila* shares a need to detect and differentiate between high and low concentrations of specific compounds present in food substrates, engaging different receptors and neuronal cells with distinct coding logic. Furthermore, the detection of bacteria by flies requires integrating inputs from multiple subsets of sensory neurons to generate appropriate responses to bacterial PGN.

Results

Bacterial PGN is Attractive to Flies at High Concentrations

We have previously shown that Diaminopimelic-type peptidoglycan (Dap-type PGN), which forms the cell wall of all Gram-negative bacteria (specifically *Escherichia coli* PGN in our study), activates neurons responsible for aversive behaviors in flies (Montanari et al., 2024). Specifically, Gr66a+ (Wang et al., 2004) and ppk23+ (Jaeger et al., 2018) neurons located on the proboscis respond to PGN (Masuzzo et al., 2022; Montanari et al., 2024). Moreover, overexpression of IMD pathway components in Gr66a+ cells alters fly behavior (Masuzzo et al., 2022). Finally, using the Proboscis Extension Reflex (PER) assay, which quantifies gustatory reflexes in immobilized animals (Shiraiwa and Carlson, 2007), we showed that *E. coli* PGN can evoke an aversive response in flies (Montanari et al., 2024).

In the present study, since bacteria might also be perceived as beneficial by the adult fly, we investigated the potential attractive effect of PGN. To assess whether brief contact with PGN could trigger an attractive response, we focused on the proboscis extension response (PER), part of the sensorimotor taste circuit in adult flies (Figure 1A) (Schwarz et al., 2017; Shiraiwa and Carlson, 2007). To measure the attractiveness of a compound, we tested the molecule without mixing it with anything other than its solvent. When the fly's labellum (analogous to the tongue) is stimulated by a molecule like sucrose, it immediately extends its proboscis towards the food (Video 1). A chemical is considered more or less attractive based on its response relative to the reference solvent and a prototypical attractive substance, sucrose.

To determine if PGN could be attractive to flies, we tested this bacterial molecule alone, diluted in water at various concentrations. When the labellum was exposed to 1 mM sucrose, approximately 70% of flies extended their proboscis in an attempt to feed, illustrating the appetitive value of sucrose (Video 1 and Figure 1B). Water alone triggered a PER in 20% of

113 flies, and since the animals were allowed to drink to satiety before the test, we considered
114 this response to water to be neutral, likely due to mechanical stimulation (Video 2 and Figure
115 1B). We then tested the fly's response to *E. coli* DAP-type PGN. At concentrations of 10 µg/ml
116 (E.c.10) and 100 µg/ml (E.c.100), only 20% of flies extended their proboscis, similar to the
117 response to water, suggesting that PGN is not palatable at these concentrations (Figure 1B).
118 However, exposure to PGN at 200 µg/ml (*E.c.200*) showed a trend toward increased response,
119 with around 40% of flies performing a PER (Figure 1B). At 500 µg/ml (*E.c.500*), 70% of flies
120 extended their proboscis, a response similar to that seen with 1 mM sucrose (Video 3, Video
121 4 and Figure 1B). Thus, PGN at 500 µg/ml is as attractive to flies as 1 mM sucrose.
122 When DAP-type PGN from the Gram-positive bacterium *Bacillus subtilis* (*B.s.500*) and Lysine-
123 type PGNs from *Staphylococcus aureus* (*S.a.500*), *Micrococcus luteus* (*M.l.500*), and *E. coli* at
124 an OD of 100 (OD100) were used as elicitors, the response was comparable to that of sucrose
125 or *E. coli* PGN at 500 µg/ml (Figure 1B). Moreover, when we exposed animals to the bacterium
126 *E. coli* at an OD of 100, we observed an attractive response, recapitulating the phenotype
127 observed with PGN (Figure 1B).The attractive effect of *E. coli* PGN at 500 µg/ml (*E.c.500*) was
128 observed in flies from different genetic backgrounds, including *w-, yw,* and *CantonS* strains, as
129 well as in *w-* flies reared on either protein-rich (prot+) or sugar-rich (suc+) media,
130 demonstrating the robustness of the phenotype (Figure 1C and 1D).
131 These findings demonstrate that, at high concentrations, both Lysine- and DAP-type PGNs are
132 attractive to the fly's gustatory system.
133
134 **Axenic flies are still capable of triggering the PER response to PGN.**
135 Given that both larvae and adults live in a non-sterile environment, and that we recently
136 reported that larval cohabitation with bacteria is crucial for adults to perceive DAP-type PGN

137 as aversive, we tested whether the palatability of PGN *E.c.500* in adult flies was also
138 dependent on the presence of larval microbiota (Montanari et al., 2024). When flies were
139 reared on antibiotic-containing medium throughout their lifespan (axenic), the PER response
140 to DAP-type PGN *E.c.500* solutions was similar to that observed with sucrose and
141 indistinguishable from the response measured in non-axenic animals (Figure 1E). Thus,
142 contrary to our findings regarding aversion to DAP-type PGN (Montanari et al., 2024), neither
143 larval cohabitation with bacteria nor adult life in a non-aseptic medium influenced attractive
144 behavior toward high concentrations of DAP-type PGN.
145
146 **PGN Detection by the Gustatory System Involves Gr5a Neurons**
147 To identify which neurons respond to PGN *E.c.500*, attractive proboscis extension reflexes
148 (PER) were performed in flies where specific neurons were inactivated by overexpressing the
149 Kir2.1 potassium channel (UAS-Kir2.1), which blocks the generation of action potentials. The
150 expression of the Gr5a receptor is characteristic of neurons involved in the detection of sweet
151 molecules (Figure 2A), and these Gr5a+ neurons play a role in attractive behaviors (Dahanukar
152 et al., 2001). Importantly, inactivation of Gr5a+ neurons using the cell-specific driver Gr5a-
153 Gal4 (Gr5a-Gal4/UAS-Kir2.1) abolished the attractive response to both 1mM sucrose and DAP-
154 type PGN (Figure. 2B). Thus, sweet neurons are essential for triggering a PER response to high
155 concentrations of PGN in adult flies. Notably, the PER response of Gr5a-Gal4/UAS-Kir2.1 flies
156 to PGN *E.c.500* was significantly lower than the baseline response to water, with less than 10%
157 of flies showing PER (Figure 2B). This suggests that in the absence of sweet neuron activity
158 (Gr5a-Gal4/UAS-Kir2.1), PGN *E.c.500* is perceived as aversive by the flies. This finding is
159 consistent with our previous observation that DAP-type PGNs can induce ppk23+ neuron-
160 dependent aversion (Montanari et al., 2024). A possible explanation is that, in the absence of

161 attractive PGN perception (Gr5a-Gal4/UAS-Kir2.1), the aversion to this molecule becomes
162 apparent. These results suggest an integration of positive and negative inputs that influence
163 the fly's decision-making process regarding the taste of different DAP-type PGN
164 concentrations.
165
166 **Gr5a Neurons Are Required in Adults to Detect PGN *E.c.500***
167 In a previous study, we showed that Gr66a+ larval neurons are essential for priming the adult
168 behavioral response to DAP-type PGN (Montanari et al., 2024). Despite the fact that Gr5a+
169 neurons do not seem to be present in larvae (Kwon et al., 2011), we wished to test in an
170 unbiased way whether putative larval Gr5a+ cells could influence adult attraction to PGN
171 *E.c.500*. We used the Gal4/Gal80ts binary system, which allows spatial and temporal control
172 of neuron inactivation (Figure 2C). When 1mM sucrose or PGN *E.c.500* solutions were used as
173 stimuli, conditional inactivation of Gr5a+ neurons only in adults was as effective as lifelong
174 inactivation of these neurons. In contrast, inactivating Gr5a+ neurons only during the larval
175 stage had no impact on the fly's ability to respond to sucrose or PGN *E.c.500* (Figure 2D).
176 Therefore, adult PER behavior towards PGN *E.c.500* requires functional Gr5a+ neurons in the
177 adult stage.
178
179 **DAP-type PGN Directly Activates Gr5a Sweet Neurons**
180 The observation that Gr5a+ neurons are required for the PER response to PGN stimulation led
181 us to investigate whether these cells could be directly activated by DAP-type PGN. Using in
182 vivo GCaMP imaging, we monitored the neuronal activity of Gr5a+ axonal projections in the
183 suboesophageal zone (SEZ) of the brain in Gr5a-Gal4/UAS-GCaMP6s flies, which were exposed
184 to either water (Video 5), sucrose 1mM or (Video 6) or a drop of PGN. Exposure of the labellum

185 of *Gr5a-Gal4/UAS-GCaMP6s* flies to DAP-type PGN at 100µg/ml (Video 7) and 500µg/ml led
186 to an increase in intracellular calcium levels in axonal projections in the SEZ (Figure 3A and
187 3B). These results were confirmed with another pan-sweet taste neuron driver, Gr64f-Gal4
188 (Jiao et al., 2008) (Videos 8-10 and Figure S1). These findings demonstrate that PGN can
189 directly activate sweet taste neurons at high concentrations.
190

191 **Sweet Neurons Mediating Attraction to PGN E.c.500 Require Gr5a and Gr64 Receptors but**
192 **Are Independent of the IMD Pathway**

193 Having identified the neurons that respond to PGN and are required for the PER, we next
194 investigated the receptors and pathways involved. Previous studies have shown that the Gr5a
195 and Gr64a-f family proteins are receptors expressed in sweet neurons and are involved in
196 detecting sweet molecules such as sucrose and fructose (Dahanukar et al., 2001; Jiao et al.,
197 2008). To identify the receptors involved in PGN sensing, we used mutants lacking both Gr5a
198 and the Gr64a-f family receptors (Gr5a-Gr64a-fCRISPR). The PER response of these mutants
199 to sucrose and PGN *E.c.500* was comparable to the response to water (Figure 4A). Since these
200 mutant animals no longer respond to sweet molecules, we used a low salt concentration
201 (50mM NaCl) as a positive attractive control to ensure proper selection of responsive animals.
202 The lack of increased PER upon exposure to PGN *E.c.500*, compared to the baseline water
203 response, indicates that the tested sugar receptors are involved in PGN *E.c.500* perception.
204 Regarding the immune response, DAP-type PGN has been shown to be a major inducer of the
205 PGRP/NF-kB-IMD pathway, leading to the production of immune effectors and regulators
206 (Leulier et al., 2003) (Figure 4B). Our previous data indicated that elements of the IMD
207 pathway are required for DAP-type PGN signal transduction in adult bitter taste neurons
208 (Gr66a+ cells) (Masuzzo et al., 2022). To explore the role of the IMD pathway in PGN *E.c.500*

209 response, we used RNA interference to downregulate components of the IMD pathway. Flies
210 with downregulated PGRP-LC receptor and the IMD transducer FADD specifically in Gr5a+
211 neurons (*Gr5a-Gal4/UAS-pgrp-LC_RNAi* and *Gr5a-Gal4/UAS-Fadd_RNAi*) showed sensitivity
212 to PGN E.c.500 similar to that of control flies, indicating that neither PGRP-LC nor FADD is
213 required for the PER response to PGN *E.c.500* (Figure 4C).
214
215 **Integration of Attractive and Aversive Signals from PGN Exposure Modulates the PER**
216 Our data show that PGN E.c.500 elicits a Gr5a+ neuron-dependent PER response, comparable
217 to that induced by 1mM sucrose. We also demonstrated that DAP-type PGN at concentrations
218 of 100µg/ml and 500µg/ml are capable of activating Gr5a+ neurons (Figure 3A-C and Video 7).
219 We previously reported that DAP-type PGN at 200µg/ml antagonizes the PER response to
220 1mM sucrose by activating adult *ppk23+* neurons (Montanari et al., 2024). Taken together,
221 these data suggest that DAP-type PGN concentrations ranging from 100µg/ml to 200µg/ml
222 can activate both attractive and aversive neurons. Thus, depending on its concentration,
223 *E. coli*-derived DAP-type PGN can either trigger or inhibit a PER via the activation of two
224 independent sets of neurons (Figure 5A).
225 Therefore, when exposed to PGN, opposing signals from different neurons can be integrated
226 by the fly, and the resulting PER will be the outcome of this integration. In this context, the
227 PER to PGN E.c.500 is likely the sum of inputs from Gr5a+ neurons (attraction) as well as from
228 Gr66a+ or ppk23+ neurons (aversion). Blocking the input from sweet neurons using Gr5a-
229 Gal4/UAS-Kir2.1 should abolish the attraction to PGN and may reveal an aversion.
230 Consistently, the PER to PGN *E.c.500* was lower than the water baseline in Gr5a-Gal4/UAS-
231 Kir2.1 flies (Figure 2B). This observation was further confirmed by performing the opposite
232 experiment, where blocking the activity of aversive neurons led to an increase in attraction.

233 While the attractive PER to DAP-type PGN at 100 µg/mL or 200 µg/mL in wild-type flies was
234 not statistically different from the solvent control (Figure 1B and 5B), blocking the activity of
235 Gr66a+ or ppk23+ neurons (Gr66a-Gal4/UAS-Kir2.1 and ppk23-Gal4/UAS-Kir2.1) resulted in a
236 significantly higher PER to PGN compared to the water baseline (Figure 5A and 5B).
237 We previously reported that aversion to PGN requires the activity of adult ppk23+ neurons,
238 and that these neurons' ability to respond to PGN is dependent on developmental diet, and in
239 particular to larval cohabitation with bacteria (Montanari et al., 2024). Based on this, we
240 hypothesized that rearing animals in axenic conditions would impair PGN aversion in adults,
241 making them more attracted to PGN. While the PER to water or 1mM sucrose was not
242 influenced by rearing conditions, axenic animals exposed to DAP-type PGN at 200µg/ml
243 exhibited an increased PER compared to controls, regardless of whether the axenic period
244 lasted throughout their lives or was restricted to the larval stage (Figure 5C). This result
245 supports a model in which the PER to PGN is determined by an integration of attractive and
246 aversive signals from independent neuronal networks.
247
248 **Discussion**
249 Our results demonstrate that at concentrations above 200 µg/ml, both DAP-type and Lys-type
250 PGN, essentially the majority of PGNs, are capable of stimulating sweet neurons through the
251 activation of receptors previously identified as sugar receptors. This activation is likely due to
252 the sugar components of PGN, specifically the long polymers of the disaccharides N-
253 acetylmuramic acid and N-acetylglucosamine. Since these sugar components are common to
254 all PGNs, it is logical that both Lys- and DAP-type PGNs are able to activate sugar neurons, as
255 observed in this study (Vollmer et al., 2008). PGN can be modified by enzymes like amidases
256 and lysozymes, which fragment the polymer into smaller units called muropeptides. Previous

257 studies in insects and mammals have shown that different muropeptides can activate specific
258 receptors with varying degrees of efficacy (Fioriti et al., 2024; Leulier et al., 2003; Tosoni et al.,
259 2019).
260 What remains to be determined is the minimum structural unit of PGN capable of activating
261 sugar neurons, and whether the level of neuronal activation depends on the specific structure
262 of the muropeptides. Interestingly, the activation we observed appears to be independent of
263 the IMD pathway, as the proboscis extension responses (PER) to PGN were similar in both
264 control flies and those mutant for certain components of the IMD/NF-kB pathway. This
265 response contrasts with previous findings where PGN, at concentrations near 200 µg/ml, was
266 shown to activate bitter sensing Gr66a+ neurons via part of the IMD pathway, though not
267 involving the Relish transcription factor (Masuzzo et al., 2022). It is also worth noting that the
268 ability to perceive PGN as a bitter molecule has only been observed with DAP-type PGNs,
269 which are typically found in Gram-negative bacteria, making this response specific to a subset
270 of bacterial species, unlike the response observed in our study.
271 In contrast, the sweet neuron response we report here more closely resembles the response
272 observed in ppk23+ neurons, which is also independent of the IMD pathway (Montanari et al.,
273 2024). However, there is a notable difference between the attractive response mediated by
274 Gr5a+ neurons and the aversive response mediated by ppk23+ neurons. The aversive
275 response to PGN requires bacterial priming during the larval phase and is absent in axenic
276 flies, while the Gr5a+ neuron-mediated attraction to PGN occurs even in flies reared in the
277 complete absence of bacteria. These findings suggest that the detection and response to PGN
278 by Gr5a+ neurons in adults are not dependent on prior exposure to bacteria.
279 By measuring PERs in flies with blocked bitter or sweet circuits, we show that the fly's taste
280 system integrates signals from at least three distinct types of neurons—Gr66a+, ppk23+, and

281 Gr5a+—to determine how to respond to PGN. Notably, the activation of these neurons is not
282 triggered by the same concentrations of PGN. Similar dose-dependent behavioral responses
283 have been described for other compounds. For example, NaCl is attractive at 50 mM through
284 the Gr5a+ network, but aversive at 250 mM via ppk23+ neurons (Zhang et al., 2013; Jaeger et
285 al., 2018; McDowell et al., 2022). Likewise, hexanoic acid is attractive at low concentrations
286 via Gr5a+ cells, but aversive at higher concentrations through the bitter network (Pradhan et
287 al., 2023). These examples support the idea that different concentrations of the same
288 compound can trigger opposite behaviors, depending on the involved neuronal circuits.
289 Some studies suggest a more complex scenario, where a molecule can be attractive as an odor
290 but aversive as a tastant. A prime example of this is acetic acid, which is attractive when
291 detected by the gustatory system but repulsive when sensed by the olfactory system (Devineni
292 et al., 2019; Joseph et al., 2009). Additionally, systems that integrate opposite inputs from
293 both sweet and bitter signals have been identified, such as the inhibition of sweet neurons by
294 bitter neurons through GABA signaling (Chu et al., 2014) or by the OBP49a protein, which
295 attenuates sweet neuron firing when bitter chemicals are combined (Jeong et al., 2013). These
296 mechanisms help facilitate the avoidance of harmful compounds when sweet molecules are
297 present in mixtures. In our case, the concentration that triggers aversion (200 µg/ml) is lower
298 than the one that triggers attraction (500 µg/ml), suggesting a unique pattern of integration
299 for PGN.
300 Our findings also highlight the fact that PGN concentrations at 100 µg/ml can activate Gr5a+
301 neurons and, in the absence of the aversive response (i.e., in Gr66a-Gal4/UAS-Kir2.1 flies),
302 trigger attraction. This suggests that sweet neurons are tuned to respond to PGN
303 concentrations lower than those that elicit aversion, and that the integration of bitter signals
304 can override the attraction. In a more complex, physiological context where both sweet and

305 bitter molecules are present, the overall influence of microbial molecules like PGN may be
306 balanced by the other chemicals present.
307 Finally, it remains to be explored what these PGN concentrations represent in terms of
308 bacterial populations in natural environments, and whether the physiological state of the
309 bacteria influences their ability to release PGN and if so, in which forms and at what
310 concentrations. This information would be critical for understanding the role of PGN detection
311 systems in nature.
312 This task is further complicated by the fact that PGN perception is just one small piece of the
313 sensory information that flies use to navigate and make decisions in an environment teeming
314 with bacteria and other microorganisms. This complex sensory integration is key to the fly's
315 ability to balance the need for food, reproduction, and immunity in an ever-changing microbial
316 landscape.
317

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327 **Data availability statement**

328 Genotypes, raw data and statistics are available at:
329 https://figshare.com/ndownloader/files/50813955 DOI: 10.6084/m9.figshare.27908889
330

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430 **Material and methods**

431 **Fly husbandry**

432 Flies were grown at 25°C on a yeast/cornmeal medium in 12h/12h light/dark cycle-controlled
433 incubators. For 1 L of food, 8.2 g of agar (VWR, cat. #20768.361), 80 g of cornmeal flour
434 (Westhove, Farigel maize H1) and 80 g of yeast extract (VWR, cat. #24979.413) were cooked
435 for 10 min in boiling water. 5.2 g of Methylparaben sodium salt (MERCK, cat. #106756) and 4
436 mL of 99% propionic acid (CARLOERBA, cat. #409553) were added when the food had cooled
437 down (prot+ media).

438 suc+ media: for 1 L of food, 11 g of agar (VWR, cat. #20768.361), 80 g of cornmeal flour
439 (Westhove, Farigel maize H1), 20 g of yeast extract (VWR, cat. #24979.413) and 30g of Sucrose
440 were cooked for 10 min in boiling water. 2.5g of Moldex and 5 mL of 99% propionic acid
441 (CARLOERBA, cat. #409553) were added when the food had cooled down.

442

443 **Fly stocks**

444 The reference strain in this study is noted w^{-} and corresponds to $w^{[1118]}$ (Bloomington #5905).
445 Canton S (Bloomington #64349) and $y^{[1]}$, $w^{[1118]}$ and *Gr5A*$^{-}$; *Gr64a-f*$^{-}$ (gift from Bernard
446 Charroux) and *Gr5a-Gal4* (Bloomington #57992) and *Gr64f-Gal4* (Bloomington #57669) and
447 *UAS-GCaMP6S* (Bloomington #42746) and *UAS-Kir2.1* (Bloomington #6595) and *Tub-Gal80ts*
448 (Bloomington #7016) and *UAS-GFP* (Bloomington #32195) and *UAS-pgrp-LC_RNAi* (VDRC
449 #101636) and *UAS-Fadd_RNAi* (Leulier et al., 2002) and *ppk23-Gal4* (Bloomington #93026) and
450 *Gr66a-Gal4* (Bloomington #28801).

451

452 **PER assay**

453 All flies used for the test were females between 5 and 7 days old. Unless experimental
454 conditions require it, the flies are kept and staged at 25°C to avoid any temperature changes
455 once they are put into starvation. The day before, the tested flies are starved in an empty tube
456 with water-soaked plug for 24h at 25°C.
457 Eighteen flies are tested in one assay, 6 flies are mounted on one slide and in pairs under each
458 coverslip. To prepare the slide, three pieces of double-sided tape are regularly spaced on a
459 slide. Two spacers are created on the sides of each piece of tape by shaping two thin cylinders
460 of UHT paste. To avoid the use of carbon dioxide flies are anesthetized on ice. Under the
461 microscope, two flies are stuck on their backs, side by side, on same piece of tape so that their
462 wings adhere to the tape. A coverslip is then placed on top of the two flies and pressed onto
463 the UHT paste, blocking their front legs and immobilizing them.
464 Once all slides are prepared, they are transferred to a humid chamber and kept at 25°C for 1.5
465 hours to allow the flies to recover before the assay.
466
467 Flies are tested in pairs, the test is carried out until completion on a pair of flies (under the
468 same coverslip), and then move on to the next pair.
469 Before the test, water is given to each pair of flies to ensure that the flies are not thirsty and
470 do not respond with a PER to the water in which the solutions are prepared. Stimulation with
471 the test solution is always followed by a control stimulation with a sweet solution, to assess
472 the fly's condition and its suitability for the test. During the test small strips of filter paper are
473 soaked in the test solution and used to contact the fly's labellum (three consecutive times per
474 control and test phase). Contact with the fly's proboscis should be as gentle as possible. Ideally
475 the head should not move. A stronger touch may prevent the fly from responding to

476 subsequent stimulation. Based on the protocol needed the test is done following the sequence

477 and the timing in the table below.

478 **Tab.1 Attraction protocol**

Water To satiety	Wait 1 min	**TEST** Repeated 3 times	Wait 1 min	Water one time	Wait 1 min	**CONTROL** Sucrose 500mM Repeated 3 times

479

480

481

482 All solutions to be tested are prepared the test day and stored at room temperature. The

483 control stimulation is performed with 500mM sucrose (D(+)-sucrose ≥99.5 %, p.a. Carl Roth

484 GmbH + Co. KG). After each control or test stimulation, a water-soaked strip is used to tap the

485 proboscis and clean it.

486 The response of the fly to each stimulation is recorded and averaged. Flies that respond

487 positively (PER) to at least one of the control stimulations are considered for further analysis,

488 the others are discarded. The PER index is calculated as the percentage of flies tested that

489 responded with a PER to the TEST stimulation and represented as ± 95% CI. In case of stage

490 dependent experiments, flies are shifted from one condition to another upon hatching.

491

492

493 **Microscopy**

494 No immunostaining was performed with proboscises. Proboscises of adult females were
495 dissected in PBS, rinsed with PBS, and directly mounted on slides using Vectashield fluorescent
496 mounting medium. The tissues were visualized directly after. Images were captured with a
497 LSM 780 Zeiss confocal microscope (20 air objective was used).

498

499 ***In vivo* calcium imaging**

500 *In vivo* adult calcium imaging experiments were performed on 5-7 day-old starved mated
501 females. Animals were raised on conventional media with males at 25°C. Flies were starved
502 for 20-24 h in a tube containing a filter paper soaked in water prior to experiments. Flies of
503 the appropriate genotype were anesthetized on ice for 1 h. Female flies were suspended by
504 the neck on a plexiglass block (2 x 2 x 2.5 cm), with the proboscis facing the center of the block.
505 Flies were immobilized using an insect pin (0.1 mm diameter) placed on the neck. The ends of
506 the pin were fixed on the block with beeswax (Deiberit 502, Siladent, 1345 209212). The head
507 was then glued on the block with a drop of rosin (Gum rosin, Sigma-Aldrich 1346 -60895-,
508 dissolved in ethanol at 70 %) to avoid any movements. The anterior part of the head was thus
509 oriented towards the objective of the microscope. Flies were placed in a humidified box for 1
510 h to allow the rosin to harden without damaging the living tissues. A plastic coverslip with a
511 hole corresponding to the width of the space between the two eyes was placed on top of the
512 head and fixed on the block with beeswax. The plastic coverslip was sealed on the cuticle with
513 two-component silicon (Kwik-Sil, World Precision Instruments) leaving the proboscis exposed
514 to the air. Ringer's saline (130 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 36 mM
515 saccharose, 5 mM HEPES, pH 7.3) was placed on the head. The antenna area, air sacs, and the
516 fat body was removed. The esophagus was cut without damaging the brain and taste nerves

517 to allow visual access to the anterior ventral part of the sub-esophageal zone (SEZ). The
518 exposed brain was rinsed twice with Ringer's saline. GCaMP6s fluorescence was viewed with
519 a Leica DM600B microscope under a 40x water objective. Stimulation was performed
520 manually using a pipette with gel loading tip by applying 140 µL of tastant solution diluted in
521 water on the proboscis. The gustatory stimulation was continuous as the tastant is in the drop
522 contacting the proboscis. The recording started before the addition of the tastant and the
523 calcium response could be observed immediately following the contact with the sensilla. For
524 each condition, n=7 to 9.
525
526 GCaMP6s was excited using a Lumencor diode light source at 482 nm ± 25. Emitted light was
527 collected through a 505-530 nm band-pass filter. Images were collected every 500ms using a
528 Hamamatsu/HPF-ORCA Flash 4.0 camera and processed using Leica MM AF 2.2.9. Each
529 experiment consisted of a recording of 70-100 images before stimulation and 160 images after
530 stimulation. Data were analyzed as previously described [38] by using FIJI (https://fiji.sc/).
531
532 **Different PGN used**
533 *E. coli* K12: Invivogen, catalog code #tlrl-kipgn
534 *Staphylococcus aureus :* Invivogen, catalog code #tlrl-kipgn
535 *Micrococcus luteus* : Sigma Aldrich, catalog #53243
536 *Bacillus subtilis*: Sigma Aldrich, catalog #SMB00288
537
538 **Bacterial strains and maintenance**
539 For this study, the following bacterial strain was used: *E. coli* K12 ATCC
540 Grown in LB liquid, in aerobic conditions, 37°C, shaking at 200 rpm.

541

542 **Statistics and data representation**

543 The Prism software was used for statistical analyses.

544 For *in vivo* calcium imaging, the D'Agostino–Pearson test to assay whether the values are
545 distributed normally was applied. As not all the data sets were considered normal, non-
546 parametric statistical analysis such as non-parametric unpaired Mann–Whitney two-tailed
547 tests was used for all the data presented.

548 For PER datasets. As the values obtained from one fly are categorical data with a *Yes* or *No*
549 value, we used the Fisher exact t-test and the 95% confidence interval to test the statistical
550 significance of a possible difference between a test sample and the related control.

551 For PER assays, at least 2 independent experiments were performed. The results from all the
552 experiments were gathered and the total amount of flies tested is indicated in the graph. In
553 addition, we do not show the average response from one experiment representative of the
554 different biological replicates, but an average from all the data generated during the
555 independent experiments in one graph. However, each open circle represents the average
556 PER of 1 experiment.

557

558 **Detailed lines, conditions and statistics for the figure section**

559 All these data are downloadable with the source data file.

560 DOI: 10.6084/m9.figshare.27908889

561 https://figshare.com/ndownloader/files/50813955

562

563 **Figure Legends**

564

565 **Figure 1 *Dose-dependent attraction to PGN***

566 (A) Drosophila feeding behavior is characterized by a sequence of distinct behavioral modules.
567 A starved fly initiates a search for food, and the detection of food odors stops this search. The
568 initial gustatory cues are acquired by sampling food using the proboscis. If the fly encounters
569 a potentially appetizing stimulus, it will begin feeding; if the stimulus is aversive, the fly will
570 retract its proboscis.

571 (B) PER index of w- flies to control solutions of 1mM sucrose, water, and increasing
572 concentrations of PGN from *E. coli* K12 (E.c.), *S. aureus* (S.a.), *M. luteus* (M.l.), and *B. subtilis*
573 (B.s.), or an *E. coli* culture at OD100. The numbers below the x-axis represent the final PGN
574 concentrations in µg/mL and the OD value for the *E. coli* culture.

575 (C) Attraction to PGN is independent of genetic background. PER index of yw- and CantonS
576 flies to control solutions of water, 1mM sucrose, and PGN from *E. coli* K12 at 500µg/mL.

577 (D) Raising medium has no effect on attraction to PGN. PER index of w- flies raised on protein-
578 enriched (prot+) or sucrose-enriched (suc+) media, to control solutions of water, 1mM
579 sucrose, and PGN from *E. coli* K12 at 500µg/mL. Data for w- prot+ flies are the same as in panel
580 1A.

581 (E) Germ-free status does not affect attraction to PGN. PER index of w- germ-free (GF) and
582 conventionally raised (CR) flies to control solutions of water, 1mM sucrose, and PGN from *E.*
583 *coli* K12 at 500µg/mL. Data for w- CR flies are the same as in panel 1A. The PER index is
584 calculated as the percentage of flies that responded with a PER to the stimulation ± 95%
585 confidence interval (CI). A PER value of 1 indicates that 100% of the tested flies extended their
586 proboscis in response to the stimulus, while a value of 0.2 indicates that 20% of the flies
587 responded. The number of flies tested (n) is shown above each bar. For each condition, at
588 least three groups of 10 flies each were tested. "ns" indicates $p>0.05$, * indicates $p<0.05$, **
589 indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$, Fisher Exact Test.
590 Further details are available in the figure section on lines, conditions, and statistics.

591

592 **Figure 2 Gr5a+ neurons are required for attraction to PGN**

593 (A) Confocal image of the proboscis of Gr5a-Gal4/UAS-GFP flies showing gustatory neurons in
594 the fly proboscis. LP refers to Labellum-Proboscis, CB to cellular body; A denotes axons

595 emanating from neurons in the proboscis. D, V, P, and A stand for Dorsal, Ventral, Posterior,
596 and Anterior.
597 (B) Impairing the activity of Gr5a+ neurons via UAS-Kir2.1 eliminates the attraction to PGN.
598 PER index of flies to control solutions of water, 1mM sucrose, and PGN from *E. coli* at
599 500µg/mL.
600 (C) Graph showing the life periods during which flies are shifted from 18°C (green) to 29°C
601 (red).
602 (D) Gr5a+ neurons are functionally required for PGN attraction in adults but are not needed
603 during the larval stage. PER index of flies to 1mM sucrose and PGN from *E. coli* K12 at
604 500µg/mL. The ubiquitously expressed Tub-Gal80ts inhibits the activity of Gal4 at 18°C and is
605 inactivated at 29°C, allowing the expression of UAS-Kir2.1 and the inhibition of Gr5a+ neuron
606 activity. The PER index is calculated as the percentage of flies responding with a PER to the
607 stimulus ± 95% CI. The number of flies tested (n) is shown above each bar. "ns" indicates
608 p>0.05, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001, **** indicates
609 p<0.0001, Fisher Exact Test.
610 Further details on conditions and statistics are provided in the figure section.
611
612 **Figure 3 Real-time calcium imaging with GCaMP6s reflects the *in vivo* neuronal activity of**
613 **Gr5a+ neurons**
614 (A) Representative images showing the GCaMP6s intensity before and after the addition of
615 control water, 100mM sucrose, or PGN from *E. coli* K12 at 100µg/mL. Scale bar is 10µm.
616 (B) Averaged fluorescence intensity of negative peaks ± SEM for Gr5a+ neurons in response
617 to water (n=7), 100mM sucrose (n=16), or PGN from *E. coli* K12 at 100µg/mL (n=7) or
618 500µg/mL (n=7). In (B), ** indicates p<0.01, *** indicates p<0.001, **** indicates p<0.0001,
619 non-parametric t-test, Mann-Whitney test.
620 Further details on conditions and statistics can be found in the figure section.
621
622
623 **Figure 4 Sugar receptors are involved in PGN attraction**
624 (A) PER index of mutant flies lacking the Gr5a receptor as well as the Gr64a-f family to water,
625 1mM sucrose, and PGN from *E. coli* K12 at 500µg/mL, compared to w- flies. Data for w- flies
626 are the same as in Fig. 1A.

627 (B) Diagram of the IMD pathway.
628 (C) RNAi-mediated inactivation of PGRP-LC (UAS-pgrp-LC RNAi) or Fadd (UAS-Fadd RNAi) in
629 Gr5a+ cells does not affect attraction to PGN. PER index of flies to water, 1mM sucrose, and
630 PGN from *E. coli* K12 at 500µg/mL. For (A) and (C), the PER index is calculated as the
631 percentage of flies responding with a PER to the stimulus ± 95% CI. The number of flies tested
632 (n) is shown above each bar. "ns" indicates p>0.05, * indicates p<0.05, ** indicates p<0.01,
633 *** indicates p<0.001, **** indicates p<0.0001, Fisher Exact Test.
634 Further details on conditions and statistics are available in the figure section.
635
636 **Figure 5 Integration of Attractive and Aversive Signals from PGN Exposure Modulates the**
637 **PER**
638 (A) Schematic illustrating the balance between attraction and aversion to PGN and the effects
639 of genetic manipulations in Gr66a+ and ppk23 neurons on this balance. Our previous work
640 showed that Gr66a+ neurons are not required during adulthood for PGN aversion, but their
641 activity during the larval stage is necessary for ppk23+ neurons to recognize and show aversion
642 to PGN in adulthood. At a PGN concentration of 500µg/mL, input from Gr5a+ neurons shifts
643 the balance from aversion to attraction. Impairing Gr66a+ or ppk23+ neuron activity (Gr66a-
644 Gal4/UAS-Kir2.1 and ppk23-Gal4/UAS-Kir2.1) abolishes their input, leading to increased
645 attraction to PGN.
646 (B) Impairing the activity of Gr66a+ or ppk23+ neurons with UAS-Kir2.1 increases attraction to
647 PGN at 200µg/mL. PER index of flies to control solutions of water and PGN from *E. coli* K12 at
648 100µg/mL or 200µg/mL.
649 (C) PER index of flies raised as conventional (CR), germ-free (GF), or germ-free only during the
650 larval stage (GF larvae CR adult) to control solutions of water and PGN from *E. coli* K12 at
651 200µg/mL. Data for w- CR flies are the same as in Fig. 1A. For (B) and (C), the PER index is
652 calculated as the percentage of flies responding with a PER to the stimulus ± 95% CI. The
653 number of flies tested (n) is shown above each bar. "ns" indicates p>0.05, * indicates p<0.05,
654 ** indicates p<0.01, *** indicates p<0.001, **** indicates p<0.0001, Fisher Exact Test.
655 Further details on conditions and statistics are available in the figure section.
656

Figure S1 Real-time calcium imaging with GCaMP6s reflects the *in vivo* neuronal activity of Gr64f+ neurons

Averaged fluorescence intensity of negative peaks ± SEM for Gr64f+ neurons in response to water (n=7), 100mM sucrose (n=6), or PGN from *E. coli* K12 at 100µg/mL (n=6). ** indicates p<0.01, *** indicates p<0.001, **** indicates p<0.0001, non-parametric t-test, Mann-Whitney test. Further details on conditions and statistics can be found in the figure section.

Video 1 PER is triggered following exposure to sucrose 1mM

DOI: 10.6084/m9.figshare.27929532

https://figshare.com/articles/media/Video_1_PER_suc_1mM/27929532?file=50876001

Response following proboscis contact with a stripe of paper soaked in a 1mM solution (water as solvent). 7-days old females starved 15h prior to the assay.

Video 2 PER is not triggered following exposure to water

DOI: 10.6084/m9.figshare.27929517

https://figshare.com/articles/media/Video_2_PER_water/27929517?file=50875968

Absence of response following proboscis contact with a stripe of paper soaked in water. 7-days old females starved 15h prior to the assay.

Video 3 PER is triggered following exposure to PGN of *E. coli* at 500µg/ml

DOI: 10.6084/m9.figshare.27929547

https://figshare.com/articles/media/Video_3_PER_PGN500/27929547?file=50876025

Response following proboscis contact with a stripe of paper soaked in an *E. coli* PGN solution at 500µg/ml (water as solvent). 7-days old females starved 15h prior to the assay.

Video 4 Comparison of Behavioral Response to Water and E. coli PGN in the Same Fly

DOI: 10.6084/m9.figshare.27929553

https://figshare.com/articles/media/Video_4_water_then_PGN500/27929553?file=50876226

Using the same fly, this experiment compares the absence of response to a proboscis contact with a paper strip soaked in water, versus a clear response to a paper strip soaked in

688 an *E. coli* PGN solution (500 µg/ml, with water as the solvent). The test was conducted on 7-
689 day-old females, starved for 15 hours prior to the assay.
690
691 **Video 5 Gr5a+ neurons do not respond to water**
692 **DOI: 10.6084/m9.figshare.27908901**
693 **https://figshare.com/articles/media/Video_5_Gr5a_GCaMP6s_vs_water/27908901?file=5**
694 **0814009**
695 Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s
696 under the control of the Gr5a-Gal4 driver to assess the in vivo neuronal activity of labellar
697 Gr5a+ neurons following exposure to water. The water was added at t+ 2 seconds.
698
699 **Video 6 Gr5a+ neurons respond to sucrose**
700 **DOI: 10.6084/m9.figshare.27908928**
701 **https://figshare.com/articles/media/Video_6_Gr5a_GCaMP6s_vs_sucrose_100mM/27908**
702 **928?file=50814042**
703 Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s
704 under the control of the Gr5a-Gal4 driver to assess the in vivo neuronal activity of labellar
705 Gr5a+ neurons following exposure to sucrose 100mM. The sucrose was added at t+2 seconds.
706
707 **Video 7 Gr5a+ neurons respond to DAP-type peptidoglycan**
708 **DOI: 10.6084/m9.figshare.27908949**
709 **https://figshare.com/articles/media/Video_7_Gr5a_GCaMP6s_vs_PGN_100/27908949?fil**
710 **e=50814069**
711 Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s
712 under the control of the Gr5a-Gal4 driver to assess the in vivo neuronal activity of labellar
713 Gr5a+ neurons following exposure to *E. coli* PGN solution at 100µg/mL. The PGN was added
714 at t+2 seconds.
715
716 **Video 8 Gr64f+ neurons do not respond to water**
717 **DOI: 10.6084/m9.figshare.27908979**
718 **https://figshare.com/articles/media/Video_8_Gr64f_GCaMP6s_vs_water/27908979?file=**
719 **50814099**

720 Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s
721 under the control of the Gr64f-Gal4 driver to assess the in vivo neuronal activity of labellar
722 Gr64f+ neurons following exposure to water. The water was added at t+2 seconds.
723
724 **Video 9 Gr64f+ neurons respond to sucrose**
725 **DOI: 10.6084/m9.figshare.27908991**
726 **https://figshare.com/articles/media/Video_9_Gr64f_GCaMP6s_vs_Sucrose_100mM/2790**
727 **8991?file=50814126**
728 Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s
729 under the control of the Gr64f-Gal4 driver to assess the in vivo neuronal activity of labellar
730 Gr64f+ neurons following exposure to sucrose 100mM. The sucrose was added at t+2 seconds.
731
732 **Video 10 Gr64f+ neurons respond to DAP-type peptidoglycan**
733 **DOI: 10.6084/m9.figshare.27909015**
734 **https://figshare.com/articles/media/Video_10_Gr64f_GCaMP6s_vs_PGN_100/27909015?f**
735 **ile=50814165**
736 Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s
737 under the control of the Gr64f-Gal4 driver to assess the in vivo neuronal activity of labellar
738 Gr64f+ neurons following exposure to *E. coli* PGN solution at 100µg/mL. The PGN was added
739 at t+2 seconds.
740
741
742
743

Figure 1

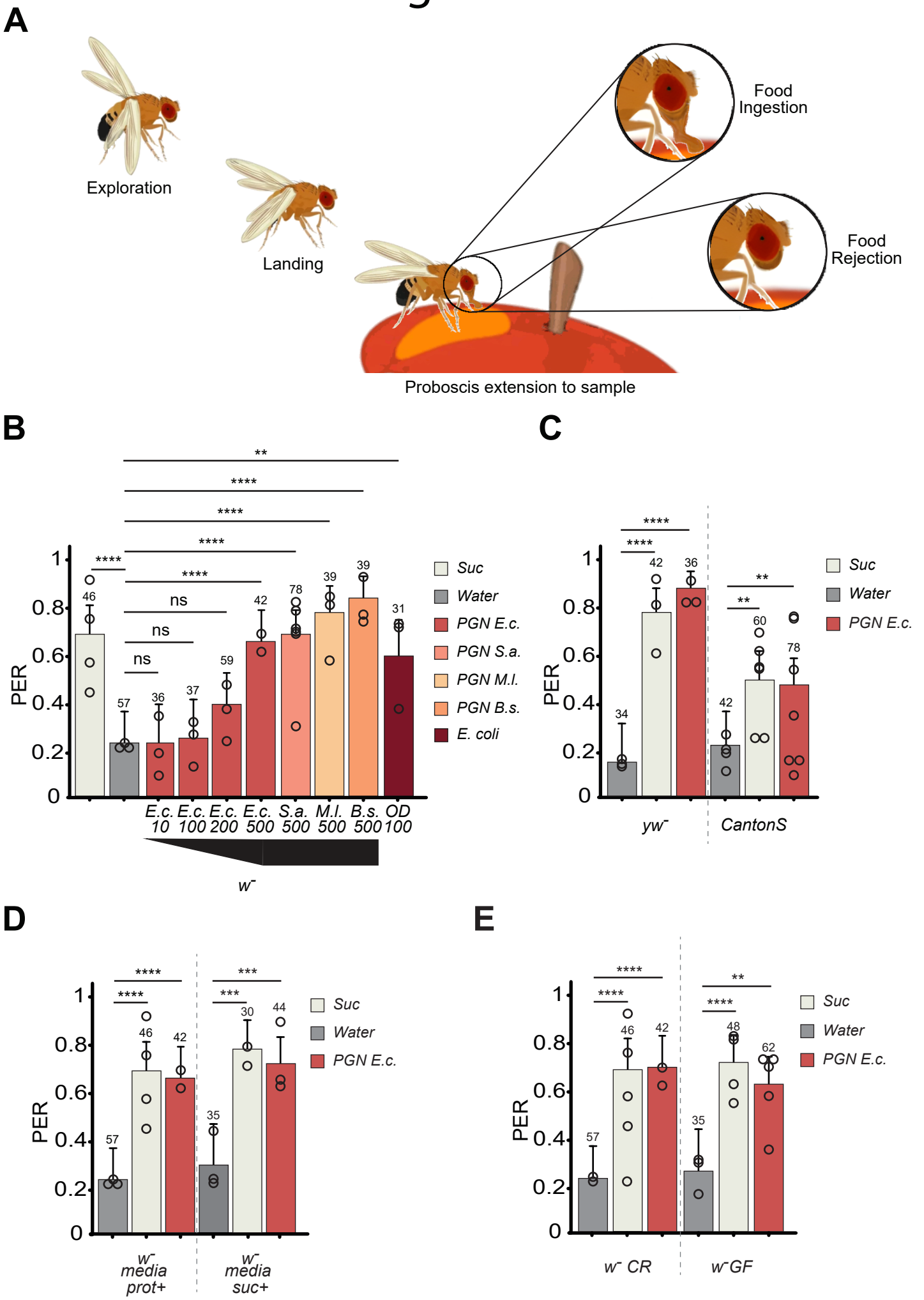

A
Exploration
Landing
Food Ingestion
Food Rejection
Proboscis extension to sample
B
PER
E.c. 10
E.c. 100
E.c. 200
E.c. 500
S.a. 500
M.l. 500
B.s. 500
OD 100
w-
Suc
Water
PGN E.c.
PGN S.a.
PGN M.l.
PGN B.s.
E. coli
ns
C
yw-
CantonS
D
w- media prot+
w- media suc+
E
w- CR
w-GF

Figure 2

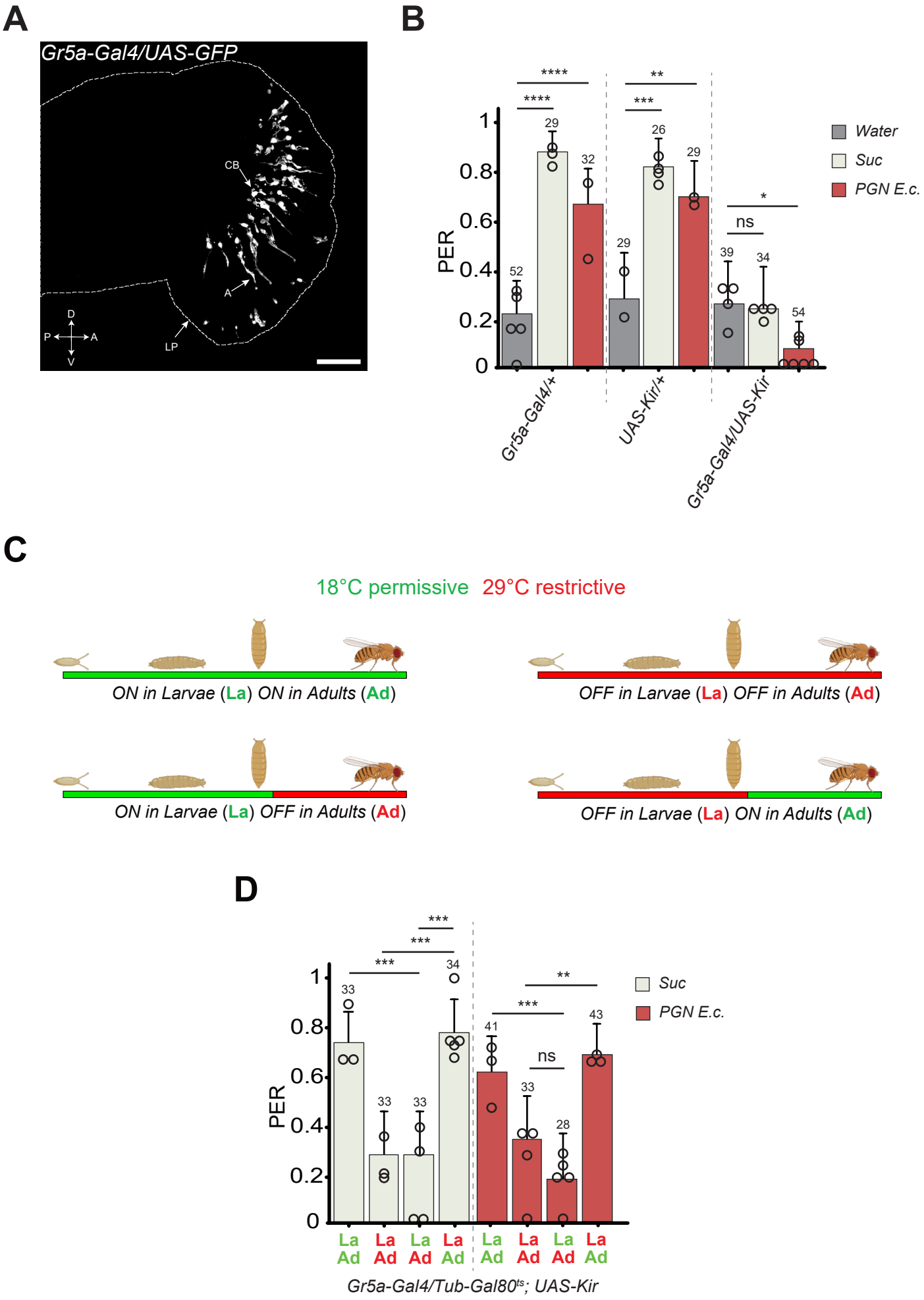
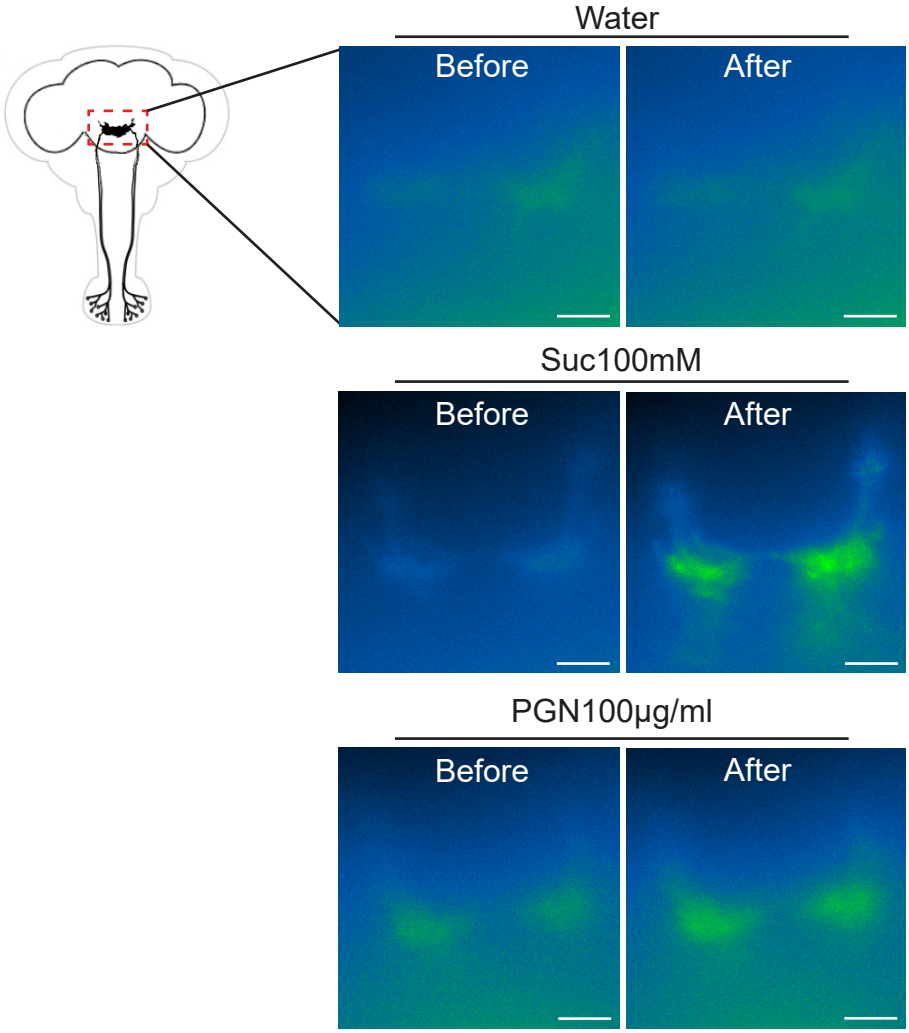

A
Gr5a-Gal4/UAS-GFP
CB
A
LP
D
P
A
V
B
PER
Water
Suc
PGN E.c.
Gr5a-Gal4/+
UAS-Kir/+
Gr5a-Gal4/UAS-Kir
ns
C
18°C permissive 29°C restrictive
ON in Larvae (La) ON in Adults (Ad)
OFF in Larvae (La) OFF in Adults (Ad)
ON in Larvae (La) OFF in Adults (Ad)
OFF in Larvae (La) ON in Adults (Ad)
D
PER
Suc
PGN E.c.
ns
La Ad
Gr5a-Gal4/Tub-Gal80ts; UAS-Kir

Figure 3

A


Water
Before
After
Suc100mM
Before
After
PGN100µg/ml
Before
After

B

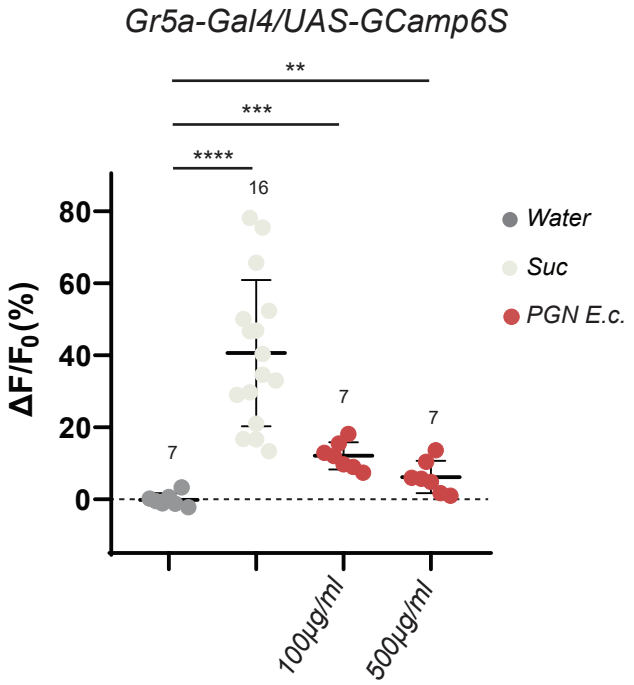
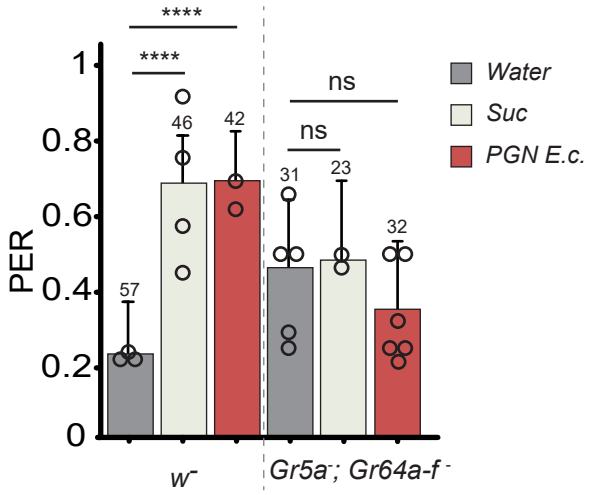
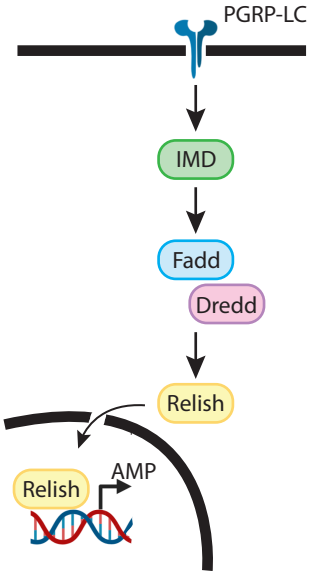

Gr5a-Gal4/UAS-GCamp6S
**
***
****
16
7
7
7
80
60
40
20
0
ΔF/F0(%)
Water
Suc
PGN E.c.
100µg/ml
500µg/ml

Figure 4

A


****
****
ns
ns
1
0.8
0.6
0.4
0.2
0
PER
57
46
42
31
23
32
Water
Suc
PGN E.c.
w-
Gr5a-; Gr64a-f-

B


PGRP-LC
IMD
Fadd
Dredd
Relish
Relish
AMP

C

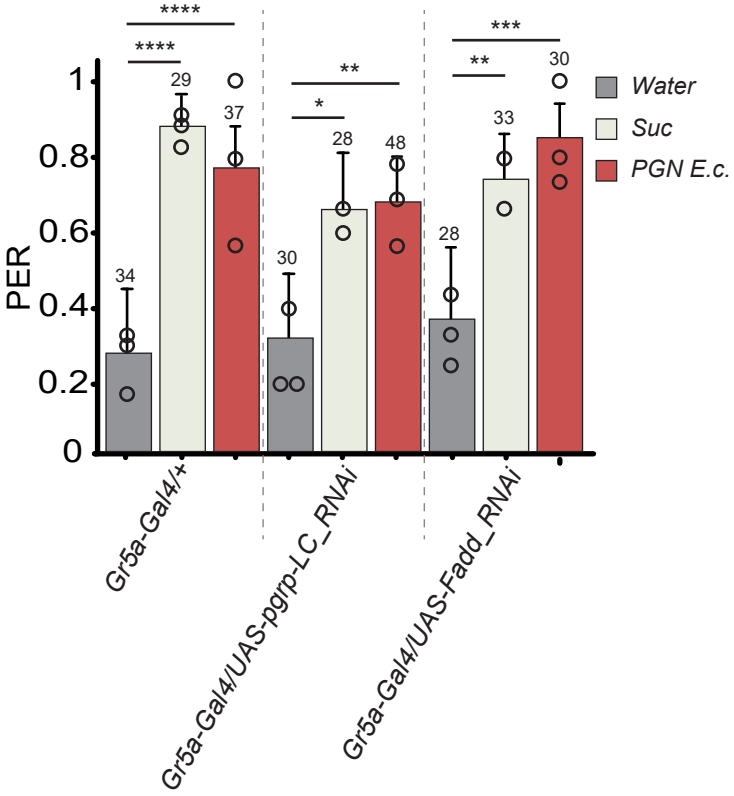

****
****
**
*
***
**
1
0.8
0.6
0.4
0.2
0
PER
34
29
37
30
28
48
28
33
30
Water
Suc
PGN E.c.
Gr5a-Gal4/+
Gr5a-Gal4/UAS-pgrp-LC_RNAi
Gr5a-Gal4/UAS-Fadd_RNAi

Figure 5

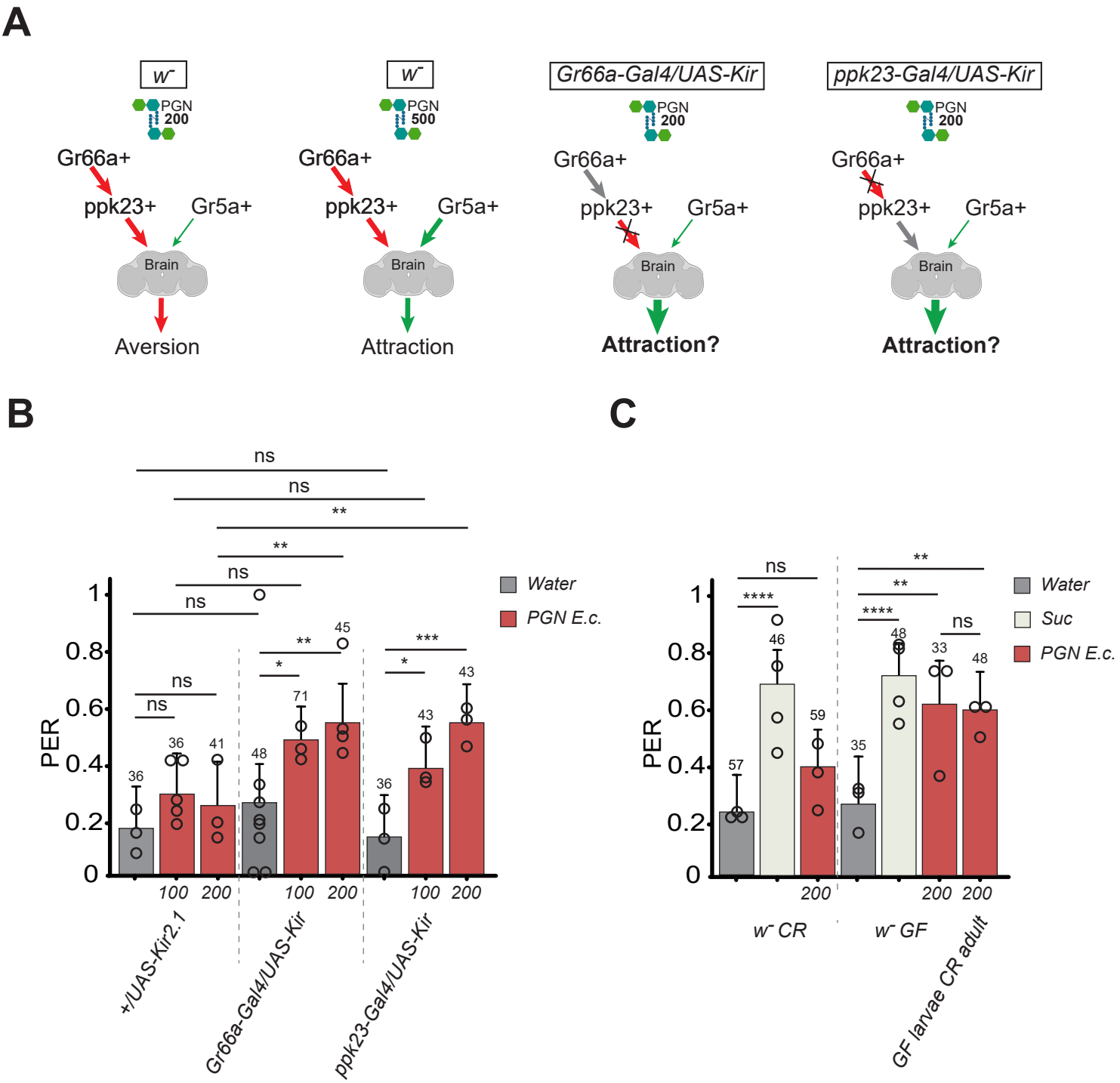
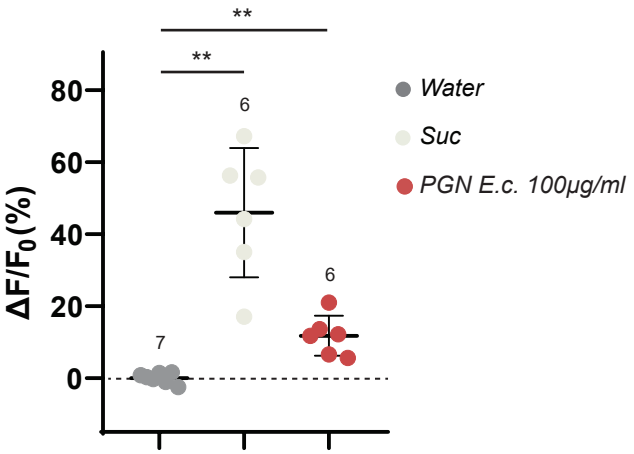

A
w-
PGN 200
Gr66a+
ppk23+
Gr5a+
Brain
Aversion
w-
PGN 500
Gr66a+
ppk23+
Gr5a+
Brain
Attraction
Gr66a-Gal4/UAS-Kir
PGN 200
Gr66a+
ppk23+
Gr5a+
Brain
Attraction?
ppk23-Gal4/UAS-Kir
PGN 200
Gr66a+
ppk23+
Gr5a+
Brain
Attraction?
B
PER
Water
PGN E.c.
+/UAS-Kir2.1
Gr66a-Gal4/UAS-Kir
ppk23-Gal4/UAS-Kir
C
PER
Water
Suc
PGN E.c.
w- CR
w- GF
GF larvae CR adult

Figure S1

Gr64f-Gal4/UAS-GCamp6S