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 components,
58 such as lipopolysaccharides (LPS) and peptidoglycan (PGN), which are well-characterized
59 ligands for receptors expressed by immune cells, might also modulate host neuronal activity.
60 Such molecular dialogue could allow the host to "sample" its surroundings via its sensory
61 system and adopt behaviors that limit contact with pathogenic microbes or, in case of
62 infection, reduce its impact on the host and its offspring (Kobler et al., 2020; Yanagawa et al.,
63 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 sensory
69 system. The detection of LPS by bitter taste 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).

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.

87 **Results**

88 **Bacterial PGN is Attractive to Flies at High Concentrations**

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

98 In the present study, suspecting that bacteria might also be perceived as beneficial by the
99 adult fly, we investigated the potential attractive effect of PGN. To assess whether brief
100 contact with PGN could trigger an attractive response, we focused on the proboscis extension
101 response (PER), part of the sensorimotor taste circuit in adult flies (Figure 1A) (Schwarz et al.,
102 2017; Shiraiwa and Carlson, 2007). To measure the attractiveness of a compound, we tested
103 the molecule without mixing it with anything other than its solvent. When the fly's labellum
104 (analogous to the tongue) is stimulated by a molecule like sucrose, it immediately extends its
105 proboscis towards the food (Video 1). A chemical is considered more or less attractive based
106 on its response relative to the reference solvent and a prototypical attractive substance,
107 sucrose.

108 To determine if PGN could be attractive to flies, we tested this bacterial molecule alone,
109 diluted in water at various concentrations. When the labellum was exposed to 1 mM sucrose,
110 approximately 70% of flies extended their proboscis in an attempt to feed, illustrating the

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

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

159 apparent. These results suggest an integration of positive and negative inputs that influence
160 the fly's decision-making process regarding the taste of different DAP-type PGN
161 concentrations.
162
163 **Gr5a Neurons Are Required in Adults to Detect PGN E.c.500**
164 In a previous study, we showed that Gr66a+ larval neurons are essential for priming the adult
165 behavioral response to DAP-type PGN (Montanari et al., 2024). Despite the fact that Gr5a+
166 neurons do not seem to be present in larvae (Kwon et al., 2011), we wished to test in an
167 unbiased way whether putative larval Gr5a+ cells could influence adult attraction to PGN
168 E.c.500. We used the Gal4/Gal80ts binary system, which allows spatial and temporal control
169 of neuron inactivation (Figure 2C). When 1mM sucrose or PGN E.c.500 solutions were used as
170 stimuli, conditional inactivation of Gr5a+ neurons only in adults was as effective as lifelong
171 inactivation of these neurons. In contrast, inactivating Gr5a+ neurons only during the larval
172 stage had no impact on the fly's ability to respond to sucrose or PGN E.c.500 (Figure 2D).
173 Therefore, adult PER behavior towards PGN E.c.500 requires functional Gr5a+ neurons in the
174 adult stage.
175
176 **DAP-type PGN Directly Activates Gr5a Sweet Neurons**
177 The observation that Gr5a+ neurons are required for the PER response to PGN stimulation led
178 us to investigate whether these cells could be directly activated by DAP-type PGN. Using in
179 vivo GCaMP imaging, we monitored the neuronal activity of Gr5a+ axonal projections in the
180 suboesophageal zone (SEZ) of the brain in Gr5a-Gal4/UAS-GCaMP6s flies, which were exposed
181 to either water (Video 5), sucrose 1mM or (Video 6) or a drop of PGN. Exposure of the labellum
182 of *Gr5a-Gal4/UAS-GCaMP6s* flies to DAP-type PGN at 100µg/ml (Video 7) and 500µg/ml led

183 to an increase in intracellular calcium levels in axonal projections in the SEZ (Figure 3A and
184 3B). These results were confirmed with another pan-sweet taste neuron driver, Gr64f-Gal4
185 (Jiao et al., 2008) (Videos 8-10 and Figure S1). These findings demonstrate that PGN can
186 directly activate sweet taste neurons at high concentrations.
187

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

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

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

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

255 receptors with varying degrees of efficacy (Fioriti et al., 2024; Leulier et al., 2003; Tosoni et al.,
256 2019).
257 What remains to be determined is the minimum structural unit of PGN capable of activating
258 sugar neurons, and whether the level of neuronal activation depends on the specific structure
259 of the muropeptides. Interestingly, the activation we observed appears to be independent of
260 the IMD pathway, as the proboscis extension responses (PER) to PGN were similar in both
261 control flies and those mutant for certain components of the IMD/NF-kB pathway. This
262 response contrasts with previous findings where PGN, at concentrations near 200 µg/ml, was
263 shown to activate bitter Gr66a neurons via part of the IMD pathway, though not involving the
264 Relish transcription factor (Masuzzo et al., 2022). It is also worth noting that the ability to
265 perceive PGN as a bitter molecule has only been observed with DAP-type PGNs, which are
266 typically found in Gram-negative bacteria, making this response specific to a subset of
267 bacterial species, unlike the response observed in our study.
268 In contrast, the sweet neuron response we report here more closely resembles the response
269 observed in ppk23+ neurons, which is also independent of the IMD pathway (Montanari et al.,
270 2024). However, there is a notable difference between the attractive response mediated by
271 Gr5a+ neurons and the aversive response mediated by ppk23+ neurons. The aversive
272 response to PGN requires bacterial priming during the larval phase and is absent in axenic
273 flies, while the Gr5a+ neuron-mediated attraction to PGN occurs even in flies reared in the
274 complete absence of bacteria. These findings suggest that the detection and response to PGN
275 by Gr5a+ neurons in adults are not dependent on prior exposure to bacteria.
276 By measuring PERs in flies with blocked bitter or sweet circuits, we show that the fly's taste
277 system integrates signals from at least three distinct types of neurons—Gr66a+, ppk23+, and
278 Gr5a+—to determine how to respond to PGN. Notably, the activation of these neurons is not

279 triggered by the same concentrations of PGN. Similar dose-dependent behavioral responses
280 have been described for other compounds. For example, NaCl is attractive at 50 mM through
281 the Gr5a+ network, but aversive at 250 mM via ppk23+ neurons (Zhang et al., 2013; Jaeger et
282 al., 2018; McDowell et al., 2022). Likewise, hexanoic acid is attractive at low concentrations
283 via Gr5a+ cells, but aversive at higher concentrations through the bitter network (Pradhan et
284 al., 2023). These examples support the idea that different concentrations of the same
285 compound can trigger opposite behaviors, depending on the involved neuronal circuits.
286 Some studies suggest a more complex scenario, where a molecule can be attractive as an odor
287 but aversive as a tastant. A prime example of this is acetic acid, which is attractive when
288 detected by the gustatory system but repulsive when sensed by the olfactory system (Devineni
289 et al., 2019; Joseph et al., 2009). Additionally, systems that integrate opposite inputs from
290 both sweet and bitter signals have been identified, such as the inhibition of sweet neurons by
291 bitter neurons through GABA signaling (Chu et al., 2014) or by the OBP49a protein, which
292 attenuates sweet neuron firing when bitter chemicals are combined (Jeong et al., 2013). These
293 mechanisms help facilitate the avoidance of harmful compounds when sweet molecules are
294 present in mixtures. In our case, the concentration that triggers aversion (200 µg/ml) is lower
295 than the one that triggers attraction (500 µg/ml), suggesting a unique pattern of integration
296 for PGN.
297 Our findings also highlight the fact that PGN concentrations at 100 µg/ml can activate Gr5a+
298 neurons and, in the absence of the aversive response (i.e., in Gr66a-Gal4/UAS-Kir2.1 flies),
299 trigger attraction. This suggests that sweet neurons are tuned to respond to PGN
300 concentrations lower than those that elicit aversion, and that the integration of bitter signals
301 can override the attraction. In a more complex, physiological context where both sweet and

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

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422

423 **Material and methods**

424 **Fly husbandry**

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

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

435

436 **Fly stocks**

437 The reference strain in this study is noted w^{-} and corresponds to $w^{[1118]}$ (Bloomington #5905).
438 Canton S (Bloomington #64349) and $y^{[1]}$, $w^{[1118]}$ and *Gr5A*$^{-}$; *Gr64a-f*$^{-}$ (gift from Bernard
439 Charroux) and *Gr5a-Gal4* (Bloomington #57992) and *Gr64f-Gal4* (Bloomington #57669) and
440 *UAS-GCaMP6S* (Bloomington #42746) and *UAS-Kir2.1* (Bloomington #6595) and *Tub-Gal80ts*
441 (Bloomington #7016) and *UAS-GFP* (Bloomington #32195) and *UAS-pgrp-LC_RNAi* (VDRC
442 #101636) and *UAS-Fadd_RNAi* (Leulier et al., 2002) and *ppk23-Gal4* (Bloomington #93026) and
443 *Gr66a-Gal4* (Bloomington #28801).

444

445 **PER assay**

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

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

470 and the timing in the table below.

471 **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

472

473

474

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

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

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

478 proboscis and clean it.

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

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

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

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

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

484

485

486 **Microscopy**

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

491

492 ***In vivo* calcium imaging**

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

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

534

535 **Statistics and data representation**

536 The Prism software was used for statistical analyses.

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

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

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

550

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

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

553 DOI: 10.6084/m9.figshare.27908889

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

555

556 **Figure Legends**

557

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

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

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

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

570 (D) Raising medium has no effect on attraction to PGN. PER index of w- flies raised on protein-
571 enriched (prot+) or sucrose-enriched (suc+) media, to control solutions of water, 1mM
572 sucrose, and PGN from *E. coli* K12 at 500µg/mL. Data for w- prot+ flies are the same as in panel
573 1A.

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

584

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

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

588 emanating from neurons in the proboscis. D, V, P, and A stand for Dorsal, Ventral, Posterior,
589 and Anterior.
590 (B) Impairing the activity of Gr5a+ neurons via UAS-Kir2.1 eliminates the attraction to PGN.
591 PER index of flies to control solutions of water, 1mM sucrose, and PGN from *E. coli* at
592 500µg/mL.
593 (C) Graph showing the life periods during which flies are shifted from 18°C (green) to 29°C
594 (red).
595 (D) Gr5a+ neurons are functionally required for PGN attraction in adults but are not needed
596 during the larval stage. PER index of flies to 1mM sucrose and PGN from *E. coli* K12 at
597 500µg/mL. The ubiquitously expressed Tub-Gal80ts inhibits the activity of Gal4 at 18°C and is
598 inactivated at 29°C, allowing the expression of UAS-Kir2.1 and the inhibition of Gr5a+ neuron
599 activity. The PER index is calculated as the percentage of flies responding with a PER to the
600 stimulus ± 95% CI. The number of flies tested (n) is shown above each bar. "ns" indicates
601 $p>0.05$, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates
602 $p<0.0001$, Fisher Exact Test.
603 Further details on conditions and statistics are provided in the figure section.
604
605 **Figure 3 Real-time calcium imaging with GCaMP6s reflects the *in vivo* neuronal activity of**
606 **Gr5a+ neurons**
607 (A) Representative images showing the GCaMP6s intensity before and after the addition of
608 control water, 100mM sucrose, or PGN from *E. coli* K12 at 100µg/mL. Scale bar is 10µm.
609 (B) Averaged fluorescence intensity of negative peaks ± SEM for Gr5a+ neurons in response
610 to water (n=7), 100mM sucrose (n=16), or PGN from *E. coli* K12 at 100µg/mL (n=7) or
611 500µg/mL (n=7). In (B), ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$,
612 non-parametric t-test, Mann-Whitney test.
613 Further details on conditions and statistics can be found in the figure section.
614
615
616 **Figure 4 Sugar receptors are involved in PGN attraction**
617 (A) PER index of mutant flies lacking the Gr5a receptor as well as the Gr64a-f family to water,
618 1mM sucrose, and PGN from *E. coli* K12 at 500µg/mL, compared to w- flies. Data for w- flies
619 are the same as in Fig. 1A.

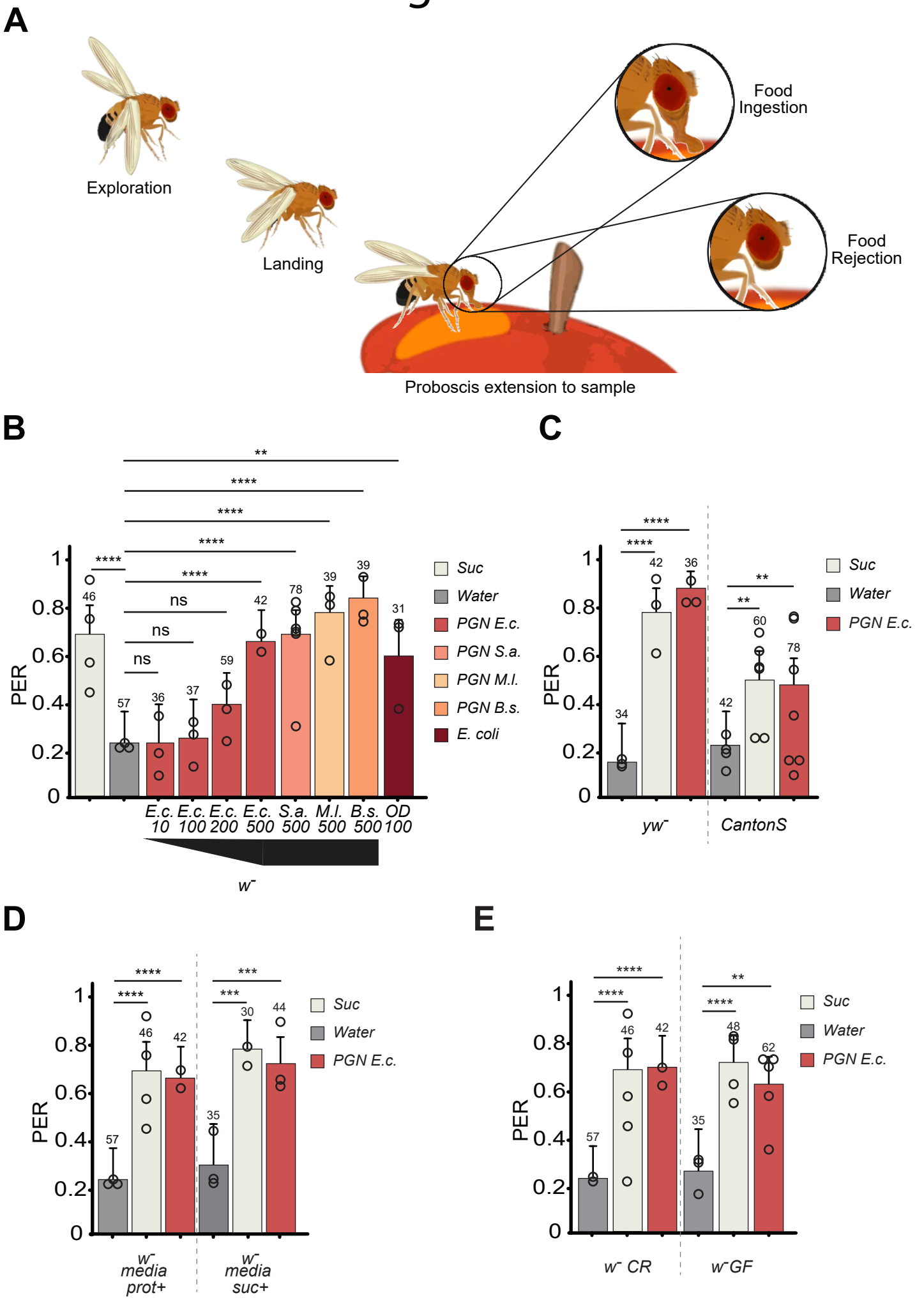
620 (B) Diagram of the IMD pathway.
621 (C) RNAi-mediated inactivation of PGRP-LC (UAS-pgrp-LC RNAi) or Fadd (UAS-Fadd RNAi) in
622 Gr5a+ cells does not affect attraction to PGN. PER index of flies to water, 1mM sucrose, and
623 PGN from *E. coli* K12 at 500µg/mL. For (A) and (C), the PER index is calculated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

650 **Figure S1 Real-time calcium imaging with GCaMP6s reflects the *in vivo* neuronal activity of**
651 **Gr64f+ neurons**
652 Averaged fluorescence intensity of negative peaks ± SEM for Gr64f+ neurons in response to
653 water (n=7), 100mM sucrose (n=6), or PGN from *E. coli* K12 at 100µg/mL (n=6). ** indicates
654 p<0.01, *** indicates p<0.001, **** indicates p<0.0001, non-parametric t-test, Mann-
655 Whitney test. Further details on conditions and statistics can be found in the figure section.
656
657 **Video 1 PER is triggered following exposure to sucrose 1mM**
658 **DOI: 10.6084/m9.figshare.27929532**
659 **https://figshare.com/articles/media/Video_1_PER_suc_1mM/27929532?file=50876001**
660 Response following proboscis contact with a stripe of paper soaked in a 1mM solution (water
661 as solvent). 7-days old females starved 15h prior to the assay.
662
663 **Video 2 PER is not triggered following exposure to water**
664 **DOI: 10.6084/m9.figshare.27929517**
665 **https://figshare.com/articles/media/Video_2_PER_water/27929517?file=50875968**
666 Absence of response following proboscis contact with a stripe of paper soaked in water. 7-
667 days old females starved 15h prior to the assay.
668
669 **Video 3 PER is triggered following exposure to PGN of *E. coli* at 500µg/ml**
670 **DOI: 10.6084/m9.figshare.27929547**
671 **https://figshare.com/articles/media/Video_3_PER_PGN500/27929547?file=50876025**
672 Response following proboscis contact with a stripe of paper soaked in an *E. coli* PGN solution
673 at 500µg/ml (water as solvent). 7-days old females starved 15h prior to the assay.
674
675 **Video 4 Comparison of Behavioral Response to Water and E. coli PGN in the Same Fly**
676 **DOI: 10.6084/m9.figshare.27929553**
677 **https://figshare.com/articles/media/Video_4_water_then_PGN500/27929553?file=50876**
678 **226**
679 Using the same fly, this experiment compares the absence of response to a proboscis
680 contact with a paper strip soaked in water, versus a clear response to a paper strip soaked in

681 an *E. coli* PGN solution (500 µg/ml, with water as the solvent). The test was conducted on 7-
682 day-old females, starved for 15 hours prior to the assay.
683
684 **Video 5 Gr5a+ neurons do not respond to water**
685 **DOI: 10.6084/m9.figshare.27908901**
686 **https://figshare.com/articles/media/Video_5_Gr5a_GCaMP6s_vs_water/27908901?file=5**
687 **0814009**
688 Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s
689 under the control of the Gr5a-Gal4 driver to assess the in vivo neuronal activity of labellar
690 Gr5a+ neurons following exposure to water. The water was added at t+ 2 seconds.
691
692 **Video 6 Gr5a+ neurons respond to sucrose**
693 **DOI: 10.6084/m9.figshare.27908928**
694 **https://figshare.com/articles/media/Video_6_Gr5a_GCaMP6s_vs_sucrose_100mM/27908**
695 **928?file=50814042**
696 Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s
697 under the control of the Gr5a-Gal4 driver to assess the in vivo neuronal activity of labellar
698 Gr5a+ neurons following exposure to sucrose 100mM. The sucrose was added at t+2 seconds.
699
700 **Video 7 Gr5a+ neurons respond to DAP-type peptidoglycan**
701 **DOI: 10.6084/m9.figshare.27908949**
702 **https://figshare.com/articles/media/Video_7_Gr5a_GCaMP6s_vs_PGN_100/27908949?fil**
703 **e=50814069**
704 Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s
705 under the control of the Gr5a-Gal4 driver to assess the in vivo neuronal activity of labellar
706 Gr5a+ neurons following exposure to *E. coli* PGN solution at 100µg/mL. The PGN was added
707 at t+2 seconds.
708
709 **Video 8 Gr64f+ neurons do not respond to water**
710 **DOI: 10.6084/m9.figshare.27908979**
711 **https://figshare.com/articles/media/Video_8_Gr64f_GCaMP6s_vs_water/27908979?file=**
712 **50814099**

713 Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s
714 under the control of the Gr64f-Gal4 driver to assess the in vivo neuronal activity of labellar
715 Gr64f+ neurons following exposure to water. The water was added at t+2 seconds.
716
717 **Video 9 Gr64f+ neurons respond to sucrose**
718 **DOI: 10.6084/m9.figshare.27908991**
719 **https://figshare.com/articles/media/Video_9_Gr64f_GCaMP6s_vs_Sucrose_100mM/2790**
720 **8991?file=50814126**
721 Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s
722 under the control of the Gr64f-Gal4 driver to assess the in vivo neuronal activity of labellar
723 Gr64f+ neurons following exposure to sucrose 100mM. The sucrose was added at t+2 seconds.
724
725 **Video 10 Gr64f+ neurons respond to DAP-type peptidoglycan**
726 **DOI: 10.6084/m9.figshare.27909015**
727 **https://figshare.com/articles/media/Video_10_Gr64f_GCaMP6s_vs_PGN_100/27909015?f**
728 **ile=50814165**
729 Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s
730 under the control of the Gr64f-Gal4 driver to assess the in vivo neuronal activity of labellar
731 Gr64f+ neurons following exposure to *E. coli* PGN solution at 100µg/mL. The PGN was added
732 at t+2 seconds.
733
734
735
736

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-
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D
w- media prot+
w- media suc+
E
w- CR
w-GF

Figure 2

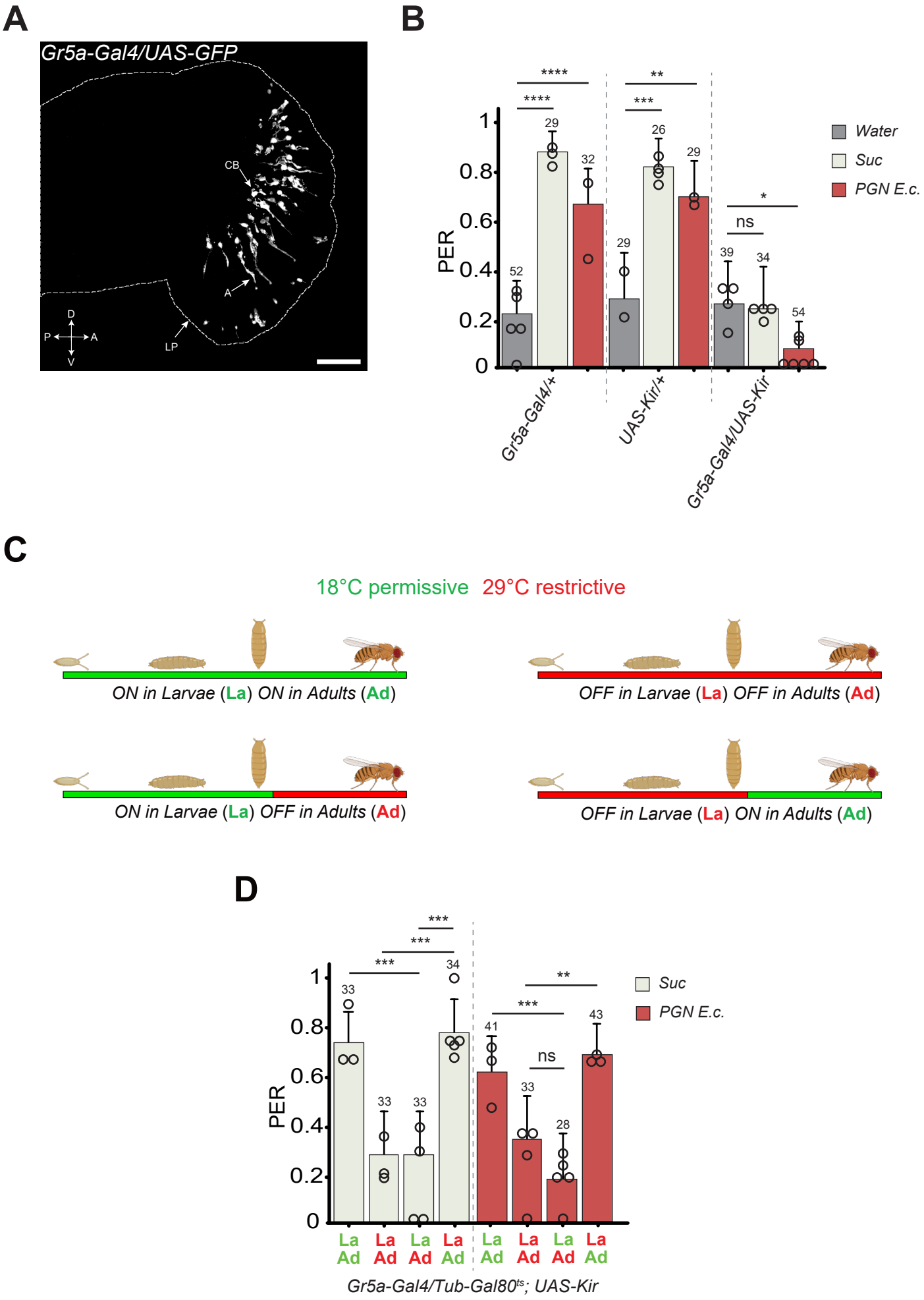
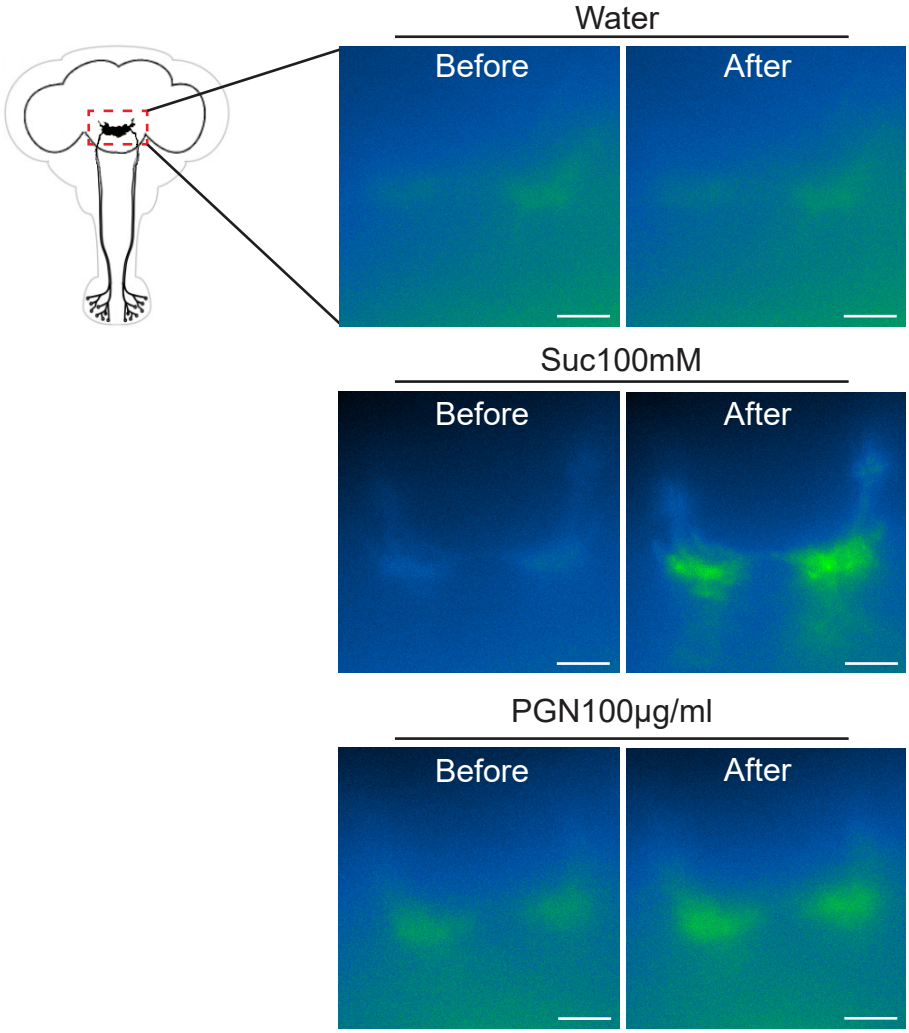

A
Gr5a-Gal4/UAS-GFP
CB
A
LP
D
P
A
V
B
PER
Water
Suc
PGN E.c.
Gr5a-Gal4/+
UAS-Kir/+
Gr5a-Gal4/UAS-Kir
ns
C
18°C permissive 29°C restrictive
ON in Larvae (La) ON in Adults (Ad)
OFF in Larvae (La) OFF in Adults (Ad)
ON in Larvae (La) OFF in Adults (Ad)
OFF in Larvae (La) ON in Adults (Ad)
D
PER
Suc
PGN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

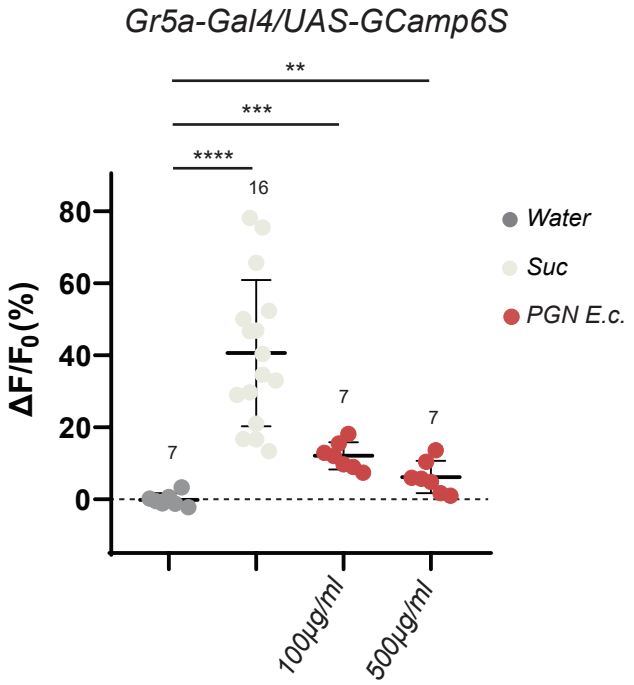
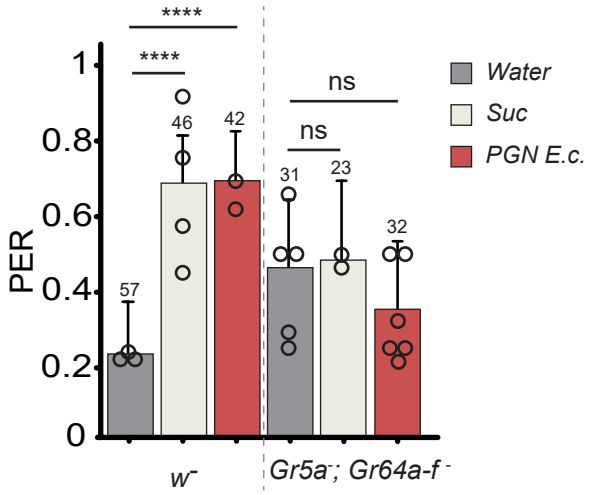
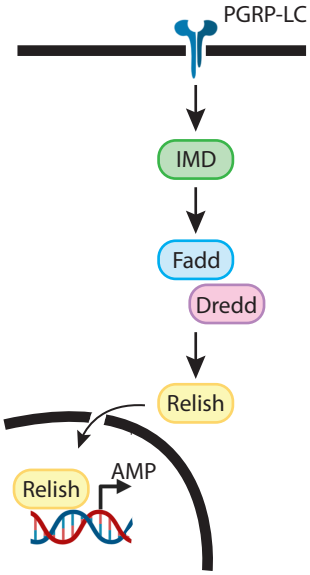

Gr5a-Gal4/UAS-GCamp6S
**
***
****
16
7
7
7
80
60
40
20
0
ΔF/F0(%)
Water
Suc
PGN E.c.
100µg/ml
500µg/ml

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

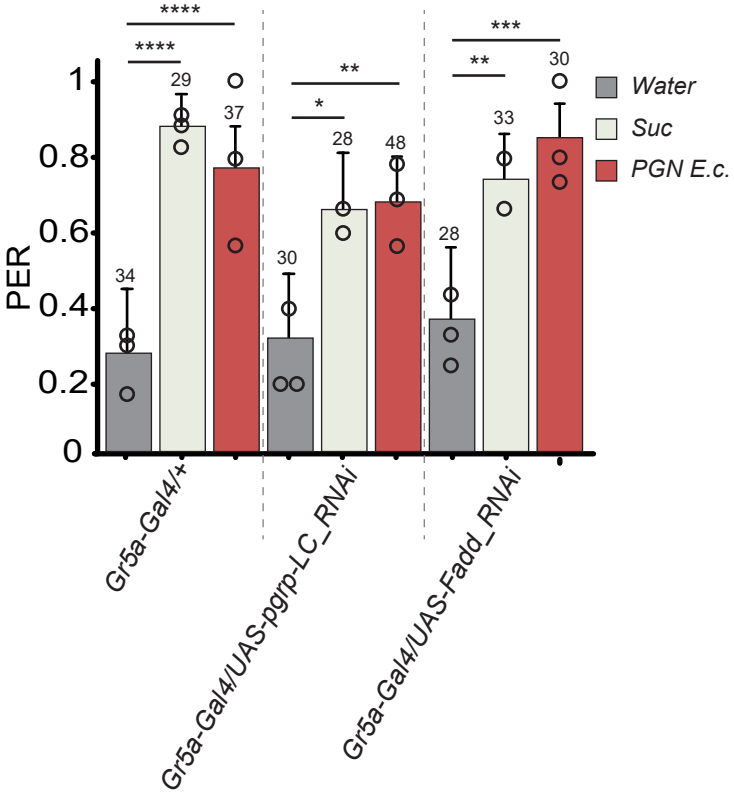

****
****
**
*
***
**
1
0.8
0.6
0.4
0.2
0
PER
34
29
37
30
28
48
28
33
30
Water
Suc
PGN E.c.
Gr5a-Gal4/+
Gr5a-Gal4/UAS-pgrp-LC_RNAi
Gr5a-Gal4/UAS-Fadd_RNAi

Figure 5

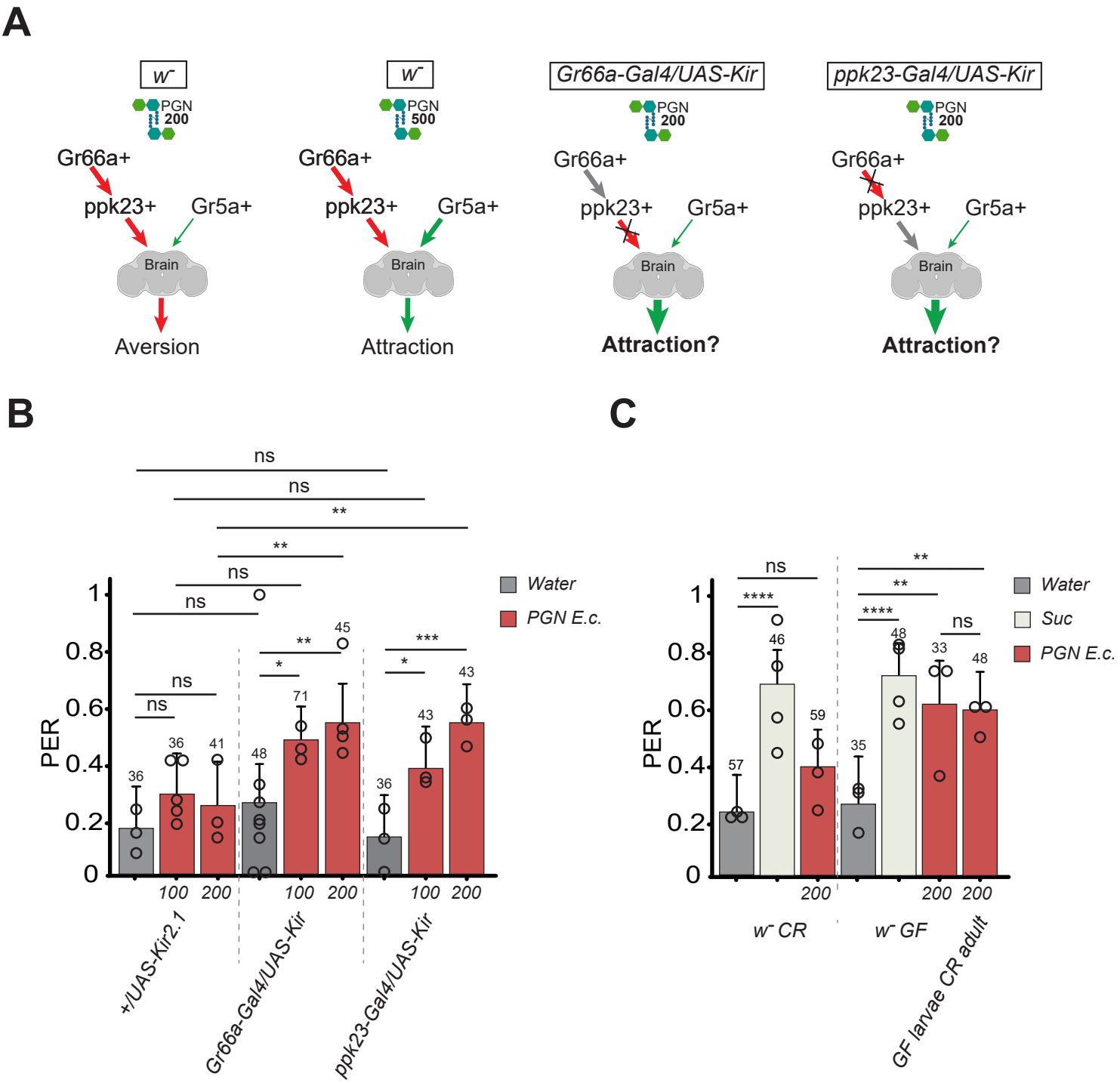
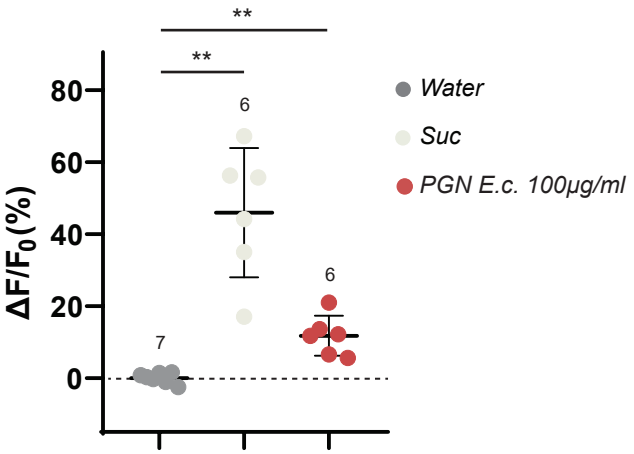

A
w-
PGN 200
Gr66a+
ppk23+
Gr5a+
Brain
Aversion
w-
PGN 500
Gr66a+
ppk23+
Gr5a+
Brain
Attraction
Gr66a-Gal4/UAS-Kir
PGN 200
Gr66a+
ppk23+
Gr5a+
Brain
Attraction?
ppk23-Gal4/UAS-Kir
PGN 200
Gr66a+
ppk23+
Gr5a+
Brain
Attraction?
B
PER
Water
PGN E.c.
+/UAS-Kir2.1
Gr66a-Gal4/UAS-Kir
ppk23-Gal4/UAS-Kir
C
PER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

Figure S1

Gr64f-Gal4/UAS-GCamp6S