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3 **A subset of sweet-sensing neurons identified by IR56d are necessary and**
4 **sufficient for fatty acid taste**

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1 **Abstract**

2 Fat represents a calorically potent food source that yields approximately twice the
3 amount of energy as carbohydrates or proteins per unit of mass. The highly
4 palatable taste of FAs promotes food consumption, activates reward circuitry, and is
5 thought to contribute to hedonic feeding underlying many metabolism-related
6 disorders. Despite a role in the etiology of metabolic diseases, little is known about
7 how dietary fats are detected by the gustatory system to promote feeding.
8 Previously, we showed that a broad population of sugar-sensitive taste neurons
9 expressing Gustatory Receptor 64f (*Gr64f*), is required for reflexive feeding
10 responses to both FAs and sugars. Here, we report a genetic silencing screen to
11 identify specific populations of taste neurons that mediate fatty acid taste. We find
12 neurons identified by expression of Ionotropic Receptor 56d (*IR56d*) are necessary
13 and sufficient for reflexive feeding response to FAs. Functional imaging reveals that
14 *IR56d*-expressing neurons are responsive to short- and medium-chain FAs. Silencing
15 *IR56d* neurons selectively abolishes fatty acid taste, while their activation is
16 sufficient to drive feeding responses. Analysis of co-expression with *Gr64f* identifies
17 two subpopulations of *IR56d*-expressing neurons. While physiological imaging
18 reveals that both populations are responsive to FAs, *IR56d/Gr64f* neurons are
19 activated by medium-chain FAs and are sufficient for reflexive feeding responses to
20 FA. Moreover, flies can discriminate between sugar and FAs in an aversive taste
21 memory assay, indicating that FA taste is a unique modality in *Drosophila*. Taken
22 together, these findings localize fatty acid taste within the *Drosophila* gustatory center
23 and provide an opportunity to investigate discrimination between different categories of
24 appetitive tastants.

25

1 **Authors Summary**

2 Fat represents a calorically potent food source that yields approximately twice the
3 amount of energy as carbohydrates or proteins per unit of mass. Dietary lipids are
4 comprised of both triacylglycerides and FAs, and growing evidence suggests that it
5 is the free FAs that are detected by the gustatory system. The highly palatable taste
6 of FAs promotes food consumption, activates reward centers in mammals, and is
7 thought to contribute to hedonic feeding that underlies many metabolism-related
8 disorders. Despite a role in the etiology of metabolic diseases, little is known about
9 how dietary fats are detected by the gustatory system to promote feeding. We have
10 identified a subset of sugar-sensing neurons in the fly that also respond to medium-
11 chain FAs and are necessary and sufficient for behavioral responses to FAs. Further,
12 we find that despite being sensed by shared neuron populations, flies can
13 differentiate between the taste of sugar and FAs, fortifying the notion that FAs and
14 sugar represent distinct taste modalities in flies.

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2 **Introduction**

3 Fat represents a calorically potent food source that yields approximately twice the
4 amount of energy as carbohydrates or proteins per unit of mass. In mammals, dietary
5 lipids are detected by taste cells, mechanosensory and olfactory neurons, as well as by
6 post-ingestive feedback [1–4]. Dietary lipids are comprised of triacylglycerides and FAs
7 (FAs), and growing evidence suggests that it is the free FAs that are detected by the
8 gustatory system [5–7]. Sensing of FAs promotes food consumption, activates reward
9 circuitry, and is thought to contribute to hedonic feeding that underlies many metabolism-
10 related disorders [8,9]. Despite a role in the etiology of metabolic diseases, little is known
11 about how dietary fats are detected by the gustatory system to promote feeding.

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13 In flies and mammals, tastants are sensed by dedicated gustatory receptors that localize
14 to the taste cells or taste receptor neurons [10–12]. These cells are sensitive to different
15 taste modalities such as sweet, bitter, salty, sour or umami, and project to higher order
16 brain structures for processing [10,13,14]. While these taste modalities have been
17 extensively studied, much less is known about how FAs are detected and how
18 information about this sensory stimulus is processed. Taste neurons in *Drosophila* are
19 housed in gustatory sensilla located on the tarsi (feet), proboscis (mouth) and wings.
20 Each sensillum contains dendrites of up to four gustatory receptor neurons (GRNs),
21 which are activated by different categories of tastants [16]. Two main classes of neurons
22 include one group that senses sweet tastants and promotes feeding, and a second, non-
23 overlapping group that senses bitter tastants and promotes food avoidance [17,18]. We
24 have previously shown that *Drosophila* is attracted to medium-chain FAs [19].
25 Consumption of FAs relies on taste, rather than smell, as it is not impaired by surgical

1 ablation of olfactory organs [19]. Additionally, FA consumption is abolished in *pox-neuro*
2 mutants in which all external taste hairs are converted to mechanosensory bristles,
3 indicating that the chemical signature rather than oily texture of FAs is associated with
4 perception [19]. Silencing *Gr64f* neurons, which are required for sugar sensing [20,21],
5 abolishes behavioral response to FAs, suggesting that shared populations of gustatory
6 neurons detect FAs and sugars [19]. Unlike sugars, FAs sensing is dependent on
7 functional Phospholipase C (PLC) indicating independent sensory pathways for FA and
8 sugar perceptions [18]. However, any further characteristics of the physiological
9 response or the specific neuronal identity of the neurons mediating FA response are
10 unknown.

11 Taste neurons from the legs and proboscis predominantly project to the subesophageal
12 zone (SEZ), the primary taste center of the *Drosophila* central nervous system, but the
13 downstream central brain circuitry contributing to taste processing is less well
14 understood [22–25]. Determining how diverse tastants activate GRNs that convey
15 information to the SEZ, and how this information is represented in higher order brain
16 centers, is central to understanding the neural basis for taste processing and feeding
17 behavior. Identifying the neural principles underlying FA taste processing requires
18 identifying the precise populations of taste neurons responsive to FAs, and their
19 innervation of the primary taste center. Recent studies in *Drosophila* have identified taste
20 neurons that are responsive to diverse modalities including salt, sugar, amino acids,
21 water, carbonation, bitter, polyamines, and electrophilic tastants [17,26–32], yet little is
22 known about the populations underlying FA taste. Here, we show that the IR56d
23 population of gustatory neurons, which partially overlaps with *Gr64f* neurons, is
24 necessary and sufficient to produce a feeding response to FAs. The IR56d-*Gr64f*
25 population of neurons is selectively responsive to medium-short-chain FAs. Our results

- 1 reveal a defined channel that senses FAs to promote food consumption, providing a
- 2 mechanism for differentiation between attractive tastants of different modalities.
- 3

1 **Results**

2 **Hexanoic acid elicits calcium activity in Gr64f sensory neurons**

3 We previously reported that silencing the Gr64f population of primary taste neurons
4 abolishes behavioral response to both sugars and FAs [10]. To investigate the
5 responsiveness of these neurons to FAs directly, the Ca²⁺ sensor GCaMP5 was
6 expressed under control of Gr64f-GAL4 [33,34] (Fig. 1A). The Ca²⁺ response to
7 proboscis application of water, sucrose, or the medium-chain fatty acid hexanoic acid
8 (HxA) was monitored *in vivo* in the axonal projections of Gr64f neurons within the SEZ
9 (Fig. 1B). Flies were provided either 10mM sucrose or 1% HxA, as these concentrations
10 induce comparable levels of proboscis extension reflex behavior. Both 10mM sucrose
11 and 1% HxA induced robust activity in the SEZ, while little response was observed to
12 water alone (Fig. 1C-F). The temporal dynamics of Ca²⁺ activity differed between the two
13 tastants, with HxA eliciting a broader response (Fig. 1E, F), yet both elicited comparable
14 peak changes in fluorescence (Fig 1C). Therefore, both sugars and FAs activate Gr64f
15 sweet-sensing GRNs, fortifying the notion that this neuronal class is generally
16 responsive to attractive tastants.

17 To localize FA-sensitive neurons within the broad Gr64f population, we selectively
18 silenced populations of taste neurons predicted to overlap with Gr64f neurons and
19 examined the proboscis extension reflex (PER) in response to sucrose and HxA. The
20 synaptobrevin cleavage peptide Tetanus Toxin-Light Chain (TNT) was expressed under
21 the control of Gustatory Receptor or Ionotropic Receptor promoters known to overlap
22 with Gr64f (Table S1) [20,30,35,36]. Of the 10 GAL4 lines tested, silencing with GAL4
23 drivers for Gr61a, IR56b, and Gr64f resulted in PER defects to sucrose and HxA, By
24 contrast, silencing Gr64e neurons significantly reduced response to sucrose without

1 affecting response to HxA, and silencing Gr5a, Gr43a, or IR56d neurons significantly
2 reduced PER to HxA without affecting sucrose response.

3 We chose to further investigate the role of IR56d neurons in FA sensing, since IRs have
4 been found to be involved in detection of non-sweet appetitive tastants, including salt
5 and amino acids [28,37,38]. *IR56d* neurons have previously been reported to project to
6 two distinct regions in the SEZ. One region overlaps with sweet-sensing projections, and
7 the other is indicated to originate in the taste pegs of the proboscis [36]. Outside of
8 sensing carbonation, little is known about ligands that activate the taste pegs or the role
9 they play in the *Drosophila* gustatory system. The sweet-sensing projections of IR56d
10 resembled the region of Gr64f projections that was activated by HxA. Consistent with
11 previous reports, expression of GFP in IR56d neurons (*IR56d-GAL4>cd8::GFP*)
12 revealed two populations of projections: one innervating the anterior SEZ and previously
13 defined as emanating from labellar bristles, and a second population, emanating from
14 the taste pegs, innervating the posterior SEZ (Fig 2A-C) [36]. To further analyze the role
15 of IR56d neurons, we silenced them using TNT and tested PER upon application of
16 tastants to the proboscis for consistency with live imaging experiments. To control for
17 genetic background and any potential non-specific effects of TNT, we compared PER in
18 flies with silenced IR56d neurons (*IR56d-GAL4>UAS-TNT*) to flies expressing an
19 inactive variant of TNT (*IR56d-GAL4>imp-TNT*) [35]. Expression of *imp-TNT* in IR56d
20 neurons did not affect PER in response to sucrose or HxA, while expression of TNT
21 selectively inhibited HxA response (Fig 2D). Therefore, IR56d neurons are required for
22 behavioral response to HxA, but dispensable for response to sucrose.

23 Broad activation of sweet-sensing neurons expressing the trehalose receptor Gr5a
24 induces feeding response in the absence of tastants [17,39,40]. To determine whether
25 activation of IR56d neurons is sufficient to induce PER, we selectively expressed the

1 thermo-sensitive cation channel *transient receptor potential* A1 (TRPA1) in IR56d
2 neurons, or Gr5a neurons as a positive control, and measured heat-induced PER [41,42].
3 TRPA1 expression induces neuronal activity at temperatures above 28°C, but has little
4 effect on neuronal activity in flies at 22°C, allowing for thermogenetic modulation of
5 neuronal activity [41,42]. In agreement with previous findings, broad activation of sweet-
6 sensing neurons induced robust PER (Fig 2F) [27,43,44]. Similarly, selective activation
7 of IR56d neurons robustly induced PER as compared to control flies harboring *UAS-*
8 *TRPA1* or *IR56d-GAL4* alone (Fig 2F). Therefore, activation of IR56d neurons alone is
9 sufficient to induce PER.

10 IR56d neurons project to both taste peg and sweet-sensing regions of the SEZ, and
11 each region can be distinguished anatomically (Fig 2A-C; [36]). To determine whether
12 sugars and FAs can differentially activate the two regions, we expressed GCaMP5 in
13 IR56d neurons (*IR56d-GAL4>GCaMP5*) and measured tastant-evoked activity
14 separately within the areas of anterior and posterior projections. The anterior region,
15 which overlaps with Gr64f neurons, responded to both HxA and sucrose with similar
16 magnitude (Fig 3A-B). The projections from the taste pegs, however, showed a strong
17 response to HxA, but did not respond to sucrose (Fig 3C-D). Therefore, while IR56d
18 neurons that project posteriorly are responsive to both sucrose and HxA, those
19 originating in taste pegs are selectively responsive to HxA, revealing two distinct
20 functional classes of IR56d taste neurons.

21 Flies exhibit feeding responses upon presentation of several FA classes [19]. It is
22 possible that the IR56d neurons are broadly responsive to different classes of FAs.
23 Alternatively, different classes of FAs may be sensed by distinct, or partially overlapping,
24 populations of sensory neurons. To test the responsiveness of IR56d neurons to
25 different classes of FAs, we first compared behavioral responses of IR56d-silenced flies

1 to short-chain pentanoic acid (5-carbon), medium-chain octanoic acid (8 carbon), and
2 long chain oleic acid (18-carbon). As compared to control IR56d>imp-TNT flies, IR56d-
3 silenced flies exhibited weaker responses to octanoic acid, but retained robust PER to
4 pentanoic acid (Fig 4A), suggesting that PER to short chain FAs is not dependent on
5 IR56d neurons. Oleic acid did not elicit strong PER in either. We directly examined
6 activity induced by different FAs in IR56d neurons with *in vivo* Ca²⁺ imaging in IR56d-
7 GAL4>GCaMP5 flies. Octanoic acid robustly activated posterior IR56d projections, while
8 both pentanoic and octanoic acid activated anterior IR56d projections (Fig 4B,C). Oleic
9 acid, which did not induce PER, also did not activate IR56d projections in either posterior
10 or anterior regions (Fig 4B,C). Together, these findings show that IR56d neurons
11 respond to short and medium chain FAs, and further, that sub-populations of IR56d
12 neurons that are separately represented in anterior and posterior locations in the SEZ
13 have distinct FA response profiles.

14 Since silencing of either IR56d or Gr64f neurons abolishes PER to hexanoic and
15 octanoic acid, one possibility is that neurons co-expressing both IR56d and Gr64f are
16 required for medium-chain FA response (Fig 5A). To validate co-expression of IR56d
17 and Gr64f, we used the LexA system to label Gr64f neurons (Gr64f-LexA>LexOp-
18 CD8:GFP) and the GAL4 system to label IR56d neurons (IR56d-GAL4>UAS-RFP) (Fig
19 5A). Examining SEZ projections revealed co-localization within the posterior SEZ, with
20 no co-localization detected in the anterior SEZ, suggesting the posterior IR56d neurons
21 co-express Gr64f and IR56d. To determine whether the IR56d+/Gr64f+ co-expressing
22 neurons are required for FA taste, we used intersectional strategies to selectively
23 silenced anterior IR56d neurons of the taste pegs. Specifically, we repressed TNT
24 expression in IR56d+/Gr64f+ using Gr64f-LexA to drive expression of the GAL80
25 repressor (IR56d-GAL4>UAS-TNT; Gr64f-LexA>LexAop-GAL80) (Fig 5B). Selectively

1 silencing IR56d neurons that do not overlap with Gr64f did not affect PER to HxA, or as
2 expected, to sugar, and no difference was observed between flies expressing imp-TNT
3 and TNT, suggesting the taste peg neurons are dispensable for the reflexive feeding
4 response to FAs (Fig 5B). Flies lacking Gr64f-LexA, but still harboring a copy of
5 LexAop-GAL80 (IR56D-GAL4-TNT; LexAop-GAL80/+), showed reduced PER as
6 expected (Fig S1).

7 Although both sugars and FAs induce feeding behavior, it is unclear whether flies can
8 qualitatively differentiate between these two classes of appetitive tastants. We have
9 developed an assay in which an appetitive tastant is applied to the tarsi, paired with
10 bitter quinine application to the proboscis, and the suppression of PER in subsequent
11 response to the appetitive tastant is measured [40,45]. To determine whether flies can
12 differentiate between sugars and FAs, we applied sucrose (conditioned stimulus) to the
13 tarsi followed immediately with quinine application (unconditioned stimulus) to the
14 proboscis. Following three training trials, memory was tested by application of sucrose
15 (control) or HxA to the tarsi, in the absence of quinine, and measuring PER (Fig 6B). We
16 also performed reciprocal experiments in which flies were trained with HxA and tested
17 for PER to sucrose or HxA.

18 As previously reported, pairing sugar with quinine significantly reduced PER over the
19 course of three training trials and this memory persisted in the absence of quinine
20 ([46,47]; Fig 6B). Further, there were no differences in PER to HxA between flies
21 repeatedly provided sucrose in the absence of quinine and flies trained with sucrose-
22 quinine pairing, indicating that the aversive taste memory formed to sucrose is not
23 generalized to HxA. Conversely, the pairing of HxA and quinine resulted in a robust PER
24 reduction that was not generalized to sucrose (Fig. 6C). Quantification of the percentage
25 reduction in PER reveals that the 'matched' groups, where quinine is paired with an

1 attractive tastant suppressed PER by 79-94%, while there was little PER suppression
2 when the tested tastant was unmatched from the one previously paired with quinine (Fig
3 6D). This reciprocal discrimination between sucrose and HxA is different from the
4 unilateral discrimination between two sugars reported previously [45] and indicates that
5 flies can discriminate between sucrose and HxA based on their identity. Thus, sugars
6 and FAs act as independent taste modalities in flies.

7

8 **Discussion**

9 Here, we identify a small population of neurons within the taste system that is responsive
10 to FAs. Other taste modalities, such as sweet and bitter, have been well studied in flies
11 and mammals, but much less is known about FA taste. We previously identified a
12 requirement for Gr64f-GAL4 neurons in the taste of FA [19]. The findings presented here
13 further localize the reflexive feeding response to taste of hexanoic and octanoic acid,
14 both medium-chain FAs, to a subpopulation of IR56d neurons. 'Sweet-sensing' neurons
15 in *Drosophila* have been broadly classified as ones responding to sugars and other
16 sweet tastants such as glycerol, subsets of which may also be involved in sensing other
17 appetitive tastants such as amino acids [17,28,48]. Our findings indicate that IR56d
18 neurons represent channels for FA detection, and likely allow flies to discriminate
19 between different attractive tastants.

20 **Localization of fatty acid-sensitive neurons**

21 We identify a subpopulation of sweet sensing neurons that are responsive to FAs. We
22 have previously shown that genetic silencing or ablation of most sweet-sensing neurons
23 using Gr64f-GAL4 abolished FA taste, suggesting shared neurons detect sugar and FA
24 taste [19]. In flies, subpopulations of Gr64f neurons are selectively sensitive to different

1 tastants including a Gr64e population that is sensitive to glycerol [48] and Gr5a subset
2 that is sensitive to trehalose [17]. To localize the Gr64f neurons responsible for FA taste
3 we conducted a targeted screen and silenced neurons that are believed to overlap with
4 Gr64f neurons, which led us to study the IR56d population of neurons. ISilencing Ir56d
5 neurons appears to selectively disrupt HxA taste without affecting response to sucrose,
6 supporting the notion that independent channels within the Gr64f population mediate
7 response to sugar and FAs. It is possible that FA-sensitive neurons are broadly tuned to
8 FAs, or selectively respond to distinct classes of FAs. Our Ca²⁺ imaging experiments
9 indicate that IR56d neurons are responsive to medium-chain HxA (six carbons (C6),
10 saturated) and octanoic acid (C8, saturated) in both anterior and posterior regions, and
11 to short-chain pentanoic acid (C5, saturated), but only in the anterior projections. We do
12 not find IR56d neurons responsive to long chain, unsaturated oleic acid (C18, mono-
13 unsaturated). These findings are supported by behavioral data revealing that flies exhibit
14 robust PER in response to pentanoic acid, HxA, and octanoic acid, but not oleic acid.
15 Therefore, it seems likely that flies are selectively responsive to short/medium chain FAs,
16 but less responsive to long-chain and/or unsaturated FAs. It is possible that a population
17 of taste neurons independent from IR56d are activated by pentanoic acid because it
18 elicited a robust behavioral response that was independent of Ir56d neurons. Further,
19 IR56d neurons may be activated by long-chain FAs that were not tested, and these
20 could modulate feeding response and induce PER. Nevertheless, the findings presented
21 here reveal specificity for short/medium chain FAs within a defined population of taste
22 neurons, and further specificity for medium chain FAs in a subset of those neurons.

23 **Discrimination between attractive substances**

24 The robust PER response induced by two different medium-chain FAs, hexanoic and
25 octanoic acid, suggests they may be part of the fruit flies diet. Typical dietary fats,

1 including many plant based oils, such as coconut oil, are rich in longer chain FAs
2 including palmitic acid, oleic acid and linoleic acid [49]. However medium chain FAs are
3 present in fermenting fruits such as guava and pollen, and it is possible this provides a
4 substrate for *Drosophila* in the wild [50,51]. While less is known about the food
5 substrates of *D. melanogaster*, the closely related *D. sechelia* consume Morinda fruit,
6 which is rich in FAs [52,53]. Further, we have previously shown that a diet of HxA alone
7 is sufficient for survival [19]. Therefore, it is possible that FA attraction evolved to
8 promote consumption of calorically rich fruits consumed by *Drosophila*.

9

10 Our aversive memory assay indicates flies can discriminate between these attractive
11 tastants. Sugar induces broad activation of Gr64f neurons, while the activation induced
12 by HxA appears more restricted, and therefore it is possible that differences in activation
13 allow for differentiation [54]. Alternatively, we find that HxA also activates IR56d neurons
14 that emanate from the taste pegs and do not co-express Gr64f, so it is possible that the
15 activation of these neurons by HxA allows discrimination. Finally, considering the
16 different biochemical pathways involved in sugar and FA sensing [19], their identity can
17 be coded by unique temporal and spatial dynamics of sensory neuron activation
18 [16,55,56]. Differences in activation are suggested to provide a mechanism for olfactory
19 discrimination within defined neural populations, and it is possible that similar
20 mechanisms are utilized for attractive tastants [53]. In *Drosophila*, attractive tastants
21 have been found to induce a wide range of excitatory responses ranging from acute to
22 prolonged firing [29,57], providing a potential mechanism for discrimination. While the
23 sensillar response to FAs has not been reported, the differences in Ca²⁺ response to
24 sugar or HxA presentation within the SEZ suggest differences in temporal activation.

25

1 We find that flies can discriminate between sugar and FAs, but it is not known whether
2 they can discriminate qualitatively between different classes of FAs. A previous study
3 examining discrimination between different sugars found that flies are unable to
4 discriminate based on quality, but could discriminate based on perceived palatability [45].
5 Here, we find that pentanoic acid elicits a robust PER response that is independent of
6 IR56d neurons. The findings that distinct populations of neurons respond to FAs from
7 different classes raises the possibility that flies may discriminate based on pattern of
8 neuronal activation.

9

10 **Role of Phospholipase C in fatty acid taste**

11 We have previously reported evidence that Phospholipase C (PLC) signaling in sweet-
12 sensing Gr64f neurons is required for FA taste [19]. Flies with mutation or knockdown of
13 the PLC- β ortholog *no receptor potential A* (*norpA*) do not respond to HxA or octanoic
14 acid but respond normally to sugars, indicating that distinct intercellular signaling
15 mechanism underlie response to FAs and sugars [19,58]. Our findings that the IR56d-
16 Gr64f co-expressing neurons respond to both sugars and FAs provide a system to
17 directly test the role of *norpA* in neuronal response within a defined neuronal population.
18 Further, our findings show that PER response to pentanoic acid does not require IR56d
19 neurons, suggesting distinct populations of taste neurons regulate response to short-
20 chain (pentanoic acid) and medium-chain FAs (HxA and octanoic acid). Testing the
21 response of *norpA* deficient flies to short-chain FAs will inform whether PLC is more
22 generally required for FA taste, or is only specific to medium-chain FAs detected by
23 IR56d neurons.

24 **Higher order neurons involved in fatty acid perception**

1 While much is known about taste coding within the SEZ, less is known about the higher
2 order circuits governing taste. Sweet sensing neurons project to the antennal
3 mechanosensory and motor center (AMMC) and downstream PAM dopaminergic
4 neurons that are activated by sugar [39,59]. In addition, a separate populations of
5 dopamine neurons, the PPL1 cluster, is required for olfactory appetitive memory and
6 taste aversive conditioning [60–62]. A central question will be whether these higher order
7 neurons downstream of the SEZ are also sensitive to FAs, or selectively tuned to sugar.
8 To date, higher order neurons responsive to FA taste have not been identified. It is
9 possible that sugar and FA taste signal through shared higher order dopaminergic
10 neurons or, alternatively, each taste modality may activate distinct populations of higher
11 order neurons that convey valence to the mushroom bodies, the memory and sensory
12 integration center in *Drosophila* [63–65].

13 **Conclusions**

14 Taken together, this study provides insight into the coding of FAs within the fly gustatory
15 system. Our results reveal a population of sweet-sensing neurons that are tuned for
16 medium-chain FAs, but not short- or long-chain FAs. Further, the finding that flies are
17 capable of discriminating between FAs and sugars suggests coding differences, either at
18 the level of spatial or temporal neuronal activation, that provides a mechanism to
19 distinguish between tastants of the same valence. Understanding how FAs are coded
20 within the fly brain provides a model for understanding taste in more complex systems
21 and will offer insight into generalizable mechanisms for taste discrimination.

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1 **Methods**

2 ***Drosophila* maintenance and fly stocks**

3 Flies were grown and maintained on standard food (New Horizon Jazz Mix, Fisher
4 Scientific). Flies were maintained in incubators (Powers Scientific; Dros52) at 25°C on a
5 12:12 LD cycle, with humidity set at 55-65%. The background control line used in this
6 study is the w^{1118} fly strain unless otherwise noted. The following fly strains were ordered
7 from The Bloomington Stock Center, UAS-imp-TNT (28840), UAS-TNT (28838), UAS-
8 TRPA1 (26263); UAS-CaMPARI; (58762); UAS-GFP (32186); UAS-GCaMP5 (42037);
9 +;; Gr43a-GAL4 (57637), Gr5a-GAL4 (57591), Gr61a-GAL4 (57658), Gr64a-GAL4
10 (57662), Gr64c-GAL4 (57663), Gr64d-GAL4 (57665), Gr64e-GAL4 (57666), Gr64f-GAL4
11 (57668), IR56b-GAL4 (60706), IR56d-GAL4 (60708), LexAop-Gal80 (32213). Gr64f-
12 LexA was kind gift from H. Tanimoto and previously described in [20]. Seven to nine day
13 old mated female flies were used for all experiments in this study, except when noted.

14 .

15 **Proboscis Extension Reflex**

16 For the initial screen using TNT, and specific testing of tarsal response, PER was
17 measured by applying tastant to the tarsi, as previously described [19]. For all other PER
18 experiments, including validation of *Ir56d* phenotypes, tastant was applied to the
19 proboscis to match behavioral results with functional imaging. Flies were anesthetized
20 on CO₂, and mounted in a pipette tip so that their head and proboscis, but not tarsi, were
21 exposed. After a 30-60m acclimation period, flies were presented with water and allowed
22 to drink freely until water satiated. Flies that did not stop responding to water within 5
23 minutes were discarded. A small Kimwipe (Kimberley Clark) wick saturated with tastant
24 was manually applied to the tip of the proboscis for 1-2s and proboscis extension reflex
25 was monitored. Only full extensions were counted as a positive response. Each tastant
26 was presented three times, with 10 seconds between each trial. Between tastant trials,

1 the proboscis was washed with water and flies were allowed to drink. PER response was
2 calculated as a percentage of proboscis extensions to total number of tastant
3 stimulations. For experiments examining the effects of TRPA1 activation on PER, flies
4 were mounted on a microscope slide using nail polish as described previously [19]. Flies
5 were then placed on a heat plate heated to 34 °C and video of activity was recorded for
6 1 minute. The number of flies for each genotype showing PER within the 1 minute trial
7 period was counted and the percentage of flies showing PER calculated.

8

9 ***In vivo* functional imaging**

10 Flies were anaesthetized on ice and placed in a small chamber with the head and
11 proboscis accessible. A small hole was cut in tin foil and fixed to the stage leaving a
12 window of cuticle exposed, then sealed using dental glue (Tetric Flow – Ivoclar
13 Vivadent). The proboscis was extended and a small amount of dental glue was used to
14 secure it in place, ensuring the same position throughout the experiment.

15 The cuticle and connective tissue was dissected to expose the SEZ, which was bathed
16 in artificial hemolymph (140 mM NaCl, 2mM KCl, 4.5 mM MgCl₂, 1.5mM CaCl₂, and
17 5mM HEPES-NaOH with [pH](#) = 7.0). Mounted flies were placed under a confocal
18 microscope (Nikon A1) and imaged using a 25x water-dipping objective lens. The
19 pinhole was opened to allow a thicker optical section to be monitored. Recordings were
20 taken at 4Hz with 256 resolution. Tastants were delivered to the proboscis for 1-2s with
21 a KimWipe wick operated by microcontroller (brand, type). For analysis, regions of
22 interest (ROI) were drawn manually, taking care to capture the same area between
23 control and experimental. Baseline fluorescence was recorded over 5 frames, 10s prior
24 to tastant application. $\Delta F/F$ was calculated for each frame as (fluorescence - baseline

1 fluorescence)/baseline fluorescence * 100. Average fluorescence traces were created by
2 taking the average and standard error of $\Delta F/F$ for all samples per frame.

3

4

5 **Immunohistochemistry**

6 Fly brains were dissected in ice-cold PBS and fixed in 4% formaldehyde, PBS, 0.5%
7 Triton-X 100 for 30 minutes. Brains were rinsed 3X with PBS, Triton-X for 10 minutes
8 and incubated overnight at 4°C in NC82 (Iowa Hybridoma Bank [66]). The brains were
9 rinsed again in PBS-TritonX, 3X for 10 minutes and placed in secondary antibodies
10 (Donkey anti-Mouse 555; Life Technologies) for 90 minutes at room temperature. The
11 brains were mounted in Vectashield (VectorLabs) and imaged on confocal microscope.
12 Brains were imaged in 2µm sections and are presented as the Z-stack projection
13 through either the entire brain, or anterior and posterior regions of IR56d projections in
14 the SEZ.

15

16 **Aversive Taste Memory**

17 PER induction was performed in one-week old mated females as described previously [5,
18 16]. One-week-old mated females were collected and placed on fresh food for 24 h, then
19 starved for 48 h in food-vials on wet Kimwipe paper. Flies were later anaesthetized on
20 CO₂ pad and glued using nail polish (Cat#72180, Electron Microscopy Science) by their
21 thorax and wing base on a microscopy slide and left to recover in a box with wet
22 Kimwipe paper for 3-6 h prior to experiments. For experiments, the slide was mounted
23 vertically under the dissecting microscope (Olympus SZX12) and PER was observed.
24 Flies were satiated with water before and during the experiment. Flies that did not
25 initially satiate within 5 minutes were excluded from conditioning. We used a 1ml syringe

1 (Tuberculine, Becton Dickinson & Comp) for tastant presentation. We used purified
2 water, 10 mM and 1000 mM sucrose, 0.4% hexanoic acid and 10 mM quinine
3 hydrochloride solutions. The protocol was adapted from [40]. Here, for pretest, each fly
4 was given 10 mM sucrose or 0.4% HxA on their tarsi three times with 10 sec inter-trial
5 interval and the number of full proboscis extensions was recorded. During training, same
6 stimulation as before was followed by a droplet of 10 mM quinine was placed on
7 extended proboscis and flies were allowed to drink it for up to 2 sec or until they
8 retracted their proboscis. After each session, tarsi and proboscis were washed with
9 water and flies were allowed to drink to satiation. After training, flies were tested with
10 either that same substance without quinine or with the untrained substance (matched or
11 opposite trained groups). Another group of flies was measured as described above but
12 quinine was never presented (naïve groups). At the end of each experiment, flies were
13 given 1000mM sucrose to check for retained ability of PER and non-responders were
14 excluded [11].

15

16 **Reagents**

17 Sucrose was purchased from Fisher Scientific (FS S5-500). All other tastants were
18 purchased from Sigma Aldrich. Sucrose, hexanoic acid (SA 153745), octanoic acid (SA
19 C2875), pentanoic Acid (SA 240370) and quinine hydrochloride (SA 145904) were
20 diluted in H₂O. Oleic Acid (SA O1008) was diluted in DMSO 1% DMSO (Sigma).

21

22 **Statistical Analysis**

23 All statistical tests were performed in R. For normally distributed data, Student's t-test or
24 ANOVA with Tukey's post-hoc comparison was performed. For data that did not fit a
25 normal distribution, nonparametric tests were used: Wilcoxon Rank-Sum or Kruskal-

1 Wallis with Dunn's post-hoc. Fisher's Exact Test was used for binary categorical data.

2 For all tests with multiple comparisons, Bonferroni p-value adjustment was performed.

3

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14

15

1 Figure Legends

2 **Fig 1. Gr64f gustatory receptor neurons respond to sucrose and HxA.**

3 (A) Expression of GFP in Gr64f-GAL4 neurons reveals axon terminals in the sub-
4 oesophageal zone (SEZ). Scale bar = 50 μ m. (B) Diagram of live-imaging experimental
5 protocol. Cuticle above the SEZ is removed and GCaMP5 fluorescence is recorded
6 while tastant is applied to the proboscis. (C) Average peak $\Delta F/F$ during response to
7 water, 1% HxA, and 10mM sucrose (n = 11, 11, 10 respectively). Error bars indicate
8 SEM. One-way ANOVA with Tukey's HSD - ***p<0.001 (D) Average $\Delta F/F$ traces and
9 representative images of calcium activity in Gr64f neurons responding to water, (E) 1 %
10 HxA, and (F) 10mM sucrose. Scale bar = 50 μ m. Shaded region of trace indicates +/-
11 SEM.

12

13 **Fig 2. IR56d neurons are necessary and sufficient for PER to FAs.**

14 (A) Expression pattern of IR56d neurons visualized with GFP. Distinct regions of
15 projection are seen in (B) posterior and (C) anterior regions of SEZ. Scale bar = 50 μ m.
16 (D) Blocking synaptic release by genetic expression of light-chain tetanus toxin (TNT) in
17 IR56d neurons significantly reduces PER to HxA, but not sucrose, compared to control
18 flies expressing an inactive form of tetanus toxin (imp-TNT). (imp-TNT n = 26; TNT n =
19 29). Wilcoxon Rank Sum Test - ***p<0.001. (E) Schematic for heat activation of TRPA1.
20 Temperature was elevated for a period of 1 minute and the number of flies showing a
21 PER during this period was counted. (F) Heat activation of IR56d neurons with TRPA1
22 induces significant PER compared to either transgene alone (n = 27 for all genotypes).
23 Fisher's Exact Test with Bonferroni correction for multiple comparisons - ***p< 0.001

24

25 **Fig 3. Response to sugar and fatty acid differs in anterior and posterior 26 IR56d projections.**

27 (A) Activity traces and representative images of calcium activity in IR56d posterior
28 projections in response to water, 1% HxA, and 10mM sucrose (n = 13 for each tastant).
29 Shaded region of trace indicates +/- SEM (B) Average peak $\Delta F/F$ for data shown in (A).
30 Error bars indicate SEM. One-way ANOVA with Tukey's HSD - **p< 0.01, ***p< 0.001.
31 (C) Activity traces and representative images of calcium activity in IR56d posterior
32 projections in response to water, 1% HxA, and 10mM sucrose (n = 12 for each tastant).
33 Shaded region of trace indicates +/- SEM (D) Average peak $\Delta F/F$ for data shown in (C).
34 Error bars indicate SEM. One-way ANOVA with Tukey's HSD - ***p< 0.001.

35

36 **Fig 4. IR56d neurons are selectively responsive to short and medium chain 37 FAs.**

38 (A) PER in response to short chain pentanoic acid (PeA, n = 46), medium chain octanoic
39 acid (OcA, n = 26), and long-chain oleic acid (n = 46) in control imp-TNT flies. Blocking
40 synaptic release in IR56d neurons with TNT significantly decreases PER to octanoic
41 acid (n = 29), but does not affect PER for pentanoic (n = 42) or oleic acid (n = 42).
42 Wilcoxon Rank-Sum Test - ***p<0.001. (B) Average peak $\Delta F/F$ for the posterior
43 projections of IR56d neurons in response to water, 1% DMSO, pentanoic acid,
44 octanoic acid, and oleic acid (n = 9, 8, 9, 9, 8, respectively), and (C) the anterior
45 projections (n = 9, 7, 9, 9, 7, respectively). Error bars indicate SEM. One way
46 ANOVA with Tukey's HSD - ***p<0.001

47

48 **Fig 5. IR56d anterior projections are not required for PER to FAs.**

1 (A) IR56d and Gr64f neurons visualized with IR56d-GAL4 driving RFP and Gr64f-LexA
2 driving GFP. Overlap can be seen in the posterior projections, but is minimal in anterior
3 projections. (B) Driving the GAL4 repressor Gal80 with Gr64f-LexA limits GFP
4 expression to the non-overlapping IR56d anterior projections. (C) Restricting TNT
5 expression to the non-overlapping anterior projection neurons does not significantly
6 impact PER to sugar or HxA (n = 23 imp-TNT; n = 22 TNT) Error bars indicate SEM.
7 Wilcoxon Rank Sum Test (Sucrose: $p > 0.98$; HxA: $p > 0.96$)
8

9 **Fig 6. *Drosophila* discriminate between sucrose and HxA.**

10 (A) Taste memory protocol to determine sugar and FA discrimination. Flies are trained
11 by pairing HxA or sucrose on tarsi with quinine on proboscis. PER in response to HxA
12 and sucrose is then tested following training to sucrose or HxA in the absence of quinine.
13 In control experiments, the same procedure is followed but quinine is never offered for
14 measurement of naïve decline of tastant responses. (B) The pairing of sucrose and
15 quinine (dark green circles) results in a significant reduction in PER across all three
16 training trials compared to unpaired naïve flies provided sucrose alone (light green
17 circles). PER response to sucrose in the test is significantly lower in trained flies
18 compared to naïve flies ($p < 0.001$, $n = 7, 11$), but no differences in response to HxA
19 (triangles) is detected between the experimental and naïve groups ($p > 0.31$, $n = 11, 12$).
20 (C) The pairing of HxA and quinine (dark blue triangles) results in a significant reduction
21 in PER across all three training trials compared to unpaired naïve flies provided HxA
22 alone (light green triangles). The test PER response to HxA alone is significantly lower in
23 trained flies compared to naïve flies ($p < 0.001$, $n = 13, 16$), but no differences in response
24 to sucrose (circles) is detected between the groups ($p > 0.59$, $n = 12, 12$). (D) Comparing
25 the percentage change in PER suppression reveals that flies train and tested to the
26 same tastant (either sucrose or HxA) significantly reduced PER compared to flies trained
27 and tested with different tastants.

28

1 **Supplemental Data**

2 **Table S1. A neuronal silencing screen for taste neurons sensitive to HxA**

3 **(A)** Data for PER in response to 100mM sucrose in flies with silenced populations of
4 gustatory neurons by driving expression of TNT with the indicated. The 'normalized'
5 column represents the PER value for experimental fly divided by the result for w1118.

6 **(B)** Data for PER in response to 1% HxA. p-values computed by Kruskal-Wallis followed
7 by Dunn's post-hoc with Bonferroni p-value adjustment, with comparison to w1118
8 control flies. *p<0.05, **p<0.01, ***p<0.001.

9

10

11 **Fig S1. Flies continue to suppress PER in response to FAs in absence of Gr64f to**
12 **drive LexAop-Gal80**

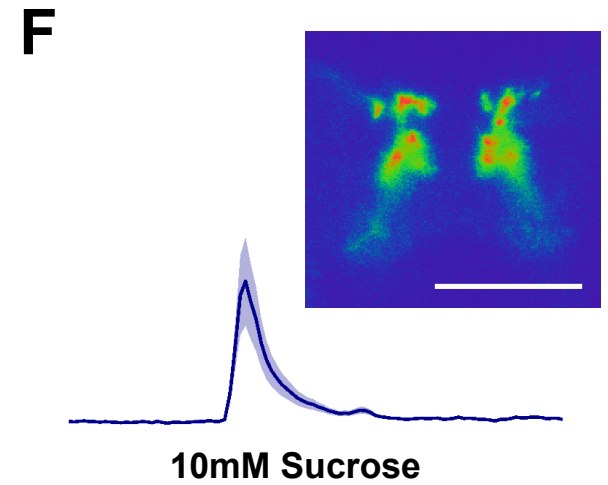
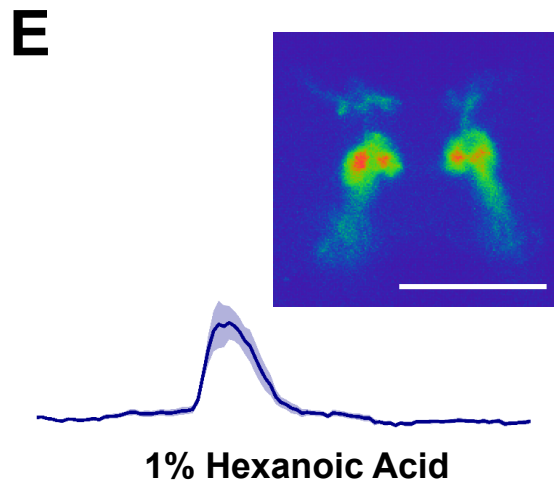
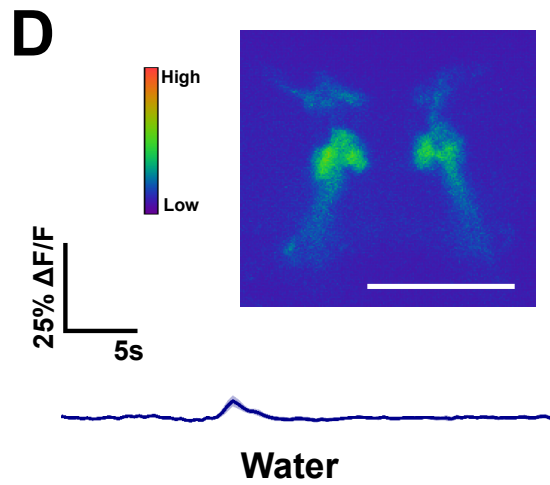
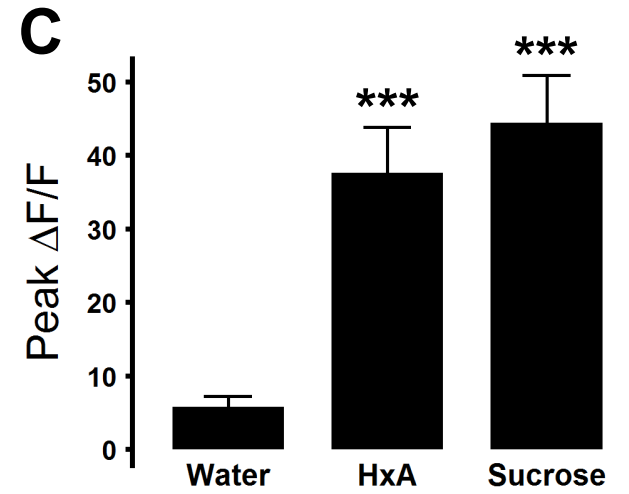
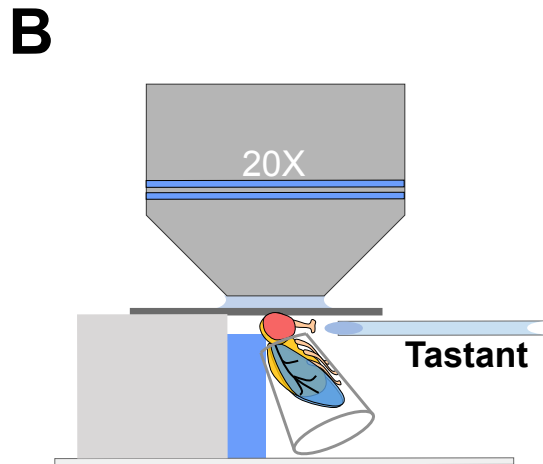
13 **(A)** PER for flies expressing either imp-TNT or TNT in IR56d neurons, (n = 31 for both
14 groups), Without Gr64f-LexA to drive the LexAop-GAL80, as in Fig 5c, TNT is expressed
15 in all IR56d neurons and PER to HxA is suppressed. Wilcoxon Rank Sum Test
16 ***p<0.001

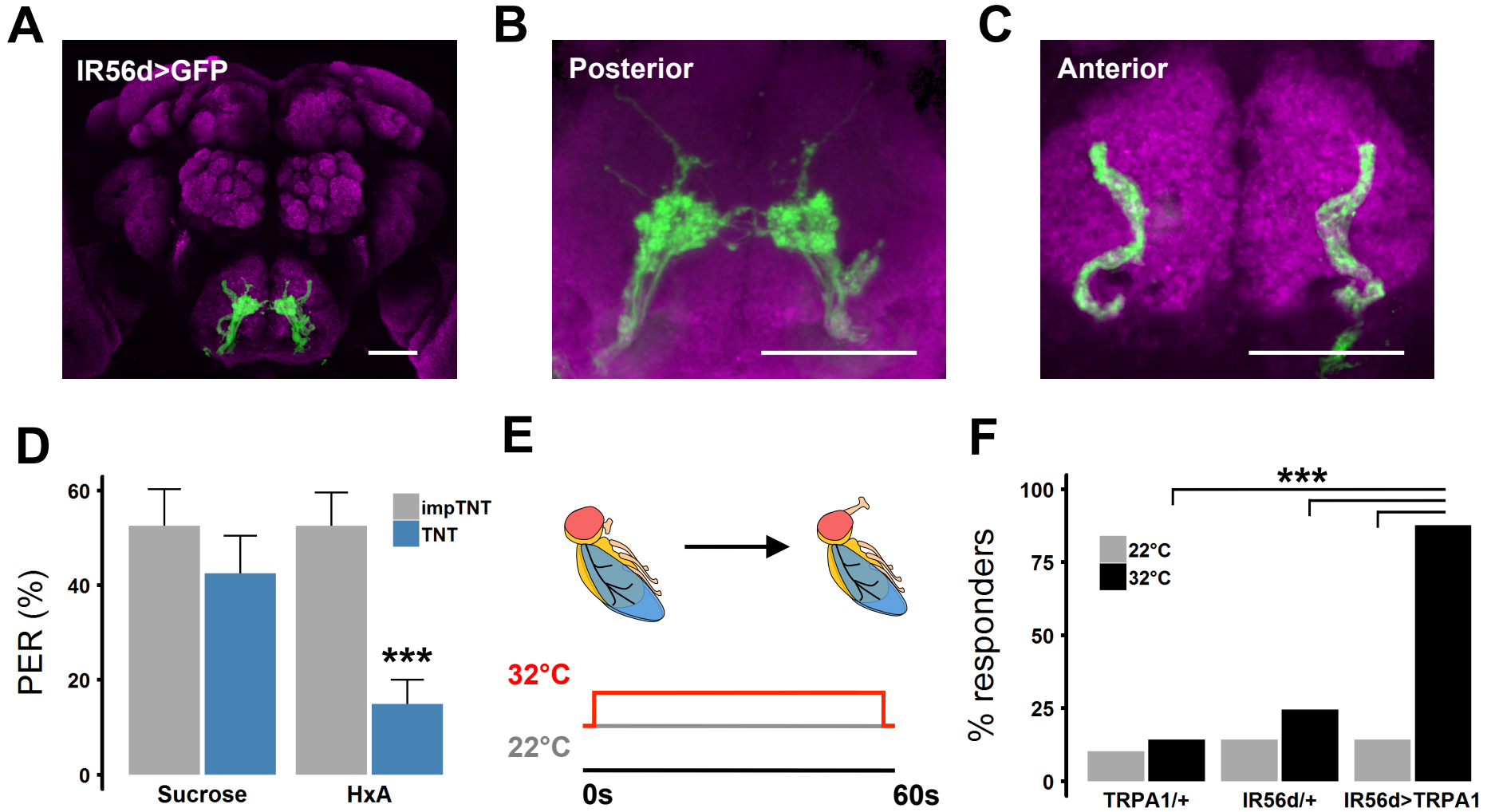
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18

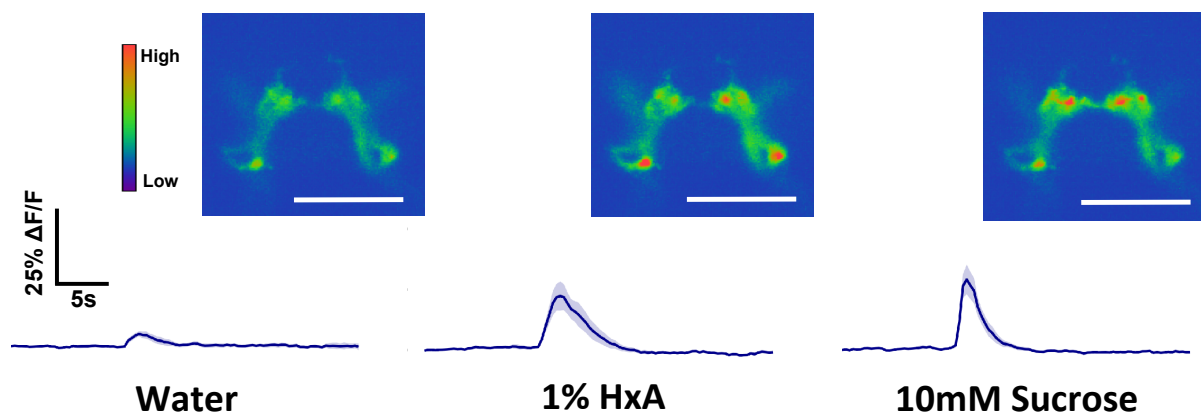
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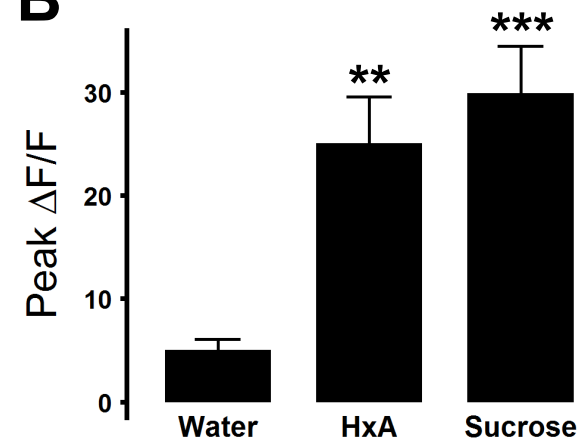




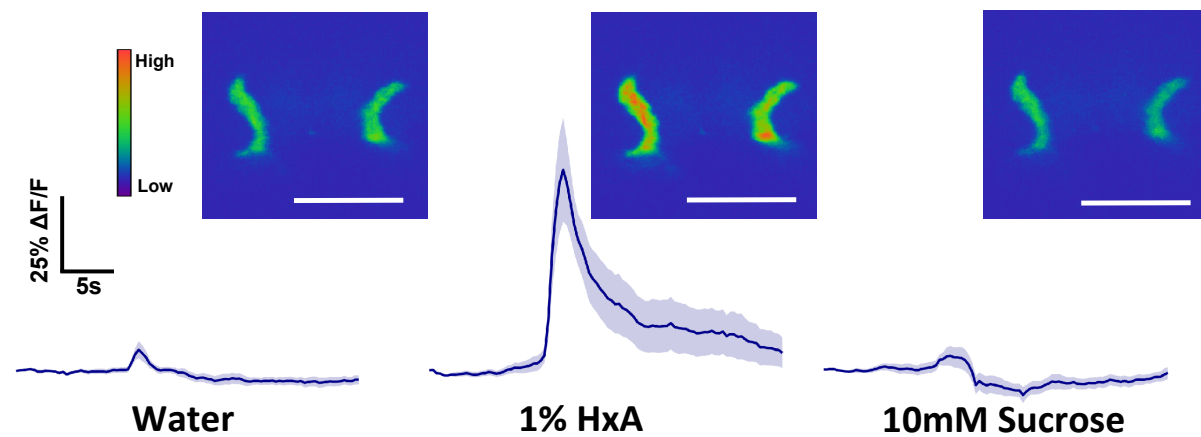
A Ir56d>GCaMP - Posterior



B



C Ir56d>GCaMP - Anterior



D

