Ir56d-dependent fatty acid responses in *Drosophila* uncovers taste discrimination between different classes of fatty acids

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Abstract

Chemosensory systems are critical for evaluating the caloric value and potential toxicity of food prior to ingestion. While animals can discriminate between 1000's of odors, much less is known about the discriminative capabilities of taste systems. Fats and sugars represent calorically potent and innately attractive food sources that contribute to hedonic feeding. Despite the differences in nutritional value between fats and sugars, the ability of the taste system to discriminate between different rewarding tastants is thought to be limited. In *Drosophila*, sweet taste neurons expressing the Ionotropic Receptor 56d (*IR56d*) are required for reflexive behavioral responses to the medium-chain fatty acid, hexanoic acid. Further, we have found that flies can discriminate between a fatty acid and a sugar in aversive memory assays, establishing a foundation to investigate the capacity of the *Drosophila* gustatory system to differentiate between various appetitive tastants. Here, we tested whether flies can discriminate between different classes of fatty acids using an aversive memory assay. Our results indicate that flies are able to discriminate medium-chain fatty acids from both short- and long-chain fatty acids, but not from other medium-chain fatty acids. Characterization of hexanoic acid-sensitive *Ionotropic receptor 56d* (Ir56d) neurons reveals broad responsive to short-, medium-, and long-chain fatty acids, suggesting selectivity is unlikely to occur through activation of distinct sensory neuron populations. However, genetic deletion of *IR56d* selectively disrupts response to medium chain fatty acids, but not short and long chain fatty acids. These findings reveal Ir56d is selectively required for fatty acid taste, and discrimination of fatty acids occurs through differential receptor activation within shared populations of neurons. These findings uncover a capacity for the taste system to encode tastant identity within a taste category.

Introduction

Animals detect food primarily through taste and olfactory systems. Across phyla, there is enormous complexity in olfactory receptors and downstream processing mechanisms that allow for detection and differentiation between odorants (Keller et al., 2017; Nara, Saraiva, Ye, & Buck, 2011; Parnas, Lin, Huetteroth, & Miesenböck, 2013). By contrast, taste coding is thought to be simpler, with most animals possessing fewer taste receptors and a diminished ability to differentiate between tastants (Freeman & Dahanukar, 2015; Scott, 2018; Yarmolinsky, Zuker, & Ryba, 2009). Most early studies in different species have focused on characterization of a limited number of taste modalities largely defined by human percepts (sweet, bitter, sour, umami, salt), though there is growing appreciation that additional taste pathways are likely to influence gustatory responses and feeding (Chaudhari & Roper, 2010; Scott, 2018). Between studies of *Drosophila* and mammals, cells or receptors that are involved in sensing water, carbonation, fat, electrophiles, polyamines, metal ions, and ribonucleotides have been identified, suggesting a previously underappreciated complexity in the coding of tastants (Cameron, Hiroi, Ngai, & Scott, 2010; Kang et al., 2010; Mishra, Thorne, Miyamoto, Jagge, & Amrein, 2018; Y. V. Zhang, Ni, & Montell, 2013). Elucidating the underlying mechanisms of tastant detection can provide fundamental insight into the molecular and cellular basis of tastant recognition and taste processing.

In flies and mammals, tastants are sensed by dedicated gustatory receptors that are expressed in gustatory receptor neurons (GRNs) or taste cells respectively. In both systems, distinct subsets of taste sensory cells are activated by compounds belonging to distinct taste modalities such as sweet or bitter, and convey information to discrete areas of higher order brain structures (Vosshall & Stocker, 2007; Yarmolinsky et al., 2009; Yifeng Zhang et al., 2003). Given the conserved logic of taste processing, flies provide a powerful system for studying sensory processing and principles of taste circuit function (Freeman & Dahanukar, 2015; Scott, 2018;

Yarmolinsky et al., 2009). Further, a number of genes and biochemical pathways that regulate feeding behavior are conserved across phyla (Vosshall & Stocker, 2007; Yarmolinsky et al., 2009). Notably, the gustatory system of *Drosophila* is amenable to *in vivo* Ca^{2+} imaging and electrophysiology, both of which can be coupled with robust behavioral assays that measure reflexive taste responses and food consumption (Wisotsky, Medina, Freeman, & Dahanukar, 2011). Taste neurons are housed in gustatory sensory structures called sensilla, which are located in the distal segments of the legs (tarsi), in the external and internal mouth organs (proboscis and pharynx), and in the wings. Each sensillum contains dendrites of multiple gustatory receptor neurons, each of which can be distinguished from the others based on its responses to various categories of tastants. Two main classes of non-overlapping gustatory neurons that have been identified are sweet-sensing and bitter-sensing neurons. Sweet-sensing GRNs promote feeding, whereas bitter-sensing GRNs act to deter (Marella et al., 2006; Thorne, Chromey, Bray, & Amrein, 2004). Both sweet and bitter GRNs express subsets of 68 G-protein-coupled gustatory receptors (GRs) (Clyne, Warr, & Carlson, 2000; Scott et al., 2001). In addition, the *Drosophila* genome encodes 66 glutamate-like Ionotropic Receptors (IRs), a recently identified family of receptors implicated in taste, olfaction, and temperature sensation (Benton, Vannice, Gomez-Diaz, & Vosshall, 2009; Rytz, Croset, & Benton, 2013). GRNs predominantly project to the subesophageal zone (SEZ), the primary taste center, but the higher order circuitry downstream of the SEZ contributing to taste processing is poorly understood (Flood et al., 2013; Marella, Mann, & Scott, 2012; Pool et al., 2014; Wang, Singhvi, Kong, & Scott, 2004). Determining how tastants activate GRNs that convey information to the SEZ, and how these signals are transmitted to higher order brain centers, is central to understanding the neural basis for taste and feeding.

In *Drosophila,* GRNs in the labellum and tarsi detect hexanoic acid (Pavel Masek & Keene, 2013). Mutation of Ionotropic receptor 56d (*IR56d*) disrupts hexanoic acid taste, implicating

IR56d as a fatty acid receptor, or as part of a complex involved in fatty acid taste (Ahn, Chen, & Amrein, 2017; Sánchez-Alcañiz et al., 2018). *IR56d* is co-expressed with Gr64f (Ahn et al., 2017; Tauber et al., 2017), which broadly labels sweet GRNs (Dahanukar, Lei, Kwon, & Carlson, 2007; Jiao, Moon, Wang, Ren, & Montell, 2008; Slone, Daniels, & Amrein, 2007). *IR56d*-expressing GRNs are responsive to both sugars and fatty acids, suggesting that these neurons may respond to diverse appetitive substances including multiple classes of fatty acids (Tauber et al., 2017). Notably, overlapping populations of sweet GRNs that are responsive to different appetitive modalities and confer feeding behavior.

Is it possible that flies are capable of differentiating between tastants of the same modality, or is discrimination within a modality exclusively dependent on concentration? Taste discrimination can be assayed by training flies to pair a negative stimulus with a tastant, and determining whether the acquired aversion generalizes to another tastant (Keene & Masek, 2012; Pavel Masek & Scott, 2010). A previous study employing such experiments found that flies are unable to discriminate between different sugars (Pavel Masek & Scott, 2010). Conversely, we reported that flies can discriminate between sucrose (sugar) and hexanoic acid (fatty acid), revealing an ability to discriminate between appetitive stimuli of different modalities (Tauber et al., 2017). Here, we find that flies are capable of discriminating between different classes of fatty acids, despite broad tuning of fatty-acid sensitive neurons to short, medium and long chain FAs.

Results

Sugars and medium chain fatty acids are sensed by an overlapping population of gustatory neurons, and flies can discriminate between these attractive tastants (Ahn et al., 2017; Tauber et al., 2017). To test whether flies are capable of discriminating within a single modality, we measured the ability of flies to discriminate between different types of fatty acids. We have used an appetitive taste memory assay in which an appetitive tastant is paired with bitter quinine,

resulting in an associative memory that inhibits responses to the appetitive tastant (Pavel Masek, Worden, Aso, Rubin, & Keene, 2015). A modified version of this assay, in which training with one tastant is followed by testing with another, allows us to determine whether flies can discriminate between these tastants (Fig 1A). We first sought to determine whether flies are capable of differentiating between short (3C-5C), medium (6C-8C) and long (>9C) FAs. We found that flies that were trained with pairing of quinine and hexanoic acid (6C) exhibited PER to subsequent application of 5C fatty acids (Fig 1B). Thus, aversive memory to 5C was not formed by training with 6C, suggesting that flies can discriminate between these short- and medium-chain fatty acids. Similarly, flies trained with 6C did not generalize aversive memory to 9C, consistent with the idea that flies can also discriminate between medium- and long-chain fatty acids (Fig 1C). To rule out the possibility that flies are unable to form aversive taste memories to short- and long-chain fatty acids, we trained with 5C and found robust aversive taste memory, which did not generalize to 9C (Fig 1D). Together, these results suggest that flies are capable of distinguishing between short, medium and long-chain classes of fatty acids.

To determine whether flies can discriminate between compounds within a single class of fatty acid, we tested the ability of flies to differentiate between different medium-chain fatty acids. We trained flies to associate 6C with quinine, while the medium-chain fatty acids 7C or 8C were not reinforced. In both cases, flies formed aversive memories to 6C, and this generalized to 7C and 8C, suggesting that flies cannot discriminate between different medium chain fatty acids (Fig 1E,F). To fortify these findings, we trained flies to 7C and measured the response to 8C. Again, flies formed aversive memory to 7C that was generalized to 8C (Fig 1G). Our findings reveal that flies cannot discriminate between different medium chain fatty acids, although they are able to discriminate medium-chain fatty acids from short- or long-chain fatty acids.

143 The short, medium, and long-chain fatty acids that we tested have distinctly different smells
144 (Hallem & Carlson, 2006), raising the possibility that flies can discriminate between these
145 compounds using a combination of olfactory and gustatory information. To exclude the effects of
146 olfactory input, we surgically ablated the antennae, the maxillary palps, or both structures, and
147 measured the ability of flies to discriminate between a representative tastant from each short-,
148 medium-, or long-chain fatty acid (Fig 2A). All test groups were able to distinguish between
149 sucrose and hexanoic acid, confirming that the ablation itself does not generally impact taste or
150 memory formation (Fig 2B). Further, flies trained to a medium-chain fatty acid (6C) did not
151 generalize aversion to short- (5C) or long-chain (9C) fatty acids, regardless of the absence of
152 one or both olfactory organs (Fig 2C,D). Taken together, our findings reveal an ability of the
153 taste system to encode the identity of different classes of fatty acids.
154
155 The finding that flies cannot discriminate between medium chain fatty acids raises the possibility
156 that *IR56d* is required for the taste of medium-chain fatty acids, but not short and long-chain
157 fatty acids. To determine whether *IR56d*-expressing GRNs mediate taste perception to other
158 classes of fatty acids, we silenced *IR56d*-expressing neurons using the synaptobrevin cleavage
159 peptide tetanus toxin light chain (TNT) (Sweeney, Broadie, Keane, Niemann, & Kane, 1995) and
160 measured proboscis extension response (PER) to multiple classes of fatty acids, including
161 short-, medium-, and long-chain fatty acids (Figure 3A). To control for any non-specific effects of
162 TNT, we compared PER in flies with silenced *IR56d* GRNs (*IR56d*-GAL4>UAS-TNT) to flies
163 expressing the inactive variant of TNT in *IR56d*-expressing GRNs (*IR56d*-GAL4>UAS-impTNT).
164 Consistent with previous findings (Tauber et al., 2017), we observed no effect of silencing
165 *IR56d*-expressing neurons on PER to sucrose (Figure 3B). Next, we measured PER to a panel
166 of saturated FAs ranging from 4C (butanoic acid) to 10C (decanoic acid) in length (Figure 3C).
167 Control flies exhibited a robust PER to all seven fatty acids, revealing that at least at a 1%
168 concentration, many diverse classes of fatty acids can trigger this behavioral response. To

169 determine whether *IR56d* is generally required for detection of fatty acids, or selectively required
170 for sensing hexanoic acid, we next measured PER in flies with *IR56d*-expressing neurons
171 silenced. Silencing *IR56d*-expressing neurons significantly reduced PER to the three medium
172 chain fatty acids (6C, 7C, and 8C). Conversely, there was no difference in PER between control
173 and *IR56d*-silenced flies in response to short chain (4C and 5C) and long-chain (9C and 10C)
174 fatty acids. Therefore, *IR56d*-expressing neurons are required for medium-chain fatty acid taste
175 perception, but are dispensable for responses to both short- and long-chain fatty acids.
176
177 To directly assess whether the *IR56d* receptor mediates responses to medium chain fatty acids,
178 we used the CRISPR/Cas9 system to generate an *IR56d* allele in which a GAL4 element is
179 inserted into the *IR56d* locus (*IR56d*^{GAL4}; Figure 4A), thereby allowing expression of UAS-
180 transgenes under the control of the *IR56d* promoter. To confirm that the GAL4 knock-in element
181 is indeed expressed in *IR56d* neurons, we generated flies carrying both UAS-mCD8:GFP and
182 the *IR56d*^{GAL4} allele (*IR56d*^{GAL4}>UAS-mCD8:GFP) and mapped the expression of GFP.
183 Consistent with previous findings, we found GFP expression in labellar neurons that projected
184 axons to both the taste peg and sweet taste regions of the SEZ (Figure 4B-E; (Koh et al., 2014;
185 Tauber et al., 2017). In agreement with previous findings from genetic silencing of *IR56d*-
186 expressing neurons, PER to sucrose did not differ between *IR56d*^{GAL4} and control flies (Figure
187 4F), suggesting that *IR56d*^{GAL4} is dispensable for response to sucrose. To examine the role of
188 *IR56d* in fatty acid taste, we measured PER to fatty acids ranging from 4C to 10C in length
189 (Figure 4G). Consistent with the results of *IR56d*-silenced flies, PER to medium chain fatty acids
190 was disrupted in *IR56d*^{GAL4} flies (6C-8C), whereas PER to short- (4C and 5C) and long-chain
191 fatty acids (9C and 10C) was not affected. Flies that were heterozygous for the *IR56d* deletion
192 (*IR56d*^{GAL4}/+) exhibited similar responses to those of control flies for all tastants measured. The
193 observed decrease in PER to medium chain fatty acids was rescued by transgenic expression
194 of *IR56d* in the *IR56d*^{GAL4} mutant background (*IR56d*^{GAL4}; UAS-*IR56d*/+), confirming that the

195 behavioral deficit of *IR56d*^{GAL4} flies is in fact due to loss of *IR56d* function. Therefore, *IR56d*
196 appears to be selectively required for taste sensing of medium-chain fatty acids.
197
198 In previous work we found that *IR56d*-expressing neurons are activated by both sucrose and
199 hexanoic acid (Tauber et al., 2017). To determine whether other classes of fatty acids can also
200 activate these neurons, and if so, whether their activity is dependent on *IR56d*, we measured
201 Ca^{2+} responses to a panel of tastants. We expressed the Ca^{2+} sensor GCaMP6.0 under the
202 control of *IR56d*^{GAL4} and measured tastant-evoked activity (Figure 5A-D). In flies heterozygous
203 for *IR56d*^{GAL4}, the labeled neurons were activated by sucrose and all fatty acids tested, which
204 ranged from 4C-10C (Figure 5E). Thus, *IR56d* neurons respond to diverse appetitive stimuli.
205 Flies with a deletion of *IR56d*^{GAL4} (*IR56d*^{GAL4}; *UAS*-GCaMP6.0) lacked responses exclusively to
206 medium chain fatty acids (6C-8C), while responses to short- (4C and 5C) and long-chain fatty
207 acids (9C and 10C) remained intact (Figure 5F). Consistent with the rescue of behavioral
208 defects, inclusion of an *IR56d* rescue transgene (*IR56d*^{GAL4}; *UAS*-GCaMP6.0/*UAS-IR56d)*
209 restored the physiological response to medium chain fatty acids (Figure 5G). Quantification of
210 the responses to all tastants confirmed that Ca^{2+} responses to 6C-8C fatty acids are disrupted in
211 *IR56d*^{GAL4} flies, and restored to levels observed in control flies by expression of *IR56d* (Figure
212 5H). Overall, these results demonstrate that at both behavioral and physiological levels,
213 *IR56d*^{GAL4} is required for taste responses to medium chain fatty acids. Therefore, these findings
214 support the notion that medium chain fatty acids are detected through a shared sensory
215 channel, allowing flies to distinguish medium chain from short or long-chain, but not between
216 different medium-chain fatty acids.
217
218 **Discussion**
219 Receptors for sweet and bitter taste have been well defined in both flies and mammals
220 (Carleton, Accolla, & Simon, 2010; Hallem, Dahanukar, & Carlson, 2006; Scott, 2018), but less

is known about detection of fats. Previous studies identified *IR56d* as a receptor for hexanoic acid and carbonation (Ahn et al., 2017; Sánchez-Alcañiz et al., 2018). Our findings suggest that *IR56d* is selectively involved in responses to medium-chain fatty acids, including 6C, 7C, and 8C fatty acids, and dispensable for responses to shorter and longer-chain fatty acids. Such receptor specificity for different classes of fatty acids based on chain length has not been documented in other systems. In flies, both sugars and fatty acids activate neurons that co-express the receptors *Gr64f* and *IR56d*. The finding that short- and long-chain fatty acids also activate *IR56d*-expressing neurons posits that additional fatty acid receptors are present in these neurons. Previously, we found that deletion of PLC signaling selectively impairs hexanoic acid response while leaving sweet taste intact, raising the possibility that activation of distinct intracellular signaling pathways could serve as a mechanism for discrimination of sucrose and hexanoic acid (Pavel Masek & Keene, 2013; Tauber et al., 2017). Examining whether or not short- and long-chain fatty acids also signal through phospholipase C may provide insight into whether signaling mechanisms are shared between different fatty acid receptors expressed in *IR56d* neurons.

Our aversive taste memory assay confirmed previous findings that flies can discriminate between sugars and fatty acids (Tauber et al., 2017), and led to the surprising observation that flies can distinguish between different classes of fatty acids. This contrasts with the results of a previous study that applied a similar assay and found that flies were unable to discriminate between different sugars or bitter compounds (Kirkhart & Scott, 2015). One possibility is that this is due to differences in fatty acid detection, which is dependent on IRs, and sweet and bitter tastant detection, which relies on GRs (Chen & Dahanukar, 2020). These results suggest the ability of the *Drosophila* the taste system to discriminate may be more like the olfactory system than previously appreciated. Flies are able to distinguish between many different odorants, likely due to the complexity of olfactory coding at the level of the receptor as well as in the antennal

lobe (Amin & Lin, 2019; Cognigni, Felsenberg, & Waddell, 2018; Guven-Ozkan & Davis, 2014). However, flies can also discriminate between odorants sensed by a single olfactory receptor, suggesting that temporal coding also plays a role in discrimination (DasGupta & Waddell, 2008). It is possible that similar mechanisms underlie discrimination between different classes of fatty acid tastants.

The *Drosophila* genome encodes 66 Ionotropic Receptors (IRs), which comprise a recently identified family of receptors implicated in taste, olfaction, and temperature sensation (Benton et al., 2009; Rytz et al., 2013). Ionotropic receptors are involved in the detection of many different tastants, and function as heteromers that confer sensory specificity (Rytz et al., 2013; van Giesen & Garrity, 2017). While *IR56d* expression is restricted to a subset of sweet taste neurons, it likely functions in a complex with *IR25a* and *IR76b*, all three of which are required fatty acid taste (Ahn et al., 2017; Sánchez-Alcañiz et al., 2018). Other tastants whose responses are mediated by IR receptors are also likely to be detected by IR complexes. For example, roles for *IR25a*, *IR62a* and *IR76b* have been described for Ca^{2+} taste (Thakur, Kim, Poudel, Montell, & Lee, 2017). The broad degree of co-expression of IRs in the brain and periphery can provide candidates for those involved in detecting short- and long-chain fatty acids.

The identification of taste discrimination between different classes of fatty acids provides the opportunity to identify how different tastants are encoded in the brain, and how these circuits are modified with experience. Although projections of primary taste neurons to the SEZ have been mapped in some detail, little is known about connectivity with downstream neurons and whether sensory neurons activated by different appetitive tastants can activate different downstream circuits. Recent studies have identified a number of interneurons that modify feeding, including IN1, a cholinergic interneuron activated by sucrose (Yapici, Cohn, Schusterreiter, Ruta, & Vosshall, 2016), E564 neurons that inhibit feeding (Mann, Gordon, & Scott, 2013), and *Fdg*

neurons that are required for sucrose-induced feeding (Flood et al., 2013). Future work can investigate whether these, and other downstream neurons, are shared for fatty acid taste. Previous studies have found that incoming sensory information is selectively modulated within the antennal lobe in accordance with feeding state (Chu, Chui, Mann, & Gordon, 2014; LeDue et al., 2016). It will be interesting to determine if similar modulation promotes differentiation of sugars and fatty acids, which are sensed by shared gustatory neurons. Large-scale brain imaging has now been applied in flies to measure responsiveness to different tastants (Harris, Kallman, Mullaney, & Scott, 2015), and a comparison of brain activity patterns elicited by different classes of fatty acids may provide insight into differences in their sensory input and processing.

All experiments in this study tested flies under starved conditions, which is necessary to elicit the PER that is used as a behavioral readout of taste acceptance. However, responses to many tastants and odorants are altered in accordance with feeding state (LeDue et al., 2016; Root et al., 2008). For example, the taste of acetic acid is aversive to fed flies but attractive to starved flies, revealing a hunger-dependent switch (Devineni, Sun, Zhukovskaya, & Axel, 2019). Similarly, hexanoic acid activates both sweet and bitter sensing taste neurons, and the activation of bitter taste neurons is dependent on different receptors from those involved in the appetitive response (Ahn et al., 2017). Further, hunger enhances activity in sweet taste circuits, and suppresses that of bitter taste circuits, providing a mechanism for complex state-dependent modulation of response to tastants that activate both appetitive and deterrent neurons (Inagaki, Panse, & Anderson, 2014; LeDue et al., 2016).

The neural circuits that are required for aversive taste memory have been well defined for sugar, yet little is known about how fatty acid taste is conditioned. The pairing of sugar with bitter quinine results in aversive memory to sugar. Optogenetic activation of sweet taste

299 neurons, which are activated by both sugar and fatty acids, in combination with quinine
300 presentation is sufficient to induce sugar avoidance, suggesting that aversive taste memory
301 does not depend on post-ingestive feedback (Keene & Masek, 2012). Further studies have
302 elucidated that aversive taste memories are dependent on mushroom body neurons that form
303 the gamma and alpha lobes, the PPL1 cluster of dopamine neurons, and alpha lobe output
304 neurons, revealing a circuit regulating taste memory that differs from that controlling appetitive
305 olfactory memory (Kirkhart & Scott, 2015; P Masek, Worden, Aso, Rubin, & Keene, 2015). It will
306 be interesting to determine whether shared components regulate conditioning to fatty acids, or
307 whether distinct mushroom body circuits regulate sweet taste and fatty acid conditioning.
308 Further, examination of the central brain circuits that regulate aversive taste conditioning to
309 different classes of fatty acids will provide insight into how taste discrimination is processed
310 within the brain.
311
312 **Materials and Methods**
313 ***Drosophila* stocks and maintenance**
314 Flies were grown and maintained on standard food media (Bloomington Recipe, Genesee
315 Scientific, San Diego, CA). Flies were housed in incubators (Powers Scientific, Warminster, PA,
316 USA) on a 12:12 LD cycle at 25°C with humidity of 55-65%. The following fly strains were
317 ordered from the Bloomington Stock Center: w^{1118} (#5905; (Levis, Hazelrigg, & Rubin, 1985));
318 *IR56D*-GAL4 (#60708; (Koh et al., 2014)), UAS-impTNT (#28840; (Sweeney et al., 1995)), UAS-
319 TNT (#28838; (Sweeney et al., 1995)), UAS-GFP (#32186; (Pfeiffer et al., 2010)); UAS-
320 GCaMP5 (#42037; (Akerboom et al., 2012)). UAS-Ir56d was generated using *Ir56d* cDNA,
321 amplified with primers that generated a NotI-KpnI fragment that was cloned in the pUAS vector.
322 The $IR56d^{GAL4}$ line was generated by WellGenetics (Taipei City, Taiwan) using the
323 CRISPR/Cas9 system to induce homology-dependent repair. At the gRNA target site, a dsDNA
324 donor plasmid was inserted containing a GAL4::VP16 and RFP cassette. This line was

generated in the w^{1118} genetic background and was validated by PCR and sequencing. All lines were backcrossed to the w^{1118} fly strain. For all experiments, mated female flies aged 7-to-9 days were used. For ablation experiments, the antenna and/or maxillary palp were removed two days post-eclosion.

Reagents

The following fatty acids were obtained from Sigma Aldrich (St Louis, MO, USA): butyric acid (4C; #B103500), valeric acid (5C; #240370), hexanoic acid (6C; #21530), heptanoic acid (7C; #75190), octanoic acid (8C; #O3907), nonanoic acid (9C; #N5502), and decanoic acid (10C; #C1875). All fatty acids were tested at a concentration of 1% and were dissolved in water. Quinine hydrochloride was also obtained from Sigma Aldrich (#Q1125), while sucrose was purchased from Fisher Scientific (#FS S5-500; Hampton, New Hampshire, USA).

Immunohistochemistry

Brains were prepared as previously described (Kubrak, Lushchak, Zandawala, & Nässel, 2016). Briefly, brains of 7-9 day-old female flies were dissected in ice-cold PBS and fixed in 4% formaldehyde, PBS, and 0.5% Triton-X for 30 minutes at room temperature. Brains were rinsed 3X with PBS and 0.5% Triton-X (PBST) for 10 minutes at room temperature and then incubated overnight at 4°C. The next day, brains were incubated in primary antibody (1:20 mouse nc82; Iowa Hybridoma Bank; The Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) diluted in 0.5% PBST at 4°C for 48 hrs. Next, the brains were rinsed 3X in 0.5% PBST 3X 10 minutes at room temperature and placed in secondary antibody (1:400 donkey anti-mouse Alexa Fluor 647; #A-31571; ThermoFisher Scientific, Waltham, Massachusetts, USA) for 90 minutes at room temperature. The brains were again rinsed 3X in PBST for 10 min at room temperature and then mounted in Vectashield (VECTOR Laboratories, Burlingame, CA). Brains were imaged in 2µm sections on a Nikon A1R confocal microscope (Nikon, Tokyo, Japan) using

a 20X oil immersion objective. Images presented as the Z-stack projection through the entire brain and processes using ImageJ2 (Tauber et al., 2017).

Proboscis Extension Response

Female flies were starved for 48 h prior to each experiment and then PER was measured as previously described (Pavel Masek & Keene, 2013; Tauber et al., 2017). Briefly, flies were anesthetized on CO_2 and then restrained inside of a cut 200 µL pipette tip (#02-404-423; Fisher Scientific) so that their head and proboscis were exposed while their body and tarsi remain restrained. After a 60 min acclimation period in a humidified box, flies were presented with water and allowed to drink freely until satiated. Flies that did not stop responding to water within 5 minutes were discarded. A wick made of Kimwipe (#06-666; Fisher Scientific) was placed partially inside a capillary tube (#1B120F-4; World Precision Instruments; Sarasota, FL) and then saturated with tastant. The saturated wick was then manually applied to the tip of the proboscis for 1-2s and proboscis extension reflex was monitored. Only full extensions were counted as a positive response. Each tastant was presented a total of three times, with 1 min between each presentation. PER was calculated as the percentage of proboscis extensions divided by the total number of tastant presentations. For example, a fly that extends its proboscis twice out of the three presentation will have a PER response of 66%.

***In vivo* calcium imaging**

Female flies were starved for 48 h prior to imaging, as described (Tauber et al., 2017). Flies were anaesthetized on ice and then then restrained inside of a cut 200 µL pipette tip so that their head and proboscis were accessible, while their body and tarsi remain restrained. The proboscis was manually extended and then a small amount of dental glue (#595953WW; Ivoclar Vivadent Inc.; Amherst, NY) was applied between the labium and the side of the pipette tip, ensuring the same position throughout the experiment. Next, both antennae were removed. A

small hole was cut into a 1 cm^2 piece of aluminum foil and then fixed to the fly using dental glue, creating a sealed window of cuticle exposed. Artificial hemolymph (140 mM NaCl, 2mM KCl, 4.5 mM MgCl2, 1.5mM CaCl2, and 5mM HEPES-NaOH with pH = 7.1) was applied to the window and then the cuticle and connective tissue were dissected to expose the SEZ. Mounted flies were placed on a Nikon A1R confocal microscope and then imaged using a 20X water-dipping objective lens. The pinhole was opened to allow a thicker optical section to be monitored. All recordings were taken at 4Hz with 256 resolution. Similar to PER, tastants were applied to the proboscis for 1-2s with a wick, which was operated using a micromanipulator (Narishige International USA, Inc.; Amityville, NY). For analysis, regions of interest were drawn manually around posterior IR56D projections. Baseline fluorescence was calculated as the average fluorescence of the first 5 frames, beginning 10 sec prior to tastant application. For each frame, the % change in fluorescence (%ΔF/F) was calculated as: (peak fluorescence - baseline fluorescence)/baseline fluorescence * 100. Average fluorescence traces were created by taking the average and standard error of %ΔF/F for each recording of a specific tastant.

Aversive Taste Memory

Taste discrimination was assessed my measuring aversive taste memory, as described previously(Tauber et al., 2017). Female flies were starved for 48 h prior to each experiment. Flies were then anaesthetized on CO_2 and the thorax of each fly was glued to a microscope slide using clear nail polish (#451D; Wet n Wild, Los Angeles, CA). Flies were acclimated to these conditions in a humidified box for 60 min. For each experiment, the microscope slide was mounted vertically under a dissecting microscope (#SM-1BSZ-144S; AmScope; Irvine, California). Flies were water satiated prior to each experiment and in between each test/training session. For tastant presentation, we used a 200 µL pipet tip attached to a 3ml syringe (#14-955-457; Fisher Scientific). For the pretest, 1% fatty acid was presented to the proboscis 3 times, with 1 min in between each presentation, and the number of full proboscis extensions

was recorded. During training, a similar protocol was used except that each tastant presentation was immediately followed by 50mM quinine presentation which flies were allowed to drink it for up to 2 sec or until an extended proboscis was retracted. A total of 3 training sessions were performed. In between each session, the proboscis was washed with water and flies were allowed to drink to satiation. To assess taste discrimination, flies were tested either with that same tastant without quinine or with an untrained tastant. Another group of flies were tested as described above but quinine was never presented (naïve). At the end of each experiment, flies were given 1M sucrose to check for retained ability to extend proboscis and all non-responders were excluded.

Statistical Analysis

All measurements are presented as bar graphs showing mean ± standard error. Measurements of PER and aversive taste memory were not normally distributed and so the non-parametric Kruskal-Wallis test was used to compare two or more genotypes. To compare two or more genotypes and two treatments, a restricted maximum likelihood (REML) estimation was used. For data that was normally distributed (calcium imaging data), a one-way or two-way analysis of variance (ANOVA) was used for comparisons between two or more genotypes and one treatment or two or more genotypes and multiple treatments, respectively. All post hoc analyses were performed using Sidak's multiple comparisons test. Statistical analyses and data presentation were performed using InStat software (GraphPad Software 8.0; San Diego, CA).

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597

Figure Legends

Figure 1. *Drosophila* can discriminate between short-, medium-, and long-chain fatty acids, but not among medium-chain fatty acids. **A** An aversive taste memory assay was used to assess FA taste discrimination. First, initial responses to a short-, medium-, or long-chain FA was assessed (Pretest). Next, flies were trained by pairing this FA with quinine (Training). PER in response to either the same or different FA was then tested in the absence of quinine (Test). In control experiments (Naïve), the same procedure was followed, but quinine was not applied to the proboscis. **B** The pairing of medium-chain hexanoic acid (6C) and quinine (red) results in a significant reduction in PER compared to naïve flies. After training, PER response to 6C was significantly lower in trained flies compared to naïve flies (P<0.0001), but there was no difference in PER to short-chain valeric acid (5C; P=0.6864). REML: $F_{1,80}$ = 7.329, P=0.0003, with Sidak's Test for multiple comparisons; N=40-42. **C** The pairing of medium-chain hexanoic acid (6C) and quinine (red) results in a significant reduction in PER compared to naïve flies. After training, PER response to 6C was significantly lower in trained flies compared to naïve flies (P<0.0001), but there was no difference in PER to long-chain nonanoic acid (9C; P=0.3346). REML: $F_{1,64}$ = 6.296, P=0.0146, with Sidak's Test for multiple comparisons; N=33. **D** The pairing of short-chain valeric acid (5C) and quinine (red) results in a significant reduction in PER compared to naïve flies. After training, PER response to 5C was significantly lower in trained flies compared to naïve flies (P=0.0014), but there was no difference in PER to long-chain nonanoic acid (9C; P=0.0789). REML: $F_{1,46}$ = 2.721, P=0.0105, with Sidak's Test for multiple comparisons; N=24. **E** The pairing of medium-chain hexanoic acid (6C) and quinine (red) results in a significant reduction in PER compared to naïve flies. After training, PER to both 6C and medium-chain heptanoic acid (7C) was significantly lower in trained flies compared to naïve flies (6C: P<0.0001; 7C: P<0.0001). REML: $F_{1,81}$ = 45.88, P<0.0001, with Sidak's Test for multiple comparisons; N=41-42. **F** The pairing of medium-chain hexanoic acid (6C) and quinine (red) results in a significant reduction in PER compared to naïve flies. After training, PER to both

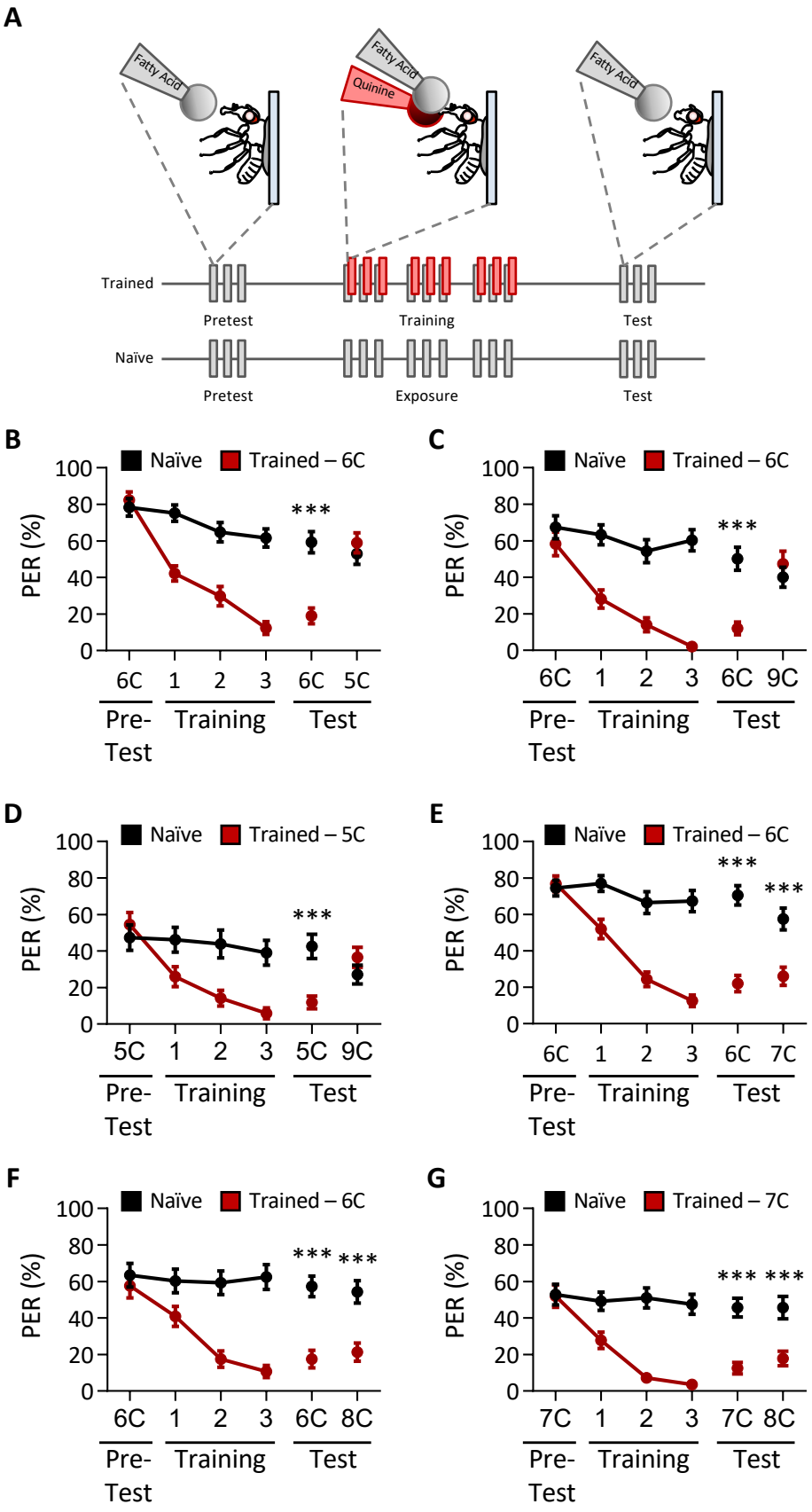
6C and medium-chain octanoic acid (8C) was significantly lower in trained flies compared to naïve flies (6C: $P<0.0001$; 8C: $P<0.0001$). REML: $F_{1,65} = 32.76$, $P<0.0001$, with Sidak's Test for multiple comparisons; N=33-34. **G** The pairing of medium-chain heptanoic acid (7C) and quinine (red) results in a significant reduction in PER compared to naïve flies. After training, PER to both 7C and medium-chain octanoic acid (8C) was significantly lower in trained flies compared to naïve flies (7C: $P<0.0001$; 8C: $P<0.0001$). REML: $F_{1,72} = 33.67$, $P<0.0001$, with Sidak's Test for multiple comparisons; N=37.

Figure 2. Ablation of chemosensory organs has no effect on the ability of *Drosophila* to discriminate between short-, medium-, and long-chain fatty acids. Aversive taste memory was measured as described in Figure 4A. Flies were trained by pairing medium-chain hexanoic acid (6C) with quinine (Training; See Figure S3) and then PER in response to either sucrose, short-chain valeric acid (5C), or long-chain nonanoic acid (9C) was measured in the absence of quinine (Test). **A** Aversive taste memory was measured in unmanipulated control flies (first panel), in flies without antennae (second panel), maxillary palps (third panel), or both antennae and maxillary palps (fourth panel). **B** For all ablation treatments, taste memory to medium-chain hexanoic acid (6C) was significantly lower in trained flies compared to naïve flies, but there was no difference in PER to sucrose. REML: $F_{1,86} = 42.41$, $P<0.0001$, with Sidak's Test for multiple comparisons; N=13-26. **C** For all ablation treatments, taste memory to 6C was significantly lower in trained flies compared to naïve flies, but there was no difference in PER to short-chain valeric acid (5C). REML: $F_{1,103} = 51.87$, $P<0.0001$, with Sidak's Test for multiple comparisons; N=19-31. **D** For all ablation treatments, taste memory to 6C was significantly lower in trained flies compared to naïve flies, but there was no difference in PER to long-chain nonanoic acid (9C). REML: $F_{1,97} = 11.47$, $P=0.0010$, with Sidak's Test for multiple comparisons; N=22-27.

649 **Figure 3.** Silencing *IR56D*-expressing neurons reduces taste perception to medium chain fatty
650 acids. **A** Proboscis extension response (PER). PER was measured in female flies after 48 hrs of
651 starvation. Either sucrose or fatty acid was applied to the fly's labellum for a maximum of two
652 seconds and then removed to observe proboscis extension reflex. **B** Blocking synaptic release
653 by genetic expression of light-chain tetanus toxin (UAS-TNT) in *IR56D*-expressing neurons has
654 no effect on PER to sucrose compared to control flies expressing an inactive form of tetanus
655 toxin (UAS-impTNT). Mann Whitney Test: U = 595, P=0.8410; N=35. **C** Silencing *IR56D*-
656 expressing neurons significantly reduces PER to medium chain fatty acids (6C-8C), but has no
657 effect on PER to either short- (4C,5C) or long-chain fatty acids (9C,10C). REML: $F_{1,406}$ = 25.03,
658 P<0.0001, with Sidak's Test for multiple comparisons; N=24-45.
659
660 **Figure 4.** *IR56D* mediates taste perception to medium-chain fatty acids. **A** *IR56d*^{GAL4} was
661 generated using the CRISPR/Cas9 system. In *IR56d*^{GAL4} flies, the *IR56D* gene was replaced by
662 GAL4 and RFP elements (red boxes). The relative location and orientation of genes in the
663 region are represented as gray arrows. **(B-E)** Expression pattern of *IR56d*^{GAL4} is visualized with
664 GFP**.** *IR56D*-expressing neurons are located on the **(B)** labellum and project to the **(C)**
665 subesophagael zone of the brain. Distinct regions of projection include the **(D)** posterior and **(E)**
666 anterior subesophagael zones. Background staining is NC82 antibody (magenta). Scale bar =
667 50µm. **F** Sucrose taste perception is similar in control and *IR56d*^{GAL4} mutant flies. Kruskal-Wallis
668 Test: H = 0.1758, P=0.9814, with Dunn's Test for multiple comparisons; N=33-40. **G** The
669 *IR56d*^{GAL4} flies have reduced PER to medium-chain fatty acids (6C-8C) relative to control,
670 *IR56d*^{GAL4} heterozygotes, and *IR56d*^{GAL4} rescue flies. However, all genotypes respond similarly
671 to both short- and long-chain fatty acids (4C,5C; 9C,10C). REML: $F_{3,850}$ = 17.80, P<0.0001, with
672 Tukey Test for multiple comparisons; N=28-40.
673

Figure 5. Neuronal activity of *IR56d*^{GAL4} mutant flies is reduced in response to medium-chain fatty acids. **A** Diagram of live-imaging experimental protocol. A tastant is applied to the proboscis while florescence is recorded simultaneously. **(B-D)** Representative pseudocolor images of calcium activity of the posterior projections of *IR56D* neurons in response to water **(B)**, 10mM sucrose **(C)**, or 1% hexanoic acid **(D)**. Scale bar = 50μm. **(E-G)** Activity traces of the posterior projections of *IR56D* neurons in response to each tastant in the **(E)** *IR56d*^{GAL4} heterozygote controls, **(F)** *IR56d*^{GAL4} mutants, and **(G)** *IR56d*^{GAL4} rescue flies. The shaded region of each trace indicates ±SEM. **H** Average peak change in fluorescence for data shown in E-G. Neuronal responses of to medium-chain fatty acids (6C-8C) are significantly reduced in *IR56d*^{GAL4} mutants compared to *IR56d*^{GAL4} heterozygote controls and *IR56d*^{GAL4} rescue flies. All genotypes respond similarly to both short- and long-chain fatty acids (4C,5C; 9C,10C), was well as to water and sucrose. Two-way ANOVA: $F_{2,256}$ = 23.67, P<0.0001, with Sidak's Test for multiple comparisons; N=8-14.

Figure 1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Pre-Test
Training
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Test

Figure 2

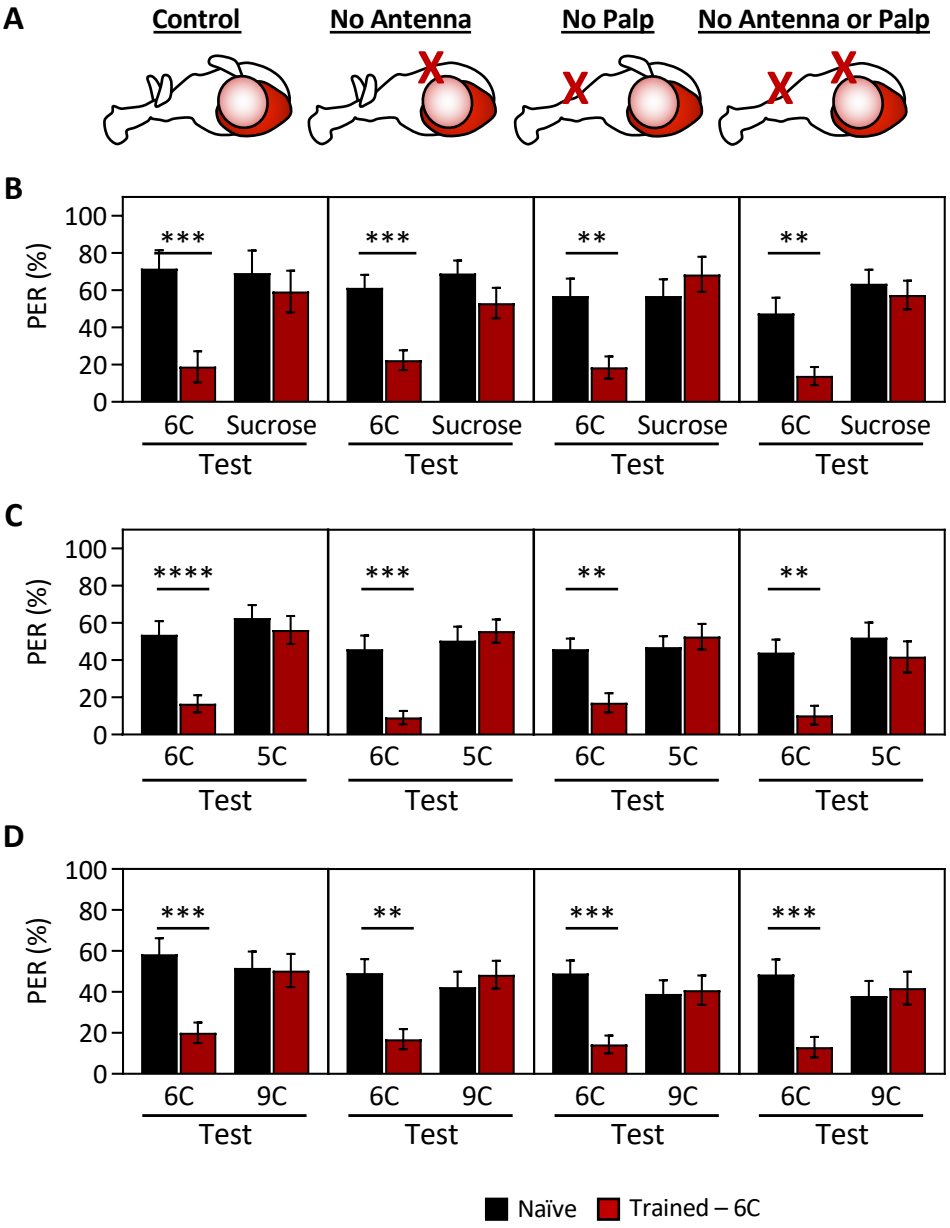

A
Control
No Antenna
No Palp
No Antenna or Palp
B
C
D
PER (%)
100
80
60
40
20
0
***
**
****
6C
Sucrose
5C
9C
Test
Naïve
Trained – 6C

Figure 3

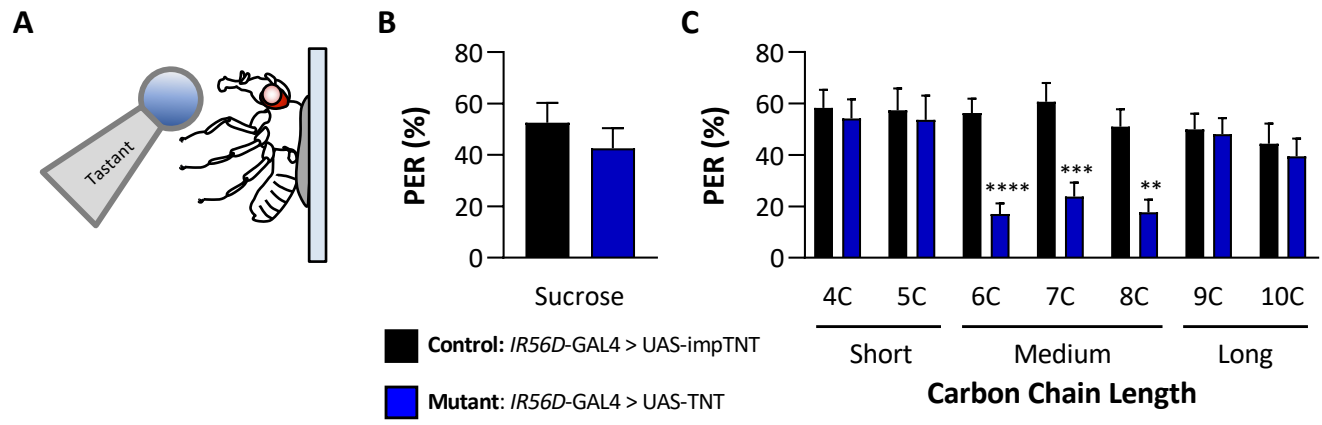

A
Tastant
B
PER (%)
80
60
40
20
0
Sucrose
C
PER (%)
80
60
40
20
0
****
***
**
4C
5C
6C
7C
8C
9C
10C
Short
Medium
Long
Carbon Chain Length
Control: IR56D-GAL4 > UAS-impTNT
Mutant: IR56D-GAL4 > UAS-TNT

Figure 4

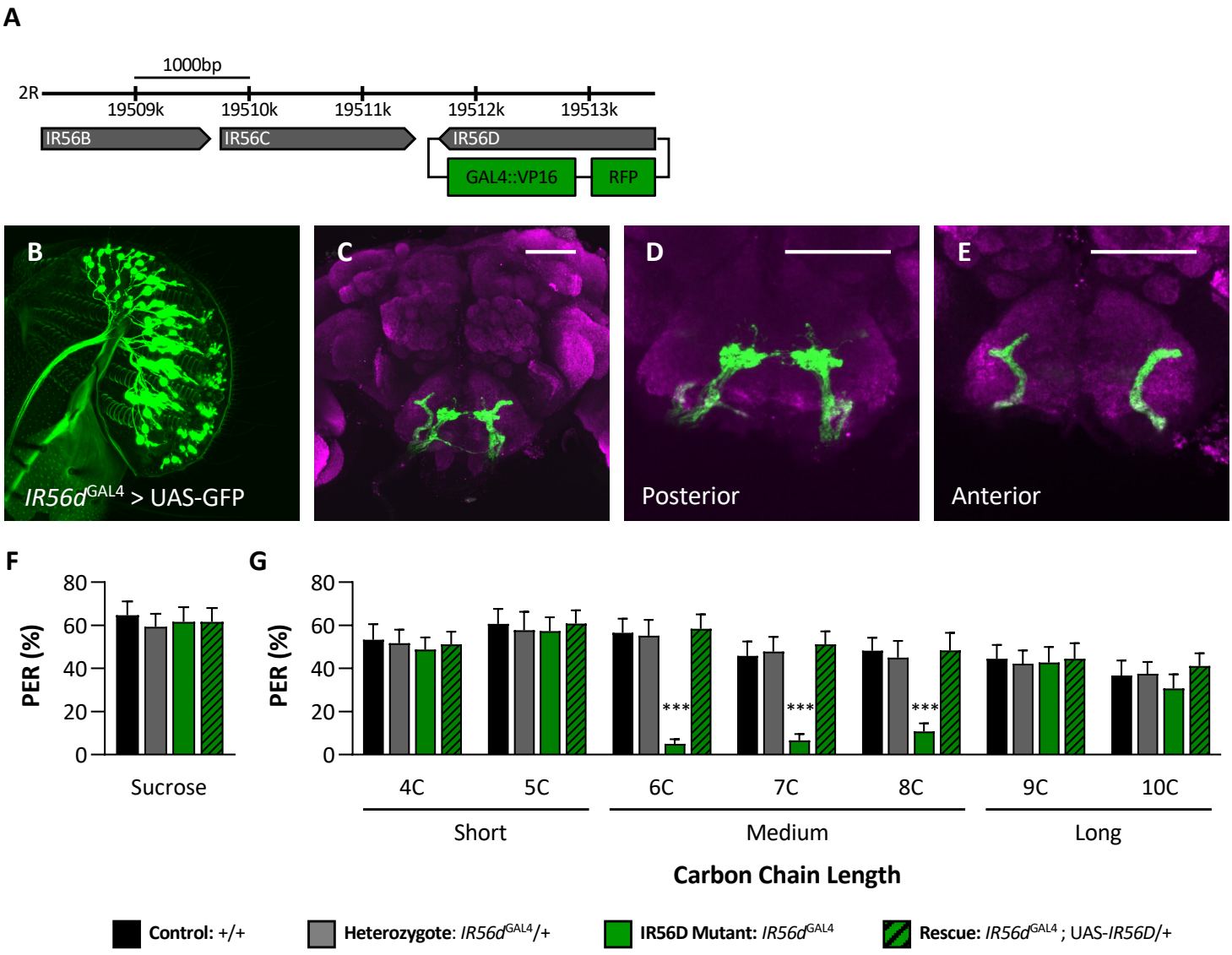
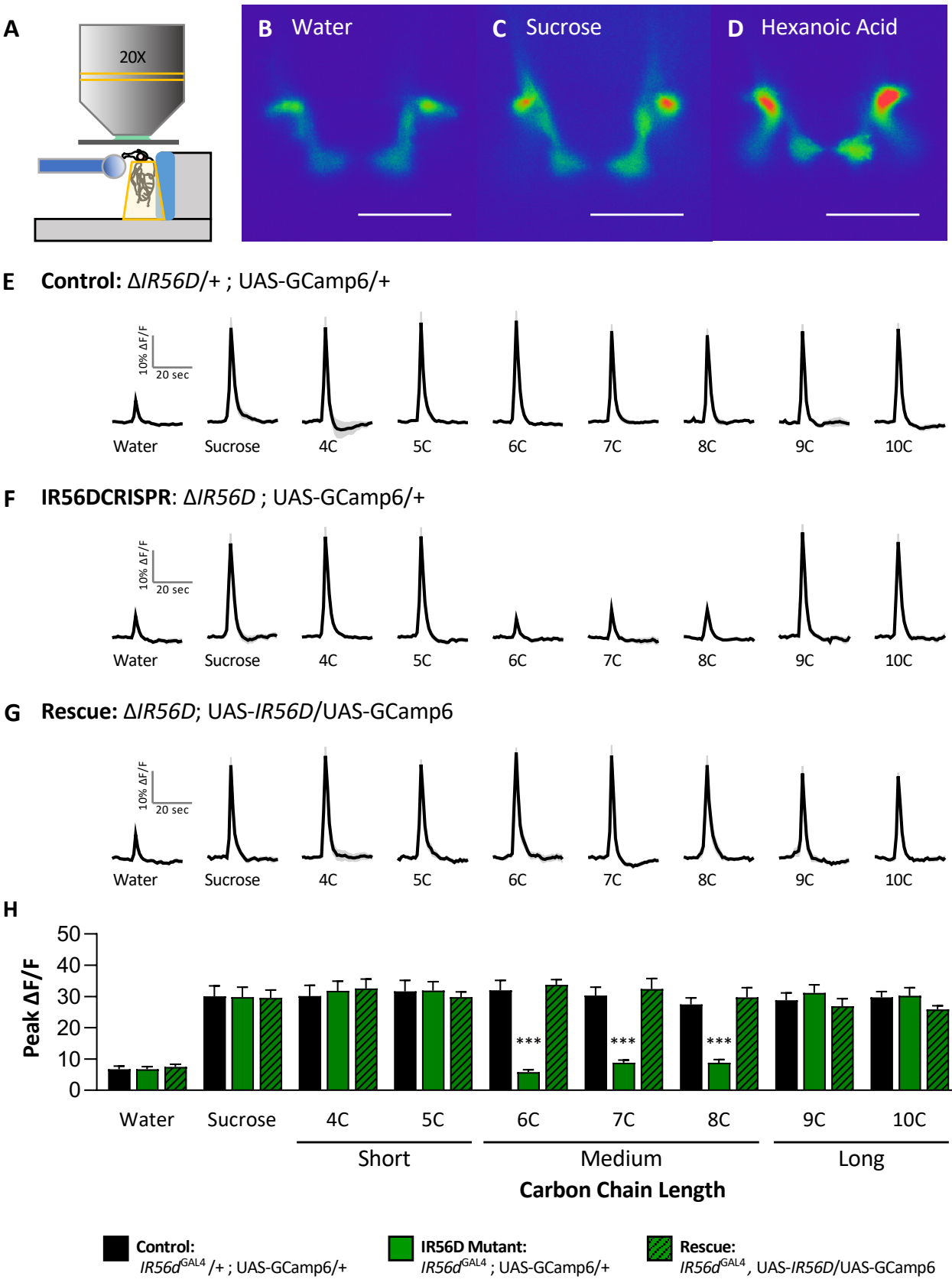

A
1000bp
2R
19509k
19510k
19511k
19512k
19513k
IR56B
IR56C
IR56D
GAL4::VP16
RFP
B
IR56d^GAL4 > UAS-GFP
C
D
Posterior
E
Anterior
F
PER (%)
Sucrose
G
PER (%)
4C
5C
6C
7C
8C
9C
10C
Short
Medium
Long
Carbon Chain Length
Control: +/+
Heterozygote: IR56d^GAL4/+
IR56D Mutant: IR56d^GAL4
Rescue: IR56d^GAL4 ; UAS-IR56D/+

Figure 5


A
20X
B Water
C Sucrose
D Hexanoic Acid
E Control: ΔIR56D/+ ; UAS-GCamp6/+
10% ΔF/F
20 sec
Water
Sucrose
4C
5C
6C
7C
8C
9C
10C
F IR56DCRISPR: ΔIR56D ; UAS-GCamp6/+
G Rescue: ΔIR56D; UAS-IR56D/UAS-GCamp6
H
Peak ΔF/F
50
40
30
20
10
0
***
Short
Medium
Long
Carbon Chain Length
Control: IR56d^GAL4 /+ ; UAS-GCamp6/+
IR56D Mutant: IR56d^GAL4 ; UAS-GCamp6/+
Rescue: IR56d^GAL4 , UAS-IR56D/UAS-GCamp6