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1 **Adult *Drosophila* aversion to caffeine requires a unique TrpA1 isoform and the PLC signaling**
2 **cascade**

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- 5
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1 **ABSTRACT**

2 Taste in *Drosophila melanogaster* is crucial to survival, influencing feeding, mating, and egg-laying
3 behaviors. Taste organs are located on various parts of the body, including the legs, proboscis,
4 wings, and ovipositor. Taste neurons detect chemicals via receptors like GRs, IRs, and TRPs, with
5 bitter and sweet tastes linked to specific neurons (*Gr66a+* and *Gr5a+*). Bitter substances such as
6 caffeine activate neurons, resulting in rejection behavior. TrpA1 channels, associated with
7 aversive responses, are involved in complex behaviors and could interact with taste receptors.
8 Our results show that caffeine mixed with sucrose reduces proboscis extension in flies compared
9 to sucrose alone, a response that requires only the TrpA1-E isoform out of the five possible ones.
10 Furthermore, our data demonstrate that this avoidance requires TrpA1 and signaling via PLC and
11 IP3-receptors in adult *Gr66a+* neurons.

1 INTRODUCTION

2 The sense of taste plays a fundamental role in environmental assessment, allowing organisms to
3 make crucial survival-related decisions. In *Drosophila melanogaster*, gustatory perception
4 regulates key behaviors such as feeding (Dweck & Carlson, 2023; Thorne et al., 2004), mating
5 (Miyamoto and Amrein, 2008), and oviposition (Chen and Amrein, 2017; Joseph et al., 2009;
6 Montell, 2009). A central challenge in food selection lies in distinguishing caloric sources (Dus et
7 al., 2011; Stafford et al., 2012) from toxic compounds (Delventhal et al., 2017) and evaluating
8 mixtures of both (French et al., 2015).

9 The peripheral taste organs of *D. melanogaster* are distributed across several body parts,
10 including the legs (Liman et al., 2014; Ling et al., 2014), wings (Raad et al., 2016), ovipositor (Yang
11 et al., 2008), and the proboscis—a long appendage functionally analogous to the human tongue
12 (Dweck and Carlson, 2023; Stocker, 1994). Taste neurons housed within bristles on these organs
13 directly interact with chemical stimuli through dendrites that contact solid or liquid surfaces.
14 Although individual neurons can detect multiple compounds, their behavioral output is typically
15 consistent (Sung et al., 2017; Wang et al., 2004). This specificity arises from the co-expression of
16 multiple receptor classes, including gustatory (GR), ionotropic (IR), and transient receptor
17 potential (TRP) channels (Chen and Amrein, 2017; Freeman and Dahanukar, 2015; Koh et al.,
18 2014).

19 Among the best-characterized gustatory neurons are those expressing the Gr66a receptor (cells),
20 which mediate bitter taste (Thorne et al., 2004; Wang et al., 2004), and Gr5a-expressing neurons
21 (*Gr5a+* cells), which respond to sugars (Dahanukar et al., 2001; Ueno et al., 2001). Sweet

1 compounds such as sucrose activate *Gr5a+* neurons, whereas bitter substances like caffeine
2 stimulate neurons, where Gr66a serves as a co-receptor (Amrein and Thorne, 2005; Lee et al.,
3 2009; Moon et al., 2006). Activation of these distinct neuronal populations leads to acceptance
4 or rejection of food, respectively (Thorne et al., 2004).

5 Beyond gustation, neurons contribute to aversive responses to environmental stressors such as
6 UV radiation through the TRP channel TrpA1 (Guntur et al., 2017; Rosenzweig et al., 2005). TrpA1
7 has been widely studied in *Drosophila*, where it mediates responses to temperature extremes (Gu
8 et al., 2019), mechanical stimuli (Gong et al., 2023, 2022), and chemical irritants (Kim et al., 2010).
9 While caffeine modulates TRPA1 activity in mammals (Nagatomo and Kubo, 2008), *Drosophila*
10 TrpA1 does not appear to influence caffeine avoidance in binary choice assays or taste sensilla
11 recordings (Kim et al., 2010). Nonetheless, TrpA1 may participate in more complex aversive
12 mechanisms through interactions with gustatory receptors (Kwon et al., 2010).

13 Feeding behavior in *Drosophila* follows a sequence of sensory checkpoints—from olfactory
14 detection to leg contact, proboscis engagement, ingestion, and consumption (Thoma et al., 2017).
15 Given TrpA1's role in detecting environmental threats, we investigated whether this channel
16 mediates caffeine avoidance at specific stages of feeding. We focused on the proboscis and its
17 associated feeding reflex, the Proboscis Extension Reflex (PER), in which flies extend the proboscis
18 in response to palatable stimuli (Schwarz et al., 2017).

19 We found that adding caffeine to sucrose significantly reduced PER compared to sucrose alone.
20 Of the five known *Drosophila* TrpA1 isoforms, only TrpA1-E was required for this effect. Caffeine-

1 mediated suppression of PER depends on *TrpA1* expression in neurons and involves a signaling
2 cascade including phospholipase C (PLC) and the inositol trisphosphate receptor (IP3R).

3 These findings reveal a more nuanced role for TrpA1 in taste aversion, extending its function
4 beyond nociception to cooperative signaling with gustatory pathways during feeding-related
5 decision-making.

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1 RESULTS

2 Caffeine Elicits Aversive Responses in a Proboscis Extension Reflex Assay

3 Caffeine is widely recognized as an aversive compound in *Drosophila*, with behavioral
4 quantification typically performed using two-choice assays (Lee et al., 2009) or the multiCAFE
5 assay (Sellier et al., 2011). These paradigms are effective for assessing attraction or aversion to
6 specific chemicals and for quantifying consumption when flies freely explore their environment
7 in groups over periods ranging from minutes to hours.

8 To investigate whether TrpA1 is necessary for caffeine-induced aversion and to delineate the
9 neuronal populations and molecular pathways involved, we focused on a reflexive feeding
10 behavior: the Proboscis Extension Reflex (PER). PER is triggered when taste sensilla on the legs or
11 proboscis contact a stimulus. A palatable substance induces immediate proboscis extension,
12 whereas aversive compounds suppress it. In this study, we selectively stimulated the proboscis.
13 To distinguish active aversion from simple disinterest, caffeine was tested in combination with 1
14 mM sucrose, which reliably induces PER (Video 1). A significant reduction in PER when caffeine is
15 added to sucrose indicates an aversive response rather than a lack of interest.

16 Historically, two-choice arenas, multiCAFE assays, and electrophysiology have been the preferred
17 methodologies for assessing responses to caffeine, with PER used primarily to validate its aversive
18 properties (Mi et al., 2023). To establish the optimal caffeine concentration for our study, 5- to 7-
19 day-old w^- females were exposed to increasing caffeine concentrations mixed with 1 mM sucrose.
20 Consistent with previous findings (Montanari et al., 2024), sucrose alone elicited PER in

1 approximately 80% of flies (Fig. 1a; Video 1). Addition of 1 mM (S1C1) or 5 mM (S1C5) caffeine
2 did not significantly affect PER levels. At 7.5 mM caffeine (S1C7.5), PER decreased to 50%,
3 suggesting partial aversion. At 10 mM caffeine (S1C10), only 20% of flies extended their proboscis,
4 demonstrating a robust, reproducible aversive response (Fig. 1a; Video 2). Therefore, S1C10 was
5 selected as the optimal test solution for subsequent TrpA1 experiments.

6 **Flies from both sexes and of different genetic backgrounds show Caffeine-Induced Aversive** 7 **Responses**

8 Previous studies on taste perception and behavior in *Drosophila* focused primarily on female flies
9 (Kurz et al., 2017; Masuzzo et al., 2022, 2019; Montanari et al., 2024), largely due to females'
10 greater resistance to starvation protocols required to increase responsiveness in PER assays. To
11 assess potential sexual dimorphism, we measured PER responses in males exposed to S1C10. Both
12 sexes exhibited caffeine aversion, with no significant difference between males and females (Fig.
13 1b). Under these conditions, sexual dimorphism in caffeine-induced PER suppression was not
14 detected. Consequently, all subsequent experiments were conducted using 5- to 7-day-old female
15 flies. Genetic background can affect behavioral responses in *Drosophila* (Zimmerman et al., 2012).
16 To ensure that caffeine-induced PER suppression was not strain-specific, we tested control lines
17 including *w⁻*, *yw*, Canton-S, and Oregon-R. All genotypes exhibited strong PER to 1 mM sucrose
18 and significant PER reduction upon addition of 10 mM caffeine (S1C10) (Fig. 1c). These results
19 demonstrate that caffeine-induced aversion is conserved across these genetic backgrounds.

20

1 **The TrpA1 and PLC Pathways in Neurons Are Essential for Caffeine-Induced Aversion**

2 In *Drosophila*, taste perception has been studied extensively, and the cellular and molecular bases
3 of avoidance behaviors are relatively well understood (Weiss et al., 2011). A central aspect of
4 aversive taste responses is the activity of neurons expressing specific gustatory receptors (GRs).
5 These neurons can be visualized and targeted based on their gene expression profiles. Among
6 them, *Gr66a+* neurons are widely used as markers for bitter-sensitive cells, and Gr66a itself is
7 essential for caffeine detection (Lee et al., 2009). Accordingly, *Gr66a+* neurons play a key role in
8 mediating aversive feeding behaviors (Joseph and Heberlein, 2012; Moon et al., 2006) (Fig. 2a and
9 a').

10 In our previous work, we confirmed that *Gr66a+* cells are necessary for caffeine-induced aversion
11 in the PER assay. To test this, we inactivated these neurons by overexpressing the inward rectifier
12 potassium channel Kir2.1, which impairs action potential generation throughout development
13 (*Gr66a-Gal4/UAS-Kir2.1*) (Baines et al., 2001; Hodge, 2009; Montanari et al., 2024). As expected,
14 this manipulation strongly reduced PER responses to S1C10, confirming that *Gr66a+* neurons are
15 required for caffeine perception (Moon et al., 2006).

16 Having established their importance, we then investigated the signaling pathways within *Gr66a+*
17 neurons that mediate caffeine aversion. Both Gr66a and Gr93a proteins have been shown to be
18 essential for caffeine responses in preference tests, electrophysiology, and PER assays (Lee et al.,
19 2009; Montanari et al., 2024; Moon et al., 2006). Given the involvement of the TRP channel TrpA1
20 in caffeine aversion in mammals (Nagatomo and Kubo, 2008), we hypothesized that it could also
21 contribute to PER suppression in flies. The *TrpA1* gene produces five protein isoforms (A–E)

1 through alternative splicing (Gu et al., 2019; Zhong et al., 2012). To test whether *TrpA1* in *Gr66a+*
2 neurons is required for caffeine-induced aversion, we used a cell-specific RNA interference
3 approach. Because the different isoforms share high sequence homology, isoform-specific RNAi
4 was not feasible; instead, we employed a pan-isoform RNAi under the *Gr66a-Gal4 driver*. Flies
5 carrying only the RNAi construct (without the driver) or only the driver displayed normal PER
6 responses, extending the proboscis to sucrose and reducing it when caffeine was added. By
7 contrast, when *TrpA1* was selectively knocked down in *Gr66a+* neurons (*Gr66a-Gal4/UAS-*
8 *TrpA1_IR*), flies failed to discriminate between sucrose and the sucrose-caffeine mixture, showing
9 no reduction in proboscis extension (Fig. 2b). These results confirm that *Gr66a+* neurons mediate
10 the aversive response to S1C10 and demonstrate that *TrpA1* activity within these neurons is
11 essential for the immediate behavioral response to caffeine.

12 In the context of taste perception, intracellular signaling cascades downstream of receptor
13 activation—particularly those involving phosphodiesterase (PDE) and phospholipase C (PLC)—
14 have been implicated in the neuronal transduction of taste stimuli (Kwon et al., 2010; Poole and
15 Tordoff, 2017). To assess whether these canonical pathways contribute to caffeine-induced
16 aversion in the PER assay (S1C10), we selectively silenced *rutabaga*, which encodes PDE (Levin et
17 al., 1992), and *plc21C* (Shortridge et al., 1991) transcripts in *Gr66a+* neurons using RNA
18 interference. Flies were then tested for their proboscis extension reflex in response to sucrose
19 alone or to a sucrose-caffeine mixture. Knockdown of *plc21C* in *Gr66a+* neurons markedly
20 impaired the aversive response to sucrose + caffeine, whereas silencing *rutabaga* had no
21 detectable effect (Fig. 2b). These findings identify PLC signaling as a critical component of the
22 molecular cascade underlying caffeine-induced suppression of the PER in *Gr66a+* cells.

1 Stage-Specific Requirement of PLC and IP3 Receptor in Caffeine Aversion

2 In our previous work, we demonstrated that early-life experiences can shape adult taste
3 discrimination in *Drosophila*, particularly for molecules of bacterial origin (Montanari et al., 2024).
4 To determine whether the role of PLC in caffeine-induced aversion is required specifically in
5 adulthood or influenced by its absence during development, we conditionally inactivated *plc21C*
6 in *Gr66a+* neurons at distinct life stages using the thermosensitive Gal4 repressor *Tub-Gal80^{ts}*. As
7 shown in Fig. 2c, silencing *plc21C* during the adult stage markedly impaired the aversive PER
8 response to sucrose + caffeine, whereas its inactivation during the larval stage had no detectable
9 effect on the adult phenotype. These findings indicate that PLC signaling in *Gr66a+* neurons is
10 required specifically during adulthood to mediate caffeine-induced aversion. To further dissect
11 this signaling cascade, we tested whether the inositol 1,4,5-trisphosphate receptor (IP3R) is also
12 required in adulthood for the aversive response. Using the same conditional approach (*Gr66a-*
13 *Gal4; Tub-Gal80^{ts}/UAS-ip3R_IR*), we found that adult-stage inactivation of IP₃R similarly abolished
14 the reduction in PER upon caffeine exposure (Fig. 2c). Together, these results identify PLC and
15 IP3R as key, adult-specific components of the intracellular signaling pathway mediating caffeine
16 aversion in *Gr66a+* neurons.

1

2 **TrpA1-E isoform mediates the response to caffeine.**

3 After establishing through cell-specific RNA interference that TrpA1 and the PLC pathway are
4 required for caffeine aversion in *Gr66a+* neurons, we next asked which TrpA1 isoform mediates
5 this response (Fig. 3a). Genetic tools exist in which the entire *TrpA1* gene is deleted and replaced
6 with constructs allowing selective expression of one or more isoforms (Gu et al., 2019). These
7 lines enable functional studies to determine whether a given isoform is necessary or sufficient for
8 a particular behavior (Gong et al., 2023, 2022; Gu et al., 2019). Importantly, prior work using these
9 genetic tools demonstrated that all *TrpA1+* cells are also *Gr66a+* (Sato et al., 2023). We first
10 examined the PER in *TrpA1* null mutants lacking the entire gene. In control flies, sucrose (1 mM)
11 induces robust proboscis extension, whereas the sucrose–caffeine mixture (S1C10) elicits
12 aversion, reflected by suppressed extension. By contrast, *TrpA1* mutants extended their proboscis
13 equally to both solutions (Fig. 3b), confirming that *TrpA1* is essential for caffeine-induced aversion
14 and corroborating our RNAi results (Fig. 2b). To identify the isoform responsible for caffeine
15 avoidance, we tested three isoform-specific knockout (KO) lines: *AD-KO*, *BC-KO*, and *E-KO* (Fig. 3a
16 and 3b). All lines extended their proboscis to sucrose alone but only the *E-KO* line failed to reduce
17 PER in response to the sucrose–caffeine mixture, indicating a loss of aversion (Fig. 3b). These
18 results suggest that isoforms A–D are dispensable, whereas the E isoform is critical for caffeine-
19 induced aversion. Notably, *E-KO* flies were tested in other studies and do not exhibit general
20 deficits in TrpA1-dependent behaviors, such as responses to heat or reactive oxygen species (ROS)

1 (Gu et al., 2019; Zhong et al., 2012), suggesting a specific role for the E isoform in bitter taste
2 signaling.

3 To further validate this finding, we tested knock-in (*KI*) lines that can only express one specific
4 TrpA1 isoform (A–E) (Gu et al., 2019). Flies expressing isoforms A–D displayed PER responses
5 similar to *TrpA1* null mutants, showing no aversion to sucrose–caffeine mixtures. In contrast, the
6 *E-KI* line behaved like wild-type flies, suppressing PER in response to caffeine, confirming that the
7 E isoform is necessary for this behavior (Fig. 3b). Finally, crossing the *A-KI* and *D-KI* lines to
8 generate *BCE-KO* flies, which lack isoforms B, C, and E, recapitulated the *E-KO* phenotype, further
9 supporting the specific requirement of TrpA1-E for caffeine avoidance.

10 Taken together, these data demonstrate that the TrpA1-E isoform is necessary for PER
11 suppression when caffeine is added to a sucrose solution. To determine whether the TrpA1-E
12 isoform was necessary for aversion to other chemicals, we tested the PER responses of *TrpA1-E-*
13 *KO* and *TrpA1-E-KI* flies to quinine. The results demonstrate that the TrpA1-E isoform is required
14 for aversion to quinine, as well as to caffeine (Supplementary Fig. S1). As all *TrpA1+* cells also
15 express *Gr66a* (Sato et al., 2023), this finding supports a central role for TrpA1-E in *Gr66a+* cells
16 in mediating avoidance behavior toward aversive compounds.

17 The requirement of the TrpA1-E isoform for caffeine-induced aversion suggests that the cells
18 expressing this isoform are directly responsible for the observed behavior. To test this, we
19 performed PER assays in flies in which *TrpA1-E+* cells were silenced by overexpressing the inward-
20 rectifier potassium channel Kir2.1 throughout development (larvae, pupae, and adults),
21 effectively impairing action potential generation (*TrpA1-E-Gal4/UAS-Kir2.1*). Control flies carrying

1 the UAS transgene without the driver (*+ / UAS-Kir2.1*) or only the driver (*TrpA1-E-Gal4/+*) extended
2 their proboscis in response to sucrose but not to the sucrose–caffeine mixture (S1C10), consistent
3 with expected aversion (Fig. 3c). In contrast, flies with silenced *TrpA1-E+* cells responded normally
4 to sucrose but failed to suppress PER when caffeine was added, indicating that these neurons are
5 necessary for the reduction of proboscis extension in the presence of caffeine (Fig. 3c).

6 To confirm that this effect depends specifically on TrpA1 activity in *TrpA1-E+* cells, we used a pan-
7 isoform RNAi construct under the control of the *TrpA1-E-Gal4* driver. Flies expressing only the
8 RNAi transgene (*+ / UAS-TrpA1_IR*) behaved like controls, extending their proboscis to sucrose and
9 reducing PER in response to sucrose–caffeine. By contrast, *TrpA1-E+* cells-specific RNAi (*TrpA1-E-*
10 *Gal4/UAS-TrpA1_IR*) completely abolished caffeine avoidance, phenocopying the *TrpA1* null
11 mutants (Fig. 3b and 3c).

12 To further substantiate the role of *TrpA1-E+* cells in caffeine aversion, we performed two-choice
13 feeding assays using 96-well plates with colored agar overlaid with test solutions (Weiss et al.,
14 2011). Freely moving females were allowed to feed, and abdominal coloration was used to assess
15 substrate preference (Supplementary Fig. S2). Control (*w⁻*) flies strongly preferred 1 mM sucrose
16 over water and over sucrose–caffeine mixtures (preference index > 0.79) (Fig. 3d). In contrast,
17 *TrpA1-KO* and *TrpA1-E-KO* flies showed markedly reduced avoidance of the sucrose–caffeine
18 solution (preference index < 0.6), with most individuals consuming both solutions (Fig. 3d). *TrpA1-*
19 *E-KI* flies behaved like controls, demonstrating robust avoidance (preference index > 0.75).
20 Interestingly, the *TrpA1¹* allele used in the pioneer study (Kim et al., 2010) also responded similarly
21 to wild-type flies, suggesting that this mutation may not completely abolish TrpA1 function or

1 may differentially affect specific isoforms (Fig. 3d). Together, these findings provide compelling
2 evidence that *TrpA1-E+* cells are required for caffeine aversion, likely mediated specifically by the
3 *TrpA1-E* isoform. Furthermore, results from cell-targeted RNAi and multiple mutants, including
4 *TrpA1¹*, suggest that the *TrpA1¹* mutation may not produce a complete loss of function or may
5 differentially affect *TrpA1* isoforms.

6 ***TrpA1-E+* cells are taste neurons of the proboscis that respond to caffeine.**

7 Having established that *TrpA1-E+* cells are necessary for the PER response to the sucrose–caffeine
8 mixture (S1C10), and noting that previous work using a pan-isoform *TrpA1* driver showed that all
9 *TrpA1+* cells are also *Gr66a+* (Sato et al., 2023), we next investigated the specific localization of
10 *TrpA1-E+* cells within the proboscis, focusing on their association with taste sensilla. Such neurons
11 have previously been implicated in aversive behaviors triggered by chemical contact with
12 proboscis taste sensilla (Liman et al., 2014) To investigate this, we imaged the proboscis of female
13 flies expressing GFP under the control of the *TrpA1-E-Gal4* driver. On average, we observed ~10
14 *TrpA1-E+* cells per labellum, each sending a distinct projection backward into the pharynx.
15 Additional processes extended into the taste sensilla, reaching their tips (Fig. 4a–4a’). These
16 morphological features are consistent with previously described proboscis taste neurons (Liman
17 et al., 2014). Because the PER is a rapid reflex triggered by contact of the proboscis taste sensilla
18 with a stimulus-soaked surface, our anatomical and functional data strongly suggest that the
19 reduction of PER in response to sucrose–caffeine (S1C10) is mediated by *TrpA1-E* expressed in
20 these specific proboscis taste neurons. Thus, *TrpA1-E+* cells within the proboscis are integral to
21 caffeine-induced aversion.

1 To further confirm that *TrpA1-E+* cells directly respond to caffeine, we monitored intracellular
2 calcium dynamics using GCaMP (Jayaraman and Laurent, 2007). Proboscises were exposed to
3 caffeine, and fluorescence changes in the cell bodies of *TrpA1-E+* neurons were quantified (*TrpA1-*
4 *E-Gal4/UAS-GCaMP*). These experiments revealed that *TrpA1-E+* cells respond directly to caffeine
5 (Fig. 5). Importantly, this calcium response was abolished when *TrpA1* was knocked down
6 specifically in *TrpA1-E+* cells using RNAi (*TrpA1-E-Gal4/UAS-GCaMP; UAS-TrpA1_IR*) (Fig. 5),
7 confirming that *TrpA1* is required for the direct detection of caffeine by these neurons.

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2 **DISCUSSION**

3 Previous studies have examined the effects of caffeine on adult flies using various approaches,
4 including the CAFé assay (Sellier et al., 2011), two-choice assays (Kim et al., 2010), and
5 electrophysiological recordings (Kim et al., 2010). These studies identified key receptors, such as
6 Gr66a (Moon et al., 2006) and Gr93a (Lee et al., 2009), as essential for caffeine perception. TrpA1,
7 a well-characterized sensor in flies, has also been implicated in aversion to certain chemicals,
8 including aristolochic acid (Kang et al., 2010; Kim et al., 2010; Kwon et al., 2010). Interestingly,
9 when assayed in some of these studies, the *TrpA1* mutants used still exhibited caffeine avoidance
10 in behavioral assays and electrophysiological responses, leading to the conclusion that TrpA1
11 might not be required for caffeine aversion in adult flies (Kim et al., 2010). In contrast, our findings
12 show that TrpA1 is indeed necessary for caffeine avoidance in PER and two-choice assays, as well
13 as for *TrpA1+* cell activity following caffeine exposure, as demonstrated by our experiments using
14 knockout (KO), knock-in (KI) mutants, RNAi and GCaMP.

15 The primary assay in our study was the proboscis extension reflex (PER), which differs from two-
16 choice assays. In a typical two-choice assay, food-deprived flies select between two solutions, one
17 containing caffeine. The PER assay, however, measures a fly's immediate reflex upon contact with
18 a substance, providing a more direct readout of taste response. Notably, caffeine (10 mM) mixed
19 with 5 mM sucrose causes aversion in two-choice assays, whereas in the PER assay, caffeine mixed
20 with sucrose concentrations above 2 mM is no longer aversive (Montanari et al., 2024). While
21 two-choice assays capture physiologically relevant, group-based decision-making over a longer

1 period and can involve additional sensory inputs such as leg-mediated taste or odor, the PER
2 reflects rapid, reflexive responses. In our tests, *TrpA1* mutants failed to respond to caffeine in
3 two-choice assays, whereas the *TrpA1*¹ allele did, suggesting that this allele may not completely
4 disrupt all TrpA1 functions or isoforms.

5 Electrophysiological measurements, which assess immediate neuronal responses, also differ from
6 behavioral PER outcomes. *TrpA1* is expressed in several taste neurons, including those of the S6
7 sensilla (Kwon et al., 2010). Bitter neurons in these sensilla may respond to caffeine
8 independently of TrpA1, as observed with citronellal, which triggers both TrpA1-dependent and -
9 independent responses (Kwon et al., 2010). The discovery of five TrpA1 isoforms with distinct
10 functions further complicates the interpretation of activation patterns. Genetic compensation is
11 unlikely to explain discrepancies, as our mutants and RNAi treatments produced consistent
12 phenotypes (Dy et al., 2024). Therefore, differences between our results and prior studies likely
13 reflect variations in assay type, sensilla tested, and the specific effects of mutations on the newly
14 described TrpA1 isoforms.

15 **TrpA1 and PLC/IP3R signaling**

16 Our study supports a conserved relationship between TrpA1 and PLC signaling observed in both
17 mammalian (Zhang et al., 2003) and *Drosophila* models (Kim et al., 2010; Kwon et al., 2010).
18 Gustatory responses to sweet and bitter stimuli often involve TRP channels, such as TRPM5, and
19 phospholipase C (PLC β 2) (Zhang et al., 2003). In insects, TrpA1 functions downstream of PLC
20 (Kwon et al., 2008). PLC activity generates inositol trisphosphate (IP3) and diacylglycerol (DAG);
21 DAG may directly activate TrpA1 to allow ion influx, while IP3 releases calcium from ER stores via

1 the IP3 receptor (IP3R), altering cytoplasmic calcium levels and triggering action potentials. In
2 taste neurons, this cascade likely involves upstream elements, including G-alpha proteins and
3 caffeine-activated GPCRs, and enhances signal specificity. TrpA1 acts as a molecular integrator,
4 converting PLC-induced Ca²⁺ fluxes into depolarizing currents with temporally regulated
5 dynamics. Rapid IP3-mediated Ca²⁺ spikes may drive acute aversion, while sustained DAG
6 signaling could mediate adaptation.

7 **The E-isoform of TrpA1**

8 First described in 2019, the E-isoform of TrpA1 had not been assigned a specific function, unlike
9 the B- and C-isoforms (Gu et al., 2019). Our data show that TrpA1-E is essential for the reflexive
10 response to caffeine. While it may not act as a direct caffeine sensor, TrpA1 channels can function
11 as “alarm signals,” depolarizing neurons following receptor-ligand interactions. Thus, both the
12 expression of TrpA1 and its interaction with gustatory receptors are crucial. The functional
13 distinctions of each isoform likely dictate these interactions. Notably, TrpA1-E is required for PER
14 aversion to caffeine and is expressed in fewer than ten neurons, compared to approximately
15 thirty *Gr66a+* cells per labellum involved in this response, suggesting that the E-isoform defines a
16 critical subset of *Gr66a+* neurons essential for caffeine detection.

17 MATERIAL AND METHODS

18 **Fly husbandry**

19 Flies were grown at 25°C on a yeast/cornmeal medium in 12h/12h light/dark cycle-controlled
20 incubators. For 1 L of food, 8.2 g of agar (VWR, cat. #20768.361), 80 g of cornmeal flour

1 (Westhove, Farigel maize H1) and 80 g of yeast extract (VWR, cat. #24979.413) were cooked for
2 10 min in boiling water. 5.2 g of Methylparaben sodium salt (MERCK, cat. #106756) and 4 mL of
3 99% propionic acid (CARLOERBA, cat. #409553) were added when the food had cooled down. We
4 use a protein-rich rearing media, in contrast to the sugar-rich media used in some other
5 laboratories. Consequently, our animals may be more sensitive to low sucrose concentrations (1
6 mM) compared to flies raised on sugar-rich media. Therefore, assays using 1 mM sucrose may not
7 yield reproducible results when performed with flies reared on sugar-rich media.

8 **Fly stocks**

9 The reference strain in this study corresponds to $w^{[1118]}$ (Bloomington #5905). Other lines were
10 Canton S (Bloomington #64349) and $y^{[1]}$, $w^{[1118]}$ (gift from Bernard Charroux) and *Gr66a-Gal4*
11 (Bloomington #28801) and *UAS-Kir2.1* (Bloomington #6595) and *Tub-Gal80^{ts}* (Bloomington
12 #7016) and *UAS-Rutabaga_IR* (Bloomington #27035) and *UAS-GFP* (Bloomington #32195) and
13 *UAS-PLC21C_IR* (Bloomington #31270) and *UAS-TrpA1_IR* (Bloomington #36780) and *UAS-*
14 *IP3R_IR* (Bloomington #25937) and *TrpA1¹* (Bloomington #26504) and *UAS-GCaMP7s* (Gift from
15 Matthieu Cavey). The *UAS-TrpA1_IR* (Bloomington #36780) should target all the isoforms as it
16 corresponds to a sequence in exon 17, an exon shared by all the isoforms. All the *TrpA1* isoform-
17 specific genetic tools (*TrpA1-KO* and *TrpA1-AD-KO* and *TrpA1-BC-KO* and *TrpA1-E-KO* and *TrpA1-*
18 *A-KI* and *TrpA1-B-KI* and *TrpA1-C-KI* and *TrpA1-D-KI* and *TrpA1-E-KI* and *TrpA1-E-Gal4*) were
19 kindly provided by the Yang Xiang laboratory. The *TrpA1-BCE-KO* is an F1 obtained after having
20 crossed together the *TrpA1-A-KI* with the *TrpA1-D-KI*. These *TrpA1* genetic tool kit lines are based
21 on a complete *TrpA1* gene deletion, replaced in situ with constructs allowing the specific
22 expression of one or several isoforms, including *Gal4* driver constructs (Gu et al., 2019). These fly

1 lines make it possible, for example, to investigate whether a given isoform is necessary or
2 sufficient for a particular mechanism (Gong et al., 2023).

3 **RNAi timely controlled**

4 The ubiquitously expressed *Tub-Gal80^{ts}*, that inhibits the activity of Gal4, is temperature sensitive:
5 it's active at 18°C and inactivated at 29°C, allowing the expression of *UAS* when animals are raised
6 at 29°C and preventing it at 18°C. For RNAi in larvae only, animals were raised from eggs to early
7 pupae at 29°C and then shifted at 18°C. for RNAi in adults only, eggs, larvae and pupae were raised
8 at 18°C and the virgin adults shifted to 29°C.

9 **PER assay**

10 All flies used for the test were females between 5 and 7 days old. Unless experimental conditions
11 require it, the flies are kept and staged at 25°C to avoid any temperature changes once they are
12 put into starvation. The day before, the tested flies are starved in an empty tube with water-
13 soaked plug for 24h at 25°C.

14 Eighteen flies are tested in one assay, 6 flies are mounted on one slide and in pairs under each
15 coverslip. To prepare the slide, three pieces of double-sided tape are regularly spaced on a slide.
16 Two spacers are created on the sides of each piece of tape by shaping two thin cylinders of UHT
17 paste. To avoid the use of carbon dioxide flies are anesthetized on ice. Under the microscope, two
18 flies are stuck on their backs, side by side, on same piece of tape so that their wings adhere to the
19 tape. A coverslip is then placed on top of the two flies and pressed onto the UHT paste, blocking
20 their front legs and immobilizing them.

1 Once all slides are prepared, they are transferred to a humid chamber and kept at 25°C for 1.5
 2 hours to allow the flies to recover before the assay.

3 Flies are tested in pairs, the test is carried out until completion on a pair of flies (under the same
 4 coverslip), and then move on to the next pair.

5 Before the test, water is given to each pair of flies to ensure that the flies are not thirsty and do
 6 not respond with a PER to the water in which the solutions are prepared. Stimulation with the
 7 test solution is always preceded and followed by a control stimulation with a sweet solution, to
 8 assess the fly's condition and its suitability for the test. During the test small strips of filter paper
 9 are soaked in the test solution and used to contact the fly's labellum (three consecutive times per
 10 control and test phase). Contact with the fly's proboscis should be as gentle as possible. Ideally
 11 the head should not move. A stronger touch may prevent the fly from responding to subsequent
 12 stimulation. Based on the protocol needed the test is done following the sequence and the timing
 13 in table 1 below.

14 **Table .1 Aversion protocol**

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

15

1 All solutions to be tested are prepared the test day and stored at room temperature. In the
2 aversion protocol, the control stimulation is performed with 1mM sucrose (D(+)-sucrose $\geq 99.5\%$,
3 p.a. Carl Roth GmbH + Co. KG). This concentration is sufficient to elicit a PER but is not so high as
4 to influence the response to the subsequent test stimulation. After each control or test
5 stimulation, a water-soaked strip is used to tap the proboscis and clean it. The caffeine mixed
6 with the sucrose is Caffeine powder ReagentPlus ref C0750 Sigma Aldrich and quinine Q1125
7 Sigma Aldrich.

8 The response of the fly to each stimulation is recorded and averaged. We distinguish between
9 two types of flies: those able to respond and those unable to respond. To make this distinction,
10 we use three control stimulations before the test and three after the test. Flies that do not
11 respond during the control stimulations (1 mM sucrose) are classified as “unable to respond” and
12 are excluded from the analysis, regardless of their responses during the tests. Flies that respond
13 during the control stimulations are classified as “able to respond,” and only their responses during
14 the tests are included in the statistical analyses. The PER index is calculated as the percentage of
15 flies “able to respond” tested that responded with a PER to the TEST stimulation and represented
16 as $\pm 95\%$ CI.

17 **Choice assay**

18 Two-way choice assays were performed essentially as described (Meunier et al., 2003). Briefly, 5-
19 to 7-day-old female flies (30 ± 10 flies per experiment) were starved for ~ 18 h at 25°C and placed
20 into a 96-well plate with 1% agarose in each well. Alternating wells contained either red
21 (sulforhodamine B, 0.2 mg/mL; Sigma-Aldrich) or blue dye (bleu E133 meilleur du chef, 0.3%).
22 Then we deposit $10\mu\text{L}$ of solution containing sucrose 1mM or Sucrose1mM + Caffein 10mM or

1 water on top of the well. Each experiment is carried out in duplicate by placing the components
2 on two different colors (*e.g.*, sucrose 1mM on blue wells for plate A and on red wells for plate B)
3 to verify whether there is an effect of color. The flies were allowed to feed in the dark for 90 min
4 at ~ 23°C. We transferred the plates to -20°C for 15min in order to rapidly freeze the flies in order
5 to count the flies based on their abdominal color under a binocular microscope. The numbers of
6 flies with blue, red, or purple (mixed red and blue) abdomens were tabulated (Supplementary Fig.
7 S2), and the P.I. was determined: $(N_{\text{suc}} + 0.5N_{\text{mix}})/(N_{\text{suc}} + N_{\text{test}} + N_{\text{mix}})$. The flies without colors in
8 their abdomen were discarded. The number of flies indicated corresponds to animals with colored
9 abdomens. With *Trpa1*¹ allele, the proportion of uncolored flies was remarkably higher than with
10 the other genotypes.

11 **Microscopy**

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

16 **Calcium imaging**

17 *In vivo* adult calcium imaging experiments were performed on proboscises of 5-7 day-old starved
18 mated females. Animals were raised on conventional media with males at 25°C. Flies were starved
19 for 20-24 h in a tube containing a filter paper soaked in water prior to experiments. Flies of the
20 appropriate genotype were anesthetized on ice for 1 h. The heads of the animals were gently
21 harvested and immobilized using insect pins (0.1 mm diameter) placed on the head and on the
22 proboscis. 200 µL of water was placed on the head and proboscis. GCaMP7s fluorescence was

1 viewed with a Leica DM600B microscope under a 25x water objective. Stimulation was performed
2 manually using a pipette with gel loading tip by applying 200 μ L of water or 200 μ L of Caffeine
3 (20mM of caffeine diluted in water for a final caffeine concentration on the preparation of
4 10mM). The gustatory stimulation was continuous as the tastant is in the solution contacting the
5 proboscis. The recording started before the addition of the tastant and the calcium response
6 could be observed following the contact with the proboscis. GCaMP7s was excited using a
7 Lumencor diode light source at 482 nm \pm 25. Emitted light was collected through a 505-530 nm
8 band-pass filter. Images were collected every 500ms. Each experiment consist of the recording of
9 30 images before stimulation to determine a F0 (mean of the 30th images previous stimulation).
10 Then a fluorescence variation in a Region Of Interest (ROI) is calculated as $(F_i - F_0) / F_0 * 100$ from
11 F1 to F131 and substrated to the fluorescence of another ROI that represents the background
12 from F1 to F131. Data were analyzed as previously described (Silbering et al., 2012) by using FIJI
13 (<https://fiji.sc/>).

14 **Statistics and data representation**

15 The Prism software was used for statistical analyses.

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

19 For PER assays, at least 3 independent experiments were performed. The results from all the
20 experiments were gathered and the total amount of flies tested is indicated in the graph. In
21 addition, we do not show the average response from one experiment representative of the

1 different biological replicates, but an average from all the data generated during the independent
2 experiments in one graph. However, each open circle represents the average PER of 1 experiment.

3
4 For choice assays datasets. As the values obtained from one fly are categorical data with a *Red* or
5 *Blue* or *Mixed* value, we used the Chi-square test to take the number of tested flies into account.
6 At least 6 independent experiments were performed and the preference index shown is an
7 average from the PI of each independent experiment.

8 For in vivo calcium imaging, the D'Agostino–Pearson test to assay whether the values are
9 distributed normally was applied. As not all the data sets were considered normal, non-
10 parametric statistical analysis such as Kruskal–Wallis H test was used for all the data presented.
11 For a given genotype, the reference for the comparisons is the exposure to water.

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

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

14 Private link: <https://figshare.com/s/31cce8c3f518b30956c9>

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3 **Ethics statements**

4 N/A

5 **Conflict of interest statement**

6 No conflict of interest

7 **Funding statement**

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11 Economical Development), and the European Council (ERC starting grant, GliSFCo-311403).

12

13 **Data availability**

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

15 Private link: <https://figshare.com/s/31cce8c3f518b30956c9>

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- 18

1 FIGURES

2 Figure 1

3 Dose-dependent caffeine inhibition of PER. (a) Dose dependent aversion to a mixture combining
4 sucrose and caffeine. PER index of w^{1118} female flies to control solutions of sucrose and sucrose +
5 increasing concentrations of caffeine. Below the x-axis, S1 corresponds to sucrose 1mM, S1C1
6 corresponds to a mixture with sucrose 1mM and caffeine 1mM, S1C5 corresponds to a mixture
7 with sucrose 1mM and caffeine 5mM, S1C7.5 corresponds to a mixture with sucrose 1mM and
8 caffeine 7.5 mM and S1C10 corresponds to a mixture with sucrose 1mM and caffeine 10mM. (b
9 and c) Aversion to a mixture combining sucrose and caffeine is not sexually dimorphic and
10 independent of genetic background. PER index of females and males w^{1118} (b) and $y^1 w^{1118}$ or
11 *CantonS* or *Oregon* female flies (c) to control solutions of sucrose and sucrose +caffeine. Labellar
12 PER was measured to 1 mM sucrose plus or minus the indicated chemical. The PER index is
13 calculated as the percentage of flies tested that responded with a PER to the stimulation \pm 95%
14 confidence interval (CI). A PER value of 1 means that 100% of the tested flies extended their
15 proboscis following contact with the mixture, a value of 0,2 means that 20% of the animals
16 extended their proboscis. The number of tested flies (n) is indicated on top of each bar. For each
17 condition, at least 3 groups with a minimum of 10 flies per group were used. ns indicates $p > 0.05$,
18 ** indicates $p < 0.01$, **** indicates $p < 0.0001$ Fisher Exact Test. Further details can be found in
19 the material and methods section and in the source data file.

20

21

22

1 **Figure 2**

2 In cells the TrpA1 channel, a pathway involving Phospholipase C (PLC) and Inositol-3-phosphate
3 receptor (IP3-R) are necessary for the caffeine-triggered aversion. **(a)** Representative confocal
4 fluorescence image of the proboscis of *Gr66a-Gal4/UAS-GFP* flies. **(a')** Merged image with
5 transmitted light. **(b)** While RNAi-mediated inhibition of AMPc synthesis (*UAS-rutabaga_IR*) in
6 cells (*Gr66a-Gal4/UAS-rutabaga_IR*) does not affect caffeine-induced aversion, RNAi-mediated
7 inhibition of *TrpA1* (*TrpA1_IR*) in the cells (*Gr66a-Gal4/UAS-TrpA1_IR*) and of PLC21C (*UAS-*
8 *plc21C_IR*) in cells (*Gr66a-Gal4/UAS-plc21C_IR*) does impair this response. PER index of flies to
9 control solution of sucrose and to sucrose + caffeine. **(c)** PLC21C and IP3-R are functionally
10 required in adult neurons for caffeine-triggered aversion, but are dispensable during larval life.
11 RNAi-mediated inhibition of PLC21C (*UAS-plc21C_IR*) in cells (*Gr66a-Gal4/UAS-plc21C_IR*) and of
12 IP3-R (*UAS-ip3R_IR*) in cells (*Gr66a-Gal4/UAS-ip3R_IR*) was controlled in a precise and timely
13 manner, turning *on* when needed and *off* when required using *Tub-Gal80^{ts}*. The ubiquitously
14 expressed *Tub-Gal80^{ts}*, that inhibits the activity of Gal4, is temperature sensitive: it's active at
15 18°C and inactivated at 29°C, allowing the expression of RNAi constructions in neurons. For **(b)**
16 and **(c)**, PER index is calculated as the percentage of flies tested that responded with a PER to the
17 stimulation \pm 95% CI. The number of tested flies (n) is indicated on top of each bar. For each
18 condition, at least 3 groups with a minimum of 10 flies per group were used. ns indicates $p > 0.05$,
19 **** indicates $p < 0.0001$ Fisher Exact Test. Further details can be found in the material and
20 methods section and in the source data file.

21

22

1 **Figure 3**

2 The TrpA1-E-isoform and the *TrpA1-E+* cells are necessary for the caffeine-triggered aversion. **(a)**

3 Graphical representation of the characterized TrpA1 isoforms produced by the *TrpA1* gene.

4 A/B/C/D/E isoforms share the blue and grey exons, C/D/E share the exons 1 and 2, A and B share

5 the exon 3, A and D share the exon 12, B and C share the exon 13. E is the only isoform not having

6 exon 12 or exon 13. On top is a representation of the *TrpA1* gene with all the exons. **(b)** The TrpA1-

7 E isoform is necessary for the caffeine-triggered aversion in adult flies. Mutant lines impaired for

8 the production of all the isoforms (*TrpA1-KO*) or producing only one specific isoforms (-KI) were

9 tested. For every line assayed, below the x-axis, the TrpA1 isoforms still produced are indicated.

10 **(c)** Impairing the activity of *TrpA1-E+* cell via *UAS-Kir2.1* (*TrpA1-E-Gal4/UAS-Kir2.1*) abrogates

11 caffeine-triggered aversion and RNAi-mediated *TrpA1* inactivation (*UAS-TrpA1_IR*) in the *TrpA1-*

12 *E+* cells (*TrpA1-E-Gal4/UAS-TrpA1_IR*) abrogates the caffeine-induced aversion. **(d)** The TrpA1-E

13 isoform is necessary for the caffeine-triggered aversion in a 2-choices assay. Shown are the

14 preference indexes for different mutants as well as the fraction of flies that ingested the proposed

15 mixtures. For **(b and c)** PER index is calculated as the percentage of flies tested that responded

16 with a PER to the stimulation \pm 95% CI. The number of tested flies (n) is indicated on top of each

17 bar. ns indicates $p > 0.05$, **** indicates $p < 0.0001$ Fisher Exact Test. Further details can be found

18 in the material and methods section and in the source data file. For **(d)**, the averaged preference

19 index is in red with the standard error of the mean obtained from 6 to 8 independent

20 experiments. The total amount of females tested is indicated on top of each assay. A preference

21 index (PI) over 0.5 indicates a preference toward sucrose while a PI of 0.5 indicates either that

22 two opposite behaviors occurred in the population or that a majority of animals consumed both

1 solutions. The fraction of flies that ingested one, the other or both solutions allow to discriminate.
2 In light colors are the fractions of flies that ingested one solution or both solutions. The animals
3 from all the experiments were pooled for statistical analyses. ns indicates $p > 0.05$, **** indicates
4 $p < 0.0001$ Chi-square Test. Further details can be found in the material and methods section and
5 in the source data file.

6

7 **Figure 4**

8 The *TrpA1-E+* cells in the labellum have the characteristics of peripheral taste neurons. **(a)**
9 Representative confocal fluorescence image of the proboscis of *TrpA1-E-Gal4/UAS-GFP* flies. **(a')**
10 Merged image with transmitted light. **(a'')** Magnified view of the area delineated by the white
11 dashed line, showing a cell with an extension inserting into a short sensillum (S) and another
12 extension running posteriorly.

13 **Figure 5**

14 *TrpA1-E+* cells located in the proboscis respond to caffeine exposure. **(a)** Averaged \pm SEM time
15 course of the GCaMP intensity variations ($\Delta F/F_0$ %) for proboscis *TrpA1-E+* cells of *TrpA1-E-*
16 *Gal4/UAS-GCaMP* and *TrpA1-E-Gal4/UAS-GCaMP; UAS-TrpA1_IR* females. The addition of water
17 (n = 5 and 5 flies) or caffeine 10mM (n = 4 and 6 flies) at a specific time is indicated by the arrow.
18 Several neurons in the focal plan located in the same proboscis were used for the quantification.
19 **(b)** Averaged fluorescence intensity of peaks \pm SEM in response to water (n = 5 and 5 flies),
20 caffeine 10mM (n = 4 and 6 flies). * $p = 0.046$, Kruskal-Wallis test comparing the 4 independent
21 sets of values.

1

2 **VIDEOS**

3 **Video 1 PER is triggered following exposure to sucrose 1mM**

4 Private link: <https://figshare.com/s/d5dc0d81a2a1a3b19365>

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

7

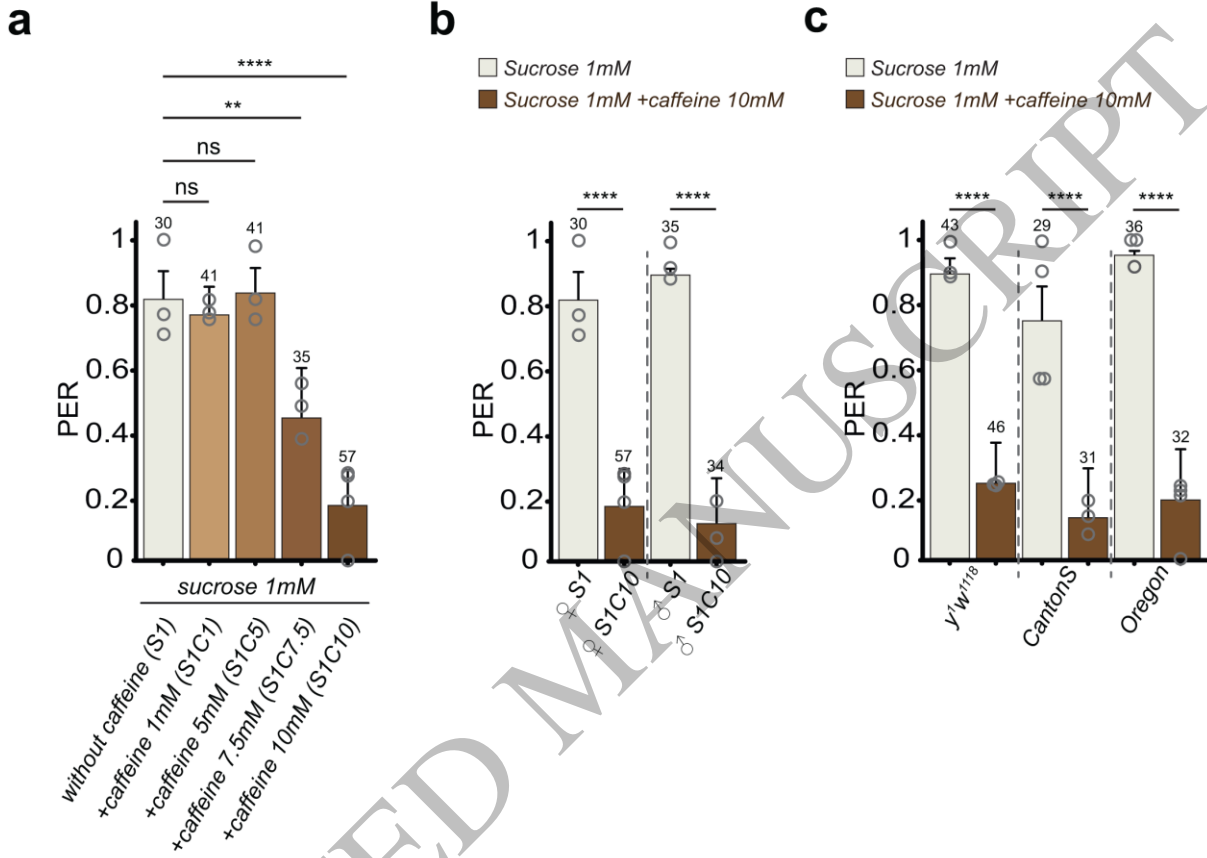
8 **Video 2 PER is not triggered following exposure to a mixture combining sucrose 1mM and**
9 **caffeine 10mM**

10 Private link: <https://figshare.com/s/e5ce4ca699699046e076>

11 Absence of response following proboscis contact with a stripe of paper soaked in sucrose 1mM
12 combined with caffeine 10mM. 7-days old females starved 15h prior to the assay.

13

FIGURE 1



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FIGURE 2

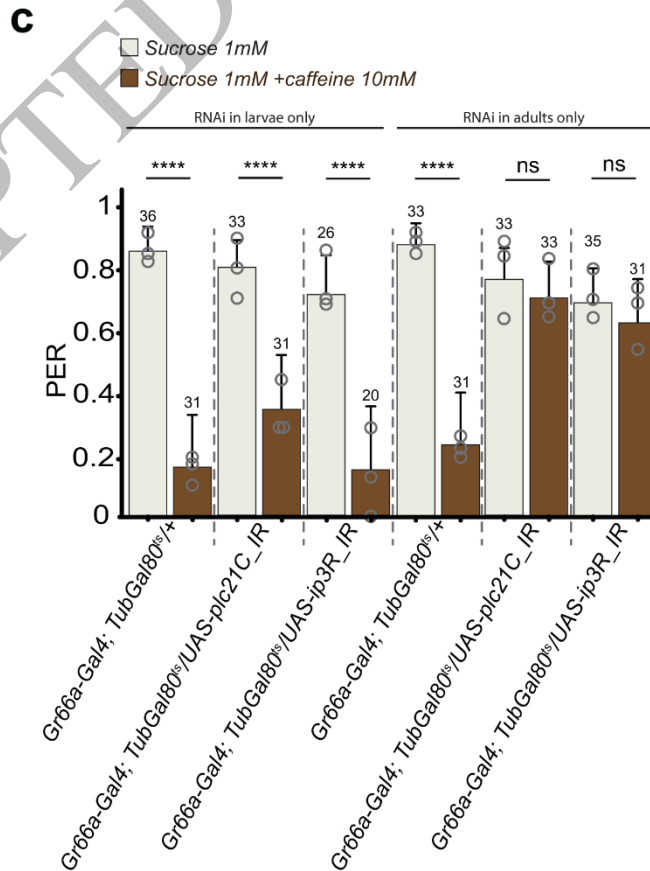
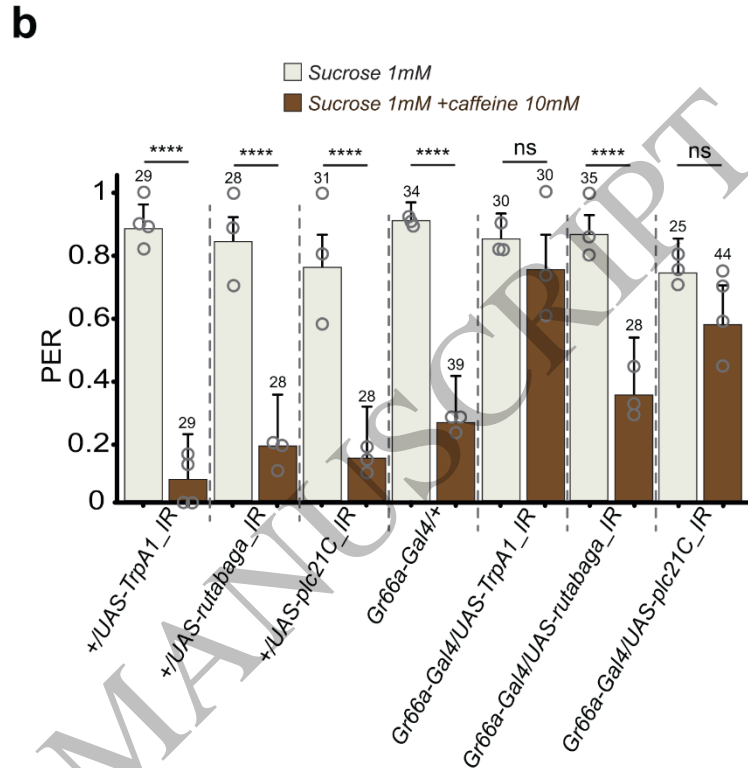
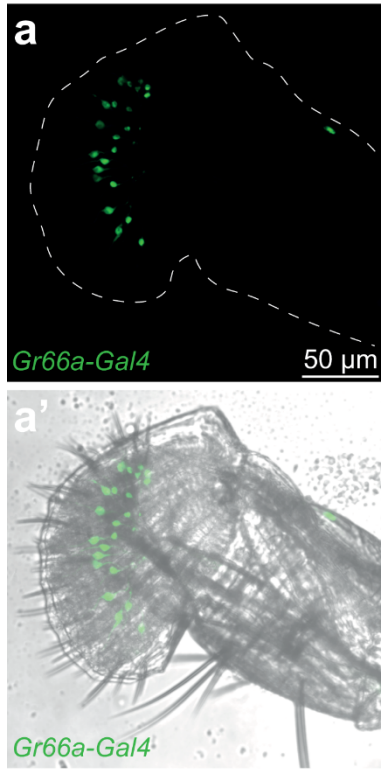
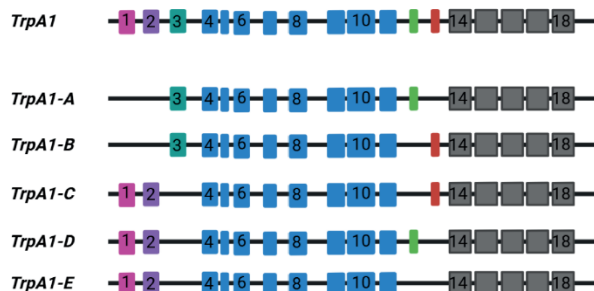
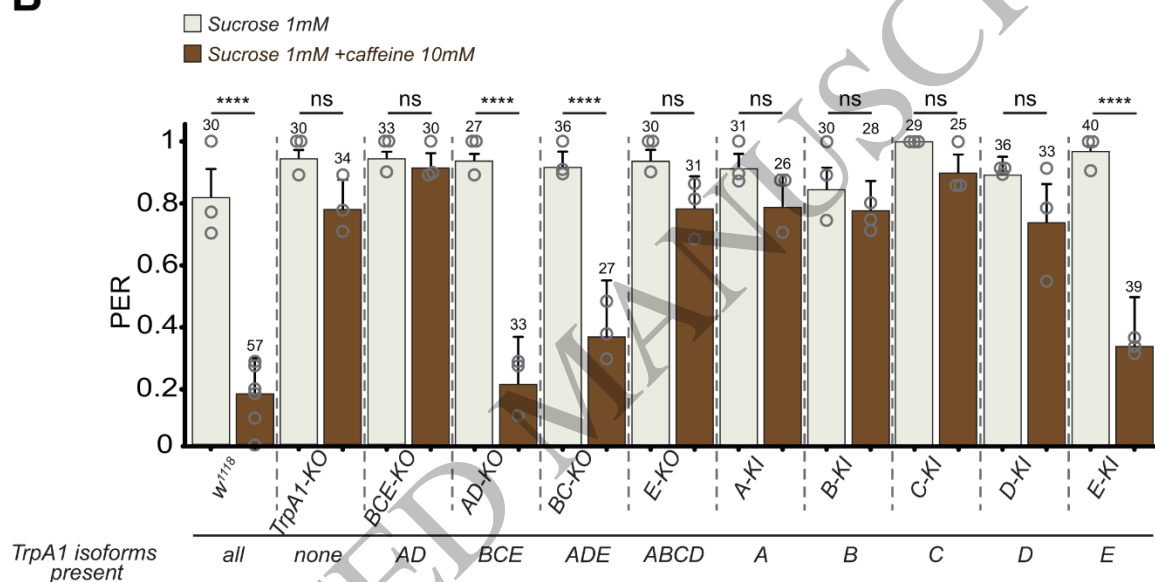


FIGURE 3

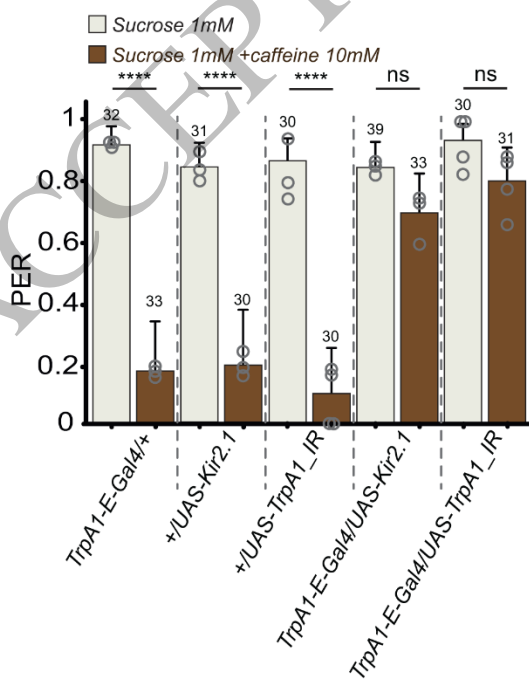
A



B



C



D

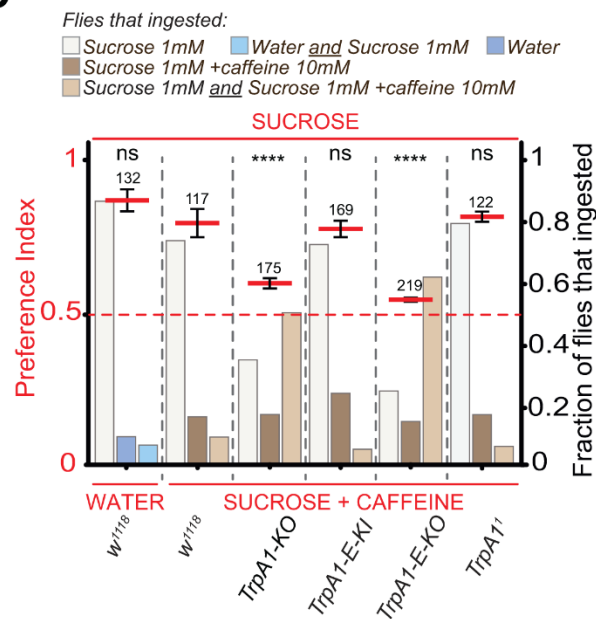
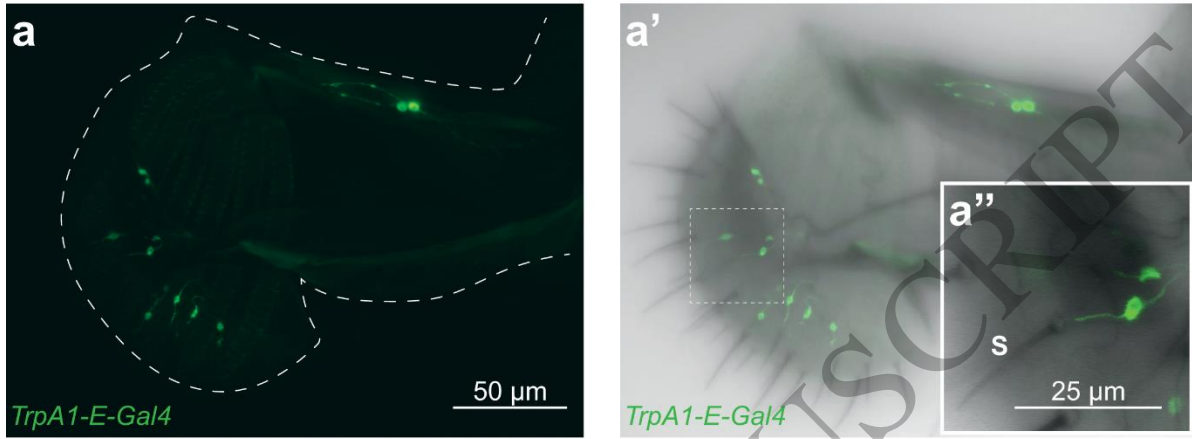


FIGURE 4



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ACCEPTED MANUSCRIPT

FIGURE 5

