A DROSOPHILA CIRCUIT FOR HABITUATION OVERRIDE

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ABSTRACT:

Habituated animals retain a latent capacity for robust engagement with familiar stimuli. In most instances, the ability to override habituation is best explained by postulating: (a) that habituation arises from the potentiation of inhibitory inputs onto stimulus-encoding assemblies; and (b) fast habituation override occurs through disinhibition. Previous work has shown that inhibitory plasticity contributes to specific forms of olfactory and gustatory habituation in *Drosophila*. Here we analyze how exposure to a novel stimulus causes override of gustatory (proboscis-extension reflex or "PER") habituation. While brief sucrose contact with tarsal hairs causes naïve *Drosophila* to extend their proboscis, persistent tarsal exposure to sucrose reduces PER to subsequent sucrose stimuli. We show that in so habituated animals, either brief exposure of the proboscis to yeast or direct thermogenetic activation of sensory neurons restores the PER response to tarsal sucrose stimulation. Similar override of PER habituation can also be induced by brief thermogenetic activation of a population of TH (Tyrosine-Hydroxylase) positive neurons, a subset of which send projections to the SEZ. Significantly, sensory-neuron induced habituation override requires transmitter release from these TH-positive cells. Treatments that cause override specifically influence the habituated state, with no effect on the naïve sucrose response across a range of concentrations. Taken together, these and other findings are consistent with a model in which novel taste stimuli trigger activity in dopaminergic neurons which, directly or indirectly, inhibit GABAergic cells that drive PER habituation. The implications of these findings for general mechanisms of attentional and sensory override of habituation are discussed.

SIGNIFICANCE STATEMENT:

Behavioral habituation can be overcome when a new context requires an enhanced response to a familiar stimulus. However, the underlying mechanisms remain incompletely understood. Previous studies have provided evidence that habituation of the sucrose-induced proboscis extension reflex (PER) in Drosophila occurs through potentiation of inhibition onto the PER pathway. This work defines controlled protocols for override of PER habituation and uses them to outline the underlying circuit mechanisms. The results presented support a model in which novel taste stimuli cause dishabituation by activating a subset of tyrosine-hydroxylase-expressing neurons that inhibit GABAergic neurons whose potentiation underlies PER habituation. At a general level, these findings further highlight a central role for inhibition and disinhibition in the control of behavioral flexibility.

INTRODUCTION.

Habituation is a form of non-associative learning in which the response to a stimulus reduces with repeated or extended passive exposure. However, a latent ability to respond to the innocuous stimulus remains. Indeed, the phenomenon of “dishabituation” is a classical, defining feature of the habituated state, distinguishing it from sensory or synaptic fatigue (Thompson and Spencer, 1966; Rankin et al., 2009; Ramaswami, 2014). In this article we often use the term “override” in place of dishabituation, which while largely synonymous, better acknowledges that behavioral responses can be reinstated not only by classic dishabituating (novel) stimuli, but also by attentional mechanisms recruited during task engagement ((COOKE and RAMASWAMI, 2020)

Both habituation and the associated phenomenon of dishabituation/ override have been described across different phyla of animal kingdom (Glanzman et al., 1989; Zaccardi et al.,

2004; Smith et al., 2009; Ramaswami, 2014). Two broad observations are relevant here. First, mechanisms underlying very short and longer lasting forms of habituation may differ, with the latter more likely to involve inhibitory potentiation (Ramaswami, 2014; Shen et al., 2020). Second, largely due to the difficulty of necessary behavioral experiments, in most instances where habituation override has been reported, it is not rigorously distinguished from a potential confounding process of response sensitization (Castellucci et al., 1970; Hawkins et al., 1998; Asztalos et al., 2007a; Asztalos et al., 2007b). Thus, although arguments and evidence support a disinhibitory mechanism (Fischer et al., 1997; Das et al., 2011; Kato et al., 2015; Ogg et al., 2018) the neural pathways and mechanisms of habituation override remain incompletely characterized. Here we address this issue in the gustatory system of *Drosophila*.

Drosophila sample and taste potential foods via chemosensory hairs on their tarsi (distal leg segments) and their proboscis (the main feeding organ) (Stocker, 1994; Montell, 2009). Sugars detected by sensory hairs trigger reflexive extension of proboscis to enable feeding. This proboscis extension reflex (PER) can be conveniently induced in insects by experimental application of sucrose or other sweet tastants to tarsal hairs (Minnich, 1921; Dethier, 1976). The neural circuit for PER is only partially understood. It involves transmission of sensory information to the subesophagial zone (SEZ) where it is processed and communicated to command neurons whose activation triggers the motor programme required for coordinated contraction and relaxation of muscles that drive proboscis extension and retraction (Flood et al., 2013). When the tarsus is repeatedly stimulated with sucrose under conditions where proboscis extension is futile, then the PER response is reduced through a process that shows several classic features of behavioral habituation (Duerr and Quinn, 1982; Le Bourg, 1983; Fois et al., 1991; Engel and Wu, 2009; Paranjpe et al., 2012). Importantly, in habituated animals, a strong PER to sucrose is quickly restored if the fly is presented with a strong, novel sensory stimulus (Le Bourg, 1983; Fois et al., 1991; Paranjpe et al., 2012). Here, we build on

these and other previous studies to investigate circuit mechanisms that drive override of PER habituation.

We first independently reproduced prior experiments providing key support for increased inhibition in the PER pathway being the core mechanism for PER habituation (Paranjpe et al., 2012). Thereafter, to address mechanisms of habituation override, we developed better defined protocols for habituation override, which we achieved by: (a) yeast stimulation of the proboscis; (b) thermogenetic activation of yeast-responsive or bitter responsive sensory neurons; or (c) thermogenetic activation of a subpopulation of dopaminergic neurons. Crucially, each of these treatments specifically affects the response of habituated animals; none sensitize naïve animals to the taste of sucrose. We further show that both sensory stimulation procedures work through dopaminergic neurons to affect habituation override. While the data do not yet conclusively define all elements of the dishabituation circuit or the mechanism by which dopaminergic neurons trigger override, they support a model in which novel stimuli induce dopamine release in the SEZ, which acts to directly or indirectly inhibit inhibitory neurons that drive PER habituation. We suggest that this work: (a) circumscribes core elements of a sensory-central circuit for habituation override; (b) provides evidence for a new disinhibitory pathway in the Drosophila brain; and (c) supports an emerging framework in which latent perceptions, memories and behaviors may be generally activated through disinhibition (Sridharan and Knudsen, 2015; Barron et al., 2017; Wang and Yang, 2018).

MATERIALS AND METHODS

Drosophila stocks*:* Fly stocks were maintained on standard corn meal media. Canton S (CS) flies were used as wild-type controls unless otherwise stated. The stocks were obtained either from stock centres or as generous gifts from following sources: *Ir25a-Gal4* was provided by

Carlos Ribeiro (Champalimaud Centre for the Unknown, Lisbon, Portugal), *TH-C'-Gal4*, *TH-D'-Gal4* and *TH-C-Gal80* were generously provided by Mark Wu (Johns Hopkins University, Baltimore, MD), *Gad1-Gal4* was from Gero Miesenbock (Oxford University, Oxford, UK), *TH-Gal4* was provided by Gaiti Hassan (National Centre for Biological Sciences, Bangalore, India), *Gr66a-LexA, lexAop-CD4::spGFP11; UAS-CD4::spGFP1-10* and *LexAop-TRPA1* were provided by Kristin Scott (University of California, Berkley). rut^{2080} and *UAS-*rut^{+} were provided by Martin Heisenberg. *UAS-*Shi^{ts} was obtained from Toshi Kitamoto (University of Iowa, Iowa city, IA), *UAS-TRPA1* was provided by Paul Garrity (Brandeis University, Waltham, MA)**.** *Wg/CyO; Gr66a-Gal4* (BL 57670), *UAS-mCD8::GFP* (BL 5130), *UAS-CD8::RFP*, *LexAop-CD8::GFP* (BL 32229) were obtained from Bloomington Drosophila Stock Centre.

Proboscis extension behaviour*:* Proboscis extension behaviour was carried out as described in (Paranjpe et al., 2012). Briefly, 3-4 days old female flies were mounted individually, ventral side up, on a cover slip. The flies were kept in a humidified chamber for 2 hours for recovery. Tarsal hairs were stimulated with 2% sucrose using 1ml syringe needle. The naïve response score was determined after stimulating tarsal hairs five times. Failed PER response was scored as zero and complete proboscis extension was scored as one. Flies that showed naïve PER response less than three out of five times were discarded and not used for habituation experiments.

Habituation of proboscis extension reflex: Tarsal hairs were stimulated with 10% sucrose for 10 minutes after which the legs were washed using distilled water. The habituated response or post exposure response was recorded by stimulating tarsus with 2% sucrose five times, similar to naïve response.

Habituation Override 10% yeast was presented to the proboscis for 1-minute in a spaced manner in order to prevent consumption of yeast and satiation. After exposure to yeast, flies were allowed to groom themselves for 1 minute so that any sticking yeast on the proboscis could be cleaned. PER response was recorded by stimulating tarsal hairs five times as described above.

For habituation override using mechanical stimulus, flies mounted on the cover slip were placed in a petri dish (35mm) and vortexed for 1 minute. Flies were allowed to recover from the shock for 1 minute before testing the response.

To distinguish override from sensitization, naïve response of flies were recorded at concentrations of sucrose lower than 2%. This was done as the PER response is maximum at 2%, any difference as a result of manipulation would have been difficult to observe. Four concentrations of sucrose were tested- 0.1%, 0.5%, 1% and 2%. Flies were then exposed to either stimulus used for override or heat, for 1 minute. Post response was tested after 1 minute rest at RT.

Heat-mediated manipulation For TRPA1 and Shi^{ts} experiments, flies were transferred to 32° and 34°C respectively on a dry bath for 1 minute. In case of Shi^{ts} experiments, proboscis was stimulated with 10% yeast while at 34°C. Flies were given 1 minute rest at RT before testing PER response.

Immunohistochemistry: Adult brains were dissected in 1X PBS and fixed in 4% paraformaldehyde diluted in 1X PBS with 0.3% Triton-X (PTX) for 30 minutes at room temperature. Samples were washed with 0.3% PTX for 20 minutes three times at room temperature and incubated with primary antibody for 48 hours at 4°C on a shaker. Samples were again washed three times with 0.3% PTX for 20 minutes each. Secondary antibodies conjugated with Alexa Fluor-488, Alexa Fluor- 568 and Alexa Fluor- 647 (1:500, Invitrogen),

diluted in 0.3% PTX, were added and samples were incubated for 24 hours. Brain samples were again washed repeatedly three times for 20 minutes each and mounted in Vectashield (H-1000, Vector laboratories) on a glass slide with spacers. Images were acquired using Zeiss LSM 510 Meta microscope and Olympus Flouview (FV- 3000).

Following primary antibodies were used: rabbit anti-GFP (1:100, invitrogen), mouse anti-Bruchpilot (n82) (1:20, DSHB), mouse anti-GFP (Sigma, 1:100), rabbit anti-TH (1:1000, Invitrogen), chick anti-GFP (Abcam, 1:5000), rabbit anti-DsRed (1:100, Clontech).

Statistical analysis*:* Non parametric tests were used to analyse data. Friedman test was used to analyse habituation override experiments whereas Wilcoxon sign rank test was used for testing sensitization at different concentrations of sucrose. All the data was analysed using GraphPad Prism v8.4.2 software.

RESULTS.

Habituation of the proboscis-extension reflex to sucrose

Two-percent sucrose applied to tarsal hairs of immobilized, naïve flies induces robust and reproducible proboscis extension response. However, following extended 10-minute exposure to 10% sucrose solution, PER decreases from 92.41% to 28.96% (***$p<0.0001$, Wilcoxon test) (Fig 1A-B). No change in naïve PER response is seen if water is presented for 10 minutes instead of 10% sucrose (Fig1B). A previous study concluded that plasticity in central GABAergic neurons is required for PER habituation (Paranjpe et al., 2012). Given their significance for the inferred inhibitory mechanism for PER habituation, we independently repeated key experiments to re-examine: first, the need for the *rutabaga*-encoded adenyl cyclase in PER habituation; and second, its reported sufficiency in *GAD1-Gal4* expressing,

predominantly GABAergic neurons for this function. Our results were consistent with and confirmed previously reported observations (Figure 1C).

Behavioral habituation is often distinguished from sensory or muscular fatigue by demonstrating the rapid reinstatement of the sensory response by strong, novel stimuli (Rankin et al., 2009; Ramaswami, 2014). Consistently, PER-habituated animals retain the ability to respond relatively robustly to sucrose, as evidenced following strong or novel sensory stimulation. Similar to olfactory habituation, strong mechanical stimulation (vortexing in a dish) for 1 minute substantially reinstates (*p= 0.01, Friedman test) the sucrose-induced PER in habituated flies, without affecting baseline sucrose sensitivity in naïve animals (Figure 1D-E; (Das et al., 2011; Paranjpe et al., 2012). Thus, the effect mechanical stimulation on PER is selective on the habituated state and represents a form of habitation override and not sensitization. In order to address the underlying circuit mechanisms, we first looked to identify more precisely defined sensory stimuli that could cause override of PER habituation.

Novel stimuli override PER habituation

Drosophila are attracted by the taste of yeast. Gustatory receptor neurons (GRNs) that respond to yeast components have been recently identified (Fischler et al., 2007; Wisotsky et al., 2011; Ganguly et al., 2017); (Steck et al., 2018). To examine whether the novel taste of yeast could influence PER habituation to sucrose, we applied a 10% yeast solution to the fly labellum, which has previously been shown to possess yeast-responsive GRNs (Steck et al., 2018), and tested if this resulted in override of sucrose habituation. Application of yeast to the labellum substantially reinstated the PER response to tarsal sucrose stimulation in PER-habituated animals (*p= 0.014, Friedman test; Fig 2A-B). In contrast, 10% sucrose solution, which should be familiar to the flies, when applied to the labellum of habituated flies had no effect

on PER (Fig 2B). Thus, a brief experience of a novel and in this case, attractive stimulus appears capable of inducing habituation override.

We further looked to identify a single GRN class responsive to yeast components that may be sufficient to drive dishabituation. To do this, we expressed heat-activated cation-permeable TRPA1 channels in yeast-responsive GRNs and tested if heat-induced activity in these cells, which bypassed the need for normal ligand-receptor interactions, would result in dishabituation (Hamada et al., 2008). Such experiments showed that "thermogenetic" activation of the Ir25a class of sensory neurons is sufficient to cause PER habituation override.

TRPA1 channels open at temperatures above 25°C; thus, activation of TRPA1-expressing neurons can be temporally controlled by exposing experimental animals to temperatures where the channel is either closed (RT) or open (above 25°C). We expressed TRPA1 in yeast responsive *Ir25a-Gal4* positive sensory neurons (Steck et al., 2018). These flies were habituated to sucrose at room temperature (RT, 21°C), transferred to 32°C post-habituation for 1 minute and then tested for PER. Thermogenetic activation of Ir25a-expressing GRNs was sufficient to cause rapid override of sucrose habituation (Fig 2C). Thus, after Ir25a activation, PER-habituated animals showed significantly increased PER to tarsal sucrose stimulation (**p=0.0012, Friedman test). Similar 32°C exposure of genetic control animals not expressing TRPA1 did not affect PER habituation (Fig 2C).

To distinguish between the novelty and attractiveness of yeast taste as being primarily instrumental in override, we also examined, whether thermogenetic activation of bitter-compound responsive Gr66a expressing GRNs could similarly affect override of PER habituation. Thus, we expressed TRPA1 in *Gr66a-Gal4* positive neurons and examined how sucrose-responsiveness of flies habituated at RT was altered after a brief 1-minute shift to 32°C. Sucrose induced PER increased from 6.67% to 56.29% (**p = 0.0042, Friedman test)

indicating that brief activation of bitter-taste sensing *Gr66a*-positive neurons is sufficient to induce dishabituation. The observation that perception of novel bitter taste as well as novel yeast taste can promote override of PER habituation strongly indicates that it is the novelty of the dishabituating stimulus, rather than its attractiveness, that drives habituation override (Fig 2D).

Does novelty-induced override of PER habituation represent a specific effect on the habituated state or a general sensitization of the sensory response? To differentiate between these possibilities, we tested whether and how yeast exposure, *Ir25a*-neuron activation or *Gr66a*-neuron activation altered PER in naïve flies to a range of sucrose concentrations. If the increase in PER response in habituated flies were due to sensitization, then we would expect an increase in PER after the novel stimulus is applied to naïve animals. However, as shown in 3A, 3B and 3C, there is no significant difference in PER response before and after presentation of yeast or *Ir25a* or *Gr66a*-GRN activation respectively. Thus, it can be concluded that the reinstatement of PER response that we observe in our experiments is due to a process that specifically acts on neural correlates of PER habituation, not broadly on taste perception.

Artificial activation of Tyrosine-Hydroxylase (TH) expressing neurons overrides PER habituation.

Across species, novel stimuli trigger central release of neuromodulators that confer or enhance their salience (Ranganath and Rainer, 2003; Kafkas and Montaldi, 2018). In particular, the activity of dopaminergic neurons has been implicated in the novelty response both in insects and in mammalian systems, (Hattori et al., 2017; Morrens et al., 2020). We therefore investigated whether thermogenetic activation of *Drosophila* tyrosine-hydroxylase (TH)

expressing central dopaminergic neurons would be (a) sufficient and (b) necessary for novel-taste induced override of PER habituation.

In PER-habituated *TH-Gal4>UAS-TRPA1* animals, 1-minute exposure to 32°C to drive thermogenetic activation of *TH-Gal4* expressing neurons resulted in rapid habituation override (Fig 4A). In PER-habituated animals, sucrose-induced PER was close to 12%; in these same animals, activation of TH expressing neurons increased PER to 60% (***p = 0.0004, Friedman test). Significantly, activation of TH Gal4 neurons had no effect on the innate response to sucrose in naïve flies (Fig 4B). Thus, activation of these modulatory neurons causes habituation override, not general taste sensitization.

TH-Gal4 labels around 200 neurons in the Drosophila brain (Friggi-Grelin et al., 2003). In order to more tightly define *TH*-expressing cells involved in PER-habituation override, we tested two non-overlapping subsets of *TH-Gal4* positive neurons, marked by *TH-D'-Gal4* and *TH-C'-Gal4* drivers (which labels ~54±5 and ~45±3 neurons respectively) for their potential roles. While 1-minute thermogenetic activation of *TH-D'* neurons had no effect on PER habituation, similar activation of neurons labelled by *TH-C'-Gal4* significantly increased PER response from 28.72% in control habituated flies, to 57.81% after *TH-C'* activation (**p= 0.0042, Friedman test Fig 4C). Moreover, *TH-Gal4*-driven habituation override required activity in *TH-C'* cell population: thus, 32°C exposure did not trigger PER habituation override in *TH-C-Gal80; TH-Gal4> UAS TRPA1* flies, in which Gal80 expression prevented TRPA1 expression in the *TH-C'* subset of *TH-Gal4* neurons (Fig 4D). This indicates that activity in *TH-C'* subset of cells is necessary and sufficient to cause habituation override. To further support this conclusion, we checked and confirmed that activation of *TH-C'* neurons had no significant effect on the PER of naïve flies across a range of sucrose concentrations (Fig 4E) confirming that *TH-C'* neurons specifically influence mechanisms of habituation, rather than general taste perception.

Significantly, a small subset of *TH-C'* neurons (~13±6) are present locally in the SEZ (suboesophagial zone), an area which not only receives inputs from taste sensory neurons, but also houses interneurons and motor neurons involved in proboscis extension (Gordon and Scott, 2009; Kain and Dahanukar, 2015). Thus, some *TH-C'* neurons are well positioned to mediate sensory-driven novelty signals that influence mechanisms of PER habituation.

A subset of TH-expressing neurons mediate novelty-induced habituation override.

In order to test whether the activity of *TH-Gal4* and *TH-C'-Gal4* neurons is necessary for novel-taste induced override of PER habituation, we examined whether novel-taste stimulation could cause habituation override under conditions where synaptic output from *TH-Gal4* or *TH-C'-Gal4* neurons was blocked. To achieve this, we expressed the temperature-sensitive, dominant-negative Shi^{ts1} mutant form of dynamin in these neurons and tested whether a one-minute exposure to 10% yeast at 34°C could override habituation in *TH-Gal4, UAS-*Shi^{ts1} and *TH-C'-Gal4 UAS-*Shi^{ts1} at temperatures restrictive for Shi^{ts1} dynamin function.

At permissive (room) temperatures, *TH-Gal4, UAS-*Shi^{ts1} and *TH-C'-Gal4, UAS-*Shi^{ts1} flies behaved similarly to wild-type flies, showing both robust PER habituation after 10 minutes of tarsal sucrose exposure (Fig 5A) and significant PER dishabituation following brief (1 min) exposure of their labella to 10% yeast solution (Fig 5A). The respective efficiency of PER in habituated and dishabituated animals (before and after yeast exposure) were 34.05% and 77.29% (***p = 0.0008, Friedman test) and 13.46% and 48.46% (*p = 0.045, Friedman test) respectively (Fig 5A).

In contrast, if after PER habituation the same flies were shifted to and exposed to 10% yeast at 34°C, where the essential function of dynamin in transmitter release would be compromised in Shi^{ts1}-expressing cells, then habituation override was significantly impaired as compared to

controls (Fig 5A). Thus, yeast-induced dishabituation of control flies not expressing *Shi^{ts1}* at 34°C was substantially more efficient (**p= 0.0015), than of *TH-Gal4, UAS-Shi^{ts1}* flies at the same temperature (Fig 5A). Similarly, dishabituation of control flies not expressing *Shi^{ts1}* was also substantially more efficient at 34°C (***p = 0.0001, Friedman test), than of *TH-C'-Gal4, UAS-Shi^{ts1}* flies, expressing temperature-sensitive mutant dynamin in *THC'-Gal4* neurons. These data argue that presynaptic activity in *TH-C'-Gal4* and *TH-Gal4* positive cells is required for yeast-induced override of PER habituation (Fig 5A).

An important caveat to the above conclusion is that dopaminergic neuron activation may be associated with motivation or reward prediction and therefore be fundamentally required for high levels of PER. In such a scenario, inactivation of TH-expressing cells would be expected to generally reduce levels of PER. To address this issue and test whether the apparently reduced override in Fig 5A is an artifact of blocking dopaminergic neurons during yeast exposure, we measured the innate response to sucrose in naïve flies with and without blockage of synaptic transmission in dopaminergic neurons (Fig 5B). There was no significant difference in the innate response to 2% sucrose, confirming a more restricted role for these TH positive cells in the override of habituation.

Sensory neurons projections in SEZ overlap spatially with projections of TH-neurons

In the mushroom body, dopaminergic neurons potentially compute novelty by combining excitatory inputs from olfactory sensory channels with inhibitory inputs driven by familiarity encoding interneurons (Zhao et al., 2021) (and see Discussion). If TH-positive cells were to play a similar role in the SEZ, then they would be predicted to receive direct or indirect inputs from taste sensory neurons as well as input from familiarity-representing inhibitory neurons. Previous work has shown that processes from some TH-positive neurons are present in the SEZ which also contains presynaptic endings of taste sensory neurons, processes of local

interneurons and dendrites of motor neurons involved in proboscis extension (Figure 6A; (Liu et al., 2012)). Therefore, we considered the possibility that taste sensory neurons make contacts with *TH*-positive processes.

To test whether taste-sensory neurons carrying dishabituating signals form connections, direct or indirect, with TH-neuron processes in the SEZ, we used the GFP-reconstitution across synaptic partners (GRASP) technique, which requires the use of dual binary transcription systems (based on LexA and Gal4 transcription factors) to separately express two complementing fragments of GFP, one in sensory neurons and the other in TH neurons. Limited by the immediate availability of transgenes, we were technically restricted to analyzing TH connections with neurons in which gene expression could be controlled by LexA. This was possible for bitter-taste responsive *Gr66a* neurons but not for the yeast-responsive *Ir25a* class.

We first examined whether *Gr66a* axonal projections and *TH-Gal4* marked processes were present in close proximity within the SEZ, by examining the relative localization of GFP driven in *Gr66a*-expressing neurons with RFP in *TH*-positive cells. Processes of sensory neurons expressing *Gr66a-LexA*-driven GFP and dopaminergic neurons expressing *TH-Gal4* driven RFP showed close proximity (Fig6A). Further, GRASP experiments showed that two halves of split GFP, one expressed in *Gr66a* neurons and the other in *TH*-positive cells could combine to reconstitute GFP fluorescence within the SEZ (Fig 6B). Robust fluorescence reconstitution was seen in 8/13 experimental animals compared to 0/15 total animals expressing only one split-GFP component. Thus, GRASP experiments confirm that the processes of *Gr66a* axons and tyrosine-hydroxylase expressing cells come in close proximity of each other. However, due to the limitation of the GRASP technique used it cannot be established certainly if there is a direct synapse between the two cell types.

These data predict that habituation override induced by thermogenetic activation of Gr66a cells should require activity in *TH-Gal4* positive cells. We tested this prediction by creating and analysing PER habituation and dishabituation in *Gr66a-LexA/ LexAop-TRPA1; TH-Gal4/ UAS-Shi^{ts1}* and control *Gr66a-LexA/LexAop-TRPA1; TH-Gal4/+* lines at temperatures permissive and restrictive for Shi^{ts1} dynamin. As shown (Fig 6C), both lines showed robust PER and PER habituation. One minute-exposure to 34°C in control flies permissive for synaptic transmission from *TH*-positive neurons resulted in significant (*p = 0.02, Friedman test) override of habituation. However, similar 34°C thermogenetic stimulation of *Gr66a* neurons in experimental flies where synaptic transmission from TH-positive neurons is blocked did not result in override of PER habituation. Together the anatomical and behavioral data indicate that *Gr66a*-expressing bitter taste sensory neurons are functionally connected to *TH*-expression modulatory neurons whose activity is required for PER habituation override.

Control experiments demonstrating that blocking synaptic output from *TH*-expressing cells has no effect on basal PER response in naïve animals further confirm that transmitter release from *TH*-neurons is required for override of neuronal mechanisms of habituation, not for the innate response to sucrose (Fig 5B).

DISCUSSION

Animal behavior is profoundly flexible. Thus, at any time, multiple potential behavioral programmes remain dormant, while a subset relevant to specific contexts are active. A growing body of evidence suggests that inhibitory inputs play a major role in preserving perceptions, behaviors and memories in dormant form until required (Barron et al., 2017). Thus, a given context, by recruiting disinhibitory circuits may override inhibition to release latent

372 perceptions, motor programmes and memories appropriate to that context. While the
373 overarching principles are increasingly appreciated, there is still limited understanding of how
374 these are implemented in cells and circuits. While there are many potential reasons for this,
375 the difficulty in studying mechanisms of override has been substantially caused by the paucity
376 of systems in which both mechanisms of habituation or cognitive silencing can be addressed
377 as well as where robust override can be experimentally achieved.

378 The results we present outline essential elements within a *Drosophila* circuit that overrides
379 habituation, in this case of the sucrose-evoked proboscis extension reflex. In doing so, they
380 connect sensory neurons mediating override to neuromodulatory neurons projecting to the
381 SEZ, which houses interneurons and motor neurons that control proboscis extension. When
382 taken together with published work showing that increased inhibition underlies PER
383 habituation, these findings are significant because they circumscribe a central circuit for
384 habituation override and add to the growing literature on the role and mechanism of
385 disinhibition in gating animal cognition.

386 Previous work in *Drosophila* concluded that PER habituation arises from increased sucrose-
387 evoked inhibition onto neurons that drive proboscis extension (Paranjpe et al., 2012). Two
388 findings, which closely mirror observations on olfactory habituation, provided key support for
389 this conclusion (Das et al., 2011; Paranjpe et al., 2012). First, the *rutabaga*-encoded adenyl
390 cyclase is required specifically in inhibitory neurons for PER habituation, an observation that
391 we have independently confirmed in this study (Figure 1). Second, experimental silencing of
392 inhibitory neurons causes override of habituation. Together these observations indicate first,
393 that increased GABAergic activity is required for the expression of PER habituation and
394 second, that disinhibition could serve as strategy for habituation override. How might such
395 disinhibition be biologically achieved? Our current experiments show that novel sensory
396 experience induces override of PER habituation through a pathway that requires activity in the

TH-C' class of dopaminergic neurons. As thermogenetic activation of *TH-C'* cells is also sufficient to override PER habituation, the data suggest a framework in which sensory stimuli activate *TH-C'* neurons, which directly or indirectly, inhibit GABAergic neurons responsible for habituation.

An important question to address is why novel but not familiar taste stimuli are effective for override? In physiological terms, how could activation of a novel subset of (*Ir25a or Gr66a*) sensory-neurons result in strong excitation of *TH-C'* cells, while similar levels of activation of sucrose-response sensory-neurons causes weaker excitation of the same *TH-C'* cells? We suggest that this occurs because familiar stimuli additionally recruit higher levels of inhibition onto *TH-C'* neurons. At a circuit level, there are multiple ways in which such a mechanism could be implemented, the simplest of which is shown schematically in Figure 7.

The model proposes that in the naïve state, sensory neurons (SN) excite excitatory projection neurons (PN) that activate the PER. These SNs also excite cognate inhibitory local interneurons (iLNs) and *TH-C'* neurons (either directly or indirectly via yet to be identified neuron). The iLNs are postulated to make weak inhibitory synapses onto PNs and *TH-C'* cells, while also receiving strong inhibition from *TH-C'*. Thus, novel stimuli result in weak total iLN excitation, strong PN excitation and robust PER. The imposition of a synaptic learning rule that coincident presynaptic and postsynaptic activity at inhibitory synapses leads to inhibitory synapse potentiation, generates the postulated habituated state, wherein PNs receive additional increased inhibition resulting in lower net excitation and reduced PER. At the same time the same learning rule also results in increased inhibition and therefore decreased net excitation of *TH-C'* neurons by "familiar" SNs. In this habituated state, the familiar tastant, causing only weak *TH-C'* excitation, would not disinhibit the PER pathway. However, a novel tastant would still trigger strong *TH-C'* cell activation, effective disinhibition of PN and thereby override of PER habituation. The above model places some constraints on SN-LN connectivity which have

yet to be examined or established: most significantly suggesting that different classes of SNs activate different subgroups of iLNs in the SEZ.

While our experiments lead to a comprehensive model for a habituation override circuit, they do not yet clarify several important issues. Most obviously many aspects of the proposed connectivity are inferred, rather than demonstrated by direct anatomical or physiological methods. The exact subset of *TH-C'* cells involved as well as the subtypes and connectivities of relevant inhibitory neurons in the SEZ remain unknown and crucially important to establish. The mechanism by which relevant *TH-C'* cells function has not been formally proven. While these cells are marked by three independent dopaminergic reporter lines, *TH-Gal4*, *TH-C'* and *TH-C-Gal80*, and therefore probably dopaminergic, it remains unclear whether dopamine release is required for habituation override. Our attempts to address this via knockdown of tyrosine hydroxylase through RNAi, or by various genetic manipulations of dopamine receptor expression did not yield definitive results, often because these manipulations affected baseline levels of PER and PER habituation.

However, given that the SEZ, which contains gustatory neurons axons as well as dendrites of motor neurons that drive PER, is likely to be numerically simple compared, the most parsimonious model for override would posit that taste sensory neurons trigger direct excitation of dopaminergic processes, which in turn acts within the SEZ to directly inhibit GABAergic cells (Pimentel et al., 2016) whose potentiation drives PER habituation.

The model above is consistent with observations on habituation override in mouse and Aplysia brains (Bristol and Carew, 2005; Smith et al., 2009; Kato et al., 2015; Ogg et al., 2018). In mouse, long term auditory habituation to a passively experienced tone is accompanied by increased activity in tone-responsive SOM+ neurons that inhibit similarly tuned pyramidal cells in the auditory cortex. However, if habituated mice are coaxed to attend to the same tone

(by a reward for successful engagement), then the behaving mice show overriding inhibition of SOM+ neurons and increased activity of downstream L2/3 pyramidal neurons (Kato et al., 2015). It appears likely that this disinhibition is accomplished by modulatory inputs onto upstream VIP+ neurons. A more recent analysis showed that cholinergic inputs into the mouse olfactory bulb could cause override of a fast form of olfactory habituation. Thus, electrical or optogenetically induced acetylcholine release into the bulb caused mice to override habituation and investigate a previously ignored odor (Ogg et al., 2018). While sensitization is not formally excluded here, these studies are consistent with an emerging theme wherein neuromodulators released in response to novel or meaningful stimuli (Vankov et al., 1995; Giovannini et al., 2001; Ranganath and Rainer, 2003; Hattori et al., 2017; Kafkas and Montaldi, 2018; Morrens et al., 2020) result in disinhibition which can either enhance learning or override habituation.

The experimental results described here provides multiple lines of circumstantial evidence in support of a novelty-induced dopaminergic pathway for disinhibition of sensory perception. It outlines a habituation override circuit all the way from sensory neurons that detect stimulus, to motor neurons that mediate behavioral response. In context of the increasingly widely appreciated role for disinhibition in the control of perception, cognition and behavior (Letzkus et al., 2015; Sridharan and Knudsen, 2015; Barron et al., 2017; Wang and Yang, 2018) we suggest that this work provides a valuable intellectual and biological foundation for future studies to comprehensively identify neurons and mechanisms involved in a central pathway for behaviorally important disinhibition.

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595

596

FIGURE LEGENDS

Figure 1. Gustatory habituation of PER to sweet taste.

(A) Behavioural protocol of PER habituation to tarsal stimulation following sucrose exposure. **(B)** Tarsal exposure to 10% sucrose for 10 minutes leads to decrease in PER response (***p< 0.0001, Wilcoxon signed rank test) whereas exposure to water does not affect the response (p> 0.05). **(C)** As previously described (Paranjpe et al, 2012), gustatory habituation is dependent on the Rutabaga adenylate cyclase since it is impaired in *rut*^{2080} mutants. Habituation can be restored by expressing wild type *rut* in inhibitory *Gad1-Gal4* neurons (**p < 0.006, Mann Whitney U test). **(D)** The habituated response can be overridden by a novel mechanical stimulus like vortexing (*p = 0.01, Friedman test). **(E)** Vortexing does not have an effect on naïve response to sucrose (p = 0.0518, p= 0.67, p= 0.16, Wilcoxon signed rank test) as, determined by testing PER response pre and post vortexing at different sucrose concentrations. Therefore, the enhanced PER response after vortexing habituated animals is not a result of general sensitization but instead represents a specific override of habituation to sucrose. Bars represent mean±SEM, ns represents not statistically significant, p>0.05.

Figure 2. Habituation override mediated by novel stimulus

(A) Behaviour protocol of PER habituation and its override by presenting stimulus to the labellum **(B)** Presentation of novel 10% yeast stimulus to labellum restores PER response (*p = 0.014 Friedman test) whereas 10% sucrose, which is a familiar stimulus, does not have an effect on habituated response (p > 0.99, Friedman test). **(C1)** Expression pattern of *Ir25a-Gal4* in the adult brain. Ir25a-Gal4 is expresssed in gustatory receptor neurons, predominantly bitter and sweet sensory neurons in the subesophageal zone (SEZ), as well as some olfactory receptor neurons in the antennal lobe (AL). **(C2)** Directly activating yeast responsive *Ir25a-Gal4* cells

expressing heat activated channel *TRPA1* for 1 minute after habituation at 32°C, is sufficient to override PER habituation to sucrose (**p = 0.0012, Friedman test) (*Ir25a-Gal4/+* p > 0.99, *UAS-TRPA1/+*, p > 0.99, Friedman test). **(D1)** Expression pattern of *Gr66a-Gal4* in the subesophageal zone (SEZ) region of adult brain. The bitter sensory neurons marked by Gr66a form a ringed structure; they project to the anterior region of the SEZ. **(D2)** Thermogenetic activation of bitter *Gr66a*-marked GRNs for 1 minute after habituation at 32°C, also overrides sweet-taste habituation (**p = 0.0042, Friedman test) whereas no difference is observed in controls (*Gr66a-Gal4/+* > 0.99, *UAS-TRPA1/+*, p > 0.99, Friedman test). Together the data show that stimulus-novelty drives override of habituation. Bars represent mean±SEM, ns represents not statistically significant, p>0.05.

Figure 3. Novelty induced habituation override does not induce sensitization of sensory response

(A) Labellar exposure to 10% yeast does not increase the naïve PER response to 0.1%, 0.5%, 1% or 2% sucrose stimulation (Probabilities of PER being unchanged using the Wilcoxon sign rank test: p = 0.64, p = 0.67, p = 0.19 and p = 0.12, at the 4 respective sucrose concentrations). **(B)** Thermogenetic activation of *Ir25a-Gal4* does not sensitize response to sucrose tested at concentration 0.1% (p > 0.99, Wilcoxon sign rank test), 0.5% (p = 0.5938, Wilcoxon sign rank test), 1% (p = 0.7588, Wilcoxon sign rank test), 2% (p = 0.4814, Wilcoxon sign rank test). **(C)**Thermogenetic activation of *Gr66a-Gal4* does not have an effect on response to 0.1% (p = 0.5, Wilcoxon sign rank test), 0.5% (p > 0.99, Wilcoxon sign rank test), 1% (p = 0.375, Wilcoxon sign rank test), 2% (p = 0.3828, Wilcoxon sign rank test) concentrations of sucrose. Points represent mean±SEM, ns represents not statistically significant, p>0.05.

Figure 4. Activation TH expressing neurons overrides habituation

(A) Thermogenetic activation of TH expressing neurons for 1 minute at 32°C after habituation results in habituation override (***p = 0.0004, Friedman test) (*TH-Gal4/+*, p >0.99, *UAS-TRPA1/+*, p > 0.99, *TH-Gal4/UAS-TRPA1* temperature control, p = 0.8665, Friedman test). **(B)** Activation of TH expressing neurons does not lead to sensitization of gustatory response tested at 0.1% (p = 0.09, Wilcoxon sign rank test), 0.5% (p = 0.1, Wilcoxon sign rank test), 1% (p = 0.14, Wilcoxon sign rank test), 2% (p = 0.14, Wilcoxon sign rank test). **(C)** Activation of a subset of TH expressing cells marked by *TH-C'-Gal4* for 1 minute after habitation at 32°C is sufficient to override habituated response to sucrose (**p = 0.0042, Friedman test) (*TH Gal4/+*, p = 0.4914, *UAS-TRPA1/+*, p > 0.99, Friedman test) (*TH-C'- Gal4* temperature control, p > 0.99, Friedman test). **(D)** Activation of *TH-D'* subset of neurons, which marks subset of neurons that does not overlap with *TH-C,'* for 1 minute after habituation at 32°C does not override habituation (p > 0.99, Friedman test). **(E)** Combining *TH-C-Gal80* along with *TH Gal4* blocks the expression in *TH-C'* subset of neurons only, as observed by GFP expression (SEZ represents subesophageal zone). **(F)** The flies carrying *TH-C-Gal80* along with *TH-Gal4* fail to show habituation override when these are activated at 32°C after habituation further confirming the role of *TH-C'* subset of neurons (p >0.99, Friedman test) (*TH-Gal4/ UAS-TRPA1*, **p = 0.0025, Friedman test) (*TH-C-Gal80/+*, p = 0.9061, Friedman test) (*UAS-TRPA1/+*, p > 0.99, Friedman test). **(G)** Activation of *TH-C'* Gal4 neurons does not affect the naïve response to sucrose tested at 0.1% (p = 0.56, Wilcoxon sign rank test), 0.5% (p = 0.37, Wilcoxon sign rank test), 1% (p = 0.73, Wilcoxon sign rank test), 2% (p = 0.31, Wilcoxon sign rank test). Bars represent mean ±SEM, points represent mean ±SEM, ns represents not statistically significant, p>0.05.

669 **Figure 5. Activity of *TH-C'* subset of neurons is necessary for novelty induced override**

670 **(A**) At permissive temperature (RT = 21°C), presentation of 10% yeast to the labellum
671 overrides habituation in both *TH-C' Gal4/ UAS-Shit^{s1}* (***p = 0.0008, Friedman test) and *TH-*
672 *Gal4/+;UAS-Shi^{ts1}/+* flies and (*p = 0.0457, Friedman test) respectively. However, at
673 restrictive temperature (34°C) blocking the synaptic transmission of *TH-Gal4* and *TH-C'-Gal4*
674 neurons by expressing *Shi^{ts1}* during presentation of novel yeast stimulus after habituation to
675 sucrose, impairs habituation override (*TH-Gal4/+;UAS-Shi^{ts1}/+*, p = 0.3897, Friedman test)
676 (*TH-C'-Gal4/ UAS-Shit^{s1}* , p > 0.99, Friedman test) respectively. The genotypic controls show
677 override of habituation to 10% yeast presented to the labellum (*TH-Gal4/+*, **p = 0.0015,
678 Friedman test) (*UAS-Shi^{ts1}/+*, ***p = 0.0009, Friedman test), (*TH-C'-Gal4/+*, ***p = 0.0001,
679 Friedman test) **(B)** Blocking *TH-Gal4* neurons and *TH-C'-Gal4* neurons does not have an effect
680 on PER itself (p = 0.1909 and p = 0.914, respectively, Wilcoxon sign rank). Bars represent
681 mean ± SEM, ns represents not statistically significantly, p>0.05.

682

683 **Figure 6. TH Gal4 neurons are functionally connected to GRNs**

684 **(A)** Expression pattern of Gr66a with respect to TH expressing neurons. **(B)** GRASP between
685 Gr66a and TH expressing neurons show GFP signal in animals expressing both the components
686 of split GFP. No signal is observed in controls lacking either of split GFP component. **(C)**
687 Controls do not show any significant increase in response at 34°C (*Gr66a LexA/ +; TH-Gal4/+*,
688 p = 0.9079, *LexAop-TRPA1/+; UAS-Shits1/+*, p = 0.0580, Friedman test). Activation of Gr66a
689 while inhibiting TH expressing neurons simultaneously fails to reinstate habituated response
690 (*Gr66a LexA/ LexAop-TRPA1; TH-Gal4/ UAS-Shits1*, p= 0.9494, Friedman test), whereas
691 activating Gr66a solely overrides habituation (*Gr66a LexA/ LexAop-TRPA1; TH-Gal4/ +*, *p
692 = 0.02). Control at permissive temperature does not show any difference in habituated response

693 (*Gr66a LexA/ LexAop-TRPA1; TH-Gal4/ UAS-Shit*^{s1} , p > 0.99, Friedman test). Bars represent
694 mean ± SEM, ns represents not statistically significantly, p>0.05, SEZ represents
695 subesophageal zone.

696

697 **Figure 7: Circuit model for PER habituation override.**

698 Model for habituation override in gustatory system of *Drosophila.* As shown in Paranjpe et al,
699 2012, plasticity of GABAergic neurons underlies habituation. During habituation override,
700 excitation of sensory neurons responsive to novel stimulus activates *TH-C'* subset of
701 dopaminergic neurons either directly or indirectly via a probable intermediate neuron. As there
702 is weak inhibition mediated by local inhibitory interneurons (iLNs) onto *TH-C'* neurons in this
703 pathway, these dopaminergic neurons exhibit strong excitation and in turn inhibit iLNs that are
704 potentiated during habituation. As a result, disinhibition leads to strong excitation of projection
705 neurons (PNs) and therefore, strong PER in response to familiar stimulus. In contrast,
706 excitation of sensory neurons responsive to familiar stimulus activates *TH-C'* neurons but due
707 to strong iLN inhibition onto *TH-C'* neurons resulting from habituation in this pathway,
708 dopaminergic neurons fail to disinhibit. This leads to weak PN excitation and therefore, weak
709 PER in response to the familiar stimulus.

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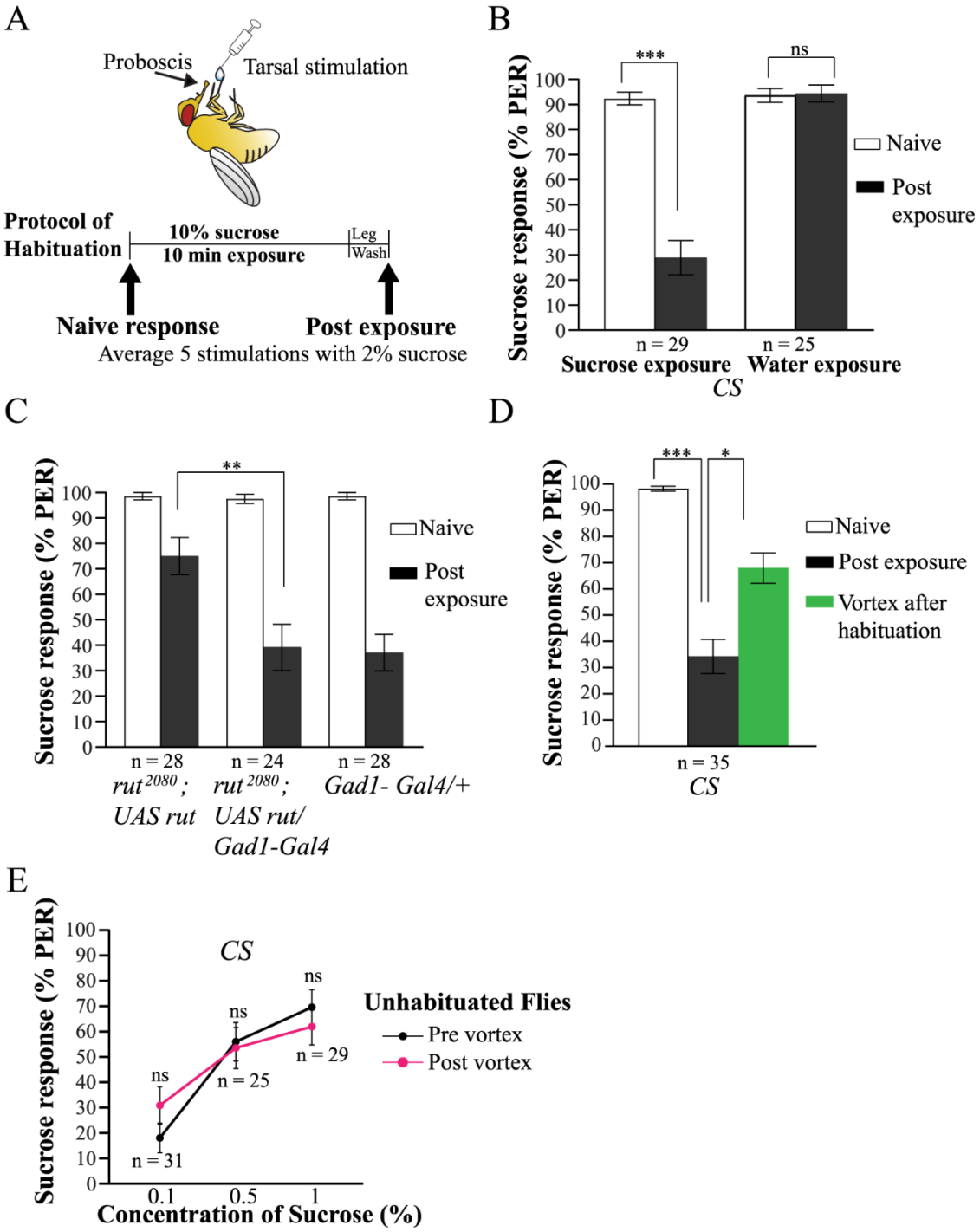

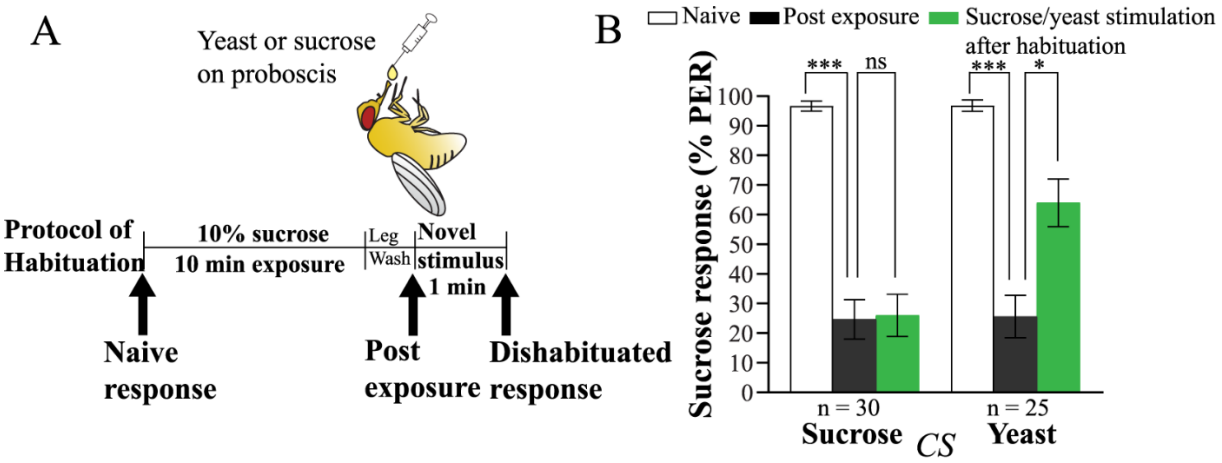

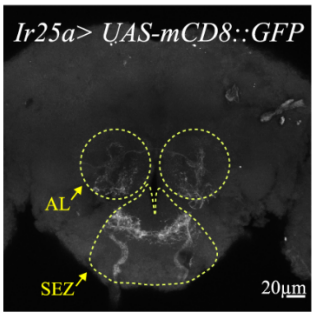

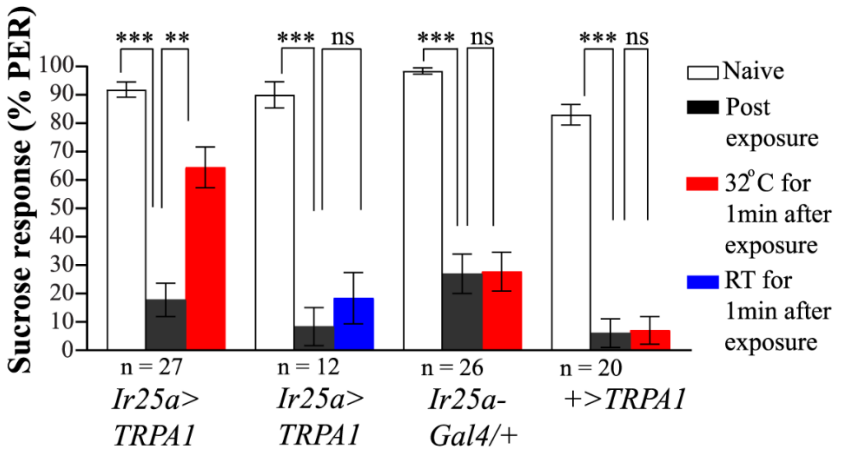

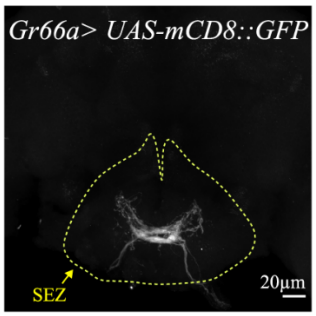

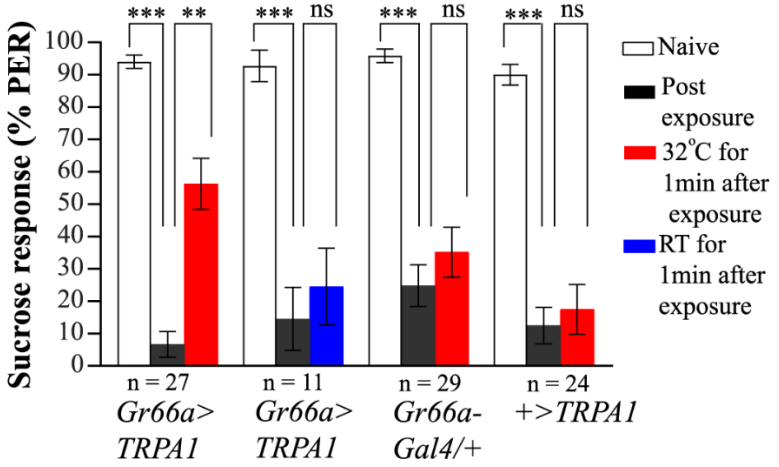

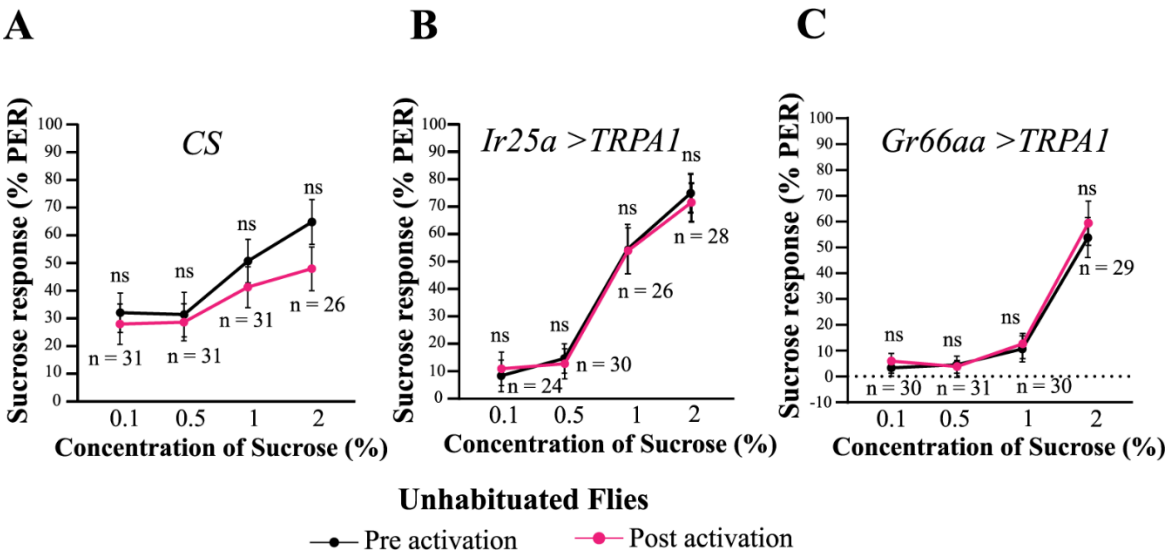

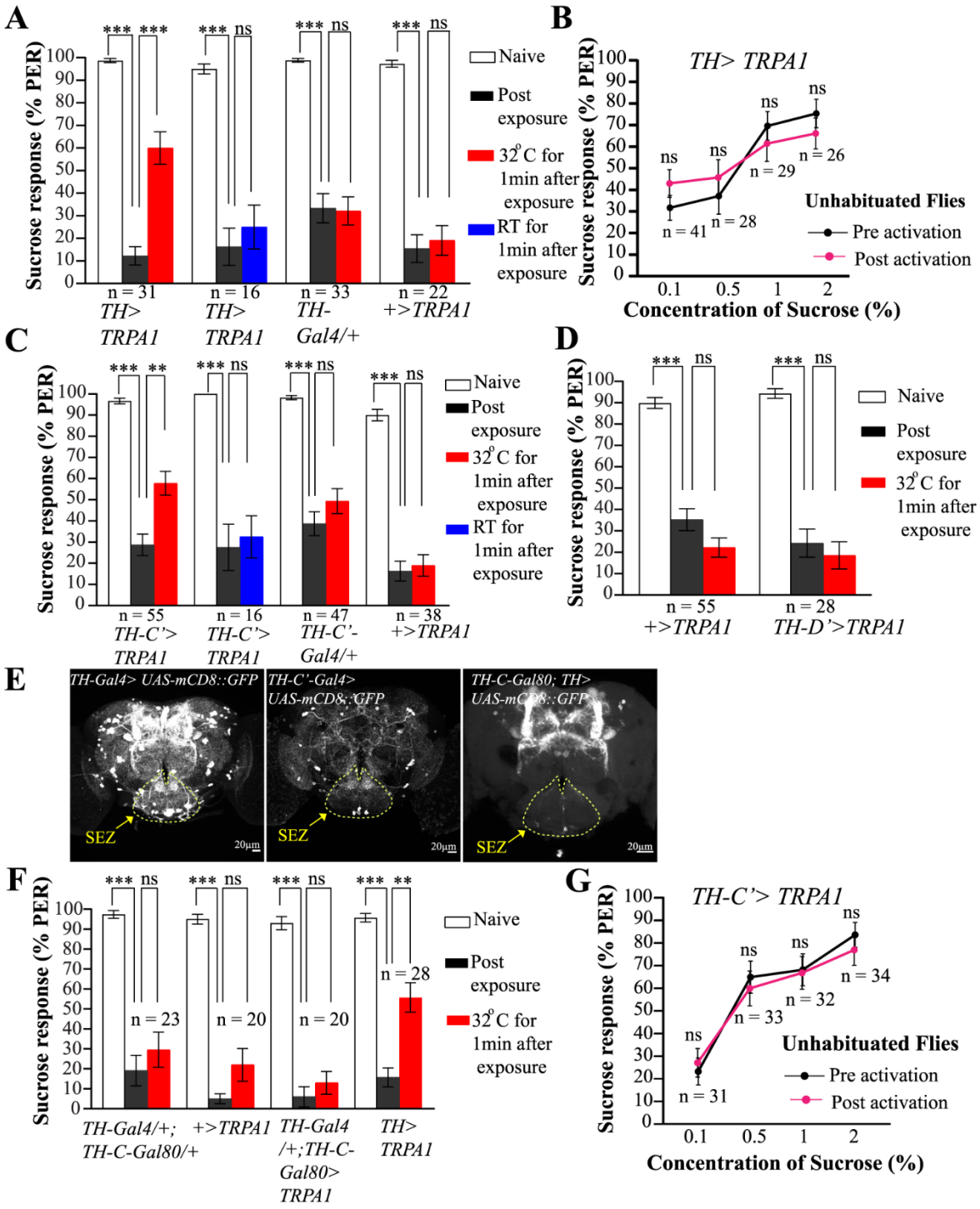

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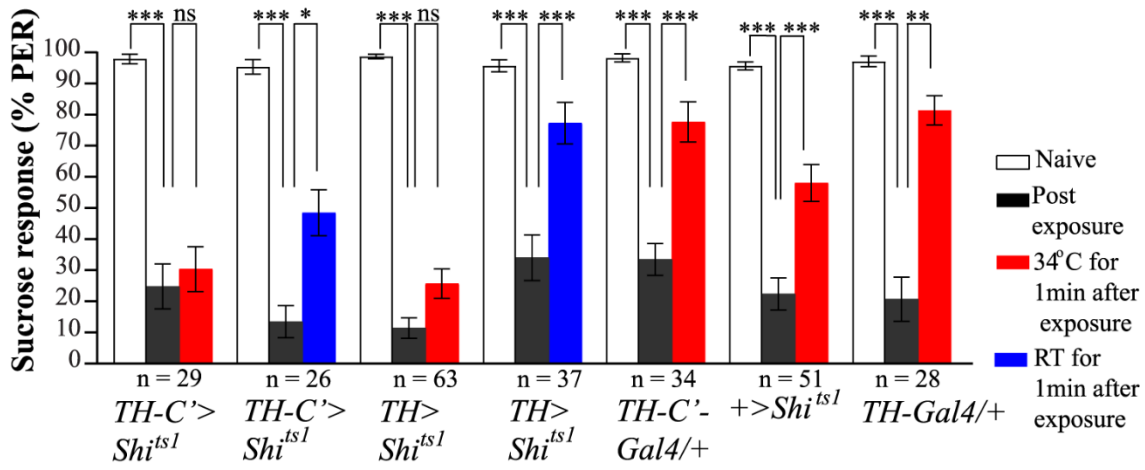

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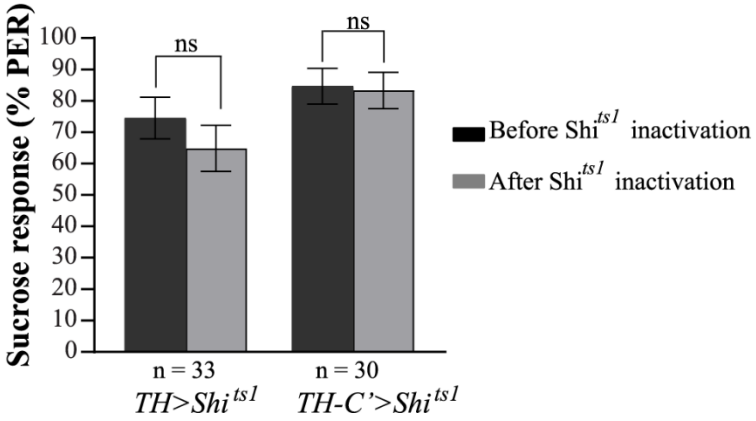

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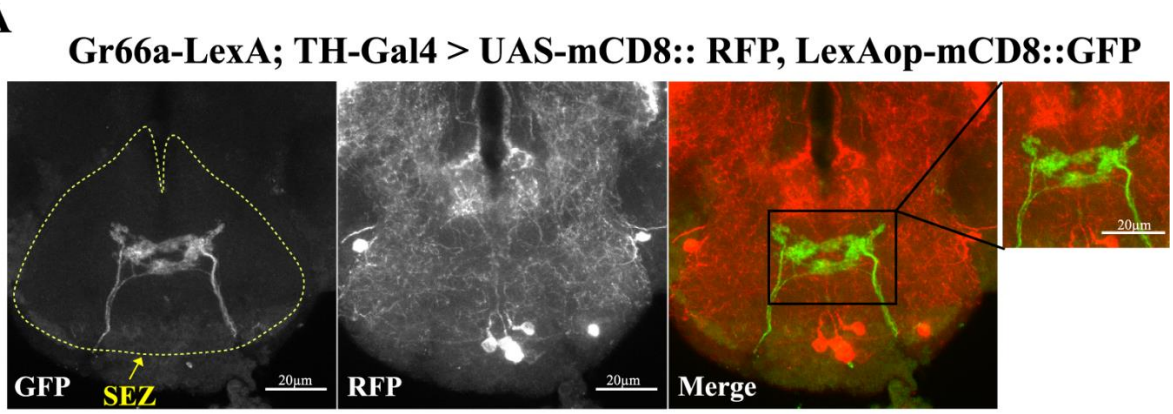

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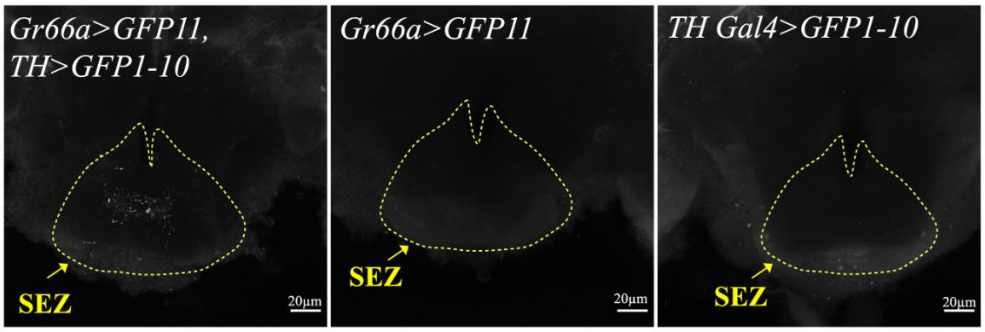

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