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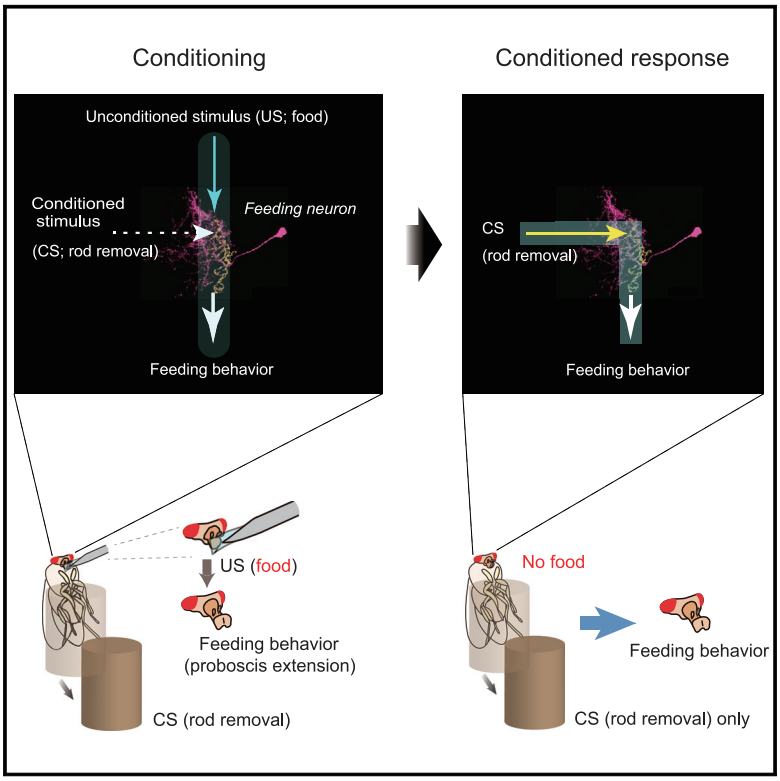
Alteration in information flow through a pair of feeding command neurons underlies a form of Pavlovian conditioning in the *Drosophila* brain

Graphical abstract



Highlights

- A new paradigm for Pavlovian conditioning using tactile CS is established
- The feeding command neuron acquires responsiveness to the CS during conditioning
- Activity of the feeding command neuron is required to form the conditioned response
- During the conditioning, CS input is intensified probably through Hebbian plasticity

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In brief

To define causal relationship between circuit plasticity to memory, Sakurai et al. establish a new protocol for Pavlovian conditioning between tactile CS and gustatory US. During the conditioning, a pair of feeding command neurons acquires the responsiveness to the CS through change in the signal flow through these neurons.

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Report

Alteration in information flow through a pair of feeding command neurons underlies a form of Pavlovian conditioning in the *Drosophila* brain

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SUMMARY

Pavlovian conditioning[1] is a broadly used learning paradigm where defined stimuli are associated to induce behavioral switching. To define a causal relationship between activity change in a single neuron and behavioral switching, we took advantage of a "command neuron" that connects cellular function to behavior.[2] To examine the cellular and molecular basis of Pavlovian conditioning, we previously identified a pair of feeding command neurons termed "feeding neurons" in the adult *Drosophila* brain[3] using genetic screening[4] and opto- and thermo-genetic techniques.[5–7] The feeding neuron is activated by sweet signals like sucrose and induces the full complement of feeding behaviors, such as proboscis extension and food pumping. Ablation or inactivation of the pair of feeding neurons abolishes feeding behavior, suggesting that this single pair of neurons is indispensable for natural feeding behaviors.[2,3] Here, we describe a novel conditioning protocol to associate a signal-mediating rod removal from legs (conditioned stimulus [CS]) to feeding behavior induced by sucrose stimulation (unconditioned stimulus [US]). Calcium imaging of the feeding neuron demonstrated it acquires responsiveness to CS during conditioning, with inactivation of the feeding neuron during conditioning suppressing plasticity. These results suggest conditioning alters signals flowing from the CS into the feeding circuit, with the feeding neuron functioning as a key integrative hub for Hebbian plasticity.

RESULTS

A novel paradigm for Pavlovian conditioning

To correlate circuit plasticity to memory during Pavlovian conditioning, a novel conditioning protocol that shows feeding-associated learning by a physically fixed animal with optical access to the CNS[8] was needed (Figures 1A and S1A–S1F). In this experimental configuration, a fly with its brain exposed to perfused saline for stable observation and manipulation can be maintained for more than 24 h. As an efficient conditioned stimulus (CS), we adopted "rod removal" because many insects show vigorous responses to tactile input in a stereotypic manner (Video S1). In all insects tested, rod removal immediately triggered flailing of the legs, presumably to regrasp the missing rod. When a rod held by a fly is removed, a typical leg movement similar to other insects is observed (Video S1). Immediately after starting rod removal as a CS, which continues for 5 s, sucrose (1 M) was applied to its proboscis as an unconditioned stimulus (US), and the fly vigorously extended its proboscis while displaying simultaneous leg movement driven by the CS (Figure 1A; Video S2). The US was restricted to sweet sensing at the proboscis without nutrition because the esophagus was cut and sucrose solution was provided through touching the proboscis with a sucrose-wetted paper strip to avoid ingestion (see STAR Methods). Whereas a naive fly rarely shows proboscis extension only to rod removal, 5 repetitions of the associated sucrose US every 30 s formed a conditioned response, with proboscis extension triggered by rod removal alone (Figures 1A–1D; Video S3). The CS-induced behavior after conditioning was indistinguishable from US-induced behavior by sucrose (Figure S1G), suggesting that the feeding motor program was induced. Similarity of the conditioned response to thermo-genetically induced feeding behavior by direct activation of the feeding neuron[3] indicates this command neuron sits at the top of the feeding motor program that is now under control of the CS. In addition to the tactile stimulus from rod removal, the CS might also represent proprioception signals responding to the flailing movement of legs or a startled stimulus secondary to sudden rod removal.

Control protocols with unpaired CS and US did not give rise to CS-induced proboscis extension (Figure 1D), demonstrating the response is caused by coincidence between the CS and US. The conditioned response was observed in flies for up to 20 min after pairing but became less robust beyond this time frame. The time course shown in Figure 1D resembles that of *Drosophila* olfactory learning, where repeated CS without US causes memory extinction.[9] We speculate the reduced response observed in

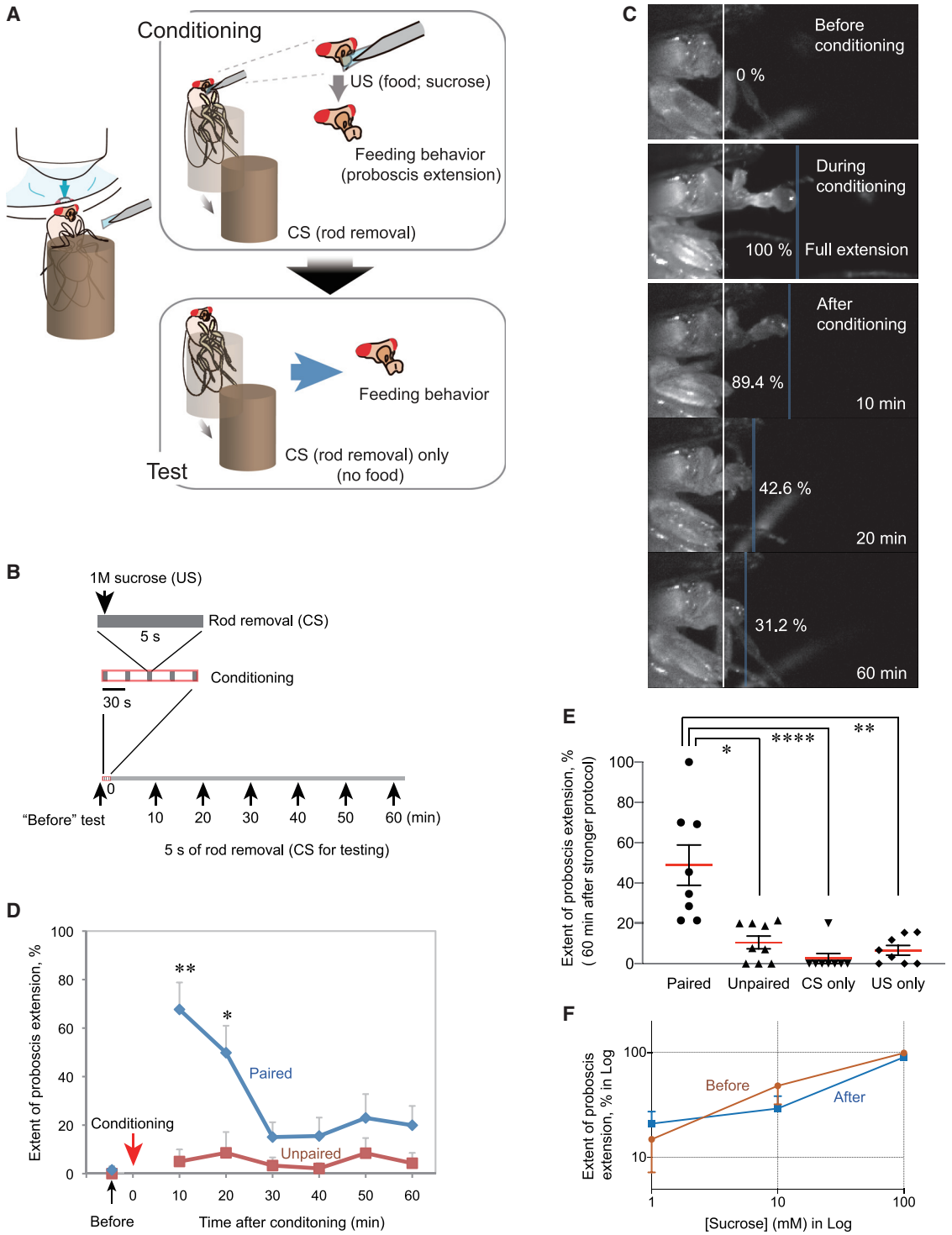

Figure 1. A novel paradigm for Pavlovian conditioning

(A) Schematic diagram of a newly designed behavioral experiment using a single head-opened fly for Pavlovian conditioning. Left: a *Drosophila* adult placed within a chamber where the head of the fly is open to perfused saline and the brain is exposed for microscopy is shown. Right: conditioning and testing procedures are shown. 1 M aqueous sucrose solution was applied from a paper strip soaked with sucrose solution to induce feeding behaviors like proboscis extension (see STAR Methods and Figures S1A–S1F).

(B) Conditioning time course. Rod removal followed by 1 M sucrose stimulation was applied 5 times every 30 s during conditioning.

(C) Sequential changes in an individual fly in response to CS except for the second panel (response to 1 M sucrose during conditioning) to demonstrate how proboscis extension was measured for (D), (E), (F), and other proboscis extension data in the following figures. All proboscis extension data are shown as % ratios

(legend continued on next page)

the current assay also reflects CS-induced extinction, which can be tested with extinction-free experiments without repeated CS in future studies. To maintain robust conditioned responses for longer periods, 7.6 s of rod removal was applied with four 0.3-s pauses to refresh the CS, instead of continuous rod removal as before. The conditioned response lasted longer using this stronger protocol, with CS-induced proboscis extension remaining statistically significant for up to 1 h after conditioning (Figure 1E) and lasting for more than 3 h in some cases. Even with the ''stronger'' protocol, conditioned responses in the paired group were significantly different from the control groups (unpaired, CS only, and US only). These results suggest that both CS and US and their association are indispensable to form conditioned responses. Because the testing CS was applied every 10 min also in the stronger protocol, the repeated CS may have caused memory extinction, leading to reduced expression during both protocols, although distinct memory can still be recognized at 60 min that is more resistant to extinction than in Figure 1D.

Is it possible that sensitization of the feeding neuron could explain the conditioned response? Two findings argue against this possibility. First, the results from ''unpaired'' and ''US only'' responses, which have the same chance of sensitization by the same US, did not result in a conditioned response. Second, sensitivity to sucrose was similar before and after conditioning (Figure 1F). Thus, the conditioned response is unlikely to be caused by non-specific sensitization of the feeding neuron during the paradigm, as the input from the CS signal is specifically intensified for the feeding behavior compared to the sucrose signal. These results demonstrate this conditioning protocol allows tracking of behavioral associations in an individual animal with its brain exposed for observation (Figure 3) and optogenetic manipulation (Figure 4).[8]

Defective conditioned response in memory mutants

To compare the properties of this conditioning paradigm with other learning assays, we tested *dunce* (*dnc*)[10] and *rutabaga* (*rut*)[11] mutants of *Drosophila* that have memory deficits in olfactory valence assays[9] associated with defective cyclic AMP (cAMP) signaling.[12,13] Both *dnc* and *rut* showed reduced conditioned responses after the protocol (Figure 2A), although CS-induced response and US-induced response during conditioning were similar in *dnc* and *rut* mutants to wild type (WT) (Figures 2B and 2C). These results are consistent with the hypothesis that an associative memory was not formed normally during the conditioning protocol in these mutant backgrounds, although we cannot exclude defects in functions other than memory acquisition that may also contribute.[2] Future single-cell analyses and manipulations available with this experimental system should elucidate how cAMP signaling contributes to the Pavlovian memory pathway.

The feeding neuron acquires responsiveness to CS during conditioning

The emergence of CS-induced feeding behavior after conditioning suggests generation of a functional link between the CS-stimulated neuronal network and the US-stimulated network, leading to CS-induced excitation of motor neurons driving feeding behavior. Given a paper strip with an aqueous sucrose solution was applied as the US, water and mechanical stimulation might also contribute to this signal. Thus, we tested gustatory contribution in the US through conditioning using a wet paper strip without sucrose. When sucrose was missing from the US, proboscis extension was much less than observed during conditioning with a 1 M sucrose strip (Figure S2A), resulting in a residual conditioned response even at 10 min after the stronger protocol (Figure S2B), as expected from Figure S2A. Thus, although water and mechanical stimulation may contribute a small amount to the US, the sweet gustatory stimulus is indispensable for forming a robust conditioned response. Sweet stimuli, which are critical for inducing feeding behavior, are sensed by Gr5a neurons[14] that extend axons into the subesophageal zone (SEZ). Gr5a neurons terminate in the area where dendrites of the feeding neurons are expanding, with Gr5a neurons activating the feeding neuron through indirect connections.[3] By integrating these multiple inputs, the feeding neuron is hypothesized to trigger the entire feeding behavior[3] through activation of downstream components that culminates in responses from specific motor neuron groups.[15] These properties suggest the feeding neuron may function as a hub for information processing at the center of the feeding circuit. Although the identity of CS-conveying neurons is not yet known, sensory neurons detecting tactile information and chordotonal organs sensing mechanical stimuli terminate in the SEZ.[16,17] Thus, we

of each proboscis extension in measured length/full extension in measured length in response to 1 M sucrose (second panel). A fly's head with its extending proboscis and anterior end of the prothorax with moving forelegs is shown. The head is attached to a chamber, with the top exposed from the upper plane and dissected in saline.

(D) Time course of conditioned behavior indicated as extent of proboscis extension (ratio to full extension as shown in C). Paired group conditioned with schedule in (B) and unpaired group, where CS was given 10 min before US, were analyzed at each time point with Mann-Whitney *U* test. Significant differences between the two groups were found at 10 (**$p < 0.01$) and 20 (*$p < 0.05$) min. The number of animals analyzed for each time point were seven for paired and five for unpaired. Error bars are SEM.

(E) Conditioned response at 60 min after conditioning induced through stronger CS (7.6 s of rod removal with four times of 0.3-s pauses to refresh the CS instead of continuous rod removal) performed with the same protocol as in (B), where CS was applied every 10 min. Extent of proboscis extension was measured and shown as a ratio to full extension as shown in (C). Paired ($n = 8$), unpaired ($n = 9$), CS-only ($n = 8$), and US-only ($n = 8$) groups were tested with Kruskal-Wallis test, and significant difference between the four groups was found (***$p < 0.001$). Dunn's post hoc analysis gave differences between paired and unpaired (*$p < 0.05$), between paired and CS only (***$p < 0.001$), and between paired and US only (**$p < 0.01$). Error bars are SEM.

(F) Comparison of sucrose-induced behavior before and after the conditioning. Responses to 1 mM, 10 mM, and 100 mM sucrose stimulation to the proboscis were sequentially measured before and after conditioning as extent of proboscis extension. The tip of the proboscis was rinsed with Milli-Q water to remove remaining sucrose after conditioning before this assay. Significant differences were not found between before and after conditioning using Student's t test at each concentration ($p > 0.05$). Six animals were analyzed. Error bars are SEM.

See also Figure S1 and Videos S1, S2, and S3.

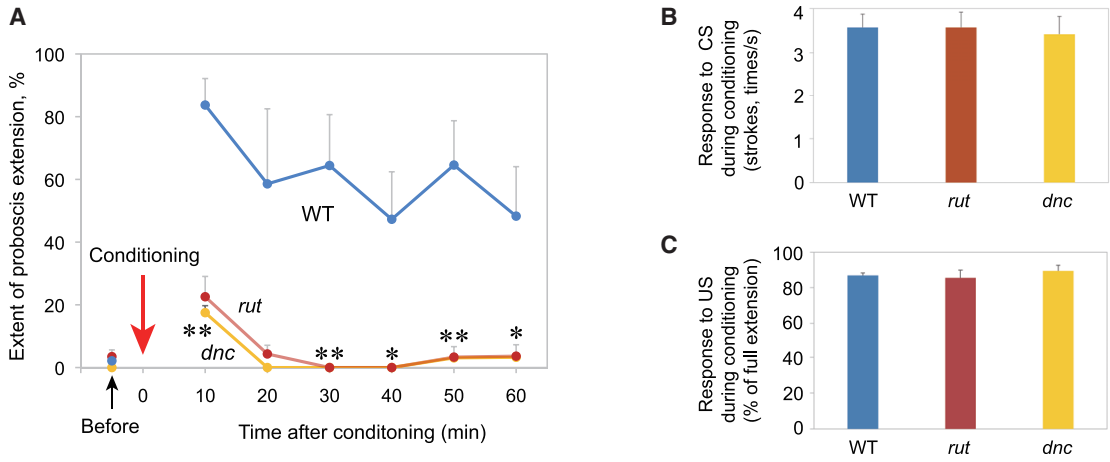

Figure 2. Reduced conditioned response with stronger CS in *dnc* and *rut* mutants

(A) Time course of conditioned behavior indicated as extent of proboscis extension. WT, dnc^1, and rut^2 flies were analyzed at each time point with Kruskal-Wallis test, and significant difference between the three groups was found at 10 (**$p < 0.01$), 30 (***$p < 0.001$), 40 (*$p < 0.05$), 50 (***$p < 0.001$), and 60 (**$p < 0.01$) min. Dunn's post hoc analysis indicated differences between WT and dnc^1 at 10 (**$p < 0.01$), 30 (**$p < 0.01$), 40 (*$p < 0.05$), 50 (**$p < 0.01$), and 60 (*$p < 0.05$) min and between WT and rut^2 at 30 (**$p < 0.01$), 40 (*$p < 0.05$), 50 (**$p < 0.01$), and 60 (**$p < 0.01$) min (only * is shown for both mutants at 60 min in the graph). Five animals of each genotype were analyzed for each time point. Error bars are SEM.

(B and C) Responses to the CS and the US in WT, dnc^1, and rut^2 during conditioning. See also Figure 1E.

(B) Strokes of mid-leg at the camera side were counted during each rod removal, and averages of their frequency (times/s) through five pairings are shown for each group as a response to the CS.

(C) Sucrose-induced proboscis extension (% of full extension) as a response to the US. WT, dnc^1, and rut^2 flies were analyzed with one-factor ANOVA. Significant difference between the three groups was not found either in (B) or in (C) ($p > 0.05$). Five animals were analyzed for each genotype, which passed Kolmogorov-Smirnov test for normality. Error bars are SEM.

hypothesize that tactile CS signals sensed by these neurons send information into the feeding circuit, allowing integration with Gr5a neuronal input onto feeding neurons that ultimately drive motor neurons to control feeding behavior. Using genetic techniques to selectively image and manipulate the feeding neuron as a single cell, we examined how this command neuron changes during Pavlovian conditioning that links somatosensory inputs to sucrose-induced feeding behavior.

To observe the activity of the feeding neuron throughout conditioning experiments, we monitored the fluorescence of the calcium indicator protein, GCaMP6m,[18] which increases fluorescence following calcium influx into excited cells (Figure 3A). We expressed the calcium indicator in the feeding neuron using the GMR81E10-*GAL4* driver, which has more restricted expression than the *GAL4* driver used in our original study[3] and allowed exclusive imaging and manipulation of only the feeding neuron (Figures S2C and S2D). GCaMP6m shows robust responses to direct sucrose application to the proboscis (Figure S2E).[3] Before conditioning, CS-induced excitation of the feeding neuron was not detected, consistent with the lack of proboscis extension (Figures 3B and 3C). After conditioning, excitation of the feeding neuron was detected as an increase in GCaMP6m fluorescence in response to the CS alone without any sweet signal, consistent with the observed proboscis extension triggered by CS alone (Figures 3B and 3C; Video S4). These results indicate that the CS signal flows into the feeding circuit at the level of the feeding neuron or above after conditioning, rather than to downstream motor neurons. To assess the strength of the conditioned response, we next compared the conditioned response of the feeding neuron to its response to sucrose directly, which is the normal default trigger for activating it and initiating the feeding motor program.[3] We stimulated the proboscis of a fly with 1 mM, 10 mM, and 100 mM sucrose and monitored GCaMP fluorescence. The conditioned response at 20 min after conditioning was similar to 10 mM sucrose-triggered response in terms of both GCaMP fluorescence and feeding behavior (Figures 3D and 3E; Video S4). These results demonstrate that Pavlovian conditioning alters the feeding neuron's responsiveness to the CS, which substitutes for the default sucrose US in a manner similar to artificial activation of the feeding neuron through opto- and thermo-genetics substituting for the sucrose signal.[3]

Inactivation of the feeding neuron during conditioning suppresses plasticity

The change in responsiveness of the feeding neuron indicates a change in information processing by the cell after CS-US pairing. However, the alteration in responsiveness might represent changes occurring in upstream neurons instead of the feeding neuron itself, for example, in sweet (US)-sensing neurons that drive feeding behaviors (Figure S3A). If the activity of the postsynaptic neuron is required for plastic changes in connection strength as Hebb proposed (see Discussion),[19] inactivation of the feeding neuron should block the conditioned response only if the CS signal flows into the feeding circuit at the level of the feeding neuron itself or downstream motor neurons (Figure S3B). To determine whether the CS-driven activation of the feeding neuron is due to changes generated in the feeding neuron or only in upstream neurons of the circuit, we inactivated the feeding neuron during conditioning and tested behavior with CS alone

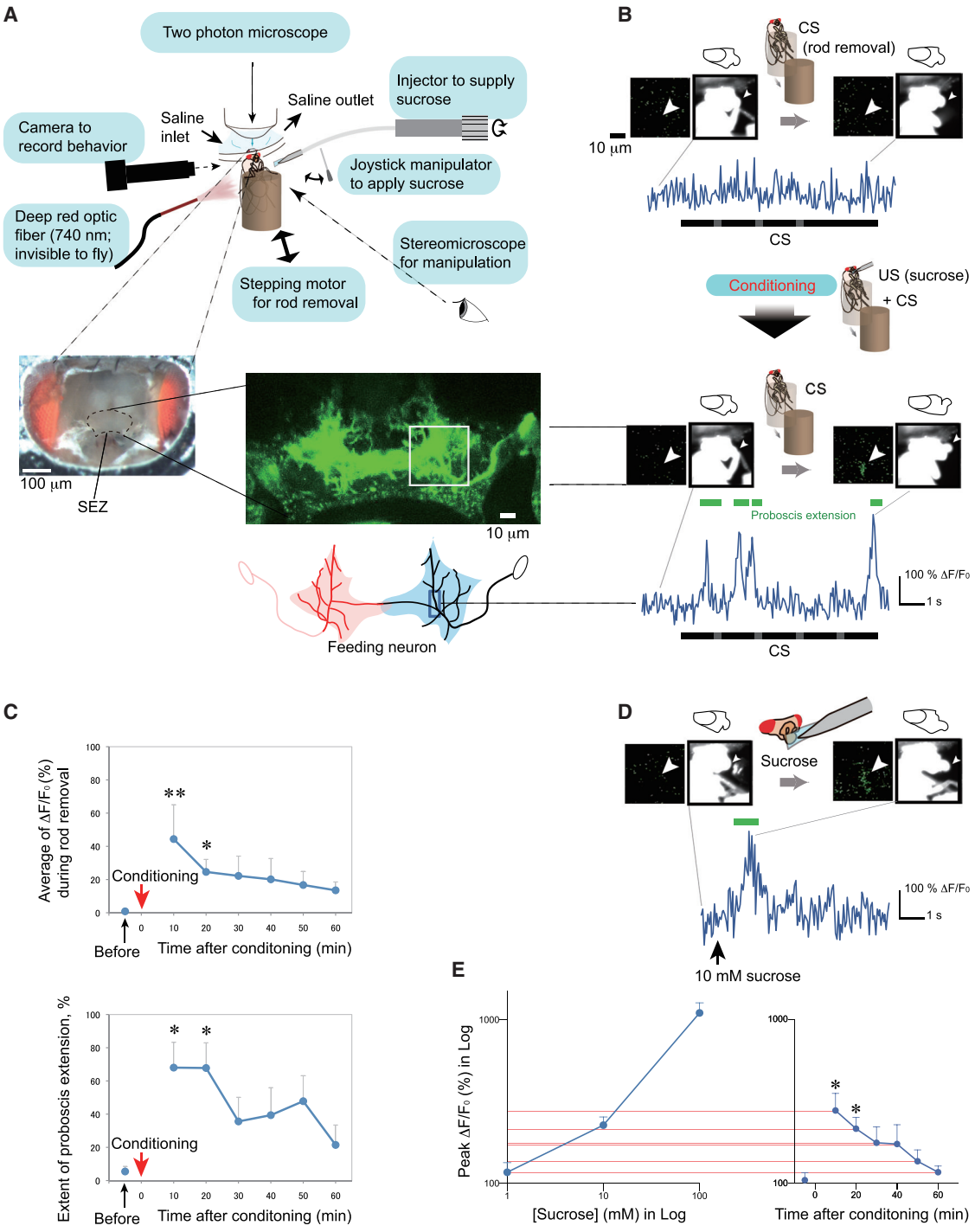

Figure 3. Monitoring activity of the feeding neuron by Ca^{2+} imaging with GCaMP fluorescence during Pavlovian conditioning with stronger CS

(A) (Upper) Schematic drawing of the experimental setup for simultaneous monitoring of feeding behavior and GCaMP fluorescence in the fly brain. See STAR Methods for details. (Lower left) Top view of a dissected brain set in a chamber is shown. The subesophageal zone (SEZ) is outlined. (Lower right) Location of the feeding neurons within the SEZ is shown. The image was a Z projection of 8 two-photon optical sections (2.25 μm increment) from GMR81E10-*GAL4* > GFP-expressing animals. The white square is area shown for GCaMP fluorescence in (B). Branching of each feeding neuron is drawn below in red (left) and black (right). Navy blue rectangle (4.97 μm × 9.93 μm) is the area used for GCaMP quantification shown as traces in (B).

(B) GCaMP fluorescent imaging and feeding behavior before (upper) and 10 min after (lower) conditioning in GMR81E10-*GAL4* > GCaMP6m flies. GCaMP imaging in the left square is shown next to the image of feeding behavior (right) at the time point shown as a connected line in the lower traces. Arrowheads highlight the main dendritic trunk and the tip of the proboscis. Illumination for GCaMP fluorescence brightens the fly, with drawings tracing the outline of the fly

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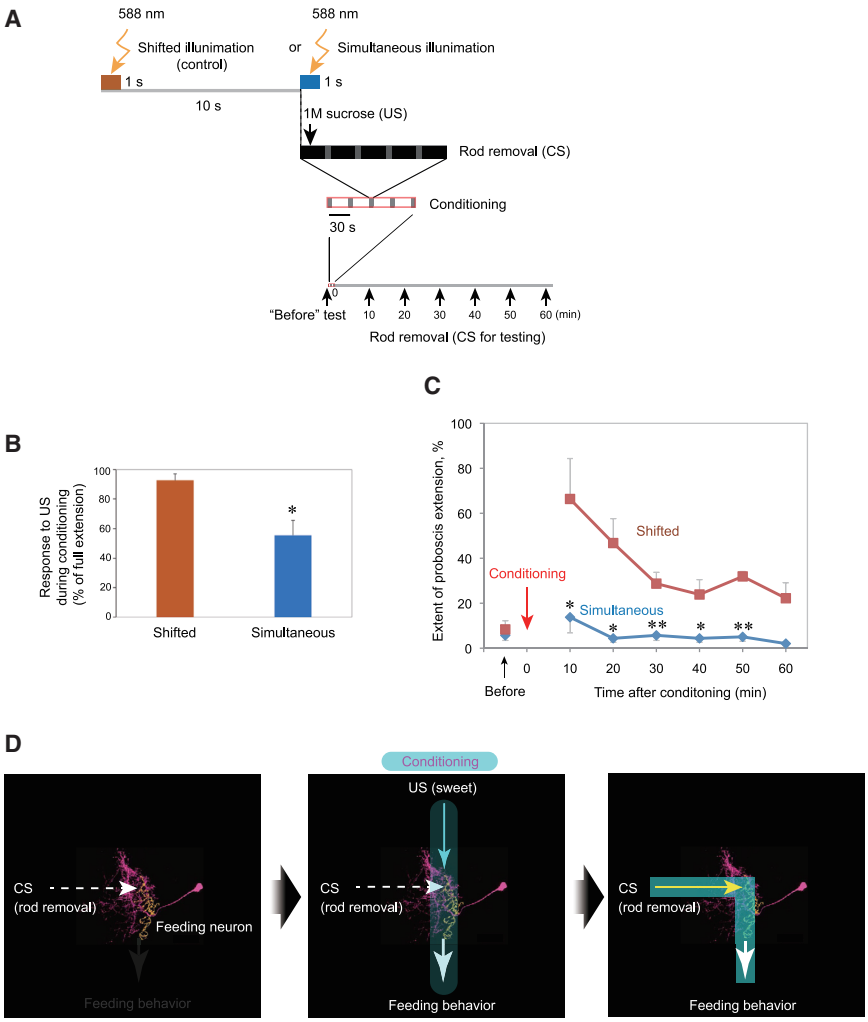

Figure 4. Effect of inactivation of the feeding neuron with halorhodopsin on conditioned responses with stronger CS

(A) Timetable for the experiment to inactivate the feeding neuron. Illumination with a 588-nm laser was applied to the dorsal feeding neuron dendrites on both sides.

(B) Suppression of US-induced response by inactivation of the feeding neuron with halorhodopsin expressed in the feeding neuron using GMR81E10-*GAL4*. Significant difference between the two groups was found (*$p < 0.05$). Averages of five sucrose stimulation trials were compared between the two groups with Student's t test. Five animals were analyzed for each group, which passed Kolmogorov-Smirnov test for normality.

(C) Time course of conditioned response. The simultaneous and shifted groups shown in (A) were analyzed at each time point with Student's t test. Significant differences between the two groups, which passed Kolmogorov-Smirnov test for normality, were found (*$p < 0.05$ at 10, 20, and 40 min; **$p < 0.01$ at 30 and 50 min). At 60 min, the simultaneous group did not pass normality test, and comparison at 60 min with Mann-Whitney *U* test was not significant enough ($p = 0.0635$). Five animals were analyzed for each time point. Error bars are SEM.

(D) Schematic diagram to demonstrate changes during the Pavlovian conditioning assay to associate the US (sucrose) with a tactile CS (rod removal). After conditioning, information processing by the feeding neuron changes such that the feeding behavioral program is now activated by CS alone. The images of the feeding neuron are from our previous report.[3]

See also Figures 1E and S3.

after conditioning every 10 min (Figure 4A). The US-induced response during conditioning was suppressed with halorhodopsin[20] at the level of feeding neuron and downstream targets (Figure 4B) although the CS-induced response was not altered with halorhodopsin (Figure S3D). Halorhodopsin-mediated inactivation at every CS/US pairing led to disruption of the plastic change in CS-induced proboscis extension, whereas inactivation using a shifted timing protocol did not abolish the plastic change (Figures 4A and 4C). These results indicate activity of the feeding neuron is required to form the conditioned response, consistent

head. Below the side-by-side images are quantification of GCaMP fluorescence for 10 s shown as fluorescence change as a proportion of baseline fluorescence ($\Delta F/F_0$) measured and averaged in the navy-blue rectangle in (A), where the nerve trunk was in focus. The period required to provide 7.6 s of rod removal as CS is shown as black bars below the traces. Green bars indicate periods when proboscis extension was observed.

(C) Time course of Pavlovian conditioning. (Top) $\Delta F/F_0$ averaged for the 7.6-s rod removal period shown as averages for 7 sample flies at each time point. Averaged $\Delta F/F_0$ through rod removal at 30 s before pairing and at 10 min and 20 min after pairing were analyzed with non-parametric repeated-measures ANOVA (Friedman test). Significant difference between the three groups was found (**$p < 0.01$). Dunn's post hoc analysis revealed differences between before and 10 min (**$p < 0.01$) and between before and 20 min (*$p < 0.05$). Error bars, SEM. (Bottom) The furthest proboscis extension in the rod removal period was quantified as the ratio to full extension. The furthest proboscis extensions before and 10 and 20 min after conditioning were analyzed with non-parametric repeated-measures ANOVA (Friedman test). Significant difference between the three groups was found (**$p < 0.01$). Dunn's post hoc analysis revealed differences between before and 10 min and between before and 20 min (*$p < 0.05$). Error bars, SEM.

(D) Activation of feeding neuron shown by GCaMP fluorescence following 10 mM sucrose stimulation. The data format and scale are the same as those in (B). Green bars indicate periods when proboscis extension was observed.

(E) (Left) Peak $\Delta F/F_0$ responses to 1 ($n = 16$), 10 ($n = 10$), and 100 mM ($n = 8$) sucrose are plotted in log as function of sucrose concentration in log. Error bars, SEM. (Right) Time course of Pavlovian conditioning for the highest peak of $\Delta F/F_0$. The highest peaks in the 7.6-s rod removal period are shown as averages for 7 sample flies at each time point. Peak $\Delta F/F_0$ before (the isolated filled circle) and 10 and 20 min after conditioning were analyzed with non-parametric repeated-measures ANOVA (Friedman test). Significant difference between the three groups was found (*$p < 0.05$). Dunn's post hoc analysis indicated differences between before and 10 min and between before and 20 min (*$p < 0.05$). Error bars, SEM.

See also Figures 1E, S2, and Video S4.

with plasticity creating functional connections onto the feeding neuron (Figures 4D and S3C).

DISCUSSION

In the current study, we demonstrate Pavlovian conditioning between tactile (CS) and gustatory (US) stimuli results in altered information processing by a pair of command neurons that control the *Drosophila* feeding circuit. This conditioning paradigm creates CS-induced excitement of the feeding neuron that commands feeding behavior in this animal, with the conditioned response requiring activity of the feeding neuron during pairing. Pioneering studies by Kandel and colleagues demonstrated the first synaptic and cellular mechanism underlying classical conditioning using the *Aplysia* gill withdrawal response.[21] In *Aplysia*, the presynaptic terminal of a sensory neuron innervating the motor neuron was modulated by serotonin. Presynaptic modulation as a mechanism to generate *Drosophila* valence behaviors has been extensively studied, and recent progress indicates presynaptic terminals innervating mushroom body output neurons are modulated by dopaminergic neurons to establish *Drosophila* valence through appetitive and aversive olfactory association.[22] Neither *Aplysia* plasticity nor *Drosophila* valence in these paradigms requires postsynaptic activity during learning. In contrast, Hebb proposed general principles to explain mechanisms for memory formation[19] that better match results from commonly used mammalian experimental models, such as hippocampal long-term potentiation (LTP).[23] Hebb postulated sequential firing of a presynaptic neuron and postsynaptic partner strengthens their connection. The requirement of feeding neuron activity for the conditioned response observed in this study fits well to a Hebbian mechanism if the underlying change is manifested in altered synaptic properties (although we cannot exclude the possibility that inactivation of the feeding neuron and subsequent behavioral changes also alter neuromodulation, influencing memory formation). During association, CS-conveying neurons and the feeding neuron driven by sucrose stimulation would now fire together, resulting in strengthened connection between CS-conveying neurons and the feeding neuron according to a Hebbian mechanism. The response to US, however, did not change during conditioning (Figure 1F), suggesting that connections between US-conveying neurons and the feeding neuron were not altered. Thus, one can hypothesize that the CS-feeding neuron circuit was newly established, whereas the pre-existing US-feeding neuron connection was not changed, as depicted in Figure 4D. These results suggest that Pavlovian conditioning is established through a change in information processing by the command neuron, which functions as the integrative hub of the feeding circuit.

This Pavlovian conditioning mechanism can also accommodate presynaptic modulation as demonstrated in *Aplysia* plasticity and *Drosophila* valence if reward signals are coupled to Hebbian plasticity through presynaptic neuromodulation. For *Drosophila* valance memory, reward signals consist of both sweet sensing and nutrition.[24,25] We speculate similar reward signals are likely to be relevant *in vivo* for Pavlovian conditioning, although the nutrition reward is eliminated in the current study due to removal of the esophagus from the preparation and application of a sucrose-wet paper strip only to the sensilla of the proboscis (see STAR Methods). Therefore, reward signals are likely constant between the groups we tested, even for different US responses in the halorhodopsin experiments (Figure 4). Thus, differences in reward signal can be excluded from the altered conditioned responses observed between the groups. We hypothesize that inactivation of the feeding neuron results in weaker memory due to postsynaptic activity in this neuron contributing to memory formation independent of changes in the reward signal. We speculate reward signals in the current model may also be mediated by dopamine, octopamine, or serotonin, similar to their role as reward signals in the mushroom bodies for *Drosophila* valence memory.[22] In *Aplysia*, presynaptic adenylyl cyclase, which synthesizes cAMP, is believed to associate CS and US in this conditioning paradigm through US-driven serotonin modulation of the presynaptic terminal of the CS-conveying neuron.[26] Adenylyl cyclase is encoded by *rut*,[12] while *dnc* encodes a cAMP phosphodiesterase that degrades cAMP.[13] As demonstrated in *Aplysia*[27] and *Drosophila*,[28,29] cAMP functions as a signal to modulate synaptic transmission. Given its role in LTP,[30] cAMP is likely to play a critical role in Hebbian plasticity as well, consistent with the disruption of CS-US pairing in *rut* and *dnc* mutants. Considering the involvement of postsynaptic cells in Hebbian plasticity, retrograde signals from the postsynaptic cell can also be coupled to presynaptic cAMP signaling, as demonstrated previously at the *Drosophila* neuromuscular junction.[31]

In the original experiment conducted by Pavlov, we speculate there are groups of neurons that command feeding behavior in the dog. CS/US association may change responsiveness of a subset of those neurons that result in sound-induced saliva secretion, even in the absence of food signals. Electrophysiological studies have shown neural responses to CS are altered after Pavlovian conditioning in cat red nucleus[32] and rabbit cerebellum,[33] although how this kind of plastic change leads to alterations in command neuron function is unknown. Neurons with command function have been identified across many species.[2] A command neuron is pivotally located within the sensorimotor watershed of a neuronal circuit and triggers a behavioral program after integrating numerous sensory inputs. Command neurons were first identified in crayfish through experiments where electrical stimulation of a certain neuron switched on or off behaviors, such as rhythmical movement of the swimmeret[34] or escape responses.[35] After identification of command neurons in invertebrate CNSs,[2,36] Mauthner cells were demonstrated to command escape behavior in fish.[37] Recently, a group of neurons commanding feeding behavior have been identified in the mouse brain.[38] Therefore, the scheme shown in Figure 4D may represent a common mechanism underlying Pavlovian conditioning across species, given the role of command neurons as an integrative hub within the sensorimotor watershed of neuronal circuits.[2]

The feeding neuron in the *Drosophila* brain functions as a single command neuron pair that triggers the entire feeding program.[3] This feature allowed us to reliably demonstrate that CS-induced activation of the feeding neuron after conditioning was as robust as US-induced activation (Figure 3E), suggesting the CS-induced activation of the feeding neuron can trigger the conditioned behavior. Thus, neurophysiological changes can be unambiguously correlated with behavioral change, making

the causal relationship clear and allowing reliable manipulation.[2] Our results are consistent with the assumption that both the CS signal and the US signal converge at a single identified neuron through a Hebbian mechanism. Taking advantage of the defined circuit with the feeding neuron at the center, we can now define the cellular and molecular mechanisms for synaptic plasticity using this experimentally accessible neuron within the CNS. This approach, coupled with real-time live imaging, may allow us to track changes in the structure or activity of identified synapses responsible for memory formation once CS-conveying neurons are defined in the experimental system. If so, we may be able to directly observe pre- and/or postsynaptic changes mediating memory formation on the dendrite of the feeding neuron. Whether a new circuit is generated by strengthening a rudimentary pre-existing connection or a new connection forms *de novo* during associative conditioning will require future analysis. Molecular and cellular mechanisms underlying this plastic change can be investigated in detail as previously characterized at neuromuscular junctions.[31] Taken together, the study of synaptic plasticity in the feeding neuron provides a model system to characterize basic principles of memory formation at the single-cell level.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Fly strains
- METHOD DETAILS
  - Imaging Preparation and Saline
  - Pavlovian Conditioning
  - Calcium Imaging and Laser Inactivation
  - Video/image Processing
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2021.07.021.

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AUTHOR CONTRIBUTIONS

A.S. and M.Y. conceived and designed the experiments. A.S. designed and carried out all genetic manipulations. A.S. and M.Y. developed the conditioning paradigm and SY2020 saline. M.Y. developed tools and method to make long-lasting preparations. A.S. conducted conditioning experiments of wild type and mutants and analyzed data. M.Y. and A.S. conducted calcium imaging experiments and halorhodopsin experiments. H.K. set up equipment for optical manipulation and provided critical advice. J.T.L. provided setup for preliminary calcium imaging experiments and useful advice. A.S. and M.Y. interpreted all data and wrote the manuscript with input from the other authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Raw data from Figures 1D–1F, 2A–2C, 3C, 3E, 4B, 4C, S2A, S2B, and S3D	Current study	Mendeley Data: https://doi.org/10.17632/w37cr4z67t.1
Experimental models: organisms/strains		
D. melanogaster: Canton-S	Gift from Yoshiki Hotta	http://flybase.org/reports/FBsn0000274.html
D. melanogaster: dunce^{1}	Gift from Hiroshi Ishimoto	Dudai et al.[10]
D. melanogaster: rutabaga^{2}	Gift from Hiroshi Ishimoto	Bellen et al.[39]
D. melanogaster: GMR81E10-*GAL4*	Bloomington *Drosophila* Stock Center	BDSC:48367
D. melanogaster: UAS-mCD8-gfp	Gift from Tzumin Lee	Lee and Luo[41]
D. melanogaster: 20x*UAS-IVS-GCaMP6m*	Bloomington *Drosophila* Stock Center	BDSC:42750
D. melanogaster: 20x*UAS-eNpHR3.0.YFP*	Bloomington *Drosophila* Stock Center	BDSC:36350
Software and algorithms		
LabView 2017	National Instruments	N/A
GraphPad Prism 8.4.3	GraphPad Software	N/A
Final cut Pro X 10.5.1.	Apple	N/A
Adobe Photoshop 2020 21.2.2.	Adobe	N/A
Adobe Illustrator 2020 24.3.	Adobe	N/A
Other		
A new saline termed “SY2020” that contained (in mM): NaCl, 85; KCl, 1; $MgCl_2$, 2.5; $CaCl_2$, 3; $NaHCO_3$, 10; HEPES-NaOH, 5; Trehalose, 5; Sucrose, 35 (pH 7.2).	Current study	N/A
Tetric N-Flow (Light curing glue)	Ivoclar vivadent	REF#604046AN A1

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Motojiro Yoshihara (motojiro@nict.go.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Raw data from Figures 1D–1F, 2A–2C, 3C, 3E, 4B, 4C, S2A, S2B, and S3D were deposited on Mendeley at https://doi.org/10.17632/w37cr4z67t.1.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila were kept in a 12/12h light-dark cycle at 25°C according to standard protocols. Females were used in all experiments.

Fly strains

Canton S was used for wild-type, with the *dnc*^{1}[10] and *rut*^{2} [39] alleles as established memory mutants for the Pavlovian assay. GMR81E10-*GAL4* was isolated by Janelia Farm[40] and used to express genes in the Feeding neuron. Adults expressing GMR81E10-*GAL4* and *UAS-mCD8-gfp*[41] were used as heterozygotes to visualize Feeding neuron anatomy.

METHOD DETAILS

Imaging Preparation and Saline

Modifications to a previous method for simultaneous recording of behavior and imaging with a recording chamber termed as FLIES (Fly brain Live Imaging and Electrophysiology Stage)[8] was used for the current experiments. For dissection, modified forceps were used as ultra-fine scissors, allowing 10 micron-size objects to be cut. Forceps with a rectangular tip (approx. 100 μm width) was used for removing internal organs and cuticle. The esophagus was removed to avoid satiation and nutrition reward. Air sacs in the adult head were kept as intact as possible during dissection. For imaging and optical manipulations performed in Figures 3 and 4, a drop of light-curing glue was placed underneath the proboscis to prevent the fulcrum from covering the SEZ, forcing the proboscis to often move up rather than forward during extension. Experiments were performed with a new saline termed "SY2020" that contained (in mM): NaCl, 85; KCl, 1; $MgCl_2$, 2.5; $CaCl_2$, 3; $NaHCO_3$, 10; HEPES-NaOH, 5; Trehalose, 5; Sucrose, 35 (pH 7.2). SY2020 was modified from prior haemolymph-like solutions[42–46] and functioned optimally to keep preparations healthy for more than 24 hours of experimental manipulation.

Pavlovian Conditioning

A toothpick fragment (0.65 ± 0.05mg) was glued to the back thorax of 3-5 day old female adults, allowing movement of the animal and attachment to the recording chamber without anesthetization. Wild-type animals were pre-starved for 27-28 hours in a plastic vial containing 4 mL of Milli-Q water soaked on a paper towel to induce a strong proboscis extension during conditioning. GCaMP6m animals[18] needed longer starvation for 47 hours, while halorhodopsin animals[47] required 40-44 hours of starvation. Preparations were used for conditioning assays only if air sacs in the head of the dissected animal were pulsating vigorously with good aeration through the tracheal system.

After starvation, animals were placed in the recording chamber as shown in Figure 3A. The animal was illuminated with a 740 nm red light (fiber coupled LED M740F2, Thorlabs) from an optic fiber (core diameter 200 μm, NA 2.2, Thorlabs) to allow manipulation for applying sucrose, avoiding visual stimulation to the fly. Fly behavior was recorded from the side using a Video-Zoom-microscope XV-440 (Wraymer) attached to a CMOS camera (DMK23U445, Imaging Source). IC capture 2.4 software (Imaging Source) was used for video acquisition. Rod removal was performed using a stepping motor (TAMM40-10C and GSC-01, SigmaKoki) driven by LabView 2017. A small manipulator (YOU-2, Narishige) was loaded onto the stepping motor. The rod (2 mm in diameter, 10 mm long) was connected to a longer stick for flexible orientation and clamped at the appropriate angle to the manipulator. The rod was positioned near the fly with the manipulator, allowing the animal to grasp the rod with its legs. Movement of the stepping motor removed the rod from the fly's legs, and returned the rod to the original position to allow regrasping.

Sucrose stimulation was performed by touching a strip of paper containing sucrose solution[8]. A rectangular strip (400 μm wide and a few mm long) of Japanese paper (Gampi-shi (Haibara)) was placed in a hypodermic needle with a bent pin holding the paper strip[8]. The needle was connected to a silicon tube and connected to an injector (IM-11-2, Narishige) for supplying sucrose solution (Figure 3A). A joystick manipulator (MN-151, Narishige) was used to position the needle under a stereomicroscope, SMZ-800, loaded on an arm-stand (Nikon), or with a stereomicroscope, LW-820 (Wraymer), loaded on an arm-stand F10 (Wraymer). This method does not allow the fly to ingest sucrose as a reward, restricting reward detection to sweet sensing sensilla on the proboscis. The stage and condenser on the microscope were replaced with the recording chamber[8] attached to a movable stage assembled with a manipulator M-152 (Narishige) and a height adjustment plate (P-1A, Narishige). Saline was perfused for aeration by a pump (TP10-SA, As-One) or gravity. Perfusion was continued for more than two hours after dissection until the fly was stabilized (spontaneous proboscis extension subsided and sucrose response was constantly strong). Conditioning experiments without either two photon imaging or optical activation were performed at 25°C.

Calcium Imaging and Laser Inactivation

For calcium imaging experiments, a FVMPE-RS two-photon microscope (Olympus) was used for visualization and an InSight DS Dual-OL (Spectra-Physics) for excitation. Although GMR81E10-*GAL4* expression in mostly restricted to the paired Feeding neurons in the anterior SEZ[48], it also labels sensory neurons that send processes through the labial nerves to the ventro-posterior SEZ (https://flweb.janelia.org/cgi-bin/flew.cgi and Figure S5B in Pool et al.[48], Figure S2D in this paper). Regions where sensory neurons terminate were excluded from imaged and manipulated areas. GCaMP fluorescence was quantified on the main dendritic trunk of the Feeding neuron in heterozygotes with *UAS-GCaMP6m*[18] and GMR81E10-*GAL4* on the 3rd chromosome. Before conditioning, the main dendritic trunk of the Feeding neuron was identified (see Figure S11 of Flood et al.[3]) with the Galvano scanning mode and a 15 pixel (4.97 μm) by 30 pixel (9.93 μm) ROI was placed for quantification. Using the same ROI, the microscope was switched to resonant scanning mode for rapid scanning. In resonant scanning mode, a 142 pixel (47.07 μm) by 512 pixel (169.71 μm) area that included the 15 pixel by 30 pixel ROI (Figure S2E) was scanned for 50 msec (single scan) for 200 cycles (10 s total). During a conditioned stimulus lasting for 7.6 s in the stronger protocol, the preparation was scanned with a 900 nm IR laser with 5% power for 10 s starting one second before onset of CS. Averaged ROI fluorescence was quantified with FV31S-SW software (Olympus) associated with FVMPE-RS.

Homozygotes containing a halorhodopsin transgene, *20XUAS-eNpHR3.0–EYFP*[47,49] on the 2nd chromosome with GMR81E10-*GAL4* on the 3rd chromosome were used for inhibition studies. Illumination was limited to the area where dendrites of the Feeding neurons spread dorsally from the level of the medial branch[3]. Before conditioning, the SEZ of animals was scanned in Galvano

scanning mode at 925 nm to identify the Feeding neurons using YFP fused to halorhodopsin. For inactivation, illumination with the 588 nm laser was applied to the dorsal dendritic field of the Feeding neurons on both sides. For maintenance of lasers, calcium imaging and inactivation studies were performed at 21°C.

Video/image Processing

All images and videos were processed in exactly same ways for comparison using Adobe Photoshop 2020 21.2.2. or Final cut Pro X 10.5.1.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed according to standard methods[50] using GraphPad Prism 8.4.3 (GraphPad Software). All the behavioral data and imaging data, which passed Kolmogorov-Smirnov test for normality, were analyzed by parametric test such as Student's t test. Data, which did not pass Kolmogorov-Smirnov test for normality, were analyzed by non-parametric test such as Mann-Whitney *U* test or Kruskal-Wallis test. Non-parametric repeated-measures ANOVA (Friedman test) was used to analyze transitions in Figure 3.

Current Biology, Volume *31*

Supplemental Information

Alteration in information flow through a pair of feeding command neurons underlies a form of Pavlovian conditioning in the *Drosophila* brain

Akira Sakurai, J. Troy Littleton, Hiroaki Kojima, and Motojiro Yoshihara

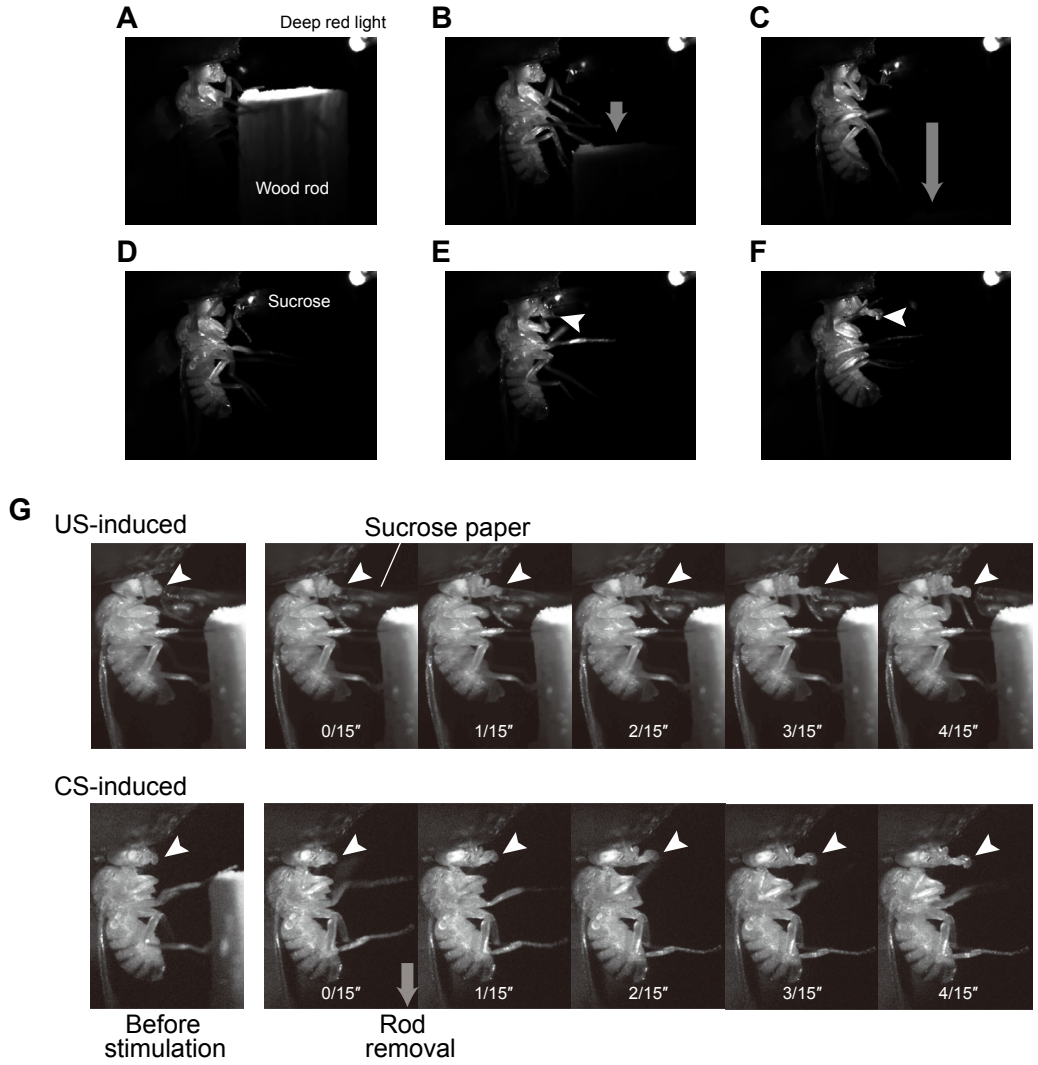

Figure S1. A novel paradigm for Pavlovian conditioning. Related to Figure 1. Video snapshots to show procedures used for Pavlovian conditioning of a wild-type (WT) fly. (A) A fly is holding a wooden rod (2 mm in diameter). (B and C) The rod is pulled down (arrow) with a stepping motor controlled by LabView to remove the rod from the fly's grasp. (D and E) Immediately after removing the rod, the fly's proboscis (arrowhead) is touched with a strip of paper soaked with sucrose solution from a hypodermic needle connected to a injector that supplies the sucrose solution. Just before touching, the sucrose solution was ejected to form a drop, and then pulled back to generate sucrose solution on the paper strip at the given concentration. (F) The fly extends its proboscis (arrowhead) as a feeding behavior in response to sucrose application. See METHOD DETAILS for more experimental detail. (G) Comparison between US (1 M sucrose)-induced response and CS (rod removal)-induced response acquired through the conditioning. Upper panels, time-lapse photographs of WT show natural feeding behavior induced by touching the proboscis (arrowhead) with a sucrose-wetted paper strip. Lower panels, conditioned response acquired from the protocol in Figure 1B is shown as a time lapse photograph. Immediately after the rod removal, proboscis extension was induced in an indistinguishable manner from that of upper panels when extension was 100%. Behavior is shown every 1/15 s.

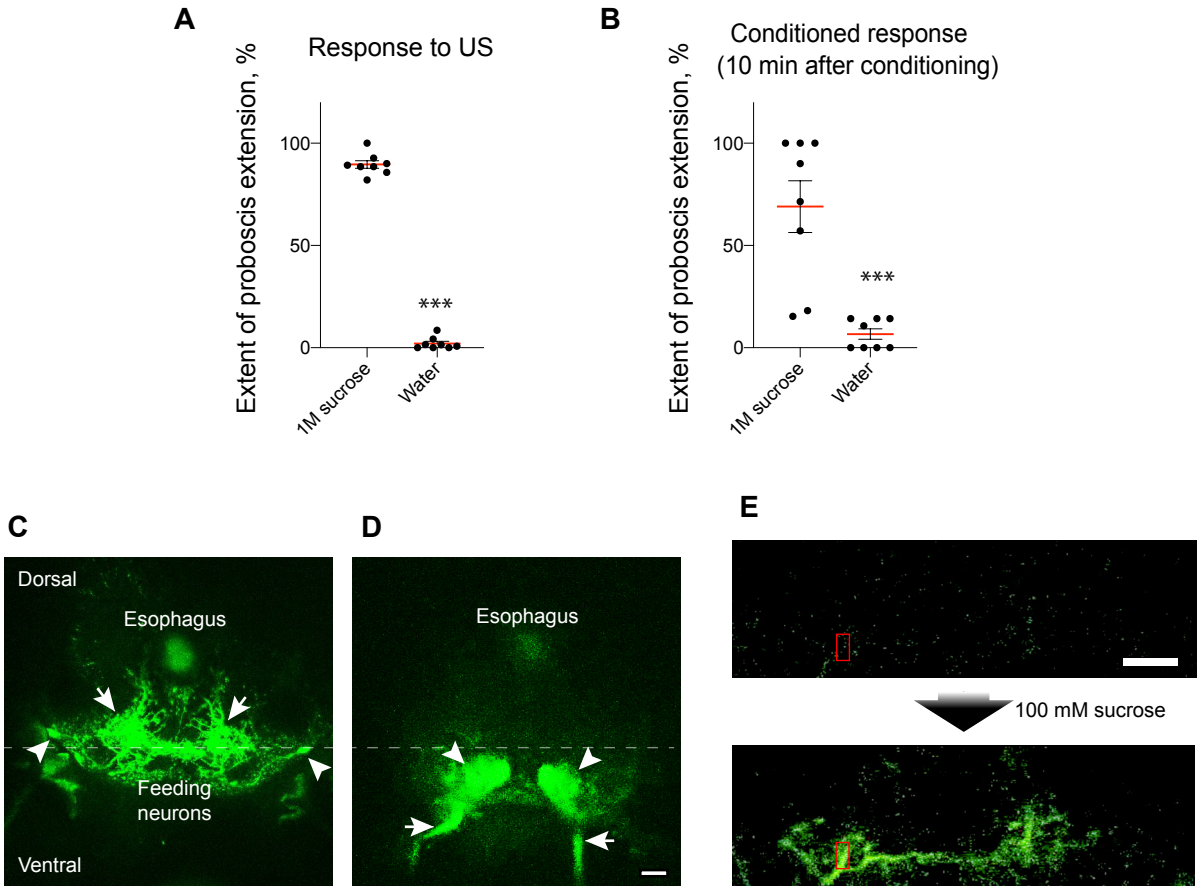

Figure S2. Conditioning with water as a US, expression pattern of GMR81E10-*GAL4*, and calcium imaging of sucrose response at the Feeding neuron. Related to Figure 3. (A) Comparison of response to US between 1M sucrose and water during conditioning (stronger protocol) was analyzed with Mann-Whitney *U* test. Significant difference between the two groups was found (*** P<0.001). Eight animals were analyzed for each group. Proboscis extension (% of full extension) is shown. (B) Comparison of conditioned response after conditioning between 1M sucrose and water as US was analyzed with Mann-Whitney *U* test. Significant difference between the two groups was found (*** P<0.001). Eight animals were analyzed for each group. Proboscis extension (% of full extension) in response to CS at 10 min after association is shown. (C) and (D) Expression pattern of the GMR81E10-*GAL4* line to label the Feeding neuron with GMR81E10-*GAL4* > *UAS-mCD8-gfp*. (C) Z projection of the anterior 7 optical sections (2.25 μm increment) of GFP fluorescence acquired through scanning the SEZ with a two photon microscope that includes a pair of Feeding neurons in a dissected animal set in the recording chamber. Arrowheads denote cell bodies of the Feeding neurons. Arrow denotes the dendrites of the Feeding neurons. (D) Z projection of the posterior 15 optical sections (2.25 μm increment) from the same animal to show sensory neurons that run through the labial nerve (arrows) and terminate in the ventro-posterior region of the SEZ (arrowheads). The dashed line shows the dorso-ventral level where medial branches from both Feeding neurons project. Scale, 20 μm. (E) An example of GCaMP live imaging before and after 100 mM sucrose stimulation. ROI rectangles (4.97 μm X 9.93 μm) used for quantification in Figure 3E are shown in red. Scale, 20 μm.

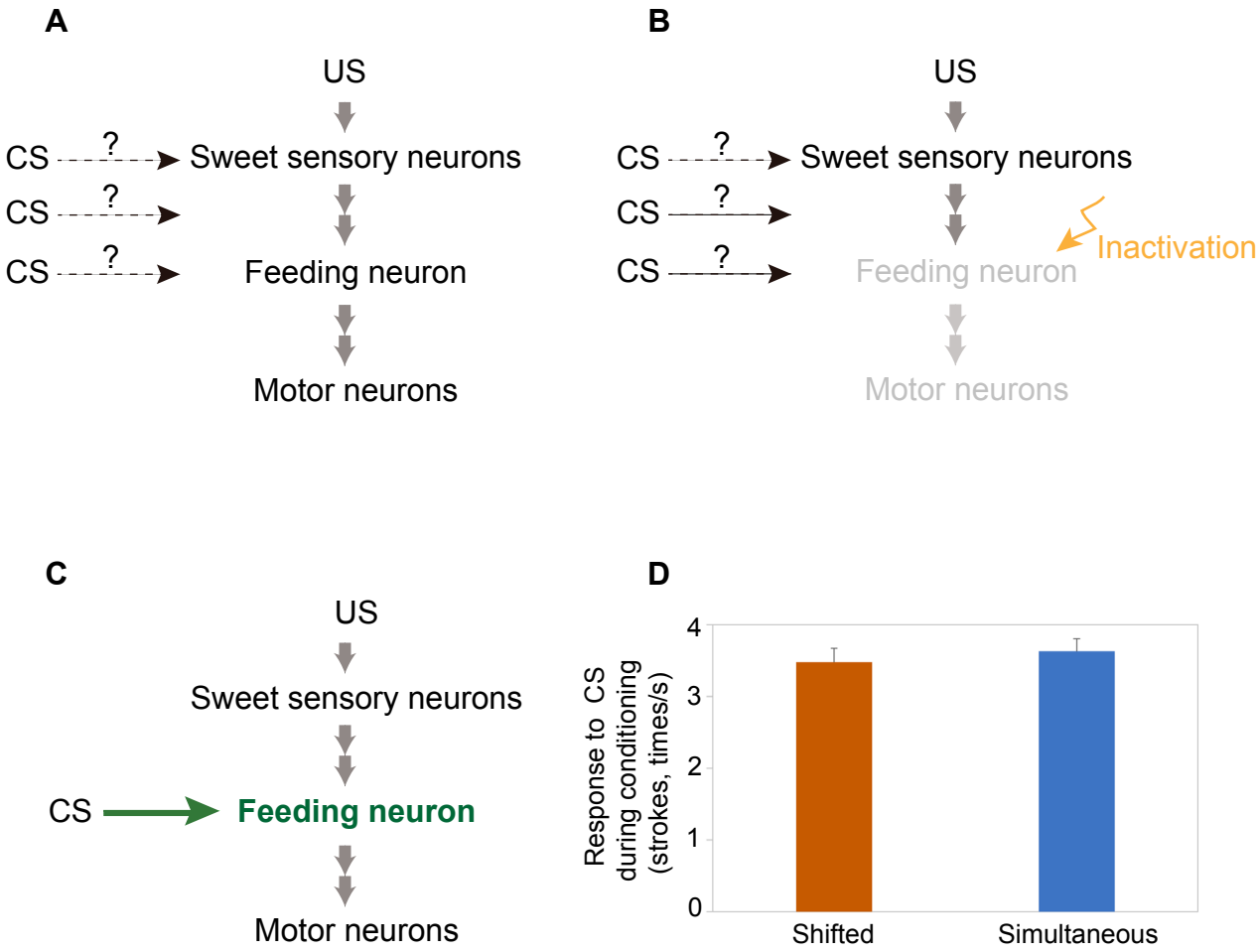

Figure S3. Inactivation of the Feeding neuron during conditioning suppresses plasticity. Related to Figure 4. (A)-(C) Schematic diagram to demonstrate signal flow through the Feeding neuron for integrating US and CS signals. (A) The alteration in responsiveness might represent changes occurring in upstream neurons instead of the Feeding neuron itself, for example, in sweet (US) sensing neurons that drive feeding behaviors. (B) Inactivation of the Feeding neuron should block the conditioned response only if the CS signal flows into the feeding circuit at the level of the Feeding neuron itself or downstream motor neurons. (C) Activity of the Feeding neuron is required to form the conditioned response, consistent with plasticity creating functional connections onto the Feeding neuron. (D) Reponses to the CS during conditioning in halorhodopsin experiments in Figure 4. Strokes of mid-leg at the camera-side was counted during each rod removal, and averages of their frequency (times/ s) through five pairings are shown for each group as a response to the CS. Significant difference was not recognized between "shifted" group and "simultaneous" group with Student's t-test ($P>0.05$). Five animals were analyzed for each group, which passed Kolmogorov-Smirnov test for normality. Error bars are SEM.