Report

Current Biology

A Subset of Serotonergic Neurons Evokes Hunger in Adult *Drosophila*

Highlights

- Activation of a small set of neurons induces a hunger response in sated flies
- The behaviors promoted include feeding and appetitive memory performance
- These serotonergic brain neurons project broadly and mediate the hunger sensation

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In Brief

Albin et al. have identified a small set of neurons that can induce sated flies to feed as though starved, as well as provide the hunger signal required for appetitive memory performance. The serotonergic subset of these neurons is responsible for conveying the sensation of hunger.

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A Subset of Serotonergic Neurons Evokes Hunger in Adult *Drosophila*

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SUMMARY

Hunger is a complex motivational state that drives multiple behaviors. The sensation of hunger is caused by an imbalance between energy intake and expenditure. One immediate response to hunger is increased food consumption. Hunger also modulates behaviors related to food seeking such as increased locomotion and enhanced sensory sensitivity in both insects [1–5] and vertebrates [6, 7]. In addition, hunger can promote the expression of food-associated memory [8, 9]. Although progress is being made [10], how hunger is represented in the brain and how it coordinates these behavioral responses is not fully understood in any system. Here, we use *Drosophila melanogaster* to identify neurons encoding hunger. We found a small group of neurons that, when activated, induced a fed fly to eat as though it were starved, suggesting that these neurons are downstream of the metabolic regulation of hunger. Artificially activating these neurons also promotes appetitive memory performance in sated flies, indicating that these neurons are not simply feeding command neurons but likely play a more general role in encoding hunger. We determined that the neurons relevant for the feeding effect are serotonergic and project broadly within the brain, suggesting a possible mechanism for how various responses to hunger are coordinated. These findings extend our understanding of the neural circuitry that drives feeding and enable future exploration of how state influences neural activity within this circuit.

RESULTS AND DISCUSSION

Animals require nourishment for survival, growth, and reproduction. Depletion of an animal's nutrient stores leads to physiological changes that result in the sensation of hunger. Most prior studies of feeding in *Drosophila* have used chronic manipulations, such as genetic mutation [11] or neuropeptide overexpression [12], but these can lead to compensatory metabolic and behavioral effects, confounding the study of hunger. Here, we use acute neuronal activity manipulations and short-term feeding assays to perform a behavioral screen to identify neurons whose acute activation evokes feeding in sated flies. First, we modified existing feeding assays to better differentiate between sated and starved animals [13, 14]: flies were exposed to blue-colored food for 15 min and the amount ingested was assessed qualitatively by visual inspection of the abdomen or quantitatively from whole-fly extracts by spectrophotometry. By several metrics, sated control flies consumed significantly less food than their siblings that were starved for 24 hr (Figures 1A, S1A, and S1B).

We expressed the temperature-sensitive cation channel dTrpA1 (*UAS-dTrpA1*) [15] in different populations of neurons using the Gal4/UAS system [16] and tested food consumption at 32°C, a temperature at which dTrpA1 activates neurons. Our Gal4 collection included lines expressed in several neuropeptidergic systems reported to regulate aspects of feeding in *Drosophila*, including sNPF-, NPF-, hugin-, and insulin-expressing neurons [12, 17–19], but activation of these neurons was not sufficient to induce feeding, and thus they do not meet our criteria for encoding the hunger state. From ~2,760 Gal4 lines with distinct expression patterns [20], we identified 20 Gal4 lines that demonstrated increased feeding (data not shown). The line with the strongest phenotype was R50H05-Gal4.

R50H05-Gal4 Expresses in Neurons that Induce Feeding in Sated Flies

Activating R50H05 neurons triggered starvation-like levels of feeding in sated flies (Figures 1A, 1B, S1A, S1B, and S1D). Moreover, activating R50H05 neurons did not result in indiscriminate feeding: these flies do not ingest abnormally large amounts of either water or bitter foods (Figure S1E), suggesting that their sense of taste is unimpaired.

Next, we asked whether activating R50H05 neurons could lead to changes in feeding behavior that persist beyond the duration of activation. Following the experimental design shown in Figure 1C, we activated R50H05 neurons in sated flies in empty vials. Flies that were transferred to colored food at the activation temperature ate as if starved, but those that were transferred to room temperature food did not, indicating that R50H05 neuron activation did not induce a persistent state change (Figure 1C).

To visualize the neurons labeled by R50H05-Gal4, we expressed the membrane-bound fluorescent reporter mCD8-GFP detected by an antibody to GFP, which revealed a small number of central brain neurons (40 per brain hemisphere), many with broadly projecting arbors (Figure 1D). There was no expression

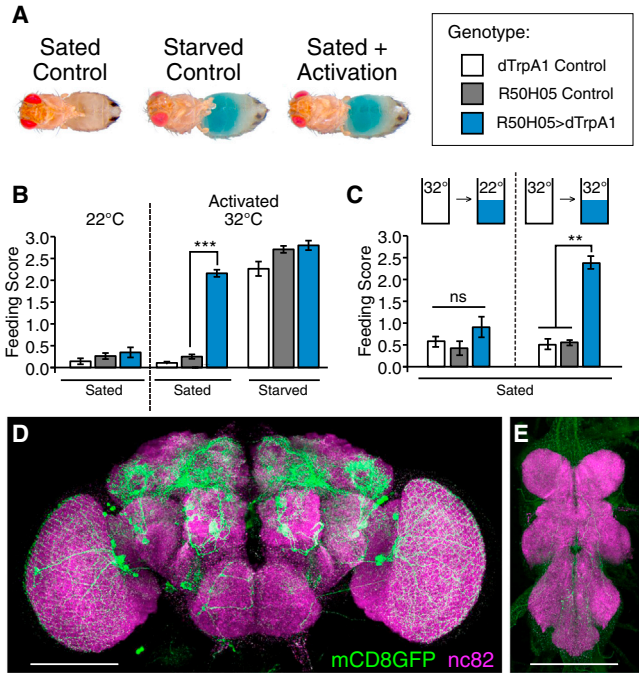

Figure 1. Activation of Central Brain R50H05 Neurons Induces Feeding

(A) Sated flies do not consume blue food, but large amounts are detected in the abdomens of starved control flies (center) or fed experimental (right) flies upon neuronal activation.

(B) Activation of R50H05 neurons increases the average feeding score in sated flies, a phenotype not seen in genetic or temperature controls (n = 7–22 groups of ten flies).

(C) Activation of R50H05 neurons in the absence of food does not lead to an increased feeding score when flies are assayed after the activation signal is terminated using room temperature food (n = 5–12 groups of ten flies).

(D and E) Adult brain (D) and ventral nerve cord (E) from *UAS-mCD8-GFP*/+; *R50H05-GAL4*/+ flies double immunostained with antibodies to GFP (green) and the neuropil marker nc82 (magenta).

All data in (B) and (C) are shown as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 using Mann-Whitney rank-sum test. The scale bars in (D) and (E) represent 100 mm. Genotypes are as follows: dTrpA1 control (*UAS-dTrpA1*/+; *BDPGal4U*/+); R50H05 control (*R50H05-Gal4*/+); and R50H05 > dTrpA1 (*UAS-dTrpA1*/+; *R50H05-Gal4*/+). See also Figure S1.

in cell bodies located in the ventral nerve cord (Figure 1E) or in sensory neurons of the proboscis, antenna, and gut (data not shown). These neurons appear to be distinct from those previously implicated in taste and feeding [17–19, 21–23].

Activating R50H05 Neurons Mimics Starvation

To determine whether activating R50H05 neurons can trigger other starvation-induced behaviors, we assayed for changes in the proboscis extension response (PER), food preference, and memory performance. PER provides an alternate measure of hunger: increasing concentrations of sucrose are presented to immobilized flies, which respond by extending their proboscis to feed. Sated flies rarely respond in this assay, but starvation increases the likelihood of PER proportional to the level of starvation [5, 24, 25]. Activating R50H05 neurons in sated animals mimicked the shift in PER responsiveness shown by starved animals (Figure 2A, dashed blue versus solid lines).

Starved flies ingest more food, but they also prioritize caloric content. We tested whether activating R50H05 neurons in fed flies induced this change in food preference using a modified two-choice assay [13]. When presented the options of food or water (from 1% agar), sated control flies showed a slight preference for water, but starved control flies and flies with activated R50H05 neurons strongly preferred to ingest food (Figure 2B). Next, we asked whether flies with activated R50H05 neurons show preference for nutritional value when selecting a food source. Although both D-glucose (a nutritive sugar) and L-glucose (a non-metabolizable sugar) taste sweet, starved flies display a clear preference for the nutritive sugar, mediated by a taste-independent metabolic sensor [21]. Activation of R50H05 neurons in sated flies also mimics this shift in nutritive preference (Figure 2C).

Hunger can also motivate an animal to initiate behaviors that may assist in food seeking but which are removed from the act of feeding itself [10]. *Drosophila* can be trained to associate odors with a sucrose reward in appetitive learning assays [26]. We tested whether activation of R50H05 neurons can substitute for starvation as the motivation for memory performance. Starved flies were trained by sequentially presenting two odors, the second of which was paired with a sucrose reward. Memory was tested at least 3 hr later as a preference for the sugar-associated odor. Flies must be food deprived for efficient appetitive learning and memory performance [8, 9], as we observed in control flies (Figures S2A and S2B). Consistent with the hypothesis that R50H05 neuronal activity signals hunger, we found that activating R50H05 neurons during testing restored appetitive memory performance in sated flies (Figure 2D; see Figures S2C and S2D for experiment controls). Surprisingly, activating R50H05 neurons during training suppressed memory performance in starved flies (Figure S2E). We speculate that flies in which R50H05 neurons were activated during training remain in a starvation-like state and therefore fail to form a positive association between odor and sucrose. In a final control experiment, pairing an odor with activation of R50H05 neurons in the absence of a sucrose reward did not result in any change in odor preference (Figures S2G–S2I).

Activating R50H05 Neurons Does Not Affect Locomotion

Starvation induces hyperactivity in many organisms, including *Drosophila* [2, 27]. Because activating R50H05 neurons mimics hunger to induce feeding, we asked whether activating these neurons caused an increase in locomotion. We assayed the percentage of time flies spent walking using an open field assay (FlyBowl; adapted from [28]). Starved control flies displayed the expected increase in locomotion relative to sated controls, as did starved flies with activated R50H05 neurons, but activating R50H05 neurons in sated flies had no effect (Figure 2E). Hence, activating R50H05 neurons selectively evokes only a subset of hunger-induced behaviors (Figure 2F).

Blocking Synaptic Transmission in R50H05 Neurons Reduces Starvation-Induced Feeding and Appetitive Memory Performance

We next tested whether R50H05 neurons are required for starvation-induced feeding by expressing the temperature-sensitive dynamin mutant Shi^{ts1} (*UAS-Shi ^{ts1}*) [29] to block synaptic

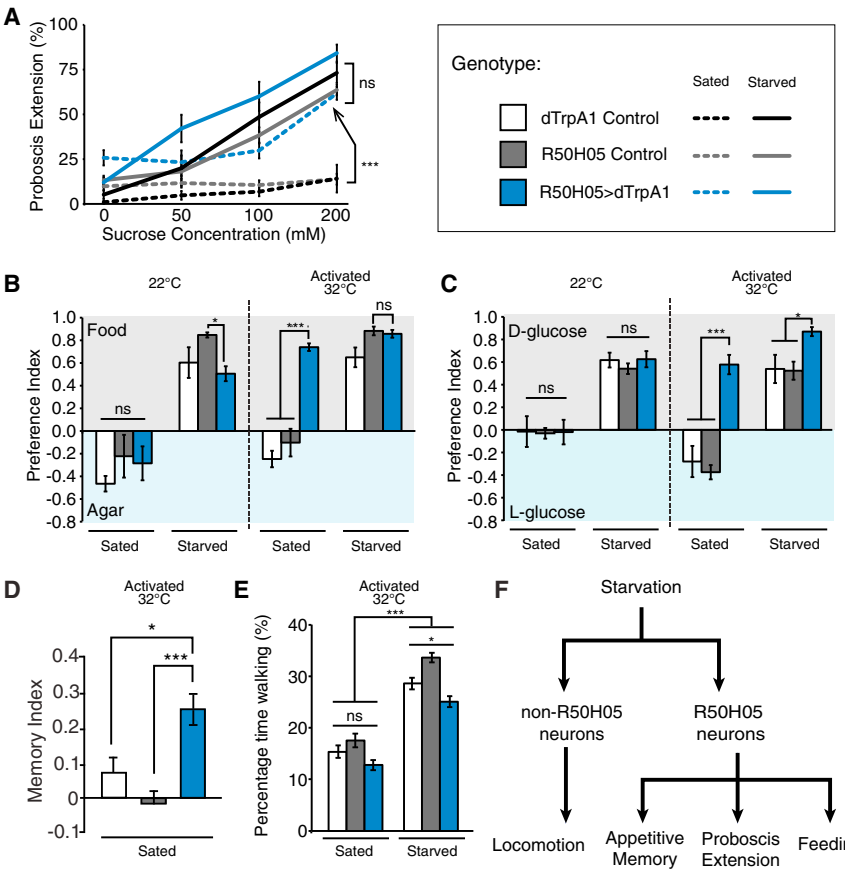

Figure 2. Activation of R50H05 Neurons Mimics Starvation-Induced Changes in PER, Food Preference, and Memory Performance, but Not Increases in Locomotion

(A) PER response in sated TrpA1-activated R50H05 (blue, dashed line) flies is increased compared to sated controls at 100 mM and 200 mM sucrose concentrations (n = 5–10 groups of 15–20 flies).

(B) Activation of R50H05 neurons in sated flies promotes a shift in preference for food over 1% agar similar to the shift seen in starved animals (22°C: n = 3 or 4 groups of 20–25 flies; 32°C: n = 8 or 9 groups of 20–25 flies).

(C) Activation of R50H05 neurons also promotes a shift in preference for nutritive D-glucose over non-metabolizable L-glucose, which is also seen in starved animals (n = 6 or 7 groups of 20–25 flies).

(D) Stimulation of R50H05 neurons prior to and during appetitive memory testing produces memory performance in sated flies (n = 14–16 groups of 50 flies). Diagram of experimental conditions depicted in Figure S2F.

(E) Activation of R50H05 neurons neither induces nor inhibits hunger-evoked changes in locomotion (n = 8–24 groups of 20 flies).

(F) Our data support a model in which R50H05 neurons are downstream of metabolic signals (starvation) and upstream of most food-related behavioral outputs.

All data in (A)–(E) are shown as mean ± SEM; *p < 005, **p < 0.01, ***p < 0.001 using Mann-Whitney rank-sum test (A–C and E) or ANOVA followed by Tukey's post hoc comparison (D). Genotypes are as follows: dTrpA1 control (*UAS-dTrpA1*/+; *BDPGal4U*/+); R50H05 control (*R50H05-Gal4*/+); and R50H05 > dTrpA1 (*UAS-dTrpA1*/+; *R50H05-Gal4*/+). The genotype for (E) is dTrpA1 control (*UAS-dTrpA1* /+) because the BDPGal4U insert has an effect on baseline locomotion. See also Figure S2.

transmission from these neurons during the feeding assay. This resulted in a modest decrease in the amount of food ingested by starved animals (Figures 3A and S1D). We do not see a complete block of feeding, perhaps due to incomplete silencing of the neurons by Shi^{ts1}. Alternatively, there may be redundancy or compensation in the hunger circuitry, a suggestion supported by the data that chronic inhibition of activity in these neurons is not lethal and does not significantly alter blue food consumption after starvation (Figure S3A). The fact that actual starvation increases food consumption in R50H05-activated flies is also consistent with the existence of additional hunger circuitry (Figure 1B; compare bars 6 and 9; $p < 0.001$).

We also tested whether R50H05 activity was required for the shift in PER responsiveness and nutritive food preference seen in starved animals but saw no effect (Figures 3B and 3C). These behaviors may be evoked by different hunger thresholds or use redundant neural circuits. Because PER and nutritive preference are normal when activity in R50H05 neurons is blocked in starved flies, these neurons are unlikely to contribute directly to taste, nutrient sensing, or the feeding motor program, supporting our proposal that they lie between these circuit elements and instead convey the sensation of hunger itself.

We also synaptically silenced R50H05 neurons throughout the duration of the appetitive memory assay. This manipulation blocked appetitive memory performance completely compared to genetic and temperature controls (Figure 3D; see Figures S3C and S3D for controls). Silencing R50H05 neurons did not affect sucrose ingestion during training (Figure S3E), ruling out this explanation for failure to learn the odor-sucrose association. The block in memory performance could occur because the synaptic silencing of R50H05 neurons causes a decrease in the hunger motivation required for appetitive memory formation and performance, but alternatively, these neurons may have a distinct role in learning and memory.

The Serotonergic Subset of R50H05 Neurons Is Responsible for Activation-Induced Feeding

To gain insight into the neurons responsible for the above behavioral phenotypes, we further characterized the R50H05-Gal4 expression pattern. The regulatory element driving expression of R50H05-Gal4 is derived from an intron of the *Drosophila melanogaster* serotonin transporter gene, which raises the possibility that some of these neurons release the neuromodulator serotonin. Co-labeling with antibodies that recognize serotonin and GFP revealed that R50H05-Gal4 expresses in 25 serotonergic neurons and 15 non-serotonergic neurons in each brain hemisphere (Figure 4A) including the serotonergic SE1, ALP, LP2, PLP, and PMP clusters [30, 31]. We named the

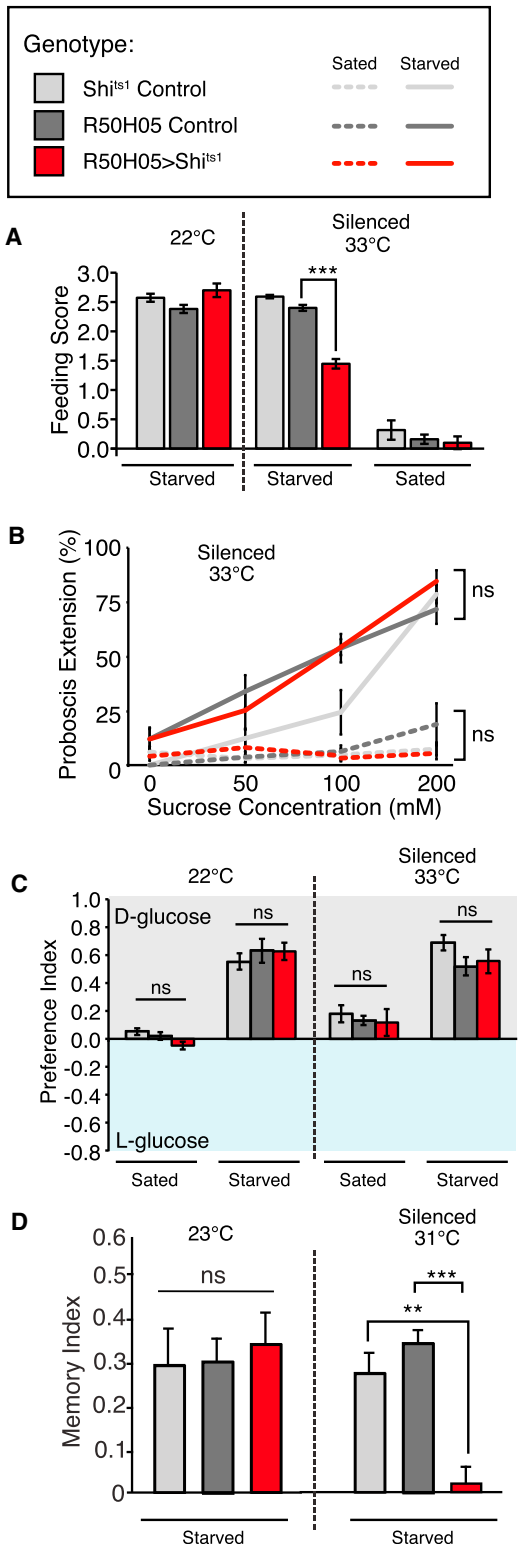

Figure 3. R50H05 Neurons Are Required for Normal Starvation-Induced Feeding and Appetitive Memory Performance but Are Dispensable for Changes in PER and Food Preference

(A) Silencing R50H05 neurons in starved flies decreases the average feeding score as compared to controls (n = 6–15 groups of ten flies). Diagram of experimental conditions is depicted in Figure S3A.

non-serotonergic R50H05 clusters based on anatomical position: neurons in the anterior medial protocerebrum were designated AMP2; those in the lateral protocerebrum LP2; and those in the posterior lateral protocerebrum PLP2. See Table S1 for cell counts of neurons in each cluster.

Serotonin has been implicated in regulating feeding in many systems, including *Drosophila* [32, 33]. We therefore inquired whether the serotonergic neurons in the R50H05-Gal4 pattern might underlie the feeding phenotypes we observed. Activation of all serotonergic neurons does not induce feeding and can even inhibit feeding in starved animals (Figure S4A); activation of the primarily serotonergic subset of R50H05 neurons recapitulated the feeding, PER, and nutritive sugar preference phenotypes induced by activation of all R50H05 neurons (Figure S4).

A single neuron may release multiple neurotransmitters [34], but we show that serotonin is necessary for the feeding phenotype displayed by flies in which R50H05 neurons are activated using RNAi to disrupt tryptophan hydroxylase, an enzyme required for serotonin biosynthesis (Trh) [35]. Expressing *UAS-Trh-RNAi* with R50H05-Gal4 eliminated anti-serotonin staining specifically in R50H05-positive serotonergic clusters. Serotonergic neurons that were not in the R50H05 expression pattern still labeled with anti-serotonin, verifying the efficacy of targeted RNAi knockdown (compare Figures 4B and 4C). Expressing Trh-RNAi in R50H05 neurons did not alter baseline ingestion (Figures 4D, third and fourth bars, and S4A1), but co-expressing it with dTrpA1 suppressed the feeding phenotype (Figure 4D, compare blue and green bars). These data are consistent with the proposal that serotonin released by R50H05 neurons transmits hunger state information to multiple brain regions, thereby promoting feeding behavior.

Identifying the neural representations of hunger is a prerequisite for understanding how an animal's internal hunger state translates to adaptive modifications of its behavior. In the current study, we identified a small set of mostly serotonergic neurons whose acute activation can induce a sated fly to perform multiple starvation-associated behaviors that include feeding, increased proboscis extension, and preference for nutritive food. We propose that these neurons encode hunger signals in the brain because their activation coordinates a range of starvation responses and also can motivate appetitive olfactory memory.

Our data imply that R50H05 neurons are not the sole mediators of hunger state information: silencing R50H05 neurons reduces, but does not abolish, starvation-induced feeding, and flies with activated R50H05 neurons do not exhibit increased locomotion. Considering the fundamental importance of feeding to an animal's survival, it is not surprising that distributed and

(B) PER response in starved R50H05 flies is unchanged upon silencing of synaptic transmission (n = 3–7 groups of 15–20 flies).

(C) Synaptic silencing of R50H05 neurons in starved flies does not affect the starvation-induced preference for nutritive D-glucose over non-metabolizable L-glucose (n = 5–11 groups of 20–25 flies).

(D) Synaptic silencing of R50H05 neurons throughout training and testing blocks appetitive memory performance (n = 16 groups of 50 flies).

All data in are shown as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 using Mann-Whitney rank-sum test (A–C) or ANOVA followed by Tukey's post hoc comparison (D). Genotypes are as follows: Shi^{ts1} control (*UAS-Shi^{ts1}/+; BDPGal4U/+*); R50H05 control (*R50H05-Gal4/+*); and R50H05 > Shi^{ts1} (*UAS-Shi^{ts1}/+; R50H05-Gal4/+*). See also Figure S3.

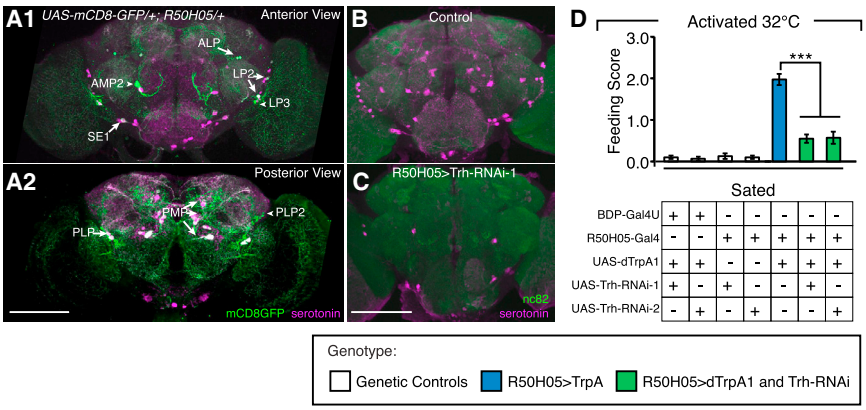

Figure 4. Some R50H05 Neurons Are Serotonergic, and Serotonin Is Required for TrpA-Activation-Induced Feeding

(A) Adult brain from R50H05 fly expressing mCD8GFP double immunostained with antibodies to GFP (green) and serotonin (magenta), shown as anterior (A1) and posterior (A2) confocal sections for clarity. Resulting expression patterns were analyzed, and R50H05 neuronal clusters were labeled with arrows if serotonergic and arrowheads if non-serotonergic (see Table S1 for cell counts).

(B) Adult *UAS-dTrpA1*/+; *R50H05*/+ brain with all serotonergic clusters stained by anti-5HT (magenta).

(C) Adult *UAS-dTrpA1*/+; *R50H05*/+ brain co-expressing Trh-RNAi1 in R50H05 neurons. Serotonergic neurons that are not in the R50H05-Gal4 line are still labeled by anti-5HT (magenta).

(D) Expression of Trh-RNAi decreases the average feeding score in fed dTrpA1-activated R50H05 flies (green versus blue bars). Expression of TrhRNAi lines in R50H05 neurons alone does not affect feeding behavior in sated flies (n = 5–9 groups of ten flies).

All data in (D) are shown as mean ± SEM; ***p < 0.001 using Mann-Whitney rank-sum test. The scale bars in (A)–(C) represent 100 mm. Note that *BDPGal4U* is an "empty" Gal4 driver, used as a control, which does express Gal4 in the adult CNS. Genotypes are as follows: R50H05 > GFP (*UAS-mCD8GFP*/+; *R50H05-Gal4*/+) and R50H05 > GFP and UAS-Trh-RNAi-1 (*UAS-mCD8GFP*/+; *R50H05-Gal4*/*UAS-Trh-RNAi-1*). See also Figure S4.

partially redundant neural circuitry has evolved to regulate hunger. Whereas activation of R50H05 neurons in sated animals induces strong phenotypes in a range of assays, synaptic silencing of R50H05 in starved animals produces more variable effects. This is perhaps due to differences in the sensitivity of each assay to hunger levels and, as suggested by recent published results [5], an escalation of behavioral responses as hunger increases. Within just 4 hr of starvation, a fly increases its sensitivity to food odors [36]. With longer starvation, flies increase their preference for nutritive over non-nutritive sugars [21]. After more extreme starvation, flies may increase locomotion and food search behaviors and eventually begin to sample increasingly bitter food [5]. At the output level, these behaviors must be mediated by different neurons, but whether the escalation of hunger is achieved by recruiting separate circuits or increasing activation levels in one remains to be determined.

Serotonin has been implicated in regulating feeding behavior in organisms as diverse as nematode, leech, mouse, and human [37–39], but it has different, even contradictory, effects on feeding in different species. For example, most (but not all) pharmacological and genetic manipulations in mammals show that serotonin signaling generally leads to a decrease in food intake [40]. In *C. elegans,* serotonin promotes feeding in the presence of familiar food by directly activating pharyngeal motor neurons [41]. Serotonin also promotes feeding in *Drosophila* larva, in which the serotonin receptor antagonist metitepine specifically and reversibly inhibited feeding [42]. In adults, our findings demonstrate that serotonin released by R50H05 neurons evokes feeding. In contrast, activating the entire serotonergic population decreases feeding, even in starved animals (Figure S4A1). This suggests that subsets of cells that release the same neuromodulator can have different—even opposing—effects on feeding behavior.

The serotonergic subset of R50H05 neurons is anatomically and behaviorally distinct from neurons previously identified as important for feeding behavior (Figures S4D–S4H). Looking forward, access to R50H05 neurons enables investigation of how the local action of serotonin controls neural activity and behavior. Hunger and neuromodulators have been shown to modulate sensory gain [25, 36], but R50H05 neurons act differently, perhaps by lowering the activation thresholds for command neurons such that a feeding motor program is more readily evoked in the presence of appropriate taste and nutritional cues. Further subdivision of the serotonergic subset of R50H05 neurons will address whether there are functionally distinct classes of serotonergic neurons that are responsible for inducing particular behaviors. Alternatively, broadly projecting neurons may coordinate evoked behaviors with different thresholds. Here, we have identified neurons whose activity can convey the sensation of hunger. This enables a greater understanding of how the global state of the animal influences its neural circuits and coordinates the repertoire of behaviors it performs.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.08.005.

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