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The leucokinin pathway and its neurons regulate meal size in *Drosophila*

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SUMMARY

Background—Total food intake is a function of meal size and meal frequency, and adjustments to these parameters allow animals to maintain a stable energy balance in changing environmental conditions. The physiological mechanisms that regulate meal size have been studied in blowflies, but have not been previously examined in *Drosophila*.

Results—Here we show that mutations in the *leucokinin neuropeptide (leuc)* and *leucokinin receptor (lkr)* genes cause phenotypes in which *Drosophila* adults have an increase in meal size and a compensatory reduction in meal frequency. Since mutant flies take larger but fewer meals, their caloric intake is the same as that of wild-type flies. The expression patterns of the *leuc* and *lkr* genes identify small groups of brain neurons that regulate this behavior. Leuc-containing presynaptic terminals are found close to Lkr neurons in the brain and ventral ganglia, suggesting that they deliver Leuc peptide to these neurons. Lkr neurons innervate the foregut. Flies in which Leuc or Lkr neurons are ablated have defects identical to those of leucokinin pathway mutants.

Conclusions—Our data suggest that the increase in meal size in *leuc* and *lkr* mutants is due to a meal termination defect, perhaps arising from impaired communication of gut distension signals to the brain. Leucokinin and the leucokinin receptor are homologous to vertebrate tachykinin and its receptor, and injection of tachykinins reduces food consumption. Our results suggest that the roles of the tachykinin system in regulating food intake might be evolutionarily conserved between insects and vertebrates.

Introduction

In mammals, nutrient intake is regulated to keep body weight constant over long periods of time. Most animals consume food in discrete bouts called meals, and total food intake is a function of both meal size and meal frequency. Identification of the pathways that regulate these meal related parameters is essential for the understanding of the relationships between body weight regulation and caloric intake [1,2].

Signals that control meal size and frequency fall into three categories: those that initiate a meal, those that maintain feeding once a meal has begun, and those that terminate a meal. In hungry

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mammals, the smell and taste of food initiate feeding. As feeding continues, the level of gastric distension is conveyed to the brain *via* stomach wall stretch receptors. When the extent of stomach distension passes a threshold, the meal is likely to terminate [3-5]. Also, during the course of a meal, some nutrients are absorbed in the small intestine, allowing a post-gastric evaluation of the caloric content of ingested food that can also contribute to meal termination [6].

The steps involved in physiological regulation of feeding behavior in flies have been elucidated primarily through studies on the blowfly *Phormia regina* [7,8]. As the hungry fly walks, taste hairs on its legs sample the surface. When a food source is detected, the fly extends its proboscis and begins to feed. During ingestion, liquid food passes through the foregut into a collapsible food-storage sac called the crop. Eventually, the fly becomes satiated and stops feeding. A number of factors contribute to termination of a feeding bout, and thus determine meal size. First, stretch receptors monitoring gut distension provide a negative feedback signal to the brain. Second, neurons in the taste hairs habituate and become less responsive to food [7,8].

Leucokinin (Leuc) is a myotropic neuropeptide found in most invertebrate species [9]. It was initially identified as a neurohormone that increases malpighian tubule fluid secretion and hindgut motility in some insect species [10-14]. The biological activity of leucokinin requires an amidated C-terminal pentapeptide motif called FXXWG-amide, a feature that it shares with the related vertebrate tachykinin neuropeptides. The tachykinin family includes substance P, substance K/neurokinin A, and neuropeptide K /neurokinin B [14,15]. Although the *Drosophila* genome encodes another peptide whose sequence is somewhat closer to vertebrate tachykinins than is leucokinin [16], the observation that the *Drosophila* leucokinin receptor, Lkr, is homologous to vertebrate tachykinin receptors confirms the homology between the leucokinin and tachykinin pathways [17].

In this paper, we report that the leucokinin pathway is involved in meal size regulation in *Drosophila*. Flies with reduced leucokinin pathway signaling due to mutations in the genes encoding either the *leucokinin ligand* (*leuc*) or the *leucokinin receptor* (*lkr*) have an abnormal increase in meal size. This increase is associated with a reduction in meal frequency that causes mutant flies to consume the same total amount of food as wild-type flies. The functions of the leucokinin pathway in regulation of meal size are executed in neurons, since pan-neuronal expression of *leuc* or *lkr* rescues the phenotypes. *leuc* and *lkr* are expressed in distinct patterns of neurons, and ablation of these neurons phenocopies the effects of the *leuc* and *lkr* mutations.

Results

leucokinin and *leucokinin receptor* mutant flies eat excessively after starvation

To obtain insights into the molecular mechanisms involved in control of meal size, we performed a screen for mutations that cause adults of the genetically tractable insect *Drosophila melanogaster* to consume abnormally large amounts of food. A number of different assays have been used to monitor food consumption in *Drosophila* [18,19]**.** For our screen, we developed a two-dye feeding assay, in which five-day old male flies in groups of twenty were starved for one day on 1% agarose, then transferred into a vial containing 1% sucrose in 1% agarose with acid red food dye. After 20 minutes, the flies were tapped into a new vial containing the same food but with acid blue dye instead of red dye, and left for 15 more minutes. Wild-type starved flies became satiated during their exposure to red food, and had an exclusively red abdomen, since they did not consume any of the blue food. Flies with a defect in meal size regulation either ate excessive amounts of red food, making them visibly bloated, and/or continued feeding during exposure to the blue food, which caused them to have a purple (red+blue) abdomen.

Since our primary interest is in the neural control of feeding behavior, we screened a set of about 150 transposable element insertion mutations in genes encoding proteins involved in neuronal function, including neuropeptides and their receptors. We identified two PiggyBac elements that caused strong meal termination defects when homozygous. One of these is $leuc^{c275}$, an insertion 929 base pairs (bp) 5′ to the transcription start site of the *leucokinin* gene, which encodes the neuropeptide leucokinin. The other is lkr^{c003}, an insertion in the third intron of the *lkr* gene encoding the leucokinin receptor. Both mutations produced abdominal bloating, usually associated with a red abdomen, when tested in the two-dye feeding assay, and dissection of the digestive tracts of bloated flies revealed overfilled crops (Figures 1A-F).

We measured the starved flies' food intake during their initial 20 minute exposure to food by mixing the sucrose/acid red food with ^{14}C-labeled-leucine [20]. Radiation measurements were taken right after testing the flies in the two-dye assay. Since the entire assay takes only about 35 minutes, it is unlikely that a loss of ^{14}C-leucine due to excretion of digested food products would occur during this time period. Therefore, ^{14}C measurements should reflect the amount of food consumed by the flies. Indeed, we found that both $leuc^{c275}$ and lkr^{c003} flies incorporated about twice as much radioactivity as controls, indicating that the bloating and expanded crop phenotypes are due to an increase in post-starvation food intake (Figure 1G).

The overeating phenotypes in $leuc^{c275}$ and lkr^{c003} flies were fully rescued by using an Elav-Gal4 driver to confer pan-neuronal expression of UAS-linked transgenes encoding a wild-type copy of either gene in its corresponding mutant background (Figures 1H-U). These results indicate that loss of leucokinin and its receptor are responsible for the phenotypes, and that both genes are required only in neurons for rescue of this phenotype.

The increase in meal size in *leuc* and *lkr* mutants is not associated with an overall increase in food intake

To examine if $leuc^{c275}$ and lkr^{c003} mutants also overeat under non-starvation conditions, we measured their food consumption by providing them with regular fly food mixed with ^{14}C-labeled-leucine for 48 hours. Surprisingly, neither mutant showed any significant difference in total food intake relative to controls (Figure 2A). There are two possible explanations for these results: First, the mutants have a defect in meal size regulation that is associated with a compensatory reduction in meal frequency, so that they consume normal amounts of food during non-starvation conditions. Second, the leucokinin pathway is only involved in a post-starvation adaptive mechanism that prevents engorgement of starving flies when they finally find food.

To measure meal size using the ^{14}C-labeled-leucine assay, the fly must be sacrificed after consuming its meal, so this assay cannot be used for long-term evaluation of meal size. Proboscis extension assays can be used over an extended period, but they do not directly measure food consumption [19]. Accordingly, to monitor the size and frequency of fly meals over a multiday period, we used the café feeding assay [18]. In this assay, a single fly is placed in a well of a 24 well tissue culture plate. The bottom of the well is covered with 0.5 ml of 1% agarose to provide moisture. The fly is then provided, on a daily basis, with a capillary tube filled with 5% sucrose and 2% yeast extract inserted through a hole in the roof of the chamber. The capillary tube provides the only source of food for the tested fly. After a 5 day acclimation period, the amount of food ingested is directly measured every hour by observing the reduction in fluid level in the capillary tube. This provides a direct measure of food intake with minimum interruption of the fly's normal behavior patterns.

During a 12 hour daytime period, wild-type control flies**,** on average**,** took 7-8 meals, with the majority of meals having a volume between 0.1 and 0.2 μl. In contrast, both $leuc^{c275}$ and lkr^{c003} flies took only 4-5 meals, with a significant reduction in the number of normal size

meals and a corresponding increase in abnormally large meals (more than 0.4 μl), which rarely occur in wild-type flies (Figures 2B-C). Because the numbers of meals that they take are reduced, the total food intake of the two mutants was similar to that of wild-type at the end of the experiment (Figure 2D), even though most of their meals were larger. This defect, like the post-starvation overeating/bloating phenotype, was rescued by pan-neuronal expression of the appropriate transgene for both the $leuc^{c275}$ and lkr^{c003} mutants (Figures 2E-J).

These results suggest that the short-term increase in food intake after starvation in $leuc^{c275}$ and lkr^{c003} (Figure 1) is due to the meal size defect observed in the café assay (Figure 2). If so, overall food consumption by starved mutants should return to wild-type levels after a normal energy balance is achieved. To examine this issue, we subjected starved mutant flies to the café assay. We observed that they consumed more food than wild-type flies during the first 12 hr. period, due to their abnormally large initial meals. However, their total food intake had returned to wild-type levels by 60 hr. (Figure 2K).

The *leuc* and *lkr* gene expression patterns identify neurons that regulate meal size

To examine expression of leucokinin and its receptor, we generated an anti-Leuc antibody and obtained an anti-Lkr antibody. We performed Western blots with these antibodies on tissue extracts from wild-type, $leuc^{c275}$, and lkr^{c003} flies. The antibodies bound to bands of molecular weights similar to those of the proteins predicted to be encoded by either gene (~10 kDa for leucokinin, and ~75 kDa for Lkr). Western blot signals showed significant reductions, as compared to wild-type, when $leuc^{c275}$ and lkr^{c003} extracts were analyzed (Figures 3A-B), indicating that the $leuc^{c275}$ and lkr^{c003} mutations produce reductions in the amount of synthesized protein.

To evaluate the expression patterns of the genes, we generated flies with transgenes composed of upstream promoter regions (3.6 kbp for *leuc*, ~2 kbp for *lkr*) of *leuc* or *lkr* driving the expression of the Gal4 transcription factor (Leuc-Gal4 and Lkr-Gal4, respectively). We mated these driver flies with flies carrying a UAS-mCD8-Green Fluorescent Protein transgene (UAS-mCD8-GFP), and examined GFP, Leuc, and Lkr expression in adult progeny.

The expression pattern of the *leuc* gene has been previously reported [22], and a Leuc-Gal4 line with a somewhat shorter promoter fragment was recently described in detail [23]. We observed brain and ventral ganglion neurons that express both Leuc and Leuc-Gal4::mCD8-GFP and appear to correspond to those described in ref. [23]. Two large neurons with soma located in the lateral horn, the LHLK neurons (Figures 3C-D), innervate the protocerebrum and the calyx and peduncle of the mushroom body [23]. The subesophageal ganglion (SOG) contains two or three pairs of Leuc and Leuc-Gal4-positive neurons, denoted as SELKs (Figures 3C, 3E). Thin neurites from these neurons ramify inside the SOG, and their long axons project into the ventral ganglion. There are also seven prominent pairs of Leuc/Leuc-Gal4 neurons in the abdominal ventral ganglion, the ABLKs (Figures 3F-G) [22,23]. In addition to these cells, de Haro et al. [23] found neurons in the brain (ALKs or ‘ghost cells’) and in the midgut that did not express Leuc, but did express their Leuc-Gal4 driver. We did not see any of these neuronal groups with our driver. No non-neuronal expression of leucokinin or Leuc-GAL4::GFP was observed by de Haro et al. [23] or by us (Figure 4A). When Leuc-Gal4 was used to drive expression of the presynaptic marker UAS-Synaptobrevin-GFP, the GFP signal colocalized with the leucokinin signal in the brain and ventral ganglion (Figures 3H-I), suggesting that leucokinin is localized to presynaptic terminals. This was also observed by de Haro et al. [23].

In Lkr-Gal4::mCD8-GFP flies, neurons stained by both anti-Lkr and anti-GFP are found in the dorsal region of the brain. Some of these send axonal processes to the fan-shaped body at the

brain midline, which is brightly labeled by both antibodies (Figures 3J-K). We also observed Lkr-positive neurons in the ventral ganglion (Figure 3L).

When we double-stained Lkr-Gal4::UAS-mCD8-GFP brains and ventral ganglia with anti-Leuc and anti-GFP, we observed that there are red Leuc-positive spots, presumably presynaptic terminals, in close proximity to or in contact with green Lkr neurons axons and cell bodies in the lateral horn area of the brain and ventral gangila (Figures 3M-N). Adjacent red and green spots can be visualized in single confocal slices (Figure 3O). These data indicate that Leuc neurons are close enough to Lkr neurons to deliver Leuc peptide to them.

When we examined cryostat sections of the thorax, we observed both Lkr-Gal4::mCD8-GFP and anti-Lkr signals in the foregut, and also on axonal tracts that connect the brain to the foregut (Figure 4B and inset). In whole-mount foregut preparations, there is extensive staining with anti-Lkr, and some of this colocalizes with GFP, especially in the region near the proventricular valve (Figure 4C). In preparations triple-stained for the neuronal nuclear marker Elav, we observed that some of the Elav-positive cell bodies (presumably those of enteric neurons) also express GFP and Lkr (Figure 4C). Our data suggest that Lkr is expressed in both neuronal and non-neuronal cells in the gut region. However, since the lkr^{c003} meal size phenotype can be fully rescued by neuronal expression of Lkr (Figures 1U, 2H), our data suggest that leucokinin regulates meal size by functioning as a neuropeptide and not as a humoral factor. Consistent with this model, we found that injection of synthetic leucokinin into the abdominal cavity did not rescue the $leuc^{c275}$ bloating defect (data not shown).

To show that leucokinin and Lkr expression in the specific neurons expressing the Leuc-Gal4 and Lkr-Gal4 drivers is required for meal size regulation, we used these drivers to direct expression of UAS-transgenes with wild-type copies of *leuc* or *lkr* in the corresponding mutant background. The mutant phenotypes were fully rescued when *leuc*-Gal4 was used to drive leucokinin in the $leuc^{c275}$ background, or when Lkr-Gal4 was used to drive Lkr in the lkr^{c003} background (Supp. Figure 2).

Finally, to confirm the relevance of the identified Leuc and Lkr neurons to control of meal size, we used *leuc*-Gal4 and *Lkr*-Gal4 to drive the cell death gene *reaper*, so as to ablate the expressing neurons. To monitor ablation, we drove *reaper* together with GFP for each Gal4 driver, and we observed that *reaper* expression completely eliminated anti-Leuc and anti-Lkr positive cells (data not shown). All aspects of the feeding behavior defects observed in both mutants were replicated in flies with ablated Leuc-Gal4 or Lkr-Gal4 neurons (Figure 5).

de Haro et al. [23] observed Leuc-Gal4::mCD8-GFP signals (but not anti-Leuc staining) in sensory cells in the leg, and in taste organs of the mouth. The taste organ signals do not have the appearance of sensory cell bodies, and we did not observe either leg or taste organ GFP expression with our driver. Nevertheless, their results suggest that leucokinin/Lkr signaling might affect meal size by altering the taste quality of the food. To address this question, we used a proboscis-extension-reflex assay [8,24-27], to evaluate the responses of $leuc^{c275}$ and lkr^{coo3} mutants to sucrose, which is the only tastant present in the two-dye feeding assay with which we detected the bloating/meal size phenotype. We observed no differences between wild-type and the mutants, suggesting that gustatory defects due to lack of peripheral Leuc expression do not account for the meal size phenotype (Supp. Figure 1).

We have also examined two other sets of neurons involved in feeding for their relevance to meal size regulation. Inhibiting hugin expressing neurons in adults causes rapid meal initiation [28] while ablating NPF pathway neurons alters larval feeding behavior [29-31]. We examined flies with ablated hugin or NPF neurons using both the two-dye and ^{14}C-labeled-leucine assays, but found no defects (Supp. Figure 3).

Discussion

Like other animals, *Drosophila* adults consume food in separated bouts known as meals. When measured using the café assay[18], wild-type flies take 7-8 meals in a 12 hr. daytime period, most of 0.1-0.2 μl in size. The molecular mechanisms by which meal size and frequency are determined are unknown. To study meal size regulation, we screened a set of insertion mutants to identify lines that overate after a starvation period. We discovered that mutants with reduced expression of the leucokinin neuropeptide or its receptor both consume excess food immediately after starvation (Figure 1), but do not eat more than normal flies when continuously supplied with food. This finding is explained by the fact that *leuc* and *lkr* mutants consume abnormally large meals, but at a reduced frequency (Figure 2).

Leucokinin is known to function as a hormone to regulate diuresis and hindgut motility, and *lkr* is expressed in the malpighian tubules, the fly excretory organ [10-14]. However, the effects of leucokinin on meal size regulation are likely to be due to its action as a peptide neurotransmitter rather than to humoral effects on malpighian tubule Lkr, because the *leuc* and *lkr* meal size phenotypes are fully rescued by pan-neuronal expression of these genes (Figure 1). This shows that control of meal size by *lkr* is due to reception of a leucokinin signal by neurons and does not involve Lkr signaling in malpighian tubules.

We examined the expression patterns of *leuc* and *lkr* by antibody staining and by constructing promoter-Gal4 fusions. Both genes are expressed in small subsets of neurons in the brain and ventral ganglia (Figure 3), and Lkr is also expressed in the foregut, which is known to be involved in meal termination (Figure 4). Ablation of *leuc* neurons using cell death genes produces the same meal size phenotype as loss of leucokinin, indicating that this neuronal circuit is essential for control of food intake (Figure 5).

What are the mechanisms by which leucokinin and Lkr regulate meal size? Since ablation of Lkr neurons causes the same phenotype as a reduction in Lkr expression, our data suggest that the activities of Lkr neurons are reduced in *leuc* and *lkr* mutants. Also, since reductions in either leucokinin or Lkr cause the same phenotype, it is likely that the Lkr neurons that are relevant to the phenotype include the brain and/or ventral ganglion neurons that are near leucokinin-positive synaptic boutons (Figure 3). Direct or indirect input of Lkr neurons to the foregut could modulate the signals emanating from gut stretch receptors, so that when Lkr neurons are absent or fire less frequently the fly's brain becomes less sensitive to gut stretch signals that indicate satiety.

Other neuropeptides and neuronal circuits have been demonstrated to affect feeding in *Drosophila*. However, our analysis suggests that their functions are distinct from those of the leucokinin pathway. In adult flies, inhibiting hugin expressing neurons causes rapid meal initiation and crop bloating [28], and ablating NPF neurons affects larval feeding [29-31]. We examined meal size in adults with ablated hugin or NPF neurons, but found no changes from wild-type (Supp. Figure 3). Two distinct neuronal populations, defined by the expression patterns of the Fru-GAL4 and c673a-GAL4 drivers, control long-term energy homeostasis. Flies in which these neurons are silenced store excess fat, while those in which they are hyperactivated lose fat. c673a-GAL4 silenced flies also consume more food than controls [32]. Sulfakinins and allatostatins inhibit contraction of insect visceral muscles, and these peptides can inhibit feeding when injected into a variety of insects [33-36]. Finally, male sex peptide increases post-fertilization feeding by females [37].

Possible relevance to mammalian systems

A variety of mammalian peptides have been implicated in food intake regulation. Some, like leptin, measure the status of the body's energy stores and are believed to influence long term

food intake. Other neuronal and gastrointestinal tract peptides regulate meal related parameters such as initiation, size, and frequency. Neuronally produced neuropeptide Y, endocannabinoid, and orexin, along with gastrically secreted ghrelin, are thought to be involved in meal initiation, while gastrointestinal tract peptides such as cholecystokinin (CCK), pancreatic peptide Y (3-36), and glucagon-like peptide 1 are believed to regulate meal size and frequency [1,2].

In mice, a reduction in CCK pathway signaling causes feeding defects (meal size increases associated with compensatory reductions in meal frequency) that are similar to those we see in *leuc* and *lkr* mutants [38]. This probably does not represent a conserved pathway, since leucokinin and its receptor have little sequence homology with mammalian CCK pathway components. However, *Drosophila* CCK-related peptides called sulfakinins do inhibit feeding when injected into flies [33].

Leucokinin and its receptor are homologous to vertebrate tachykinin and tachykinin receptor, and tachykinins cause reductions in food intake when injected into vertebrates [39-43]. Tachykinins and their receptors are expressed within or near brain centers that regulate body weight and food intake, such as the arcuate nucleus [44]. Our findings in *Drosophila* suggest that the roles of tachykinins in regulating food intake might be evolutionarily conserved between insects and vertebrates.

Experimental Procedures

Fly stock maintenance

The *leuc*^{c273} and *lkr*^{c003} lines were obtained from the Bloomington *Drosophila* stock center. They were kept on regular fly food (8% corn meal, 5% sucrose, 2% yeast, 1% propionic acid, 0.5% agar) at 25 °C. All behavioral analysis was preformed on 5-7 day old male flies.

Immunocytochemistry

Anti-Lkr was obtained from Julian Dow (University of Glasgow), while anti-leuc was generated against the full length amidated leucokinin peptide by YenZym Antibodies, LLC (San Francisco, CA). For staining, 2-4 day old male brains were dissected and fixed in 4% paraformaldehyde in 1X PBS for 1 hour at room temperature, followed by 5 washes of 30 minutes each in 1X PBT with 0.1% Triton X-100. The brains were incubated for 1 hour in a blocking solution composed of 1% preimmune goat serum in 1X PBT. They were then incubated overnight at 4° C in 1:100 dilutions of rabbit anti-leuc or anti-Lkr, with anti-GFP-Alexa Fluor-463. The brains were then washed in 1X PBT 5 times for 1 hour each followed by a 1 hour incubation in the blocking solution. They were then incubated in a 1:500 dilution of goat anti-rabbit Alexa Fluor-568 (Invitrogen, #A21069) for 2 hours followed by 5 washes in PBT for 1 hour each. The brains were mounted in Vectashield and visualized with a Zeiss LSM 510 NLO confocal microscope. The GFP label was excited with a laser beam at 488nm and the images were captured with a 500-530nm bandpass filter. The Alexa Fluor-568 label was excited with a laser beam at 561nm with capture using a 575-615nm band pass filter. Autofluorescence images captured with 488nm excitation and collected with a longpass 575nm filter were used as background.

For cryostat immunocytochemistry, flies were embedded in sagittal position and cut with a cryostat at 16 μm thickness. The sections were collected on SuperFrost/Plus microscope slides (Fisher Scientific), and were defrosted in a desiccation box for 15 minutes, followed by fixing in 4% paraformaldehyde in 1xPBS buffer for 10 minutes. The sections were washed three times for 5 minutes each in 1X PBT with 0.1% triton X-100 followed by incubation in minutes. The

stained sections were washed three times for five minutes each in 1X PBS, and were then mounted in glycerol and examined using the Cy3 fluorescence channel.

^{14}C-leucine assay

50 ml of hot 1% sucrose in 1% agarose mixed with acid red dye was mixed with 250 μl of 50 μCi/ml ^{14}C-leucine and aliquoted as 5 ml portions into regular fly food vials. A group of 20 flies of a given genotype were exposed to this food for 20 minutes. 10 flies from each genotype were transferred into scintillation vials containing 250 μl of a 1:1 mixture of perchloric acid and hydrogen peroxide and incubated at 75° C for 30 minutes, which dissolved flies to a clear fluid. The resulting mixture was mixed with 5 ml of scintillation fluid and radiation was counted using a scintillation counter.

Café assay

A single fly is placed in a well of a 24 well tissue culture plate. The bottom of the well is covered with 0.5 ml of 1% agarose to provide moisture. The fly is then provided, on a daily basis, with a capillary tube filled with 5% sucrose and 2% yeast extract inserted through a hole in the roof. After a 5 day acclimation period, the amount of food ingested is directly measured every hour by observing the reduction in fluid level in the capillary tubes.

For the post-starvation response café assay, 24 hour males starved on 1% agarose were anesthetized by chilling, and then transferred in group of five to the café apparatus. Their food intake was measured every 12 hours for 3 days.

Transgene generation

Transgene containing 2 kbp promoter sequence or open reading frame (ORF) of either *leuc* or *lkr* genes were generated using high fidelity PCR. Primer pair ACGGTACCACATGTTTGGG CGTTG and GCAGCCCTGCTTATATATAGCCACTC were used to generate *leuc* promoter amplicon on wild-type fly DNA template, while primer pair ATCGAGATCTGAAGCCCAT TTG GCGGACTCAACTAAC and AATAGCGGCCGCTGTGCTTTTTGTGTCTGTTGTTA TGGC were used to generate *lkr* promoter amplicon on wild-type fly DNA template. For ORF transgene, primer pair AACGCAGTTGGCCGAGAGGATTA and CGCTTCTCGGTTTGC AATCATCG were used to generate *leuc* ORF amplicon on wild-type cDNA template, while *lkr* ORF was generated using primer pair ATTTGCGGCCGCAGTTGACTTCGGGAGCTTTAATC G and TAATGGTACCTGGCCGGATCCATTACTGGAGAG on full length *lkr* cDNA clone obtained from DGRC. Promoter *leuc* amplicon was digested with Not I and Stu I, while promoter *lkr* amplicon was digested with Bgl II and Not I. For *leuc* ORF and *lkr* ORF amplicons, they were digested with Not I and Kpn I restriction enzyme then cloned into the PUAST vector. Successful clones were sent to Rainbow transgenic flies Inc. for transformation in w^{1118} flies (Newbury Park, CA).

Injection assay

$leuc^{275}$ flies starved for 22 hours in 1% agarose were anesthetized by chilling them on ice. They were then injected with 0.2 μl of 150 μg/ml amidated leucokinin peptide (NSVVLGKKQRFHSWG-amide) or with a control peptide made of scrambled leucokinin sequence (NFSLVKGWHRVQVKG-amide), both in 1XPBS. After a 2 hour recovery period, they were subjected to the two-dye feeding assay.

Proboscis-Extension Assay

Four-day-old male flies, previously starved for 24 hr on 1% agarose, were anaesthetized by chilling on ice. They were then glued by their backs to a glass slide and allowed to recover for

2 hr at room temperature. Flies that showed no sign of movement after the recovery period were discarded. The remaining flies were given water on a cotton swab until satiation and were then used for the proboscis-extension assay. In this assay, each fly was briefly touched for 5 s on the legs with a cotton swab soaked in the test solution, and the presence or absence of extension was recorded. This stimulus was repeated five times, with a 2 min rest period between repetitions.

Highlights

1. Mutations in *leucokinin* or *leucokinin receptor* genes affect meal size in *Drosophila*
2. Mutant flies take fewer but larger meals, so their total food consumption is normal
3. Leucokinin and its receptor function in small groups of neurons to regulate meal size
4. Ablation of the neurons expressing the peptide or its receptor increases meal size

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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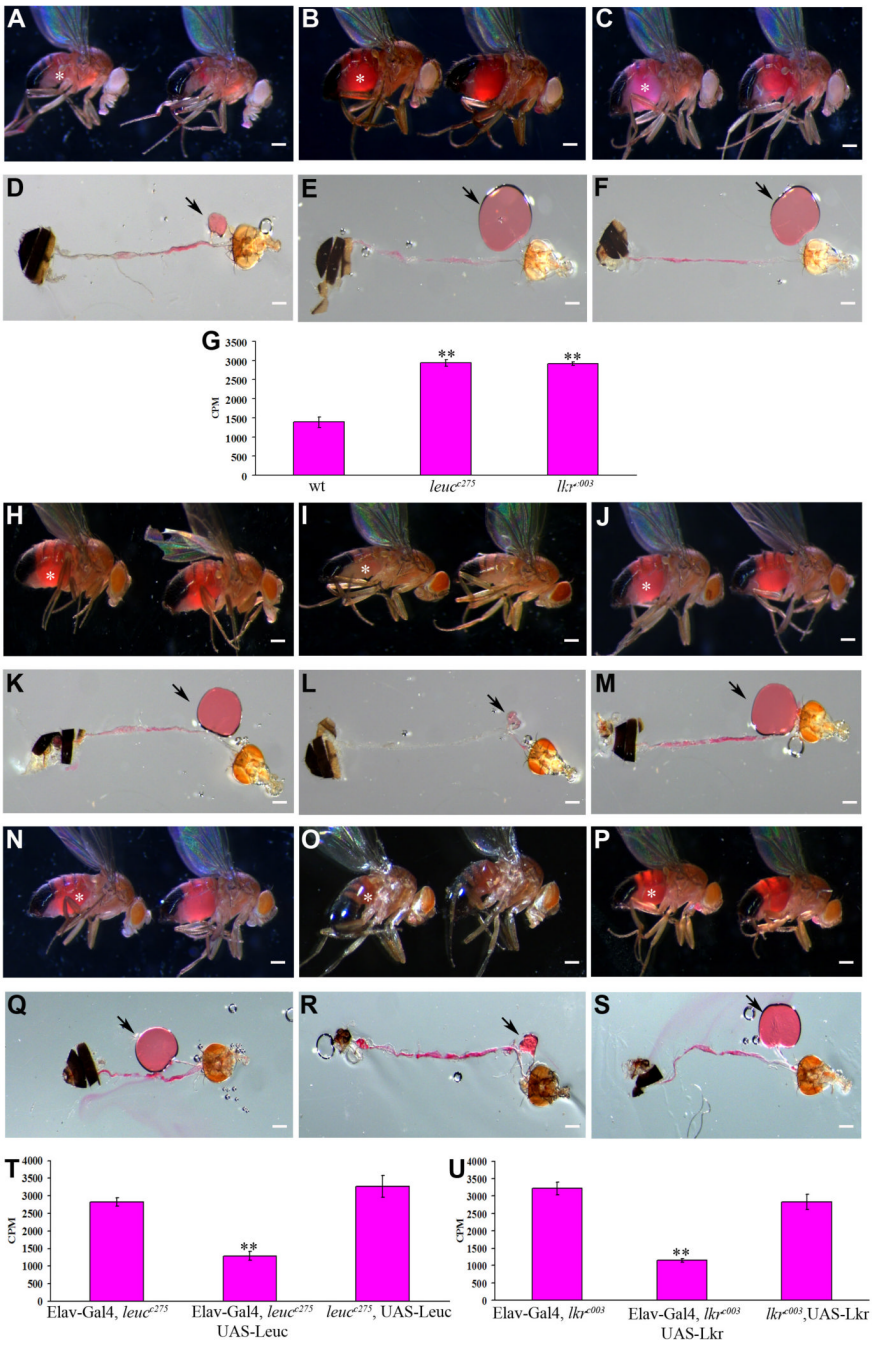

Figure 1. Mutation of *leuc* or *lkr* causes increases in post-starvation food intake

Wild-type flies have a normally sized abdomen (A, asterisk) and crop (D, arrow) when subjected to the two-dye feeding assay after starvation. The crop of unstarved flies would be of a similar size. *leuc*^{c275} mutants have bloated abdomens (B, asterisk) with enlarged crops (E, arrow) when subjected to the same assay. The same result is observed in *lkr*^{c003} mutant flies (C, asterisk, and F, arrow, respectively). (G) When *leuc*^{c275} or *lkr*^{c003} flies are fed ^{14}C-labelled-leucine food in the two-dye feeding assay after starvation, an increase in food intake is observed as compared to wild-type. In *leuc*^{c275} mutants, panneuronal expression of UAS-*leuc* using the Elav-GAL4 driver rescues the abdominal and crop bloating phenotypes as shown by the two-dye assay (I, asterisk, and L, arrow, respectively), and the abnormal increase in post-starvation

food intake (T). No rescue is observed in control *leuc*^{c275} flies that carry the Elav-GAL4 driver alone (H, K, T) or only the UAS-*leuc* transgene (J, M, T). Pan-neuronal expression of UAS-*lkr* also rescues the phenotypes of *lkr*^{c003} (O, R, U). No rescue is observed in control mutant *lkr*^{c003} flies that carry the Elav-GAL4 driver alone (N, Q, U) or only the UAS-*lkr* transgene (P, S, U). White scale bar: 200 μm. Error bars are standard deviations of five different replicates for a given genotype. (Asterisks denote T-test statistical significance: *; P<0.05, **; P<0.01, ***; P<0.005, ****; P<0.0005).

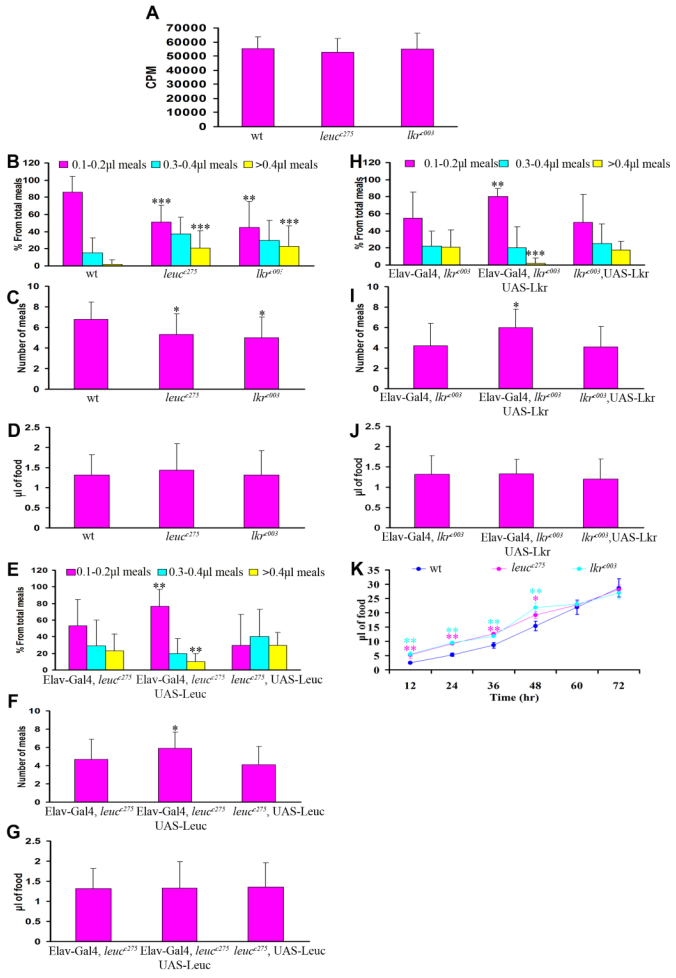

Figure 2. *leuc* and *lkr* mutants have an increase in meal size that is associated with a reduction in meal frequency

(A) *leuc*^{c275} and *lkr*^{c003} do not exhibit an increase in intake of radioactivity relative to wild-type when exposed to ^{14}C-labelled-leucine food for 48 hours without starvation. When the café feeding assay is performed on single non-starving flies, *leuc*^{c275} and *lkr*^{c003} flies have a decrease in 0.1-0.2µl meals that is associated with an increase in meals that are larger than 0.4µl. This increase in meal size is associated with a reduction in recorded meal events as compared to wild-type flies (B, and C, respectively). However, both mutants still have an overall food intake that is similar to wild-type (D). Pan-neuronal expression of UAS-*leuc* rescues the meal size and frequency defects of *leuc*^{c273} flies in the single fly café assay (E and F, respectively). No rescue of either feeding parameter is observed in control mutant *leuc*^{c273} flies that carry the Elav-Gal4 driver alone or only the UAS-*leuc* transgene (E and F, respectively). Pan-neuronal expression of UAS-*lkr* rescues the meal size and frequency defects of *lkr*^{c003} in the single fly café assay (H and I, respectively). No rescue of either feeding parameter is observed in control mutant *lkr*^{c003} flies that carry the Elav-Gal4 driver alone or only the UAS-*lkr* transgene (H, and I, respectively). No difference in total food intake is observed between the different genotypes (G and J). (K) The post-starvation increase in food intake in *leuc*^{c275} and *lkr*^{c003} is later compensated for by a reduction in food intake that ultimately causes them to have similar overall food intake as wild-type flies by ~60 hr. Error bars are standard deviations for 5-8 different replicates for a given genotype in A and K, and

for 20-25 single fly analyses in B-J. Asterisks denote T-test statistical significance: *; P<0.05, **; P<0.01, ***; P<0.005, ****; P<0.0005.

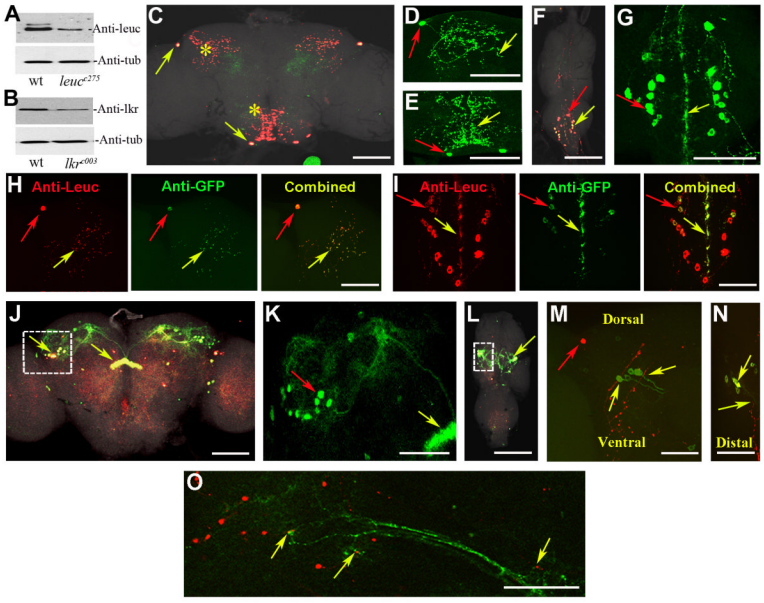

Figure 3. Expression patterns of Leucokinin and Lkr in the brain and ventral ganglia

Western blotting using antibodies against leucokinin (anti-Leuc) or Lkr (anti-Lkr) demonstrates a reduction in the level of expression of leucokinin in *leuc*^{c275} mutants (A) and of Lkr in *lkr*^{c003} mutants (B) as compared to wild-type. Antibody against tubulin (anti-tub) was used as a tissue extract loading control; these lanes show that the mutant extracts contain the same amount of protein. In Leuc-Gal4::UAS-mCD8-GFP flies, anti-Leuc (C and F, red; D and E, green) and anti-GFP (C and F, green) signals co-localize in neuronal soma in the lateral horn and the SOG (C, yellow arrows). Asterisks in (C) indicate neuropilar regions that label brightly with anti-Leuc. (D) A higher magnification view of one of the lateral horn Leuc neurons, LHLK, showing the cell body (red arrow) and puncta along neuronal processes (yellow arrow). (E) A similar view of two of the SOG neurons, the SELKs. (F) The ventral ganglia, showing two rows of Leuc neurons (ABLKs). Some of these (yellow, indicated by yellow arrow) express more GFP than others (red, indicated by red arrow. (G) A higher magnification view of the ABLKs. A cell body is labeled by a red arrow, while the line of axons and synapses along the midline are indicated by a yellow arrow. (H-I) Leuc-GAL4::n-syb-GFP brain (H) and ventral ganglia (I) stained with anti-Leuc (red) and anti-GFP (green), showing co-localization in cell bodies (red arrows) and presynaptic terminals (yellow arrows). The ABLK cell bodies in (I) have much less n-syb than the terminals. In Lkr-GAL4::UAS-mCD8::GFP flies, anti-Lkr (J and L, red) and anti-GFP signals (J-L, green) co-localize in dorsally located neuronal cell bodies, and also in the axons of the fan-shaped body in the central complex (F, arrows). We also observe expression in two large neurons in the ventral ganglia (L, arrow). (K) A higher magnification view of the brain Lkr neurons in one hemisphere. Red arrow, cell body; yellow arrow, fan-shaped body. (M-O) Brain/ventral ganglia in Lkr-GAL4::UAS-mCD8::GFP animals, stained with anti-Leuc and anti-GFP. (M) An LHLK neuron (red arrow) has neuronal processes with synaptic boutons (chains of red dots) that are close to green-stained Lkr-GAL4::UAS-mCD8::GFP neurons (yellow arrows). (N) Leuc-positive boutons are near axons (faint green lines) of Lkr-GAL4::UAS-mCD8::GFP neurons in the ventral ganglion. (O) A single confocal slice of approximately 0.3 μm in depth shows Leuc-positive synaptic terminals (red) in the lateral horn adjacent to or contacting processes of Lkr-GAL4::UAS-mCD8::GFP neurons (green). Note the paired red dots adjacent to a green profile (left arrow), and a red dot between two green dots (middle arrow. White scale bar: 200 μm.

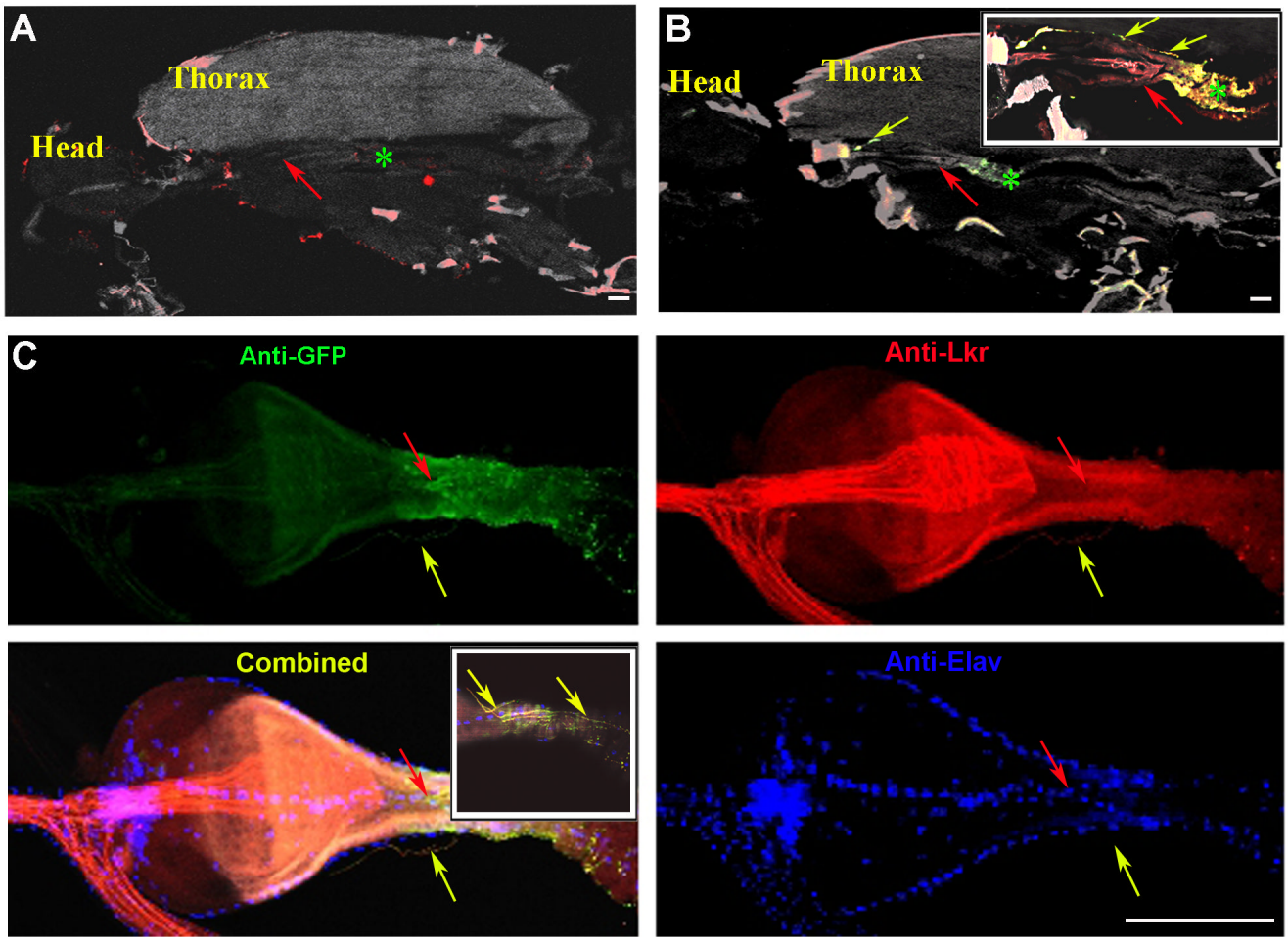

Figure 4. Lkr expression in the foregut

(A) In a sagittal cryostat section of Leuc-GAL4::UAS-mCD8-GFP flies, no expression of either GFP or Leuc is observed in the foregut region (asterisk, gut lumen; arrow, proventricular region). (B) In a sagittal cryostat section of Lkr-GAL4::UAS-mCD8-GFP flies, GFP and Lkr are observed in the foregut (B and B inset, red arrows and green asterisks). Note the GFP-positive axons that run along the dorsal side of the foregut, and may connect it with the brain (B inset, yellow arrows). (C) A dissected foregut section (anterior to the left) from Lkr-GAL4::UAS-mCD8-GFP flies, triple-stained with anti-Lkr (red), anti-GFP (green), and anti-Elav, which labels neuronal nuclei (blue). Green staining overlaps with red staining in the proventricular area (yellow arrow). Note that some of the Elav-positive neurons appear to also express Lkr and GFP (red arrows). Triple-stained foregut section (inset) also shows colocalization of Lkr and GFP on axons (yellow arrows). White scale bar: 200 μm.

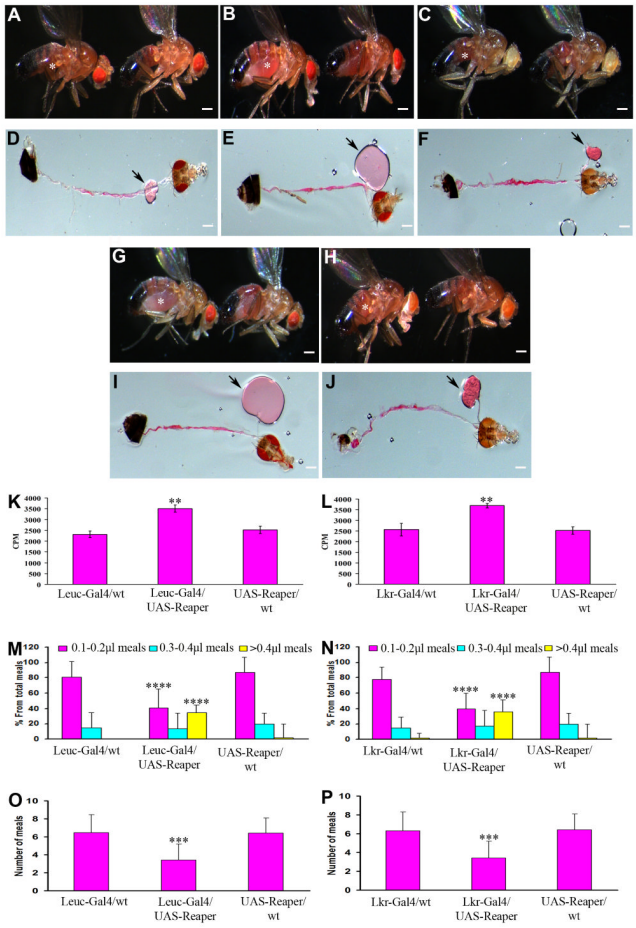

Figure 5. Ablation of Leuc-Gal4 and Lkr-Gal4 expressing neurons produces meal size and frequency defects that match those seen in *leuc*^{c275} and *lkr*^{c003} mutants

Leuc-GAL4::UAS-*reaper* (B, E, K) and Lkr-GAL4::UAS-*reaper* (G, I, L) flies have bloated abdomens (asterisks) and overfilled crops (arrows), when subjected to the two-dye feeding assay after starvation. (K-L) They also exhibit an increase in ^{14}C -leucine food intake. Leuc-GAL4::UAS-*reaper* (M) and Lkr-GAL4::UAS-*reaper* (N) flies exhibit decreases in 0.1-0.2µl meals that are associated with increases in meals that are larger than 0.4 µl when examined by the single fly café feeding assay. This increase in meal size is associated with a reduction in the number of meals taken (O and P, respectively). Control UAS-*reaper*/+ (A, D), Leuc-GAL4/+ (C, F), and Lkr-GAL4/+ (H, J) flies do not show any of the above defects in feeding behavior when examined by the same assays. White scale bar: 200 µm. Error bars are standard deviation of five different replicates for a given genotype in K and L, and of 20-25 single fly analyses in M-P. Asterisks denote T-test statistical significance: *; P<0.05, **; P<0.01, ***; P<0.005, ****; P<0.0005.