Enteric neurons increase maternal food intake during reproduction

Reproduction induces increased food intake across females of many animal species\(^1\)\(^-\)\(^4\), providing a physiologically relevant paradigm for the exploration of appetite regulation. Here, by examining the diversity of enteric neurons in \textit{Drosophila melanogaster}, we identify a key role for gut-innervating neurons with sex- and reproductive state-specific activity in sustaining the increased food intake of mothers during reproduction. Steroid and enteroendocrine hormones functionally remodel these neurons, which leads to the release of their neuropeptide onto the muscles of the crop—a stomach-like organ—after mating. Neuropeptide release changes the dynamics of crop enlargement, resulting in increased food intake, and preventing the post-mating remodelling of enteric neurons reduces both reproductive hyperphagia and reproductive fitness. The plasticity of enteric neurons is therefore key to reproductive success. Our findings provide a mechanism to attain the positive energy balance that sustains gestation, dysregulation of which could contribute to infertility or weight gain.

Internal state has profound effects on brain function\(^5\)\(^-\)\(^9\)\(^4\). Despite increasingly recognized roles for the gut–brain axis in maintaining energy balance\(^10\)\(^-\)\(^15\), links between internal state and gastrointestinal innervation remain poorly characterized. Progress has been hindered by neuroanatomical complexity, which is only beginning to be parsed in mammals\(^16\)\(^-\)\(^20\). The simpler—yet physiologically complex—intestine of \textit{Drosophila} provides an alternative entry point into the study of gastrointestinal innervation.

**Innervation of the stomach-like crop**

Innervation of the main digestive portion of the adult fly intestine, which encompasses the anterior midgut and the crop\(^21\)\(^,\)\(^22\) (Extended Data Fig. 1a, b), emanates from an enteric hypocerebral ganglion (HCG) (Extended Data Fig. 1a, c, e, g, i, j) and central neurons of the pars intercerebralis (PI) in the brain (Extended Data Fig. 1a, d, f, g). PI neurons directly innervate the anterior midgut and the crop, and include insulin-producing neurons\(^23\)\(^-\)\(^25\) and other peptidergic subtypes\(^26\) (Extended Data Fig. 1a, d, f, g). The crop is further populated by processes that emanate from cells of the corpora cardiaca, which produce the glucagon-like adipokinetic hormone and are adjacent to the HCG\(^27\)\(^,\)\(^28\) (Extended Data Fig. 1h). Also adjacent to both the HCG and the corpora cardiaca are the corpus allatum cells, which produce juvenile hormone and extend short local projections (Extended Data Fig. 1c, k). The thoraco-abdominal ganglion of the central nervous system might not innervate these gut regions (Extended Data Fig. 1c, k). The thoracico-abdominal ganglion of the central nervous system might not innervate these gut regions (Extended Data Fig. 1c, k). The thoracico-abdominal ganglion of the central nervous system might not innervate these gut regions (Extended Data Fig. 1c, k). The thoracico-abdominal ganglion of the central nervous system might not innervate these gut regions (Extended Data Fig. 1c, k). The thoracico-abdominal ganglion of the central nervous system might not innervate these gut regions (Extended Data Fig. 1c, k).

The crop—an expandable structure found in the intestines of insects\(^22\)—might be disregarded as a passive food store, but several observations suggest active regulation of its physiology. Refeeding flies after starvation results in enlarged, food-filled crops\(^29\) (Extended Data Fig. 2a, d, e), pointing to modulation of food ingestion into and out of the crop. Live imaging or temporal dissections of flies revealed that food always enters the crop before proceeding to the midgut (Extended Data Fig. 2b, c, Supplementary Video 1). Additionally, food transit through the crop is dependent on both its palatability and its nutritional value (Extended Data Fig. 2f).

Therefore, in adult flies, all food transits through the crop, which is nutrient-sensitive and shows chemically and anatomically diverse innervation.

**Control of the crop by myosuppressin neurons**

The crop and anterior midgut are innervated by myosuppressin (Ms)-positive neurons\(^30\)\(^,\)\(^31\), located in the PI and the HCG (Extended Data Fig. 3a, b, f, i, o). PI Ms neurons are distinct from known neuronal subsets, with the exception of eight Ms neurons that co-express the \textit{Taotie}-\textit{GAL4} marker (Extended Data Fig. 3l, m, n, p, q). Two PI Ms neuron populations can be distinguished by cell size: one comprises 18 large cells (including the \textit{Taotie}-positive subset) and another comprises 12 smaller cells (Extended Data Fig. 3i). Single-cell clones of large Ms neurons reveal a single process that bifurcates into a longer, probably axonal projection to the gut—which arborizes in the HCG and extends further to innervate the crop—and a shorter, probably dendritic process.

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\(^{1}\)MRC London Institute of Medical Sciences, London, UK. \(^{2}\)Faculty of Medicine, Imperial College London, London, UK. \(^{3}\)Australian Research Council Centre of Excellence in Plant Energy Biology, School of Molecular Sciences, The University of Western Australia, Perth, Western Australia, Australia. \(^{4}\)Harry Perkins Institute of Medical Research, Perth, Western Australia, Australia. \(^{5}\)School of Biological and Chemical Sciences, Queen Mary University of London, London, UK. \(^{6}\)Laboratory of Molecular Biology, National Institute of Mental Health, National Institutes of Health, Bethesda, MD, USA. \(^{7}\)Genes and Dynamics of Memory Systems, Brain Plasticity Unit, CNRS, ESPCI Paris, PSL Research University, Paris, France. \(^{26}\)e-mail: i.miguel-aliaga@imperial.ac.uk

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that reaches the suboesophageal zone, where the axons of peripheral gustatory sensory neurons terminate (Extended Data Fig. 3c–e). A subset of HCG Ms-expressing neurons also innervates the crop, whereas another subset projects locally (Extended Data Fig. 3b). We confirmed the expression of MsUs using an endogenously tagged Ms reporter (Ms–GFP; see Methods) and in situ hybridization (Extended Data Fig. 3j, k). We also observed Ms innervation of the hindgut, the rectal ampulla and the heart, and a subset of peripheral Ms-positive neurons innervating the female reproductive tract (Extended Data Fig. 3f–h; data not shown).

We selectively activated or silenced Ms neurons in adult flies. Activation resulted in greatly enlarged crops in flies that were fed ad libitum, consistent with the relaxant properties of Ms on insect muscles ex vivo (Fig. 1a, Extended Data Fig. 4b, d). By contrast, silencing of Ms neurons prevented crop enlargement in a starved–refed condition (Extended Data Fig. 2a) in which the crop normally expands (Fig. 1b, Extended Data Fig. 4c). Genetic downregulation or mutation of Ms (using a new mutant, see Methods) prevented crop enlargement, albeit to a lesser extent than Ms neuron silencing (Fig. 1d, Extended Data Fig. 4a, e–f). This could be due to another Ms-neuron-derived neurotransmitter or neuropeptide contributing to crop enlargement, or to loss of the Ms peptide during development in these experiments, resulting in adaptations that render the crop more active than it would be in response to acute loss of the Ms peptide. We generated a Gal4 insertion into the Ms locus that disrupts Ms production (MsSGM; see Methods). In contrast to the crop enlargement resulting from TrpA1-mediated activation from Ms-Gal4, TrpA1 expression from this (Ms mutant) MsSGM Gal4 driver failed to induce crop enlargement (Extended Data Fig. 4j, k), further confirming a requirement for Ms. Ms neuron subtype-specific downregulations and activations enabled us to establish that the P1Ms neurons (in particular, the Taotie-Gal4-positive subset of large PI Ms neurons) induce, and are indispensable for, crop enlargement through their production of Ms neuropeptide (Fig. 1d, Extended Data Fig. 4f, m).

We then explored the contributions of myosuppressor receptors 1 and 2 (MsR1 and MsR2) (Fig. 1e). We observed MsR2 expression in crop muscles, in subsets of neurons including the PI and HCG Ms-positive neurons and neurons innervating the ovary and heart; no MsR1 expression was detected in ovarian or heart muscles (Extended Data Fig. S1a–i). Expression of MsR2 was also detected in crop muscles (Extended Data Fig. S1i). To investigate the function of the Ms receptor, we downregulated MsR1 specifically in adult crop muscles using two independent driver lines (um-Gal4 and MsRF10GAL4; see Methods, Fig. 1 and Extended Data Fig. S5 for details). Both genetic manipulations led to reduced crop enlargement in a starvation–refeeding assay, comparable to that observed for Ms neuron silencing or Ms mutation (Fig. 1c, Extended Data Fig. 5k–o). Downregulation of MsR2 did not affect crop enlargement (Extended Data Fig. S5p). A role for MsR1 in mediating crop enlargement was confirmed using a MsR1SGM mutant (see Methods; Extended Data Fig. S5q–s). MsR1 is therefore identified as the crop muscle receptor through which Ms signals to modulate crop enlargement.

Neuron remodelling during reproduction

We next explored the physiological regulation of crop enlargement, and found that it is dependent on sex and on reproductive status: the crops of mated females fed ad libitum (which were used for all the experiments described above) were consistently more expanded than those of virgin female or mated male flies fed ad libitum (Fig. 2a, Extended Data Fig. 6o). Because we failed to observe post-mating changes in Ms neuron projections (Extended Data Fig. 6a, b), we wondered whether post-mating crop enlargement might result from the release of Ms preferentially in mated females. Ms peptide levels were lower in the PI neuron cell bodies of females only after mating (Fig. 2b, Extended Data Fig. 6c). In the absence of Ms transcriptional changes (Extended Data Fig. 6i) this observation is consistent with a post-mating increase in the secretion of Ms peptide in females. This effect of mating on Ms levels was specific to mating: nutrient availability did not affect intracellular Ms levels (Extended Data Fig. 6d–h). We also observed that the Ms neurons of mated females had higher cumulative calcium levels and a reduced amplitude of calcium oscillations compared to virgin females, as detected both by in vivo GCaMP6 calcium imaging and by the calcium-sensitive reporter CalExA, in which GFP expression is proportional to cumulative neuronal activity (Fig. 2c, d, Extended Data Fig. 6j–n). Physiologically, and in contrast to observations in mated females, a reduction of Ms signalling in males or in virgin female flies failed to impair crop enlargement. Consequently, when Ms signalling...
Levels of the steroid hormone ecdysone, which promotes egg production and intestinal stem-cell proliferation, increase after mating34.35. The ecdysone receptor (EcR) is expressed by all PI Ms neurons (Extended Data Figs. 7a, 8i), which suggests that they might be sensitive to circulating ecdysone. Expression of a dominant-negative EcR—which targets all EcR isoforms—confined to the Ms neurons of adult flies was found to increase intracellular Ms levels in the Ms PI neuron cell bodies of mated females to the levels observed in virgin females, whereas it had no effect on virgin females (Fig. 3, Extended Data Fig. 7b–d). Downregulation of EcR (using RNA interference lines that target all isoforms or the B1 isoform specifically; see Methods) produced comparable results. In both experiments, the amplitude of in vivo calcium oscillations in Ms neurons was increased to levels seen in virgin females (Extended Data Fig. 8n, o). Compromising EcR signalling in adult Ms neurons significantly reduced crop enlargement preferentially in mated females (Fig. 3b, Extended Data Fig. 7e–j); this phenotype was also apparent when the PI Ms neurons were targeted using Taotie-Gal4 (Extended Data Fig. 9k, l). Ecdysone therefore communicates mating status to Ms neurons through its B1 receptor.

We previously showed that the adult intestine is resized and metabolically remodelled after mating36, but did not investigate possible effects on its hormone-producing enteroendocrine cells. We now observe a post-mating increase in the number of enteroendocrine cells, including a subset that expresses the hormone bursicon α (Burs), which is known to signal to adipose tissue through an unidentified neuronal relay37 (Fig. 3c, d, Extended Data Fig. 8a–c). An endogenous protein reporter for the Burs receptor Rickets (Rk, also known as Lgr2) revealed its expression in subsets of neurons including all PI Ms neurons (including the Taotie-Gal4-positive subset) and in projections terminating in the HCG (Extended Data Fig. 8d–j). Expression in a subset of the HCG Ms neurons was observed only sporadically (Extended Data Fig. 8e).

Consistent with the regulation of Ms neurons by the increase in Burs derived from enteroendocrine cells after mating, adult-specific downregulation of the Burs receptor gene *rk* in Ms neurons reverted intracellular Ms levels in the PI Ms neurons of mated females to levels observed in virgin females; there was no effect in virgin females (Fig. 3e, Extended Data Fig. 8k–m). Like EcR downregulation, *rk* downregulation in Ms neurons also increased the amplitude of in vivo calcium oscillations in the Ms neuron cell bodies of mated females to values similar to those observed in virgin females (Extended Data Fig. 8n, o). Functionally, both the downregulation of *Burs* in intestinal enteroendocrine cells and the adult-specific *rk* downregulation in Ms neurons—either in all neurons or in the *Taotie-Gal4*-positive subset in the PI—preferentially reduced crop enlargement in mated females (Fig. 3f, Extended Data Fig. 9a–e, k, l). Conversely, stimulating the intestinal release of enteroendocrine hormones—including Burs—from enteroendocrine cells resulted in reduced Ms levels in the Ms neuron cell bodies of virgin females, similar to those observed in mated females (Extended Data Fig. 9f–h), and greatly enlarged crops (Extended Data Fig. 9i, j; see also Fig. 3 | Steroid and enteroendocrine modulation of Ms neurons and crop enlargement. a, b, Representative Ms levels (a) and crops (b) after adult-specific, Ms-Gal4-driven expression of EcR^{ΔN} (a) or EcR^{ΔN} (b) in mated females. Higher Ms levels in PI Ms neuron cell bodies (a, left) and smaller crops (b, left) are apparent relative to controls (middle and right). c, Increased expression of the enteroendocrine cell marker Prospero (Pros, white) and Burs (red) in the midguts of mated (right) compared with virgin (left) female flies. Filled arrowheads indicate Pros ‘Burs’ cells; empty arrowheads indicate Pros ‘Burs’ cells. The top images are full z projections; the bottom images are single z slices. d, There are more Pros ‘Burs’ midgut enteroendocrine (EE) cells apparent in mated females than in virgin females. e, f, Representative Ms levels (e) and crops (f) after adult-specific, Ms-Gal4-driven *rk* downregulation in mated females. Higher Ms levels in PI Ms neuron cell bodies (e, left) and smaller crops (f, left) are apparent relative to controls (middle and right). Scale bars: 20 μm (*a, e*), 50 μm (*c* and *f*, left) and 500 μm (*b, f*). In d, statistical significance was assessed using a Mann–Whitney–Wilcoxon test.

Fig. 2 | Reproductive modulation of Ms neurons. a, b, Representative dissected intestines (a) and Ms stainings of the PI region of the brain (b) of wild-type flies. Mated females have a more expanded crop (a, right) and less Ms in their cell bodies (b, right) than virgin females (a and b, middle) or mated males (a and b, left). In b, fluorescence signals are pseudocoloured: high to low intensity is displayed as warm (yellow) to cold (blue) colours here and thereafter. c, d, Temporally defined video snapshots of Ms-driven GCaMP6 activity in the PI of virgin (c) or mated (d) females, imaged over 1,000 frames (frames acquired every 427 ms). The asterisks and arrows highlight two randomly chosen Ms neurons. See also Supplementary Video 1. Scale bars, 20 μm (d–f); 500 μm (a).
Post-mating, Ms-mediated crop enlargement increases food intake and reproductive output. a, b, Adult- and crop-muscle-specific MsR1 downregulation in mated females. Reductions in the amount of dye-laced food ingested (a) and in the number of sips per fly (b) are apparent relative to controls. FCF denotes the dye Brilliant Blue FCF (also known as E133). c, d, Adult-specific EccR downregulation in Ms neurons (e) and Burs downregulation in Pros-expressing enteroneocrine cells (d) in mated females. Both downregulation experiments result in reduced food ingestion relative to controls. e, Reduced Ms signalling to crop muscles reduces fecundity. Data are the number of eggs laid by a group of 40 mated females per day over the course of 4 days. Adult- and crop-muscle-specific MsR1 downregulation is shown in red and the two genetic controls are shown in grey. Statistical significance was assessed using Kruskal–Wallis tests (a–d) or a two-way ANOVA followed by a Tukey’s multiple comparison test, with day and genotype as the two independent factors (e).

Neuron remodelling promotes food intake

To investigate the importance of Ms neuron modulation after mating, we selectively prevented post-mating crop enlargement by downregulating MsR1 in adult crop muscles using two independent strategies (Extended Data Fig. 5k, l). This had no discernible effects in males or virgin females, but specifically prevented the increase in food intake that is normally observed in female flies after mating1 (Fig. 4a, b, Extended Data Fig. 10a–e). Comparable results were obtained by blocking the post-mating ec dysome and Burs inputs into the Ms neurons (Fig. 4c, d, Extended Data Fig. 10f, g). Downregulation of MsR2 had no such effect (Extended Data Fig. 10d). The post-mating change in crop expandability, mediated by Ms and MsR1 signalling, thus causes the increased food intake observed in females after mating.

The negative pressures that have been reported in the crops of larger insects38 suggest that the crop may draw food in by generating suction. The increased crop expandability enabled by Ms release after mating could therefore increase food intake through changes in suction. We observed that mated females ingest more food per sip than virgin females (Extended Data Fig. 10h), which is consistent with mated females needing to generate a higher suction pressure to facilitate bigger sips. We therefore modelled crop enlargement using the Poiseuille equation for incompressible fluid flow in a pipe (see Methods), and found that the crop would need a suction pressure of the order of −1 kPa to achieve the previously reported intake volume per sip3. This is in reasonable agreement with previously reported values measured in cockroach crops39 of between −0.5 and −1 kPa. The model predicts that mated flies would require a modest increase in suction pressure to −1.3 kPa in order to facilitate the increased sip size.

In the model, the change in crop volume drives food intake through increased suction (Extended Data Fig. 10h). A crop that cannot enlarge, or a persistently enlarged crop, should therefore result in a comparable reduction in food intake by preventing the generation of suction. We tested this by persistently preventing crop enlargement (using crop-muscle-specific MsR1 knockdown; Extended Data Fig. 5k, l) or by persistently inducing it (using TrpA1-mediated Ms neuron activation from Ms-Gal4 or Taotie-Gal4; Extended Data Fig. 4l, m), after which the diet of these flies was switched from an undyed to a dye-laced food source to assess food intake. As predicted, both genetic manipulations reduced food intake (Extended Data Fig. 10d, e, i, j, m). Conversely, increasing the rate at which the crop expands should increase food intake. We tested this by activating the Ms neurons as in the previous experiment, but this time we provided the dye-laced food source and monitored its intake at the same time as we activated the neurons (that is, as we were inducing greater crop expansion) rather than after a persistent activation (when the crop is already maximally expanded). We observed increased food intake under these conditions in the absence of changes in the number of meals (Extended Data Fig. 10k, l, n). Although further work will be required to elucidate the full dynamics of crop enlargement, filling and emptying, these experiments support the idea that the Ms-induced enlargement of the crop after mating increases food intake at least partly by increasing the suction power of the crop.

Finally, given the links between nutrient intake and fecundity40, we propose that the Ms-driven crop enlargement after mating might be adaptive and support reproduction. We prevented crop enlargement selectively after mating by downregulating MsR1 from crop muscles, as in previous experiments (Fig. 1c, Extended Data Fig. 5m, n). This resulted in reduced egg production (Fig. 4e, Extended Data Fig. 10o), and the eggs that were produced had reduced viability (Extended Data Fig. 10p). We therefore conclude that the crop and its Ms innervation sustain the increase in food intake after mating, maximising female fecundity.

Discussion

Our findings lead us to propose that the maternal increase in food intake during reproduction is adaptive, that the crop is a key reproductivestory organ, and that Ms is a major effector of post-mating responses. In support of these ideas, the crop is absent in larvae—the juvenile stage of insects—and other Diptera have co-opted it for reproductive behaviours such as the regurgitation of nuptial gifts or the secretion of male pheromones35. Ms receptors are also closely related to the Sex peptide receptor (the ‘mating sensor’ of female flies), and both diverged after duplication of an ancestral receptor that might have responded to the Myoinhibitory peptide (Mip) in the last common ancestor of protostomes36. It will be interesting to explore possible links between Ms and Sex peptide signalling, and whether and how these mating signals affect recently described crop mechanosensing.

Extended Data Fig. 8a for co-expression of the Tkg-Gal4 enteroneocrine cell driver and Burs.

Thus, steroid and enteroneocrine hormones communicate mating status to the brain. Acting through their receptors in the PI Ms neurons, these hormones change Ms neuronal activity, promoting the release of Ms after mating (Extended Data Fig. 9m).

Extended Data Fig. 9m.
mechanisms that restrain ingestion as the crop expands in order to terminate large meals.\textsuperscript{42,43} We provide evidence for a gut-to-brain axis in \textit{Drosophila} by identifying central Ms neurons as targets of the gut-derived hormone Burs. These central neurons innervate the gut, ‘closing’ a gut–brain–gut loop that connects midgut enteroendocrine signals to the crop, a more anterior gut region. This might allow for the functional coordination of different gut portions, while enabling central modulation by sensory cues (for example, gustatory). We also identify the Ms neurons as the neural targets of ecysyne, which has been shown to promote food intake.\textsuperscript{44} Reproduction has pronounced, and in some cases lasting, effects on the human female brain\textsuperscript{45,46}; Ms neurons provide a tractable and physiologically relevant neural substrate for the investigation of the mechanisms involved.

The human digestive system might be similarly modulated by reproductive cues to affect food intake. In mammals, enteric neurons express sex- and reproductive-hormone receptors\textsuperscript{47}, and enteroendocrine hormone levels change during reproduction.\textsuperscript{1} We suggest that pregnancy and lactation represent an attractive and relatively unexplored physiological adaptation for the investigation of nutrient intake regulation, organ remodelling and metabolic plasticity—mechanisms that might eventually be leveraged to curb appetite and/or weight gain.

\textbf{Online content}

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-020-2866-8.
Fly stocks

Drivers. The following drivers were used: nSyb-Gal4 (original insert on third chromosome, gift from J. Simpson), Hpl2-3-Gal4 (ref. 52), Gt43a-Gal4 (ref. 53), Dhh4-Gal4 (ref. 54), Mip-Gal4 (ref. 55), pain-Gal4 (ref. 56), Gr28a-Gal4 (ref. 57), Aug21-Gal4 (BDSC: 30137), Ubx-Gal4 (ref. 58), and A-Gal4 (ref. 59), Ms-Gal4 (ref. 60), Taotie-Gal4 (ref. 61), Dsk-Gal4 (ref. 62), MsR1GEM-Gal4 (this study), voila-Gal4 (ref. 63), rkGEM-Gal4 (ref. 64), tub-Gal4 (ref. 65), nsp-Gal80 (ref. 66), gift from J. Simpson.

Reporters. The following reporters were used: Ms–GFP (this study), UAS-FBJ1 (ref. 67), UAS-DenMark-RFP, UAS-Venus-pm (refs. 68, 69), recombinant, gift from M. Landgraf), UAS-hs-nlsFlp (ref. 70), UAS-Tyr2 (ref. 71), UAS-Ms-RNAi (VDRC: 4874), UAS-Ms-RNAi (TRIP: JF02144), UAS-stinger GFP (ref. 72), UAS-Msr1-RNAi (VDRC: GD 9369), UAS-Msr2-RNAi (VDRC: GD 42304), UAS-Calexa (ref. 73), UAS-GcaMP6f (ref. 74), UAS-EcR-RNAi (BDSC: 9326, referred to as EcR<sup>RNAi</sup>-3), UAS-EcR-Bi-RNAi (BDSC: 9329, referred to as EcR<sup>RNAi</sup>-2), UAS-EcR-EcR-RNAi (VDRC: GD 37058, referred to as EcR<sup>RNAi</sup>-3), UAS-EcR-EcR-RNAi (BDSC: 6872), UAS-rk-RNAi (VDRC: GD 29932), UAS-dcr2 (VDRC: 60010), UAS-Burs-RNAi (VDRC: GD 3951).

Mutants. The following mutants were used: Ms<sup>α</sup> (this study), Df(3R) Xel6199 (BL7678), MsR1GEM-Gal4 (this study), Ms<sup>α</sup>-GFP (this study), Df(3L)w+1-1 (BDSC: 5411). Oregon R (Ork) and w<sup>1118</sup> were used as control flies.

Generation of Ms-GFP transgenic reporter line. The CBGt-g9060F04101D GFP-tagged clone for Ms from the fosmid library Transgeneome Resource (Source Bioscience) was used to establish transgenic lines using qPCR integrase mediated recombination (BestGene). The landing site used was aptp40 (y<sup>1</sup> w<sup>1118</sup> P{CaryPlatt40}).

Generation of Ms<sup>α</sup> null mutant. Ms<sup>α</sup> was generated using CRISPR-Cas9-assisted homologous recombination as described in ref. 75. The entire coding region of the gene was removed and replaced with an aptp site and an excisable P3–mCherry cassette. We chose to use a two-gRNA (guide RNA) approach (gRNA1: 5′-TTTTAGAGCTAGAAATAG-3′ and gRNA2: 5′-AACACCACTTTGGTCCCGA-3′), making use of the pCFD4 vector (Addgene, 49411). The two homology arms were cloned in the modified pTuV3-mCherry vector (gift from C. Alexandre). Both vectors were injected into y<sup>1</sup>; nos Cas9 (Il-attP40) flies by BestGene. The landing site used was aptp40 (y<sup>1</sup> w<sup>1118</sup> P{CaryPlatt40}).

Methods

Fly husbandry

Fly stocks were reared on a standard cornmeal/agar diet (6.65% cornmeal, 7.1% dextrose, 5% yeast, 0.66% agar supplemented with 2.2% nipagin and 3.4% propionic acid). All experimental flies were kept in incubators at 65% humidity and on a 12 h light/dark cycle, at 18 °C, 25 °C or 29 °C depending on the specific experiment. Flies were transferred to fresh vials every 3 days, and fly density was kept to a maximum of 20 flies per vial. Four day-old and 7 day-old virgin flies were used for experiments at 25 °C and 18 °C, respectively, unless otherwise indicated.

Temperature-controlled experiments. We used UAS-TrpA1 to activate Ms neurons (neuropeptide release) and to force release of peptides (including Burs) from enteroendocrine cells. For pre-activation of Ms neurons to assess crop enlargement and feeding, we transferred flies to a 29 °C incubator for 4 h before transfer to dye-laced food. For concurrent activation of Ms or Taotie-Gal4 neurons during feeding, flies were transferred to a 29 °C incubator at the same time as they were transferred to dye-laced food (to allow crop expansion during feeding before it reaches maximum size; Extended Data Fig. 10, 1k, n). In starved- or refed scenarios, feeding was monitored over the course of 15–20 min; in fed ad libitum conditions, feeding was monitored over the course of 2 h or for 1 h when comparing pre-activation with concurrent activation of Ms neurons with feeding. To force enteroendocrine peptide release we extended the incubation at 29 °C to 14–16 h. For Ms neuron silencing (neuropeptide retention) we used the ubiquitously expressed temperature sensitive Gal80 allele (ubGal80<sup>α</sup>) recombined with the UAS-Kir2.1 gene. Flies were reared, aged and mated at 18 °C. They were then transferred for 24 h at 29 °C and either starved or kept feeding ad libitum for an additional 14–16 h at 29 °C. Next, experimental assays were carried out at 29 °C.

RNAi experiments were also performed at 29 °C unless otherwise indicated. For these, flies were reared and aged at permissive temperature (18 °C) and then transferred to 29 °C for RNAi induction for 5 days. Experimental assays were carried out at 29 °C.

Ms-Gal4 Flybow clones were generated using the Flybow 1.1 construct based on the method described in ref. 63. Multiple heat-shock approach at different developmental time points was used. Each heat-shock lasted 1 h at 37 °C.

Diet

For experiments exploring the dietary regulation of crop enlargement, we used agar-based diets with a single nutrient source supplemented with 1% Brilliant Blue FCF (Sigma, S0717, also known as El33 and referred to as FCF blue). The basic recipe contained 1% agar, 1% FCF blue, 2.2% nipagin and 3.4% propionic acid. Each specific nutrient was added to the basic recipe in the following amounts: sorbitol-only 18.2% (1 M), yeast-only 5%, arabinose-only 15% (1 M) and sucrose-only 34.2% (1 M). For experiments exploring the dietary regulation of crop enlargement, we used agar-based diets with a single nutrient source supplemented with 1% Brilliant Blue FCF (Sigma, S0717, also known as El33 and referred to as FCF blue). The basic recipe contained 1% agar, 1% FCF blue, 2.2% nipagin and 3.4% propionic acid. Each specific nutrient was added to the basic recipe in the following amounts: sorbitol-only 18.2% (1 M), yeast-only 5%, arabinose-only 15% (1 M) and sucrose-only 34.2% (1 M). For details of these diets and their palatability and nutritional value see refs. 50–51. Times displayed in Extended Data Fig. 2b correspond to times after initiation of feeding of the dye-laced diets; only flies that continued to engage with the food after initiation of feeding were scored.

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To assess the effect of starvation on Ms levels, 4–5 day-old virgin female flies or female flies mated for 24 h were placed on 1% agar for 16 h before immunohistochemical analysis. For fecundity assays, which required daily egg counting, experimental flies were kept in cages on apple juice plates with a smear of live yeast. Plates required visualisation and/or quantitation of the food in the fly gut. For these, 1% FCF blue was added to the standard fly food. When pre-starvation was required, flies were kept in vials containing 1% agar in Milli-Q water, with 2.2% nipagin and 0.34% propionic acid.

FlyPAD food was pan-cooked using 1% agarose, 5% live yeast (S. cerevisiae) and 7.1% dextrose. It was dispensed into 2 ml Eppendorf tubes and stored at −20 °C until use. The food was melted to liquid form using a heat-block at 95 °C. It was then dispensed as a viscous droplet in the FlyPAD set up, where it fully solidified.

Refeeding assays required visualization and/or quantitation of the food in the fly gut. For these, 1% FCF blue was added to the standard fly food. When pre-starvation was required, flies were kept in vials containing 1% agar in Milli-Q water, with 2.2% nipagin and 0.34% propionic acid.
Generation of TCTGAT TCTCAT TACATAT TGCC.

T AGACTTGCAAA TT AAAAA TTGT A TGACTTTT AAAAA TT AGTTTCTTTG

T T TAT T TA A AT TA ATA A A A A AT TA A ACA A ATA ACAGA ATAT TCTA A ATC

ACCA TTT AA TTGCA TGTTTTT A TT AAA TT A TTTTGCCA TTCTT AAAGGT

GTTTTTTGA TTTTCAA TTTTTTTGCTTTTGCTGAAAAAGTT AAAAGTT

AGA ATGGT TA ATGGGT TCCATAGTACGCAGATAT T T TCGCTCCAT TGG

TGA A A A ATAT T TCCGA AT T T TAT TCGTATCCT TGA A ATATA AT T TCGTATG

ATGGAT TAT TGACGCTAT TGCAT T T TGT TGTACGTCAT T TGCGTA ATCT T

T TGACGGT T TCT TATACGT TA A A ACAT TCTA ATA A AGTCA AT T T TACTA A

ACAA T AAAAAAAGGT ACGAAA TTTTTTTTT ACA TTTT AA TTT A TT ACTG

T T T T TGCTCCT T TCTA ACA AGT T TATATAGTCA ATCACCATGGA ATA A

CAACAATGATTTTGGCGAGTTAGATTGTGAACTTCATACATAATTAACT

TGGTTTACATTTAATAGGAAAGTTGGGCTACTCTTTGAACAACATTCAA

CAAACTTTTCCTCTCAATTTGTGAATGCATAGCAAAATGCAATTGAAA

A ATGT T T TCA A ATCCA A ACACTAT T T TCCGT TGTATACT T TA ATA A AGA

TCTCACACATAT T TCCCTAGCATGA AGCACTAT TAT TA A ATA ACCA ACA

CTGAGTGT TATA A AGA ACTA ATA ATACGTAT T TCA ACGATGT T TA A ATATCTCACACATAT T TCCCTAGCATGA AGCACTAT TAT TA A ATA ACCA ACA

TATTTTTTTCAGTTGGTTCGGAAAAATTTGG

TTGGCTACTCTTTGAACAA.

Generation of MsR1 TGEM-Gal4 mutant/drive line. The MsTGEM-Gal4 mutant was made by inserting a Trojan Gal4 Expression Module (TGEM, ref. 27) into a PAM site (GTAATTGATAATCTATTGAG) within intron 3 of the Ms gene using CRISPR–Cas9. To make the TGEM construct, homologous arms of 700 bp were flanked by the Cas9 cleavage sites were synthesized by Integrated DNA Technologies and were homologous arms of approximately 700 bp flanking the Cas9 cleavage sites were subcloned into the pTGEM/I vector (Addgene #49411) for double strand breaks by two different gRNA's (gRNA1: 5′-GAGTCGGCAGAGGTCCGCGG-3′ and gRNA2: 5′-GAGTCGGCAGAGGTCCGCGG-3′). Similar to MsI, a two-breaks approach was used to minimize off-target breaks, and the pCFD4 plasmid (Addgene #4293) was used for gRNA expression. Homology arms flanking the Ms9 cut sites were subcloned into the pTGEM/I (Addgene #62893) plasmid. Both vectors were injected into yw; nos Cas9 (II-attP40) flies by BestGene.

5′-Homology arm:

TTTCCGTTGTATACTTTAATAAAGACAAACTTTTCCTCTCAATTTGTGA

Expression. Homology arms flanking the Ms9 cut sites were subcloned into the pTGEM/I vector (Addgene #49411) for double strand breaks by two different gRNA's (gRNA1: 5′-GAGTCGGCAGAGGTCCGCGG-3′ and gRNA2: 5′-GAGTCGGCAGAGGTCCGCGG-3′). Similar to MsI, a two-breaks approach was used to minimize off-target breaks, and the pCFD4 plasmid (Addgene #4293) was used for gRNA expression. Homology arms flanking the Ms9 cut sites were subcloned into the pTGEM/I (Addgene #62893) plasmid. Both vectors were injected into yw; nos Cas9 (II-attP40) flies by BestGene.

Generation of MsR1 TGEM-Gal4 mutant/drive line. The MsTGEM-Gal4 mutant was made by inserting a Trojan Gal4 Expression Module (TGEM, ref. 27) into a PAM site (GTAATTGATAATCTATTGAG) within intron 3 of the Ms gene using CRISPR–Cas9. To make the TGEM construct, homologous arms of 700 bp were flanked by the Cas9 cleavage sites were synthesized by Integrated DNA Technologies and were homologous arms of approximately 700 bp flanking the Cas9 cleavage sites were subcloned into the pTGEM/I vector (Addgene #49411) for double strand breaks by two different gRNA's (gRNA1: 5′-GAGTCGGCAGAGGTCCGCGG-3′ and gRNA2: 5′-GAGTCGGCAGAGGTCCGCGG-3′). Similar to MsI, a two-breaks approach was used to minimize off-target breaks, and the pCFD4 plasmid (Addgene #4293) was used for gRNA expression. Homology arms flanking the Ms9 cut sites were subcloned into the pTGEM/I (Addgene #62893) plasmid. Both vectors were injected into yw; nos Cas9 (II-attP40) flies by BestGene.

5′-Homology arm:

TTTCCGTTGTATACTTTAATAAAGACAAACTTTTCCTCTCAATTTGTGA

Generation of MsR1 TGEM-Gal4 mutant/drive line. The MsTGEM-Gal4 mutant was made by inserting a Trojan Gal4 Expression Module (TGEM, ref. 27) into a PAM site (GTAATTGATAATCTATTGAG) within intron 3 of the Ms gene using CRISPR–Cas9. To make the TGEM construct, homologous arms of 700 bp were flanked by the Cas9 cleavage sites were synthesized by Integrated DNA Technologies and were homologous arms of approximately 700 bp flanking the Cas9 cleavage sites were subcloned into the pTGEM/I vector (Addgene #49411) for double strand breaks by two different gRNA's (gRNA1: 5′-GAGTCGGCAGAGGTCCGCGG-3′ and gRNA2: 5′-GAGTCGGCAGAGGTCCGCGG-3′). Similar to MsI, a two-breaks approach was used to minimize off-target breaks, and the pCFD4 plasmid (Addgene #4293) was used for gRNA expression. Homology arms flanking the Ms9 cut sites were subcloned into the pTGEM/I (Addgene #62893) plasmid. Both vectors were injected into yw; nos Cas9 (II-attP40) flies by BestGene.

5′-Homology arm:

TTTCCGTTGTATACTTTAATAAAGACAAACTTTTCCTCTCAATTTGTGA

Expression. Homology arms flanking the Ms9 cut sites were subcloned into the pTGEM/I vector (Addgene #49411) for double strand breaks by two different gRNA's (gRNA1: 5′-GAGTCGGCAGAGGTCCGCGG-3′ and gRNA2: 5′-GAGTCGGCAGAGGTCCGCGG-3′). Similar to MsI, a two-breaks approach was used to minimize off-target breaks, and the pCFD4 plasmid (Addgene #4293) was used for gRNA expression. Homology arms flanking the Ms9 cut sites were subcloned into the pTGEM/I (Addgene #62893) plasmid. Both vectors were injected into yw; nos Cas9 (II-attP40) flies by BestGene.

5′-Homology arm:

TTTCCGTTGTATACTTTAATAAAGACAAACTTTTCCTCTCAATTTGTGA

Expression. Homology arms flanking the Ms9 cut sites were subcloned into the pTGEM/I vector (Addgene #49411) for double strand breaks by two different gRNA's (gRNA1: 5′-GAGTCGGCAGAGGTCCGCGG-3′ and gRNA2: 5′-GAGTCGGCAGAGGTCCGCGG-3′). Similar to MsI, a two-breaks approach was used to minimize off-target breaks, and the pCFD4 plasmid (Addgene #4293) was used for gRNA expression. Homology arms flanking the Ms9 cut sites were subcloned into the pTGEM/I (Addgene #62893) plasmid. Both vectors were injected into yw; nos Cas9 (II-attP40) flies by BestGene.
Higher flow rates require larger negative pressures in the crop, while higher conductivities mean the same flow can be achieved with smaller negative crop pressure. We measured the dimensions of the oesophagus and crop duct from microscopy images to estimate their conductivities, and the sip duration (0.13 s) and intake per sip (1.05 nl) were taken from ref. 40 to estimate dV/dt of the crop in mated flies. We calculate that the intake per sip for virgin females is lower by a factor of 0.6 compared to mated females, based on our own quantifications of sip number and total food intake (see Extended Data Fig. 10b). The crop pressure required to achieve the measured flow rate from −0.5 kPa to −1 kPa, which is comparable to the −0.5 kPa to −1 kPa measured in cockroach crops38, 39, suggesting that crop suction is a plausible physiological mechanism to drive food intake.

Immunohistochemistry and tissue stainings

After dissection, the central and enteric nervous systems and gut-associated secretory glands, all attached to intact intestinal tissues, were fixed at room temperature for 45 min in PBS, 4% paraformaldehyde. All subsequent washes were done in PBS, 4% horse serum, 0.3% Triton X-100 at room temperature following standard protocols. Primary antibody incubations were carried out at 4 °C overnight, whereas secondary antibody incubations were carried out at room temperature for 2 h.

The following primary antibodies were used: rabbit anti-Akh (ref. 27, 1/200), rabbit anti-Burs (ref. 30, 1/200), rat anti-Elav (DSHB, DDA2.7 1/10), goat anti-GFP (Abcam, ab5450 1/200), rabbit anti-Burs (ref. 37, 1/500), rabbit anti-Ms (ref. 29, 1/1,000), mouse anti-Pro (DSHB, MRIA 1/25).

Fluorescent secondary antibodies (FITC-, Cy3- and Cy5-conjugated) were obtained from Jackson ImmunoResearch and used at 1/200. Vectashield with DAPI (Vector Labs) was used to stain DNA. Phalloidin stainings were performed after immunohistochemistry using mussel phallolidin AlexFluor 647 probe (Life Technologies A22287, 1/200 for 45 min).

Custom-made fluorescence in situ hybridization probes were outsourced to either Stellaris RNA FISH (for Ms transcript) or Advanced Cell Diagnostics RNAscope (for MsR and Ms transcripts). Dissection tools and surfaces were treated with RNaseZAP for single RNA in situ stainings, which were generally conducted according to the standard manufacturer’s protocol after tissue dissection. For Stellaris probes, dissected samples were dehydrated in 70% EtOH overnight at 4 °C. The probes were applied in the hybridization buffer according to the manufacturers’ instructions, followed by a 4-h incubation at 45 °C. Subsequent washes were also performed at 45 °C before mounting in Vectashield. For RNAscope a negative control probe was provided, targeted against the bacterial gene dapB.

For Burs stainings, flies were pre-starved for 22 h before dissection and immunostaining to maximize retention of otherwise circulating Burs peptide in enteroendocrine cells37.

Crop and intestinal transit measurements and assays

Crop size and fullness as well as transit of dye-laced food along the alimentary canal were assessed in response to certain diets, internal states and/or genetic manipulations. Virgin flies of both sexes were collected and aged for either 4 or 7 days when raised at 25 °C or 18 °C respectively (tipped over to fresh food every 2 or 3 days respectively). Each group of flies was then either mated for 24 h or kept as a virgin control group. After mating, flies were either starved overnight (14–16 h) or kept feeding ad libitum on standard food. The next morning at 11:00 flies were gently transferred to tubes containing FCF Blue food by a single quick tap and allowed to feed ad libitum for 20 min if previously starved, or
1–2 h otherwise (see section ‘Temperature-controlled experiments’). After feeding, flies were transferred by a single quick tap to a fresh empty fly-food vial and euthanized by snap freezing them in liquid nitrogen. Frozen tissues were either used for dissection directly or kept at −80 °C for analysis at a later stage. Tissues were never thawed and re-frozen. Experimental and control flies were all raised and assayed in the same batch of food for each experiment. For temperature-sensitive experiments we devised a simple home-made solution for temperature control that enables real-time monitoring of feeding behaviour. We named this the ‘sand incubator’. This comprised an empty metallic tray for fly vials filled with sand used for reptile terrariums (Exo Terra Heatwave Desert Heat Mat, 25 × 43 cm, Large). The temperature of the mat was controlled by a thermostat (HabiStat Digital Temperature Thermostat + Timer). Fly vials were immersed in the sand for temperature control and remained available for undisturbed assaying of feeding behaviour. Tissues were dissected in 1.5× PBS (to avoid dye leaching out of the gut through small holes poked during dissection) and were either manually scored for crop size and food location, or transferred to a slide for bright-field imaging immediately after dissection.

CROP SIZE AND ENLARGEMENT QUANTIFICATIONS. Crop area and roundness measurements were conducted on segmented crops using the Fiji image analysis software.85 For crop area, we used either the ‘Polygon’ or the ‘Wand’ tracing tools, using the ‘Default’ method in ‘Threshold Colour’ to generate a binary mask that segmented blue-stained crops against a white background. Roundness corresponds to 4π.area/(π.major_axis^2), or the inverse of the aspect ratio.

For crop shape analysis, 2 landmarks and 20 semi-landmarks were annotated for each crop using the ‘multipoint tool’ in the Fiji image analysis software.85 Fixed landmarks were assigned to the base of the crop, where it meets the crop duct, and to a point diametrically opposed to this on the crop margin and along the axis of symmetry. 10 semi-landmarks were placed between each fixed landmark and allowed to slide between the immediate two neighbouring landmarks. Landmark coordinates were subjected to a generalized procrustes analysis (GPA) to standardize for size, position and orientation, assuming bilateral symmetry. We analysed variation in crop shape using principal component analysis (PCA) of the GPA aligned configurations of crop shapes and visualized these differences using thin plate spline (TPS) deformation grids. All morphometric analysis was performed using the ‘geomorph’ R package.

For a small subset of experiments (typically those that were confirmatory or negative), crop size was assessed only qualitatively; crop size was ranked as one of four categories: small (S), medium (M), large (L) and very large (VL).

IN VIVO CROP ENLARGEMENT ASSAYS. For live imaging of crop enlargement, virgin flies were collected and aged for 5 days at 25 °C and then either mated for 24 h or kept as virgin. Flies were then starved for 2–3 h before being briefly anesthetized on ice (2–5 mins) and mounted between two coverslips using a modified version of the Bellymount protocol in which the flies were positioned over the edge of the coverslip to enable access to mouthparts for feeding. Mounting enabled crop and some loops of the midgut to be visible through the ventral surface of the abdomen. Flies were positioned with ventral side up and imaged on a Leica MZ165 FC attached to an S-View SXI-130 camera. Flies were fed with liquid food containing Brilliant Blue FCF (2 g Brilliant Blue FCF, 10 g sucrose, 10 g yeast extract, 200 ml H2O) using a narrow capillary for 3–5 mins and then were imaged for a further 10 mins. Time taken from first sip, to food visible in the crop, to food visible in the midgut was calculated.

FOOD INTAKE AND FEEDING BEHAVIOUR ASSAYS. FlyPAD. FlyPAD assays were performed as described in ref. 39. Half of the wells of a given flyPAD arena were filled with 2.4 μl of food (5% yeast, 7% dextrose in 1% agar), and the other half were either loaded with an agar control (1% agar) or left empty. For all experiments, flies were individually transferred to flyPAD arenas by mouth aspiration and allowed to feed for 1 h at 25 °C or 29 °C and 65% relative humidity. The total number of sips per fly over this hour was acquired using the Bonsai framework, and analysed in MATLAB using previously described custom-written software.88 Non-eating flies (defined as having fewer than two activity bouts during the assay) were excluded from the analysis. All flyPAD experiments were performed at the same time of day between 11:00 and 13:00. Data for experimental and control genotypes used for comparison were always acquired in the same flyPAD assay. See Supplementary Information for additional information about numbers of flies and experimental conditions.

Blue dye-based assays. Quantification of ingested food was carried out using diets containing 1% FCF blue. Flies were allowed to feed (for up to 20 min if pre-starved, and for up to 2 h if previously fed ad libitum) and were then transferred by a single quick tap to a fresh empty fly-food vial for snap freezing in liquid nitrogen. Frozen flies were transferred in groups of three to a clean 2 ml PCR tube (Eppendorf, 22431048) with 0.5 ml of water and a 5 mm stainless-steel metal bead (QIAGEN, 96989). Fly tissues were homogenized using a QIAGEN TissueLyser II for 90 s at 30 Hz. The samples were centrifuged at 10.000 g for 5–10 min. Then, 0.2 ml of the supernatant per fly was directly transferred into individual wells of a 96-well, flat bottom, optically clear plate (Thermo Fisher Sterilin, 61H196). A BMG Labtech FLUOstar Omega plate reader was used to measure dye content by reading the absorbance at 629 nm. We used a standard curve of pure FCF blue dye to calculate the dye content ingested per fly.

FERTILITY AND FECUNDITY ASSAYS. Virgin females were raised and aged for 7 days at 18 °C, and then transferred to 29 °C for the experiment. A group of 40 female flies of each of the three genotypes was used and crossed to 25 or 1 male flies. The assays were performed in fly cages on apple juice plates with a smear of live yeast. The number of eggs laid per 24-h window was manually counted using a hand-held counter device. To assess egg viability, 200 freshly laid eggs (laid over a 6-h window) were collected for each genotype with a hook, split into 10 fresh food vials in groups of 20, and kept at 25 °C until eclosion. The number of adults from each tube was scored.

IMAGING. Bright-field imaging. Dissected crops and intestines were imaged using either a Leica MZ16f stereomicroscope attached to a DFC420 camera, or a Leica MZ165 FC attached to an S-View SXI-130 camera.

Confocal imaging. A Leica SP5 confocal microscope was used to generate all confocal images. The images were acquired using both Leica HyD Photon counters as well as standard photomultiplier tubes (PMTs) tailored for the fluorophores of each sample accordingly. For flybow clones we used the built-in Leica channel unmixing algorithm post-imaging.

Quantifications of Ms neuron crop axonal terminals. The number of branches in crop terminals and their diameters were analysed using the NeuronStudio software.

IN VIVO CALCIUM IMAGING. Ms-Gal4 flies were crossed to UAS-GCaMP6f (attP40) to drive the expression of the calcium reporter in Ms neurons. Virgin female flies from the progeny were collected and aged for 4–5 days. Flies were then either mated or kept virgin and used for imaging experiments. Flies were briefly anesthetized (5 s) on ice and one fly was picked and glued for surgery. The proboscis was also glued to the thorax to limit motion artefacts during image acquisition. Surgery was performed to open the cuticle and obtain optical access to...
the brain as described previously. During surgery and subsequent recordings, the aperture on the top of the fly head was bathed in an artificial haemolymph-like solution (130 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 36 mM sucrose, 5 mM HEPES-NaOH; pH 7.3: 305 mM). Confocal imaging was performed under a scanning confocal microscope (Olympus, BX61W1), using a water-immersion 20× objective (XLUMPlanFL, NA 1.0) and an excitation laser at 470 nm. The laser intensity was adjusted for each sample, but on average the laser power was similar between the two conditions (mated and virgin). Fluorescence recordings were performed at a rate of one image every 427 ms in a single plane. To collect from the maximum number of cells, multiple planes were recorded consecutively in some samples.

Image analysis was performed offline with a graphical user interface, custom-programmed with MATLAB. Regions of interest (ROI) were delimited by hand and surrounding individual cell bodies of GaMP6-expressing cells. Cells were classified as big or small on the basis of expert knowledge of PIMs neuronal anatomy (by D.H.). After background subtraction, the absolute level of the 8-bit encoded fluorescence was calculated for each ROI as the mean over a time period selected for showing minimal fluctuations. Amplitude oscillation measurements were conducted as described in ref. 98.

**Cell number quantifications, statistics and data presentation**

For each experiment, a minimum of 10 samples per group were examined per genotype or condition. Experimental and control flies were bred in identical conditions, and were randomized whenever possible (for example, with regard to housing and position in tray). Control and experimental samples were dissected and processed at the same time and on the same slides, or assessed behaviourally simultaneously. All replicates were biological rather than technical and all measurements were taken from distinct samples. Experiments were typically repeated 3 times and only those experiments for which repeats resulted in comparable outcomes are included in the manuscript. Specific details of the number of experimental repeats for each experiment are provided in the Supplementary Information. Experiments were controlled for sex, mating status, genotype and physiological state (for example, starved or ad libitum-fed). Details are provided in the Supplementary Information. No data points or outliers were excluded from our experiments and blinding was performed for a subset of experiments. Fly numbers are not limiting so no power calculations were used to pre-determine sample size.

Quantifications of fluorescence signals in the brains of virgin and mated females and males stained for the anti-Ms antibody were performed using Fiji measurements and the corrected total cell fluorescence (CTCF) metric. The brain samples used for these measurements were from flies that were raised on the same food batch, were dissected at the same time and stained on the same slide. They were then imaged applying the same imaging parameters.

For counts of Ms-positive, GaLeA-activated cells, flies were dissected and stained 22 h after mating along with virgin controls. These flies were raised on the same food batch, dissected at the same time and stained for Ms on the same slide. The same imaging parameters were applied to both groups and Ms- and GFP-positive cells were manually counted upon inspection of the entire brain.

Cell counts of enterendocrine cells in the intestines of mated and virgin flies were performed using the Malpighian tubules at the level of the hindgut as a posterior-most landmark, imaging the entire field of view within 20× or 63× magnification. The entire Z stack was used when manually counting cells using the Cell Counter plugin in Fiji.

All statistical analyses were carried out in GraphPad Prism 7.04. Statistical tests were typically two-sided. Comparisons between genotypes or conditions were analysed using Kruskal–Wallis and Mann–Whitney Wilcoxon tests for multiple or pairwise comparisons, respectively, conservatively assuming that data distributions were not parametric (as is often the case for our data outputs). For egg-laying experiments, a two-way ANOVA followed by a Tukey’s multiple comparison test was used, considering day and genotype as independent factors. All graphs were generated using GraphPad Prism 7.04. Ranked crop values are displayed as percentages. All confocal and bright-field images belonging to the same experiment and displayed together in our figures were acquired using the exact same settings. For visualization purposes, level and channel adjustments were applied using ImageJ to the confocal images shown in the figure panels (the same correction was applied to all images belonging to the same experiment), but all quantitative analyses were carried out on unadjusted raw images or maximum projections. In all experiments, n denotes the number of samples assayed and analysed for each genotype/condition (see Supplementary Information for full details). Data are presented as box plots with all data points shown and the median (line) and minimum and maximum values (whiskers) plotted. Boxes encompass the 25th to 75th percentiles as calculated by GraphPad Prism 7.04. P values are indicated as asterisks highlighting the significance of comparisons: *P < 0.05; **P < 0.01; ***P < 0.001.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request. Source data are provided with this paper.

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Correspondence and requests for materials should be addressed to I.M.-A.

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Crop / anterior midgut innervation: enteric neuronal subsets

Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Innervation of the anterior portion of the adult Drosophila intestine.  

**a**, Schematic summary of the innervation of the anterior portion of the adult fly intestine, encompassing foregut, crop and anterior midgut. **b**, Pan-neuronal nSyb-Gal4 driver expression visualized with EGFP (from UAS-FB1.1 reporter) in green. Gut muscles are highlighted in blue with phalloidin staining. In all subsequent panels, driver expression is in green and phalloidin staining in blue. Abbreviations are as per **a, c, Cell number quantifications of the enteric nervous system (ENS) ganglia and secretory glands associated with the adult anterior midgut.** **d - d″**, Direct innervation of the crop by neurons located in the central nervous system. **d**, Projections emanating from the insulin-producing neurons in the PI (labelled with Ilp2-3-Gal4-driven expression of UAS-FB1.1-derived EGFP in green) innervate the crop and anterior midgut. Neuronal nuclei are labelled with anti-Elav antibody in red, and gut muscles are labelled in blue with phalloidin. **d″**, The axonal projections of these insulinergic neurons are visualized using immunostaining for Ilp2 peptide in red. **e - e″**, Innervation of the crop by peripheral neurons. Taste receptor-expressing neurons visualized with the Gr43a-Gal4 driver; gut muscles are labelled with phalloidin. The boxed area in **e** highlights the cell bodies of ENS-like sensory neurons located in the HCG. **e″** shows their projections on the crop muscle lobes (arrow). In **d - e″**, arrowheads point to the paired nerves that innervate the crop. **f - j**, Spatially restricted Gal4 drivers or antibodies reveal distinct crop-innervating neuronal subsets. In all panels, Gal4 expression is visualized with EGFP (from UAS-FB1.1 reporter) in green, and gut muscles are highlighted in blue with phalloidin staining. **f, Dh44-Gal4** expression. Dh44-Gal4-positive cell bodies in the PI (top dashed box) project to the HCG (bottom dashed boxed) and crop through the crop nervi. They also innervate the anterior midgut. No Dh44-Gal4-positive cell bodies are apparent in the HCG. DAPI labels the nuclei of the brain–gut axis in cyan. **g, Mip-Gal4**-positive cell bodies are found in both the PI and HCG (dashed boxes). They extend projections to the anterior midgut, and along the crop nervi towards the crop. **h**, Glucagon-like adipokinetic hormone Akh (labelled with an anti-Akh antibody in red) is produced by cell bodies located in the paired corpoca cardiaca (CC) glands and is apparent in their projections along the crop nervi up to the junction between crop duct and lobes. **i**, Expression of a pain-Gal4 reporter for painless (coding for a TRPA channel that detects noxious heat and mechanical stimuli) in a subset of ENS neurons in the HCG (dashed box), pointing to their possible mechanosensory identity. **j**, Expression of a Gr28a-Gal4 reporter for Gustatory receptor 28a in two HCG cell bodies (dashed box), suggestive of chemosensory identity. Their neurites populate the anterior midgut and their putative axons project along the recurrent nerve (RN). **k**, The Aug21-Gal4 reporter reveals short local projections from the corpus allatum around the foregut and anterior midgut. **l, m**, The use of Hox gene reporters allows labelling of large population central neurons in thoracico-abdominal ganglion segments. No neurons in the Ubx-Gal4 (**l**) or abdA-Gal4 (**m**) expression domains contribute to the innervation of the crop of anterior midgut. Gal4 expression is visualized with EGFP (from UAS-FB1.1 reporter) in green, and gut muscles are highlighted in blue with phalloidin staining. Neuronal nuclei are visualized in red with anti-Elav (SG = salivary gland). Scale bars, 50 μm. See Supplementary Information for a list of full genotypes, sample sizes and conditions.
Extended Data Fig. 2 | Intestinal transit dynamics and dietary regulation of crop enlargement. a. Schematic summarizing ad libitum and starvation–re-feeding assays using dye-laced food. b–b‴. Transit of dye-laced food at specific time points after ingestion. b, Gut dissected 10 s after feeding initiation; food is apparent in the crop duct and begins to enter the crop. b′, Gut dissected 40 s after feeding initiation; food fills the crop duct, crop, and begins to enter the midgut. b″, Gut dissected 2 min after feeding initiation; food fills the crop duct and midgut. b‴, Gut dissected 40 min after feeding initiation; food fills the crop, crop duct, midgut and has now reached the hindgut and rectal ampulla. All panels show dissected adult fly intestines, anterior (left) posterior (right). c, c′. Frequency histograms derived from in vivo food ingestion videos (see Supplementary Video 1 for a representative example) showing a larger number of flies with faster transit times of food to the crop (c) compared to midgut (c′). d. Quantification of crop area revealed that re-feeding after starvation results in larger crops than ad libitum feeding. e–e‴. Representative dissected guts of a starved fly (e, 16 h starvation on 1% agar), starved–refed fly (e′, 16 h starvation on 1% agar, refeed for 20 min on dye-laced standard food), ad libitum-fed fly (e″, fed on dye-laced standard food for 2 h). f. Ability of different food sources to elicit crop enlargement. These are categorized as palatable (P) and/or nutritious (N) using filled boxes if true and empty boxes if false (see Methods for further details of the different diets). In this and all subsequent ranked data panels, crop size was ranked as one of four categories: small (S), medium (M), large (L) and very large (VL). Graphs are colour-coded from light to dark shades of red corresponding to increasing size of the crop. Data are displayed as percentages. Scale bars, 500 μm. See Supplementary Information for a list of full genotypes, sample sizes and conditions. In all box plots, line: median; box: 75th–25th percentiles; whiskers: minimum and maximum. All data points are shown. *P < 0.05; **P < 0.01; ***P < 0.001.
**Extended Data Fig. 3** | See next page for caption.
Extended Data Fig. 3 | Characterization of Ms expression. a, Schematic depicting Ms neuronal subtypes. Dashed boxes highlight the main sites of Ms expression: around 30 neuronal cell bodies in the PI, around 5 enteric neurons located in the HCG and neuronal projections in the HCG and on the crop muscles. b, c, Single-cell Flybow clones of Ms-Gal4-expressing neurons (in red); gut muscle labelled with phalloidin (in blue). b, The PI and HCG where the Ms cell bodies reside are boxed. No Ms neurons have been labelled in the PI, but a single-cell, mCitrine-positive clone (in red) reveals an HCG neuron that innervates the crop muscle. Inset shows a single-cell clone of a second type of HCG Ms-Gal4-expressing neuron that only extends local projections. c, Single-cell Flybow clone of a large PI Ms-Gal4-expressing neuron. The main projection bifurcates, with one shorter (putatively dendritic) branch projecting towards the suboesophageal zone (SEZ) (empty arrows), and a longer (axonal) branch projecting towards the midgut/crop (arrows). d, d′. Co-expression of the dendritic marker DenMark (in red) and membrane marker Venus shown (in green) from Ms-Gal4 reveals relative DenMark enrichment in their SEZ projections. d, consistent with dendritic nature. Venus enrichment is apparent in the crop nerve (d′), consistent with its axonal identity. Top left arrow points to the crop nerve, and bottom arrow points to where it terminates. e, Quantification of fluorescence for DenMark and Venus in SEZ (top) crop nerve (bottom) projections. f–j″, Ms-Gal4 expression, visualized by EGFP from the UAS-FB1.1 reporter (in green). f, Overview of Ms-Gal4-positive intestinal innervation; Ms-positive neurites are apparent on the crop, anterior midgut and posterior hindgut (rectal ampulla). Neuronal nuclei are stained with an anti-Elav antibody in red, and gut muscles are labelled in blue with phalloidin. g, Ms-Gal4 expression in heart-innervating neurons; heart muscles are labelled in blue with phalloidin. h, Ms-Gal4 expression in peripheral neurons that innervate the ovaries, oviduct and spermatheca (SP). i–i″, Ms-Gal4 and Ms peptide (in red) in a cluster of PI neurons; arrows and arrowheads point to big and small PI Ms neuron subtypes, respectively. j and f′ show single-channel images for Ms-Gal4 and anti-Ms antibody, respectively. The merged image is shown in f″. j–j″, Co-expression of Ms-Gal4 and Ms transcript (visualized using single-molecule RNA fluorescence in situ hybridization in red) in the same cluster of PI neurons. j and f′ show single-channel images for Ms-Gal4 and Ms transcript, respectively. The merged image is shown in j″. k–k″, Ms protein reporter expression (in green). Ms peptide is in red and gut muscles are labelled with phalloidin in blue. k, Co-expression between the Ms protein reporter Ms peptide in the nervous system, and in neuronal projections towards the gut. Ms and the Ms protein reporter are co-expressed by the PI Ms neurons (boxed and inset). k′, The Ms protein reporter also labels axonal projections innervating the crop muscles. l–q″, Expression (or lack thereof) of neuropeptides and other markers in the Ms-expressing neurons in the PI or HCG. For each letter, the first panel shows double staining, the second and third panels show single channels for clarity. l–l″, Ms-Gal4-expressing neuron. The main projection is denoted by arrows. m–m″, PI Ms neurons do not co-express Dsh44-Gal4, used as a marker of insulino-production neurons. n–n″, PI Ms neurons do not co-express Dsh44-Gal4, used as a marker of Diuretic Hormone 44-producing neurons. o–o″, PI Ms neurons do not co-express Mip-Gal4, used as a marker of Myoinhibiting peptide precursor-producing neurons. p–p″, Ms-Gal4 expression in three out of the five HCG Ms-expressing neurons. Phalloidin was used to label gut muscles (in blue). p–p″, A subset of PI Ms neurons co-express Taotie-Gal4; other Taotie-Gal4-positive PI neurons are Ms-negative. In the HCG, Taotie-Gal4 expression is only apparent inconsistently in one Ms neuron (data not shown). q–q″, PI Ms neurons do not co-express Dsk-Gal4, used as a marker of Drosulfakinin-producing neurons. Scale bars: b, d, f, h and k–k″ = 50 μm; i–j″, l–o″ and q–q″ = 25 μm, b (inset), c, d, p–p″ = 20 μm and k (inset) = 10 μm. See Supplementary Information for a list of full genotypes, sample sizes and conditions.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Ms neuron regulation of crop enlargement.

**a–a’** Validation of Ms Δ mutant using anti-Ms staining shown in green; PI is highlighted by dashed lines. **a**. Lack of Ms staining in the PI of Ms mutants (MsΔ/Df(3R)Exel6199(a’)) and MsΔ(a’’) heterozygous control flies. **b**. Quantifications of crop area in ad libitum-fed flies upon Ms-Gal4-driven TrpA1 expression (4 h at the permissive temperature), showing these have significantly larger crops relative to UAS and Gal4 controls. **c**. Quantifications of crop area in starved-refed flies upon Ms-Gal4-driven Kir2.1 expression (temporally confined with tub-Gal80ts), showing that these have significantly smaller crops relative to UAS and Gal4 controls. **d–e”**, Effect of neuronal activation and Ms downregulation on Ms levels in PI neurons. Thermogenic activation of Ms neurons in ad libitum fed flies depletes Ms peptide (in red) from Ms neuron cell bodies in the PI (d) compared to UAS (d’) and Gal4 (d”) controls. Adult-specific Ms downregulation in Ms neurons of starved/refed flies results in reduced Ms staining (red) in PI neurons (e), compared to UAS (e’) and Gal4 (e”) controls. **f–i**. Effect of Ms loss-of-function and adult-specific Ms neuron inactivation on crop expansion and shape, upon starvation-refeeding in mated females. **f**. Quantifications of crop area reveal that Ms neuron inactivation results in smaller crops relative to Ms mutant or w1118, UAS and Gal4 controls. **g**. Representative crop images of genotypes quantified in f. **h**. Quantifications of crop roundness reveal that crops are more round upon Ms neuron inactivation or in Ms mutant compared to w1118, UAS and Gal4 controls. **i**. PCA of landmark position variation along the crop outline, showing that crop shapes are distinct between Ms mutant (red), Ms neuron inactivation (yellow) and w1118 (grey), being more similar between Ms mutant and w1118, as highlighted by partial overlap of their 95% confidence ellipses. Wireframe deformation grids are shown to illustrate the minimum and maximum shape deviations as compared to the mean shape along each PC axis. **j, k**. Effect of Ms neuron activation on crop expansion in Ms mutant background, upon starvation-refeeding in mated females. **j**. Quantifications of crop area show that activation of Ms neurons by Ms-Gal4-driven TrpA1 expression results in larger crops relative to activation of Ms neurons by MsTGEM, driven TrpA1 expression in an heteroallelic mutant background, as well as relative to Ms mutant or UAS and Gal4 controls. **k**. Representative crop images of flies with the genotypes quantified in j. **l–m**, Effect of Ms and Taotie neuron activation on crop enlargement, upon starvation in mated females. **l**. Quantification of crop area shows that activation of either Ms neurons or Taotie neurons results in larger crops compared to respective Gal4 controls and UAS control, even in the absence of food. **m**. Representative crop images of genotypes quantified in l. Scale bars: **a–a’** = 10 μm, **d–e”** = 25 μm, **g, k, m** = 500 μm. See Supplementary Information for a list of full genotypes, sample sizes and conditions. In all box plots, line: median; box: 75th–25th percentiles; whiskers: minimum and maximum. All data points are shown. *P < 0.05; **P < 0.01; ***P < 0.001.
**Extended Data Fig. 5** See next page for caption.
Extended Data Fig. 5 | Expression of Ms receptors and their regulation of crop enlargement. a, FB1.1-derived EGFP reveals MsR1 expression in the crop muscles and nervous system, including nerves innervating the crop, hindgut and rectal ampulla. In this and subsequent panels, muscles are labelled with phalloidin (in blue). b–b″, Co-expression of MsR1 mRNA stained with single-molecule RNA fluorescence in situ hybridization (b, b″, in red) and FB1.1-derived EGFP driven by MsR1GΩ/Gal4 (b, b″, in green) is observed in crop muscles. Muscle nuclei are shown in blue with DAPI; single channels are shown for clarity. c, Detail of the HCG and corpora cardiaca (CC); the latter is extensively innervated by MsR1-expressing neuronal projections. d, FB1.1-derived EGFP reveals MsR1 expression in neurons innervating the female reproductive system, but not in its muscles. e, FB1.1-derived EGFP reveals MsR1 expression in heart-innervating neurons, but not in heart muscles. f, Higher magnification image of the central brain; nuclear GFP reveals broad MsR1 expression in neurons including the PI Ms neurons shown with Ms staining (in red). g, A subset of 2–3 MsR1-positive neurons in the HCG co-express Ms. h, Nuclear GFP driven from MsR1GΩ/Gal4 reveals co-expression of MsR1 and Akh (in red) in CC cells. i, Single-molecule fluorescence in situ hybridization of MsR1 and MsR2 mRNAs in crop muscles; MsR1 (in green) is more readily detected than MsR2 (in red). Muscle cell nuclei are shown in blue by DAPI staining. The MsR1 expression described in a–h is consistent with transcriptomics data. j–j″, F′ and F″ show single MsR1 or MsR2 channels for clarity. j–j″, Validation of adult-specific MsR1 knockdown in visceral muscles (umGal4 > MsR1 KD). Panels show high-magnification images of crop muscles. MsR1 mRNA expression is visualized by single-molecule RNA fluorescence in situ hybridization (in green) in Gal4(F′) and UAS(F″) controls, but it is reduced or absent when MsR1 is downregulated from crop muscles (um-Gal4 > MsR1 KD) (j). k, Quantifications of crop area in starved–refed flies upon downregulation of MsR1 in visceral muscles, showing that crop size is significantly reduced upon MsR1 downregulation compared to UAS and Gal4 controls. l, A similar reduction in crop area is also quantified upon MsR1 downregulation specifically in crop muscles using a different driver line (MsR1GΩ > MsR1KD). MsR1GΩ-Gal4 is MsR1-Gal4, nsyb-Gal80, in which MsR1-Gal4 neuronal expression is prevented using the pan-neuronal nsyb-Gal80 driver, rendering it a crop muscle-specific driver. m–o″, Effect of crop-muscle-specific downregulation of MsR1 on crop size. m, Quantifications of crop area in starved–refed mated females show that crop-specific downregulation of MsR1 (MsR1GΩ > MsR1KD) results in reduced crop areas compared to Gal4 and UAS controls, similar to Ms neuron inactivation (Ms > kir2.1). n–o″, Representative crop phenotypes of flies with the genotypes quantified in m. p, Quantifications of crop area upon visceral muscle-specific MsR1 and MsR2 downregulation, showing that MsR1 downregulation, but not MsR2 downregulation, results in reduced crop sizes, as compared to UAS and Gal4 respective controls. q, Quantifications of crop area in starved–refed mated females show that heteroallelic MsR1GΩ/Dif62 mutants have reduced crop areas relative to w1118 or heterozygous controls. r, Representative crop images from genotypes quantified in q. s, Validation of MsR1 mutation and MsR1 fluorescence in situ hybridization signal specificity. MsR1 mRNA (green) is absent from the crop muscle cells of MsR1GΩ/w1118 mutants, and apparent in w1118 control flies. Scale bars: b–b″, f–j″ and s = 10 μm; a, c–e = 50 μm; r = 500 μm; n–o″ = 1 mm. See Supplementary Information for a list of full genotypes, sample sizes and conditions. In all box plots, line: median; box: 75th–25th percentiles; whiskers: minimum and maximum. All data points are shown. *P < 0.05; **P < 0.01; ***P < 0.001.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Post-mating modulation of Ms neurons. a, b, Analysis of Ms neuron crop terminals in virgin and mated females. Neither the number of axonal branches (a) nor their diameter (b) is significantly different between virgin and mated females. c. Quantifications of Ms staining levels in the cell bodies of PI neurons of wild-type, ad libitum-fed males, virgin females and mated females. Mated females have less Ms peptide than virgin females or males; virgin females have less peptide than males. d–h, Comparison of Ms peptide levels in the cell bodies of PI neurons in fed versus starved virgin and mated females. Representative images of Ms staining in the cell bodies of the PI neurons of fed virgin females (d), starved virgin females (e), fed mated females (f) and starved mated females (g). h, Quantification of Ms staining in the cell bodies of PI neurons shows that Ms levels are reduced in mated females compared to virgins, irrespective of fed or starved status. i, RT-qPCR expression data for Ms transcript levels in the brain of ad libitum-fed, control males (grey column), virgin females (pink column) and mated females (red column). No significant differences are apparent between groups. j–l, CaLexA-based assessment of mating-triggered changes in PI Ms neuronal activity, achieved by adult- and Ms-confined CaLexA expression (MsΔ > CaLexA). Representative images of ad libitum-fed, wild-type virgin (j, j′), and mated females (k, k′) are shown. Ms neurons are labelled with anti-Ms antibody (in red) and CaLexA channel is shown as a single channel (in green) for clarity. i. Quantification of CaLexA-derived GFP-positive cells in PI Ms neurons of virgin (pink box) and mated (red box) females shows that fewer cells are CaLexA-positive in virgin compared to mated females; each data point corresponds to a different brain. m, n, Quantification of baseline GCaMP fluorescence (corrected for background) (m) and amplitude of GCaMP fluorescence oscillations (n) in the cell bodies of PI Ms neurons of virgin females (pink box) or mated females (red box). Each data point corresponds to an individual cell measurement. Higher GCaMP signal and reduced oscillation amplitude are detected in mated females. o, Crop area quantifications in wild-type, ad libitum-fed males, virgin females and mated females. The crop of mated females is bigger than that of virgin females or males. p, q, Effects of sex and mating status on Ms signalling contribution to crop size. p, Quantification of crop area upon adult-specific downregulation of MsR1 in visceral muscles shows that it is significantly reduced in mated females but not in males or virgin females, as compared to respective controls. q, Representative crop images of genotypes quantified in m. Scale bars: d-g, j–k′ = 20 μm; q = 500 μm. See Supplementary Information for a list of full genotypes, sample sizes and conditions. In all box plots, line: median; box: 75th–25th percentiles; whiskers: minimum and maximum. All data points are shown. *P < 0.05; **P < 0.01; ***P < 0.001.
Extended Data Fig. 7 | Ecdysone modulation of Ms neurons and crop size. 

a–a', Expression of EcR in PI Ms neurons. Ms (antibody labelled in green) is co-expressed with EcR (labelled in red with an antibody that recognizes all EcR isoforms) (a). EcR staining is shown as a single channel below for clarity (in red) (a'). b–d. Ecdysone effect on Ms levels in PI neurons. Representative images show comparable Ms levels upon expression of EcR DN in virgin females (b) relative to UAS (b') and Gal4 (b'') controls. Fluorescence signals are pseudo-coloured: high to low intensity is displayed as warm (yellow) to cold (blue) colours. c. Quantification of Ms staining intensities in PI neurons of virgin females upon expression of EcR DN shows comparable levels to UAS and Gal4 controls. d. Quantification of Ms staining intensities in PI neurons of mated females upon expression of EcR DN shows increased Ms levels relative to UAS and Gal4 controls. e. Quantification of crop area in starved–refed mated females reveals smaller crops upon adult- and Ms neuron-specific EcR downregulation compared to UAS and Gal4 controls. f–j. Classification of crop size upon expression of EcR DN (f, g), EcR-BI downregulation (h, i) or EcR (all isoforms) downregulation (j) in starved–refed female flies. The distribution of crop sizes does not significantly differ from that of UAS and Gal4 controls in virgin females (f, h, j). In mated females, the distribution shifts towards smaller crop sizes, relative to UAS and Gal4 controls (g, i). Ranked data are displayed as percentages. Scale bars, 20 μm. See Supplementary Information for a list of full genotypes, sample sizes and conditions. In all box plots, line: median; box: 75th–25th percentiles; whiskers: minimum and maximum. All data points are shown. *P < 0.05; **P < 0.01; ***P < 0.001.
Regulation of Burs-positive EE cells

rk expression

rk / Burs modulation of Ms neurons

Regulation of Ms neuron activity by Bursicon and Ecdysone
Extended Data Fig. 8 | Bursicon modulation of Ms neurons. a, Co-expression of Burs (a, in red), Pros (a”, in white) and GFP driven by Tkg-Gal4 (a”, in green) in midgut enteroendocrine cells of mated females. b, Quantifications of Pros-positive midgut cells show increased enteroendocrine cell number in mated females relative to virgins. Flies were starved for 22 h to increase Burs staining in the enteroendocrine cell bodies. Single channels for each marker are shown for clarity. c, Quantifications of enteroendocrine cells of mated females labelled by Tkg-Gal4-driven EGFP and Burs staining (such as that shown in a). More Tkg-Gal4-positive than Burs-positive enteroendocrine cells are apparent. The majority of Burs-positive enteroendocrine cells are Tkg-Gal4-positive. d–e, Co-expression of rk TGEM (driving FB1.1, in green) and Ms peptide (in red) is shown in brain and VNC neurons (d), and in the HCG ganglion (e). f–f’, Co-expression of rk TGEM (driving FB1.1-derived EGFP, in green) and Ms peptide (in red) is apparent in brain PI neurons. f, Ms staining is shown as a single channel for clarity. f’, rk fluorescence in situ hybridization signal is shown as a single channel for clarity. g–h, Co-expression of Ms-Gal4 (driving FB1.1-derived EGFP, in green) and rk mRNA (stained with fluorescence in situ hybridization, in red) is apparent in brain PI neurons. g’, rk mRNA fluorescence in situ hybridization signal is shown as a single channel for clarity. h, Co-expression of Ms-Gal4 (driving FB1.1-derived EGFP, in green) and Ms peptide (in white) and EcR (in red) is apparent in brain PI neurons. Nuclei are stained with DAPI (in blue). i–j, Co-expression of Taotie-Gal4 (driving FB1.1-derived EGFP, in green) and rk mRNA (stained with fluorescence in situ hybridization, in red) is apparent in brain PI neurons. Nuclei are stained with DAPI (in white). j’, rk mRNA fluorescence in situ hybridization signal is shown as a single channel for clarity. k–m, rk regulation of Ms levels in PI neurons. Representative images show similar Ms staining signal upon adult-specific rk downregulation in virgin females (k) relative to UAS (k’) and Gal4 (k”) controls. Fluorescence signals are pseudo-coloured; high to low intensity is displayed as warm (yellow) to cold (blue) colours. l, Quantification of Ms staining intensities in PI neurons of virgin females upon adult-specific rk downregulation showed comparable levels to UAS and Gal4 controls. m, Quantification of Ms staining intensities in PI neurons of mated females upon adult-specific rk downregulation showed increased Ms levels relative to UAS and Gal4 controls. n, Quantification of the amplitude of GCaMP oscillations in PI neurons of mated females shows that downregulation of EcR and rk in Ms neurons significantly increases the amplitude of calcium signal. o, Quantification of GCaMP baseline fluorescence levels in PI neurons of mated females reveals that downregulation of EcR in Ms neurons significantly reduces GCaMP levels, whereas downregulation of rk increases GCaMP levels, both relative to expression of EGFP. Hence, calcium oscillations become virgin-like upon both EcR or rk downregulation, whereas their effects on overall calcium fluorescence are different. Scale bars = 20 μm apart from a–a”, d, e = 50 μm. See Supplementary Information for a list of full genotypes, sample sizes and conditions. In all box plots, line: median; box: 25th – 75th percentiles; whiskers: minimum and maximum. All data points are shown. *P < 0.05; **P < 0.01; ***P < 0.001.
**Extended Data Fig. 9** | See next page for caption.
Extended Data Fig. 9 | Post-mating modulation of crop enlargement by Burs and ecdysone. **a–a′**, Classification of crop size upon rk downregulation in Ms neurons of starved–refed female flies. The distribution of crop sizes does not significantly differ from that of UAS and Gal4 controls in virgin females (a). In mated females, the distribution shifts towards smaller crop sizes, relative to UAS and Gal4 controls (a′). Ranked data are displayed as percentages.

**b–e**, Effect of Burs downregulation from enteroendocrine cells on crop enlargement in virgin (b, d) and mated (c, e) females. Representative crop images of ad libitum-fed flies virgin females show that crop size is not visibly changed upon downregulation of Burs in Pros-expressing enteroendocrine cells (b) relative to UAS (b′) and Gal4 (b″) controls. By contrast, in mated females, crops are less expanded (c), relative to UAS (c′) and Gal4 (c″) controls. Quantifications of crop area of genotypes shown in b–b″ and c–c″ are shown in d and e respectively. **f–h**, Thermogenic activation of Tkg-Gal4-positive cells (which include Burs-positive enteroendocrine cells but also a very small subset of central neurons outside the PI, not shown) results in significantly reduced Ms levels in the cell bodies on PI neurons of virgin females, relative to UAS and Gal4 virgin controls. **f–g″**, Representative images of Ms staining in PI neurons of the genotypes quantified in h. Reduced Ms staining is apparent in PI neurons of virgin females upon activation of Tkg-Gal4-positive cells (f) relative to UAS (f′) and Gal4 (f″) virgin controls. The difference between activated (g) versus control (g′; g″) flies is not apparent when female flies are mated (presumably because more Ms peptide has been released in controls). Fluorescence signals are pseudo-coloured; high to low intensity is displayed as warm (yellow) to cold (blue) colours. **i, j**, Effect of gut hormone release from enteroendocrine cells on crop enlargement. Representative crop images of ad libitum-fed female flies show that crop size is increased upon thermogenic activation of Tkg-Gal4-positive cells (i) relative to UAS (i′) and Gal4 (i″) controls, quantified in j.

We note that the Tkg-Gal4-positive cells include most Burs-positive enteroendocrine cells as well as a very small subset of central neurons outside the PI (not shown). **k, l**, Effect of ecdysone and Burs signalling in Taotie neurons on crop enlargement after mating. Representative crop images of starved–refed mated females show that, relative to the UAS control (k), downregulation of EcR (k′) or rk (k″) results in visibly smaller crops. Quantifications of crop area of genotypes shown in k–k″ are shown in l. **m**, Schematic summary of key findings. Circulating levels of Bursicon and Ecdysone increase after mating. Ecdysone and Burs signal via their receptors to Ms neurons, change their neural activity, leading to crop enlargement. Scale bars: f–g″ = 20 μm; b–c″, i–i″ = 500 μm; k–k″ = 1 mm. See Supplementary Information for a list of full genotypes, sample sizes and conditions. In all box plots, line: median; box: 75th–25th percentiles; whiskers: minimum and maximum. All data points are shown. *P < 0.05; **P < 0.01; ***P < 0.001.
Extended Data Fig. 10 | Regulation of food intake, fecundity and fertility by Ms neurons. a, b. Mated females increase their food intake. Both the amount of ingested dye-laced food (a) and the number of sips per fly (b) are increased in wild-type mated females relative to virgins. c–e. Regulation of food intake by MsR1 downregulation in crop muscles. Quantifications of ingested dye show that downregulation of MsR1 in the visceral muscles of starved–refed virgin females results in similar food intake relative to UAS and Gal4 controls (c), whereas downregulation of MsR1, but not MsR2, in mated females, results in reduced food intake relative to UAS and Gal4 controls (d). e. Quantification of the number of sips per fly shows that downregulation of MsR1 specifically in crop muscles using an independent driver line also reduces food intake relative to Gal4 and UAS controls in starved–refed virgin females (d). Comparable results were obtained in mated females. f. Quantification of the number of sips per fly shows that downregulation of MsR1 specifically in crop muscles using an independent driver line also reduces food intake relative to Gal4 and UAS controls in starved–refed mated females. g, h. In the model, food ingestion from the oesophagus is driven by crop enlargement, which is assumed to be linear during sips and constant in between sips. The observed increase in food intake in mated females compared to virgins can be explained by an increase in negative pressure from −0.8 kPa to −1.3 kPa (increased suction), leading to an increased intake during sips. See Source Data for crop morphometry and FlyPad quantifications used for this crop fluid dynamics model. i, j. Thermogenic activation of Ms neurons (Ms > TrpA1) for 4 h before the transfer of flies from undyed to dye-laced food reduces the mean amount of ingested dye during the course of 1 h (i), and reduces the mean number of sips per fly over 1 h of feeding (j) relative to Gal4 and UAS controls. k, l. Concurrent thermogenic activation of Ms neurons during feeding of dye-laced food increases the mean amount of ingested dye during the course of 1 h (k), but has no effect on the mean number of sips per fly over 1 h of feeding (l) relative to Gal4 and UAS controls. m, n. Effect of neuronal activation on the regulation of food intake by Taotie-Gal4-positive neurons. Quantification of ingested dye-laced food shows that thermogenic activation of Taotie neurons for 4 h before the switch from undyed to dye-laced food reduces the amount of ingested dye relative to Gal4 and UAS controls over the course of 1 h (m). By contrast, concurrent activation during feeding of such food increases the amount of ingested dye relative to Gal4 and UAS controls over the course of 1 h (n). o, p. Effect of Ms signalling to crop muscles on fecundity and fertility. o. Quantification of eggs laid in 24 h by mated females shows that MsR1 downregulation specifically in crop muscles results in significantly fewer eggs laid after 4 days relative to UAS and Gal4 controls. p. Quantification of adult progeny produced from a 24 h period of egg laying by mated females shows that MsR1 downregulation in visceral muscles results in significantly fewer progeny relative to UAS and Gal4 controls. Sip number measurements were taken over 1 h of feeding. See Supplementary Information for a list of full genotypes, sample sizes and conditions. In all box plots, line: median; box: 75th–25th percentiles; whiskers: minimum and maximum. All data points are shown. *P < 0.05; **P < 0.01; ***P < 0.001.
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- □ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection Bonsai framework for flyPAD data collection.

Data analysis MAFFT v7.221, trimAL v1.4.rev15, HHMNER 3.1b2, IQ-TREE 1.5.5 for sequence analysis; Fiji image analysis software and R geomorph package (R version 3.3.3, geomorph package version 3.0.6) for image analysis; MATLAB for flyPAD data and calcium imaging analyses; built-in Leica channel unmixing algorithm for FlyBow clones; NeuronStudio (ns0.9.92) software for quantitative analysis of Ms neuron projections; GraphPad Prism 7.04 for statistical analyses.

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### Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes are provided for each experiment in Supplementary Information (the first figure legend refers the reader to Supplementary Information for sample sizes, experimental repeat and specific experimental conditions). For each experiment, a minimum of 3 samples per group were examined per experiment; details are also provided in Supplementary Information. Fly numbers are not limiting so no power calculations were used to pre-determine sample size. Oversampling was mitigated by choosing sample sizes based on previous knowledge of phenotypic variability in controls and other mutants, and by testing each hypothesis using at least two completely independent experimental approaches (e.g. use of gene mutation and Gal4-UAS-mediated RNAi downregulation). Similar sample sizes for different animal groups (e.g. mutants vs controls) were tested in the same experimental design. |
|---|---|
| Data exclusions | No data points/outliers were excluded from our experiments. |
| Replication | Experiments were typically replicated 3 times and only those experiments for which repeats gave comparable outcomes are included in the manuscript. Specific details of the number of experimental repeats for each experiment are provided in Supplementary Information. |
| Randomization | Experimental and control flies were bred in identical conditions, and were randomised whenever possible (for example, with regard to housing, position in tray). Control and experimental samples were dissected and processed at the same time and on the same slides, or behaviourally assessed simultaneously. Experiments were controlled for sex, mating status, genotype and physiological state (for example starved or ad libitum-fed). Details are provided in the main text and, more systematically for each figure panel, in the Supplementary Information. |
| Blinding | Blinding was performed for a subset of experiments. |

### Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Antibodies | Antibiots used | The following primary antibodies were used: rabbit anti-Akh (ref. 25, 1/200), rabbit anti-Burs (ref. 78, 1/200), rat anti-E1av (DSHB, 7E8A1O 1/25), mouse anti-Ecr (DSHB, DDA2.7 1/10), goat anti-GFP (Abcam, ab5450 1/1000), rat anti-Ilp2 (ref. 79, 1/500), rabbit anti-Ms (ref. 80, 1/1000), mouse anti-Pro (DSHB, MR1A 1/25). Fluorescent secondary antibodies (FITC-, Cy3- and Cy5-conjugated) were obtained from Jackson Immunoresearch and used at 1/200. Vectashield with DAPI (Vector Labs) was used to stain DNA. Phalloidin stainings were performed after immunohistochemistry using mushroom phalloidin AlexFluor 647 probe (Life Technologies #A22287, 1/200 for 45min). |
|---|---|---|---|
| n/a | Involved in the study | Antibodies | | |
| | | | Eukaryotic cell lines |
| | | | Palaeontology |
| | | | Animals and other organisms |
| | | | Human research participants |
| | | | Clinical data |
| Methods | n/a | Involved in the study | ChIP-seq |
| | | | Flow cytometry |
| | | | MRI-based neuroimaging |

### Antibodies

**Validation**

Validation of Ms antibody was conducted by immunohistochemistry by revealing co-expression with known Ms neurons markers (e.g. Ms-Gal4, see Extended Data Fig. 3i-i’'), reduced expression following genetic Ms downregulation (Extended Data Fig. 4e-e’’), and absent expression in an Ms mutant (Extended Data Fig. 4a-a’’).
# Animals and other organisms

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| Category               | Description                                                                 |
|------------------------|-----------------------------------------------------------------------------|
| Laboratory animals     | Adult Drosophila melanogaster, males and females up to 2-3 weeks old.        |
| Wild animals           | The study did not involve wild animals.                                     |
| Field-collected samples| The study did not involve samples collected in the field.                   |
| Ethics oversight       | No ethical approval was required. The use of Drosophila melanogaster does not require ethical approval or guidance. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.