FAMILIAR FOOD-INDUCED FEEDING ACTIVATION IN C. ELEGANS

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DEDICATION

During my graduate study, I've met so many wonderful people. From them, I have learned the fun of science and importance of hard work. Leon, in particular, has been a big inspiration throughout my graduate study. His life filled with continuous learning and integrity has always reminded me of what being a scientist is about. I am also very grateful for all his guidance, generous support and learning opportunities that he has provided. I especially have learned a lot during the process of preparation of my papers and communications with journal editors. I cannot thank Chanhee Kang, Euiseok Kim and Young-jai You enough for the scientific interaction throughout my graduate study and for their friendship. They are such gifted scientists and I have learned a lot during discussions with them. Especially Chanhee and Young-jai helped me a lot to endure the tough times just after transferring to Leon's lab.

I would like to thank my committee members, Drs. Cameron, Huber and Kramer for their guidance. Their questions have helped me to think outside the box. I am particularly grateful to Dr. Huber, who has appreciated my food recognition project. I cannot thank Dr. Lin enough for her kindness to be my signing committee.

Last but not least, BIG THANKS to my family and to all my friends! My husband Bong-hyun and our daughter Erin are my absolute treasures. Their presence has sustained me this far. I have enjoyed my scientific journey with my husband Bong-hyun and am so thrilled about our future as scientists. I want to thank my parents and parents-in-law for their love and support. I am also thankful to all my friends, Tracey Shipman, Jing Zou, Byunghoon Lee, Jeongmi Lee, Misung Kim, Peter Changhwan Lee, Jiyeon Lee, Yoonhui Choi, Kiwoo Kim, Dongjae Jeon, Ran Song, Myungsil Lim, Jennifer Winn, Robert Pollok, Taehong Yang, Jimok Yoon, Sangbum Kim, Alex Artyukhin, Sunyoung Park, Yangsik Jeong, Steve Cho and everyone in Korean Society for being my surrogate family who I can share my life with.

FAMILIAR FOOD-INDUCED FEEDING ACTIVATION IN C. ELEGANS

by

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FAMILIAR FOOD-INDUCED FEEDING ACTIVATION IN CAENORHABDITIS ELEGANS

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The University of Texas Southwestern Medical Center at Dallas, 2011

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The growing epidemic of obesity and eating disorders demands the study of regulatory mechanisms of food intake. Studying mutants whose food intake is altered under various conditions has greatly advanced our understanding of the mechanism. However, it is still largely unknown by which mechanisms perception of food activates food intake. The simple anatomy, genetic tractability, and well-characterized and quantifiable feeding behavior and evolutionary conservation of feeding regulators make *C. elegans* an attractive model system for the study.

Food intake in *C. elegans* requires two muscle motions, pharyngeal pumping and isthmus peristalsis, and the frequencies of the two feeding motions dramatically increase in response to food as in other organisms. I attempted to understand the mechanism underlying food-induced feeding activation by

studying the mechanism and the physiological context of action of serotonin, an endogenous activator of pharyngeal pumping. Here I show that like food, serotonin increases overall feeding by activating both feeding motions. Serotonin activates the two feeding motions by activating two distinct neural pathways. A 5-HT_7 receptor activated the two motions mainly by acting in the two distinct pharyngeal motor neurons that are essential for food-induced feeding activation. Moreover, the results support that the serotonin receptor activated the two distinct neurons mainly by activating two distinct downstream G protein signaling pathways. Despite the separate regulation, isthmus peristalsis was coupled to the preceding pharyngeal pump. The separate regulation with coupling of the two feeding motions may have evolved to support efficient feeding by allowing control of the ratio of the frequencies of the two muscle motions according to density of food and by preventing futile isthmus peristalsis.

Then, which aspect of food triggers the serotonin signal that increases food intake? I found that recognition of familiar food selectively triggers the serotonin signal. Worms selectively consume particular bacteria more actively after experience and the behavioral plasticity requires serotonin signaling. By dissecting the mechanism, I found that recognition of familiar food triggers serotonin release from a pair of chemosensory neurons. The released serotonin acts as an endocrine signal to increase pharyngeal pumping rate by activating the pharyngeal motor neuron that directly triggers pharyngeal pumping. The results

suggest that worms form a memory of previously experienced food and that the memory controls food intake. Consistently, the familiar-food induced feeding was strongly dependent on duration of exposure to food to learn but not developmental timing of exposure or nutritional status. Furthermore, worms could remember the previously experienced food at least for several hours.

My study provides insight into how feeding organ operates to increase food intake in response to food and how a particular aspect of food controls the process to increase food intake in *C. elegans*. Studying familiar food-induced feeding activation may help us understand the mechanisms underlying perception of different food and encoding, retention and retrieval of the memory of familiar food.

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PRIOR PUBLICATIONS

1. Recognition of familiar food activates feeding via an endocrine serotonin signaling in *C. elegans*. (in preparation)
2. Serotonin activates overall feeding by activating two separate neural pathways in *C. elegans*. (in preparation)

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Chapter 1 Introduction

GENETIC STUDIES OF FOOD INTAKE

Animals sense and respond to environmental stimuli. Such behaviors are shaped by interaction between genes and environment. Gene changes alter behavior by affecting development or function of sensory organs, central nervous system or motor output. Analysis of mutants whose behaviors are altered by genetic mutations has advanced substantial understanding of cellular and molecular basis of behaviors by supporting the behavioral analysis in several ways, for instance, identifying molecular components that control behaviors and their way of action, dissecting complex behavioral systems and investigating the physiological or ecological context and significance of a behavior.

Due to the rapidly growing epidemic of obesity and eating disorders, understanding regulatory mechanism of food intake is of great demand. Feeding behavior, consisting of food seeking and food consumption, is regulated by various environmental and internal inputs such as sensory factors, nutritional status, emotional processing, decision-making and learning (Figure 1-1). Genetic analysis of mutants has greatly advanced our understanding how food intake is regulated in the context of overall energy balance (Figure 1-2) (Barsh and Schwartz, 2002; Coll et al., 2007). In contrast, it is largely unknown by which

mechanism cognitive or emotional inputs such as polymodal sensory representations of food, reward expectation from food intake, previous food experiences or emotion regulate food intake (Berthoud, 2004; Zheng and Berthoud, 2008). Given that food intake is regulated by integration of cognitive inputs and metabolic inputs, it is crucial to study the mechanism underlying cognitive control of food intake for better understanding of regulation of food intake.

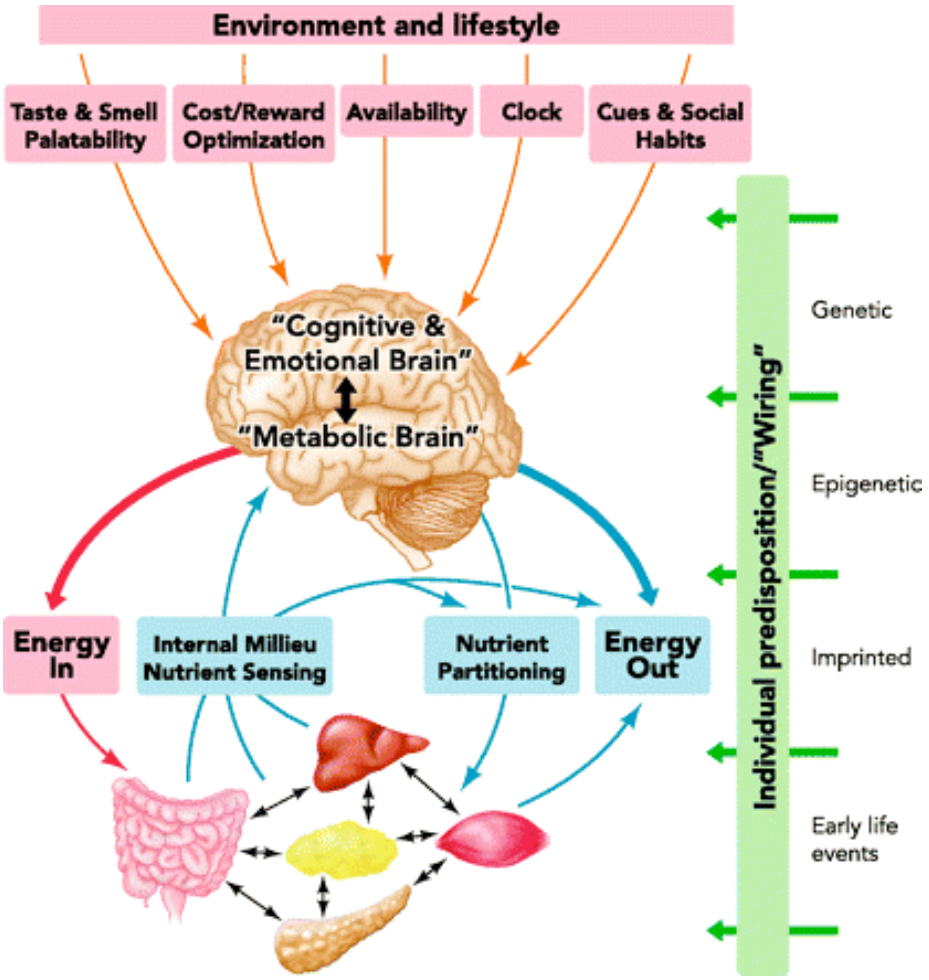


Figure 1-1. Schematic diagram of various environmental and internal inputs that are integrated to control food intake. The various inputs can be classified into two types, overall energy balance (metabolism), and cognition/emotion (taken from (Zheng and Berthoud, 2008)).

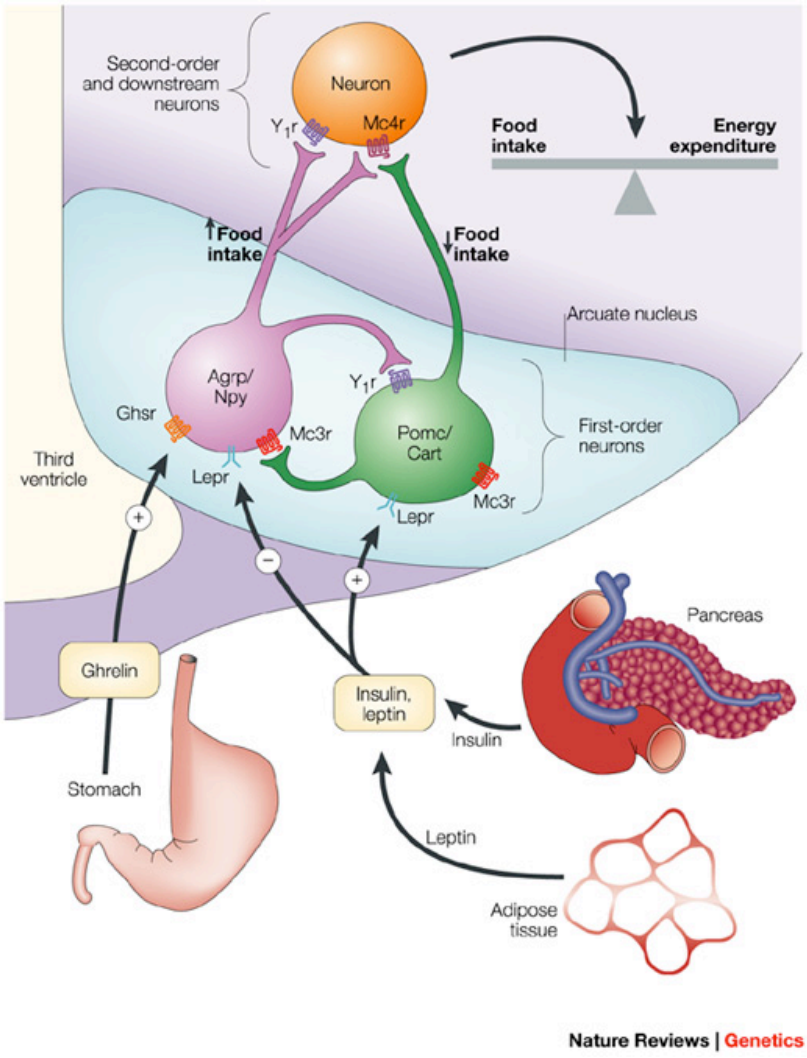


Figure 1-2. A schematic of the mechanisms underlying regulation of food intake in the context of overall energy balance. The two sets of neurons in the arcuate nucleus, orexigenic Agrp/Npy and anorexigenic Pomc/Cart neurons, play opposite roles in regulating food intake. Agrp and Npy are neuropeptides that increase food

intake and decrease energy expenditure, whereas α-melanocyte stimulating hormone (a post-translational derivative of Pomc) and Cart are neuropeptides that inhibit food intake and increase energy expenditure. Insulin and leptin, hormones that circulate in proportion to body adipose stores, decrease food intake by suppressing Agrp/Npy neurons and by stimulating adjacent Pomc/Cart neurons. Ghrelin, a circulating peptide secreted from the stomach, increases food intake by activating Agrp/Npy neurons. Agrp, agouti-related protein; Npy, neuropeptide Y; Pomc, proopiomelanocortin; Cart, cocaine-and amphetamine-regulated transcript; Ghsr, growth hormone secretagogue receptor; Lepr, leptin receptor; Mc3r/Mc4r, melanocortin 3/4 receptor; Y_1r, neuropeptide Y1 receptor (taken from (Barsh and Schwartz, 2002)).

C. ELEGANS AS A MODEL SYSTEM FOR FEEDING RESEARCH AND RESEARCH APPROACH

C. elegans, a bacteria-eating roundworm, is an attractive model organism for studying regulation of food intake. Genetic manipulation is easy and the anatomy is simple (Figure 1-3) (Schafer, 2005). Feeding behaviors in *C. elegans*

have been well characterized and are amenable to quantitative analysis. Moreover, the molecules that control food intake are also at least partly conserved in *C. elegans* (Douglas et al., 2005; Kaun and Sokolowski, 2009; You et al., 2008).

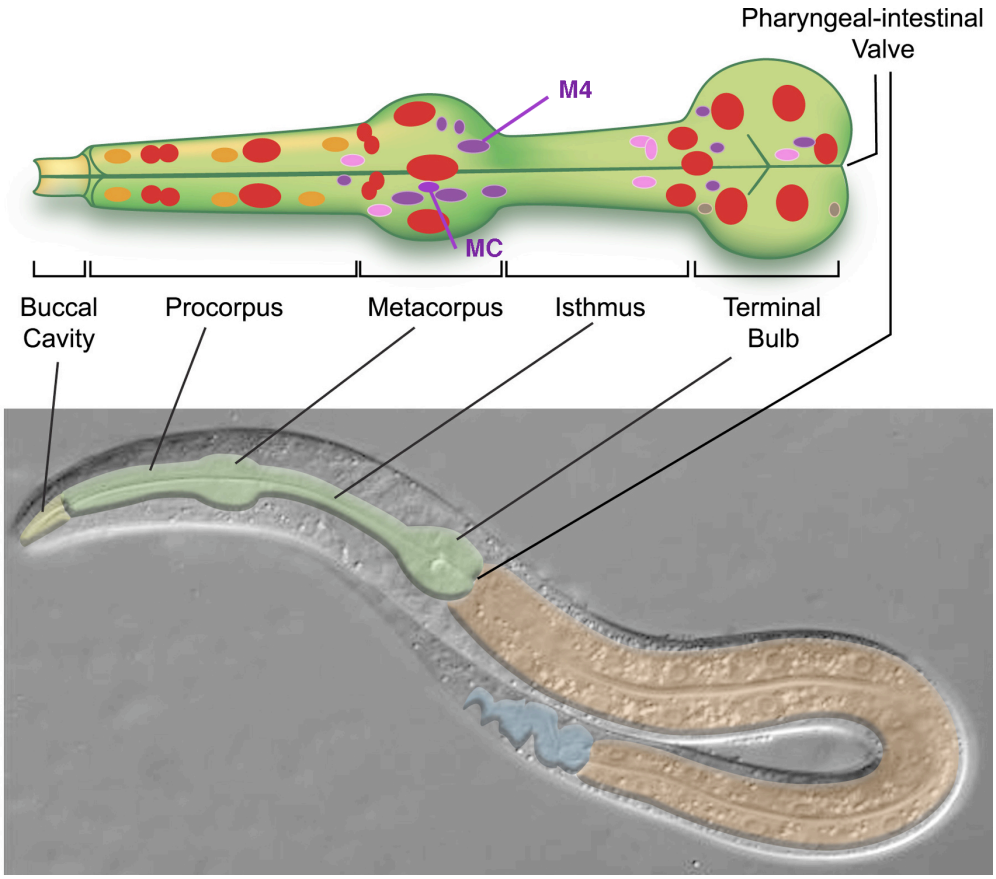


Figure 1-3. Nuclei of the pharynx. The *C. elegans* digestive tract consists of the buccal cavity (lower panel, yellow), foregut or pharynx (green), midgut or intestine (orange) and hindgut (blue). Within the pharynx (upper panel), nuclei of muscles, neurons, epithelia, marginal cells and glands are shown as red, purple, orange, pink and brown. The picture of pharynx is a lateral view of the left-side nuclei and does not show all 14 types of pharyngeal neurons. MC and M4 pharyngeal motor neurons are labeled in purple (modified from wormbook).

Food intake in humans requires a series of repetitive muscle motions such as putting food into the oral cavity, chewing and swallowing. Thus, studying mutants that are defective in increasing frequencies of the feeding motions in response to food would be informative to understand the mechanism by which perception of food, a cognitive regulator of food intake, triggers food intake. Food intake in *C. elegans* requires pharyngeal pumping and isthmus peristalsis, the two muscle motions of the feeding organ (Albertson and Thomson, 1976). Pharyngeal pumping is a synchronized muscle motion that allows worms to take up bacteria from environment and crushes them for further digestion. Isthmus peristalsis is a peristaltic motion that transports bacteria from the anterior to the posterior

pharyngeal muscle that crushes bacteria. As in mammals, food increases frequencies of the feeding motions in *C. elegans* (Avery and Horvitz, 1990; Horvitz et al., 1982).

Frequencies of the feeding motions are controlled by the nervous system of the feeding organ, the pharynx (Avery and Horvitzt, 1989). The *C. elegans* pharyngeal nervous system consists of 20 neurons of 14 types. Among the 14 types of neurons, MC and M4, two motor neurons in the feeding organ (Figure 1-3), are essential for active pharyngeal pumping and isthmus peristalsis, respectively, in response to food (Avery and Horvitz, 1987, 1989).

There are multiple humoral factors that control frequencies of the feeding motions in *C. elegans* (Papaioannou et al., 2005; You et al., 2008). Among them, serotonin is particularly interesting. Serotonin is an endogenous regulator that mimics the effects of food in controlling multiple behaviors, including pharyngeal pumping (Horvitz et al., 1982). Because of its food-mimicking pleiotropic effect, serotonin signal was proposed to act upon perception of food. Furthermore, serotonin-depleted mutants are defective in full activation of pharyngeal pumping in response to food (Sze et al., 2000). The *C. elegans* genome encodes 5 serotonin receptors: SER-1, SER-4, SER-5, SER-7 and MOD-1. SER-1, SER-4, SER-5 SER-7 encode *C. elegans* homologs of 5-HT type 2, 5-HT type 1, 5-HT type 6

and 5-HT type 7 serotonin metabotropic receptors, respectively, and MOD-1 encodes a 5-HT type 3 serotonin-gated Chloride channel. Among them, only SER-7 is necessary for full fast pumping in response to serotonin as well as food (Hobson et al., 2006). Interestingly, SER-7 is expressed in MC and M4 (Hobson et al., 2006), the two motor neurons that are crucial to activate the two feeding motions in response to food. For these reasons, I studied the mechanism and the physiological context of serotonin action in an attempt to understand the mechanism underlying food-induced feeding activation.

Chapter 2 Materials & Methods

General methods and strains

Except when stated otherwise, *C. elegans* was cultured at 19 °C as described by Brenner (1974). Except in Fig. 4-8, all worms used were hermaphrodites and the wild-type strain was *N2* (Brenner, 1974).

In Chapter 3, following alleles were used:

Null mutations: *mod-1(ok103) V*, *ser-4(ok512) III*, *ser-1(ok512) X*, *ser-7(tm1325) X*, *eat-2(ad465) II, gsa-1(pk75) I, acy-1(pk1279) III, gpa-12(pk322) X, dgk-1(sy428) X, pkc-1(nj3) V*.

Hypomorphic mutations: *kin-2(ce179) X, cha-1(p1152) IV, unc-13(s69) I, unc-31(e928) IV*.

Gain of function mutations: *gsa-1(ce81gf) I, acy-1(ce2gf) III*, *HS::gsa-1(Q227L), punc-17::gpa-12(Q205L), punc-17::rho-1(G14V), punc-17::pkc-1B(A160E).*

The mutant strains used were MT9668: *mod-1(ok103) V*, AQ866: *ser-4(ok512) III*, DA2100: *ser-7(tm1325) X*, DA1814: *ser-1(ok345) X*, KG421: *gsa-1(ce81gf) I*, DA2245: *gsa-1(ce81gf) I; ser-7(tm1325) X*, DA2301: *ser-7(tm1325) X;*

nyIs80[pflp-21::gfp(+)], DA2298: *ser-7(tm1325) X; adIs2298[pflp-21::ser-7(+) pflp-21::gfp(+)], eat-2(ad465) II; ser-7(tm1325) X; adIs2298[pflp-21::ser-7(+) pflp-21::gfp(+)],* DA2247: *gsa-1(ce81gf) I; ser-7(tm1325) X; adEx2247[pser-7b::gfp rol-6(d)]*, DA2375: *gsa-1(ce81gf) I; ser-7(tm1325) X; adEx2375[pser-7b::ser-7(+) pser-7b::gfp rol-6(d)]*, DA2297: *ser-7(tm1325) X; adEx2297[pser-7b::ser-7(+) pflp-21::gfp]*, *ser-7(tm1325) X; adEx[pflp-2::ser-7(+) pflp-21::gfp]*, KG532: *kin-2(ce179) X*, DA2191: *gsa-1(pk75) I / hT2(bli-4(e937) let(q782) qIs48[myo-2::gfp pes-10::gfp ges-1::gfp] I); + / hT2(III)*, KG761: *acy-1(pk1279) III; ceEx108[myo-3::acy-1(+)]*, DA2374: *gsa-1(pk75) I / hT2(bli-4(e937) let(q782) qIs48[myo-2::gfp pes-10::gfp ges-1::gfp] I); + / hT2(III)*; *kin-2(ce179) X*, *gsa-1(pk75) I / hT2(bli-4(e937) let(q782) qIs48[myo-2::gfp pes-10::gfp ges-1::gfp] I); + / hT2(III)*; *adEx2374[pflp-21::gsa-1(+) pflp-21::gfp],* KG621: *Is[HS::gsa-1(Q227L)],* KG518: *acy-1(ce2gf) III*, DA2246: *adIs2246[punc-17::gpa-12(Q205L) pacr-2::gfp]*, QT309: *nzIs29[punc-17:rho-1G14V punc-122::gfp]*, PS2627: *dgk-1(sy428) X*, NL594: *gpa-12(pk322) X*, QT253: *nzEx72[punc-17:rho-1(G14VF25N) pttx-3::gfp]*, *dgk-1(sy428) X;* DA2376: *gsa-1(ce81gf) I; ser-7(tm1325) X; adEx2376[pser-7b::rho-1(G14V) pser-7b::gfp rol-6(d)],* PR1152: *cha-1(p1152) IV*, DA465: *eat-2(ad465) II*, DA2189: *cha-1(p1152) IV; kin-2(ce179) X*, *unc-13(s69) I,* DA509: *unc-31(e928) IV*, *unc-31 IV; nzEx72[punc-17:gpa-12(Q205L) pttx-3::gfp],* KP2753: *pkc-1(nj3) V*, KP2841: *nuIs131[Punc-17::pkc-1B(A160E)]*, *pkc-1(nj3) V; nzEx72[punc-17:gpa-*

12(Q205L) pttx-3::gfp], DA2378: *unc-31(e928) IV; adEx2378[pser-7b::unc-31(+) pflp-21::gfp],* DA2377: *unc-31(e928) IV; adEx2377[pflp-21::gfp]*.

In Chapter 4, the following mutant alleles were used: *mod-1(ok103) V*, *mod-5(n3314) I*, *ser-4(ok512) III*, *ser-7(tm1325) X*, *tph-1(mg280) II*. In the main text only the gene name is shown. The mutant strains used were DA2100: *ser-7(tm1325) X,* MT15434: *tph-1(mg280) II,* MT9772: *mod-5(n3314) I,* DA2289: *tph-1(mg280) II; kyEx947[pceh-2::tph-1(+)::gfp punc-122::gfp(+)]*, DA2290: *tph-1(mg280) II; kyEx949[psrh-142::tph-1(+)::gfp punc-122::gfp(+)],* DA2293: *tph-1(mg280) II; ser-7(tm1325) X*, DA2294: *tph-1(mg280) II; ser-7(tm1325) X; kyEx949[psrh-142::tph-1(+)::gfp punc-122::gfp(+)]*, DA2295: *mod-5(n3314) I; tph-1(mg280) II*, DA2296: *mod-5(n3314) I; tph-1(mg280) II; kyEx949[psrh-142::tph-1(+)::gfp punc-122::gfp(+)]*, DA2301: *ser-7(tm1325) X; nyIs80[pflp-21::gfp(+)],* DA2297: *ser-7(tm1325) X; adEx2297[pser-7::ser-7(+) pflp-21::gfp]*, DA2298: *ser-7(tm1325) X; adIs2298[pflp-21::ser-7(+) pflp-21::gfp(+)],* DA2299: *mod-5(n3314) I; tph-1(mg280) II; yzIs71[ptph-1::gfp rol-6(su1006)]; kyEx949[psrh-142::tph-1(+)::gfp punc-122::gfp(+)]*, DA2300: *tph-1(mg280) II; yzIs71[ptph-1::gfp rol-6(su1006)]; kyEx949[psrh-142::tph-1(+)::gfp punc-122::gfp(+)],* GR1333: *yzIs71 [tph-1::gfp, rol-6(su1006)]* V,

OT180: *ser-4(ok512); mod-1(ok103) V; ser-7(tm1325) X*, *ser-7(tm1325) X; adEx[pflp-2::ser-7(+) pflp-21::gfp]*

C. elegans killing assay

To examine the potential pathogenicity of HB101 and DA1878, we performed fast and slow *C. elegans* killing assays as previously described (Tan et al., 1999). A pathogenic bacteria strain *Pseudomonas aeruginosa* PA14 (Tan et al., 1999) was used as a positive control for *C. elegans* killing. We transferred ~50 adult worms to each plate and prepared triplicates for each condition. After the transfer, we cultured the worms at room temperature and counted the number of dead worms every 24hrs. The assays continued for 7-10 days after the transfer. No killing was observed on either HB101 or DA1878. On the contrary, effective killing was observed on PA14 as previously described (Mahajan-Miklos et al., 1999; Tan et al., 1999).

Feeding assay

Developmentally synchronized L1 larvae were cultured until adulthood (for 54hrs at 19°C) on training food. After the training, individual animals were transferred to test food for measuring feeding rates with or without being starved for 7-8hrs

on unseeded NGM plate at room temperature (RT). We starved the animals for the following reasons: First, the 7-8hrs of starvation synchronized the nutritional status of worms. Second, the 7-8 hour gap between the test and the last exposure to the training bacteria allows us to test if *C. elegans* can actually remember the familiar bacteria. Worms were fed fluorescent bacteria, and feeding rates of individual animals were quantified by counting pharyngeal contractions 2-5 mins after the transfer to test food at RT. Feeding motions of individual animals were observed with a Zeiss Stemi SV11 Apo microscope with rhodamine fluorescence filters (excitation at 546nm and emission at 610-675nm) to measure isthmus peristalsis rate as well as the pumping rate. The isthmus peristalsis data were not used for the analyses in this paper. The feeding rate of each animal (pumps per min) was calculated by averaging the three measures from each animal (pumps per 30secs) and subsequently by multiplying by 2.

Preparation of test food for feeding assay

For all figures except Figs. 4-2 A-B and 4-3A-B, test food was prepared by seeding 10ul of bacterial culture in LB (OD=5.0) on new NGM plates and incubating the seeded plates at RT for 5.5hrs and 7hrs for mCherry-expressing HB101 and DA1878, respectively. Before seeding the culture of DA1878,

fluorescent latex beads (L3280, SIGMA-ALDRICH, INC) were mixed with the culture at a 1:800 ratio. For Figs. 4-2 A-B and 4-3 A-B, test food was prepared in the same way except that 100ul of bacterial culture of mCherry-expressing HB101 was seeded. The variation in food preparation was necessary because *tph-1* mutants would not stay on food that was prepared from 10ul of the culture.

Molecular biology and generation of transgenic strains

The truncated *ser-7* promoter (2.2kb) for expression in M4 and M2 (Hobson RJ *et al*. (2003)) [pser-7 F: 5'- CAA ACA GGT AGA CAA TGT TGT AAA CTG TGA -3' and pser-7 R: 5'- TTC ACC CCT CAG GCT GTG -3'] was cloned by PCR from wild-type(*N2*) genomic DNA. 1.3kb *ser-7* cDNA [SER-7 cDNA F primer: 5'-*CCCGGG***ATG**GCCCGTGCAGTC-3' and SER-7 cDNA R primer: 5'-*CCCGGG*CTAGACGTCACTTGGTTCGT-3'] (Hobson et al., 2003) was cloned by PCR from a cDNA pool that was reverse transcribed from *N2* mRNA extracts. *pflp-21* and *pflp-2* were a kind gift from Dr. Chris Li (Kim and Li, 2004). *pser-7b* and *pflp-21* were cloned into HindIII-BamHI digested pPD96.52. *ser-7* cDNA was cloned into the EcoRI site of the two vectors containing each of the promoters. *gsa-1* cDNA [GSA-1 cDNA F primer: 5'-*GAA TTC* **ATG** GGG TGC GTC G and GSA-1 cDNA R primer: 5'- *GAA TTC* TTA TAG AAG CTC GTA

CTG TCG TAG] was cloned by PCR from wild-type (*N2*) cDNA library. After digestion with EcoRI, the cloned *gsa-1* cDNA was inserted into the EcoRI site of pPD96.42 containing the *flp-21* promoter. Dr. Nurrish and Dr. Miller kindly provided cDNAs that encode *rho-1(G14V)* and *unc-31*, respectively. NheI-SpeI digested *rho-1(G14V)* cDNA and AgeI-NheI digested *unc-31* cDNA were inserted into the XhoI site of pPD96.52 containing the *ser-7b* promoter by blunt-end ligation. The *pflp-2::ser-7(+)* rescue construct was generated by the PCR-fusion method (Hobert, 2002) using the following primers: [pflp-2 A primer, 5'-TCTGTGTTCACTCTACCAGGAACTTTTCTCACTTTTTAATACATATTTTC ATGAAC -3', pflp-2 A' primer, 5'- TCTGTGTTCACTCTACCAGGA -3', pflp-2 B primer, 5'- GAGATATGTTGACTGCACGGGCCATGGTTTGCGACAATTG GTTTGGCAACG -3', SER-7 cDNA C primer, 5'- ATGGCCCGTGCAGTC-3', pPD9575 3'UTR D primer, 5'- GGAAACAGTTATGTT TGGTATATTGGG -3']. To generate DA2297, DA2298 and *ser-7;Ex[pflp-2::ser-7(+) pflp-21::gfp]*, germline transformation was performed with *pser-7::ser-7(+)* (100ng/ul), *pflp-21::ser-7(+)* (50ng/ul) or *pflp-2::ser-7(+)* (75ng/ul) , and *pflp-21::gfp* (50ng/ul) as a injection marker in *ser-7(tm1325).* For DA2298, the extrachromosomal array was integrated into the chromosome by gamma irradiation (6 krad). The integration line was outcrossed five times against *ser-7(tm1325).* For DA2374, a mixture of *pflp-21::gsa-1(+)* (50ng/ul) and *pflp-21::gfp* (50ng/ul) was germline transformed in wild-type worms. Then, the transgenic males were crossed with

DA2191 hermaphrodites. F1 males that carry *pmyo-2::gfp* and *pflp-21::gfp* were selected and crossed with DA2191 hermaphrodites. After crossing, F1 hermaphrodites that carry *pmyo-2::gfp* and *pflp-21::gfp* were singled. After letting the hermaphrodites self, the plates in which all adult worms had green pharynxes were selected. DA2375 and DA2376 were generated by germline transformation of DA2245 with *pser-7::ser-7(+)* (50ng/ul) or *pser-7::rho-1(G14V)* (15ng/ul). *pser-7b::gfp* (88ng/ul) and pRF4 (50ng/ul) were used as coinjection markers. For DA2377 and DA2378, *pflp-21::gfp* (50ng/ul) was germline transformed with or without *pser-7b::unc-31(+)* (50ng/ul) into *unc-31(e928).*

Laser ablation of ADF

Laser ablation of ADFs was performed by a modified procedure (Avery and Horvitz, 1987) We destroyed both ADFs in newly hatched GR1333 larvae (0-4hr old) using a MicroPoint laser ablation system (Photonic instruments, INC). For laser ablation, we mounted the larvae on 2% agarose pads containing 10mM sodium azide. To minimize the variation caused by sodium azide, we retrieved all the mock-operated and the putative ADF-ablated animals from the agarose pad after the same incubation time (12 mins). Mock-operated groups were treated in the same way except that the laser was not fired. The retrieved animals were

cultured on HB101 until adulthood (54hrs at 19°C). After 7hrs of starvation, feeding assays were performed as described in pages 10-11. Successful ablation of ADFs was confirmed by absence of the ADF GFP signals. Only data from animals in which both ADFs were specifically destroyed are included.

Immunohistochemistry

Samples of DA2299 and DA2300 were prepared as for the feeding assay. Just after the 7 hour starvation we divided each group into three equal subgroups and fixed one. The remaining two groups were separately refed on either DA1878 or HB101 and fixed after 1hr. This assay could not be done immediately after training because most of the serotonin-uptaking cells were serotonin positive in DA2300 as in wild type worms. The background was too high to detect any increase in serotonin release. Immunohistochemistry was performed by a protocol from Curtis Loer (http://home.sandiego.edu/~cloer/loerlab/anti5htlong.html) using the following antibodies: anti-serotonin rabbit IGG: S5545 (SIGMA-ALDRICH, INC), Anti-GFP chicken IGG: GFP-1020 (Aves LABS, INC.), ALEXA FLOUR 488 goat anti-chicken IGG: A11039 (Invitrogen Corporation), Cy-3 conjugated donkey anti-rabbit IGG: 711-165-152 (Jackson ImmunoResearch). Failure of the co-immunostaining against serotonin and

against GFP was 0%. The GFP signal was for identifying serotonergic neurons (*ptph-1::gfp*) and for finding transgenics that express *tph-1* cDNA in ADF (*psrh-142::tph-1::gfp*). Due to *Is[ptph-1::gfp]* in DA2299 and DA2300, GFP signal was found in NSM, ADF, HSN and sporadically in RIH and AIM in all animals. Although *ptph-1::gfp* expresses GFP in ADF, we could still recognize the transgenic animals that express *Ex[psrh-142::tph-1::gfp]* because GFP signal in ADF is much stronger in those transgenics. The strong GFP signal in ADF was perfectly correlated with serotonin signal. In DA2300, serotonin signal was found in four different classes of serotonergic neurons (ADF, NSM, RIH and AIM). In DA2299, the control strain that is defective in serotonin uptake, serotonin signal was found only in ADF. Images were obtained with a Zeiss LSM510-meta confocal microscope using a 40X oil-immersion objective.

Quantification of serotonin positive neurons

To calculate the increase in the average number of the serotonin positive serotonin-uptaking cells during 1hr of refeeding for each group (Fig. 4-4d), we first blindly counted the number of serotonin positive AIMs and RIH from each animal and calculated the average number for each group. ADF, NSM and HSN were not included because serotonin was detected in all ADFs and NSMs even before refeeding and in none of HSNs even after refeeding. To minimize

variation, only the animals expressing TPH-1 in both ADFs (as indicated by the presence of the strong GFP signal from *psrh-142::tph-1::gfp*) were considered for counting the number of serotonin positive AIMs and RIH. Then, we subtracted the baselines from each familiar and novel food group as follows (see detailed analysis of data presented in Fig. 4-4d in page 22): $(\text{Serotonin positive cell})_{HD/HH}$ = $(\text{Average number of serotonin positive AIMs and RIH})_{HD/HH}$ − $(\text{Average number of serotonin positive AIMs and RIH})_{H.}$ $(\text{Serotonin positive cells})_{DH/DD}$ = $(\text{Average number of serotonin positive AIMs and RIH})_{DH/DD}$ − $(\text{Average number of serotonin positive AIMs and RIH})_{D.}$ For data presentation, we combined the values from three independent experiments (see detailed analysis of data presented in Fig.4-4d in page 22).

Statistical analysis

To test the hypothesis that isthmus peristalsis is coupled to either the preceding pump or the following pump, we asked if the interval between isthmus peristalsis and either of the pumps correlates with the interval between the two consecutive pumps that are flanked by isthmus peristalsis (see the schematic in Fig. 3-1) using Spearman's correlation test. The interval between the two pumps decreases as pharyngeal pumping rate increases. If isthmus peristalsis is coupled to the preceding or following pump, the interval between isthmus peristalsis and the

pump should be constant regardless of pharyngeal pumping rate, i.e. there should be no correlation between the interval between isthmus peristalsis and the pump and the interval between the two consecutive pumps. In contrast, if isthmus peristalsis and either of the pump is not coupled, the two intervals should have a strong correlation.

Testing for effects of drugs or mutations on isthmus peristalsis is complicated by the fact that most treatments that affect isthmus peristalsis also affect pumping, and isthmus peristalsis depends nonlinearly on pumping. Therefore, to compare isthmus peristalsis rates in two datasets, we required that the pumping rates in those datasets overlap, then asked whether isthmus peristalsis differed between datasets at the same pumping rate. The second question was answered by fitting parabolas to the data. Both datasets were simultaneously fit to the model

$$y_i = ax_i + bx_i^2 + D_i(cx_i + dx_i^2)$$

x_i and y_i are pumping and isthmus peristalsis rates for worm i, D_i is an indicator (dummy) variable that is 0 if worm i is in dataset 1 and 1 if worm i is in dataset 2, and a, b, c, and d are the fitting parameters. If isthmus peristalsis differs between the two datasets, (c, d) will differ from (0, 0). This was tested by calculating the F statistic:

$$F_{2,n_1+n_2-2} = \frac{\frac{c^2}{\sigma_c^2} + \frac{d^2}{\sigma_d^2} - 2\rho\frac{cd}{\sigma_c\sigma_d}}{2\left(1-\rho^2\right)}$$

where:

c, d are the least-squares estimates of c and d.

σ_c, σ_d, and ρ are estimates of the standard error of c, the standard error of d, and the correlation between c and d from the linear regression.

n_1, n_2 are the numbers of points in datasets 1 and 2.

The level of significance is the upper tail probability of a standard F distribution with 2 and $n_1 + n_2 - 2$ degrees of freedom. (Since c and d are squared in computing F, any deviation from (0, 0) gives a positive F, so this is a one-tailed test.)

The data from the feeding assays either on bacteria or on serotonin were statistically analyzed by both the unpaired *t*-test and the Mann-Whitney U test (two-tailed). The two tests produced the same conclusions for all data analyses. For data presentation, the more conservative *P* value was selected. Only the familiar food group and the novel food group that were tested on the same test food were compared since the feeding rate on HB101 was significantly higher than the rate on DA1878 in both non-starved wild-type worms (P=0.006) and 7-8hrs starved wild-type worms (P=0.003). The *ser-7* effect on the feeding rate (shown in Fig. 4-2f) for each food condition was calculated by subtracting the

averaged feeding rate of the *ser-7* mutant from the rate of wild-type worms that were tested under each food condition (see detailed analysis of data presented in Fig. 4-2f in page 20). Student's *t*-test (two-tailed) was used to compare the *ser-7* effects on feeding rate between HH and DH groups, and between DD and HD groups (see detailed analysis of data presented in Fig. 4-2f in page 20). GraphPad Prism (version 5.0) was used for statistical analysis. To compare the increase in the numbers of serotonin positive cells during 1hr of refeeding on familiar food with the increase on novel food, we tested the data shown in Fig. 4-4d using Fisher's method (Fisher, 1954) for combining the results of several independent tests bearing upon the same overall hypothesis. We first compared the difference in the increase in the number of serotonin positive cells between HH and DH groups, and between DD and HD groups in each experiment using Student's *t* test (two-tailed). *P* values from three independent experiments were then combined using Fisher's method and tested by X^2 test (see see detailed analysis of data presented in Fig. 4-4d in page 22).

Drug treatment and feeding assay

To examine drug effects on feeding in the absence of bacteria, feeding rates were quantified from 3-5hr old L1 larvae that had never been exposed to bacteria. After

collecting embryos by egg preparation (Lewis and Fleming, 1995), we incubated them on unseeded NGM plates for 2hrs. Newly hatched L1 larvae (0-2hr old) were then transferred to unseeded NGM plates and incubated for 3hrs. The effect of each drug on the feeding rates was examined after 15 min incubation on 2% agarose pads containing the drug. Final concentrations of serotonin (H7752, SIGMA-ALDRICH, INC) and forskolin (F6886, SIGMA-ALDRICH, INC) in the pad were 20mM and 115.4mM, respectively. The pad in which forskolin was dissolved contained 0.25% ethanol and the controls for forskolin-treated worms were incubated with the same concentration of ethanol. 0.25% ethanol did not affect feeding rates (data not shown). Feeding motions of each larva were observed using a Zeiss Axiophot microscope with a 63x objective. 2min videos were taken from each larva with a Hitachi kP-160 CCD camera and digitized using Adobe Premiere v6.5 for quantification of feeding rates. Feeding rates were calculated by averaging 2 measures per each animal (pumps per 55sec).

Detailed analysis of data presented in Fig. 4-2f

Comparison of the differences in the feeding rates between wild-type(*N2*) and the *ser-7* null mutant animals on familiar food with the differences on novel food using Student *t*-test

STEP 1. Calculation of the parameters (means and standard error of the means of the differences in the feeding rates between *N2* and *ser-7* under HH, DH, DD and HD) for the comparisons using Student *t*-test

$$\text{Mean}(N2_{HH}\text{-ser-}7_{HH}) = \text{Mean}(N2_{HH}) - \text{Mean}(\text{ser-}7_{HH})$$

$$\text{Standard error of the mean}(N2_{HH}\text{-ser-}7_{HH})$$

$$= [\text{Var}(N2_{HH})/n(N2_{HH}) + \text{Var}(\text{ser-}7_{HH})/n(\text{ser-}7_{HH})]^{1/2}$$

$$\text{Mean}(N2_{DH}\text{-ser-}7_{DH}) = \text{Mean}(N2_{DH}) - \text{Mean}(\text{ser-}7_{DH})$$

$$\text{Standard error of the mean}(N2_{DH}\text{-ser-}7_{DH})$$

$$= [\text{Var}(N2_{DH})/n(N2_{DH}) + \text{Var}(\text{ser-}7_{DH})/n(\text{ser-}7_{DH})]^{1/2}$$

$$\text{Mean}(N2_{DD}\text{-ser-}7_{DD}) = \text{Mean}(N2_{DD}) - \text{Mean}(\text{ser-}7_{DD})$$

$$\text{Standard error of the mean}(N2_{DD}\text{-ser-}7_{DD})$$

$$= [\text{Var}(N2_{DD})/n(N2_{DD}) + \text{Var}(\text{ser-}7_{DD})/n(\text{ser-}7_{DD})]^{1/2}$$

$$\text{Mean}(N2_{HD}\text{-ser-}7_{HD}) = \text{Mean}(N2_{HD}) - \text{Mean}(\text{ser-}7_{HD})$$

$$\text{Standard error of the mean}(N2_{HD}\text{-ser-}7_{HD})$$

$$= [\text{Var}(N2_{HD})/n(N2_{HD}) + \text{Var}(\text{ser-}7_{HD})/n(\text{ser-}7_{HD})]^{1/2}$$

Mean(X_Y) is the mean of the feeding rate of animals of genotype X under Y condition

Var(X_Y) is the variance of the feeding rate of animals of genotype X under Y condition

n(X_Y) is number of animals of genotype X that were tested under Y condition

STEP 2. Comparison of the differences in the feeding rates between wild-type(*N2*) and the *ser-7* null mutant animals on familiar food with the differences on novel food using Student *t*-test

A. Comparison between HH and DH

$$t = \frac{[\{\text{Mean}(N2_{HH}) - \text{Mean}(\text{ser-}7_{HH})\} - \{\text{Mean}(N2_{DH}) - \text{Mean}(\text{ser-}7_{DH})\}]}{[\text{Var}(N2_{HH})/n(N2_{HH}) + \text{Var}(\text{ser-}7_{HH})/n(\text{ser-}7_{HH}) + \text{Var}(N2_{DH})/n(N2_{DH}) + \text{Var}(\text{ser-}7_{DH})/n(\text{ser-}7_{DH})]^{1/2}}$$

$P<0.001$

B. Comparison between DD and HD

$$t = \frac{[\{\text{Mean}(N2_{DD}) - \text{Mean}(\text{ser-}7_{DD})\} - \{\text{Mean}(N2_{HD}) - \text{Mean}(\text{ser-}7_{HD})\}]}{[\text{Var}(N2_{DD})/n(N2_{DD}) + \text{Var}(\text{ser-}7_{DD})/n(\text{ser-}7_{DD}) + \text{Var}(N2_{HD})/n(N2_{HD}) + \text{Var}(\text{ser-}7_{HD})/n(\text{ser-}7_{HD})]^{1/2}}$$

$P<0.001$

These statistical analyses concluded that the difference in the feeding rates between wild-type(*N2)* and *ser-7* is greater on familiar food than the difference on novel food, suggesting that serotonin signaling via SER-7 is more active on familiar food than novel food.

Detailed analysis of data presented in Fig. 4-4d

STEP 1. Comparison of the increase in the average number of serotonin positive serotonin-uptaking cells in the *tph-1;Is[ptph-1::gfp];Ex[ADF::tph-1(+)::gfp]* animals that were refed on familiar food with the increase in the animals that were refed on novel food using Student *t*-test

A. Calculation of the parameters (means and standard error of the means for HH-H, DH-D, DD-D and HD-H) for the comparisons using Student *t*-test

(The subtraction was for isolating the increase in the number during the 1hr of refeeding.)

Mean(HH-H) = Mean(HH) - Mean(H)

Standard error of the mean(HH-H) = $[Var(HH)/n_{HH} + Var(H)/n_H]^{1/2}$

Mean(DH-D) = Mean(DH) - Mean(D)

Standard error of the mean(DH-D) = $[Var(DH)/n_{DH} + Var(D)/n_D]^{1/2}$

Mean(DD-D) = Mean(DD) - Mean(D)

Standard error of the mean(DD-D) = $[Var(DD)/n_{DD} + Var(D)/n_D]^{1/2}$

Mean(HD-H) = Mean(HD) – Mean(H)

Standard error of the mean(HD-H) = $[Var(HD)/n_{HD} + Var(H)/n_H]^{1/2}$

Mean(X) is mean of number of serotonin-positive serotonin-uptaking cells in group X

Var(X) is variance of number of serotonin-positive serotonin-uptaking cells in group X

n_X is sample number of group X

B. Comparison between HH-H and DH-D in each experiment

$$t = \frac{[\{Mean(HH) - Mean(H)\} - \{Mean(DH) - Mean(D)\}]}{[Var(HH)/n_{HH} + Var(H)/n_H + Var(DH)/n_{DH} + Var(D)/n_D]^{1/2}}$$

Experiment 1: P=0.239
Experiment 2: P=0.380
Experiment 3: P=0.007

C. Comparison between DD-D and HD-H in each experiment

$$t = \frac{[\{\text{Mean(DD)} - \text{Mean(D)}\} - \{\text{Mean(HD)} - \text{Mean(H)}\}]}{[\text{Var(DD)}/n_{DD} + \text{Var(D)}/n_{D} + \text{Var(HD)}/n_{HD} + \text{Var(H)}/n_{H}]^{1/2}}$$

Experiment 1: P=0.155
Experiment 2: P=0.005
Experiment 3: P=0.540

STEP 2. Comparison of the increases in number of serotonin-positive serotonin-uptaking neurons (HH-H vs DH-D and DD-D vs HD-H) using χ^2 test after combining the data from 3 independent experiments using Fisher's method.

$$X^2 = -2\sum_{i=1}^{k} \log_e(p_i),$$

where p_i is the P-value for the i^{th} hypothesis test. When the P-values tend to be small, the test statistic X^2 will be large, which suggests that the null hypotheses are not true for every test.

When all the null hypotheses are true, and the p_i (or their corresponding test statistics) are independent, X^2 has a chi-square distribution with $2k$ degrees of freedom, where k is the number of tests being combined. This fact can be used to determine the P-value for X^2.

A. Comparison between HH and DH: X^2=14.63 (degree of freedom=6): P=0.023
B. Comparison between DD and HD: X^2=15.40 (degree of freedom=6): P=0.017

These statistical analyses conclude that the increases in the average number of serotonin positive serotonin-uptaking neurons of HH and DD groups are greater than the increases of DH and HD groups, respectively.

Chapter 3 Serotonin-induced feeding activation in C. elegans

ABSTRACT

Feeding in *C. elegans* requires two distinct pharyngeal muscle motions, pharyngeal pumping and isthmus peristalsis. Bacteria, the natural food of *C. elegans*, activate both feeding motions. The food-induced active pharyngeal pumping and isthmus peristalsis require MC and M4, the two motor neurons in the pharynx, respectively. However, it is largely unknown by which mechanisms the pharyngeal neurons activate the feeding motions in response to food. To understand the mechanism, we studied the mechanism of action of serotonin, an endogenous activator of pharyngeal pumping whose action is activated by bacteria. Here we show that serotonin, like bacteria, activates overall feeding by activating both feeding motions. During active feeding, the frequencies and the timing of onset of the two motions were different, but each isthmus peristalsis was coupled to the preceding pump. We found that serotonin activates the two feeding motions mainly by activating two separate neural pathways. For activating pumping, SER-7 in MC activated acetylcholine transmission from MC to the pharyngeal muscles by activating the Gsα signaling pathway. For activating isthmus peristalsis, SER-7 in M4 (and possibly M2) activated the $G_{12}\alpha$ signaling pathway in a cell-autonomous manner.

It is unclear by which neurotransmitter M4 activates the isthmus peristalsis, but release of dense core vesicles from unknown cell(s) acts in parallel with simulation by M4. Based on our results and previous calcium imaging of pharyngeal muscles, we propose that inducing isthmus peristalsis requires both muscle excitation during the pump and stimulation by M4. This model explains how the two feeding motions are separately regulated yet coupled. The pharynx may have evolved this way to support efficient feeding.

INTRODUCTION

The pharynx, a neuromuscular tube, is the feeding organ in *C. elegans*. The pharynx consists of 8 muscles and 20 neurons of 14 types. The five large pharyngeal muscles are connected by gap junctions and constitute the 3 functional parts of the pharynx, the corpus, the isthmus and the terminal bulb, which are arranged from anterior to posterior (Albertson and Thomson, 1976). The corpus takes in food from the environment and accumulates the food in the anterior isthmus by its contraction and subsequent relaxation. The accumulated food is transported to the terminal bulb by a peristaltic movement of the posterior isthmus. The grinder in the terminal bulb crushes the food for nutrient absorption in the intestine. The pharyngeal muscles show two feeding motions, pumping and isthmus peristalsis.

Pumping is a synchronized contraction and subsequent relaxation of the corpus, the anterior isthmus and the terminal bulb (Figure 3-5A). Isthmus peristalsis is a peristaltic movement of the posterior isthmus (Figure 3-5B).

The frequencies of the two feeding motions increase in response to bacteria, the natural food of *C. elegans* (Croll, 1978). Among the 14 types of pharyngeal neurons, only MC and M4 are essential for normal fast pumping and for isthmus peristalsis, respectively (Avery and Horvitz, 1987; Avery and Horvitzt, 1989). It is unknown by which mechanisms the pharyngeal neurons activate feeding in response to bacteria. Here, we attempted to understand the mechanism by studying the process by which serotonin activates feeding for the following reasons: First, serotonin is an endogenous activator of pumping whose action is induced by bacteria (Sze et al., 2000). Second, serotonin activates pumping via a 5-HT type 7 receptor SER-7, which is expressed in multiple pharyngeal neurons including MC and M4 (Hobson et al., 2006). In fact, to activate pumping, serotonin requires cholinergic transmission from MC to the pharyngeal muscles (Raizen et al., 1995). Furthermore, SER-7 expression in M4 raises the possibility that serotonin also activates isthmus peristalsis.

RESULTS

Isthmus peristalsis is coupled to the preceding pharyngeal pumping.

To ask if serotonin controls isthmus peristalsis as well as pumping, we compared the rates of the two feeding motions in presence of serotonin with the rates in its absence. Serotonin significantly increased the frequencies of both pharyngeal pumping and isthmus peristalsis. However, the following observations suggested that isthmus peristalsis is coupled to the preceding pharyngeal pump: First, isthmus peristalsis never occurred in the absence of pumping. Second, isthmus peristalsis always occurred immediately after a pump, although not every pump was followed by isthmus peristalsis (On average, isthmus peristalsis occurred every 3.4 pumps.). To test if the two feeding motions are coupled, we measured the time interval between isthmus peristalsis and the preceding or following pump. If the two motions are not coupled, both the interval between peristalsis and pump and the variance of this interval should decrease as the rate of pumping increases. This is indeed what we saw for the interval between peristalsis and the following pump (Figure 3-1B). In contrast, if pumping triggers isthmus peristalsis, one might expect the interval and its variance to remain constant as pumping rate increases. In fact, the interval between peristalsis and the preceding pump was small (~150 msec) and unaffected by the increase in pharyngeal pumping rate. The variance of the interval was

also small (50 msec) and constant (Figure 3-1A), which suggests that isthmus peristalsis is indeed coupled to the preceding pharyngeal pumping.

SER-7 serotonin receptor separately activates the two feeding motions mainly by activating pharyngeal neurons MC and M4.

Since isthmus peristalsis is coupled to the preceding pharyngeal pump, the increased isthmus peristalsis rate in response to serotonin might be caused by the increased pharyngeal pumping rate. To find out if serotonin stimulates isthmus peristalsis independent of pumping, we tested whether the isthmus peristalsis rate changed at a given pharyngeal pumping rate (see Statistical analysis in Chapter 2 for details of statistical analysis). To compare isthmus peristalsis rates at a given pharyngeal pumping rate, we used scatter plots in which the two feeding rates of each worm were plotted as a point (Figure 3-1C). In subsequent plots we summarize the data as ellipses for clarity (see Figure 3-1D legend for details). We found that serotonin stimulated isthmus peristalsis as well as pumping, and the effect on

isthmus peristalsis was greater than what could be accounted for by the effect on pumping alone (Figure 3-1 C and D). These data imply that serotonin directly activates both feeding motions in a coordinated manner.

We asked which receptor mediates the serotonin effect by examining the feeding rates of serotonin receptor null mutants in response to serotonin. Among 5 serotonin receptor null mutants, only the ser-7 mutant failed to pump rapidly in presence of serotonin (Figure 3-1E and Figure 4-6), confirming the previous report that SER-7 is the major serotonin receptor that mediates serotonin action on pumping (Hobson et al., 2006). On the contrary, it was not obvious which serotonin receptor activates isthmus peristalsis. The pharyngeal pumping rate of the *ser-7* null mutant was too low to compare the isthmus peristalsis rate of the mutant with the rate of wild-type and the other serotonin receptor mutants had small or no change in isthmus peristalsis rate in response to serotonin (Figure 3-1E and Figure 3-6). To learn whether SER-7 affects isthmus peristalsis, we compared *gsa-1(gf)* and *gsa-1(gf); ser-7* mutant animals. *gsa-1* encodes $G_s\alpha$. The *gsa-1* gain of function mutation was sufficient to activate pumping, even in the absence of SER-7 (Figure 3-1F and Figure 3-2B). Although *gsa-1(gf)* and *gsa-1(gf); ser-7* pumped at the same rate in the presence of serotonin, the isthmus peristalsis rate was lower in *gsa-1(gf); ser-7* than in *gsa-1(gf)* (Figure 3-1F) These results

suggest that serotonin signaling via SER-7 activates both feeding motions and that serotonin activates isthmus peristalsis separately from pharyngeal pumping.

We next asked in which cells SER-7 acts to activate the feeding motions. Based on a previous cell ablation study (Avery and Horvitz, 1987, 1989) and expression pattern of SER-7 (Hobson et al., 2006), we hypothesized that SER-7 acts in MC and M4 to activate pharyngeal pumping and isthmus peristalsis, respectively. Consistent with the hypothesis that SER-7 in MC stimulates pumping, expression of SER-7 driven by the *flp-21* or the *flp-2* promoter fully restored serotonin-stimulated pumping in the *ser-7* null mutant (Figure 3-1G and H). The *flp-21* and the *flp-2* promoters drive expression in several neurons, and the expression patterns of *flp-21* and *flp-2* overlap only in MC and M4 (Hobson et al., 2006; Kim and Li, 2004). Furthermore, expression of SER-7 in M4 failed to change the pumping rate of the *ser*-7 null mutant in response to serotonin (Figure 3-1I). The failure is unlikely due to insufficient expression because a significant increase in isthmus peristalsis rate was observed in the transgenic animals (Figure 3-1I). To test if SER-7 indeed acts mainly in MC to stimulate pumping, we tested if blocking neurotransmission from MC blocks the rescue effect of *pflp-21*::*ser-7(+)* in the *ser-7* mutant. EAT-2 is a nicotinic acetylcholine receptor subunit (Raizen et al., 1995). It is specifically localized in the pharyngeal muscles postsynaptic to MC (McKay et al., 2004) and thus an *eat-2* null

mutation selectively blocks acetylcholine transmission from MC to pharyngeal muscles. *eat-2* suppressed the rescue effect of *pflp-21*::*ser-7(+)* (Figure 3-1J), suggesting that SER-7 in MC activates pumping. Then, we tested if SER-7 acts in M4 to stimulate isthmus peristalsis. The *ser-7b* promoter drives expression in M4 and occasionally in M2 (Hobson et al., 2003). As expected, expression of *pser-7b::ser-7(+)* rescue construct fully restored serotonin-stimulated isthmus peristalsis in the *gsa-1(gf);ser-7* mutant (Figure 3-1K), supporting that SER-7 in M4 (and possibly in M2) activates isthmus peristalsis.

Serotonin activates pharyngeal pumping in response to bacteria, a natural food of *C. elegans* (Sze et al., 2000). Thus, we tested the physiological relevance of serotonin-stimulated feeding by testing if SER-7 in MC and M4 activates pharyngeal pumping and isthmus peristalsis, respectively in response to bacteria. As expected, SER-7 was essential to fully activate isthmus peristalsis and pumping in response to bacteria—pumping and isthmus peristalsis decreased 25% compared to wild type in the *ser-7* null mutant (Figure 3-1L-N). Consistent with our results from exogenous serotonin treatment, the *pflp-21*:: *ser-7(+)* construct fully restored pumping and isthmus peristalsis rates (Figure 3-1M) to the wild-type level in response to familiar bacteria. In contrast, the *pser-7b:: ser-7(+)* construct fully restored stimulated isthmus peristalsis with a small effect on pumping (Figure 3-1N). The small yet significant increase in pharyngeal

pumping suggests that the two regulatory pathways that activate the feeding motions are not entirely distinct—one possible explanation is a mechanosensory effect of isthmus peristalsis on MC (Raizen et al., 1995). Together, these results suggest that SER-7 acts mainly in MC and M4 to stimulate pumping and isthmus peristalsis in response to serotonin, respectively.

SER-7 cell-autonomously activates pharyngeal pumping via $G_s\alpha$.

SER-7 is a type 7 serotonin receptor (5-HT_7). The 5-HT_7 receptor is a G protein coupled receptor that is thought to be coupled to $G_s\alpha$ and $G_{12}\alpha$ (Kvachnina et al., 2005). In favor of the hypothesis that $G_s\alpha$ signaling is downstream of SER-7, heterologous expression of *ser-7* cDNA increased intracellular cAMP level (Hobson et al., 2003), an indication of $G_s\alpha$ signaling. Thus, we hypothesized that active SER-7 in MC and M4 increases feeding rates by activating $G_s\alpha$ and $G_{12}\alpha$ signaling pathways.

We first tested if genetic manipulation of the conserved $G_s\alpha$ signaling pathway (Figure 3-2A) (Reynolds et al., 2005) alters the feeding rate as the hypothesis predicts. GSA-1, ACY-1 and KIN-2 encode $G_s\alpha$, an adenylyl cyclase, and a

regulatory subunit of Protein Kinase A (PKA), respectively (Figure 3-2A). A constitutively active *gsa-1* mutation increased pumping as effectively as serotonin did (Figure 3-2B). Furthermore, null mutations of *gsa-1* and *acy-1* completely abolished and substantially attenuated activation of pumping, respectively (Figure 3-2E and F). We also found that treatment with forskolin, an activator of adenylyl cyclases, activated pumping as effectively as serotonin did (Figure 3-2C). However, an *acy-1* gain-of-function mutation did not activate pumping (Figure 3-2N), suggesting that multiple adenylyl cyclases including ACY-1 act together to activate pumping. *ce179*, a hypomorphic allele of *kin-2*, also increased pharyngeal pumping compared to wild type in presence of serotonin (Figure 3-2D). *ce179* by itself was not sufficient to activate pharyngeal pumping (data not shown), suggesting that the defective KIN-2 bearing the mutation still has some residual activity. In support of this hypothesis *ok248,* a *kin-2* null mutation causes embryonic lethality (Barstead). Next, we confirmed the $G_s\alpha$ signaling pathway shown in (***Figure 3-2***A). Both forskolin treatment (Figure 3-2G) and *ce179* (Figure 3-2H) in part restored the pumping rate in the *gsa-1* null mutant, supporting that activated $G_s\alpha$ indeed acts by activating adenylyl cyclases and PKA in response to serotonin. Together, these data imply that activation of $G_s\alpha$ signaling is necessary and sufficient for serotonin to activate pharyngeal pumping.

Next, we tested if $G_s\alpha$ signaling acts downstream of SER-7. If so, activation of the $G_s\alpha$ signaling pathway should bypass the *ser-7* null mutation block in activating pumping in response to serotonin. As expected, both the constitutively active *gsa-1* mutation (Figure 3-2I) and forskolin treatment (Figure 3-2J) fully restored pharyngeal pumping rate in the *ser-7* null mutant. These data suggest that $G_s\alpha$ signaling indeed acts downstream of SER-7.

Finally, we asked if SER-7 activates $G_s\alpha$ signaling in a cell-autonomous manner by testing if expression of GSA-1 in *flp-21*-expressing cells (9 types of neurons including MC and M4 (Kim and Li, 2004)) is sufficient for normal serotonin-stimulated pumping. Consistent with the hypothesis that SER-7 in MC cell-autonomously stimulates feeding, the rescue construct fully restored pharyngeal pumping in the *gsa-1* null mutant (Figure 3-2E).

Together, these data support the conclusion that SER-7 in MC activates pharyngeal pumping by activating $G_s\alpha$ signaling pathway in a cell-autonomous manner in response to serotonin.

SER-7 serotonin receptor in M4 activates isthmus peristalsis mainly by activating the $G_{12}\alpha$ signaling pathway in a cell-autonomous manner.

Contrary to the dramatic and consistent effect on pumping, the $G_s\alpha$ signaling cascade had a relatively small effect on isthmus peristalsis. Constitutively active *acy-1* was sufficient to increase isthmus peristalsis rate, but the size of the increase was small (Figure 3-2N). *kin-2(ce179)* (Figure 3-2D) and acute expression of heat-shock-inducible constitutively active *gsa-1(Q227L)* (Figure 3-2L) also significantly increased isthmus peristalsis rate in response to serotonin. However, activation of no component of the $G_s\alpha$ signaling pathway by itself increased isthmus peristalsis rate as effectively as serotonin (Figure 3-2K and N). Moreover, isthmus peristalsis in the *gsa-1* null mutant increased in response to serotonin (Figure 3-2M), suggesting that an additional regulatory pathway exists. Since 5-HT_7 receptors also activate $G_{12}\alpha$ (Kvachnina et al., 2005), we hypothesized that $G_s\alpha$ and $G_{12}\alpha$ act in parallel to increase isthmus peristalsis.

We first tested if activation of the $G_{12}\alpha$ signaling pathway increases isthmus peristalsis. *gpa-12*, *rho-1* and *dgk-1* encode $G_{12}\alpha$, the small GTPase RhoA and diacylglycerol kinase components of the $G_{12}\alpha$ signaling cascade, respectively (Figure ***3-3***A) (Hiley et al., 2006; McMullan et al., 2006). In favor of the hypothesis, expression of

constitutively active GPA-12 or RHO-1 in cholinergic neurons was sufficient to increase isthmus peristalsis rate (Figure ***3-3***B and C) in absence of serotonin. A *dgk-1* null mutation also increased the isthmus peristalsis rate greatly in the presence of serotonin (Figure ***3-3***D). It is unclear why activation of $G_{12}\alpha$ signaling by expression of constitutive GPA-12 or constitutive RHO-1 but not by depletion of *dgk-1* activated isthmus peristalsis. Together, these data suggest that active $G_{12}\alpha$ signaling activates isthmus peristalsis.

The *gpa-12* null mutation did not affect isthmus peristalsis rate in response to serotonin, suggesting that a redundant regulatory pathway exists (Fig. 3E). Since activation of $G_s\alpha$ signaling stimulated isthmus peristalsis, we speculated that $G_{12}\alpha$ signaling and $G_s\alpha$ signaling pathways redundantly activate isthmus peristalsis. To test this possibility, we tested if blocking both $G_s\alpha$ and $G_{12}\alpha$ signaling pathways suppress the serotonin-stimulated increase in isthmus peristalsis rate. Indeed, isthmus peristalsis rate was lower in the *gsa-1; gpa-12* null mutant than in *gsa-1* null mutant (Figure ***3-3***F), confirming that $G_s\alpha$ and $G_{12}\alpha$ signaling pathways redundantly stimulate isthmus peristalsis.

We next confirmed that $G_{12}\alpha$ signaling activates isthmus peristalsis through the previously reported pathway (Figure ***3-3***A) (Hiley et al., 2006; McMullan et al., 2006). RHO-1 activates neurotransmission by directly binding to and inhibiting DGK-1 in *C. elegans* (McMullan et al., 2006). We tested if RHO-1 increases isthmus peristalsis by the same

mechanism by testing whether F25N, a mutation of RHO-1 that disrupts the domain essential for DGK-1 inhibition, blocks the G14V (a constitutively active mutation) induced increase in isthmus peristalsis rate. Indeed, *punc-17::*RHO-1(G14VF25N) failed to increase isthmus peristalsis (Figure ***3-3***C). Furthermore, the *dgk-1* null mutation restored the isthmus peristalsis rate of the transgenics expressing *punc-17*::RHO-1(G14VF25N) to the rate of the transgenics expressing *punc-17::rho-1*(G14V) (Figure ***3-3***G), suggesting that RHO-1 activates isthmus peristalsis by inhibiting DGK-1.

Finally, we tested if SER-7 activates isthmus peristalsis by activating $G_{12}\alpha$ signaling in M4 in a cell-autonomous manner. For this, we asked if *pser-7b::* RHO-1(G14V) restores serotonin-stimulated isthmus peristalsis in *gsa-1(gf); ser-7(tm1325*). The construct fully restored isthmus peristalsis (Figure ***3-3***H). Together, these data suggest that SER-7 in M4 (and possibly in M2) activates isthmus peristalsis rate by activating the $G_{12}\alpha$ signaling pathway in a cell-autonomous manner.

The $G_s\alpha$ signaling pathway activates pumping via cholinergic transmission from MC to the pharyngeal muscles.

Activation of either the $G_s\alpha$ signaling or $G_{12}\alpha$ signaling cascade increases neurotransmission in a cell-autonomous manner in *C. elegans* (Hiley et al., 2006; McMullan et al., 2006; Reynolds et al., 2005; Sieburth et al., 2007). To understand by which mechanism MC and M4 activate the pharyngeal muscles to increase feeding rates in response to serotonin, we asked which neurotransmitters mediate the neural stimulation. We first confirmed the previous result (Raizen et al., 1995) that serotonin requires cholinergic transmission from MC to the pharyngeal muscles to increase pumping rate. A hypomorphic *cha-1(p1152)* mutation attenuated pumping in response to serotonin (Figure ***3-4***A). *cha-1* encodes choline acetyltransferase, an enzyme required for biosynthesis of acetylcholine, and *p1152* decreases choline acetyltransferase activity by 99% (Rand and Russell, 1984). Then, we confirmed that the cholinergic transmission from MC to the pharyngeal muscles stimulates pharyngeal pumping by testing whether an *eat-2* null mutation suppresses the serotonin-stimulated pharyngeal pumping. Consistent with the previous report (Raizen et al., 1995), the *eat-2* null mutation suppressed the serotonin-stimulated pumping (Figure ***3-4***C). Based upon our results that SER-7 activated pumping by activating $G_s\alpha$ signaling, we hypothesized that $G_s\alpha$ signaling activates pumping by activating cholinergic

transmission from MC to the pharyngeal muscles. Indeed, the hypomorphic *cha-1(p1152)* and *eat-2* null mutations attenuated or completely suppressed the forskolin-stimulated pumping (Figure ***3-4***C and D), suggesting that the $G_s\alpha$ signaling pathway activated by SER-7 increased pumping by activating acetylcholine release from MC to the pharyngeal muscles.

$G_s\alpha$ and $G_{12}\alpha$ signaling pathways activate isthmus peristalsis via acetylcholine and neuromodulators packaged in dense core vesicles.

Next, we tested if M4 also activates isthmus peristalsis by releasing acetylcholine. We suspected acetylcholine for the following reasons. First, both M4 and M2 express genes necessary for cholinergic transmission (Alfonso et al., 1993). Second, acute treatment with arecoline, an activator of muscarinic acetylcholine receptors, restored isthmus peristalsis in M4- worms (data not shown). We found that *cha-1(p1152)* mutation attenuated the isthmus peristalsis rate in *kin-2(ce179)* (Figure ***3-4***E) but not in wild-type (Figure ***3-4***A) in response to serotonin. This result could be explained in several ways. First, it might be that acetylcholine is indeed the major neurotransmitter by which M4 activates isthmus

peristalsis, but the residual acetylcholine synthesized by the defective CHA-1 barely supports the activation of isthmus peristalsis in wild type animals. As a result, the effect of *cha-1(p1152)* is visible only in *kin-2(ce179),* where isthmus peristalsis occurs more frequently and thus more acetylcholine is required. Consistent with this possible explanation, we found no difference in the isthmus peristalsis rates between *cha-1(p1152); kin-2(ce179)* and *cha-1(p1152)* mutant animals (Figure ***3-4***E).

Alternatively, but not mutually exclusively, M4 might activate isthmus peristalsis by releasing additional neurotransmitters. To test this possibility, we examined whether isthmus peristalsis rate is attenuated in *unc-31* and *unc-13* null mutants. Neurotransmitters are secreted from neurons through two types of vesicles, synaptic vesicles and dense core vesicles. Classical neurotransmitters such as GABA, acetylcholine and glutamate are packaged in synaptic vesicles whereas dense core vesicles contain neuropeptides and monoamines. *unc-13* and *unc-31* null mutations selectively yet completely block exocytosis of synaptic vesicles and dense core vesicles, respectively (Richmond et al., 2001; Zhou et al., 2007). The *unc-13* result was not informative. Although *unc-13* did not decrease isthmus peristalsis rate in presence of serotonin, isthmus peristalsis in the *unc-13* mutant was not affected by serotonin (data not shown).

In contrast, the *unc-31* null mutation substantially attenuated isthmus peristalsis in response to serotonin (Figure ***3-4***F), suggesting that the neuromodulators packaged in dense core vesicles control isthmus peristalsis rate.

Next, we tested if $G_{12}\alpha$ signaling activates isthmus peristalsis by releasing dense core vesicles. As expected, an *unc-31* null mutation completely suppressed the increased isthmus peristalsis rate in the transgenics expressing constitutively active GPA-12 in cholinergic neurons (Figure ***3-4***G). Since SER-7 and $G_{12}\alpha$ signaling act in M4, we asked if dense core vesicles released from M4 are responsible for activating isthmus peristalsis. However, *pser-7b::unc-31(+)* failed to restore isthmus peristalsis rate in the *unc-31* mutant (Figure ***3-4***H), suggesting that UNC-31 acts in the cells that are regulated by M4 or in cells that act in parallel with M4. Considering that M4 synapses directly onto the isthmus muscle, it is likely that UNC-31 acts in a parallel pathway (Figure 3-5B).

Together, these data imply that both releases of acetylcholine and dense core vesicles contribute to activating isthmus peristalsis. The release of acetylcholine is activated by the $G_s\alpha$ signaling pathway but the site of action is unidentified. Which neurotransmitter M4 uses to activate isthmus peristalsis in response to serotonin is unclear, but dense core vesicle release from unknown cell(s) is essential in addition to the activation by M4.

DISCUSSION

Here we show that serotonin activates the two feeding motions in *C. elegans*, pharyngeal pumping and isthmus peristalsis. Like food, serotonin separately activates pumping and isthmus peristalsis mainly by activating SER-7 serotonin receptor in MC and M4 (and possibly M2), respectively. Activated SER-7 in MC and M4 activates neurotransmission mainly by activating $G_s\alpha$ and $G_{12}\alpha$ signaling pathways, respectively in a cell-autonomous manner (Figure 3-5A and B). The cholinergic transmission from MC to the pharyngeal muscles is the major trigger to activate pharyngeal pumping (Figure 3-5A). In contrast, activation of isthmus peristalsis may require more than activation of M4 (Figure 3-5B). Restoring SER-7 or activating $G_{12}\alpha$ in M4 fully restored isthmus peristalsis rate in the mutants lack of *ser-7*, supporting that M4 is the major neuron by which serotonin activates isthmus peristalsis. However, restoring UNC-31 (required for serotonin-stimulated isthmus peristalsis) in M4 failed to restore isthmus peristalsis in the *unc-31* null mutant. Although it is still possible that M4 stimulates isthmus peristalsis by releasing dense core vesicles, the result suggests that activating isthmus peristalsis requires dense core vesicle release from unidentified cells in addition to M4 activation. Considering SER-7 mainly acts in M4 to activate isthmus peristalsis in response to serotonin, it is possible that secretion of dense core vesicles from the unidentified cells may be constitutively active rather than being

triggered by serotonin. The $G_s\alpha$ pathway, which contributes to the stimulation of isthmus peristalsis via cholinergic transmission (Figure 3-5B), may cell-autonomously act as a downstream of SER-7 in M4 in parallel with the $G_{12}\alpha$ pathway but the possibility has not been tested (Figure 3-5C).

Our discovery that serotonin activates MC and M4 by activating $G_s\alpha$ and $G_{12}\alpha$ signaling pathways, together with previous studies (McMullan et al., 2006; Reynolds et al., 2005), suggests that the mechanism by which G protein signaling pathways increase neuronal activity is widely conserved throughout the nervous system in *C. elegans*.

How do $G_s\alpha$ and $G_{12}\alpha$ signaling cascades activate neurotransmission? Several studies (Aravamudan and Broadie, 2003; McMullan et al., 2006; Reynolds et al., 2005) suggested that PKA and diacylglycerol, downstream of $G_s\alpha$ and $G_{12}\alpha$ signaling cascades (Jose and Koelle, 2005), enhance synaptic efficacy by increasing the abundance of UNC-13 at the neuromuscular junction. UNC-13 is a primary determinant of synaptic vesicle priming and neurotransmitter release probability. However, several observations suggest that $G_s\alpha$ signaling in MC may activate pumping by a different mechanism that increases the firing rate (Raizen and Avery, 1994). An MC-dependent depolarization of pharyngeal muscle precedes every pump during normal fast pumping. Additionally, incubation of the pharynx with nicotine, an agonist of the nicotinic acetylcholine receptor, increased the duration but not frequency of pharyngeal contractions,

suggesting that MC increases the pumping rate by increasing its firing rate. To increase pumping rate in response to serotonin, $G_s\alpha$ signaling may increase the firing rate of MC by modulation of ion channel activity. In fact, we observed that a null mutation of *shl-1*, a voltage-gated A type potassium channel (Fawcett et al., 2006), dramatically increased pharyngeal pumping (data not shown). The inhibition of mammalian voltage-gated A type potassium channels by PKA (Schrader et al., 2002) and the expression of *shl-1* in MC (Fawcett et al., 2006) suggest the possibility that $G_s\alpha$ signaling may stimulate pharyngeal pumping by inhibiting SHL-1. Another interesting question to be solved is why SER-7 in MC and M4 activated the two neurons mainly by activating two distinct G protein signaling cascades. It may be because components of $G_{12}\alpha$ are not expressed in MC. It is notable that expression of constitutively active RHO-1 in cholinergic neurons did not alter pharyngeal pumping rate (Figure 3-3C).

We also found that, despite their separate neuronal regulation, isthmus peristalsis was coupled to the preceding pump. How are the two feeding motions coupled? A previous calcium imaging study reported that an anterior-to-posterior calcium wave travels in the posterior isthmus selectively during isthmus peristalsis (Shimozono et al., 2004), suggesting that the calcium wave dictates the muscle contraction. When a pump occurs, the electrical signal that excites the pharyngeal muscles to elicit pumping travels from the corpus to the terminal bulb (Raizen and Avery, 1994;

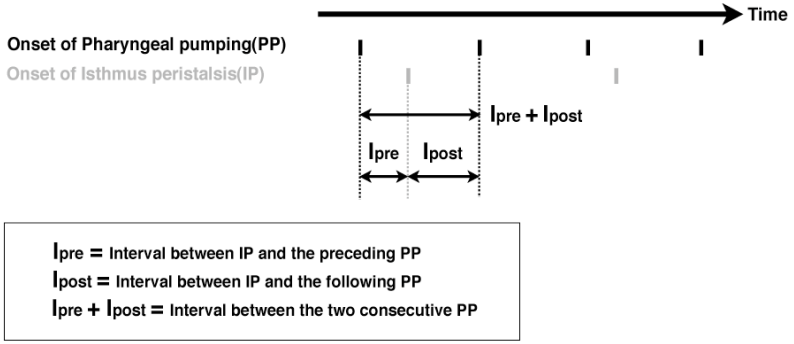
Shimozono et al., 2004). The observations that M4 killing results in failure of isthmus peristalsis (Avery and Horvitz, 1987) and that peristalsis requires pumping suggest that induction of the calcium wave that causes peristalsis requires both muscle excitation during pumping and stimulation by M4 (Figure 3-5B and C). This model explains how the feeding motions can be separately regulated yet coupled.

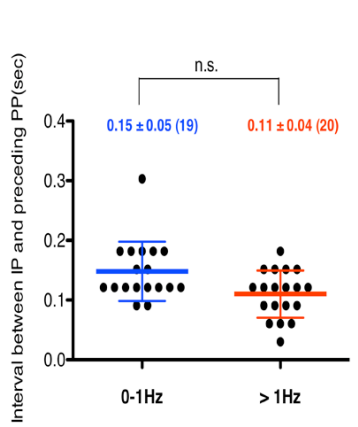
The pharynx may have evolved to regulate feeding motions in this way to support efficient feeding. Separate regulation of the two feeding rates allows worms to adjust the ratio of isthmus peristalsis to pharyngeal pumping (IP-to-PP-ratio) according to the density of food. When food is scarce, the IP-to-PP-ratio would decrease because worms need to take in food as actively as possible, but they do not need to swallow frequently. When food is abundant, however, the ratio would increase because worms do not need to work so hard to take in food, but they need to swallow frequently so that the anterior pharynx does not become full. In fact, we observed that the average IP-to-PP-ratio tends to be higher when the pharynx pumps slowly (Figure 3-1C). Furthermore, the regulators of feeding that we found can be classified into two groups: The first group, such as components of the $G_s\alpha$ or *dgk-1* signaling pathways increases both feeding rates when activated or inhibited. The second group, such as *pkc-1* (data not shown) and *unc-31* (Figure

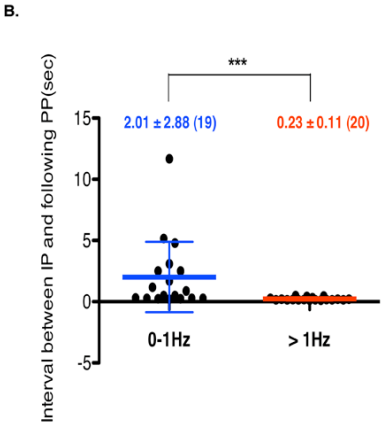
3-4F), decreased isthmus peristalsis but increased pharyngeal pumping when suppressed. To adjust the IP-to-PP-ratio according to food density, activity of the second group may be selectively suppressed when food is scarce.

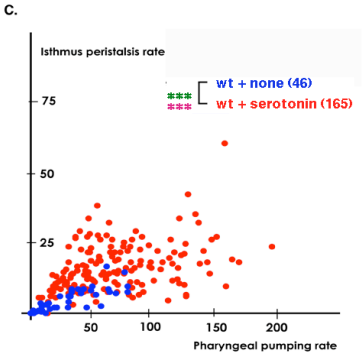
The coupling of the two feeding motions also supports efficient feeding by coordinating them. Food accumulated by pumping is transported from the anterior isthmus to the grinder in the terminal bulb by isthmus peristalsis. Thus, isthmus peristalsis would be futile in absence of pumping and the coupling of isthmus peristalsis to the preceding pump may serve to prevent such futile feeding motions.

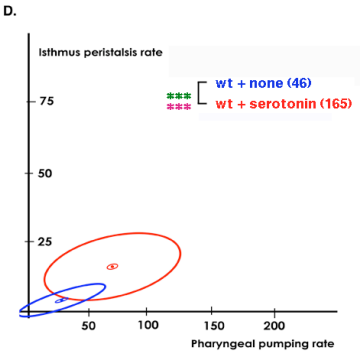
Several interesting questions remain to be answered. For instance, by what mechanisms are the frequency and timing of onset of the feeding motions determined? How does a single isthmus muscle show two different feeding motions? Further studies to answer these questions may lead us to understand how nervous system and muscle interact for sophisticated regulation of muscle motions.

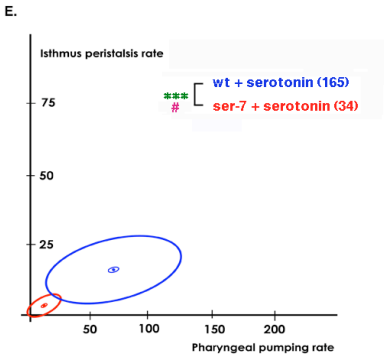

Time
Onset of Pharyngeal pumping(PP)
Onset of Isthmus peristalsis(IP)
Ipre + Ipost
Ipre
Ipost
Ipre = Interval between IP and the preceding PP
Ipost = Interval between IP and the following PP
Ipre + Ipost = Interval between the two consecutive PP

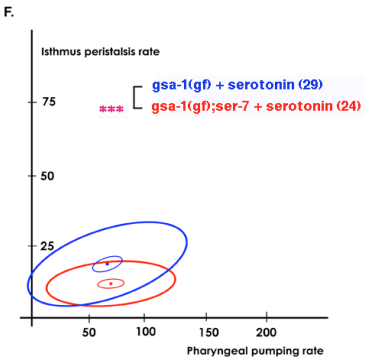

A.
n.s.
0.15 ± 0.05 (19)
0.11 ± 0.04 (20)
Interval between IP and preceding PP(sec)
0.4
0.3
0.2
0.1
0.0
0-1Hz
> 1Hz


B.
***
2.01 ± 2.88 (19)
0.23 ± 0.11 (20)
Interval between IP and following PP(sec)
15
10
5
0
-5
0-1Hz
> 1Hz


C.
Isthmus peristalsis rate
wt + none (46)
wt + serotonin (165)
75
50
25
50
100
150
200
Pharyngeal pumping rate


D.
Isthmus peristalsis rate
wt + none (46)
wt + serotonin (165)
75
50
25
50
100
150
200
Pharyngeal pumping rate


E.
Isthmus peristalsis rate
wt + serotonin (165)
ser-7 + serotonin (34)
75
50
25
50
100
150
200
Pharyngeal pumping rate


F.
Isthmus peristalsis rate
gsa-1(gf) + serotonin (29)
gsa-1(gf);ser-7 + serotonin (24)
75
50
25
50
100
150
200
Pharyngeal pumping rate

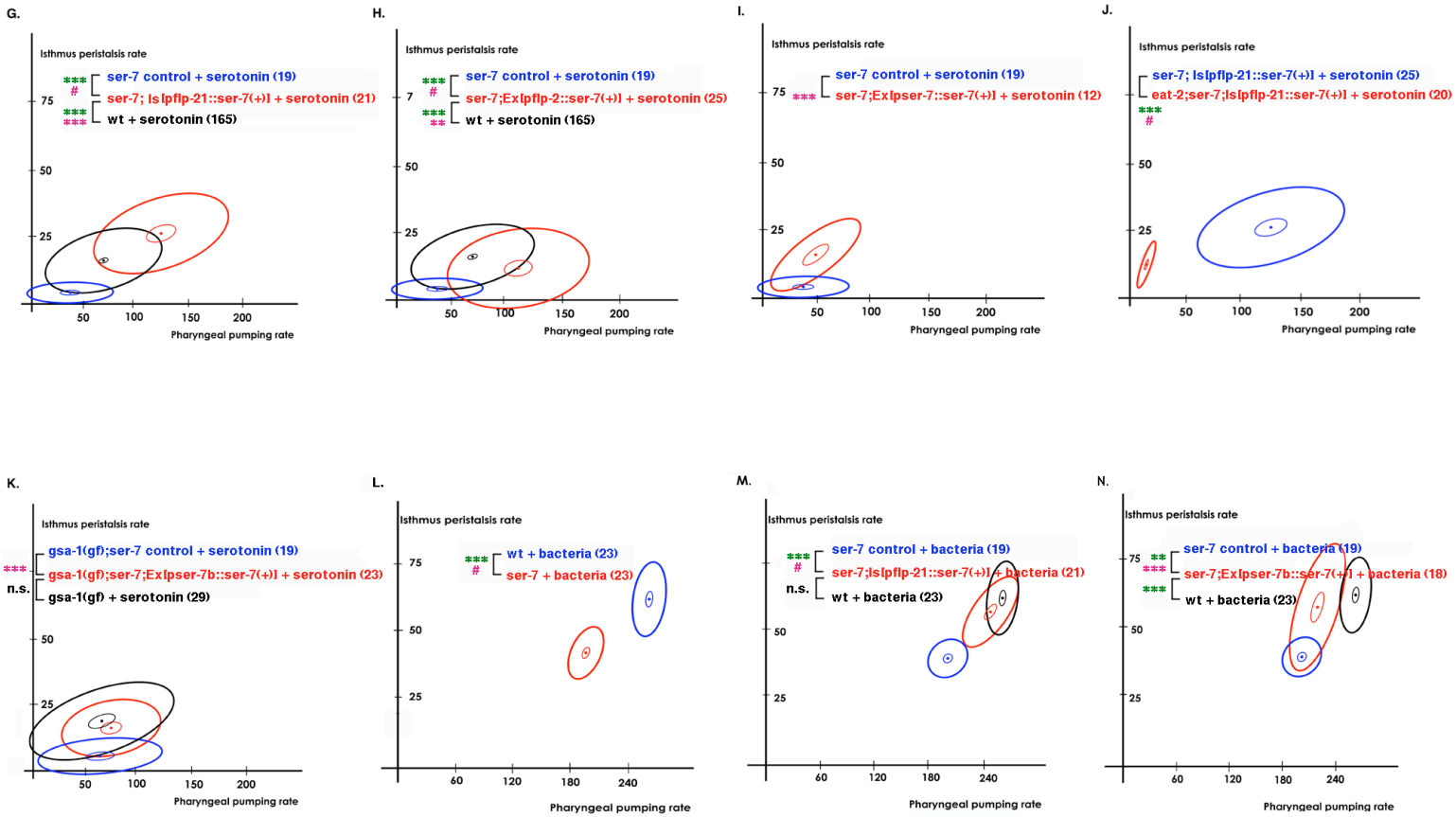
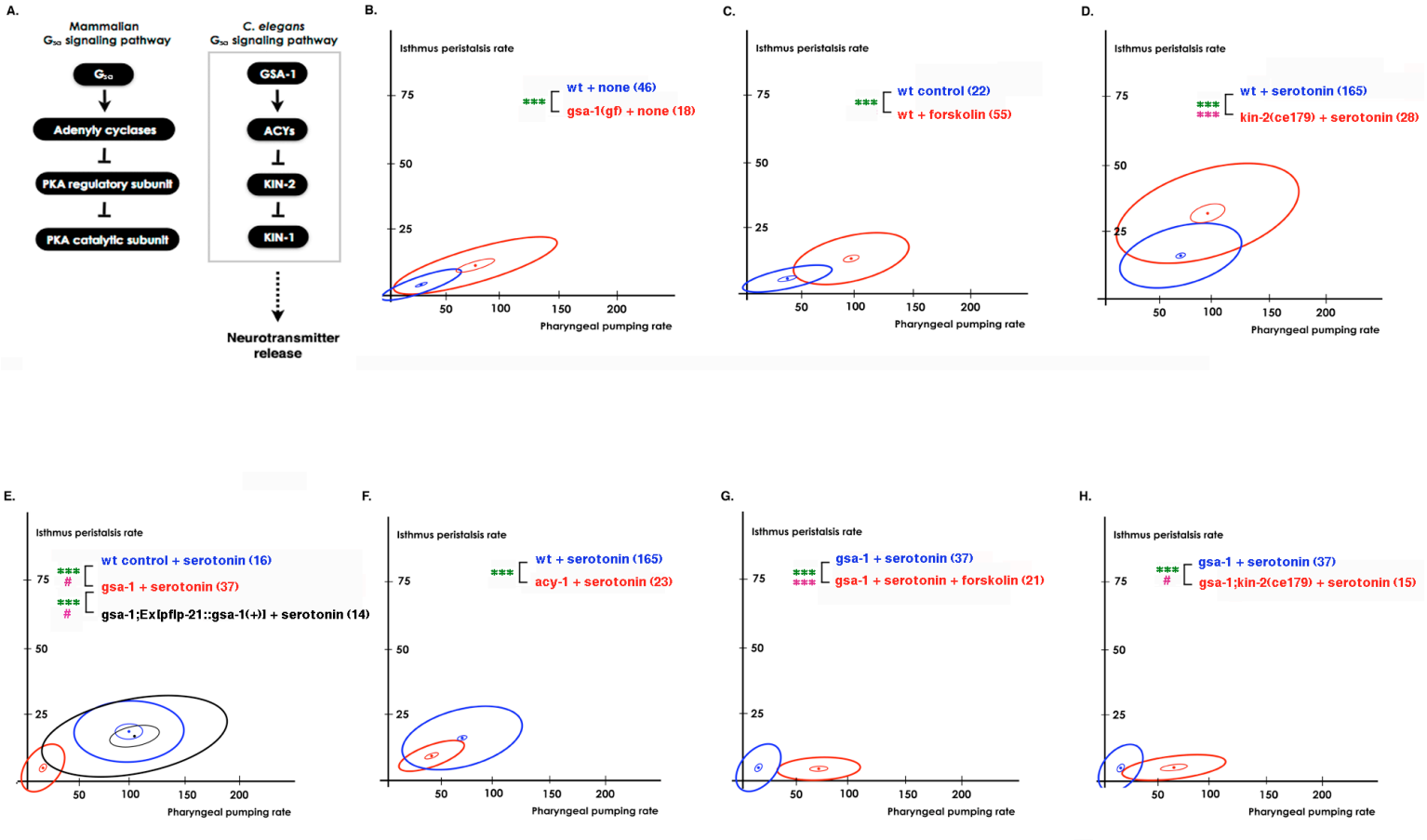
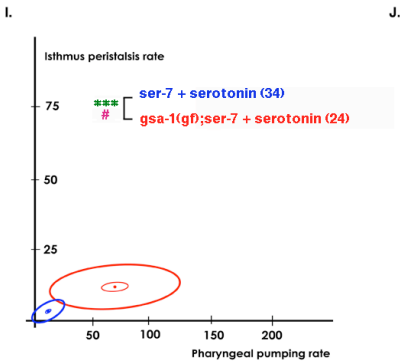

G.
Isthmus peristalsis rate
*** # ser-7 control + serotonin (19)
ser-7; lsIpflp-21::ser-7(+)l + serotonin (21)
*** *** wt + serotonin (165)
Pharyngeal pumping rate
H.
Isthmus peristalsis rate
*** # ser-7 control + serotonin (19)
ser-7;Exlpflp-2::ser-7(+)l + serotonin (25)
*** ** wt + serotonin (165)
Pharyngeal pumping rate
I.
Isthmus peristalsis rate
*** ser-7 control + serotonin (19)
ser-7;Exlpser-7::ser-7(+)l + serotonin (12)
Pharyngeal pumping rate
J.
Isthmus peristalsis rate
ser-7; lsIpflp-21::ser-7(+)l + serotonin (25)
eat-2;ser-7;lsIpflp-21::ser-7(+)l + serotonin (20)
*** #
Pharyngeal pumping rate
K.
Isthmus peristalsis rate
gsa-1(gf);ser-7 control + serotonin (19)
*** gsa-1(gf);ser-7;Exlpser-7b::ser-7(+)l + serotonin (23)
n.s. gsa-1(gf) + serotonin (29)
Pharyngeal pumping rate
L.
Isthmus peristalsis rate
*** # wt + bacteria (23)
ser-7 + bacteria (23)
Pharyngeal pumping rate
M.
Isthmus peristalsis rate
*** # ser-7 control + bacteria (19)
ser-7;lsIpflp-21::ser-7(+)l + bacteria (21)
n.s. wt + bacteria (23)
Pharyngeal pumping rate
N.
Isthmus peristalsis rate
** *** ser-7 control + bacteria (19)
ser-7;Exlpser-7b::ser-7(+)l + bacteria (18)
*** wt + bacteria (23)
Pharyngeal pumping rate

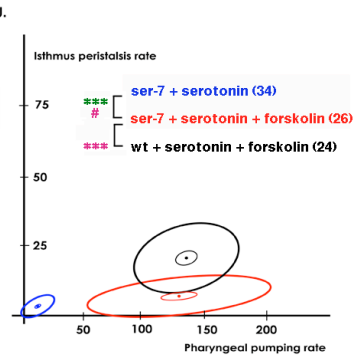
Figure 3-1. Serotonin activates both pharyngeal pumping and isthmus peristalsis. SER-7 in MC and M4 (and possibly M2) separately activates pumping and isthmus peristalsis, respectively. Despite separate regulation, isthmus peristalsis is coupled to the preceding pump. (A-B) Isthmus peristalsis(IP) was selectively coupled to the preceding (A) but not to the following (B) pharyngeal pump (PP) in wild type worms. Each filled circle indicates the interval between isthmus peristalsis and the indicated pump in one worm. *** $P<0.001$; Spearman's correlation test, n.s. indicates not significant ($P\geq0.05$), data shown as the mean ± standard deviation (C-D) Serotonin activated both pumping and isthmus peristalsis. (C) A scatter plot of feeding rates of wild type(wt) with or without serotonin treatment. Each dot represents one worm. Thus, the x and y values of each dot indicate pumping rate and isthmus peristalsis rate, respectively, of one worm. (D) For simplicity, we transformed scatter plots into ellipse plots. The central dot is plotted at the mean pumping and isthmus peristalsis rate for each tested strain. The large ellipse, a plot of root-mean-square deviation from the mean, shows the extent of variation from animal to animal. The width of the ellipse is $\sqrt{2}$ times the standard deviation of pumping rate, the height is $\sqrt{2}$ times the standard deviation of isthmus peristalsis rate, and narrowness or breadth is related to correlation. For normally distributed data, 63% of data points would be within the ellipse. The small ellipse similarly shows the likely range of the mean. (E) The *ser-7(tm1325)* null

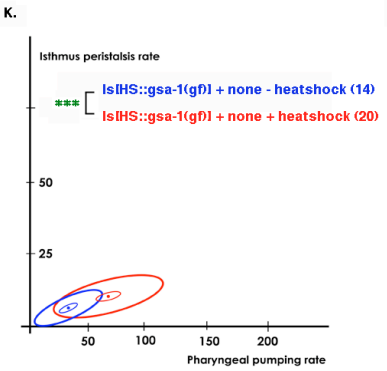
mutant was defective in activating pharyngeal pumping in response to serotonin. (F) The *ser-7* null mutation decreased isthmus peristalsis rate in the *gsa-1(ce81)* gain of function mutant in response to serotonin. (G-I) SER-7 in MC cell-autonomously activates pharyngeal pumping in response to serotonin. (G-H) Expression of *ser-7* cDNA using the *flp-21* promoter(G) or the *flp-2* promoter(H) fully restored pumping in the ser-7 null mutant in response to serotonin. The *pflp-2::ser-7(+)* only partly restored isthmus peristalsis rate (H). This might be because the *flp-2* promoter drives a weak and occasional expression in M4 (Kim and Li, 2004). (I) Expression of *ser-7* cDNA using the *ser-7* promoter increased isthmus peristalsis rate but not pumping rate in the *ser-7* null mutant in response to serotonin. (J) The *eat-2(ad465)* null mutation that specifically blocks cholinergic transmission from MC to pharyngeal muscles suppressed the rescue effect of *pflp-21::ser-7(+)* in the *ser-7* mutant in response to serotonin. Pharyngeal pumping rate was lower in *eat-2(ad465); ser-7(tm1325)* than in *eat-2(ad465) or ser-7(tm1325)* (Figure 4-3c). These data suggest that acetylcholine marginally activates pumping in an eat-2-independent manner and that there is residual acetylcholine release in absence of SER-7 in response to serotonin. (K) SER-7 acts mainly in M4 to stimulate isthmus peristalsis in response to serotonin. (L) Both feeding rates of the ser-7 null mutant decreased to 75% of wild type animals in response to familiar bacteria, a physiologically relevant stimulant of feeding. (M) As seen in response to serotonin,

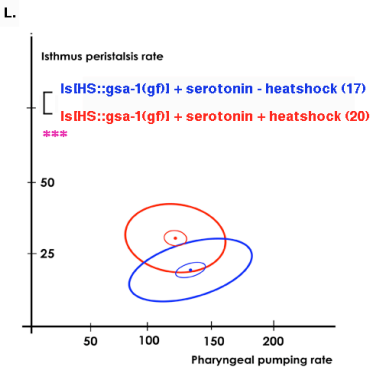
expression of ser-7 cDNA from the flp-21 promoter fully restored pumping in the ser-7 null mutant in response to familiar bacteria. (N) Expression of ser-7 cDNA in M4 fully restored isthmus peristalsis rate in the ser-7 null mutant with a small effect on pharyngeal pumping in response to familiar bacteria. *** $P<0.001$ ** $P<0.01$ n.s. not significant ($P>0.05$); unpaired t test (two-tailed test) was used for comparison of pumping rates and the level of significance is indicated by green asterisks, isthmus peristalsis rates were compared as described in methods and the level of significance is indicated by asterisks in pink, # Comparison of isthmus peristalsis rates was infeasible due to lack of overlap in pumping rates between the two samples compared. The number of animals tested ($n\geq3$ independent experiments per each group) is shown in parentheses. For the comparisons with the transgenics expressing rescue constructs, transgenic animals expressing only co-injection marker(s) were used as controls.

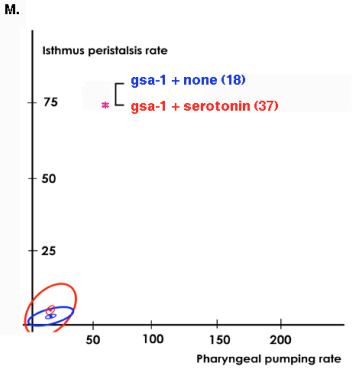

A.
Mammalian
$G_{s\alpha}$ signaling pathway
$G_{s\alpha}$
Adenyly cyclases
PKA regulatory subunit
PKA catalytic subunit
C. elegans
$G_{s\alpha}$ signaling pathway
GSA-1
ACYs
KIN-2
KIN-1
Neurotransmitter release
B.
Isthmus peristalsis rate
wt + none (46)
gsa-1(gf) + none (18)
Pharyngeal pumping rate
C.
Isthmus peristalsis rate
wt control (22)
wt + forskolin (55)
Pharyngeal pumping rate
D.
Isthmus peristalsis rate
wt + serotonin (165)
kin-2(ce179) + serotonin (28)
Pharyngeal pumping rate
E.
Isthmus peristalsis rate
wt control + serotonin (16)
gsa-1 + serotonin (37)
gsa-1;Ex[pflp-21::gsa-1(+)] + serotonin (14)
Pharyngeal pumping rate
F.
Isthmus peristalsis rate
wt + serotonin (165)
acy-1 + serotonin (23)
Pharyngeal pumping rate
G.
Isthmus peristalsis rate
gsa-1 + serotonin (37)
gsa-1 + serotonin + forskolin (21)
Pharyngeal pumping rate
H.
Isthmus peristalsis rate
gsa-1 + serotonin (37)
gsa-1;kin-2(ce179) + serotonin (15)
Pharyngeal pumping rate


I.
Isthmus peristalsis rate
75
50
25
50
100
150
200
Pharyngeal pumping rate
*** #
ser-7 + serotonin (34)
gsa-1(gf);ser-7 + serotonin (24)


J.
Isthmus peristalsis rate
75
50
25
50
100
150
200
Pharyngeal pumping rate
*** #
ser-7 + serotonin (34)
ser-7 + serotonin + forskolin (26)
***
wt + serotonin + forskolin (24)


K.
Isthmus peristalsis rate
50
25
50
100
150
200
Pharyngeal pumping rate
***
IsIHS::gsa-1(gf)I + none - heatshock (14)
IsIHS::gsa-1(gf)I + none + heatshock (20)


L.
Isthmus peristalsis rate
50
25
50
100
150
200
Pharyngeal pumping rate
IsIHS::gsa-1(gf)I + serotonin - heatshock (17)
IsIHS::gsa-1(gf)I + serotonin + heatshock (20)
***


M.
Isthmus peristalsis rate
75
50
25
50
100
150
200
Pharyngeal pumping rate
*
gsa-1 + none (18)
gsa-1 + serotonin (37)

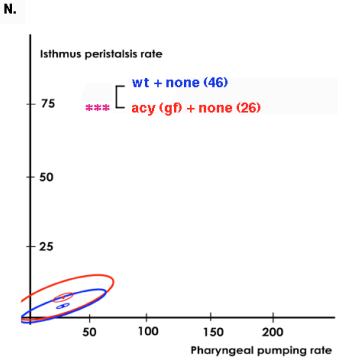

N.
Isthmus peristalsis rate
75
50
25
50
100
150
200
Pharyngeal pumping rate
***
wt + none (46)
acy (gf) + none (26)

Figure 3-2. SER-7 activates pharyngeal pumping by activating the $G_s\alpha$ signaling pathway in response to serotonin. Activation of $G_s\alpha$ signaling has a small effect in activating isthmus peristalsis. (A) Schematic of the $G_s\alpha$ signaling pathway in *C. elegans.* (B) The constitutively active *gsa-1(ce81)* mutation activated pumping as effectively as serotonin did. (C) Activation of adenylyl cyclases by forskolin treatment activated pumping as effectively as serotonin did. (D) *ce179,* a hypomorphic allele of kin-2, increased both feeding rates in response to serotonin. (E) The *gsa-1(pk75)* null mutation completely suppressed the serotonin-stimulated pharyngeal pumping. Expression of *gsa-1* using the *flp-21* promoter fully restored the pumping rate in the *gsa-1(pk75)* null mutant in response to serotonin. (F) An *acy-1(pk1279)* null mutation attenuated pumping in response to serotonin. (G-H) Forskolin (G) and *ce179* (H), a hypomorphic allele of *kin-2*, increased pumping in the *gsa-1(pk75)* null mutant in response to serotonin. (I-J) Activation of $G_s\alpha$ signaling by constitutively active *gsa-1(ce81)* (I) or by forskolin treatment(J) fully restored pumping in the *ser-7(tm1325)* null mutant in response to serotonin. (K) Acute expression of constitutively active GSA-1(Q227L) using heat shock was sufficient to activate pharyngeal pumping, but not isthmus peristalsis in absence of serotonin. (L) Acute expression of constitutively active GSA-1(Q227L) using heat shock activated both feeding motions in response to serotonin. (M) The *gsa-1(pk75)* null mutation failed to suppress serotonin-stimulated isthmus peristalsis. (N) The

constitutively active acy-1(ce2) mutation activated isthmus peristalsis but not pumping in the absence of serotonin. *** P<0.001 * P<0.05 n.s. not significant (P>0.05) ; The unpaired t test (two-tailed test) was used for comparison of pumping rates and the difference indicated in green, isthmus peristalsis rates were compared as described in methods and the level of significance is indicated by asterisks in pink, # Comparison of isthmus peristalsis rates was infeasible due to lack of overlap in pumping rates between the two samples compared. The number of animals tested (n≥3 independent assays per each group) is shown in parenthesis.

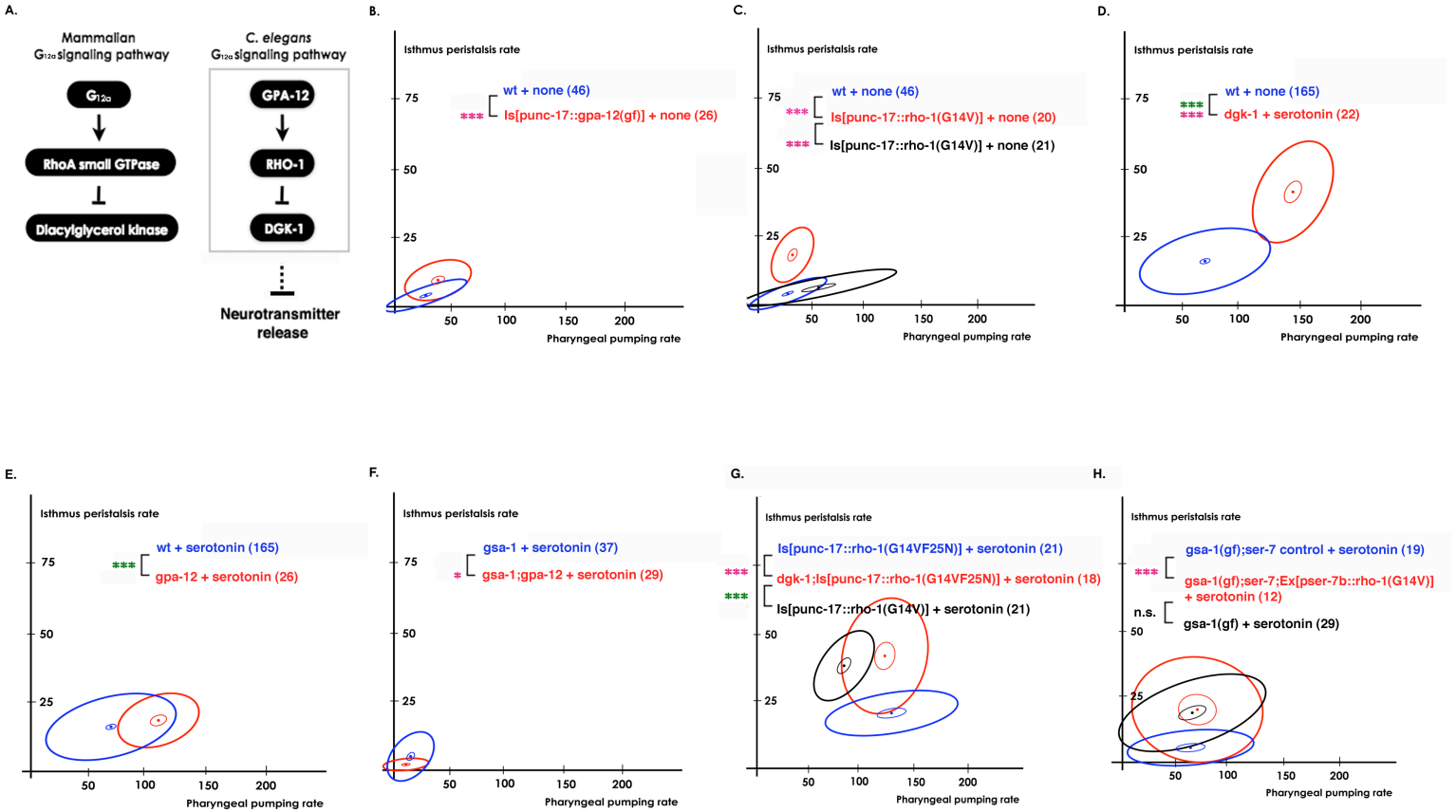

A.
Mammalian
$G_{12\alpha}$ signaling pathway
$G_{12\alpha}$
RhoA small GTPase
Diacylglycerol kinase
C. elegans
$G_{12\alpha}$ signaling pathway
GPA-12
RHO-1
DGK-1
Neurotransmitter release
B.
Isthmus peristalsis rate
wt + none (46)
*** ls[punc-17::gpa-12(gf)] + none (26)
Pharyngeal pumping rate
C.
Isthmus peristalsis rate
wt + none (46)
*** ls[punc-17::rho-1(G14V)] + none (20)
*** ls[punc-17::rho-1(G14V)] + none (21)
Pharyngeal pumping rate
D.
Isthmus peristalsis rate
*** wt + none (165)
*** dgk-1 + serotonin (22)
Pharyngeal pumping rate
E.
Isthmus peristalsis rate
wt + serotonin (165)
*** gpa-12 + serotonin (26)
Pharyngeal pumping rate
F.
Isthmus peristalsis rate
gsa-1 + serotonin (37)
* gsa-1;gpa-12 + serotonin (29)
Pharyngeal pumping rate
G.
Isthmus peristalsis rate
ls[punc-17::rho-1(G14VF25N)] + serotonin (21)
*** dgk-1;ls[punc-17::rho-1(G14VF25N)] + serotonin (18)
*** ls[punc-17::rho-1(G14V)] + serotonin (21)
Pharyngeal pumping rate
H.
Isthmus peristalsis rate
gsa-1(gf);ser-7 control + serotonin (19)
*** gsa-1(gf);ser-7;Ex[pser-7b::rho-1(G14V)] + serotonin (12)
n.s. gsa-1(gf) + serotonin (29)
Pharyngeal pumping rate

Figure 3-3. SER-7 activates isthmus peristalsis by activating $G_{12}\alpha$ signaling in M4. (A) Schematic of the $G_{12}\alpha$ signaling pathway in *C. elegans.* (B-C) Expression of constitutively *gpa-12(Q205L)*(B) or constitutively active *rho-1(G14V)*(C) in cholinergic neurons was sufficient to activate isthmus peristalsis in the absence of serotonin. (C) The F25N mutation completely suppressed the increase in isthmus peristalsis rate caused by the constitutively active *rho-1(G14V)* mutation. (D) The *dgk-1(sy428)* null mutation increased both feeding rates in response to serotonin. (E-F) The $G_{12}\alpha$ and $G_s\alpha$ signaling pathways act redundantly to activate isthmus peristalsis in response to serotonin. The *gsa-1(pk75); gpa-12(pk322)* double null mutations(F), but not the *gpa-12(pk322)* single null mutation(E), decreased isthmus peristalsis in response to serotonin. (G) *dgk-1(sy428)* fully restored isthmus peristalsis rate in the transgenic animals expressing *rho-1(F25NG14V)* in cholinergic neurons to the level of the transgenics expressing *rho-1(G14V)* in response to serotonin. (H) Expression of constitutively active *rho-1(G14V)* in M4(M2) fully restored isthmus peristalsis in *gsa-1(ce81);ser-7(tm1325)* in response to serotonin. The number of animals tested ($n \geq 3$ independent assays per each group) is shown in parenthesis. *** $P<0.001$ * $P<0.05$ n.s. not significant ($P>0.05$) ; unpaired t test (two-tailed test) was used for comparison of pharyngeal pumping rates and the difference was marked in green, isthmus peristalsis rates were compared as described in methods and the level of significance is indicated by asterisks in pink, the number of

animals tested (n≥3 independent assays per each group) is shown in parenthesis. For the comparisons with the transgenics expressing rescue constructs, the transgenic animals expressing only co-injection marker(s) were used as controls.

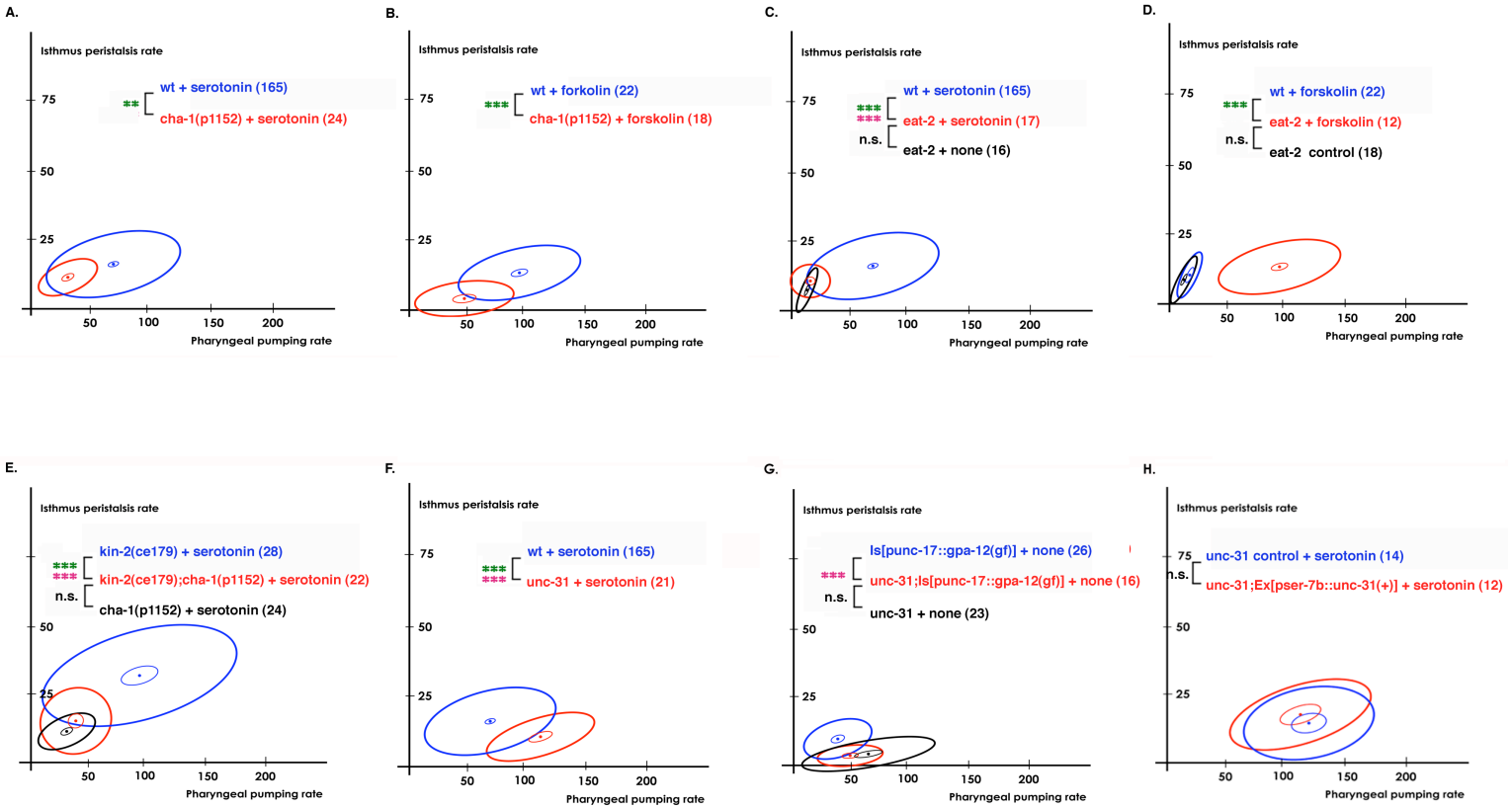

A.
Isthmus peristalsis rate
75
50
25
50
100
150
200
Pharyngeal pumping rate
**
wt + serotonin (165)
cha-1(p1152) + serotonin (24)
B.
Isthmus peristalsis rate
75
50
25
50
100
150
200
Pharyngeal pumping rate
***
wt + forkolin (22)
cha-1(p1152) + forskolin (18)
C.
Isthmus peristalsis rate
75
50
25
50
100
150
200
Pharyngeal pumping rate
***
***
wt + serotonin (165)
eat-2 + serotonin (17)
n.s.
eat-2 + none (16)
D.
Isthmus peristalsis rate
75
50
25
50
100
150
200
Pharyngeal pumping rate
***
wt + forskolin (22)
eat-2 + forskolin (12)
n.s.
eat-2 control (18)
E.
Isthmus peristalsis rate
50
25
50
100
150
200
Pharyngeal pumping rate
***
***
kin-2(ce179) + serotonin (28)
kin-2(ce179);cha-1(p1152) + serotonin (22)
n.s.
cha-1(p1152) + serotonin (24)
F.
Isthmus peristalsis rate
75
50
25
50
100
150
200
Pharyngeal pumping rate
***
***
wt + serotonin (165)
unc-31 + serotonin (21)
G.
Isthmus peristalsis rate
50
25
50
100
150
200
Pharyngeal pumping rate
***
ls[punc-17::gpa-12(gf)] + none (26)
unc-31;ls[punc-17::gpa-12(gf)] + none (16)
n.s.
unc-31 + none (23)
H.
Isthmus peristalsis rate
75
50
25
50
100
150
200
Pharyngeal pumping rate
n.s.
unc-31 control + serotonin (14)
unc-31;Ex[pser-7b::unc-31(+)] + serotonin (12)

Figure 3-4. $G_s\alpha$ signaling increased pumping by activating acetylcholine transmission from MC to the pharyngeal muscles. $G_s\alpha$ signaling and $G_{12}\alpha$ signaling increased isthmus peristalsis by causing the releases of acetylcholine and dense core vesicle, respectively. (A-B) The hypomorphic *cha-1(p1152)* mutation attenuated pumping in response to serotonin (A) or forskolin (B). (C-D) The *eat-2(ad465)* null mutation suppressed the increase in pumping induced by serotonin (C) or by forskolin (D). (E) *cha-1(p1152)* attenuated the *kin-2(ce179)*-stimulated isthmus peristalsis. (F) The *unc-31(e928)* null mutation attenuated isthmus peristalsis rate in response to serotonin. (G) The *unc-31(e928)* null mutation completely suppressed the cholinergic GPA-12(Q205L)-stimulated isthmus peristalsis. (H) Expression of *unc-31* cDNA in M4 failed to restore isthmus peristalsis rate in the *unc-31(e928)* mutant. The number of animals tested ($n\geq3$ independent assays per each group) is shown in parenthesis. *** $P<0.001$ ** $P<0.01$ n.s. not significant ($P>0.05$); unpaired t test (two-tailed test) was used for comparison of pharyngeal pumping rates and the difference was marked in green, isthmus peristalsis rates were compared as described in methods and the level of significance is indicated by asterisks in pink, the number of animals tested ($n\geq3$ independent assays per each group) is shown in parenthesis. For the comparisons with the transgenics expressing rescue constructs, the transgenic animals expressing only co-injection marker(s) were used as controls.

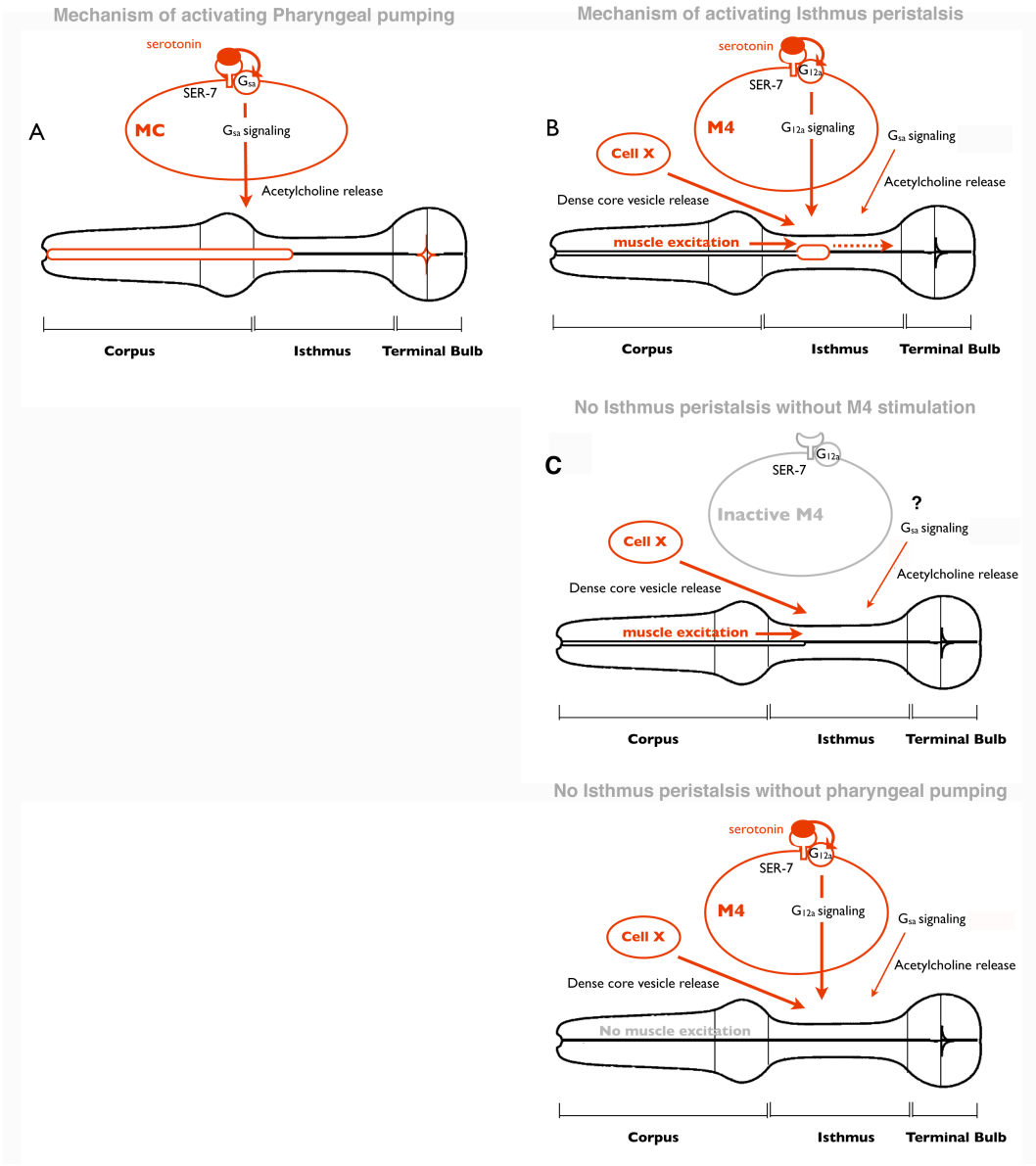

Mechanism of activating Pharyngeal pumping
serotonin
SER-7
G_{sa}
A
MC
G_{sa} signaling
Acetylcholine release
Corpus
Isthmus
Terminal Bulb
Mechanism of activating Isthmus peristalsis
serotonin
SER-7
G_{12a}
B
M4
G_{12a} signaling
G_{sa} signaling
Cell X
Acetylcholine release
Dense core vesicle release
muscle excitation
Corpus
Isthmus
Terminal Bulb
No Isthmus peristalsis without M4 stimulation
C
SER-7
G_{12a}
Inactive M4
?
G_{sa} signaling
Cell X
Acetylcholine release
Dense core vesicle release
muscle excitation
Corpus
Isthmus
Terminal Bulb
No Isthmus peristalsis without pharyngeal pumping
serotonin
SER-7
G_{12a}
M4
G_{12a} signaling
G_{sa} signaling
Cell X
Acetylcholine release
Dense core vesicle release
No muscle excitation
Corpus
Isthmus
Terminal Bulb

Figure 3-5. Model of regulation of feeding by serotonin. (A) In response to serotonin, SER-7 in MC cell-autonomously activates its downstream $G_s\alpha$ signaling pathway, which subsequently stimulates pharyngeal pumping by activating cholinergic transmission from MC to the pharyngeal muscles. (B) In response to serotonin, SER-7 in M4 (and possibly in M2) activates its downstream $G_{12}\alpha$ pathways in a cell autonomous manner, which subsequently activates M4. The stimulus from active M4, along with dense core vesicle release that is caused by an unidentified pathway, activates isthmus peristalsis. $G_s\alpha$ signaling pathway and its downstream cholinergic transmission also contribute to activating isthmus peristalsis but their action sites have not been characterized. Given that expression of SER-7 in M4 fully restored isthmus peristalsis rate in the *ser-7* null mutant, it is plausible that release of dense core vesicles from the unidentified cells are constitutively active rather than triggered by serotonin. (C) Stimuli from an active M4 neuron and from the anterior part of the pharynx, excited by pumping, are both required to activate isthmus peristalsis. In the absence of either stimulus, isthmus peristalsis does not occur.

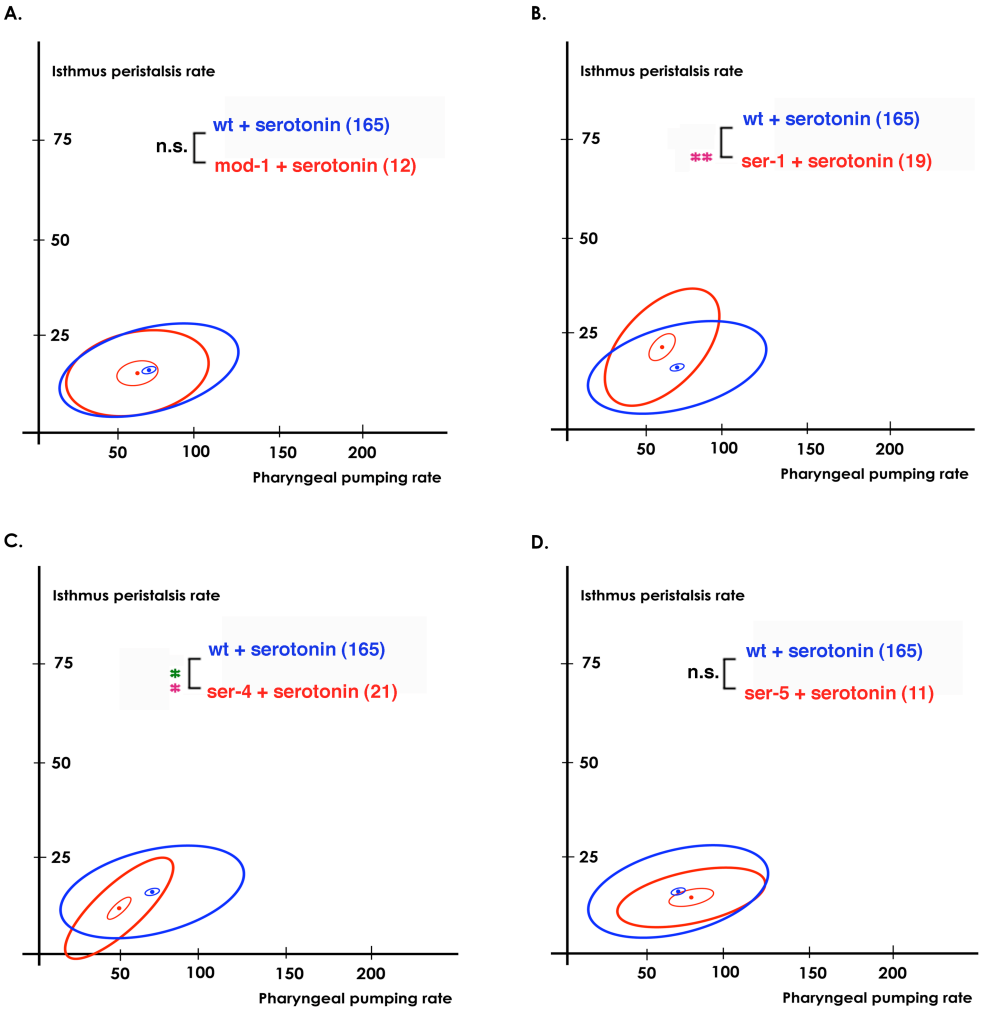

A.
Isthmus peristalsis rate
75
50
25
n.s.
wt + serotonin (165)
mod-1 + serotonin (12)
50
100
150
200
Pharyngeal pumping rate
B.
Isthmus peristalsis rate
75
50
25
**
wt + serotonin (165)
ser-1 + serotonin (19)
50
100
150
200
Pharyngeal pumping rate
C.
Isthmus peristalsis rate
75
50
25
**
**
wt + serotonin (165)
ser-4 + serotonin (21)
50
100
150
200
Pharyngeal pumping rate
D.
Isthmus peristalsis rate
75
50
25
n.s.
wt + serotonin (165)
ser-5 + serotonin (11)
50
100
150
200
Pharyngeal pumping rate

Figure 3-6. Null mutations in serotonin receptors other than SER-7 have little or no effect in the feeding rates.

Chapter 4
Recognition of familiar activates feeding via an endocrine serotonin signaling in C. elegans

ABSTRACT

Familiarity discrimination allows animals to respond appropriately to previously encountered stimuli by altering behavior. It is poorly understood by which mechanisms the recognition memory alters behavior. Here, we show that the nematode *C. elegans* is capable of familiarity discrimination. Worms form a memory of particular bacteria after experience and display behavioral plasticity, increasing the feeding response when they subsequently recognize the familiar bacteria. Using this behavior, we identified a neural pathway that is activated by recognition of familiar bacteria and delineated the mechanism by which the pathway increases the feeding response. Recognition of familiar food increases serotonin release from the pair of ADF chemosensory neurons. The released serotonin acts humorally and directly activates SER-7, a type 7 serotonin receptor, in MC (and possibly M4) motor neurons in the feeding organ whose activation increases the feeding response. Our study provides insight into the mechanism by which familiarity discrimination alters behavior.

INTRODUCTION

Recognition is the ability to identify and to judge a recently encountered item as having been presented previously (Brown and Aggleton, 2001). The ability allows animals to respond to previously encountered stimuli with an altered behavioral output that is beneficial for their survival. One of the recognition memories that elicits such behavioral plasticity is familiarity discrimination, knowledge of prior occurrence without memory of a specific episode (Brown and Aggleton, 2001). Although familiarity discrimination (Barker et al., 2006; Brown and Aggleton, 2001; Seoane et al., 2009; Uslaner et al.) and subsequent behavioral plasticity (Kandel and Schwartz, 1982; Kravitz, 1988) has been extensively studied, the mechanism by which the recognition memory controls behavioral plasticity is poorly understood.

Familiarity discrimination and subsequent behavioral plasticity require multiple biologically important neural computations such as learning and memory. Although recognition memory including familiarity discrimination has been mostly studied in higher vertebrate models and in humans, the complexity of the nervous system and the long generation time of vertebrates call for a simple animal model, from which we can learn the principles of neural computation and molecular mechanisms underlying familiarity discrimination and its control of subsequent behavioral plasticity. With its simple nervous system and our

unparalleled knowledge of its neuronal wiring, combined with powerful genetics and imaging techniques in a transparent animal (Schafer, 2005), the nematode *C. elegans* would be an attractive model system for analyses of this challenging problem. Here, we suggest that *C. elegans* is a proper model for familiarity discrimination and its control of subsequent behavioral plasticity by showing that a mere exposure to a nonpathogenic bacterium alters feeding behavior selectively in response to that particular bacterium in later encounter. Using the behavioral pattern that we identified, we uncover the mechanism by which familiarity discrimination alters the feeding behavior.

RESULTS

Recognition of familiar bacteria increases the feeding response in *C. elegans.*

To test if *C. elegans* can discriminate familiarity, we tested if familiarity of food alters its feeding response. For this assay, we trained wild-type animals to develop familiarity either to *Escherichia coli* HB101 (H) or to *Pseudomonas* DA1878 (D) by exposing the animals to one or the other bacterium from the first larval stage (L1) until adulthood (Figure 4-1a). HB101 and DA1878 were chosen because these bacterial strains support the growth of *C. elegans* at a similar rate and show no obvious signs of pathogenicity (see *C elegans* killing assay in Chapter 2 for details). Once the animals reached adulthood, we compared feeding

rates on previously experienced bacteria (HH and DD groups) to feeding rates on novel bacteria (DH and HD groups) (Figure 4-1a; see Feeding assay and Statistical analysis in Chapter 2 for details). We found that the feeding rates of the animals on familiar bacteria were significantly higher than the rates on novel bacteria, regardless of bacterial type (Figure 4-1b and c). The increased feeding rates on familiar food compared to the rates on novel food persisted 7-8hrs after the training was over (Figure 4-1d and e). The data imply that *C. elegans* forms a recognition memory of bacteria after experience, which allows the worms to discriminate familiar bacteria from novel bacteria, and that the recognition of familiar bacteria increases the feeding response.

Recognition of familiar food increases the feeding response by activating serotonin signaling via SER-7.

The neurotransmitter serotonin increases feeding in *C. elegans* (Avery and Horvitz, 1990; Croll, 1975; Horvitz et al., 1982) and serotonin has long been suggested to be a food signal in *C. elegans* (Horvitz et al., 1982). However, the serotonin effect on feeding was tested only on familiar bacteria; thus it is unknown if the serotonin feeding signaling is activated by novel bacteria. We thus hypothesized that recognition of familiar bacteria might increase the feeding response by activating serotonin signaling. To test our hypothesis, we tested if a

tph-1 null mutation suppresses the difference in the feeding rates between animals on familiar bacteria and novel bacteria. *tph-1* encodes a tryptophan hydroxylase required for serotonin biosynthesis (Sze et al., 2000). We found no difference in the feeding rates between the *tph-1* null mutant animals on familiar bacteria and novel bacteria (Figure 4-2a and 2b), suggesting that recognition of familiar bacteria indeed increased feeding rate by activating serotonin signaling. To find out which serotonin receptors mediate the serotonin action on feeding, we compared the feeding rates between wild-type and serotonin receptor null mutants in response to serotonin. Consistent with the previous report that serotonin activates feeding via a type 7 serotonin receptor SER-7 (Hobson et al., 2006), only the *ser-7* null mutant failed to activate feeding in response to serotonin (Figure 4-6). Only a small difference was found between feeding rates in the *tph-1* single null mutant and the *ser-7* single null mutant and no difference was found between the feeding rates in the *tph-1* single null mutant and the *tph-1; ser-7* double null mutant in response to familiar bacteria, suggesting that serotonin increases feeding via SER-7 in response to familiar food (Figure 4-3a). Since SER-7 is the major receptor to mediate the serotonin action, we initially expected no difference between feeding rates of the *ser-7* null mutant on familiar food and novel food like the *tph-1* null mutant. However, the feeding rate of the *ser-7* mutant was lower on familiar food than the rate on novel food (Figure 4-7a and b). Serotonin also suppressed feeding in the *ser-7* mutant (Figure 4-2e). Thus we

hypothesized that there is also an inhibitory serotonin signal that is more active on familiar food. Indeed, we found that null mutations in *ser-4*, a type 1 serotonin receptor (Olde and McCombie, 1997), and *mod-1*, serotonin gated Cl^- channel (Ranganathan et al., 2000), completely relieved the suppression of feeding by serotonin in the *ser-7* mutant (Figure 4-2e), suggesting that serotonin inhibited feeding by acting on SER-4 and MOD-1. Furthermore, the feeding rates of the *ser-4; mod-1; ser-7* triple null mutant on familiar food were not different from the rates of the *ser-7* single null mutant and the *ser-4; mod-1; ser-7* triple null mutant on novel food (Figure 4-2c and d and Figure 4-7c and d), suggesting that the inhibitory serotonin signal is active only on familiar food. This explains why the difference in feeding rates between wild-type and the *ser-7* null mutant on familiar food was even larger and more easily measured than the simple effect of familiarity in wild-type worms (Figure 4-1d and e and Figure 4-7a and b).

We then tested if serotonin feeding signaling via SER-7 gets activated by recognition of familiar food by comparing the differences between feeding rates of wild-type and the *ser-7* null mutant animals (the SER-7 effect) on familiar food with the differences on novel food (see Figure 4-7e and Chapter 2 for details). Any feeding rate difference between wild-type and the *ser-7* mutant animals indicates active serotonin signaling via SER-7 because *ser-7* specifically affects serotonergic signaling, with some contribution from basal activity of SER-7 in the absence of ligand, serotonin (Hobson et al., 2003) (Figure 4-7e). If serotonin

signaling is equally active on familiar food and novel food, we expect the SER-7 effect to be similar on familiar food and novel food. However, on familiar food the SER-7 effect was far greater than on novel food (Figure 4-2f), suggesting that serotonin signaling via SER-7 is indeed more active on familiar food than novel food. We concluded that recognition of familiar food increases the feeding response mainly by activating serotonin signaling via SER-7.

Serotonin from ADF chemosensory neurons directly activates the feeding circuit.

To gain insight into how serotonin signals familiar bacteria, we asked which serotonergic neurons regulated the feeding response. Serotonin is detected in 5 types of neurons in *C. elegans* hermaphrodites: NSM, ADF, HSN, RIH and AIM (Sze et al., 2000). RIH and AIM obtain serotonin by taking up extracellular serotonin (J. Y. Sze, personal communication) although they occasionally express *tph-1,* the essential enzyme for de novo synthesis of serotonin (Sze et al., 2000). HSN is unlikely to be necessary for the behavioral plasticity because feeding rates of males, which do not have HSN, were also greater on familiar food than the rates on novel food (Figure 4-8). We therefore hypothesized that either NSM or ADF uses serotonin to control feeding. The NSM neurons are a pair of secretory neurons located in the pharynx, whereas the ADF neurons are a pair of

chemosensory neurons located outside the pharynx (Sze et al., 2000) that have been suggested to sense bacteria (Bargmann CI, 1991b). We asked if serotonin either in ADF or in NSM regulates the feeding response by expressing *tph-1* cDNA in the *tph-1* null mutant using either the *srh-142* promoter or the *ceh-2* promoter. The *srh-142* promoter drives expression specifically in ADF and the *ceh-2* promoter drives expression in NSM and 3 additional neurons (Liang et al., 2006). We found that restoring serotonin synthesis in ADF, but not in NSM, rescued the feeding response in the *tph-1* mutant (Figure 4-3a), suggesting that ADF regulates feeding in response to familiar bacteria. Laser killing of ADF also decreased feeding on familiar food (Figure 4-3b)—the difference in feeding rates between ADF-minus and mock-operated animals (49.8 ± 7.4; feeding rates of ADF-minus and mock-operated animals were 199 ± 7.7 and 249 ± 3.5, respectively) was comparable to the difference in the feeding rates between *tph-1* and wild-type animals (58.4 ± 3.5; feeding rates of *tph-1* and wild-type animals were 207.7 ± 1.9 and 266.1 ± 3.0, respectively), further supporting the idea that ADF regulates feeding in response to familiar bacteria.

In *tph-1; Ex[ADF::tph-1(+)]* animals, in which serotonin synthesis activity was restored only in ADF, serotonin was detected in other serotonergic neurons in addition to ADF (Figure 4-4b; see Immunohistochemistry in Chapter 2 for details). This suggests that serotonin synthesized by ADF might act in either of two possible ways: it could activate SER-7 directly, or it could be taken up and

subsequently released by other serotonergic neurons. To distinguish between these possibilities, we compared the feeding rates of *tph-1; Ex[ADF::tph-1(+)]* animals with or without *mod-5*. MOD-5 is a serotonin transporter required to take up extracellular serotonin into some serotonergic neurons (Ranganathan et al., 2001) (J. Y. Sze, personal communication). In *mod-5; tph-1; Ex[ADF::tph-1(+)]*, serotonin was detected only in ADF (Figure 4-4d), suggesting that *mod-5* loss blocks serotonin uptake into other cells. If serotonin synthesized by ADF acts through other serotonergic neurons, *mod-5* loss should substantially decrease the feeding rate of *tph-1; Ex[ADF::tph-1(+)]*. However, we found that ADF could activate feeding as effectively in the absence of *mod-5* as in its presence (Figure 4-3a), suggesting that serotonin from ADF directly activates SER-7.

Next, we confirmed that ADF regulates feeding through SER-7. A *ser-7* null mutation suppressed the rescue effect of restoring serotonin in ADF in the *tph-1* null mutant (Figure 4-3a). To understand how serotonin increases feeding at a neural circuit level, we asked where SER-7 acts. SER-7 is expressed mostly in pharyngeal neurons (Hobson et al., 2006), which regulate the motions of pharyngeal muscles (Avery and Horvitz, 1989). Among the pharyngeal neurons, MC is particularly interesting because it is essential for normal fast feeding on bacteria (Avery and Horvitz, 1989), and SER-7 was suggested to activate MC (Hobson et al., 2006). To ask if SER-7 acts in MC, we expressed SER-7 in the *ser-7* null mutant using the *flp-21* and the *flp-2* promoters. The *flp-21* and the *flp-*

2 promoters drive expression in several neurons, and the expression patterns of the two promoters overlap only in MC and M4 (Kim and Li, 2004). We found that both *pflp-21::*SER-7 and *pflp-2::*SER-7 fully rescued the feeding rate in the *ser-7* mutant in response to familiar food as well as serotonin (Figure 4-3c and d). In contrast, expression of SER-7 in M4 and occasionally in M2 using the *ser-7b* promoter failed to alter the pumping rate in the *ser-7* null mutant in response to serotonin, suggesting that SER-7 in MC activates pharyngeal pumping. The failure in rescue is unlikely due to an insufficient expression because expression of the rescue construct significantly activated isthmus peristalsis, the other feeding motion in *C. elegans* (Figure 4-3c). To test whether SER-7 indeed acts through MC, we used an *eat-2* null mutation to test if blocking neurotransmission from MC suppresses the rescue effect of *pflp-21*::SER-7 in the *ser-7* mutant. *eat-2* encodes a nicotinic acetylcholine receptor subunit specifically localized in the pharyngeal muscles postsynaptic to MC (McKay et al., 2004). Thus, an *eat-2* null mutation selectively blocks acetylcholine transmission from MC to the pharyngeal muscles. We found that the *eat-2* null mutation suppressed the rescue effect of *pflp-21*::*ser-7(+)* in response to serotonin (Figure 4-3c), supporting our hypothesis that SER-7 in MC increases the feeding response. In summary, we conclude that serotonin released from extrapharyngeal ADF increased feeding in response to familiar bacteria mainly by activating SER-7 in MC directly, which in turn activate cholinergic transmission from MC to the pharyngeal muscles.

Recognition of familiar bacteria increases serotonin release from ADF.

We next asked how ADF increases feeding response only on familiar bacteria. A simple explanation is that only familiar bacteria can activate ADF, and this activation causes increased serotonin release. Since direct measurement of serotonin release from ADF in response to food is infeasible, we developed a method to detect released serotonin indirectly by its uptake into other serotonergic neurons. As mentioned above, in *tph-1; Ex[ADF::tph-1(+)]* animals, serotonin signal is detected in other cells in addition to ADF (Fig. 4b; see immunohistochemistry in Chapter 2 for details). Since no serotonin was detected in the *tph-1* null mutant animals (Figure 4-9a) and since the ADFs are the only cells capable of synthesizing serotonin in *tph-1; Ex[ADF::tph-1(+)]*, all the serotonin in these animals must have been synthesized in ADF, and its appearance in other serotonergic neurons must have occurred after release from ADF and uptake into other neurons. In confirmation of this hypothesis, in *mod-5; tph-1; Ex[ADF::tph-1(+)],* serotonin is detected only in ADF (Figure 4-4c). Thus, the presence of serotonin in cells other than ADF in *tph-1; Ex[ADF::tph-1(+)]* animals is an indication of serotonin release from ADF.

We thus tested if ADF releases more serotonin in response to familiar bacteria than novel bacteria by comparing the numbers of serotonin positive serotonin-uptaking cells in *tph-1; Ex[ADF::tph-1(+)]* animals on familiar bacteria and novel bacteria. As for the feeding assay, we trained the animals on HB101 or DA1878 and tested them on HB101 or DA1878 after a 7hr interval (Figure 4-4a; see Immunohistochemistry in Chapter 2 for details). Consistent with our hypothesis, the increase in the number of serotonin positive serotonin-uptaking cells during the 1 hour incubation on familiar food was greater than the increase on novel food (Figure 4-4d; see Quantification of serotonin positive neurons in Chapter 2 for details), suggesting that ADF releases more serotonin in response to familiar food than novel food. We conclude that recognition of familiar food increases serotonin release in ADF, which in turn increases the feeding response.

DISCUSSION

Here, we show that *C. elegans* discriminates familiar food from novel food and that recognition of familiar food stimulates feeding. Using this response, we uncover a mechanism by which familiarity discrimination alters behavior. When a previously encountered bacterium is sensed, chemosensory neurons ADF in the head increase serotonin release. The released serotonin, acting humorally, directly activates the serotonin receptor SER-7 in MC (and possibly in M4)

pharyngeal motor neurons, whose activation increases the feeding rate via cholinergic transmission from MC to the pharyngeal muscles (Figure 4-5).

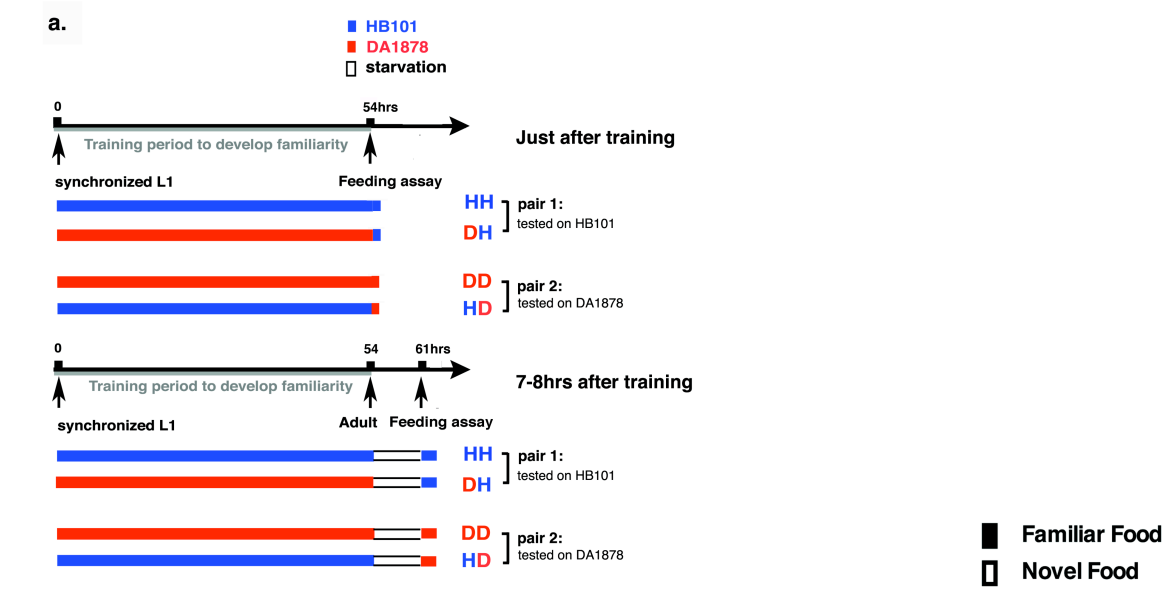
Our result that serotonin transmission from ADF chemosensory neurons increases in response to familiar food suggests that ADF is activated by recognition of familiar food. Because the neural computation for recognizing familiar food should occur before serotonin in ADF is released, the neurons involved in the computation should be ADFs, or potentially other neurons regulating ADF. That ADFs are amphid neurons, which sense environmental stimuli (Bargmann CI, 1991a, b), and that they are connected by chemical synapses and gap junctions to neurons involved in learning and memory (Kuhara and Mori, 2006; Stetak et al., 2009; White), also support the hypothesis that ADFs or the neurons that regulate their activity are involved in recognizing familiar food.

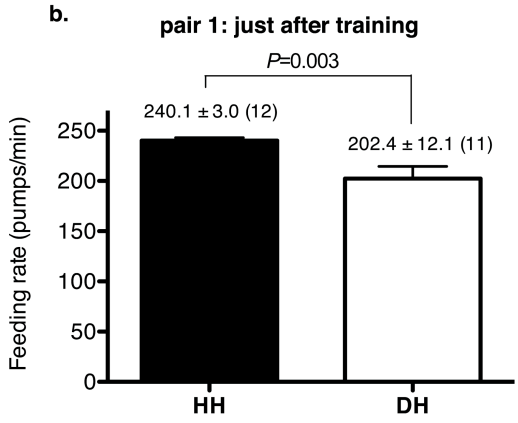
Our finding that recognition of familiar food employs endocrine serotonin signaling to increase feeding suggests that serotonin may serve to control multiple behaviors and physiological adaptations such that *C. elegans* responds appropriately when familiar food is present. To test this possibility, it would be informative to study whether familiarity of food affects behaviors (Avery and Horvitz, 1990; Horvitz et al., 1982; Sawin et al., 2000; Sze et al., 2000) and various aspects of physiology (Liang et al., 2006; Petrascheck et al., 2007; Srinivasan et al., 2008) that are controlled by serotonin in response to food (e.g.

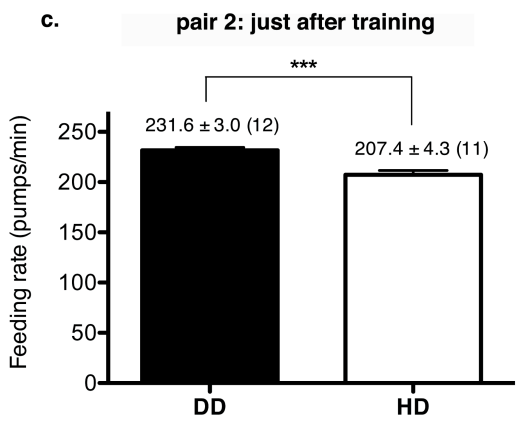
the systemic suppression of stress response that requires serotonin from ADF (Ranganathan et al., 2001). Further studies will be helpful to understand how recognition of familiar food contributes to survival in *C. elegans*.

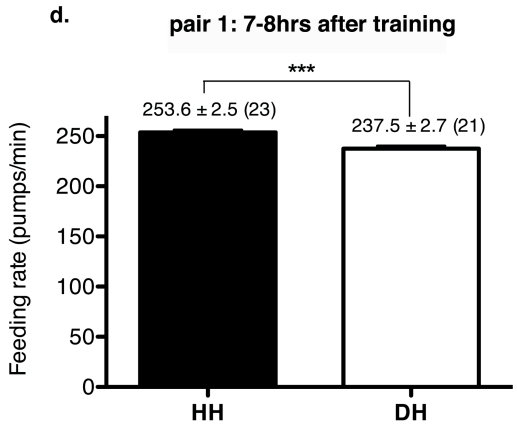
A previous study reported that *C. elegans* avoids the smell of pathogenic bacteria after experience (Zhang et al., 2005), and serotonin in ADF was suggested to be critical for this olfactory learning. This aversive change in olfactory preference is opposite in direction to the appetitive change in feeding behavior that we describe here. It would be interesting to understand how serotonin signaling from ADF and the physiological context are integrated to produce seemingly opposite experience dependent behaviors.

Many questions remain to be answered to fully understand the mechanism underlying recognition of familiar bacteria. For instance, which sensory modalities are important for *C. elegans* to discriminate different bacteria—touch, taste, smell? Additional questions arise from the discovery that *C. elegans* retains the memory of familiar bacteria for at least 7 hours, a long time in the life of an animal that grows from hatching to adulthood in just 2 days. How is the memory encoded, maintained and retrieved? Forward and reverse genetics in *C. elegans* may be useful in finding answers to these questions.


a.
HB101
DA1878
starvation
0
54hrs
Training period to develop familiarity
Just after training
synchronized L1
Feeding assay
HH
DH
pair 1:
tested on HB101
DD
HD
pair 2:
tested on DA1878
0
54
61hrs
Training period to develop familiarity
7-8hrs after training
synchronized L1
Adult
Feeding assay
HH
DH
pair 1:
tested on HB101
DD
HD
pair 2:
tested on DA1878
Familiar Food
Novel Food


b.
pair 1: just after training
P=0.003
240.1 ± 3.0 (12)
202.4 ± 12.1 (11)
Feeding rate (pumps/min)
250
200
150
100
50
0
HH
DH


c.
pair 2: just after training
***
231.6 ± 3.0 (12)
207.4 ± 4.3 (11)
Feeding rate (pumps/min)
250
200
150
100
50
0
DD
HD


d.
pair 1: 7-8hrs after training
***
253.6 ± 2.5 (23)
237.5 ± 2.7 (21)
Feeding rate (pumps/min)
250
200
150
100
50
0
HH
DH

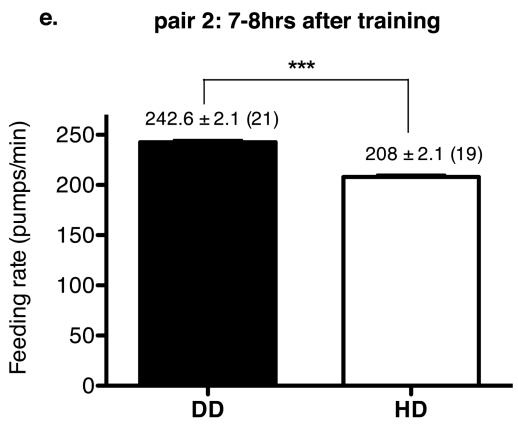

e.
pair 2: 7-8hrs after training
***
242.6 ± 2.1 (21)
208 ± 2.1 (19)
Feeding rate (pumps/min)
250
200
150
100
50
0
DD
HD

Figure 4-1. Recognition of familiar food increases feeding response in C. elegans. The memory of familiar food lasts for at least 7 hrs. (a) Experimental design for the feeding assay. The periods during which animals were exposed to HB101, DA1878, and starvation are denoted blue, red, and white, respectively. Each condition is coded by two letters representing the training and test foods in order. H and D represent HB101 and DA1878. (b-c) Feeding rates of wild-type (+) worms on HB101 (b) and DA1878 (c) just after training the animals on one or the other bacterium. (d-e) Feeding rates of wild-type(+) worms on HB101 (d) and DA1878 (e) after a 7-8hr interval from training the animals on one or the other bacterium. Data shown as mean ± SEM, *** $P<0.001$, unpaired t-test and Mann-Whitney U test (two-tailed). The number of animals tested ($n\geq3$ independent assays per each group) is shown in parenthesis.

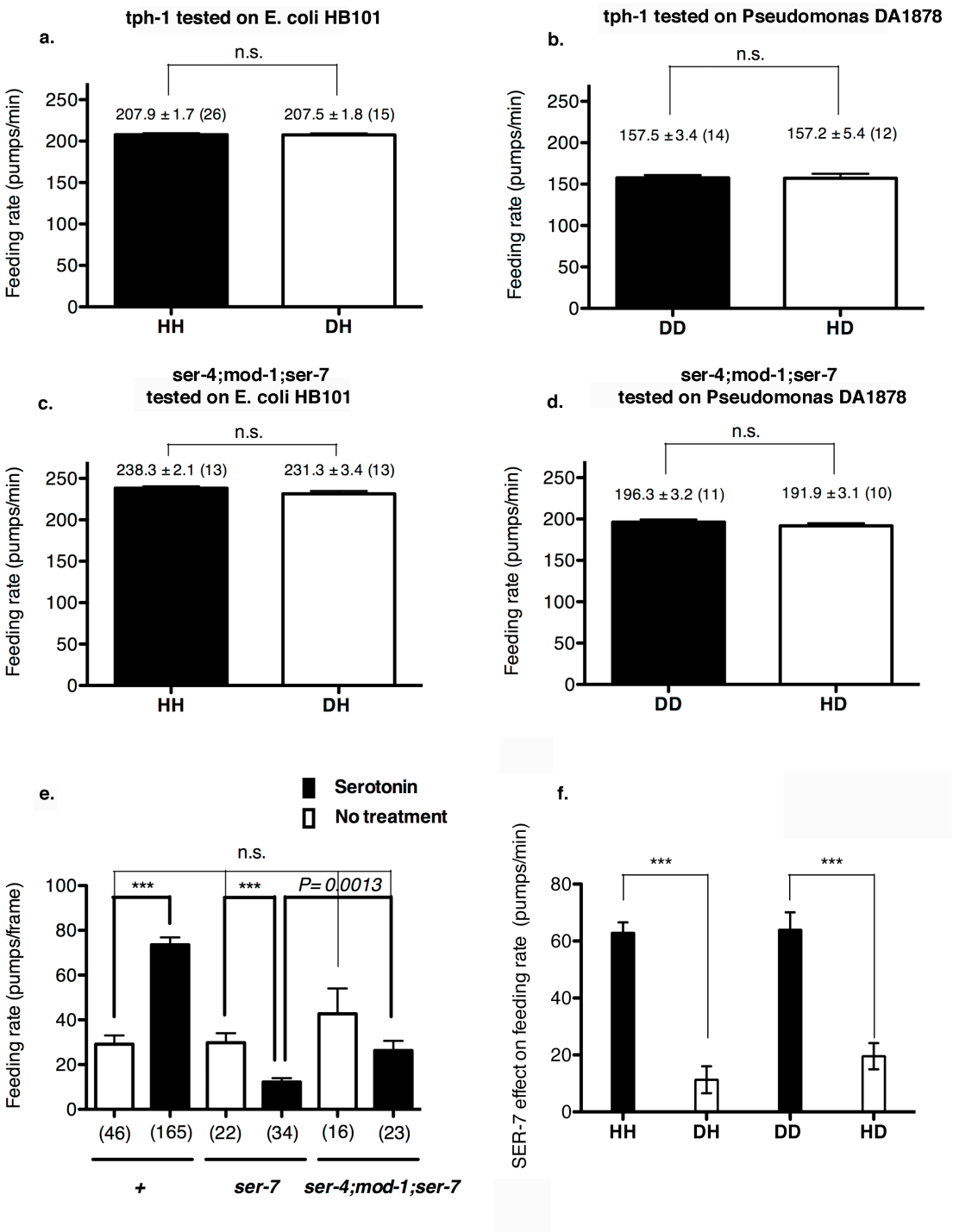

a.
tph-1 tested on E. coli HB101
n.s.
207.9 ±1.7 (26)
207.5 ±1.8 (15)
Feeding rate (pumps/min)
HH
DH
b.
tph-1 tested on Pseudomonas DA1878
n.s.
157.5 ±3.4 (14)
157.2 ±5.4 (12)
DD
HD
c.
ser-4;mod-1;ser-7
tested on E. coli HB101
238.3 ±2.1 (13)
231.3 ±3.4 (13)
d.
ser-4;mod-1;ser-7
tested on Pseudomonas DA1878
196.3 ±3.2 (11)
191.9 ±3.1 (10)
e.
Serotonin
No treatment
***
P= 0.0013
Feeding rate (pumps/frame)
(46) (165) (22) (34) (16) (23)
+
ser-7
ser-4;mod-1;ser-7
f.
SER-7 effect on feeding rate (pumps/min)

Figure 4-2. Recognition of familiar food increases the feeding response by activating serotonin signaling via SER-7. (a-b) Familiarity of food does not alter the feeding rates in *tph-1(mg280).* (c-d) Familiarity of food does not alter feeding rate in *ser-4(ok512); mod-1(ok103); ser-7(tm1325).* (e) Serotonin controls feeding positively via SER-7 and negatively via SER-4 and MOD-1. The feeding rate of the *ser-4; mod-1; ser-7* triple null mutant is not altered by serotonin treatment. (f) Serotonin signaling via SER-7 that activates the feeding response is more active on familiar food than novel food. The y axis indicates the difference in the feeding rates between wild-type (+) and *ser-7(tm1325)* animals. Each value corresponds to the difference in the feeding rates between wild-type (+) and the *ser-7* null mutant presented in Figure 4-7 a and b. Data shown as mean ± SEM, *** $P<0.001$; for Figure 4-2a-e, unpaired t-test and Mann-Whitney U test (two-tailed) and for Figure 4-2f, Student's t test (see Supplementary Methods for details), n.s., not significant ($P\geq0.05$). The number of animals tested ($n\geq3$ independent assays per each group) is shown in parenthesis.

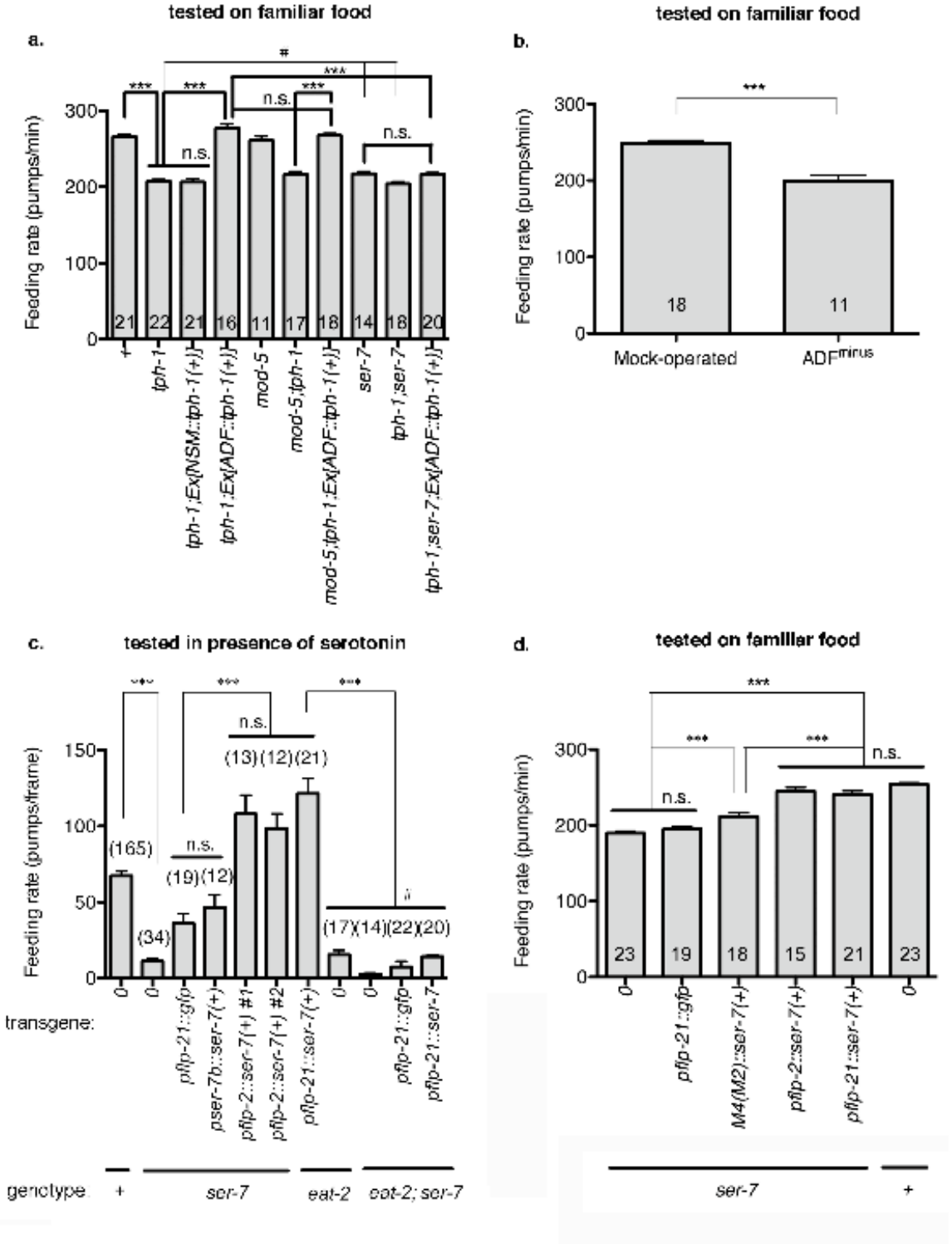
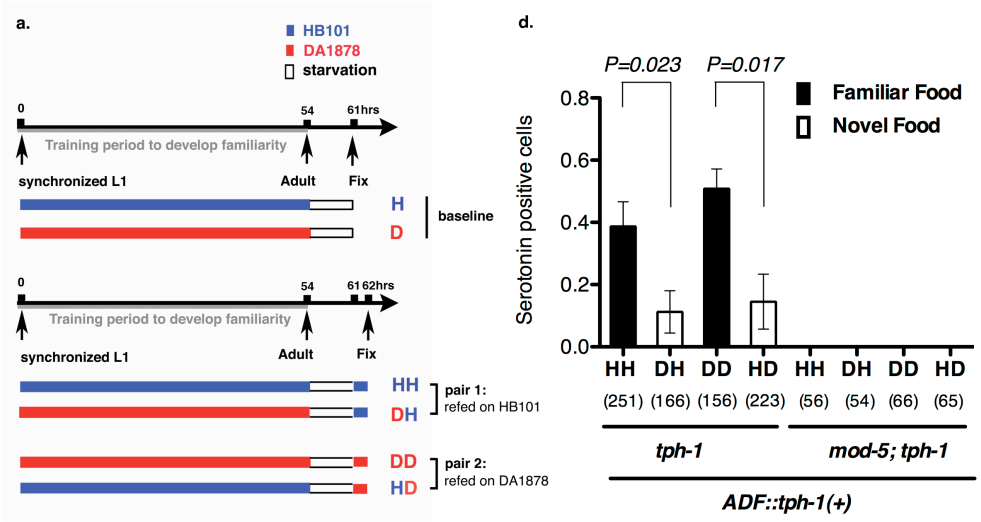
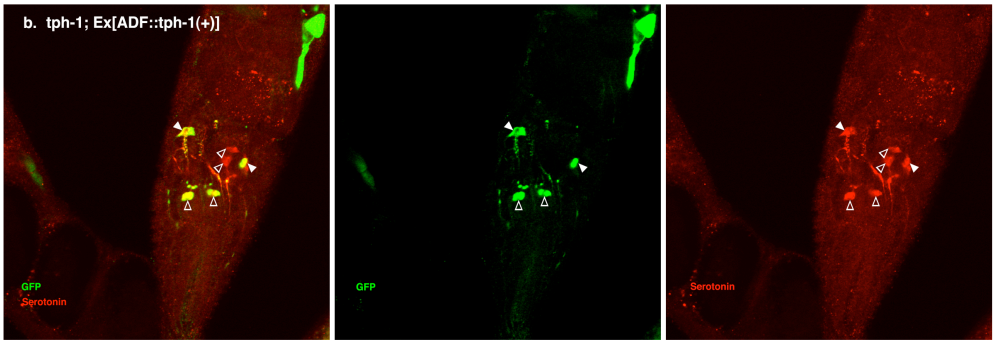

a.
tested on familiar food
Feeding rate (pumps/min)
300
200
100
0
***
***
***
n.s.
n.s.
n.s.
#
21
22
21
16
11
17
18
14
18
20
+
tph-1
tph-1;Ex[NSM::tph-1(+)]
tph-1;Ex[ADF::tph-1(+)]
mod-5
mod-5;tph-1
mod-5;tph-1;Ex[ADF::tph-1(+)]
ser-7
tph-1;ser-7
tph-1;ser-7;Ex[ADF::tph-1(+)]
b.
tested on familiar food
Feeding rate (pumps/min)
300
200
100
0
***
18
11
Mock-operated
ADF^minus
c.
tested in presence of serotonin
Feeding rate (pumps/frame)
150
100
50
0
***
***
***
n.s.
n.s.
(165)
(34)
(19)(12)
(13)(12)(21)
(17)(14)(22)(20)
transgene:
0
0
pflp-21::gfp
pser-7b::ser-7(+)
pflp-2::ser-7(+) #1
pflp-2::ser-7(+) #2
pflp-21::ser-7(+)
0
0
pflp-21::gfp
pflp-21::ser-7
genotype
+
ser-7
eat-2
eat-2; ser-7
d.
tested on familiar food
Feeding rate (pumps/min)
300
200
100
0
***
***
***
n.s.
n.s.
23
19
18
15
21
23
0
pflp-21::gfp
M4(M2)::ser-7(+)
pflp-2::ser-7(+)
pflp-21::ser-7(+)
0
ser-7
+

Figure 4-3. Serotonin from ADF activates feeding in response to familiar food mainly by activating SER-7 in MC pharyngeal motor neurons directly. Active SER-7 in MC (and possibly in M4) acts mainly via cholinergic transmission from MC to the pharyngeal muscles. (a) *tph-1* expression in ADF, but not in NSM, restores feeding rate in the *tph-1* null mutant. The rescue effect is suppressed by loss of *ser-7*, but not by loss of *mod-5*. # The feeding rate of the ser-7 single null mutant was significantly different from both the tph-1 single null mutant ($P< 0.01$) and the *tph-1; ser-7* double null mutant ($P< 0.01$). No difference was found in feeding rates between the tph-1 single null mutant and the *tph-1; ser-7* double null mutant. (b) ADF-minus animals feed significantly less in response to familiar food. (c) Expression of ser-7 cDNA driven either by the *flp-2* promoter or by the *flp-21* promoter but not by the ser-7b promoter fully restored the feeding rate in the *ser-7* null mutant in response to serotonin. The rescue effect was suppressed by blocking acetylcholine transmission from MC to the pharyngeal muscles. Pharyngeal pumping rate was lower in the *eat-2;ser-7* double null mutant than the *eat-2* single null mutant ($P< 0.001$) and the *ser-7* single null mutant ($P=0.0023$). The difference suggests that acetylcholine marginally activates pumping in an EAT-2-independent manner and that there is residual acetylcholine release in absence of SER-7 in response to serotonin. No difference in feeding rates was found between the *eat-2; ser-7* mutant expressing *pflp-21::gfp* and the mutant expressing *pflp-21::ser-7* cDNA. (d) Expression of *ser-7*

cDNA driven either by the *flp-2* promoter or by the *flp-21* promoter fully restored the feeding rate in the ser-7 null mutant in response to familiar food. Expression of *ser-7* cDNA in M4 (and occasionally in M2) driven by the *ser-7b* promoter also increased the feeding rate, but the effect was relatively small. Data shown as mean ± SEM, *** $P<0.001$; unpaired t-test and Mann-Whitney U test (two-tailed) n.s., not significant ($P\geq0.05$). The number of animals tested ($n\geq3$ independent assays per each group) is shown at each bar.


a.
HB101
DA1878
starvation
0
54
61hrs
Training period to develop familiarity
synchronized L1
Adult
Fix
H
D
baseline
0
54
61 62hrs
Training period to develop familiarity
synchronized L1
Adult
Fix
HH
DH
pair 1:
refed on HB101
DD
HD
pair 2:
refed on DA1878
d.
P=0.023
P=0.017
Familiar Food
Novel Food
Serotonin positive cells
0.8
0.6
0.4
0.2
0.0
HH DH DD HD HH DH DD HD
(251) (166) (156) (223) (56) (54) (66) (65)
tph-1
mod-5; tph-1
ADF::tph-1(+)


b. tph-1; Ex[ADF::tph-1(+)]
GFP
Serotonin
GFP
Serotonin

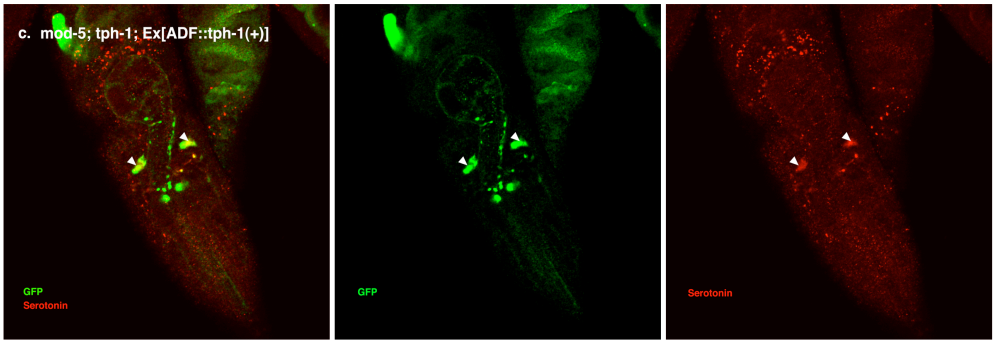

c. mod-5; tph-1; Ex[ADF::tph-1(+)]
GFP
Serotonin
GFP
Serotonin

Figure 4-4. ADF serotonergic neurons release more serotonin in response to familiar food than novel food. (a) Schematic of experimental design for anti-serotonin staining. Coding is as in Figure 4-1a. (b-c) Serotonin immunoreactivity in *tph-1; Is[ptph-1::gfp]; Ex[ADF::tph-1(+)::gfp]* (b) and in *mod-5; tph-1; Is[ptph-1::gfp]; Ex[ADF::tph-1(+)::gfp]*, the paired control animals defective in serotonin uptake (c). The *Is[ptph-1::gfp]* allows the identification of NSM and ADF by GFP expression. Filled arrowheads and open arrowheads indicate ADFs and serotonin-uptaking cells, respectively. The serotonin signals not marked by arrowheads are neuronal processes. (d) Increase in the average number of serotonin positive serotonin-uptaking cells during the 1 hour refeeding on familiar or novel food in tph-1; Is[ptph-1::gfp]; Ex[ADF::tph-1(+)::gfp] and in *mod-5; tph-1; Is[ptph-1::gfp]; Ex[ADF::tph-1(+)::gfp]*(see Immunohistochemistry and Quantification of serotonin positive neurons in Chapter 2 for details). Data shown as mean ± SEM. The baselines, the average numbers of serotonin positive AIMs and RIH in the animals trained on HB101 and DA1878 after starvation, were 2.06±0.07 and 1.99±0.05, respectively. The number of animals examined (n=3 independent assays per each group) is shown under each bar.

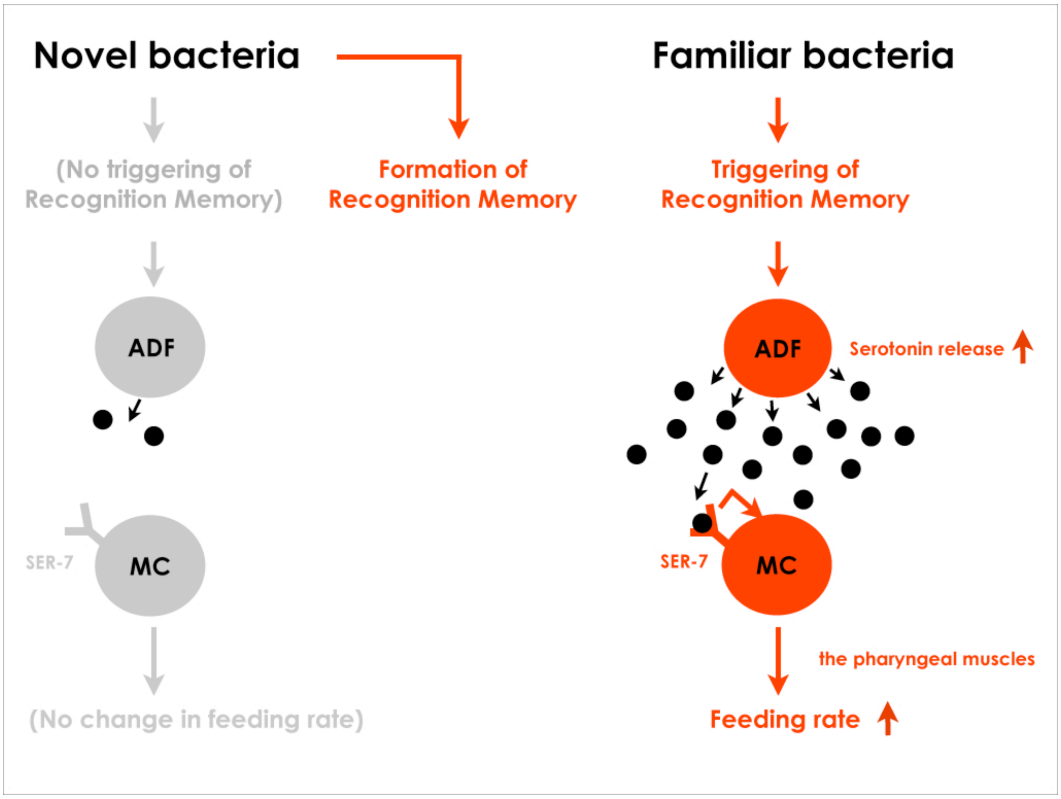


Figure 4-5. Model of activation of the feeding response by recognition of familiar food in *C. elegans*. (SER-7 in M4 may also contribute to the increased feeding in response to familiar food.)

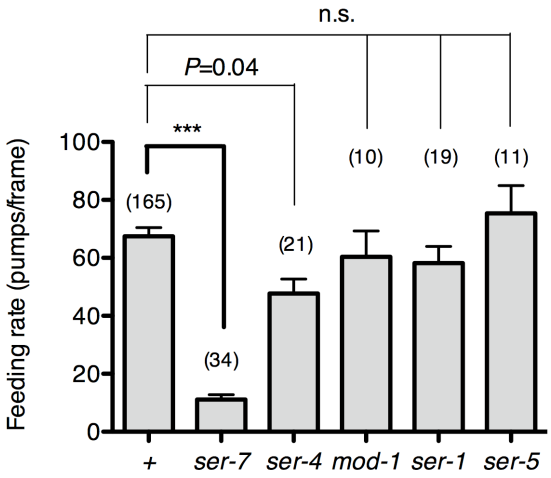
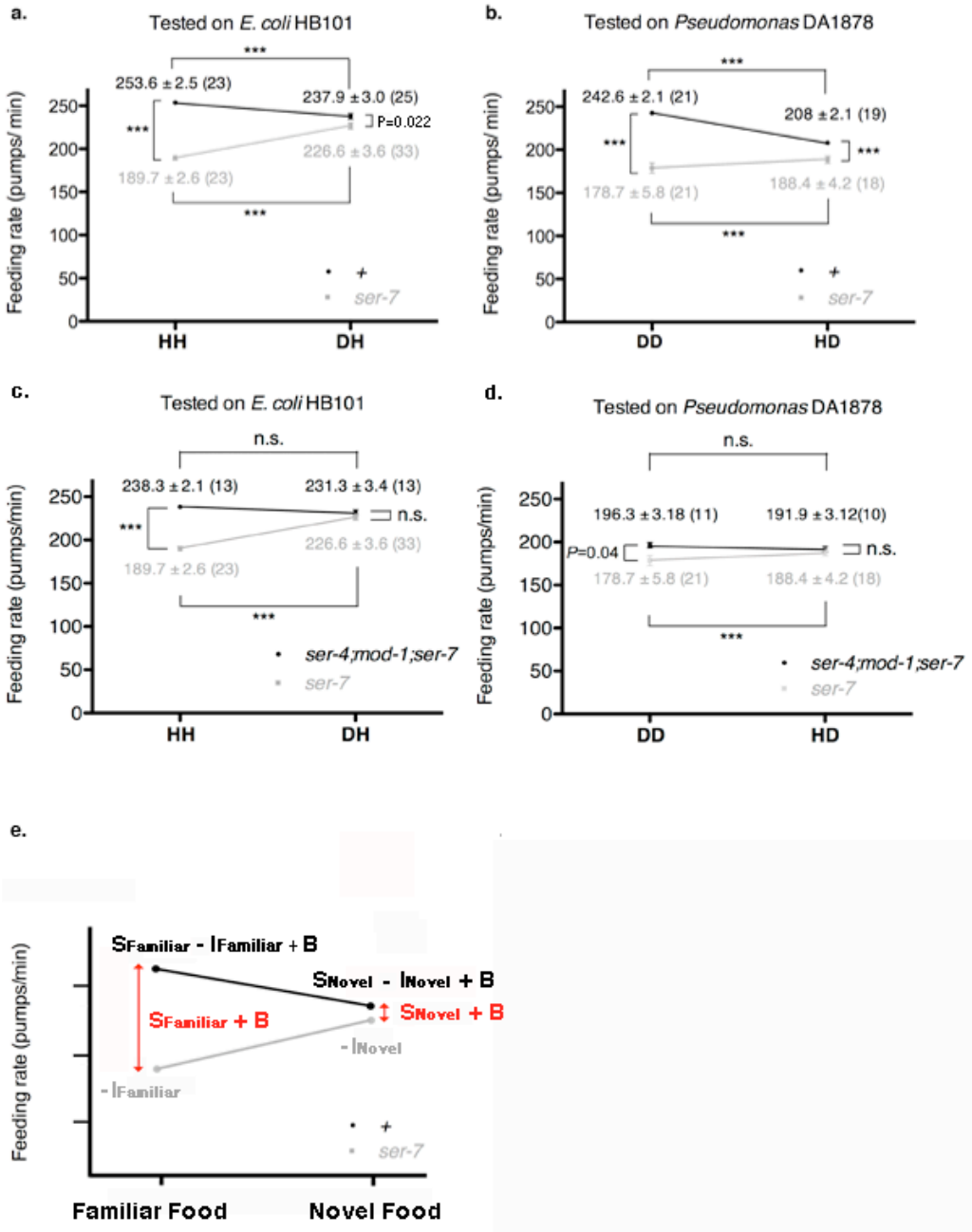


Figure 4-6. Serotonin increases feeding mainly by acting on SER-7 serotonin receptor. Among 5 serotonin receptor null mutants, only *ser-7(tm1325)* failed to activate feeding in response to serotonin. *** P<0.001; unpaired t-test and Mann-Whitney U test (two-tailed); n.s., not significant (P≥0.05). The number of animals tested (n≥2 independent assays per each group) is shown in parentheses.



$S_{Familair/Novel}$ = serotonin-induced increase in the feeding rate on familiar/novel food

$-I_{Familiar/Novel}$ = serotonin-induced decrease in the feeding rate on familiar/novel food

B = increase in the feeding rate caused by basal activity of SER-7

Figure 4-7. Feeding rates of wild-type, *ser-7* single, *ser-4;mod-1;ser-7* triple null mutant on HB101 and DA1878 and model of feeding regulation by serotonin. a-b. Feeding rates of wild-type(+) and *ser-7(tm1325)* on HB101 (a) and DA1878 (b) after a 7-8hr interval from training the animals on one or the other bacterium. Wild-type worms feed more quickly on familiar food than novel food. On novel food the feeding rate of wild-type is slightly higher than that of the ser-7 null mutant. The difference may be due to constitutive activity of SER-7; i.e., SER-7 is active to some extent even in absence of its ligand, serotonin (Hobson et al., 2003). c-d. Feeding rates of *ser-4(ok512); mod-1(ok103); ser-7(tm1325)* and *ser-7(tm1325)* on HB101 (c) and DA1878 (d) after a 7-8hr interval from training the animals on one or the other bacterium. The feeding rate of the ser-7 null mutant on familiar food is lower than the rate on novel food because there is still inhibitory serotonin signal via SER-4 and MOD-1. Like the positive SER-7-mediated signal, the inhibitory SER-4- and MOD-1-mediated serotonin signaling is more active on familiar food than novel food, but it decreases the feeding rate. a-d. Data shown as mean ± SEM, *** $P<0.001$; unpaired t-test and Mann-Whitney U test (two-tailed), The number of animals tested ($n\geq3$ independent assays per group) is shown in parentheses. e. A simple linear model explaining how different serotonin receptors might contribute to the regulation of pumping on familiar food and on novel food. There are three effects: B: basal activity of SER-7, S: serotonin-stimulated activity of SER-7, and -I: serotonin-

stimulated activity of inhibitory serotonin receptors SER-4 and MOD-1. The net effect of serotonin on wild-type(+) pumping is S+B–I; the net effect on pumping in a mutant lacking SER-7 is –I. While it is presented as an aid to thinking about the results, none of the results presented in the paper depend on this model. Figure 4-2f, in particular, is a direct measurement of the effect of SER-7 under differing conditions, calculated as the difference in feeding rates between wild-type(+) and the *ser-7* null mutant worms. A change in this number suggests the action of serotonin via SER-7. We use this as the measure of serotonin action via SER-7 because it is model-independent and robust.

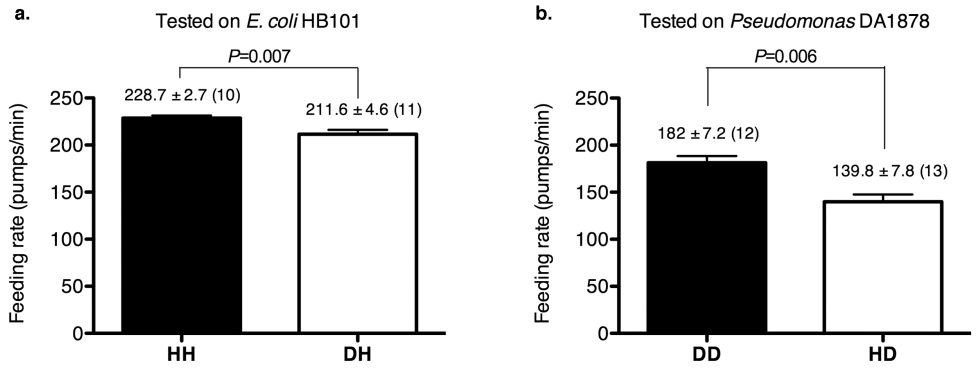


Figure 4-8. Male worms discriminate familiar food from novel food.

a-b Feeding rates of wild-type(+) male worms on HB101 (b) and DA1878 (c) after a 7-8hr interval from training the animals on one or the other bacterium. Unpaired *t*-test and Mann-Whitney U test (two-tailed), The number of animals

tested (n≥3 independent assays per each group) is shown in parentheses above each bar.

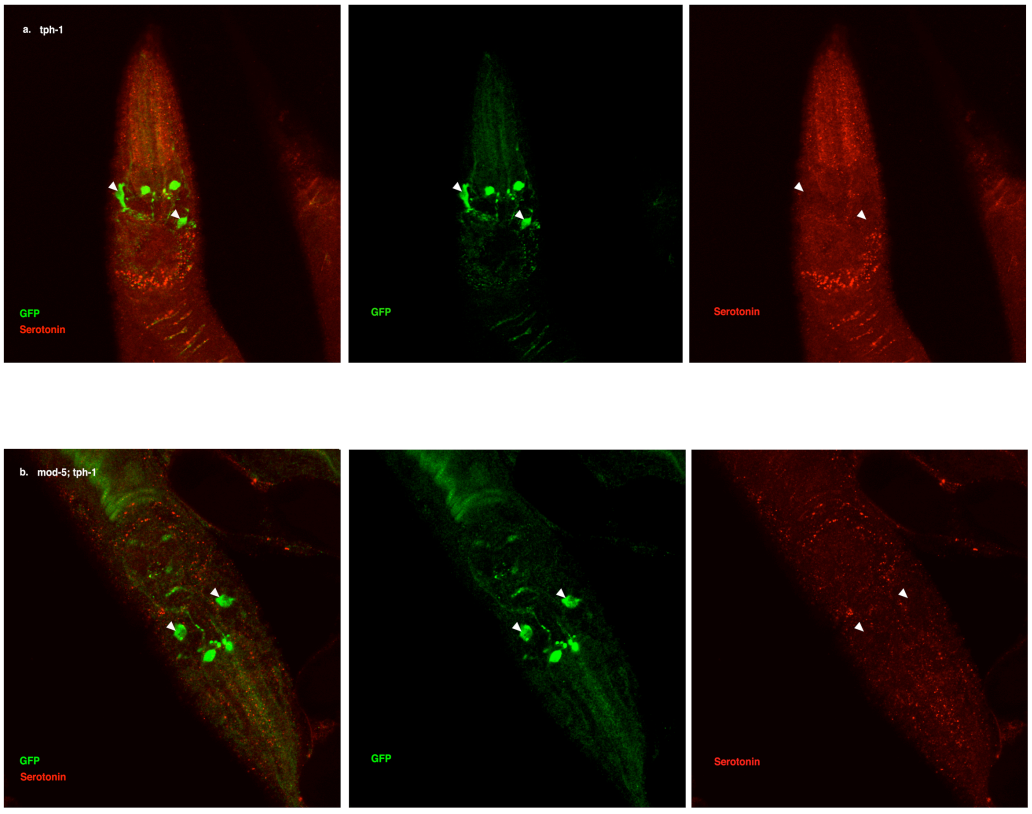


Figure 4-9. Serotonin immunoreactivity in *tph-1* single and *mod-1; tph-1* double null mutants. a-b. No serotonin signal was detected in the *tph-1(mg280);Is[ptph-1::gfp]* (a) and in the *mod-5(n3314);tph-1(mg280);Is[ptph-1::gfp]* (b) mutant animals. Filled arrowheads indicate ADFs. The *Is[ptph-1::gfp]* allows the identification of NSM and ADF by GFP expression.

Chapter 5

Conclusions and Recommendations

SEROTONIN-INDUCED FEEDING

In this study, I dissected the local circuit that directly activates feeding motions. Like food, serotonin activated overall feeding by activating isthmus peristalsis as well as pharyngeal pumping. During active feeding, the timing of onset and the frequencies of the two feeding motions were distinct (isthmus peristalsis occurs every 3.4 pumps). Consistent with the differences, the activation of the two feeding motions is mainly controlled by two separate neural pathways. A 5-HT type 7 receptor SER-7 acts in two distinct types of neurons to separately control the two feeding motions in response to food as well as serotonin. Moreover, SER-7 activates the separate types of neurons mainly by activating two distinct G protein signaling pathways.

Despite the separate regulation, isthmus peristalsis was coupled to the preceding pharyngeal pump such that every isthmus peristalsis occurs ~150ms after the preceding pump. How then are the two feeding motions that are controlled by two separate neural pathways coupled? My work and a previous study suggest that triggering isthmus peristalsis requires stimulation of the posterior isthmus both by muscle excitation during the pump and by M4, the major neuron by which serotonin activates isthmus peristalsis (for details, see

Chapter 3 discussion). Thus we propose that because of the contribution of muscle excitation during the pump, only M4 firing that occurs within a certain time window from the preceding pharyngeal pump can trigger isthmus peristalsis, resulting in the coupling between the two muscle motions. This model can be tested in transgenic animals expressing channelrhodopsin or halorhodopsin in M4 by asking the following questions: does activation or suppression of M4 activity control isthmus peristalsis as expected? If it does, does induction of isthmus peristalsis require both M4 activity and pharyngeal pumping? In the absence of the pumping activators (food or serotonin) worms pump slowly (< 1Hz) and hardly have any isthmus peristalsis, providing a good condition for testing the hypothesis.

A previous calcium imaging study on the pharyngeal muscles reported a that calcium wave occurs in the posterior isthmus selectively during isthmus peristalsis. To study the mechanisms underlying the coupling between the feeding motions, it would be informative to test if the calcium wave is essential to trigger isthmus peristalsis. This can be tested by testing whether injecting a calcium chelator into the isthmus muscle blocks serotonin-induced isthmus peristalsis. If the calcium wave in the posterior isthmus is essential, it would be informative to study whether the increased intracellular calcium level in other pharyngeal muscles during a pump contributes to triggering the calcium wave in the posterior

isthmus, assuming that isthmus peristalsis requires pharyngeal pumping. This may also be addressed by testing if injecting calcium chelator either in the corpus muscles or in the terminal bulb muscles blocks serotonin-induced isthmus peristalsis (see Figure 3-5 for details). It would be also informative to test if disruption of molecules that control intracellular calcium level affects the two feeding motions. I pursued this a little by testing if either *itr-1* or *unc-68* null mutations or thapsigargin treatment affected the frequency of isthmus peristalsis. *itr-1* and *unc-68* encode the *C. elegans* homologs of inositol trisphosphate receptor and ryanodine receptor, respectively. Thapsigargin is an inhibitor of SERCA (Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase). Inositol triphosphate receptor and ryanodine receptor increase intracellular calcium levels by releasing calcium ion from sarcoplasmic/endoplasmic reticulum to cytosol. In contrast, SERCA is a calcium ATPase that decreases intracellular calcium levels by pumping calcium from cytosol into sarco/endoplasmic reticulum. None of these attempts produced significant changes in isthmus peristalsis rate (data not shown), suggesting that the molecules play redundant roles if they control isthmus peristalsis.

RECOGNITION OF FAMILIAR FOOD ACTIVATES FEEDING VIA AN ENDOCRINE SEROTONIN SIGNALING IN C. ELEGANS.

In this study, I found the physiological context in which serotonin signal activates feeding by identifying a new behavioral pattern. Experience of a food selectively causes active consumption of the particular food by triggering serotonin release from a pair of chemosensory neurons. The released serotonin acts as an endocrine signal that subsequently activates the local feeding circuit, which subsequently activates feeding.

This finding suggests that worms discriminate familiar food from novel food and that familiarity of food affects feeding in *C. elegans*. Discrimination of familiar food shows that *C. elegans* can form a recognition memory of previously experienced food. Consistent with this, the behavioral plasticity was strongly dependent on duration of the feeding experience on particular bacteria (Figure 6-1 and Figure 6-2) but neither on developmental timing of the exposure (Figure 6-2) nor on the nutritional status of worms (Figure 4-1). Furthermore, worms could remember the previously experienced food at least for several hours after the feeding experience.

Familiar food-induced feeding is an attractive model to study perception of different bacteria, learning and memory because the behavior requires multiple neural processes such as sensing and learning particular bacteria upon exposure and recognizing familiar food in later encounters. It takes at least 9hr of exposure

to food for worms to remember the familiar food (Figure 6-1 and Figure 6-2). Searching for mutations that shorten the minimum memory acquisition time may provide insight into the process. Searching for mutations that lengthen the maximum memory retention time would be also informative to understand the mechanism underlying learning and memory required for the behavior.

My work provides insight into how the nervous system in the feeding organ operates to trigger food intake and how environmental input controls the feeding organ to control food intake. Given that killing MC and M4, the two motor neurons by which serotonin activates the feeding motions, completely blocks active pharyngeal pumping and isthmus peristalsis in response to food, it is tempting to speculate that various environmental and internal inputs that are sensed by *C. elegans* nervous system converge onto MC and M4 to control food intake.

Chapter 6 APPENDIX

FAMILIAR FOOD-INDUCED FEEDING IS DEPENDENT ON DURATION OF EXPOSURE TO FOOD.

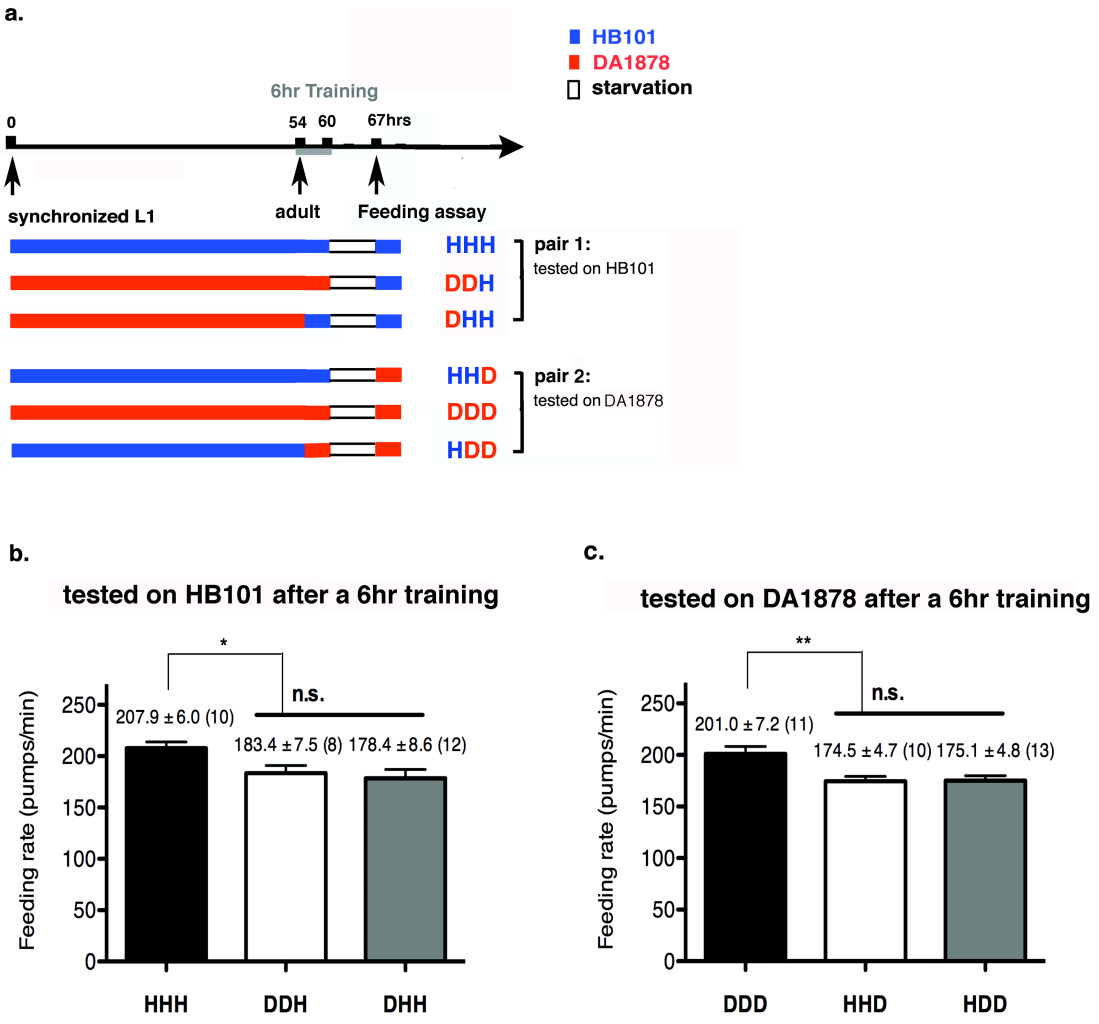
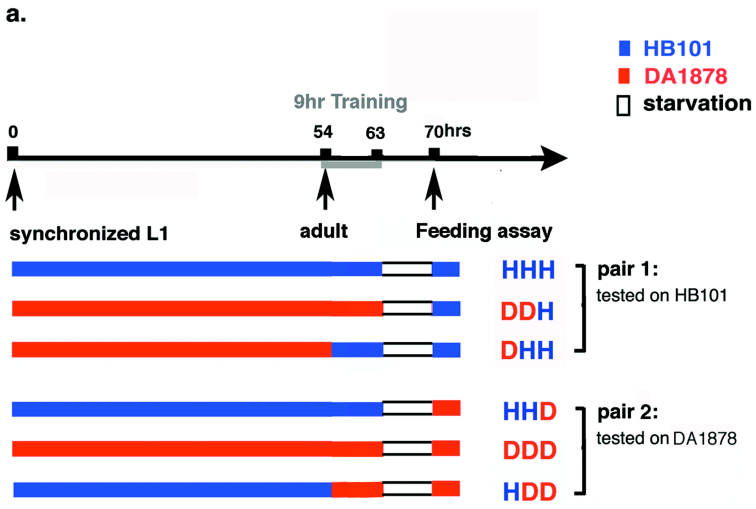
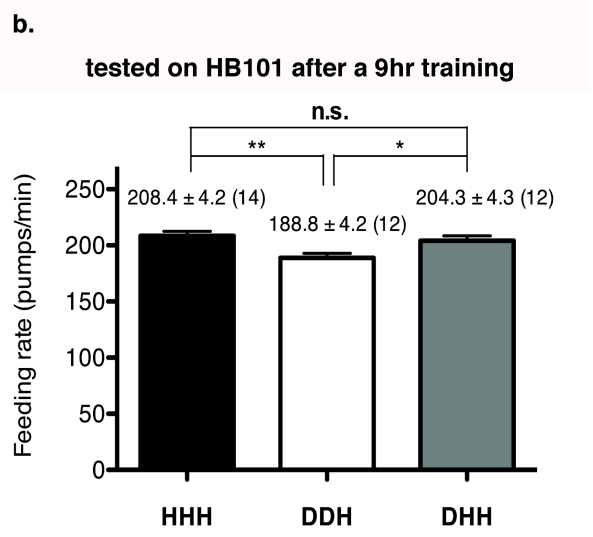


Figure 6-1. A 6hr exposure was not sufficient for worms to learn familiar food.


a.
HB101
DA1878
starvation
9hr Training
0
54
63
70hrs
synchronized L1
adult
Feeding assay
HHH
DDH
DHH
pair 1:
tested on HB101
HHD
DDD
HDD
pair 2:
tested on DA1878


b.
tested on HB101 after a 9hr training
n.s.
**
*
208.4 ± 4.2 (14)
188.8 ± 4.2 (12)
204.3 ± 4.3 (12)
Feeding rate (pumps/min)
250
200
150
100
50
0
HHH
DDH
DHH


c.
tested on DA1878 after a 9hr training
n.s.
**
*
205.0 ± 2.0 (13)
177.8 ± 4.4 (12)
194.8 ± 4.9 (13)
Feeding rate (pumps/min)
250
200
150
100
50
0
DDD
HHD
HDD

Figure 6-2. Exposure to food for 9hrs was sufficient for worms to learn familiar food.

BIBLIOGRAPHY

Albertson, D.G., and Thomson, J.N. (1976). The pharynx of Caenorhabditis elegans. Philos Trans R Soc Lond B Biol Sci *275*, 299-325.

Alfonso, A., Grundahl, K., Duerr, J.S., Han, H.P., and Rand, J.B. (1993). The Caenorhabditis elegans unc-17 gene: a putative vesicular acetylcholine transporter. Science *261*, 617-619.

Aravamudan, B., and Broadie, K. (2003). Synaptic <I>drosophila</I> UNC-13 is regulated by antagonistic G-protein pathways via a proteasome-dependent degradation mechanism. Journal of Neurobiology *54*, 417-438.

Avery, L., and Horvitz, H.R. (1987). A cell that dies during wild-type C. elegans development can function as a neuron in a ced-3 mutant. Cell *51*, 1071-1078.

Avery, L., and Horvitz, H.R. (1989). Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of C. elegans. Neuron *3*, 473-485.

Avery, L., and Horvitz, H.R. (1990). Effects of starvation and neuroactive drugs on feeding in Caenorhabditis elegans. J Exp Zool *253*, 263-270.

Avery, L., and Horvitzt, H.R. (1989). Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of C. elegans. Neuron *3*, 473-485.

Bargmann CI, H.H. (1991a). Chemosensory neurons with overlapping functions direct chemotaxis to multiple chemicals in C. elegans. Neuron *7*, 729-742.

Bargmann CI, H.H. (1991b). Control of larval development by chemosensory neurons in Caenorhabditis elegans. science *251*, 1243-1246.

Barker, G.R., Bashir, Z.I., Brown, M.W., and Warburton, E.C. (2006). A temporally distinct role for group I and group II metabotropic glutamate receptors in object recognition memory. Learn Mem *13*, 178-186.

Barsh, G.S., and Schwartz, M.W. (2002). Genetic approaches to studying energy balance: perception and integration. Nat Rev Genet *3*, 589-600.

Barstead, R.J.

(Wombase(http://www.wormbase.org/db/gene/variation?name=ok248)).

Berthoud, H.R. (2004). Neural control of appetite: cross-talk between homeostatic and non-homeostatic systems. Appetite *43*, 315-317.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71-94.

Brown, M.W., and Aggleton, J.P. (2001). Recognition memory: What are the roles of the perirhinal cortex and hippocampus? Nat Rev Neurosci *2*, 51-61.

Coll, A.P., Farooqi, I.S., and O'Rahilly, S. (2007). The hormonal control of food intake. Cell *129*, 251-262.

Croll, N.A. (1975). Indolealkylamines in the coordination of nematode behavioral activities. Can J Zool. *53*, 894-903.

Croll, N.A. (1978). Integrated behavior in the feeding phase of Caenohrabditis elegans. J. Zool *184*, 507-517.

Douglas, S.J., Dawson-Scully, K., and Sokolowski, M.B. (2005). The neurogenetics and evolution of food-related behaviour. Trends Neurosci *28*, 644-652.

Fawcett, G.L., Santi, C.M., Butler, A., Harris, T., Covarrubias, M., and Salkoff, L. (2006). Mutant analysis of the Shal (Kv4) voltage-gated fast transient K+ channel in Caenorhabditis elegans. J Biol Chem *281*, 30725-30735.

Fisher, R.A. (1954). Statistical Methods for Research Workers (Oliver and Boyd).

Hiley, E., McMullan, R., and Nurrish, S.J. (2006). The Galpha12-RGS RhoGEF-RhoA signalling pathway regulates neurotransmitter release in C. elegans. EMBO J *25*, 5884-5895.

Hobson, R.J., Geng, J., Gray, A.D., and Komuniecki, R.W. (2003). SER-7b, a constitutively active Galphas coupled 5-HT7-like receptor expressed in the Caenorhabditis elegans M4 pharyngeal motorneuron. J Neurochem *87*, 22-29.

Hobson, R.J., Hapiak, V.M., Xiao, H., Buehrer, K.L., Komuniecki, P.R., and Komuniecki, R.W. (2006). SER-7, a Caenorhabditis elegans 5-HT7-like receptor, is essential for the 5-HT stimulation of pharyngeal pumping and egg laying. Genetics *172*, 159-169.

Horvitz, H., Chalfie, M., Trent, C., Sulston, J., and Evans, P. (1982). Serotonin and octopamine in the nematode Caenorhabditis elegans. science *216*, 1012-1014.

Jose, A.M., and Koelle, M.R. (2005). Domains, amino acid residues, and new isoforms of Caenorhabditis elegans diacylglycerol kinase 1 (DGK-1) important for terminating diacylglycerol signaling in vivo. J Biol Chem *280*, 2730-2736.

Kandel, E.R., and Schwartz, J.H. (1982). Molecular Biology of Learning: Modulation of Transmitter Release. science *218*, 433-443.

Kaun, K.R., and Sokolowski, M.B. (2009). cGMP-dependent protein kinase: linking foraging to energy homeostasis. Genome *52*, 1-7.

Kim, K., and Li, C. (2004). Expression and regulation of an FMRFamide-related neuropeptide gene family in Caenorhabditis elegans. J Comp Neurol *475*, 540-550.

Kravitz, E.A. (1988). Hormonal Control of Behavior: Amines and the Biasing of Behavioral Output in Lobsters. science *241*, 1775-1781.

Kuhara, A., and Mori, I. (2006). Molecular Physiology of the Neural Circuit for Calcineurin-Dependent Associative Learning in Caenorhabditis elegans. J. Neurosci. *26*, 9355-9364.

Kvachnina, E., Liu, G., Dityatev, A., Renner, U., Dumuis, A., Richter, D.W., Dityateva, G., Schachner, M., Voyno-Yasenetskaya, T.A., and Ponimaskin, E.G. (2005). 5-HT7 receptor is coupled to G alpha subunits of heterotrimeric G12-protein to regulate gene transcription and neuronal morphology. J Neurosci *25*, 7821-7830.

Lewis, J.A., and Fleming, J.T. (1995). Basic culture methods. Methods Cell Biol *48*, 3-29.

Liang, B., Moussaif, M., Kuan, C.-J., Gargus, J.J., and Sze, J.Y. (2006). Serotonin targets the DAF-16/FOXO signaling pathway to modulate stress responses. Cell Metabolism *4*, 429-440.

Mahajan-Miklos, S., Tan, M.W., Rahme, L.G., and Ausubel, F.M. (1999). Molecular mechanisms of bacterial virulence elucidated using a Pseudomonas aeruginosa-Caenorhabditis elegans pathogenesis model. Cell *96*, 47-56.

McKay, J.P., Raizen, D.M., Gottschalk, A., Schafer, W.R., and Avery, L. (2004). eat-2 and eat-18 are required for nicotinic neurotransmission in the Caenorhabditis elegans pharynx. Genetics *166*, 161-169.

McMullan, R., Hiley, E., Morrison, P., and Nurrish, S.J. (2006). Rho is a presynaptic activator of neurotransmitter release at pre-existing synapses in C. elegans. Genes Dev *20*, 65-76.

Olde, B., and McCombie, W. (1997). Molecular cloning and functional expression of a serotonin receptor from Caenorhabditis elegans. Journal of Molecular Neuroscience *8*, 53-62.

Papaioannou, S., Marsden, D., Franks, C.J., Walker, R.J., and Holden-Dye, L. (2005). Role of a FMRFamide-like family of neuropeptides in the pharyngeal nervous system of Caenorhabditis elegans. J Neurobiol *65*, 304-319.

Petrascheck, M., Ye, X., and Buck, L.B. (2007). An antidepressant that extends lifespan in adult Caenorhabditis elegans. Nature *450*, 553-556.

Raizen, D.M., and Avery, L. (1994). Electrical activity and behavior in the pharynx of Caenorhabditis elegans. Neuron *12*, 483-495.

Raizen, D.M., Lee, R.Y., and Avery, L. (1995). Interacting genes required for pharyngeal excitation by motor neuron MC in Caenorhabditis elegans. Genetics *141*, 1365-1382.

Rand, J.B., and Russell, R.L. (1984). Choline acetyltransferase-deficient mutants of the nematode Caenorhabditis elegans. Genetics *106*, 227-248.

Ranganathan, R., Cannon, S.C., and Horvitz, H.R. (2000). MOD-1 is a serotonin-gated chloride channel that modulates locomotory behaviour in C.[thinsp]elegans. Nature *408*, 470-475.

Ranganathan, R., Sawin, E.R., Trent, C., and Horvitz, H.R. (2001). Mutations in the Caenorhabditis elegans serotonin reuptake transporter MOD-5 reveal serotonin-dependent and -independent activities of fluoxetine. J Neurosci *21*, 5871-5884.

Reynolds, N.K., Schade, M.A., and Miller, K.G. (2005). Convergent, RIC-8-dependent Galpha signaling pathways in the Caenorhabditis elegans synaptic signaling network. Genetics *169*, 651-670.

Richmond, J.E., Weimer, R.M., and Jorgensen, E.M. (2001). An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. Nature *412*, 338-341.

Sawin, E.R., Ranganathan, R., and Horvitz, H.R. (2000). C. elegans Locomotory Rate Is Modulated by the Environment through a Dopaminergic Pathway and by Experience through a Serotonergic Pathway. *26*, 619-631.

Schafer, W.R. (2005). Deciphering the Neural and Molecular Mechanisms of C. elegans Behavior. Current Biology *15*, R723-R729.

Schrader, L.A., Anderson, A.E., Mayne, A., Pfaffinger, P.J., and Sweatt, J.D. (2002). PKA modulation of Kv4.2-encoded A-type potassium channels requires formation of a supramolecular complex. J Neurosci *22*, 10123-10133.

Seoane, A., Massey, P.V., Keen, H., Bashir, Z.I., and Brown, M.W. (2009). L-type voltage-dependent calcium channel antagonists impair perirhinal long-term recognition memory and plasticity processes. J Neurosci *29*, 9534-9544.

Shimozono, S., Fukano, T., Kimura, K.D., Mori, I., Kirino, Y., and Miyawaki, A. (2004). Slow Ca2+ dynamics in pharyngeal muscles in Caenorhabditis elegans during fast pumping. EMBO Rep *5*, 521-526.

Sieburth, D., Madison, J.M., and Kaplan, J.M. (2007). PKC-1 regulates secretion of neuropeptides. Nat Neurosci *10*, 49-57.

Srinivasan, S., Sadegh, L., Elle, I.C., Christensen, A.G., Faergeman, N.J., and Ashrafi, K. (2008). Serotonin regulates C. elegans fat and feeding through independent molecular mechanisms. Cell Metab *7*, 533-544.

Stetak, A., H√∂rndli, F., Maricq, A.V., van den Heuvel, S., and Hajnal, A. (2009). Neuron-Specific Regulation of Associative Learning and Memory by MAGI-1 in <italic>C. elegans</italic>. PLoS ONE *4*, e6019.

Sze, J.Y., Victor, M., Loer, C., Shi, Y., and Ruvkun, G. (2000). Food and metabolic signalling defects in a Caenorhabditis elegans serotonin-synthesis mutant. Nature *403*, 560-564.

Tan, M.W., Rahme, L.G., Sternberg, J.A., Tompkins, R.G., and Ausubel, F.M. (1999). Pseudomonas aeruginosa killing of Caenorhabditis elegans used to identify P. aeruginosa virulence factors. Proc Natl Acad Sci U S A *96*, 2408-2413.

Uslaner, J.M., Parmentier-Batteur, S., Flick, R.B., Surles, N.O., Lam, J.S.H., McNaughton, C.H., Jacobson, M.A., and Hutson, P.H. Dose-dependent effect of CDPPB, the mGluR5 positive allosteric modulator, on recognition memory is associated with GluR1 and CREB phosphorylation in the prefrontal cortex and hippocampus. Neuropharmacology *In Press, Corrected Proof*.

White, J.G., Southgate, E., Thomson, J. N. & Brenner, S. In WormAtlas.

You, Y.J., Kim, J., Raizen, D.M., and Avery, L. (2008). Insulin, cGMP, and TGF-beta signals regulate food intake and quiescence in C. elegans: a model for satiety. Cell Metab *7*, 249-257.

Zhang, Y., Lu, H., and Bargmann, C.I. (2005). Pathogenic bacteria induce aversive olfactory learning in Caenorhabditis elegans. Nature *438*, 179-184.

Zheng, H., and Berthoud, H.R. (2008). Neural systems controlling the drive to eat: mind versus metabolism. Physiology (Bethesda) *23*, 75-83.

Zhou, K.M., Dong, Y.M., Ge, Q., Zhu, D., Zhou, W., Lin, X.G., Liang, T., Wu, Z.X., and Xu, T. (2007). PKA activation bypasses the requirement for UNC-31 in the docking of dense core vesicles from C. elegans neurons. Neuron *56*, 657-669.