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The role of neuropeptide signaling in *C. elegans*
food sensation and social behavior

by

Joshua Faguet

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The role of neuropeptide signaling in *C. elegans* food sensation and social behavior

Joshua Faguet

Abstract

Neuropeptides are a diverse and ancient class of signaling molecule. Many act as neuromodulators, incorporating flexibility into the fixed circuits of the nervous system. In this capacity, neuropeptides subserve one of the nervous system’s most important functions – allowing an organism to respond adaptively to changes in its environment. Neuropeptide signaling pathways have been characterized across a range of phyla. Still, many neuropeptides remain poorly understood or altogether undiscovered, and the mechanisms by which pathways interact with each other remain unclear. *C. elegans* offers a powerful model system for studying neuropeptide signaling. Beyond its genetic tractability and compact nervous system, the worm produces a uniquely large and varied set of neuropeptides. In this work, we use genetic analysis to explore two neuropeptide signaling pathways that modulate *C. elegans* behaviors. In Part I, we examine the HEN-1 neuropeptide, originally identified for its role in sensory integration. We demonstrate that HEN-1 constitutes a novel signal of food-depletion, and that it regulates foraging, feeding, and egg-laying according to food availability. HEN-1 acts through SCD-2, a receptor tyrosine kinase, and SCD-2 ultimately modulates the activity of tyramine and octopamine, the invertebrate counterparts of epinephrine and norepinephrine. In Part II, we explore a well-known *C. elegans* social feeding behavior. Social feeding is governed by NPR-1, a member of the neuropeptide-Y receptor family. We establish that reductions in NPR-1 activity enhance the secretion of neuropeptide-containing vesicles. And we show that release of an as yet unidentified neuropeptide drives social feeding.
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PART I:

The HEN-1 neuropeptide regulates adaptations to food depletion via monoamine signaling
Chapter I:

Introduction
**C. elegans as a model organism for studying behavior**

*C. elegans* is a small, soil-dwelling nematode found across multiple continents (Barrière and Félix, 2005). Since its introduction to experimental science in the 1960’s, *C. elegans* has proven to be a powerful model for tackling wide-ranging biological questions. For several reasons, the worm is particularly well-suited to studying the neural basis of behavior.

*C. elegans* has an extremely compact nervous system. The adult hermaphrodite contains 302 neurons, which descend through an invariant lineage of precursor cells (Sulston and Horvitz, 1977; Sulston et al., 1983). Early on, serial electron microscopy was used to reconstruct the entire nervous system (Ward et al., 1975; White et al., 1986). Owing to this pioneering work in *C. elegans* development and anatomy, we now know the identity, position, and connectivity of every cell in the worm’s nervous system.

Despite the relative simplicity of its nervous system, *C. elegans* bears many similarities to mammals in the way it interacts with its environment. Worms have sensory neurons dedicated to the perception of odors, tastes, touch, temperature, and gas concentrations (Inglis et al., 2007; Bargmann, Cl., 2006). *C. elegans* also exhibits diverse behaviors which range in complexity – from simple withdrawal reflexes to sophisticated social communication (de Bono and Mariqc, 2005).

The *C. elegans* genome contains roughly 20,000 protein-coding genes. Approximately 40% of these have clear mammalian homologs (Shaye et al., 2001). Importantly, most of the major signaling pathways and neurotransmitters used by mammalian nervous systems also operate in *C. elegans*. This conservation of key signaling molecules, coupled with the worm’s rich behavioral repertoire and well-defined anatomy, make *C. elegans* an ideal platform for elucidating the molecular and cellular circuits that generate behavior.
**Food availability regulates C. elegans behavior and development**

In the natural world, food availability is unpredictable. And unpredictable environments favor flexible behavior. Like most organisms, *C. elegans* regulates its behavior according to food abundance and internal state. When food is plentiful, the worm feeds rapidly and lays eggs. As food grows scarce, the animal reallocates its energy – away from activities subserving growth and reproduction, toward those related to locating new food sources. In addition to modifying behavior, food availability dictates the developmental trajectory of *C. elegans*. Nutrient poor environments promote entry into dauer, a hibernation-like state that delays reproductive maturity (Hu, PJ., 2007).

*C. elegans* monitors food levels via multiple sensory modalities. Each modality is served by a small number of sensory neurons, and the majority of these extend specialized, ciliated dendrites into the environment (Inglis et al., 2007). The ASE neurons detect attractive, water-soluble compounds. The AWA and AWC neurons sense volatile odors. These gustatory and olfactory neurons help animals locate nearby and distant attractants, respectively (Altun and Hall, 2011). Other chemosensory neurons contribute weakly to navigation. But several, such as ASI and ADF, are potent inputs to food-sensing circuits (Bargmann and Horvitz, 1991). *C. elegans* also detects food through mechanosensation and through perception of ambient oxygen concentration. The tactile qualities of bacteria alert animals to new food. And low oxygen concentrations indicate the presence of actively metabolizing bacteria (Sawin et al., 2000; Gray et al., 2004).

**Sensory neurons communicate food condition through neuroendocrine hormones**

Sensory neurons monitor environmental food status. This information is conveyed to the rest of the nervous system through various neuroendocrine signals. Monoamine neurotransmitters have a significant role in *C. elegans* food signaling. Dopamine mediates the tactile arm of food sensation and
regulates multiple aspects of foraging behavior (Sawin et al., 2000; Hills et al., 2004). Serotonin, secreted from ADF, NSM, and other neurons, also signals food availability. The properties of bacteria that trigger serotonin release remain unclear. But the effects of serotonin signaling are broad and well-defined. Serotonergic pathways modulate locomotion, feeding rate, egg-laying, and several other food-related behaviors (Horvitz et al., 1982; Hardaker et al., 2001). Two monoamines – tyramine and octopamine – communicate food deprivation rather than food abundance (Chase and Koelle, 2007). Though not released from sensory neurons, tyramine and octopamine play an important part in behavioral responses to starvation. This topic will be amplified in Chapter III.

In addition to monoamines, neuropeptides have a significant role in transmitting information related to food availability. DAF-7 is a neuropeptide homologous to mammalian TGF-β (Gumienny and Savage-Dunn, 2013). In *C. elegans*, DAF-7 is produced in the ASI sensory neuron in response to favorable environmental conditions (Ren et al., 1996). Upon secretion from ASI, DAF-7 acts at distant sites in the nervous system to coordinate multiple aspects of the animal’s behavior, development, and metabolism. DAF-7 is perhaps best understood in the context of dauer regulation. The SMAD transcription factor DAF-3 promotes dauer formation in response to nutrient poor environments. As long as the environment is food-replete and otherwise favorable to growth, DAF-7 signaling prevents dauer entry by repressing DAF-3 (Hu, PJ., 2007). In terms of behavior, DAF-7 regulates processes including pharyngeal pumping, egg-laying, and locomotion (Greer et. al., 2008; You et al., 2008).

Many of the food signals that operate in *C. elegans* have conserved roles in mammals. Serotonin regulates food intake, and dopamine plays a major role in the control of hunger and satiety (Ramos et al., 2005). Mammalian TGF-β has also recently been implicated in the regulation of food intake and energy expenditure (Townsend et al., 2012).
Identification of neuropeptide food signals

We became interested in identifying novel C. elegans food signals. For several reasons, we focused our search on neuropeptides. In mammals, neuropeptides such as leptin, ghrelin, and neuropeptide Y are among the chief regulators of appetite and food-related behaviors (Keen-Rhinehart et al., 2013). Along with DAF-7, several C. elegans neuropeptides also modulate behaviors in a food-dependent manner. For instance, FLP-1 promotes egg-laying in response to ample food and FLP-18 alters foraging strategies according to hunger level (Waggoner et al., 2000; Cohen et al., 2009). However, relative to other classes of signaling molecules, the role of neuropeptides in C. elegans food-signaling remains largely unexplored.

C. elegans produces over 250 distinct neuropeptides (Li and Kim, 2008). Loss-of-function mutants exist for many of the corresponding genes. Though it would be possible to screen mutants, looking for defects in a particular food-related behavior, we opted for a different approach. We surveyed the existing literature for studies about learning, memory, and decision-making in C. elegans. We reasoned that while not strictly related to food sensation, these processes are influenced by an animal’s nutritional status and by its perception of food in the environment. Consequently, these topics might provide entry points into the discovery of novel food-signaling pathways.

The HEN-1/SCD-2 pathway in C. elegans

In reviewing the literature, we came across a study of simple decision-making in C. elegans. In this study, the authors devised an assay to measure how animals respond to conflicting stimuli (Ishihara et al., 2002). Worms are separated from an attractive odor (diacetyl) by a noxious barrier of heavy metal ions (Cu^{2+}). Animals initially migrate toward the odor, but only a fraction cross the barrier to reach it. The authors conducted a forward genetic screen and identified one mutant that crossed significantly
less frequently than wild-type worms. They mapped the mutation to a novel gene and named it *hen-1*, for “hesitation in crossing an aversive barrier.”

The *hen-1* gene encodes a secreted neuropeptide most closely related to the *Drosophila* protein *Jeb* (*Jelly belly*). HEN-1 is composed of 99 amino acids and like *Jeb*, it contains an LDL-receptor domain (Ishihara et al., 2002). In *Drosophila*, *Jeb* directs visceral muscle development (Weiss et al., 2001). HEN-1 does not seem to share this function, as *hen-1* mutants are morphologically wild-type. *Jeb* signals through *Alk*, a receptor tyrosine kinase homologous to human anaplastic lymphoma kinase (Lee et al., 2003). Based on its similarity to the *Drosophila* and human proteins, a *C. elegans* tyrosine kinase called *SCD-2* was predicted to be the HEN-1 receptor. This prediction has been validated by the fact that *scd-2* mutants have similar defects in the decision-making assay (Shinkai et al., 2011).

Chemosensation is the primary means by which *C. elegans* locates and navigates toward food. Notably, *hen-1* and *scd-2* mutants have no defects in chemosensation or chemotaxis. When tested for their ability to respond to diacetyl or copper as individual stimuli, the animals perform normally – they migrate toward diacetyl and avoid copper. Their phenotype emerges only when the attractive and aversive stimuli are paired. In other words, they refrain from migrating toward the odor only when faced with a significant obstacle. This phenotype may therefore reflect a deficit in motivation. And in this scenario, one explanation for why mutants choose not to breach the barrier is that they fail to sense or respond to hunger.
References


Chapter II:

HEN-1 modulates behavior according to food availability
Introduction

The *hen-1* gene encodes a secreted neuropeptide. Loss-of-function *hen-1* mutants were initially discovered based on their performance in a decision-making assay (Ishihara et al., 2002). Whereas wild-type animals readily cross a noxious barrier to reach an attractive food-related odor, *hen-1* mutants refrain. Based on this phenotype, we hypothesized that HEN-1 may signal a state of food-deprivation. Animals lacking this signal would therefore be less willing to tolerate risk in pursuit of food.

In this chapter, we test our hypothesis by examining various food-regulated behaviors. We demonstrate that *hen-1* is dispensable for the performance of these behaviors yet required for their modulation. We also characterize a mutation in *scd-2*, which encodes the receptor for *hen-1*. As with *hen-1*, *scd-2(lf)* specifically disrupts the ability of animals to coordinate their behaviors with food condition. Finally, we show that the HEN-1 neuropeptide is secreted in response to starvation.

Results

HEN-1 and SCD-2 promote dispersal upon food depletion

The initial phenotypes reported for *hen-1* and *scd-2* mutants suggest that these genes may participate in sensing or responding to food depletion. We explored this possibility by analyzing known food-dependent behaviors in mutant animals. Wild-type worms move slowly and sporadically in the presence of food. Upon exhausting a food source, the animal increases its rate of movement and disperses in search of new food. This exploratory behavior has been previously quantified using a radial dispersal assay (Tsalik and Hobert, 2003). In the assay, well-fed worms are rinsed and transferred to unseeded plates. At various timepoints, the experimenter measures the distance worms have migrated from their origin. If worms are starved prior to the assay, the magnitude of dispersal increases. Thus, the
animal’s movement is influenced not only by its perception of food availability but also by its current nutritional status.

We filmed the movement of wild-type and *hen-1(lf)* worms for five minutes following transfer to unseeded plates. Using software to reconstruct individual worm tracks, we measured the furthest radial distance each worm traveled during the experimental period. On average, wild-type worms reached a maximum distance of 17 mm from their point of origin, compared to 9.7 mm for *hen-1* mutants and 9.6 mm for *scd-2* mutants (Figure 1). Importantly, *hen-1(lf)* worms have no defect in movement per se – the rate of their sinusoidal body bends is equivalent to that of wild-type worms (Ishihara et al., 2002). Thus, their reduced dispersal suggests that *hen-1* mutants have a diminished exploratory drive.

**HEN-1/SCD-2 suppresses pharyngeal pumping in the absence of food**

*C. elegans* ingests food through a rhythmic pumping of its pharyngeal muscles. Although worms feed constitutively, the rate of feeding varies according to food availability. In the absence of food, wild-type animals lower their pumping rate by approximately 60% (Greer et al., 2008). This drop occurs rapidly and the feeding rate remains low for at least four hours during starvation. Within this timeframe, the pumping rate returns to normal if the animal is reintroduced to food. Thus, the reduction in pumping reflects physiological regulation, rather than a general deterioration of the animal’s condition.

We measured the pumping rate of individual worms by counting the number of pharyngeal contractions in a ten second period. On food, wild-type worms pumped an average of 45 times per ten seconds. For off-food conditions, we transferred worms to unseeded plates and measured pumping 90 minutes later. Consistent with earlier reports, wild-type worms pumped 65% more slowly off food (Figure 2). Next, we measured the pumping rate of *hen-1(lf)* animals, both on and off food. There was no difference between the feeding rates of wild-type and *hen-1(lf)* worms on food. However, when removed from food, *hen-1* mutants only reduced their pumping by ~45% (Figure 2). The same pattern of
results emerged when we analyzed scd-2(If) animals – on food, mutants behaved identically to wild-type worms. But like hen-1 mutants, scd-2(If) worms failed to fully depress their feeding rate off-food. This attenuated response to food deprivation further implicates hen-1 and scd-2 in signaling food status.

HEN-1/SCD-2 coordinates egg-laying with food condition

C. elegans reproduces primarily by self-fertilization. In adult hermaphrodites, maturing oocytes encounter sperm as they travel from the distal to proximal portion of the gonad. Fertilized embryos then move into the uterus, where they join the store of eggs ready for expulsion. The rate of egg-laying, like pharyngeal pumping, is strongly modulated by environmental conditions. In the absence of food, a worm lays far fewer eggs.

Based on the role of hen-1 and scd-2 in feeding regulation, we wondered whether they might also participate in the modulation of egg-laying. To measure egg-laying rates, we assayed groups of approximately ten worms during their first day of adulthood. For assays on food, worms were transferred to fresh bacterial lawns and allowed to lay eggs for one hour. The worms were removed and their progeny counted the following day. For assays off food, worms were rinsed and moved to unseeded plates. In these assays, eggs were counted immediately after the laying period. In the presence of food, wild-type worms laid approximately ten eggs per worm per hour. When assayed off food, the rate dropped by 80% (Figure 3A). On food, the egg-laying rates of hen-1 and scd-2 mutants were no different than that of wild-type animals. But mutants showed marked defects in their ability to suppress egg-laying off food – both hen-1 and scd-2 mutant animals could only reduce their rates by 50% (Figure 3A).

Differences in egg-laying rates between hen-1 and wild-type worms emerge only in the absence of food. This result suggests a role for hen-1 in the modulation of egg-laying according to food condition. However, it is conceivable that hen-1 mutants simply produce more eggs. In this scenario, if
worms were already laying eggs at maximum speed on food, the additional eggs produced by hen-1 mutants would need to be discharged in moments off of food. To rule out this possibility, we measured the number of progeny produced by hen-1 mutants over the course of their reproductive lifetime. The brood size of hen-1(1f) animals was identical to that of wild-type worms (Figure 3B). The increased egg-laying rate of hen-1 mutants therefore reflects defective regulation rather than increased production.

**Food deprivation promotes HEN-1 secretion**

HEN-1 is a neuropeptide produced and secreted by a small set of neurons (Ishihara et al., 2008). If HEN-1 signals food depletion, we might expect starvation to enhance its release. To measure HEN-1 secretion, we used a coelomocyte accumulation assay (Lee et al., 2011). C. elegans has three pairs of coelomocytes, which are macrophage-like scavenger cells. The coelomocytes reside in the worm’s body cavity and non-specifically engulf circulating macromolecules. In the accumulation assay, a fluorescently-tagged peptide is expressed from a defined neuronal source. By quantifying the fluorescence within coelomocytes, we can indirectly measure neuropeptide release.

We expressed a HEN-1::mCherry transgene under the endogenous hen-1 promoter. The resulting strain had a punctate distribution of mCherry throughout the animal’s head and within coelomocytes. We starved transgenic animals by transferring them to unseeded plates for three hours. Relative to naïve, well-fed animals, food-deprived worms had 65% more mCherry within coelomocytes (Figure 4). This result confirms what one would predict based on behavioral experiments – that food depletion initiates or enhances HEN-1 secretion.
Discussion

The HEN-1/SCD-2 pathway coordinates behaviors with food condition

We have shown that loss-of-function mutations in *hen-1* and *scd-2* impair the regulation of multiple *C. elegans* behaviors. These behaviors include foraging, feeding, and egg-laying. Disrupting the HEN-1/SCD-2 pathway has no effect on animals under well-fed conditions; phenotypes emerge only in the context of food deprivation. In addition to characterizing mutant behaviors, we also visualized HEN-1 directly and found that removing animals from food increases HEN-1 secretion. Taken together, these results indicate that HEN-1 is a state-dependent signal that tunes behaviors to food condition. In the original paper characterizing *hen-1*, the authors showed that compared to wild-type worms, *hen-1* mutants are less willing to cross an aversive barrier to reach an attractive stimulus. They also found that *hen-1(lf)* worms have defects in multiple associative learning assays. Interestingly, the learning paradigms used in the study both rely on food deprivation as an unconditioned stimulus (Ishihara et al., 2002). Our results offer a simple explanation – if HEN-1 is a sensory signal that indicates food deprivation, *hen-1* mutants would be unable to use that signal to form associations.

HEN-1 conveys information about food availability

One remaining question is what the HEN-1 signal actually reports – a lack of food in the environment or the state of hunger secondary to food deprivation. While our experiments do not directly address the issue, several lines of evidence suggest that HEN-1 communicates the absence of food in the environment. First, *hen-1* mutants exhibit a foraging defect immediately upon food removal. Removing worms from food and rinsing them free of bacteria takes less than five minutes. Though energy depletion could occur in this short timeframe, the initial stages of foraging are more likely driven by an animal’s perception of its environment. Second, *hen-1(lf)* animals show no defects in behaviors
driven by the experience of starvation per se. If a worm is briefly removed from food and then reintroduced, its pumping rate quickly returns to baseline. If the period of deprivation is prolonged, however, the animal will transiently elevate its feeding upon return to food (Avery and Horvitz, 1990). Loss-of-function hen-1 mutants have no defect in this rebound feeding response (data not shown). As with feeding, the experience of starvation also induces a temporary change in C. elegans locomotion. A previously starved worm moves more slowly on food than it would if never food-deprived. This behavior has been termed the “enhanced slowing response” and hen-1(If) animals have no impairment in it (Sawin et. al., 2000; Ishihara et al., 2002). These data highlight the fact that hen-1 mutants can accurately perceive and respond to short-term starvation. Thus, the aspect of food deprivation that HEN-1 reports is more likely related to environmental availability.

**Potential sites of HEN-1 secretion**

The hen-1 gene is expressed in three pairs of interneurons (AIY-R/L, AFD-R/L, RIC-R/L) and unilaterally in the ASE sensory neuron (ASE-R) (Ishihara et al., 2002; Johnston et al., 2005). Immunostaining reveals HEN-1 protein exclusively in ASE and AIY (Ishihara et al., 2002). Which of these cells secretes HEN-1 in response to food deprivation? Because we examined secretion using the endogenous hen-1 promoter, any of the aforementioned cells could be a site of release. We speculate, however, that ASE-R might be the primary source of regulated secretion. The ASE cells are the worm’s primary gustatory neurons, sensing the majority of soluble attractants. Furthermore, the ASE cells are functionally lateralized such that ASE-R responds specifically to decreases in chemical concentrations (Suzuki et. al., 2008). In one possible model, diminishing food levels would activate ASE-R, stimulating the release of HEN-1. Once secreted, HEN-1 could then orchestrate behavioral adaptations appropriate to food depletion.
Figure 1: Mutations in *hen-1* and *scd-2* reduce the dispersal rate of *C. elegans* off-food

(A) Radial dispersal assay. Worms were rinsed, placed on unseeded NGM plates, and allowed to move freely for five minutes. In the absence of food, animals quickly disperse. The graph shows the maximum distance a worm has traveled from its point of origin. Each bar represents the average of 14-16 worms. Experiment was repeated on at least two separate occasions with similar results. Graph shows one experiment. Asterisks indicate a statistically significant change relative to wild-type worms (p < 0.001, two-tailed t-test, Bonferroni post-test). Standard error bars are shown.

(B) Examples of individual worm tracks from the radial dispersal assay. Animals were filmed at two frames per second during the assay period. The tracks of each worm were later reconstructed. Wild-type worms (black) disperse into the new environment, whereas *hen-1* (purple) and *scd-2* (green) mutants tend to remain close to their point of origin.
FIGURE 2

![Bar chart showing pumps per 10 sec. for WT, hen-1, and scd-2 strains on and off food.](chart.png)
Figure 2: *hen-1* and *scd-2* mutants are defective in the suppression of pharyngeal pumping

Pharyngeal pumping rates. For the off-food condition, worms were rinsed and placed on unseeded NGM plates. Pumping was measured after 90 minutes. On food, the pumping rates of *hen-1* and *scd-2* mutants are indistinguishable from that of wild-type worms. In the absence of food, wild-type worms sharply reduce their pumping rate. Both *hen-1* and *scd-2* mutants exhibit defects in the modulation of pumping. Day-one adult worms were used in each condition (n = 10 worms/genotype on food; n = 15 worms/genotype off food). Experiment was repeated on at least two separate occasions with similar results. Graph shows one experiment. Asterisks indicate a statistically significant change relative to wild-type worms in the off-food condition (p < 0.01, two-tailed t-test, Bonferonni post-test). Standard error bars are shown.
FIGURE 3

(a) The graph shows the number of eggs produced per hour by worms when food is available (on food) and when food is not available (off food). The bars represent the average number of eggs produced by WT, hen-1, and scd-2 mutants. The error bars indicate the standard error. Asterisks denote significant differences between groups. 

(b) The graph compares the number of progeny produced by WT and hen-1 mutants. The bars indicate the average number of progeny per worm, with error bars showing the standard error. "ns" indicates no significant difference between the groups.
Figure 3: *hen-1* and *scd-2* mutations impair the ability of *C. elegans* to modulate egg-laying

(A) Egg-laying rates on and off food. Animals were assayed in groups of 5 – 15 individuals (n = 4 replicates per genotype for each food condition). Synchronized adult worms were allowed to lay eggs for one hour, on either a thin lawn of bacterial food or on unseeded NGM plates. For assays on food, the number of eggs laid was inferred by counting the number of L1 progeny the following day. Pilot experiments were conducted to ensure that each genotype laid 100% viable eggs. For off-food assays, eggs were counted immediately after the experiment. The number of eggs on a plate was divided by the number of egg-laying worms to yield a score of eggs per worm per hour. On food, all three genotypes lay eggs at similar rates. Off food, wild-type worms nearly cease egg-laying whereas *hen-1* and *scd-2* mutants modestly reduce their rates. Experiment was repeated on two separate occasions with similar results. Graph shows one experiment. Asterisks indicate a statistically significant change relative to wild-type worms in the off-food condition (p < 0.05, two-tailed t-test, Bonferonni corrected). Standard error bars are shown.

(B) Brood sizes of wild-type worms and *hen-1* mutants. For each genotype, 12 individual worms were followed. Graphs indicate the total number of progeny produced by worms over a lifetime. The brood sizes of wild-type animals and *hen-1* mutants were not statistically different (p = 0.23, two-tailed t-test). Standard error bars are shown.
FIGURE 4

a

b
Figure 4: Food deprivation promotes HEN-1 secretion

(A) Secretion of HEN-1. Coelomocyte accumulation assays were used to measure secretion of the HEN-1 neuropeptide during well-fed and food-deprived conditions. hen-1 gDNA was fused to mCherry and expressed under the endogenous hen-1 promoter (schematic). Steady state release was measured by quantifying the amount of mCherry scavenged by coelomocytes. Animals starved for approximately three hours prior to imaging exhibit increased HEN-1 secretion. Graph represents pooled data from two separate experiments (n = 35 coelomocytes for well-fed condition; n = 53 coelomocytes for starved condition). Asterisk indicates a statistically significant change (p < 0.001, two-tailed t-test). Standard error bars are shown.

(B) Sample images from the HEN-1 secretion assay. Images show puncta of mCherry engulfed by the animal’s anterior two coelomocytes. In separate channels (not shown), cytosolic GFP fills coelomocytes. The extent of GFP was used to trace the boundaries of cells, and the intensity of mCherry fluorescence within each coelomocytes was measured.
References


Chapter III:

HEN-1 and SCD-2 govern food-related behaviors via tyramine/octopamine regulation
Introduction

In the previous chapter, we demonstrated that HEN-1 and SCD-2 comprise a pathway that couples perception of food availability to adaptive behavioral responses. The absence of food promotes secretion of the HEN-1 neuropeptide. HEN-1 then coordinates a systemic response, signaling through its receptor SCD-2. Elements of this response include exploration for new food sources, suppression of pharyngeal pumping, and cessation of egg-laying. In this chapter, we investigate signaling downstream of the SCD-2 pathway in order to delineate a more complete neuromodulatory circuit.

Tyramine and octopamine are biogenic amines derived from the amino-acid tyrosine. They serve as both primary neurotransmitters and neuromodulators across a range of invertebrate species. Based on structural and functional similarities, tyramine and octopamine are regarded as the invertebrate counterparts of epinephrine and norepinephrine, respectively. As such, tyramine and octopamine direct body-wide responses to starvation and other stressful situations. In locusts, for instance, circulating octopamine liberates fatty acids and mobilizes other sources of stored energy (Roeder et al. 2003).

In C. elegans, tyramine and octopamine mediate several behavioral responses to food deprivation (Chase and Koelle, 2007). Earlier work from our own lab showed that in the absence of food, the DAF-7 pathway suppresses feeding via tyramine/octopamine regulation (Greer et al., 2008). A dopaminergic food-sensing pathway also converges onto octopamine producing neurons (Suo et al., 2009). In this chapter, we use genetic and pharmacological approaches to dissect the roles of tyramine and octopamine in feeding and egg-laying. We demonstrate that tyramine and/or octopamine are downstream effectors of HEN-1/SCD-2 with regard to feeding. With respect to the modulation of egg-laying, we present evidence that HEN-1/SCD-2 may rely on tyramine signaling.
Results

HEN-1/SCD-2 modulates pharyngeal pumping via tyramine/octopamine signaling

Tyramine and octopamine are derived from the amino-acid tyrosine. Tyrosine is converted to tyramine by the enzyme TDC-1, tyrosine decarboxylase. Tyramine functions as both a signaling molecule itself and as the precursor for octopamine synthesis. TBH-1, tyramine β-hydroxylase, catalyzes the conversion of tyramine to octopamine (Figure 1A) (Chase and Koelle, 2007). In the C. elegans nervous system, tdc-1 and tbh-1 expression are limited to a small number of neurons. The interneurons RIM and RIC express tdc-1. The expression of tbh-1 is restricted to RIC. Thus, RIM is capable of producing tyramine whereas RIC can synthesize both tyramine and octopamine (Alkema et. al., 2005).

Worms require both tdc-1 and tbh-1 to properly regulate pharyngeal pumping. Earlier work from our lab showed that both mutants feed at wild-type rates on food, but fail to fully suppress pumping when removed from food (Greer et. al., 2008). Mutants in hen-1 and scd-2 share this modulatory defect, prompting us to ask whether all these genes comprise a single pathway. We began by confirming the tdc-1(1f) phenotype. In the context of abundant food, tdc-1 mutants and wild-type animals fed at equal rates. Upon removal from food, tdc-1(1f) animals reduced their feeding by 33%, compared to 55% by wild-type worms (Figure 1B). We then constructed a tdc-1; hen-1 double mutant and assayed its feeding behavior. Like each single mutant, tdc-1; hen-1(1f) animals suppressed their pharyngeal pumping by approximately 33% (Figure 1B). This result suggests that tdc-1 acts in a single pathway with hen-1/scd-2.

Loss of tdc-1 function eliminates the production of both tyramine and octopamine. Mutants deficient in tbh-1 only lack octopamine. In agreement with earlier work, we found that tbh-1(1f) animals pumped normally on food but were defective in their ability to suppress pumping when removed from food (Figure 1C). We then crossed tbh-1(1f) animals to hen-1 mutants and assessed the double mutant’s
feeding behavior. As with *tdc-1(lf)*, the combination of *tbh-1* and *hen-1* mutations produced a non-additive phenotype, indicating that *tbh-1* also acts in a linear genetic pathway with *hen-1/scd-2* (Figure 1C).

Next we sought to establish the relationship between HEN-1/SCD-2 activity and tyramine/octopamine signaling. In one scenario, tyramine and octopamine could act upstream of HEN-1/SCD-2 – for instance, by stimulating the release of HEN-1 in response to food deprivation. Alternatively, HEN-1/SCD-2 activity could promote tyramine/octopamine signaling downstream. To distinguish these possibilities, we treated food-deprived *scd-2(lf)* worms with exogenous tyramine and octopamine. If tyramine and/or octopamine regulate the HEN-1/SCD-2 pathway, drug treatment should not alter the feeding rate of *scd-2* mutants. If HEN-1/SCD-2 regulates tyramine/octopamine, however, exogenous applications might be expected to normalize the *scd-2(lf)* phenotype. 500 µM of either tyramine or octopamine had no effect on the pumping rate of wild-type animals. The same concentration of both compounds inhibited *scd-2(lf)* pumping by approximately 20%. This effect rendered *scd-2* mutant pumping rates indistinguishable from those of wild-type animals (Figure 2).

**The HEN-1/SCD-2 pathway may regulate egg-laying through tyramine**

Previous pharmacological studies have implicated tyramine and octopamine in the control of egg-laying. Exogenous addition of either compound inhibits egg-laying on food. Conversely, an octopamine receptor antagonist stimulates the rate of egg-laying (Alkema et. al., 2005; Horvitz et. al. 1982). We examined the egg-laying behavior of *tdc-1* mutants. In the presence of bacteria, *tdc-1* mutants lay eggs at the same rate as wild-type animals (Alkema et. al., 2005). When tested in the absence of food, *tdc-1(lf)* animals laid eggs at twice the rate of wild-type worms, though this effect did not reach statistical significance (Figure 3A). This behavioral pattern resembles that of *hen-1* and *scd-2* mutants. On food, all three mutants lay eggs at a wild-type rate. Off food, mutants exhibit defects in
their ability to suppress egg-laying. In tdc-1; hen-1 double mutants, the defect in modulation was no more severe than in individual mutants. While further experiments will be necessary to confirm the tdc-1(lf) egg-laying phenotype, tdc-1 and hen-1 may regulate egg-laying through a common pathway.

To determine the direction of signaling between HEN-1/SCD-2 and tyramine/octopamine, we treated hen-1 mutants with exogenous compounds. 4 mM of tyramine suppressed the elevated egg-laying rate of hen-1(lf) animals off food. An equal concentration of octopamine had no effect. Tyramine treated hen-1 mutants laid eggs at a rate similar to that of untreated, wild-type animals (Figure 3B, C). This result suggests that HEN-1/SCD-2 activation could promote downstream tyramine/octopamine signaling and that HEN-1 might act through tyramine.

Discussion

The HEN-1/SCD-2 pathway regulates tyramine/octopamine signaling

In chapter two, we established that the HEN-1/SCD-2 pathway governs behavioral responses to food deprivation. In this chapter, we aimed to identify downstream effectors of HEN-1/SCD-2 signaling. We focused on tyramine and octopamine, two biogenic amines which had previously been implicated in C. elegans food signaling. Using genetic analyses and pharmacological experiments, we have shown that tyramine and octopamine mediate the effects of HEN-1/SCD-2 on feeding. Our results also provide preliminary evidence that tyramine may mediate the egg-laying effects of HEN-1/SCD-2.

Like hen-1/scd-2 mutants, tdc-1(lf) and tbh-1(lf) animals exhibit defects in the suppression of pumping off-food. Double mutants between hen-1 and either enzymatic gene are no more defective than any constituent single mutants. Thus, hen-1/scd-2 and tdc-1/tbh-1 act within a common genetic pathway. When treated with exogenous tyramine or octopamine, scd-2 mutants regain the capacity to
fully suppress pumping. This result demonstrates that tyramine and octopamine are effectors, rather than upstream regulators, of HEN-1/SCD-2. Taken together, the data support a linear circuit model in which HEN-1/SCD-2 activity promotes tyramine/octopamine signaling.

**Relative contributions of tyramine and octopamine to feeding and egg-laying behaviors**

Octopamine is required for the modulation of feeding – when removed from food, *tbh-1* mutants do not appropriately reduce pumping. Mutants in *tdc-1* share this defect, indicating that tyramine production is also required for feeding suppression. It remains unclear, however, whether worms require tyramine merely as a synthetic precursor for octopamine, or as a bona fide regulator of feeding.

Exogenous tyramine reduces the pumping rate of *scd-2* mutants off-food. This effect supports the idea that tyramine itself regulates pumping. However, exogenously added tyramine may undergo conversion to octopamine within the worm. Exogenous tyramine might also have non-specific effects, including activation of octopamine receptors. To establish the role of tyramine more conclusively, one could evaluate how tyramine affects *scd-2; tbh-1* double mutants. In addition, at least two tyramine-specific receptors have been identified – *ser-2* and *tyra-2* (Rex and Komuniecki, 2002; Rex et al., 2004; Rex et al., 2005). One could examine whether mutations in these receptors impair feeding regulation.

With regard to egg-laying, tyramine may function as the primary regulatory signal. Both exogenous tyramine and octopamine affected feeding behavior in our assays. In contrast, only tyramine was able to blunt the hyperactive egg-laying of food-deprived *hen-1* mutants. This tyramine-specific effect echoes other studies. For instance, *tdc-1* mutants lay eggs that are immature relative to those expelled by wild-type worms. Mutants in *tbh-1* lay eggs at the normal developmental stage (Alkema et al., 2005). Coupled with our pharmacological data, this finding suggests that tyramine may regulate egg-laying independent of octopamine.
FIGURE 1

a

tyrosine $\xrightarrow{TDC-1}$ tyramine $\xrightarrow{TBH-1}$ octopamine

b

<table>
<thead>
<tr>
<th></th>
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</tr>
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<tbody>
<tr>
<td>WT</td>
<td>45</td>
</tr>
<tr>
<td>tdc-1</td>
<td>40</td>
</tr>
<tr>
<td>hen-1</td>
<td>35</td>
</tr>
<tr>
<td>tdc-1; hen-1</td>
<td>30</td>
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on food  off food

C

<table>
<thead>
<tr>
<th></th>
<th>pumps / 10 sec.</th>
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<tbody>
<tr>
<td>WT</td>
<td>50</td>
</tr>
<tr>
<td>tbh-1</td>
<td>45</td>
</tr>
<tr>
<td>hen-1</td>
<td>40</td>
</tr>
<tr>
<td>tbh-1; hen-1</td>
<td>35</td>
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on food  off food
Figure 1: Tyramine and octopamine deficient mutants are defective in the modulation of pumping

(A) Schematic of tyramine/octopamine biosynthetic pathway. The amino acid tyrosine is converted to tyramine by the enzyme TDC-1, tyrosine decarboxylase. The enzyme tyramine β-hydroxylase, TBH-1, catalyzes the conversion of tyramine to octopamine.

(B) Pharyngeal pumping rates. In the presence of food, *tdc-1* and *hen-1* mutants pump at wild-type rates. Off food, both mutants are defective in suppressing pumping. *tdc-1; hen-1* double mutants showed no greater defect in pumping modulation than either single mutant. Day-one adult worms were used for both conditions. Graph represents pooled data from two separate experiments (n = 20 worms/genotype on food; n = 30 worms/genotype off food). Asterisks indicate a statistically significant change relative to wild-type worms in the off-food condition (p < 0.001, ANOVA with Bonferonni post-test). Standard error bars are shown.

(C) Pharyngeal pumping rates. On food, *tbh-1* and *hen-1* mutants pump at wild-type rates. Off food, both mutants are impaired in the modulation of pumping. *tbh-1; hen-1* double mutants are no more defective than either single mutant. Day-one adult worms were used for both conditions. Graph shows data from one unrepeated experiment (n = 10-13 worms/genotype on food; n = 15-16 worms/genotype off food). Due to high variance, the WT vs. *tbh-1* comparison only reached statistical significance when data were normalized to on-food pumping rates (cross symbol indicates p < 0.01, ANOVA with Bonferonni post-test). Other comparisons were statistically significant whether data were normalized or not (asterisks indicate p < 0.01, ANOVA with Bonferonni post-test). Data are presented non-normalized in order to preserve similarity to graph in (B). Standard error bars are shown.
FIGURE 2

a

![Graph showing pumps/10 sec for WT and scd-2 under vehicle and tyramine (500 μM) conditions.]

b

![Graph showing pumps/10 sec for WT and scd-2 under vehicle and octopamine (500 μM) conditions.]

* denotes significant difference.
Figure 2: Exogenous tyramine and octopamine suppress scd-2 mutant pumping defects

(A) Pharyngeal pumping rates off food. Tyramine hydrochloride dissolved in water was added to unseeded NGM plates to achieve a final concentration of 500 µM. Addition of tyramine allowed scd-2 mutants to reduce their pumping rate to wild-type levels. Assays were performed on day-one adult worms. Graph represents pooled data from two separate experiments (n = 35-36 worms/genotype for both vehicle and drug treated conditions). Asterisk indicates a statistically significant change relative to untreated scd-2 mutants (p < 0.01, two-tailed t-test). Standard error bars are shown.

(B) Pharyngeal pumping rates off food. Octopamine hydrochloride dissolved in water was added to unseeded NGM plates to achieve a final concentration of 500 µM. As with tyramine, addition of octopamine allowed scd-2 mutants to reduce their pumping rate to wild-type levels. Assays were performed on day-one adult worms. Graph shows data from one unrepeated experiment (n = 15 worms/genotype for both vehicle and drug treated conditions). Asterisk indicates a statistically significant change relative to untreated scd-2 mutants (p < 0.05, two-tailed t-test). Standard error bars are shown.
FIGURE 3

a

![Bar graph showing eggs/worm/hr for WT, tdc-1, hen-1, tdc-1; hen-1.](image)

b

![Bar graph showing comparison between WT and hen-1 with vehicle and tyramine at 4 mM.](image)

c

![Bar graph showing comparison between WT and hen-1 with vehicle and octopamine at 4 mM.](image)
Figure 3: Tyramine modulates the egg-laying rate of food-deprived animals

(A) Egg-laying rates off food. Mutants in tdc-1 and hen-1 lay eggs at a higher rate than wild-type worms, though the WT vs. tdc-1 comparison did not reach statistical significance. The phenotype of tdc-1; hen-1 double mutants is approximately equal in magnitude to that of each single mutant. Graph shows data from one experiment (n = 3 replicates per genotype). Experiment was repeated on two separate occasions with similar results, but data could not be pooled for analysis. Asterisks indicate statistically significant changes relative to wild-type worms (p < 0.01, ANOVA with Bonferroni post-test). Standard error bars are shown.

(B) Egg-laying rates off food. Tyramine hydrochloride dissolved in water was added to unseeded NGM plates to achieve a final concentration of 4 mM. Tyramine reduced the egg-laying rate of hen-1 mutants to near wild-type levels. Graph shows data from one unrepeated experiment (n = 3 replicates per genotype or condition). Asterisk indicates a statistically significant change relative to untreated hen-1 mutants (p < 0.05, two-tailed t-test). Standard error bars are shown.

(C) Egg-laying rates off food. Octopamine hydrochloride dissolved in water was added to unseeded NGM plates to achieve a final concentration of 4 mM. Octopamine did not alter the elevated egg-laying rate of hen-1 mutants. Graph shows data from one unrepeated experiment (n = 3 replicates per genotype or condition). Standard error bars are shown.
References


Chapter IV:

The HEN-1/SCD-2 pathway likely acts on RIC and additional neurons to regulate behavior
Introduction

In Chapter II, we demonstrated that HEN-1 is a neuropeptide hormone which signals food depletion. Acting through its receptor SCD-2, HEN-1 drives adaptive responses in foraging, pharyngeal pumping, and egg-laying. In Chapter III, we showed that the HEN-1/SCD-2 pathway exerts its effects on pumping by promoting tyramine/octopamine signaling. To modulate egg-laying, HEN-1/SCD-2 might act through tyramine. Here we discuss several experiments aimed at clarifying how the HEN-1/SCD-2 and tyramine/octopamine pathways interact.

Within the C. elegans nervous system, tyramine and octopamine are produced in the RIM and RIC interneurons. We show that SCD-2 regulates egg-laying by acting both directly in RIM/RIC and in additional neurons. With respect to pharyngeal pumping, SCD-2’s site of action remains undetermined. We present evidence that the effects of SCD-2 on pumping may be mediated by the transcription factor DAF-3. Our lab previously defined a circuit by which favorable environmental conditions repress DAF-3 in order to maintain a high feeding rate. In this circuit, abundant food promotes DAF-7 neuropeptide signaling. DAF-7 activity represses DAF-3, which in turn inhibits the release of tyramine/octopamine (Greer et al., 2008). Given the opposing effect of HEN-1 on tyramine/octopamine signaling, we explore the possibility that HEN-1 and DAF-7 act as countervailing food signals.

Results

SCD-2 is expressed in the RIC interneurons

In C. elegans, tyramine and octopamine production are limited to a small number of cells. The tdc-1 gene is expressed in the uv1 uterine cells, the gonadal sheath cells, and the RIM and RIC interneurons. The expression of tbh-1 is further restricted to the gonadal sheath cells and the RIC
interneurons (Alkema et al., 2005). RIC is therefore capable of synthesizing both tyramine and octopamine, whereas RIM can produce only tyramine.

To address the possibility that scd-2 may function directly in the RIM/RIC interneurons, we first determined its expression pattern. To do so, we generated a GFP transcriptional reporter with a promoter consisting of two kilobases of DNA immediately upstream of the scd-2 coding region. Consistent with other reports, scd-2 was expressed in numerous head neurons (Figure 1A) (Liao et al., 2004; Shinkai et al., 2011). Next, we created a strain containing both scd-2 and tdc-1 transcriptional reporters (pscd-2::gfp and ptdc-1::mCherry). We observed co-expression of the markers in RIC, but not RIM (Figure 1A, inset).

**SCD-2 acts partially in RIM/RIC to modulate egg-laying**

Having established that scd-2 is expressed in RIC, we next examined whether SCD-2 activity in RIM/RIC is sufficient to regulate behavior. To do so, we constructed a plasmid containing the full length scd-2 cDNA driven by a tdc-1 promoter. The promoter sequence we used corresponds to the two kilobases of DNA upstream of the tdc-1 coding region. This is the same sequence used in our tdc-1 transcriptional reporter, and it drives expression strongly and specifically in RIM/RIC.

We introduced the ptdc-1::scd-2 construct into scd-2(lf) mutants. When injected at high concentrations (20 - 100 ng/µL), the plasmid caused animals to arrest at the L1 stage, and appear sluggish and sickly. A similar effect was observed by a group who attempted to express a constitutively-active scd-2 allele in wild-type animals (Reiner et al., 2008). We ultimately titrated the injection concentration down to 1 ng/µL. At this concentration, transgenic animals had no growth delays or other obvious defects.

We assayed the egg-laying behavior of the transgenic animals off food. Non-transgenic scd-2(lf) mutants laid eggs at over three times the rate of wild-type animals. Reconstitution of scd-2 in RIM/RIC
partially rescued this effect. Compared to non-transgenic controls, transgenic scd-2 mutants laid 40% fewer eggs (Figure 1B). This result suggests that SCD-2 acts directly in RIM/RIC to inhibit egg-laying, but that in order to fully suppress egg-laying off food, SCD-2 may be required in other locations.

**DAF-3 and SCD-2 act in a common pathway for feeding regulation**

The *daf-3* gene encodes a co-SMAD transcription factor related to the vertebrate Smad4 proteins (Patterson et al., 1997). In cooperation with DAF-5, a nematode specific protein, DAF-3 regulates systemic adaptations to nutrient poor environments (da Graca et al., 2004). Our lab previously showed that DAF-3 activation, specifically in RIM/RIC, reduces pharyngeal pumping, promotes fat storage, and initiates dauer entry (Greer et al., 2008). Like HEN-1/SCD-2, DAF-3 affects feeding rate through tyramine/octopamine signaling. Based on this similarity, we wondered whether DAF-3 also mediates the effects of HEN-1/SCD-2 on pumping.

On food, *daf-3*(l) animals pumped at wild-type rates. When removed from food, *daf-3* mutants suppressed pumping by approximately 30%, compared to a nearly 55% reduction by wild-type animals. The feeding reduction of *daf-3* mutants was similar in magnitude to that of *scd-2*(l) animals (Figure 2). We then examined the feeding rates of *scd-2; daf-3* double mutants, both on and off food. Double mutants pumped at wild-type rates in the presence of food. In the absence of food, they suppressed pumping by roughly 25%. This level of suppression was not significantly different from that of either single mutant (Figure 2). We therefore conclude that *scd-2* and *daf-3* act in a single pathway to regulate feeding.
**DAF-7 stimulates egg-laying**

DAF-3 is a major downstream target of a pathway that signals favorable environmental conditions. When food is plentiful, the ASI sensory neuron produces DAF-7, a neuropeptide homologous to mammalian TGF-β (Ren et al., 1996). Secreted DAF-7 activates the DAF-1/DAF-4 receptor complex, which in turn leads to the repression of DAF-3. Thus, through its effect on DAF-3, the DAF-7 hormone prevents well-fed animals from initiating starvation responses. Consistent with this role, daf-7(lf) mutants exhibit a reduced feeding rate even in the presence of food (Greer et al., 2008).

Given their feeding regulatory function, we wondered whether DAF-7 and DAF-3 might also modulate egg-laying in response to food condition. Previous studies have noted that daf-7(lf) animals accumulate excessive eggs in their uteri, a phenotype known as “Egl” (Trent et al., 1983). Various defects can produce an Egl phenotype, including increased egg production, egg retention, or physical abnormalities of the vulva (Trent et al., 1983; Daniels et al., 2000). Given that DAF-7 conveys food availability, we wondered whether the daf-7(lf) Egl phenotype reflects an inappropriate retention of eggs under food-replete conditions.

On food, daf-7(lf) animals laid eggs at approximately half the rate of wild-type worms (Figure 3A). In the off-food condition, daf-7 mutants also had a 50-60% lower egg-laying rate than wild-type animals (Figure 3B). These results imply that daf-7 mutants, while defective in their baseline egg-laying rate, can still effectively modulate egg-laying in response to food deprivation.
Discussion

SCD-2 likely acts in RIC and additional neurons to promote food-deprivation responses

In previous chapters, we showed that SCD-2 suppresses egg-laying in the absence of food. This effect may occur through modulation of tyramine/octopamine signaling. A preliminary experiment suggested that tdc-1 mutants may share the egg-laying phenotype of scd-2(II) animals. In addition, exogenous tyramine rescues the egg-laying defect of scd-2 mutants. In this chapter, we showed that scd-2 is normally expressed in the tyraminergic RIC neuron. Selective reconstitution of scd-2 in RIM and RIC allowed scd-2 mutant to reduce their egg-laying rate toward wild-type levels. In one possible model, SCD-2 could act on RIC to stimulate the production or release of tyramine during food-deprivation. However, SCD-2 activity in RIM/RIC was insufficient to normalize the scd-2 phenotype. One explanation for this incomplete rescue is that scd-2 is required in other tyraminergic cells. While RIM and RIC are the only neuronal sources of tyramine, the uv1 uterine cells are tyraminergic and may regulate egg-laying through a paracrine mechanism involving tyramine (Lints and Hall, 2009).

We have not yet tested whether SCD-2 acts directly in RIM/RIC to regulate pumping. However, other data support this possibility. During food-deprivation, diminished DAF-7 signaling promotes DAF-3 activity. Via cell-autonomous effects in RIM and RIC, DAF-3 stimulates tyramine/octopamine signaling to lower pharyngeal pumping (Greer et al., 2008). We demonstrated that DAF-3 also acts in the SCD-2 pumping regulatory pathway. Furthermore, the HEN-1/SCD-2 pathway signals through tyramine and octopamine. Thus, SCD-2 may operate directly in RIM/RIC to modulate pumping.
**HEN-1 and DAF-7 are competing inputs to a feeding regulatory circuit**

HEN-1 down-regulates pharyngeal pumping in the absence of food. DAF-7 maintains a high feeding rate when food is present. These opposing functions suggest that HEN-1 and DAF-7 antagonistically regulate a common feeding circuit. If so, at what point do the HEN-1 and DAF-7 signaling pathways converge?

One possibility is that HEN-1 and DAF-7 both act through DAF-3. DAF-3 is required for the feeding regulatory effects of DAF-7. In this chapter, we showed that *scd-2* and *daf-3* also act in a single pathway. Our finding agrees with two other studies that characterized the role of *scd-2* in dauer formation (Inoue and Thomas, 2000; Reiner et al., 2008). The earlier study identified *scd-2* by screening for animals defective in dauer entry (*scd-2* = suppressor of constitutive dauer). Specifically, the authors looked for mutations that suppressed the constitutive dauer phenotype of *daf-7* pathway mutants (Inoue and Thomas, 2000). The authors of the second study visualized DAF-3 activity using a transgenic reporter. The reporter consists of a promoter rich in DAF-3 binding sequences fused to the GFP open reading frame. Mutations in *daf-7* and *scd-2* altered GFP expression in the predicted opposite directions (Reiner et al., 2008).

In regulating pumping, HEN-1/SCD-2 may antagonize DAF-7 signaling at the level of DAF-3. But regardless of whether these pathways converge on DAF-3, each ultimately regulates tyramine/octopamine signaling. The mechanism for this regulation remains unclear. Our lab previously examined *tdc-1* and *tbh-1* transcriptional reporters in the context of a *daf-7* mutation (Greer ER, 2008). Deficiency of *daf-7* did not increase expression of either enzymatic gene, as one might expect. In this study, we examined the effect of starvation on dense-core vesicle release from RIM/RIC (Supplemental Figure 1). If tyramine and octopamine are secreted in dense-core-vesicles, one prediction is that food-deprivation would elevate vesicle release. Instead, we observed a slight downward trend in secretion during starvation.
HEN-1 and DAF-7 may regulate egg-laying via separate pathways

HEN-1 and DAF-7 signaling may converge in RIM/RIC to regulate pharyngeal pumping. In modulating egg-laying, however, the two food signals probably operate in distinct circuits. We presented preliminary evidence that HEN-1 affects egg-laying through tyramine/octopamine signaling. DAF-7 regulates egg-laying by an independent pathway, as tdc-1 mutations fail to suppress the Egl phenotypes of daf-7 pathway mutants (Greer et al., 2008). HEN-1 and DAF-7 also appear to affect egg-laying in qualitatively different ways – hen-1 mutants lay eggs at a normal rate on food and are defective in lowering their rate off food. Mutation of daf-7 reduces egg-laying rates, both on and off food. These phenotypic differences suggest that DAF-7 may provide tonic input to one egg-laying circuit while HEN-1 modulates a distinct circuit according to food status.
Figure 1: SCD-2 acts partially in the RIM/RIC interneurons to regulate egg-laying

(A) Image of scd-2 and tdc-1 transcriptional reporters. Image shows expression patterns of scd-2 and tdc-1 in the head. Numerous head neurons express scd-2 whereas tdc-1 expression is restricted to the RIM and RIC interneuron. Panel shows pseudo-colored fluorescent images overlaid on a DIC image. Inset is a digital magnification of panel image. Co-expression of scd-2 and tdc-1 occurs in RIC but not RIM.

(B) Egg-laying rates off food. Reconstitution of scd-2 in RIM/RIC partially rescues the egg-laying phenotype of scd-2 mutants. scd-2 cDNA was expressed under the tdc-1 promoter. Transgenic scd-2 mutants had intermediate egg-laying rates relative to wild-type animals and non-transgenic scd-2 mutants. Graph shows one unrepeated experiment (n = 3-4 replicates per genotype). Asterisks indicate statistically significant changes (p < 0.01, ANOVA with Bonferroni post-test). Standard error bars are shown.
Figure 2: Mutations in daf-3 impair feeding regulation

Pharyngeal pumping rates. daf-3 mutants pump at wild-type rates on food. Off food, daf-3 mutants are defective in suppressing pumping, like scd-2 mutants. scd-2; daf-3 double mutants have no greater impairment than either single mutant. Day-one adult worms were used in assay (n = 10 worms/genotype on food; n = 15 worms/genotype off food). Graph shows one unrepeated experiment. Asterisks indicate statistically significant changes relative to wild-type worms in the off-food condition (p < 0.001, ANOVA with Bonferroni post-test). Standard error bars are shown.
FIGURE 3

(a) eggs/worm/hr.

(b) eggs/worm/hr.
Figure 3: Mutation of daf-7 reduces egg-laying on and off food

(A) Egg-laying rate on food. Relative to wild-type animals, daf-7 mutants lay eggs at a reduced rate. Graph shows data from one unrepeated experiment (n = 4 replicates per genotype). Asterisk indicates statistically significant change (p = 0.006, two-tailed t-test). Standard error bars are shown.

(B) Egg-laying rate off food. Relative to wild-type animals, daf-7 mutants lay eggs at a reduced rate. Graph shows data from one unrepeated experiment (n = 3 replicates per genotype). Asterisk indicates statistically significant change (p = 0.02, two-tailed t-test). Standard error bars are shown.
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Chapter V:

Models for the regulation of feeding and egg-laying by HEN-1 and DAF-7
For most organisms, food availability fluctuates over time and space. In order to regulate behaviors accordingly, an animal needs accurate and robust mechanisms to sense changes in its environment. The *C. elegans* nervous system relays information related to food status through multiple, parallel signaling pathways. While some of these pathways have been well-characterized, others remain undiscovered or poorly understood. An additional challenge is to understand how these various food signaling pathways interact with each other.

We have discovered a novel food signaling pathway. The HEN-1 neuropeptide is released from a small set of neurons in response to food depletion. Secreted HEN-1 acts in a hormonal fashion, activating its receptor SCD-2 at multiple downstream sites. Activation of SCD-2 instructs the animal to leave its nutrient poor environment, to reduce energetically wasteful pharyngeal motion, and to delay the reproductive process in the interest of its future progeny. To regulate pumping, SCD-2 enhances the production or release of tyramine and octopamine – two monoamine signaling molecules previously implicated in the *C. elegans* starvation response. To regulate egg-laying, SCD-2 may act through tyramine. Within the nervous system, tyramine and octopamine are produced exclusively in the RIM/RIC interneurons. Though untested, SCD-2 may act directly on RIM/RIC to affect pumping. To suppress egg-laying, SCD-2 is likely to act in RIM/RIC, and in one or more additional locations. The uv1 neuroendocrine cell, located adjacent to the vulva, may be this additional site.

DAF-7, a TGF-β-like neuropeptide, is produced by the ASI sensory neuron in response to abundant food or otherwise favorable conditions. DAF-7 was first characterized in the context of dauer regulation. Animals deficient in DAF-7 enter dauer constitutively and behave like starved animals, even if food is replete. These behavioral phenotypes include reduced pumping and egg-laying rates on food. DAF-7 signals through its receptors DAF-1/DAF-4, ultimately repressing the DAF-3 transcription factor. This process occurs in RIM/RIC and is also mediated by tyramine/octopamine signaling.
Based on DAF-7’s neuronal target, and its monoamine effectors, we hypothesized that SCD-2 and DAF-1/4 antagonistically regulate DAF-3. We tested this idea in the context of pumping regulation. Loss-of-function daf-3 mutants are defective in suppressing pumping off food, and scd-2; daf-3 double mutants are equally defective. While this data does not necessarily indicate that SCD-2 acts through DAF-3, it does establish that SCD-2 and DAF-3 work in the same pathway. In our proposed model of pumping regulation, food-deprivation activates SCD-2, which stimulates tyramine/octopamine signaling (Figure 1). DAF-3 may mediate this effect, or SCD-2 may regulate tyramine/octopamine signaling independently. When food is abundant, the SCD-2 pathway is shut off and the DAF-7 pathway is active. DAF-7 signaling ultimately represses DAF-3, thereby inhibiting tyramine/octopamine activity. Future experiments will be needed to determine the mechanism by which DAF-3 regulates tyramine/octopamine signaling.

HEN-1 and DAF-7 signaling also regulate egg-laying in opposing directions. Unlike with pumping, however, the two pathways may not converge upstream of the end organ (Figure 2). DAF-7 acts outside of RIC and independently of tyramine/octopamine. Preliminary data indicate that HEN-1/SCD-2 acts at least partially in RIM/RIC and that the pathway might signal through tyramine. In addition, beyond regulating egg-laying in opposite directions, the two pathways serve distinct functions. HEN-1 plays a modulatory role, reducing egg-laying in response to food-deprivation. DAF-7 stimulates egg-laying without regard to food condition.
Figure 1: Model for the regulation of pharyngeal pumping by HEN-1 and DAF-7 signaling

In response to diminishing food levels, the ASE/AIY neurons secrete the HEN-1 neuropeptide. HEN-1 activates its receptor SCD-2, possibly on the RIM/RIC interneurons but perhaps elsewhere. SCD-2 activity promotes tyramine/octopamine signaling, which in turn reduces pharyngeal pumping. In one possible scenario, SCD-2 acts in RIM/RIC to drive the production or release of tyramine/octopamine. This effect could be mediated by the DAF-3 transcription factor (dotted line), or by another mechanism (solid line). When food is abundant, the HEN-1 signaling pathway is inhibited. DAF-7, originating from the ASI neuron, activates its receptor complex DAF-1/4 (green/black) specifically on RIM/RIC. DAF-1/4 activity leads to DAF-3 repression. Normally, DAF-3 promotes the release of tyramine and octopamine. By inhibiting DAF-3 activity, DAF-7 limits tyramine/octopamine release, thereby maintaining the animal’s high pumping rate on food.
FIGURE 2
Figure 2: Model for egg-laying regulation via parallel HEN-1 and DAF-7 signaling pathways

The presence of food maintains the DAF-7 pathway in an active state. Tonic DAF-7 signaling drives egg-laying. When food becomes depleted, ASE/AIY elevate HEN-1 secretion. Signaling through its receptor SCD-2, HEN-1 suppresses egg-laying. The regulation of egg-laying by HEN-1/SCD-2 may in part depend on SCD-2 activity in RIC. In one speculative model (gray box), SCD-2 stimulates the release of tyramine from RIM/RIC and/or the neuroendocrine cell uv1. Secreted tyramine inhibits egg-laying.
PART II:

The NPR-1 neuropeptide receptor governs social behavior by modulating neuropeptide release
Chapter VI:

Enhanced neuropeptide secretion drives $npr-1$ mutant social behavior
Introduction

Social behaviors are common throughout the animal kingdom. Interaction between members of a species enables unique strategies for avoiding predation, acquiring food, and caring for offspring (Krebs and Davies, 2000). Among the simplest social behaviors is aggregation, the process by which individual members of a species form groups. In the roundworm C. elegans, aggregation is part of a polymorphic behavior known as social feeding. Whereas strains isolated from the wild tend to cluster and feed in groups, the standard laboratory strain feeds alone. These social and solitary phenotypes are attributable to a single base-pair difference within the npr-1 gene, a member of the neuropeptide-Y receptor family. The allele found in wild isolates encodes a low activity NPR-1 permissive to social feeding. High NPR-1 activity, conferred by the domesticated allele, inhibits aggregation (de Bono and Bargmann, 1998).

On a cellular level, NPR-1 operates within a “hub-and-spoke” circuit. This circuit consists of the RMG hub interneuron, and various sensory spoke neurons connected to it via gap junctions (Macosko et al., 2009). Spoke neurons transduce a number of environmental signals that drive aggregation, including pheromones, ambient oxygen, and food-derived odors (Jang et al., 2012; Gray et al., 2004; de Bono et al., 2002). In social feeding animals – those with low NPR-1 activity – sensory neurons respond strongly to these signals (Macosko et al., 2009; Busch et al., 2011). In solitary animals, high NPR-1 activity directly inhibits the sensory activation of spoke neurons (Coates and de Bono, 2002). NPR-1 also acts in the RMG hub, through which it further dampens sensory neuron excitability (Macoscko et al., 2009). Thus, via coordinated action in both hub and spoke neurons, NPR-1 governs the circuit’s overall responsiveness to aggregation-promoting signals.

Despite understanding how NPR-1 regulates this sensory circuit, we still have little insight into how the circuit generates social feeding behavior. Specifically, which neuron(s) drive aggregation and how do they signal downstream? Recently, our lab has been investigating molecular pathways involved
in dense-core vesicle release from the ADL sensory neuron. The activity of ADL and the related ASH neuron is required for social behavior. Ablating ADL/ASH in npr-1 loss-of-function mutants suppresses aggregation. Disrupting ADL sensory transduction also transforms npr-1(lf) animals into solitary feeders (de Bono et al., 2002). Given this known role for ADL in social feeding, we wondered whether NPR-1 regulates dense-core vesicle release from ADL, and whether the secreted cargoes of vesicles drive aggregation behavior. Here we present our analysis of the effects of npr-1 on dense-core vesicle release, from ADL and other neurons, and on the relationship between secretion and social behavior.

Results

Loss of NPR-1 promotes dense-core vesicle release

To monitor dense-core vesicle release from individual neurons, we used a previously established coelomocyte accumulation assay. The C. elegans pseudocoelom is a fluid-filled cavity that borders and surrounds various internal organs. Three pairs of cells within the cavity, known as coelomocytes, non-specifically engulf macromolecules and other components of the circulating fluid. Many signaling molecules that operate within the nervous system eventually diffuse into the cavity and accumulate in coelomocytes. By expressing fluorescently tagged peptides in selected neurons, and measuring their abundance in coelomocytes, we can determine the level of steady state release from defined sources (Lee et al., 2011).

To determine whether NPR-1 activity affects dense-core vesicle release, we examined neuropeptide secretion in an npr-1 loss-of-function mutant. Previous work has implicated the ADL neurons in the regulation of social behavior (de Bono et al., 2002). We therefore began by crossing npr-1(lf) to a transgenic strain in which an mCherry-tagged insulin (DAF-28::mCherry) is expressed solely in
ADL neurons. Relative to wild-type N2 animals, *npr-1* mutants exhibited 135% higher secretion from ADL (Figure 1A). The elevated secretion was not specific to the tagged neuropeptide – *npr-1* mutants showed equally high ADL secretion when a different reporter was examined (FLP-21::mCherry) (Figure 1C). These results suggest that NPR-1 regulates ADL dense-core vesicle release in general, rather than affecting particular neuropeptide cargos.

A previous report demonstrated that *npr-1* mutants differentially regulate a number of genes related to immune function (Styer et al., 2008). The ADL-specific promoter we used in our assays (*psrh-220*) does not have a known role in immunity. Nevertheless, to ensure that elevated ADL secretion was not a byproduct of increased transcription, we measured *psrh-220* activity using a fluorescent reporter. The intensity of *mCherry* driven by *psrh-220* was no greater in the ADL cell bodies of *npr-1* mutants than in those of wild-type animals (Figure 1D). Thus, loss of NPR-1 elevates neuropeptide secretion from ADL by regulating a process distal to gene expression.

NPR-1 is expressed in approximately 15-20 head neurons, along with several other body neurons (Coates and de Bono, 2002). The ADL neurons, while functionally necessary for aggregation behavior, do not themselves express NPR-1. We therefore sought to explore whether NPR-1 regulates dense-core vesicle release from additional neurons in a cell non-autonomous fashion. The ASI sensory neurons do not express NPR-1 and have not been functionally implicated in NPR-1 mediated aggregation. Unlike ADL, which dramatically increased secretion in the absence of *npr-1*, ASI exhibited a more modest 20% elevation in DAF-28 release (Figure 1B). This finding indicates that NPR-1’s robust effect on dense-core vesicle release is not present in all cells, and may be restricted to neurons involved in social feeding behavior.
Reducing dense-core vesicle release suppresses social behavior

Aggregation behavior is strongly regulated by environmental signals. These signals include bacteria-derived odors, which are processed by the ADL and ASH neurons, and ambient oxygen, whose concentration is detected by URX and other gas-sensing neurons (Cheung et al., 2004). Manipulating environmental conditions – lowering oxygen levels or killing the animals’ bacterial food source – transforms npr-1 mutants into solitary feeders (Gray et al., 2004; de Bono et al., 2002). Interfering with the physiological transduction of these signals also renders npr-1 mutants unable to aggregate. In the ADL neurons, sensory transduction occurs through a TRPV cation channel encoded by osm-9. Given that mutations in osm-9 abolish npr-1(lf) social feeding, we wondered whether osm-9(lf) would also suppress the elevated ADL secretion of npr-1 mutants (de Bono et al., 2002). Indeed, osm-9(lf) reduced the secretion of npr-1 mutants by 70%, a level approximately equal to that of osm-9 single mutants (Figure 2).

To further establish the relationship between aggregation behavior and increased dense-core vesicle release, we constructed double mutants between npr-1 and genes involved in the secretory pathway. The rbf-1 gene encodes the C. elegans homolog of Rabphillin, a mammalian protein that regulates exocytosis from neuroendocrine cells. Consistent with an earlier study, rbf-1(lf) reduced ADL secretion by 55% in wild-type animals (Lee et al., 2011). Loss of rbf-1 function also suppressed the elevated secretion of npr-1 mutants, reducing DAF-28 release to levels near those of rbf-1 single mutants (Figure 3A, B). If enhanced dense-core vesicle release promotes social feeding, rbf-1(lf) should inhibit the behavior. As expected, rbf-1; npr-1 double mutants were indistinguishable from wild-type animals in terms of aggregation (Figure 3C, D). A similar pattern emerged when we examined mutants in aex-6, a gene that encodes a Rab GTPase thought to act upstream of RBF-1/Rabphillin. Like rbf-1 mutants, aex-6(lf) strongly suppressed ADL secretion in npr-1(lf) animals (Figure 3E). In addition, the
aex-6 mutation abrogated npr-1(1f) social behavior (Figure 3F). Thus, mutations that impair ADL dense-core vesicle release also suppress aggregation.

The correlation between ADL secretion and aggregation suggests that ADL may secrete a factor governing social behavior. However, aex-6 and rbf-1 are expressed broadly throughout the nervous system. Their roles in dense-core vesicle release likely extend beyond the ADL neurons. Consequently, we sought to determine whether social behavior requires secretion specifically from ADL. To address the question, we selectively blocked dense-core vesicle release from the ADL neurons, using a dominant active form of RAB-5 (Sasidharan et al., 2012). RAB-5 is a Rab GTPase required for effective dense-core vesicle exocytosis but completely dispensable for synaptic vesicle release. A constitutively GTP-bound allele, rab-5(Q78L), reduces neuropeptide secretion by ~80% in a cell autonomous manner. We placed rab-5(Q78L) under the control of the ocr-2 promoter, which targets expression to ADL, ASH, and three additional pairs of neurons. To our surprise, directly impairing secretion from these neurons had no effect on NPR-1 mediated aggregation (Figure 4A).

In order to locate the source of secretion driving aggregation, we turned our attention to other cells previously implicated in social behavior. URX, along with AQR and PQR, comprise a small set of sensory neurons that detect environmental oxygen (Zimmer et al., 2009). Like ADL/ASH, these neurons are essential for aggregation – impairing their sensory function or ablating them altogether transforms npr-1(1f) animals into solitary feeders (Coates and de Bono, 2002). Using the gcy-32 promoter, we expressed rab-5(Q78L) exclusively in the URX/AQR/PQR neurons of npr-1 mutants. This manipulation abolished aggregation (Figure 4A). Thus, NPR-1 mediated social behavior requires one or more secreted factors, contained within the dense-core vesicles of oxygen-sensing neurons.
Social behavior requires peptidergic signaling

Our data indicate that the URX/AQR/PQR neurons secrete at least one factor, contained within dense-core vesicles, that drives aggregation behavior. Typical dense-core vesicle cargoes include neuropeptides, biogenic amines, and other neuroendocrine hormones. Since neuropeptides play a major role in modulating *C. elegans* behavior, they seemed likely to be the component mediating aggregation (Taghert and Nitabach, 2012). To validate this idea, we examined a mutation in *egl-21*. Neuropeptides undergo a series of posttranslational modifications before reaching functional maturity. One step requires a carboxypeptidase enzyme encoded by *egl-21*, such that *egl-21(lf)* dramatically reduces the profile of active neuropeptides (Husson et al., 2007). A mutation in *egl-21* fully suppressed *npr-1(lf)* aggregation, indicating that social behavior is indeed governed by one or more EGL-21-processed neuropeptide (Figure 4B). Recently, two studies demonstrated that EGL-21 dependent neuropeptides regulate NPR-1 mediated locomotion phenotypes (Busch et al., 2011; Choi et al., 2013). Thus, enhanced neuropeptide secretion may be a shared mechanism underlying various *npr-1(lf)* behaviors.

We next tried to determine whether EGL-21 in URX/AQR/PQR is sufficient to restore aggregation to *egl-21; npr-1* mutants. Pan-neuronal expression of an *egl-21* cDNA fully rescued aggregation (Figure 4B). However, the same cDNA failed to restore aggregation when expressed solely in the oxygen-sensing neurons (data not shown). This result suggests that while a secreted neuropeptide from URX/AQR/PQR governs social behavior, execution of the behavior requires neuropeptide signaling in other locations. It is additionally possible that the factor secreted from oxygen-sensing neurons does not depend on EGL-21 processing. Leaving *egl-21* intact, we then adopted a candidate gene approach, hoping to identify a specific neuropeptide that regulates aggregation and originates from URX/AQR/PQR. Guided by previously reported expression patterns, we constructed double mutants between *npr-1* and seven neuropeptide genes (*daf-28, ins-7, pdf-1, pdf-2, flp-3, flp-10, flp-19*). No mutation appreciably altered
npr-1(lf) aggregation (data not shown). This outcome may reflect a need to inactive multiple
neuropeptides in order to disrupt aggregation. Alternatively, an unexamined or as yet uncharacterized
factor may be responsible for the contribution of oxygen-sensing neurons to social behavior.

Discussion

NPR-1 regulates social behavior via neuropeptide release from O2-sensing neurons

The C. elegans neuropeptide Y-like receptor NPR-1 regulates dense-core vesicle release. In this
study, we focused on the ADL sensory neuron and found that npr-1(lf) enhances secretion of
neuropeptide-containing vesicles. Mutations in osm-9, a cation channel used by ADL for sensory
transduction, abolish the enhanced secretion of npr-1 mutants. This result suggests that mechanistically,
NPR-1 may operate by reducing sensory-evoked calcium influx. In support of this idea, mammalian
neuropeptide Y signaling has been shown to reduce catecholamine secretion by inhibiting calcium
channels (Toth et al., 1993; Boehm and Huck, 1997).

Elevated secretion in npr-1 mutants gives rise to social behavior. Disrupting components of the
secretion machinery suppressed aggregation. Mutations in egl-21 also eliminated NPR-1 mediated social
behavior, indicating that one or more secreted neuropeptide drives aggregation. However, the source of
peptidergic secretion necessary for social behavior is URX or other oxygen-sensing neurons, rather than
ADL. We attempted to identify the URX-secreted neuropeptide by crossing npr-1(lf) animals to mutants
in neuropeptide genes known to be expressed from URX/AQR/PQR. One reason this candidate approach
may not have worked is that multiple, functionally redundant peptides originate from URX.

During the course of our work, another group discovered that NPR-1 mediates a locomotion
phenotype via secretion of a neuropeptide (Choi et al., 2013). Though PDF-1 does not mediate npr-1
mutant aggregation, their work validates our own and suggests it may be possible to identify a single neuropeptide driving aggregation. One strategy would involve reconstituting EGL-21 in the oxygen-sensing neurons of egl-21 mutants (Husson et al., 2007). Using mass spectrometry to selectively detect mature neuropeptides, it might then be possible to identify new candidates for genetic analysis.

**URX neuropeptide secretion in the context of the hub-and-spoke circuit**

Aggregation is regulated by various environmental cues, processed by distinct sensory neurons. These sensory neurons share gap junctions with the common RMG interneuron, forming a “hub-and-spoke” circuit. NPR-1 activity in RMG regulates the excitability of surrounding sensory neurons, such that npr-1(lf) heightens their responsiveness to aggregation promoting signals. In the current model, the hub-and-spoke circuit signals downstream via distributed synaptic outputs from ASK, ASJ, URX, and RMG (Macosko et al., 2009).

We have shown that blocking dense-core vesicle release from URX (and two other non-spoke neurons) is sufficient to abolish social behavior. We propose that social behavior requires two conditions: a threshold level of synaptic output from the hub-and-spoke circuit, which can be met by contributions from multiple neurons, and peptidergic output specifically from URX. Supporting this model are experiments involving selective activation of hub-and-spoke neurons. The *pkc-1* gene encodes a *C. elegans* protein kinase C that promotes dense-core vesicle release and heightens neuronal excitability (Sieburth et al., 2007; Okochi et al., 2005). A *pkc-1* gain-of-function allele causes wild-type animals to aggregate when expressed in URX and RMG, but not in ASK and RMG, or RMG alone (Macosko et al., 2009).
**RMG may permit passive communication between sensory neurons**

The requirement of oxygen-sensing neurons for social behavior has been well established. The soluble guanylate cyclase GCY-35 is required for URX oxygen sensation and *gcy-35* mutations suppress aggregation (Cheung et al., 2004; Coates and de Bono, 2002; Gray et al., 2004). Genetic ablation of URX also eliminates npr-1 mutant social behavior (Chang et al., 2006). Interestingly, analogous manipulations of ADL and ASH produce the same result. Ablating both ADL and ASH, or disrupting either of their sensory functions, renders npr-1 mutants incapable of aggregation (Coates and de Bono, 2002). Given that *rab-5(Q78L)* in ADL/ASH had no effect on aggregation, how do these neurons contribute to social behavior?

During our study, we noticed that *gcy-35* mutations reduced secretion from ADL neurons (Supplemental Figure 2). Since *gcy-35* is not expressed in ADL, one possible explanation is that *gcy-35(lf)* has a cell-autonomous effect in URX that is propagated to ADL via RMG. Indeed, *gcy-35* deficiency eliminates oxygen-evoked calcium flux in URX, and consequently in RMG (Busch et al., 2011). RMG activity is known to influence the excitability of other spoke neurons. If the reduction in RMG activity induced by URX disruption can in turn reduce ADL activity, then the reverse process may also occur. In other words, disrupting ADL and ASH may suppress aggregation by modulating URX output. Passive communication between spoke neurons could endow the hub-and-spoke circuit a type of sensory redundancy, such that any one aggregation promoting signal would be sufficient to drive behavior.
FIGURE 1

a

DAF-28::mCherry (A.U.)

WT | npr-1

b

DAF-28::mCherry (A.U.)

WT | npr-1

* indicates significant difference.

ASL

npr-1

WT

npr-1

b

ASL

npr-1

WT

npr-1

* indicates significant difference.

ADL

npr-1

WT

npr-1

FLP-21::mCherry (A.U.)

WT | npr-1

* indicates significant difference.

mCherry (A.U.)

WT | npr-1

ns indicates no significant difference.
**Figure 1: npr-1 loss-of-function promotes dense-core vesicle secretion**

(A) Dense-core vesicle secretion from the ADL neuron. Coelomocyte accumulation assays were used to measure secretion from ADL. Briefly, *daf-28* gDNA was fused to *mCherry* and expressed under the ADL-specific *srh-220* promoter. Steady state release was measured by quantifying the amount of mCherry scavenged by coelomocytes. Mutations in *npr-1* increase ADL dense-core vesicle secretion. Experiment was repeated on at least five separate occasions with similar results. Graph shows one experiment (n = 26-27 coelomocytes per genotype). Images below bar graph show mCherry within the animal’s anterior coelomocyte pair. Asterisk in graph indicates a statistically significant change (p < 0.0001, two-tailed t-test). Standard error bars are shown.

(B) Dense-core vesicle secretion from the ASI neuron. The ASI-specific *daf-7* promoter was used to drive expression of *daf-28::mCherry*. Experiment was repeated on at least four separate occasions with similar results. Graph shows one experiment (n = 12-19 coelomocytes per genotype). Relative to wild-type animals, *npr-1* mutants showed a small but significant elevation in secretion (p < 0.01, two-tailed t-test). Images below bar graph show mCherry within the animal’s anterior coelomocyte pair. Standard error bars are shown.

(C) Dense-core vesicle secretion from the ADL neuron. *flp-21::mCherry* was expressed from ADL. As with DAF-28, FLP-21 secretion is elevated in *npr-1* mutants. Graph represents one experiment (n = 45-47 coelomocytes per genotype). Asterisk indicates a statistically significant change (p < 0.01, two-tailed t-test). Standard error bars are shown.

(D) *mCherry* expression under the ADL-specific promoter, *psrh-220*. The intensity of mCherry fluorescence was measured in the ADL cell body of a transcriptional reporter strain (*psrh-220::mCherry*). Graph represents one experiment (n = 15-25 cells per genotype). *npr-1* loss-of-function does not alter transcription of genes driven by the heterologous *psrh-220* promoter.
FIGURE 2
**Figure 2: Disrupting the OSM-9 sensory transduction channel reduces ADL secretion**

ADL dense-core vesicle secretion. Mutations in the TRPV cation channel *osm-9* suppress *npr-1* mutant secretion. The *osm-9* gene is expressed in a small number of sensory neurons including ADL. Mutations in *osm-9* have been previously shown to suppress *npr-1* mediated aggregation. Asterisks indicate statistically significant changes relative to *npr-1* mutants (p < 0.0001, ANOVA with Bonferroni post-test). Graph represents one experiment (n = 15-22 coelomocytes per genotype). Standard error bars are shown.
FIGURE 3

(a) DAF-28::mCherry (A.U.)

(b) Images of WT, npr-1, rbf-1, and rbf-1; npr-1.

(c) Graph showing fraction aggregating.

(d) Images of WT, npr-1, rbf-1, and rbf-1; npr-1.

(e) DAF-28::mCherry (A.U.)

(f) Graph showing fraction aggregating.
Figure 3: Mutations that reduce ADL dense-core vesicle release also suppress social behavior

(A) ADL dense-core vesicle secretion. The *rbf-1* gene encodes the *C. elegans* homolog of Rabphilin, a component of the dense-core vesicle secretion machinery. Mutations in *rbf-1* reduce ADL secretion in a wild-type background and suppress the elevated secretion of *npr-1* mutants. Graph represents one experiment (n = 14-30 coelomocytes per genotype). Asterisks indicate statistically significant changes relative to wild-type animals (p < 0.0001, ANOVA with Bonferroni post-test). Standard error bars are shown.

(B) Images of DAF-28::mCherry secretion from ADL. Images show mCherry within the animal’s anterior coelomocyte pair.

(C) Aggregation behavior. Fifty adult worms were placed on a small bacterial lawn and left undisturbed for two hours. Worms in contact with at least one other worm were scored as aggregating. An *rbf-1* mutation strongly suppresses the social behavior of *npr-1* mutants. Graph represents pooled data from two separate experiments (n = 3-5 replicates per genotype). Asterisk indicates a statistically significant change relative to *npr-1* mutants (p < 0.0001, ANOVA with Bonferroni post-test). Standard error bars are shown.

(D) Images of aggregation behavior. For each genotype, twenty worms were placed on a small bacterial lawn and photographed two hours later. *npr-1* mutants strongly aggregate on the borders of the lawn. An *rbf-1* mutation suppresses *npr-1(lf) aggregation*. Note that *rbf-1(lf)* causes more worms to reside on borders irrespective of *npr-1* genotype.

(E) ADL dense-core vesicle secretion. The *aex-6* gene encodes a Rab GTPase thought to act upstream of *rbf-1*. Mutations in *aex-6* reduce ADL secretion in a wild-type background and suppress the elevated secretion of *npr-1* mutants. Graph represents one experiment (n = 14-19 coelomocytes per genotype). Asterisks indicate statistically significant changes relative to wild-type animals (p < 0.0001, ANOVA with Bonferroni post-test). Standard error bars are shown.

(F) Aggregation behavior. An *aex-6* mutation partially suppresses *npr-1* mutant social behavior. Graph represents pooled data from two separate experiments (n = 3-6 replicates per genotype). Asterisk indicates a statistically significant change relative to *npr-1* mutants (p < 0.0001, ANOVA with Bonferroni post-test). Standard error bars are shown.
FIGURE 4

(a) 

Fraction aggregating

WT  npr-1  *

rab-5(Q78L)  -  pOCR-2  pGCI-32

(cDNA)

(b) 

Fraction aggregating

npr-1  egl-21  egl-21; npr-1  *

pEGL-3::egl-21  -  +

cDNA
Figure 4: Social behavior requires neuropeptide secretion from oxygen-sensing neurons

(A) Aggregation behavior. The *rab-5*(Q78L) gain-of-function allele blocks dense-core vesicle secretion cell-autonomously. The *ocr-2* promoter directs expression to ADL and several other neurons. The *gcy-32* gene is expressed in the oxygen-sensing neurons URX/AQR/PQR. Blocking secretion from *gcy-32*-expressing cells abolishes *npr-1* mediated social behavior. Graph represents pooled data from three separate experiments (n = 4-16 replicates per genotype). Asterisk indicates a statistically significant change relative to non-transgenic *npr-1* mutants (*p* < 0.0001, ANOVA with Bonferroni post-test). Standard error bars are shown.

(B) Aggregation behavior. Mutations in the neuropeptide processing gene *egl-21* suppress *npr-1* mutant social behavior. Pan-neuronal reconstitution of *egl-21*, using the *egl-3* promoter, restores aggregation in *egl-21; npr-1* double mutants. Experiment was performed on two separate occasions with similar results. Graph shows one experiment (n = 2-6 replicates per genotype). Asterisks indicate statistically significant changes (*p* < 0.0001 for *npr-1* vs. *egl-21; npr-1*, and for transgenic vs. non-transgenic *egl-21; npr-1* double mutants, ANOVA with Bonferroni post-test). Standard error bars are shown.
References


Appendix I:

Supplemental figures
FIGURE 1

(a) Diagram showing RIM/RIc proteins interacting with DAF-28.

(b) Bar graph showing DAF-28::mCherry (a.u.) levels in well-fed, starved (1.5 hrs.), and starved (2.5 hrs.) conditions. The graph indicates no significant difference (ns) between the conditions.
Figure 1: Food deprivation fails to promote dense-core vesicle release from RIM/RIC

(A) Schematic of strain used in secretion assay. daf-28::mCherry was expressed selectively in RIM/RIC using a tdc-1 promoter. Promoter consisted of the first two kilobases of DNA immediately upstream of the tdc-1 start codon.

(B) RIM/RIC dense-core vesicle secretion. Graph shows data from one unrepeated experiment (n = 11-17 coelomocytes per condition). Contrary to our expectation, neither 1.5 nor 2.5 hours of food deprivation increased dense-core vesicle release. Instead, starvation appeared to reduce secretion, though this trend did not reach statistical significance (p = 0.06, ANOVA with Bonferroni post-test).
FIGURE 2
Figure 2: Disrupting oxygen sensation reduces secretion non-cell autonomously

ADL dense-core vesicle secretion. The gcy-35 gene encodes a soluble guanylate cyclase necessary for oxygen sensation in URX. gcy-35 is expressed in URX/AQR/PQR and several additional neurons, but not in ADL. Mutations in gcy-35 suppress the elevated secretion of npr-1 mutants. Experiment was performed on two separate occasions with similar results. Graph shows one experiment (n = 16-25 coelomocytes per genotype). Asterisks indicate statistically significant changes relative to npr-1 mutants (p < 0.0001, ANOVA with Bonferroni post-test). Standard error bars are shown.
FIGURE 3

a

b

<table>
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<td>mgl-2</td>
<td>glc-3</td>
</tr>
<tr>
<td></td>
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<td>glc-4</td>
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<td></td>
<td>glr-5</td>
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<td>avr-14</td>
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<td></td>
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<td>avr-15</td>
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<td>glr-7</td>
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</table>
Figure 3: Social behavior requires glutamate signaling

(A) Aggregation behavior. The eat-4 gene encodes a vesicular glutamate transporter used to load glutamate into secretion-bound vesicles. Mutations in eat-4 suppress npr-1 mutant social behavior. Experiment was performed on three separate occasions. Graph shows one experiment (n = 3 replicates per genotype). Asterisk indicates a statistically significant change relative to npr-1 mutants (P < 0.0001, ANOVA with Bonferroni post-test). Standard error bars are shown.

(B) List of glutamate receptor mutants tested. In an effort to identify glutamate receptors required for npr-1 mediated social behavior, double mutants were constructed between npr-1 and genes encoding each listed glutamate receptor. C. elegans glutamate receptors are divided by functional class (NMDA, AMPA/kainite, metabotropic, and glutamate-gated chloride channels). Every known glutamate receptor was tested, with the exception of glr-3 and glc-1, for which no mutants exist. In addition, a glr-1 glr-2; npr-1 triple mutant was constructed. No glutamate receptor mutation appreciably suppressed npr-1 mutant social behavior.
Appendix II:

Methods and protocols
C. elegans maintenance and strain construction

Animals were maintained on nematode growth medium (NGM) plates seeded with OP50 *E. coli* bacteria as a food source. Animals were generally reared at room temperature, with the exception of *daf*-7 mutants, which developed at 15°C until adulthood. Strains were obtained from the C. elegans Genetics Center (CGC), OMRF Knockout Consortium, and the National BioResource Project. The *glr*-1 *glr*-2 strain was generously provided by the Maricq lab. The genotypes of double and triple mutant strains constructed in this work were confirmed by PCR and sequencing. Transgenic animals were injected with plasmids at concentrations ranging from 1-100 ng/μL.

Molecular biology

The majority of plasmids were constructed with Gateway Technology as described in previously published work (Lee et al., 2008, see Chapter VI for reference). The *hen*-1 and *scd*-2 promoters consisted of approximately two kilobases of DNA upstream of the respective start codons. Both were PCR amplified from wild-type genomic DNA and cloned into pka6, a modified pDONR-P4-P1R plasmid (Invitrogen).

The *hen*-1 ORF was PCR amplified from wild-type genomic DNA. The *rab*-5(Q78L) allele was a generous gift of the Eimer lab. *hen*-1 and *rab*-5(Q78L) were amplified with flanking Gateway recombination sequences and cloned into pka5, a modified pDONR-221 plasmid (Invitrogen). An *scd*-2 cDNA was generously provided by the Ishihara lab. The supplied plasmid was modified by excising the existing promoter at NotI/SalI sites, and inserting a PCR amplified *tdc*-1 promoter.
**Radial dispersal assays**

Radial dispersal assays were conducted on day-one adults which had been age-synchronized via hypochlorite treatment and raised at room temperature. Approximately 15 worms were rinsed three times in 100 µL volumes of S-basal. Rinses were performed in a clear-bottomed 96-well plate. Worms were pipetted onto the center of an unseeded 5 cm NGM plate which had been dried under a fume hood for one hour the night before. Assays began immediately after wicking away remaining buffer with filter paper. Locomotion was filmed for five minutes, at two frames per second, using a custom-built macroscope. After the assays, worm tracks were reconstructed using ImageJ and the MTrackJ plugin.

**Pharyngeal pumping assays**

Pharyngeal pumping assays were conducted on day-one adults which had been age-synchronized via hypochlorite treatment and raised at room temperature. One day prior to assays, L4 animals were transferred from their rearing plates to freshly seeded plates. For each genotype or condition, 50 animals were picked onto each of three new plates. These plates were maintained overnight at 20°C, removed the following morning, and allowed to acclimate back to room temperature at least one hour prior to assays. The 20°C incubation helped reduce developmental variability between experiments.

To quantify pumping, the number of pharyngeal oscillations in a ten second period was counted. Since removing the lids of petri dishes induces a transient increase in locomotion, worms were allowed to rest on the stage for one minute before scoring. In general, five worms were scored from each of the three plates prepared for a genotype or condition.

For off-food assays, worms were rinsed from plates with 10 mL S-basal/0.1% PEG and pipetted into 15 mL conical tubes. The tubes were centrifuged for 30 seconds at 4,000 RPM, the supernatant was
removed, and 10 mL of fresh buffer was added. This rinse process was repeated three additional times to ensure worms were completely free of bacteria. After the final rinse, the animals were concentrated and pipetted onto three unseeded NGM plates. Remaining buffer was then wicked away using filter paper. The unseeded plates had been previously dried under a fume hood for one hour. The drying was necessary to prevent excessive moisture from the NGM adhering to worms via capillary action. Upon plating, worms were starved for 90 minutes. The period of starvation was selected based on pilot experiments – by 90 minutes, worms have fully suppressed their pumping rate but are still several hours away from decrepitude. As with the on-food condition, five worms were scored from each of three plates.

Stock solutions of tyramine HCL and octopamine HCL (Sigma) were made in ddH₂O at concentrations of 250 mM and 100 mM, respectively. Solutions were made fresh for every experiment and further diluted in ddH₂O vehicle. The solutions were dispensed onto NGM plates the night before the assay in order to diffuse through the medium. Concentration values in text and figures reflect the final concentration of drugs in 10 mL NGM. As in experiments without drugs, the unseeded plates had been previously dried for an hour under a fume hood.

Egg-laying assays

Worms were reared the same way as those used in pharyngeal pumping assays. Briefly, synchronized populations were raised at room temperature until the L4 stage, at which point they were transferred to freshly seeded plates and incubated overnight at 20°C. On the morning of egg-laying experiments, worms were moved back to room temperature and allowed to acclimate for at least one hour. During this time, 40 µL of 4M fructose was applied to the perimeter of 5 cm unseeded NGM plates. Plates had been previously dried under a fume hood for one hour. The high osmolarity fructose deters worms from crawling off plates during assays. For experiments involving drug treatments,
compounds were dispensed onto the surface of plates in 300 μL ddH₂O vehicle. Solutions of tyramine HCl and octopamine HCl (Sigma) were made fresh for every experiment at stock concentrations of 250 mM and 100 mM, respectively. Concentration values in text and figures reflect the final concentration of drugs in 10 mL NGM.

Approximately 15 worms were picked off their rearing plate and rinsed four times in 100 μL volumes of S-basal. Rinses were performed in a clear-bottomed 96-well plate. After transferring the worms into the first well, a glass pipette was used to gently mix the buffer, dislodging clumps of bacteria from the animals. The dirty buffer was discarded and the next volume of buffer, premeasured in an adjacent well, was transferred onto the worms. This process was repeated four times. Worms were then pipetted onto the assay plate and the number of worms was recorded. The remaining buffer was wicked away with filter paper. The plate was left undisturbed on the benchtop while worms laid eggs. One hour later, the worms were vacuumeed off the assay plate and the plate was set aside.

Plates were scored once all assays had been completed. First, the edges of plates were carefully inspected for eggs. Due to the meniscus of the agar, eggs laid near the edge were in multiple focal planes and often difficult to see. After scoring edges, the rest of the plate was scanned for eggs. For each plate, the number of eggs counted was divided by the number of worms to give a value of eggs laid per worm per hour. Occasionally, worms crawled off the agar and became desiccated on the walls of the petri dish. In these cases, there was no way of knowing when in the assay the worm left the plate. But given that these occurrences were infrequent, and that they did not seem to correlate with genotype, the data was included in our analysis.

For measuring egg-laying rates on food, pre-dried 5 cm NGM plates were seeded with 100 μL OP50. The OP50 solution was allowed to dry overnight at room temperature to produce a small circular lawn in the middle of the plate. Ten day-one adult worms were picked onto the lawn, allowed to lay eggs for one hour, and then removed. Instead of counting the eggs immediately, hatched progeny were
counted the following day. One day later, plates were examined again to account for late hatching or non-viable eggs.

**Brood size measurement**

For each genotype, twelve L4 stage animals were singled onto 5 cm NGM plates seeded with OP50. Animals matured and laid eggs for approximately 24 hours, at which point parents were transferred to new seeded plates. After another day, parents were moved to fresh plates once again. The day following removal from a plate, an animal’s progeny were counted and vacuumed off the bacterial lawn. To account for late hatching eggs, plates were reexamined a day later. Worms laid the vast majority of their eggs on the first two plates. Parental worms were maintained on their third, terminal plates and these plates were checked daily for progeny until reproduction ceased.

**Coelomocyte accumulation assays**

Synchronized L1 worms were grown for two days at room temperature until reaching the L4/young adult stage. For each strain, 20-40 worms were immobilized in 1% sodium azide, mounted on a slide, and visualized through a 40x objective on a Zeiss Axioplan 2 microscope. Images of the anterior two coelomocytes were acquired with a 12-bit Hamamatsu Orca II camera.

Strains used in secretion assays express an mCherry-tagged neuropeptide from one or more neurons, and a fluorescent coelomocyte marker (*punc-122::gfp*). Images of the coelomocyte marker were used to trace the boundaries of cells and the intensity of mCherry fluorescence was then measured within each coelomocyte. For each cell, the minimum intensity value was subtracted from the mean fluorescence intensity to give a final value.
Aggregation assays

Two days before assays, standard NGM plates were seeded with 200 µL OP50 to form small circular lawns. Fifty well-fed gravid adults were picked from their rearing plates onto the center of the assay lawns. Aggregation was measured two hours later. Worms in groups of two or more were scored as aggregating.

Statistical tests

Significance was determined with either two-tailed t-tests or one-way ANOVA. Bonferroni post-tests were used to correct for multiple comparisons. Figures indicate the tests used in particular experiments.
Appendix III:

*C. elegans* strains
Strains used in Part I

CF2929  hen-1 (tm501)
RB783   scd-2 (ok565)
KQ190   tdc-1 (ok914)
RB1161  tbh-1 (ok1196)
KQ2552  tdc-1 (ok914); hen-1 (tm501)
KQ2553  tbh-1 (ok1196) hen-1 (tm501)
KQ2554  scd-2 (ok565); daf-3 (mgDf90)
CB1372  daf-7 (e1372)
KQ2555  N2; [phen-1::hen-1::mCherry; punc-122::gfp]
KQ2556  N2; Ex[pscd-2::gfp; ptdc-1::mCherry]
KQ2431  N2; Ex[ptdc-1::daf-28::mCherry; punc-122::gfp]
KQ2557  scd-2 (ok565); Ex[ptdc-1::scd-2 cDNA; pmyo-2::mCherry; punc-122::gfp]

Strains used in Part II

DA609   npr-1 (ad609)
NM1278  rbf-1 (js232)
KQ2558  rbf-1 (js232); npr-1 (ad609)
KQ2559  aex-6 (ft16); npr-1 (ad609)
MT1241  egl-21 (n611)
KQ2560  egl-21 (n611); npr-1 (ad609)
KQ2561  npr-1 (ad609) tbh-1 (ok1196)
KQ2562  tdc-1 (ok914); npr-1 (ad609)
KQ2563  tph-1 (mg280)
KQ2564  tph-1 (mg280); npr-1 (ad609); ftls25 [psrh-220::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
VC2497  flp-3 (ok3265)
RB1989  flp-10 (ok2624)
RB1902  flp-19 (ok2460)
LSC27   pdf-1 (tm1996)
FX04393 pdf-2 (tm4393)
KQ2565  flp-3 (ok3265) npr-1 (ad609)
KQ2566  flp-10 (ok2624); npr-1 (ad609)
KQ2567  flp-19 (ok2460); npr-1 (ad609)
KQ2568  pdf-1 (tm1996); npr-1 (ad609)
KQ2569  pdf-2 (tm4393); npr-1 (ad609)
KQ2570  daf-28 (tm2308); npr-1 (ad609)
KQ2571  ins-7 (ok1537); npr-1 (ad609)
MT6308  eat-4 (ky5)
KP4     glr-1 (n2461)
FX669   glr-2 (tm669)
VM6290  glr-1 (ky176); glr-2 (ak10)
FX03239 glr-4 (tm3239)
FX03506 glr-5 (tm3506)
DA1384  glc-1 (pk54); avr-14 (ad1302)
DA1370  glc-1 (pk54); avr-15 (vu227)
VC722  glc-2 (ok1047)
XA4000  glc-3 (ok321)
JD31  glc-4 (ok212)
FX3075  nmr-2 (tm3785)
KQ2572  eat-4 (ky5); npr-1 (ad609)
KQ2573  glr-1 (n2461); npr-1 (ad609)
KQ2574  glr-2 (tm669); npr-1 (ad609)
KQ2575  glr-1 (ky176) glr-2 (ak10); npr-1 (ad609)
KQ2576  glr-4 (tm3239); npr-1 (ad609)
KQ2577  glr-5 (tm3506); npr-1 (ad609)
KQ2578  glr-6 (tm2729); npr-1 (ad609)
KQ2579  glr-7 (tm2877); npr-1 (ad609)
KQ2580  glc-2 (ok1047); npr-1 (ad609)
KQ2581  glc-3 (ok321); npr-1 (ad609)
KQ2582  glc-4 (ok212); npr-1 (ad609)
KQ2583  avr-14 (ad1302); npr-1 (ad609)
KQ2584  avr-15 (ad1051); npr-1 (ad609)
KQ2585  nmr-1; npr-1 (ad609)
KQ2586  nmr-2 (tm3785); npr-1 (ad609)
KQ2587  mgI-1; npr-1 (ad609); ftIs25 [psrh-220::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
KQ2588  mgI-2 (tm355); npr-1 (ad609)
KQ2589  mgI-3 (tm1766); npr-1 (ad609)
KQ1251  N2; ftIs25 [psrh-220::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
KQ2590  npr-1 (ad609); ftIs25 [psrh-220::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
KQ1254  N2; ftIs32 [pdaf-7::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
KQ2591  npr-1 (ad609); ftIs32 [pdaf-7::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
KQ1787  N2; Ex[psrh-220::fip-21::mCherry; punc-122::gfp]
KQ2592  npr-1 (ad609); Ex[psrh-220::fip-21::mCherry; punc-122::gfp]
KQ2112  N2; Ex[psrh-220::mCherry]
KQ2593  npr-1 (ad609); Ex[psrh-220::mCherry]
KQ1330  osm-9 (yz6); ftIs25 [[psrh-220::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
KQ2594  osm-9 (yz6); npr-1 (ad609); ftIs25 [[psrh-220::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
KQ1690  gcy-35 (ak769); ftIs25 [psrh-220::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
KQ2595  gcy-35 (ak769); npr-1 (ad609); ftIs25 [psrh-220::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
KQ1657  rbf-1 (js232); ftIs25 [psrh-220::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
KQ2596  rbf-1 (js232); npr-1 (ad609); ftIs25 [psrh-220::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
KQ1569  aex-6 (ft16); ftIs25 [psrh-220::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
KQ2597  aex-6 (ft16); npr-1 (ad609); ftIs25 [psrh-220::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp]
KQ2598  aex-6 (ft16); npr-1 (ad609); ftIs25 [psrh-220::daf-28::mCherry; pmyo-2::gfp; punc-122::gfp];
   Ex[pocr-2::aex-6 cDNA::SL2::gfp; pmyo-3::gfp]
KQ2599  eat-4 (ky5); npr-1 (ad609); fTE722 [pegl-3::eat-4::SL2::gfp]
KQ2600  eat-4 (ky5); npr-1 (ad609); Ex[pocr-2::eat-4::SL2::gfp; pmyo-3::gfp]
KQ2601  eat-4 (ky5); npr-1 (ad609); Ex[pocy-32::eat-4::SL2::gfp; pmyo-3::gfp]
KQ2602  egl-21 (n611); npr-1 (ad609); Ex[pegl-3::egl-21::SL2::mCherry; pmyo-3::gfp]
KQ2603  egl-21 (n611); npr-1 (ad609); Ex[pocr-2::egl-21::SL2::mCherry; pmyo-3::gfp]
KQ2604  npr-1 (ad609); Ex[pocy-32::rab-5(Q78L)::SL2::gfp; pmyo-3::gfp]
KQ2605  npr-1 (ad609); Ex[pocr-2::rab-5(Q78L)::SL2::gfp; pmyo-3::gfp]
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