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Dopamine modulation of quiescence in dauer larvae, and other investigations on hibernation and lifespan in the nematode C. elegans

by

Marta Maria Gaglia

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

Copyright 2007

By

Marta Maria Gaglia

In loving memory of my grandfather,

who taught me reading and maths

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I would like to thank my graduate advisor, Cynthia Kenyon, who supervised the work detailed in this thesis and offered invaluable advice and inspiration. I would like to thank my thesis committee members, Ulrike Heberlein, Kaveh Ashrafi, Laurence Tecott, Elizabeth Blackburn, for their support and help throughout the Ph.D. In addition, I would like to thank my external committee member Miriam Goodman of Stanford University for allowing me to use facilities in her lab for much of the work detailed in Chapter 2 and all members of her lab for being extremely helpful and nice to me.

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Dopamine modulation of quiescence in dauer larvae, and other investigations on hibernation and lifespan in the nematode *C. elegans*

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Marta Maria Gaglia

Abstract

Studies in organisms from worms to humans have brought an increasing appreciation for a crucial role of the central nervous system in the regulation of many basic physiological processes including energy homeostasis, immune responses, development and aging. However, the mechanisms underlying such regulation are still incompletely understood. In *C. elegans,* two physiological processes that are notably modulated by the nervous system are the developmental decision to arrest in the hibernation-like dauer larval stage and the regulation of aging. In this thesis, we studied the interplay between endocrine systems controlling aging and dauer formation, such as insulin/IGF-1 signaling, and neuronal signaling, such as neurotransmitter and sensory signaling. We found that dopamine signaling may be specifically modulated by insulin/IGF-1 signaling to regulate the quiescence behavior of dauer, and it may also be involved in the process of exit from the dauer stage. Because there are physiological and molecular similarities between the dauer stage and seasonal hibernation in mammals, it is possible that dopamine signaling contributes to behavioral quiescence in hibernating mammals. In a separate study, we found that signals from the sensory system feed into the regulation of at least two different transcription factors, the FOXO homolog *daf-16*

and the steroid nuclear hormone receptor *daf-12*, both targets of endocrine pathways. In addition, we found that the sensory system is required for normal expression of immune response genes, and that sensory system mutants have increased susceptibility to a bacterial pathogen. These results indicate that sensory inputs can coordinately regulate several processes by affecting a number of important transcriptional responses. The insulin/IGF-1 pathway, the dopamine pathway and sensory inputs are crucial in the mammalian central nervous system for the regulation of many physiological as well as behavioral responses. Therefore these studies in a simpler, genetically tractable organism may offer valuable insights on how these systems may be coordinated in higher organisms.

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Chapter 1:

Introduction

All organisms, from prokaryotes to humans, have to interact appropriately with their environment to survive. Multicellular organisms have evolved complex networks of neuronal cells, which are used to acquire information about the environment, process this information and translate it into appropriate behavioral and physiological responses. In the free-living nematode *C. elegans* the importance of the nervous system is highlighted by the high percentage of neurons, 302 out of the 959 cells in a hermaphrodite worm. *C. elegans* neuronal cells are generally divided into 3 classes: sensory neurons, many of which have microtubule-based cilia, motor neurons, which connect to muscle cells, and interneurons, which do not fall in either category, but presumably are used to process sensory signals and generate behavioral outputs (Hobert, 2005). The simplified structure of the system, and the stereotypy of the connections between *C. elegans* neurons, which have been extensively mapped using electron microscopy (White et al., 1986), make *C. elegans* a great model for trying to piece together circuits, both anatomically and functionally.

Early studies focused on circuitry that controlled simple behaviors, but it was clear that the nervous system also controlled physiological processes affecting the whole organism, such as development. Right after hatching, *C. elegans* larvae can either

progress through the normal developmental stages and develop to adulthood, or, if the conditions are unfavorable, they can take a different path and develop into an alternative arrested larval stage called the dauer, resuming development later when conditions improve (Cassada and Russell, 1975). Dauer larvae have a different morphology: their gonad is developmentally arrested, their pharynx is remodeled and they have a special cuticle, among other things (Cassada and Russell, 1975). Also, their behavior is different, because they do not move spontaneously, do not feed and do not respond readily to chemosensory cues (Cassada and Russell, 1975; Albert and Riddle, 1983). Sensory input provides information about the environmental conditions and therefore is very important in the decision to enter and exit the dauer stage (Albert et al., 1981). Specific neurons have been identified in the amphid, the main olfactory/gustatory organ of the worm, that regulate dauer formation in a positive or negative direction (Bargmann and Horvitz, 1991; Schackwitz et al., 1996).

Molecules and signaling pathways that regulate the dauer decision have been studied extensively. Three main pathways that control dauer formation have been identified: a cGMP-regulated pathway, a TGFβ pathway and an insulin/IGF-1-like pathway [reviewed in Riddle and Albert (1997) and Hu (2007)]. These pathways are believed to respond to competing cues, a food odor and a pheromone that indicates crowding of the population (Golden and Riddle, 1984). In addition, temperature is also an important cue, because at high temperature wild-type animals arrest in the dauer stage at low frequency (Ailion and Thomas, 2000). Moreover, mutations that broadly compromise the function of the sensory system greatly increase the frequency of dauer

formation at high temperature (Ailion and Thomas, 2000). Quite surprisingly, however, very little is known about how these cues are translated into the characteristic physiological and behavioral changes. All three pathways act in the neurons to regulate dauer formation, and converge on the bile-acid steroid nuclear hormone receptor (NHR) gene *daf-12* (Vowels and Thomas, 1992; Antebi et al., 1998; Antebi et al., 2000). The cGMP and the TGFβ pathway are clearly involved in the sensation of the stimuli that contribute to the decision to arrest in dauer (Schackwitz et al., 1996), whereas it is possible that the insulin/IGF-1 pathway may control a later stage of the process.

Recent studies in *C. elegans* have found an extensive role for neural control of other physiological outputs as well as dauer formation. Sensory perception appears to control aging, as mutations that disrupt structure of the sensory neurons or their function result in longer lifespan (Apfeld and Kenyon, 1999). Ablation of specific neurons of the amphid organ can also affect lifespan, both positively and negatively (Alcedo and Kenyon, 2004). Interestingly, mutations in the *Drosophila* gene *Or38b*, which encodes a widespread odorant co-receptor, also result in longer lifespan (Libert et al., 2007). In mammals, the hypothalamus modulates a number of endocrine pathways presumably in response to environmental cues, and thus allows many basic physiological processes to be exquisitely responsive to changing environmental conditions. The hypothalamus receives innervation from several sensory organs, including the olfactory cortex [reviewed in Risold et al. (1997)]. Kappeler et al. (2008) found that loss of the IGF-1 receptor specifically in the brain of mice, including the hypothalamus, extends lifespan, suggesting that aging is also a process that is regulated by the hypothalamus. Thus,

studying neuronal control of aging, development, stress responses and other important physiological processses in a simple, genetically tractable organism like *C. elegans* may allow us to gain insights on fundamental regulation of physiology from the sensory system and the nervous system in general.

In this study we focused on several aspects of neural control of physiology and behavior. The first one, detailed in Chapter 2, is an investigation of the role of dopamine in the suppression of movement that characterizes the dauer larva. The dauer larva particularly interested us for a number of reasons, besides the fact that the dauer decision in an environmentally regulated developmental switch. The dauer stage is analogous to the infective larval stage of the life cycle of many parasitic worms (Cassada and Russell, 1975). Also, the dauer stage has been described as a hibernation-like state, because many of the metabolic and behavioral features of the dauer stage resemble those of mammalian hibernation (Carey et al., 2003), one of the most notable being the near absence of spontaneous movement. We were particularly interested in the dauer locomotory quiescence, which was first noted in the original Cassada and Russell study (Cassada and Russell, 1975). Nothing is known about this quiescence, and its regulation seems to be distinct from that of other quiescent behaviors, such as the quiescence during the lethargus molting stage (Raizen et al., 2008), or satiety-induced quiescence (You et al., 2008). Insulin/IGF-1 signaling, via the DAF-2/Insulin-IGF-1 receptor (InsR) (Kimura et al., 1997) and the FOXO transcription factor DAF-16 (Lin et al., 1997; Ogg et al., 1997), appears to have a direct role in controlling this behavioral quiescence, as adults carrying strong reduction-of-function mutations in *daf-2*/InsR also display dauer-like behavioral

quiescence as fully-formed adults (Gems et al., 1998). Adults carrying weaker reduction-of-function mutations in *daf-2*/InsR do not display this behavioral change, but are still long-lived and constitutively form dauers at higher temperature, thus dissociating the behavioral phenotype from other effects of the *daf-2*/InsR pathway (Gems et al., 1998). Taking a candidate approach, we examined the role of a well-established neurotransmitter pathway, the dopamine pathway, specifically in the regulation of dauer locomotory quiescence. The dopamine pathway is well conserved between *C. elegans* and higher organisms. A set of conserved enzymes is required for the synthesis of dopamine (Lints and Emmons, 1999; Hare and Loer, 2004), and multiple receptors [DOP-(1-4)] have been found, which bind dopamine and activate G-protein signaling (Suo et al., 2002, 2003; Chase et al., 2004; Sugiura et al., 2005). A conserved re-uptake transporter, DAT-1, terminates the dopamine signal by removing the neurotransmitter from the extracellular space (Jayanthi et al., 1998; Nass et al., 2002). In *C. elegans*, dopamine appears to modulate a range of behaviors in response to changes in food availability, including locomotion (Schafer and Kenyon, 1995; Sawin et al., 2000; Chase et al., 2004), egg laying (Schafer and Kenyon, 1995; Dempsey et al., 2005), and mechanosensory adaptation (Kindt et al., 2007). We found that changes in the dopamine pathway may underlie the altered response of dauers to stimulation, though perhaps not the basal quiescence behavior of dauers and dauer-like adults. Dopamine is involved in control of voluntary movement in many organisms. Thus, it is possible that modulation in dopamine signaling may underlie suppression of movement in quiescence states in higher organisms, such as hibernation or sleep in mammals.

The second aspect of neuronal control of physiology we investigated (Chapter 3) was the role of the sensory system in controlling worm physiology. Mutations that compromise the structure of the sensory cilia result both in longer lifespan (Apfeld and Kenyon, 1999) and increased dauer formation at high temperature (Ailion and Thomas, 2000). However, not much is known about which pathways the nervous system employs on to affect dauer formation and longevity, which environmental cues are important and which signaling molecules it uses to communicate with other tissues. Because the phenotypes of sensory mutants are analogous to those of insulin/IGF-1 pathway mutants, and both longevity and constitutive dauer formation of sensory mutants are partially dependent on DAF-16/FOXO (Apfeld and Kenyon, 1999; Ailion and Thomas, 2000), the insulin/IGF-1 pathway may be one of the outputs of the sensory system in these processes. Interestingly, when sensory mutations are combined with mutations in *daf-2*/InsR, they can actually shorten the lifespan of *daf-2*/InsR animals (Apfeld and Kenyon, 1999). This suggests that the sensory system may provide complex inputs into lifespan-regulating pathways, both positive and negative, and this can be seen in sensory mutants, in which a number of different neurons are impaired. To gain insight into this complex regulation, we aimed to identify transcriptional outputs that affect the aspects of worm physiology governed by the sensory system. We found three patterns of gene regulation that appeared to be under the control of the sensory system, including, unexpectedly, significant changes in immune response genes. We observed that, despite their extended longevity, sensory mutant animals are more sensitive to pathogenic infections, presumably because they improperly regulate innate immunity genes. Sensory input is

clearly relevant for all animals to formulate an optimal and continuously adaptable survival strategy in terms of both behavior and physiology. Thus studying how the simple *C. elegans* nervous system coordinately regulates aging, development, innate immunity and other process through overlapping and distinct transcriptional outputs may give us some clues to how more complex neural systems achieve similar results.

One additional aim of the microarray analysis of sensory mutant animals was to identify potential signals used by the neurons to communicate with other tissues to regulate longevity, but it is possible that such signals would not be regulated at the level of transcription. We also took a candidate approach and explored the possibility that biogenic amine neurotransmitters may be used as signals in the regulation of physiological processes. These preliminary investigations are described in Chapter 4. We focused on biogenic amines because loss of these neurotransmitters has few overt phenotypes (Lints and Emmons, 1999; Sze et al., 2000; Alkema et al., 2005), and we examined mainly their interaction with the insulin/IGF-1 pathway, as the neurons are the site of action for dauer regulation and, in part, for lifespan regulation in this pathway (Apfeld and Kenyon, 1998; Wolkow et al., 2000; Libina et al., 2003). We found some functional interaction between the dopamine and serotonin system and the insulin/IGF-1 pathway. In mammals, insulin, dopamine and serotonin are known to interact in the regulation of energy homeostasis. Perhaps they may contribute in a similar way to the control of aging.

Chapter 2:

Stimulation of Movement in a Quiescent, Hibernation-like Form of *C. elegans* by Dopamine Signaling

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Abstract

One of the characteristics of animals in hibernation is reduced behavioral activity. The *C. elegans* dauer larva is a hibernation-like state of diapause that displays a dramatic reduction in spontaneous locomotion. A similar dauer-like quiescent state is produced in adults by strong mutations in the insulin/IGF-1 receptor homolog *daf-2*. In this study, we show that mutations affecting the neurotransmitter dopamine, which regulates voluntary movement in many organisms, can stimulate movement in dauers and dauer-like quiescent adults. Unexpectedly, the movement of quiescent animals is stimulated by conditions that reduce dopamine signaling and also by conditions predicted to increase dopamine signaling. Reducing dopamine signaling is likely to stimulate movement by activating a foraging response also seen in non-dauers after withdrawal of food. In contrast, the stimulation of movement by increased dopamine is much more pronounced in quiescent *daf-2(-)* dauer and dauer-like adult animals than in wild type. This altered response to dopamine is largely due to activity of the FOXO transcription factor DAF-16 in neurons. We propose that dauer and dauer-like quiescent adults may have underlying changes in the dopamine system that enable them to respond differently to environmental cues.

Introduction

Adapting one's physiology to changing environmental conditions is essential for all living organisms. The *C. elegans* juvenile responds to conditions unfavorable for

reproductive growth by altering its developmental trajectory and entering an alternative, hibernation-like larval stage called dauer (Cassada and Russell, 1975). Dauer larvae have unique morphological and behavioral features, including a marked reduction in movement, which are thought to allow them to survive harsh conditions. Because dauers can move when stimulated mechanically (Cassada and Russell, 1975), their quiescence appears to be a behavioral rather than a morphological adaptation. However, little is known about how their quiescence is influenced by neurotransmitter signaling.

We asked whether neurotransmitter systems that influence locomotion in *C. elegans* might also modulate dauer quiescence. We focused on the biogenic amine dopamine because dopamine regulates voluntary movement in many organisms. In *C. elegans* hermaphrodites, dopamine is produced by eight neurons (Sulston et al., 1975), and signals via at least four dopamine receptors, DOP-(1-4) (Suo et al., 2002, 2003; Chase et al., 2004; Sugiura et al., 2005). In addition, a presynaptic re-uptake transporter, DAT-1, removes dopamine from the extracellular space to terminate signaling (Jayanthi et al., 1998; Nass et al., 2002).

In *C. elegans,* dopamine is best known for mediating the "basal slowing response" in which the animal's movement slows when it encounters food (Sawin et al., 2000). Dopamine is thought to be released in response to food and to act on motor neurons to modulate the animal's movement (Chase et al., 2004). Animals that cannot produce dopamine fail to slow in response to food (Sawin et al., 2000), and exogenous dopamine slows the movement of animals cultured without food (Schafer and Kenyon, 1995). The response to dopamine in this situation is complex, as the absence of specific dopamine

receptors can have either positive or negative effects on movement (Chase et al., 2004).

In this study, we have asked how conditions predicted to change the level of dopamine signaling influence the movement of dauers. Unexpectedly, we find that dauer movement can be stimulated either by increasing dopamine signaling or by decreasing the level of dopamine.

Dauers are developmentally-arrested, pre-pubescent juveniles, but a dauer-like quiescent state can also be produced in the adult. Dauer formation is regulated by several signaling pathways, including the insulin/IGF-1 pathway (Riddle et al., 1981; Gottlieb and Ruvkun, 1994; Kimura et al., 1997). Mutations that completely eliminate the function of DAF-2, the *C. elegans* homolog of the insulin/IGF-1 receptor (InsR) (Kimura et al., 1997), cause constitutive dauer formation due to increased activity of the FOXO transcription factor DAF-16 (Riddle et al., 1981; Vowels and Thomas, 1992; Gottlieb and Ruvkun, 1994; Lin et al., 1997; Ogg et al., 1997; Hu, 2007). Slightly weaker *daf-2*/InsR mutations allow the animals to reach adulthood, but produce a dauer-like quiescent behavior (Gems et al., 1998). We found that the movement of these quiescent adults was also stimulated by conditions predicted to either increase or decrease dopamine signaling.

Our findings suggest three conclusions. First, because neither reducing nor increasing the level of dopamine signaling produces dauer-like quiescence in normal animals, it is unlikely that dauer quiescence is caused simply by a change in dopamine-signaling strength. Second, because reducing food levels, like reducing dopamine levels, stimulates the movement of both dauer-like quiescent animals and non-dauers, we infer that these quiescent animals respond to food withdrawal in a manner similar to wild-type

animals. Third, because increasing dopamine signaling stimulates movement in *daf-2(-)* dauers and dauer-like adults, but not in non-dauer animals, we infer that reductions in insulin/IGF-1 signaling change the animals' response to dopamine. This altered response to dopamine is mediated in part by increased DAF-16/FOXO transcriptional activity in the nervous system.

Materials and Methods

Strains

Nematodes were raised under standard laboratory conditions on agar plates containing a lawn of *Escherichia coli* strain OP50, as described previously (Brenner, 1974). "Wild type" was the *C. elegans* strain N2. The mutant and transgenic strains used were as follows: CB1372 *daf-7(e1372),* CF1041 *daf-2(e1370),* CF1085 *daf-16(mu86); daf-2(e1370),* CF1362 *daf-16(mu86); daf-2(e1370); muEx142[rol-6D],* CF1592 *daf-2(e1370); muEx226[rol-6D],* CF1794 *daf-16(mu86); daf-2(e1370); muIs109[pdaf-16::GFP::DAF-16cDNA, rol-6D],* CF2005 *daf-16(mu86); daf-2(e1370); muIs120[pges-1::GFP::DAF-16cDNA, rol-6D],* CF2093 *daf-16(mu86); daf-2(e1370); muIs131[punc-119::GFP::DAF-16cDNA, rol-6D],* CF2102 *daf-16(mu86); daf-2(e1370); muIs126[pmyo-3::GFP::DAF-16cDNA, rol-6D],* CF2470 *dat-1(ok157),* CF2532 *daf-2(e1370) dat-1(ok157),* CF2593 *cat-2(e1112)*, CF2625 *daf-2(e1370); lin-15(n765ts); akEx248[pdat-1::GFP, lin-15(+)],* CF2626 *daf-2(e1370); lin-15(n765ts); akEx387[pdat-1::GFP, pdat-1::hICE, lin-15(+)],* CF2646 *daf-2(e1370); dop-3(vs106),*

CF2665 *daf-2(e1370); dop-1(vs100) dop-3(vs106),* CF2666 *daf-2(e1370); dop-1(vs100),* CF2805 *dop-2(vs105); dop-4(ok1321) dop-1(vs100) dop-3(vs106),* CF2831 *daf-2(e1370); dop-2(vs105); dop-4(ok1321) dop-1(vs100) dop-3(vs106),* CF3148 *daf-16(mu86); daf-2(e1370) dat-1(ok157)*, JT195 *daf-11(sa195).* All mutations were backcrossed at least 3 times to our laboratory N2 strain, except the mutations in CB1372 and JT195.

Movement assays

Wild-type animals vs. daf-2 mutant dauers

Wild-type (N2) and *daf-2(e1370)* mutant animals were grown from eggs at 25ºC. Wild-type L1/L2 larvae were scored the day after moving the eggs to 25ºC and wild-type L3/L4 the second day after transferring the eggs. Wild-type adults and *daf-2(e1370)* dauers were scored three days after transferring the eggs. All populations were scored at least 3 times.

Visual assay- Mutant dauers

Movement of dauers was scored visually using a dissecting microscope. Dauers of strains carrying *daf-2(e1370), daf-7(e1372)* and *daf-11(sa195)* mutations were obtained by growing animals from eggs at 25ºC for three days on plates containing food (*E. coli* OP50). Dauers were scored as moving if they moved at least one body bend forward or backward. A minimum of 30 animals per strain was scored in each repeat of the experiment. Experiments represented in Figure 2.1B, 2.2B and 2.7D were part of a set of experiments done in parallel, in which each strain was scored at least 11 times.

Other dauer motility assays were repeated at least 5 times. The experiment in Figure 2.2A includes both transgenic and non-transgenic animals, as it is not possible to distinguish *lin-15(-)* and *lin-15(+)* animals at the dauer stage. This is the case for both the test and the control strain.

For *daf-2(-) dat-1(-)* mutant dauers, we checked that the morphology of the pharynx, cuticle and gonad were those of a fully-formed dauer larva, and found that they were. Also, *dat-1* mutations did not impair the ability of worms to respond to dauer pheromone, and *dat-1(-)* dauers formed after exposure to dauer pheromone were also more motile than wild-type dauers. These observations indicate that there is no reason to think *dat-1* mutations impair dauer formation, or that their effect is very specific to *daf-2*/InsR mutant dauers.

Visual assay- adults

Animals were raised until the L4 stage at 15ºC, then moved to 25ºC on plates with *E. coli*, and scored three days later. CF2625 *daf-2(e1370); lin-15(n765ts); akEx248[pdat-1::GFP, lin-15(+)]* and CF2626 *daf-2(e1370); lin-15(n765ts); akEx387[pdat-1::GFP, pdat-1::hICE, lin-15(+)]* were grown at 20ºC, instead of 15ºC, in order to pick out transgenic animals. Animals were scored as moving if they moved at least one body bend forward or backward. If animals were rollers (Figure 2.5C), they were scored as moving if their head moved forward or backward in a circle. A minimum of 30 animals per strain was scored in each repeat of the experiment. Experiments in Figure 2.1C, 2.3A, 2.5A, 2.7E and 2.7F were part of a set of experiments done in parallel, in which each strain was scored at least 12 times. Strains in Figure 2.5C were tested at

least 10 times, and strains in Figure 2.7A were tested 3 times.

For the longitudinal studies (Figure 2.8A and 2.8B), animals were grown at 15ºC, shifted to 25ºC as L4, and scored every day. On the third day, they were shifted back to 15ºC and scored every day for two more days. Each strain was tested 4 times.

Adult assays using the worm tracker ("Tracker assay")

The parallel worm tracker platform developed by the laboratory of Miriam Goodman (Stanford University) was used to characterize adult movement as described (Ramot et al., 2008). Animals were grown in the same conditions used for the visual assays, and tested three days after the L4 stage. At the beginning of the experiment, animals were transferred on NGM plates seeded with *E. coli* OP50 as described (Ramot et al., 2008). The only exceptions were the "off food" assays, in which animals were transferred to unseeded plates instead. 1-minute videos were taken every 10 minutes for 90 minutes and analyzed using the parallel worm tracker tools. The "Identify paralyzed" function was used to determine the average speed of the population and the fraction of animals that were moving. The average speed was defined as the average of the instantaneous speed measurement for all tracks in a given video (Ramot et al., 2008). To determine the fraction of animals moving, animals were deemed "paralyzed" if 80% of the instantaneous speed measurements collected during the track were less than 0.015 mm/s. Each strain was tested 4-6 times.

We noticed that right after the transfer (t = 0 minute) more animals moved, and at a faster speed. Over time, both the speed and the percentage of animals moving decreased, and reached a stable value. We considered the initial movement response to

be movement in response to a stimulus, whereas we reasoned that the stable level of locomotion represented baseline (unstimulated) movement.

Long-term dopamine addition

Dopamine plates were prepared as previously described (Schafer and Kenyon, 1995; Chase et al., 2004). Test plates contained 30mM dopamine and 2mM glacial acetic acid. Control plates contained only glacial acetic acid. Animals were grown at 15ºC and shifted to 25ºC as L4 larvae. Three days later, animals were moved to dopamine plates seeded with *E. coli* OP50 for 2.5 hours. They were then shifted to fresh plates also seeded with *E. coli* containing the same dopamine concentrations and tested using the parallel worm tracker. 1-minute videos were taken every 10 minutes for 60 minutes. Each strain-concentration combination was tested 3 times.

Dopamine-induced paralysis

Acute dopamine treatment was carried out as described previously (Schafer and Kenyon, 1995), with minor modifications. Animals were grown at 15ºC, shifted to 25ºC as L4 larvae and tested the following day. They were transferred to 1.5% Difco agar (Becton-Dickinson) plates containing 2 mM glacial acetic acid and the indicated concentration of dopamine, and kept at 25ºC for 1 hour. The fraction of animals moving was then determined visually using a dissecting microscope.

Pumping

Animals were grown at 15ºC and transferred to 25ºC at L4 stage. Pumping rates

were scored three days later. The contractions of the pharynx in a 30-second interval were counted for 5 animals per strain on 3 separate days. Animals were counted whether or not they pumped, as 20% of the *daf-2(e1370)* mutant animals were observed not to pump at all.

HPLC analysis

Animals were grown at 15ºC until the L4 stage and shifted to 25ºC for three days. Samples were collected in M9 buffer and frozen immediately. HPLC analysis was done by the Neurochemistry Core facility at Vanderbilt University's Center for Molecular Neuroscience. Each strain was tested at least 3 times.

Statistics

Statistical analysis was carried out using XLSTAT 8.0 add-in software for Excel (Addinsoftware, USA). Appropriate tests were chosen as follows: 1) Student's *t*-test was used when two strains were compared to each other in visual assays; 2) One-way ANOVA followed by post-hoc Dunn-Sidak's corrected *t*-test or Dunnet's corrected *t*-test (for comparison to a single control) was used when multiple strains were compared to each other in visual assay; 3) Two-way ANOVA was used to analyze the tracker data, setting as variables time and strain, time and condition for the on-off food comparisons or time and concentration for the experiments with dopamine addition; *p* values reported are for the strain/ condition/ dopamine concentration component of the analysis (the time component had, as expected , a significant effect in all comparisons); when single data

points are reported (e.g. for t = 0 minute), the Student's *t*-test or Dunn-Sidak's corrected *t*-test was used; 4) Kruskal-Wallis test with Dunn's test was used for the pumping data, as these data are not normally distributed.

Results

Dauer larvae move less than wild-type animals

C. elegans dauer larvae share certain features with animals in hibernation, as both are states of dormancy characterized by suppressed metabolism and increased resistance to stress (Carey et al., 2003). Consistent with this, Cassada and Russell (1975) reported that dauer larvae have reduced spontaneous locomotion, but respond to stimuli. We tested whether we could quantify the quiescence of dauer larvae by observing non-dauers and dauers visually and comparing the fractions of animals that moved. We found that this was the case: whereas 56% to 60% of non-dauer, wild-type animals moved freely, only 14% of the dauer larvae did (Figure 2.1A). (Henceforth, we refer to this method of scoring as the "visual assay".)

Several signaling pathways interact to regulate dauer formation. In addition to the insulin/IGF-1 signaling pathway, a TGF-β pathway and a cGMP-dependent pathway also influence dauer formation, and mutations in either of these pathways can cause constitutive dauer formation [reviewed in Riddle and Albert (1997) and Hu (2007)]. To test whether the dauer quiescence phenotype was specific to *daf-2*/InsR mutant dauers, we assayed the movement of dauer larvae formed in response to mutations in *daf-7,* a gene encoding a TGF-β ligand (Ren et al., 1996)*,* and in *daf-11*, a gene encoding a

guanylate cyclase (GC) that acts in the cGMP pathway (Birnby et al., 2000). Dauers caused by all three mutations were quiescent (Figure 2.1B). We also examined wild-type dauers induced by exposure to dauer pheromone, which is a natural cue that induces dauer formation, and confirmed that these animals were quiescent as well (data not shown). We conclude that locomotory quiescence is a general feature of dauer larvae.

daf-2(e1370) adults display a quiescent behavior reminiscent of dauer larvae

In order to better characterize the quiescence phenotype, we sought to examine quiescence independently of dauer formation. To this end, we took advantage of the dauer-like locomotory phenotype produced by class II mutations in *daf-2/*InsR, which are thought to cause strong reduction in insulin/IGF-1-like signaling (Gems et al., 1998). Because the process of dauer formation is facilitated by high temperature, these mutations cause constitutive dauer formation at high temperature (25ºC). However, when raised at low temperature (15ºC) until the last stage of larval development and then shifted to 25ºC, these animals develop into adults, but have several phenotypes that are reminiscent of dauers, including reduced locomotion. We confirmed that *daf-2(e1370)* mutant adults progressively became quiescent when shifted to 25°C at the last stage of larval development, the L4 stage (Gems et al., 1998 and this study, Figure 2.8A). (All subsequent adult assays were carried out three days after the shift to 25ºC). The defect could be readily reversed by shifting the animals back to 15°C (Gems et al., 1998 and this study, Figure 2.8B). We also found that *daf-2(e1370)* animals had dramatically reduced rates of pharyngeal pumping three days after the shift to the restrictive temperature

(Figure 2.8C), which is reminiscent of the lack of pumping in dauer larvae. These dauer-like phenotypes can be uncoupled from the increased longevity of *daf-2(-)* animals, as weaker (class I) mutations in *daf-2*/InsR do not affect movement but still increase the lifespan of the worms (Gems et al., 1998).

Like class I *daf-2*/InsR mutations, mutations in genes from other dauer-formation pathways did not cause dauer-like phenotypes as adults. For example, both *daf-7*/TGF-β and *daf-11*/GC mutant adults moved as much as wild-type animals (Figure 2.1C) and had pumping rates similar to wild-type animals (Figure 2.8C). Although it is possible that changing both the cGMP and the TGF-β pathways in the same animal would trigger adult quiescence [these double mutants have an enhanced dauer-constitutive phenotype (Thomas et al., 1993)], the adult data suggest that the *daf-2*/InsR pathway may have a direct effect on the expression of dauer-like quiescence.

Because of their small size, dauer larvae are difficult to track with automated systems. However, we were able to use an automated tracker (Ramot et al., 2008) to analyze the quiescence of *daf-2(e1370)* adults. (We will refer to assays done using the automated tracker as "tracker assays"). Tracker assays allowed us to observe both the average speed and the percentage of animals moving over an extended period of time.

We noticed that, regardless of the strain, we could now clearly distinguish two stages of movement (Figure 2.1D and 2.8D). When first transferred to the assay plate, the animals moved faster, but later the average speed of the animals and the percentage that were moving decreased and subsequently remained more or less constant. We reasoned that the first stage represented movement in response to stimulation

(transferring to new plates, see Materials and Methods), whereas the second stage represented baseline, unstimulated movement. In principle, mutations that alter movement rates could affect the stimulated movement, the baseline movement or both. We found that *daf-2*/InsR mutations affected baseline rates of movement, because 60 minutes after the beginning of the assay the average speed of the *daf-2(-)* population had dropped to 3.3 ± 0.5 μm/s, whereas the speed of wild-type animals was 23.1 ± 4.6 μm/s (Figure 2.1D, t = 60 minutes, $p < 0.05$, Dunn-Sidak's corrected *t*-test). However, both wild-type and *daf-2(-)* animals moved much faster immediately after stimulation (Figure 2.1D, average population speed ± s.e.m at t = 0 minute: wt = 59.4 ± 6.2 μm/s vs *daf-2(-)* = 41.1 ± 4.7 μm/s, $p < 0.05$, Dunn-Sidak's corrected *t*-test), and in fact in both populations almost all the animals were classified as moving (Figure 2.8D, percentage moving ± s.e.m. at t = 0 minute: wt = 88% ± 6% vs *daf-2(-)* = 90% ± 6%). These data indicate that *daf-2*/InsR mutant adults, like dauers, are capable of moving after stimulation, consistent with the interpretation that their quiescence is likely due to an active modulation of the locomotory circuit, rather than to permanent defects in muscles or neurons or to a debilitating lack of energy.

Mutations that affect dopamine can reverse the quiescence of dauer larvae

Previous studies have shown that dopamine modulates the locomotion of *C. elegans* (Schafer and Kenyon, 1995; Sawin et al., 2000; Chase et al., 2004; Hills et al., 2004). Therefore, we asked whether changes in dopamine signaling could affect dauer

movement. To test the effect of decreased dopamine signaling, we examined a strain in which dopamine-producing neurons were genetically ablated by cell-specific expression of a cell-death caspase (Hills et al., 2004). To test the effects of increased dopamine signaling, we examined animals with a loss-of-function mutation in the dopamine-reuptake transporter *dat-1* (Nass et al., 2002), which is needed for termination of the dopamine signal (Gainetdinov and Caron, 2003). In mammals, dopamine transporter (DAT) mutations increase extracellular dopamine levels (Giros et al., 1996), and previous behavioral analysis in *C. elegans* suggests this may be the case in the worm too (Kindt et al., 2007; McDonald et al., 2007). Neither treatment appeared to affect the ability of the animals to form dauers (data not shown, see Materials and Methods). Surprisingly, we found that both conditions stimulated dauer movement: *daf-2(-)* dauers with no dopamine-producing neurons moved more than control *daf-2(-)* dauers (Figure 2.2A) and *daf-2(-) dat-1(-)* double-mutant dauer larvae moved more than *daf-2(-)* dauers (Figure 2.2B). We also found that *dat-1* mutations could increase movement in dauers obtained by exposure to dauer pheromone (data not shown). Together, these data suggest that mutations affecting the dopamine system can influence the behavioral quiescence of dauer larvae without overt effects on the decision to arrest in the dauer stage.

Decreases in dopamine levels in *C. elegans* have previously been correlated with increased locomotion in the absence of food (Sawin et al., 2000), which could potentially explain why loss of dopamine-producing neurons can increase movement in dauer larvae. In contrast, increases in dopamine signaling have not been reported to stimulate locomotion in *C. elegans* before. Therefore, we will discuss the effects of mutations

predicted to increase or decrease dopamine-signaling levels separately.

***dat-1* mutations stimulate the movement of *daf-2(e1370)* adults**

To study the effects of increasing dopamine signaling, we first asked whether *dat-1* mutations could also affect the quiescence of *daf-2*/InsR mutant adults. We found that these mutations dramatically increased the percentage of *daf-2(-)* animals moving in the visual assay (Figure 2.3A). When we characterized these mutants further using the tracker assay, we found that *dat-1* mutations caused an increase in movement after the animals were transferred to the assay plates, indicating that *dat-1* mutations affected movement in response to stimulation. However, by 40 minutes most of the animals had stopped moving, suggesting that *dat-1* mutations did not affect baseline movement of *daf-2(-)* animals (Figure 2.3B). Specifically, at time 0, *dat-1* mutations dramatically increased the average speed of the *daf-2(-)* population (average speed ± s.e.m at t = 0 minute: *daf-2(-)* = 39.5 ± 3.8 μm/s vs *daf-2(-) dat-1(-)* = 81.3 ± 8.6 μm/s, $p < 0.01$, Dunn-Sidak's corrected *t*-test), but the difference decreased over time (average speed ± s.e.m at t = 90 minutes: *daf-2(-)* = 2.8 ± 0.1 μm/s vs *daf-2(-) dat-1(-)* = 3.4 ± 0.3 μm/s, $p < 0.01$, Dunn-Sidak's corrected *t*-test). We conclude that *dat-1* mutations allow *daf-2*/InsR mutant adults to respond more vigorously to external stimuli.

***dat-1* mutations have little effect on wild-type movement**

Finding that mutations predicted to increase dopamine signaling increased

movement in response to stimulation was surprising, because in *C.elegans* loss of dopamine is known to increase movement in the presence of food (Sawin et al., 2000). However in other organisms mutations in genes encoding DAT, which prevent reuptake of the neurotransmitter and thus prolong dopamine signaling, cause hyperactivity (Giros et al., 1996; Kume et al., 2005). *dat-1* mutations in *C. elegans* have been reported not to have an effect on wild-type locomotion under standard culture conditions (McDonald et al., 2007). To investigate this further, we tested what effect *dat-1* mutations had on wild-type movement rates in the tracker assay. We found that *dat-1* mutations did not change the average population speed of *daf-2(+)* worms (Figure 2.9A), despite their dramatic effect on the speed of *daf-2(-)* animals (Figure 2.3B). *dat-1* mutations did increase the percentage of *daf-2(+)* animals moving after stimulation (Figure 2.9C) (a measure that is clearly visible with the tracker); however, the effect was relatively modest (Figure 2.9C, percentage moving ± s.e.m. at time t = 20 minutes: wt = 62% ± 4% vs *dat-1(-)* = 77% ± 5%; compare with Figure 2.10A, percentage moving ± s.e.m. at time t = 20 minutes: *daf-2(-)* = 10% ± 6% vs *daf-2(-) dat-1(-)* = 46% ± 10%).

Exogenous dopamine stimulates the movement of *daf-2*/InsR mutant adults

These data suggest that a prolonged increase in dopamine signaling can stimulate the movement of *C. elegans,* particularly that of quiescent insulin/IGF-1-receptor mutants. We decided to test this more directly by asking whether we could mimic the *dat-1* mutation by prolonged exposure to exogenous dopamine. We found that exposing

the worms to 30 mM dopamine (DA) for 3.5 hours (which included a 2.5-hour pre-exposure and 1 hour of recording on the tracker) increased the movement of quiescent *daf-2(-)* adults, both in terms of the average speed of the population (Figure 2.4A) and the percentage of worms moving (Figure 2.4C). No significant effect was detected in wild-type animals (Figure 2.4B and D). We conclude that prolonged exposure to dopamine increases the movement of quiescent *daf-2*/InsR mutant adults.

Notably, right after stimulation, at time 0, wild-type animals treated with dopamine actually moved less than normal (Figure 2.4D, percentage moving ± s.e.m. at t = 0 minute, 0 mM DA = 91% ± 11% vs 30 mM DA = 69% ± 7%, $p < 0.05$, Student's *t*-test), a decrease that may reflect the paralysis reported previously in acute assays (Schafer and Kenyon, 1995; Chase et al., 2004).

In wild-type animals treated with 30 mM dopamine, we observed a trend towards increased speed, but it was not statistically significant. We considered the possibility that we failed to see an effect on wild-type animals because their average speed was already high. However, food removal could double the speed of wild-type animals (Figure 2.9A and B). Therefore, these data indicate that increases in dopamine specifically suppress the quiescence of *daf-2*/InsR mutant animals after stimulation.

***daf-2*/InsR mutations require *daf-16*/FOXO activity in neurons to affect movement**

daf-2/InsR mutations lead to increased nuclear localization and activation of the FOXO transcription factor DAF-16 (Lin et al., 2001). *daf-16*/FOXO is required for *daf-2*/InsR mutant animals to become dauers (Riddle et al., 1981; Vowels and Thomas, 1992;

Gottlieb and Ruvkun, 1994), and Gems et al. (1998) showed that *daf-16*/FOXO is also required for the quiescence phenotype of *daf-2(e1370)* adults. Therefore, we wanted to test whether *daf-16*/FOXO was required for the enhanced response of *daf-2*/InsR mutants to *dat-1* mutations.

First, we confirmed that *daf-16*/FOXO mutations increased movement in *daf-2(e1370)* adults (Figure 2.5A). When we analyzed *daf-16(-); daf-2(-)* double mutants with the tracker, we found that *daf-16*/FOXO mutations partially rescued the phenotype of decreased baseline unstimulated movement (Figure 2.5B). At t = 60 minutes, when the worms have reached a stable speed, the average speed of wild-type animals was 23.1 ± 4.6 μm/s, whereas that of *daf-2(-)* animals was 3.3 ± 0.5 μm/s. In contrast, *daf-16(-); daf-2(-)* mutants moved at 12.5 ± 2.2 μm/s. We conclude that, although there may be additional pathways involved, *daf-16/*FOXO has a role in controlling quiescence in *daf-2(e1370)* animals.

We then asked in which tissue *daf-16*/FOXO acts to prevent animals from moving. To this end, we used transgenic lines expressing a *daf-16* cDNA under the control of tissue-specific promoters in a *daf-16(-); daf-2(-)* mutant background. Libina et al. (Libina et al., 2003) previously showed that neuronal *daf-16* expression is sufficient to restore a constitutive dauer-formation phenotype to *daf-16(-); daf-2(-)* double mutants. As expected, expression of *daf-16*/FOXO under its own promoter restored the quiescence phenotype of the *daf-2*/InsR mutant adults. Expression from the neuronal promoter *punc-119* also restored the quiescence, whereas expression from intestinal (*pges-1)* and muscle (*pmyo-3*) promoters did not (Figure 2.5C). These data suggest that DAF-16/FOXO acts

in neurons to slow the movement of *daf-2*/InsR mutants.

Loss of *daf-16*/FOXO causes *daf-2*/InsR mutants to respond more like wild type to *dat-1* mutations.

Because *daf-16/*FOXO and *dat-1* mutations both partially suppress the quiescence phenotype of *daf-2*/InsR mutant adults, and both of them act in neurons, we tested how *daf-16(-); daf-2(-)* animals behaved when the *dat-1* dopamine transporter was removed. We found that the average speed of *daf-16(-); daf-2(-) dat-1(-)* mutants was only slightly higher than that of *daf-16(-); daf-2(-)* mutants (Figure 2.6A), which is similar to the situation in wild-type animals (Figure 2.9A). In addition, as in wild type, a greater percentage of *daf-16(-); daf-2(-) dat-1(-)* triple mutants moved at early time points compared to *daf-16(-); daf-2(-)* double mutants (Figure 2.6B). These data indicate that *dat-1* mutations affect *daf-16(-); daf-2(-)* mutants similarly to the way they affect wild type. These data suggest that *daf-16*/FOXO is necessary for *daf-2*/InsR mutant animals to respond to *dat-1* mutations with increased movement.

***daf-2*/InsR mutants have a normal basal slowing response**

Our data indicate that *dat-1* mutations and exogenous dopamine addition can stimulate movement in *daf-2*/InsR mutant dauer and adults. However, as mentioned earlier, previous studies on the dopamine system in *C. elegans* have focused on the animals' requirement for dopamine in order to slow their movement in response to food, an effect that has been called the "basal slowing response" (Sawin et al., 2000). In this

context, reduction of dopamine increases the speed of the animals. We wondered what role this response played in our system. Indeed, we found that reduction in dopamine signaling due to ablation of dopamine-producing neurons also increased the movement of *daf-2*/InsR mutant dauers (Figure 2.2A) and adults (Figure 2.7A).

To explain the apparent paradox that both increasing and decreasing dopamine signaling leads to increased movement in dauers and dauer-like adults, we hypothesized that there are two different mechanisms at play. On the one hand, for reasons that are not understood, increases in dopamine preferentially stimulate the movement of *daf-2*/InsR mutant dauers and adults. On the other hand, loss of dopamine-producing neurons speeds up *daf-2*/InsR mutant adults, just as it does in wild-type animals, because loss of dopamine mimics the absence of food, which in turn stimulates movement.

To test this hypothesis, we asked whether *daf-2*/InsR mutant animals have a basal slowing response, that is, whether they move more in the absence of food. We found that this was the case, as the average speed of *daf-2(-)* adults was higher when the animals were off food than when they were on food (Figure 2.7B). However, *daf-2(-)* animals still eventually stopped moving in the absence of food (Figure 2.7B, average population speed ± s.e.m. at t = 90 minutes: on food = 3.1 ± 0.3 μm/s vs off food = 6.1 ± 2.7 μm/s, p = 0.35, Student's *t*-test), which indicated that absence of food did not alter baseline movement but specifically affected the response of animals to stimuli.

Next, we tested whether other mutations in dopamine-signaling genes known to affect the basal slowing response in wild-type animals also affected the locomotion of *daf-2*/InsR mutant animals. We found that mutations in the dopamine receptor *dop-3*

(Chase et al., 2004), which prevent slowing in response to food, increased the percentage of *daf-2(e1370)* dauers and dauer-like adults that were moving (Figure 2.7D and E). Furthermore we found that in both cases the increased motility of *daf-2(-)* animals carrying *dop-3(-)* mutations was dependent on another dopamine receptor, *dop-1* (Figure 2.7D and E). In the basal slowing response of non-dauer animals, *dop-3* mutations have a similar epistatic relationship with mutations in *dop-1* (Chase et al., 2004). Together these data suggest that dopamine-pathway mutations that prevent the slowing response to food may affect the quiescence of *daf-2*/InsR mutant animals because they mimic food withdrawal. They also suggest that *daf-2*/InsR-mutant quiescence is not simply an exaggerated response to food, as quiescence is displayed in the absence of food as well. Instead, it appears that a reduction in dopamine signaling can stimulate movement equally in *daf-2/*InsR mutant animals and in wild-type animals. In contrast, *daf-2*/InsR mutant animals may respond in a different manner from wild type to increases in dopamine signaling.

daf-2/InsR mutants lacking all four known dopamine receptors do not move as much as _daf-2_/InsR mutant animals with no dopamine-producing neurons

In the course of this study, we also analyzed the phenotype of *daf-2*/InsR mutant animals carrying mutations in all four known *C. elegans* dopamine receptors (Suo et al., 2002, 2003; Chase et al., 2004; Sugiura et al., 2005). We expected these animals to behave like animals with no dopamine-producing neurons. However, even though *daf-2(-); dop-2(-); dop-4(-) dop-1(-) dop-3(-)* mutant adults moved more than *daf-2(-)* mutant

adults (Figure 2.7F), they moved less than animals with no dopamine-producing neurons (compare with Figure 2.7A). We also examined these receptor-defective animals in a *daf-2(+)* background. We found that *dop-2(-); dop-4(-) dop-1(-) dop-3(-)* mutants still responded in a dopamine-induced paralysis assay (Figure 2.11). Although it is simply possible that some of these mutations are not null, this observation may also suggest the existence of other dopamine receptors in the worm, or, alternatively, ligand-independent activity of one or more of the receptors.

Discussion

In this study, we investigated the effects of dopamine signaling on a behavioral condition reminiscent of hibernation in *C. elegans*; namely, the quiescence of *daf-2*/InsR mutant dauer larvae and dauer-like adults. Both types of animals display a remarkable and largely uncharacterized decrease in spontaneous movement. We found that perturbations in dopamine signaling have dramatic effects on *daf-2*/InsR mutant dauer and adult movement. Paradoxically, both lack of dopamine-producing neurons, which should prevent dopamine production, and reduced clearance of dopamine from the extracellular medium, which should increase dopamine signaling, increase the percentage of *daf-2*/InsR mutant dauers and adults that move. Changes in dopamine signaling affect the animals' ability to sustain an increase in motion following stimulation, rather than their unstimulated, baseline quiescence. This finding is significant because it suggests that dopamine specifically affects the response of these animals to environmental cues.

In addition, it indicates that changes in dopamine do not affect the movement of *daf-2(-)* animals by somehow up-regulating insulin/IGF-1 signaling.

We also found that *daf-16*/FOXO, a transcription factor that mediates most of the effects of *daf-2*/InsR mutations, controls the unstimulated baseline movement in *daf-2/*InsR mutant animals. Therefore, mutations in *daf-16*/FOXO and dopamine signaling affect *daf-2*/InsR mutant quiescence in different ways.

Dauer-like quiescence is probably not caused by changes in dopamine signaling

If the quiescence of dauers and dauer-like adults were directly caused by a change in dopamine signaling within the animal, it should be possible to produce dauer-like quiescence simply by altering dopaminergic pathways. However, neither the addition of exogenous dopamine, the *dat-1* mutation, loss of dopamine-producing neurons, nor mutation of any dopamine receptors produced a quiescent dauer-like phenotype. While quiescence could conceivably be caused by a more subtle change in dopamine signaling, it seems most likely that dauer-like quiescence is caused by changes in non-dopaminergic pathways.

The basal slowing response is intact in *daf-2*/InsR mutants

The loss of dopamine-producing neurons increases the movement of dauer-like animals, and we attribute this effect to mimicry of a food-deprived state. Dopamine is required for worms to slow down when they encounter food, and as a consequence, the increased locomotion of animals that lack dopamine consistently resembles that of

animals off food (Sawin et al., 2000; Chase et al., 2004; Li et al., 2006). The slowing response to food, called the basal slowing response, is thought to ensure that animals stay on food when they encounter it. We found that *daf-2*/InsR mutant animals display a basal slowing response, as their average speed is higher off food than on food. (However, food withdrawal is not sufficient to prevent quiescence, as *daf-2*/InsR mutant animals eventually become quiescent whether they are on food or not.) In addition, we found that mutations in the *dop-3*- and *dop-1*-receptor genes affect *daf-2*/InsR mutant quiescence in a similar manner to the way they affect the basal slowing response in wild-type non-dauer animals (Chase et al., 2004). We conclude that, in *daf-2*/InR mutant animals, the dopamine circuit that controls the basal slowing response is largely intact.

***dat-1* mutations affect the quiescence of *daf-2*/InsR mutants through a novel mechanism**

Our results suggest that *dat-1* mutations increase the locomotion of *daf-2/*InsR mutant animals by a different mechanism, involving higher extracellular dopamine levels. Because *dat-1* mutant animals can modulate their locomotion in response to food similarly to wild-type animals (Figures 2.9A and 2.9B), it is unlikely that the increase in extracellular dopamine simply leads to a compensatory decrease in dopamine signaling that mimics the basal slowing response (Jones et al., 1998). In addition, other phenotypes displayed by *dat-1* mutants are consistent with their having elevated levels of dopamine signaling, such as slowed habituation on food (Kindt et al., 2007) and swimming-induced paralysis (McDonald et al., 2007). Therefore, we conclude that *dat-1* mutations increase

movement in *daf-2*/InsR mutants by a mechanism that is novel and unrelated to the basal slowing response.

Because wild-type worms do move more frequently and faster than *daf-2*/InsR animals, one possibility is that an increase in locomotory activity may be easier to detect in the quiescent *daf-2(-)* animals. However, it is possible to increase the speed of locomotion in wild type; for example, by food withdrawal, ablation of dopamine-producing neurons, and mutations in *dop-3* (Chase et al., 2004). Therefore, we reasoned that a stimulation of movement by dopamine should be detectable. We hypothesize that *daf-2*/InsR mutant dauers and adults are programmed to respond differently from non-dauers to increases in dopamine signaling.

The DAF-2/Insulin/IGF-1-like receptor signals to the nucleus by affecting the activity of the FOXO transcription factor DAF-16 (Lin et al., 2001). We observed that in *daf-16(-); daf-2(-)* double mutants, which move more like wild type, *dat-1* mutations increase the percentage of animals moving, but have only a small effect on their speed. This resembles the situation in wild-type animals. In addition, restoring *daf-16*/FOXO in the neurons is sufficient for *daf-16(-); daf-2(-)* double mutants to become quiescent. Thus, transcriptional changes controlled by DAF-16/FOXO in neurons may underlie the different responses to dopamine displayed by *daf-2*/InsR mutants.

We do not know which DAF-16-target genes may be responsible for the specific effect of dopamine on *daf-2*/InsR mutants. Of the known components of the dopamine pathway, two were downregulated in *daf-2*/InsR mutant animals compared to wild-type animals: *bas-1*, an aromatic amino acid decarboxylase required for synthesis of dopamine

and serotonin, and *dop-2*, one of the dopamine receptors (McElwee et al., 2004). However, these changes do not explain our findings, as there was no detectable difference in the overall levels of dopamine in wild type versus *daf-2(e1370)* animals (Figure 2.12, tested as day 3 adults by HPLC analysis), though the data do not rule out more subtle changes in distribution of dopamine in the animal. In addition, *daf-2(-) dat-1(-); dop-2(-)* animals still moved faster than *daf-2(-); dop-2(-)* animals (data not shown). It would be interesting to test all candidate DAF-16-regulated genes for their effects on quiescence, but because RNAi does not work well in neurons (Timmons et al., 2001), this is not a simple task. Perhaps critical genes could be recovered in a screen for mutations that stimulate the movement of quiescent *daf-2(-)* animals.

Insulin regulation of dopamine signaling in other organisms

Whereas in *C. elegans,* dopamine is known to decrease movement (Schafer and Kenyon, 1995), in most other organisms dopamine is known to increase movement (Giros et al., 1996; Kume et al., 2005). We find that, at least under conditions of limiting insulin/IGF-1-like signaling, dopamine signaling in worms may act in a fashion more similar to other organisms than previously appreciated. In addition, our data suggest that *C. elegans daf-2*/InsR mutations may alter the dopamine signaling system, so as to make animals more sensitive to the stimulatory effects of dopamine. This conclusion is compatible with the emerging idea that in the mammalian brain, hormonal signals involved in energy balance, including insulin, can affect the dopamine reward pathway to modulate feeding behavior (Figlewicz et al., 2007; Palmiter, 2007). Food-restricted

animals, which have lower circulating insulin levels, behave as though they have elevated dopamine signaling. However, the mechanism by which insulin signaling acts at the cellular level to influence dopamine signaling is still unclear.

In the mammalian brain, some of the effects of dopamine are not due to simple increase or decrease of dopamine, but rather to changes in the kinetics of dopamine release (Goto et al., 2007). In *C. elegans,* many of the subtleties of dopamine signaling kinetics that are important for dopamine signaling in other organisms (Goto et al., 2007) are still unexplored, because all studies, including this one, have relied on generalized loss of dopamine production or unspecific increases in dopamine levels. Therefore, the study of *daf-2*/InsR mutant movement in response to stimulation may be an interesting paradigm where to explore the worm's dopamine system in more detail.

Implications for hibernation

Quiescent *C. elegans* dauers resemble animals in hibernation in many ways (Carey et al., 2003), although hibernation in higher animals does not occur only at a specific developmental stage. Interestingly, neurons of hibernating mammals have decreased activity of Akt (Lee et al., 2002; Eddy and Storey, 2003; Cai et al., 2004; Abnous et al., 2008), which is one of the kinases that inhibit DAF-16/FOXO under replete conditions. Thus hibernation and the dauer diapause may be similar at a molecular level. It is even possible that hibernation is potentiated, in part, by a reduction in insulin/IGF-1 signaling caused by nutrient deprivation or other stressful conditions. During hibernation, suppression of movement is important to prevent energy expenditure,

but little is known about how suppression of movement is regulated in this context. It would be interesting to know whether hibernating animals are particularly susceptible to the stimulatory effects of dopamine. Likewise, in *C. elegans,* it would be interesting to know whether there are environmental stimulants that activate quiescent dauers by elevating dopamine signaling.

It seems possible that the ability to suppress movement is an ancient response to prolonged environmental stress that is expressed in the context of the dauer in *C. elegans*, and in the context of hibernation (and possibly sleep) in other animals. If so, then understanding the neural pathways that regulate dauer behavior, which are genetically accessible, may have more general implications for the behavior of higher organisms.

Figures

Figure 2.1. Dauer larvae have reduced spontaneous movement and mutations in the *daf-2*/InsR pathway can produce dauer-like quiescence in adult animals.

A) The percentage of *daf-2(e1370)* dauer larvae that moved upon visual observation was compared to that of wild-type (wt) larvae and adults also grown at 25ºC. Percentage moving ± s.e.m: wild-type L2/L3 larvae = 56% ± 6%, wild-type L4 larvae = 57% ± 6%, wild-type adults = 60% ± 7%, *daf-2(-)* dauer larvae = 14% ± 1%. ***$p < 0.0001$ (Dunn-Sidak's corrected *t*-test) for *daf-2(-)* vs wt (any stage).

B) The movement of dauer larvae formed at 25ºC due to mutations in genes representative of the three major dauer formation pathways was assayed: the insulin/IGF-1 receptor homolog (InsR) *daf-2* for the insulin/IGF-1-like pathway, the TGF-β homolog *daf-7* for the TGF-β pathway, and the guanylate cyclase (GC) *daf-11* for the cGMP pathway. Dauers formed by all three mutations were quiescent. Percentage moving ± s.e.m: *daf-2(e1370)* = 20% ± 6%, *daf-11(sa195)* = 36% ± 6, *daf-7(e1372)* =12% ± 2%.

C) Mutations in *daf-2*/InsR, but not mutations in *daf-7*/TGF-β or in *daf-11*/GC, decreased the percentage of animals moving. Percentage moving ± s.e.m: wt = 78% ± 3%, *daf-2(e1370)* = 25% ± 2%, *daf-7(e1372)* = 88% ± 2%, *daf-11(sa195)* = 76% ± 5%. ***$p < 0.0001$ (Dunn-Sidak's corrected *t*-test) vs wt.

D) *daf-2(e1370)* adults were compared to wild-type animals using the tracker. Both animals responded to stimulation, but the average baseline speed of the population was lower in *daf-2(e1370)* adults. $p < 0.0001$ (Two-way ANOVA) for *daf-2(-)* vs wt. Error

bars represent s.e.m.

Animals in Figure 2.1C and 2.1D were grown at 15°C, shifted to the quiescence-inducing temperature, 25°C, at the L4 stage (after the dauer-decision point) and tested at day 3 of adulthood on food.

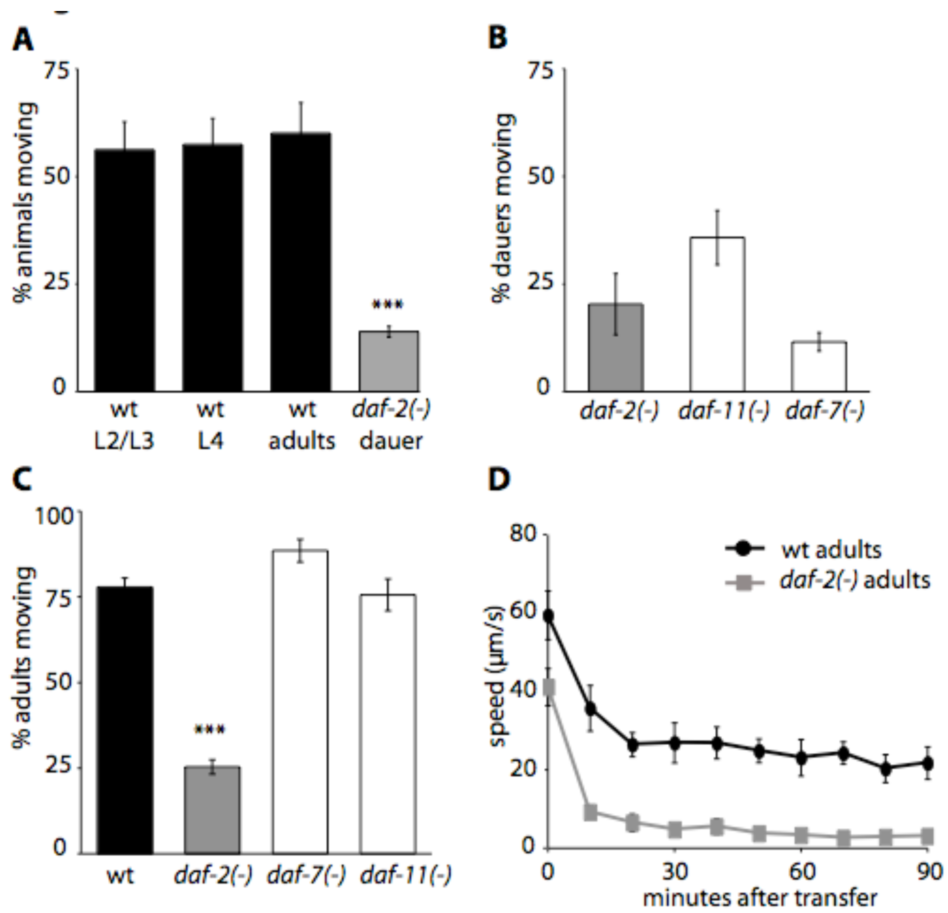


Figure 2.2. Ablation of the dopamine-producing neurons and mutations in the dopamine transporter *dat-1* increase the percentage of moving dauers.

A) *daf-2(e1370)* strains expressing GFP (+DA neurons) or a human homolog of *C. elegans* caspase (interleukin converting enzyme) (-DA neurons) in the dopamine-producing neurons (Hills et al., 2004) were tested to see the effect of genetically ablating dopamine-producing neurons. Loss of the dopamine-producing neurons increased the number of dauers that moved. Percentage moving ± s.e.m: *daf-2(-); lin-15(-); akEx248[pdat-1::GFP, lin-15(+)]*(*daf-2(-)* +DA neurons) = 19% ± 3%, *daf-2(-); lin-15(-); akEx387[pdat-1::GFP, pdat-1::hICE, lin-15(+)]*(*daf-2(-)* –DA neurons) = 45% ± 5%. **$p < 0.01$ (Student's *t*-test)

B) The effect of loss of the dopamine transporter was tested by comparing spontaneous movement in *daf-2(e1370)* and *daf-2(e1370) dat-1(ok157)* dauer larvae. *dat-1* mutations increased the percentage of dauers that were moving. Percentage moving ± s.e.m: *daf-2(-)* = 14% ± 2%, *daf-2(-) dat-1(-)* = 50% ± 3%. ***$p < 0.0001$ (Student's *t*-test).

Figure 2.2

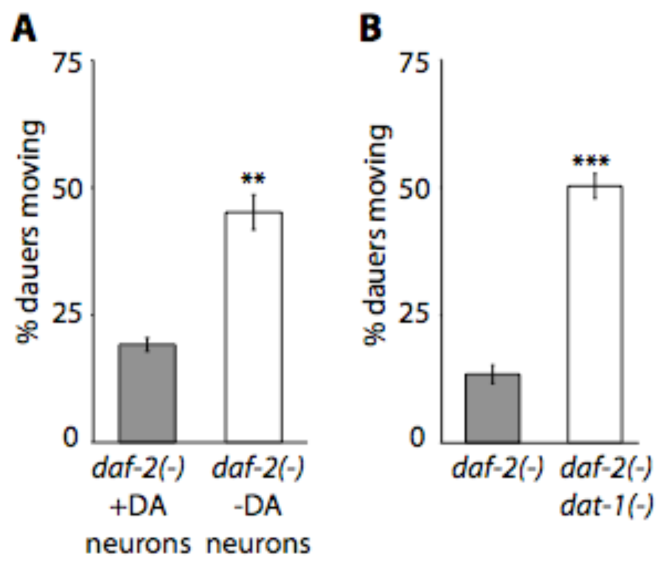


Figure 2.3. Mutations in *dat-1* increase movement in *daf-2*/InsR mutant adults by affecting their locomotory response to stimulation.

A) Mutations in the dopamine transporter *dat-1* increased the percentage of adult *daf-2(-)* animals that moved. Percentage moving ± s.e.m: *daf-2(e1370)* = 25% ± 2%, *daf-2(e1370) dat-1(ok157)* = 67% ± 3%. ***$p < 0.0001$, Student's *t*-test

B) When stimulated, *daf-2(e1370) dat-1(ok157)* animals initially moved faster than *daf-2(e1370)* mutants alone. $p < 0.0001$ (Two-way ANOVA). Error bars represent s.e.m. All animals were grown at 15°C, shifted to 25°C at the L4 stage and tested at day 3 of adulthood on food.

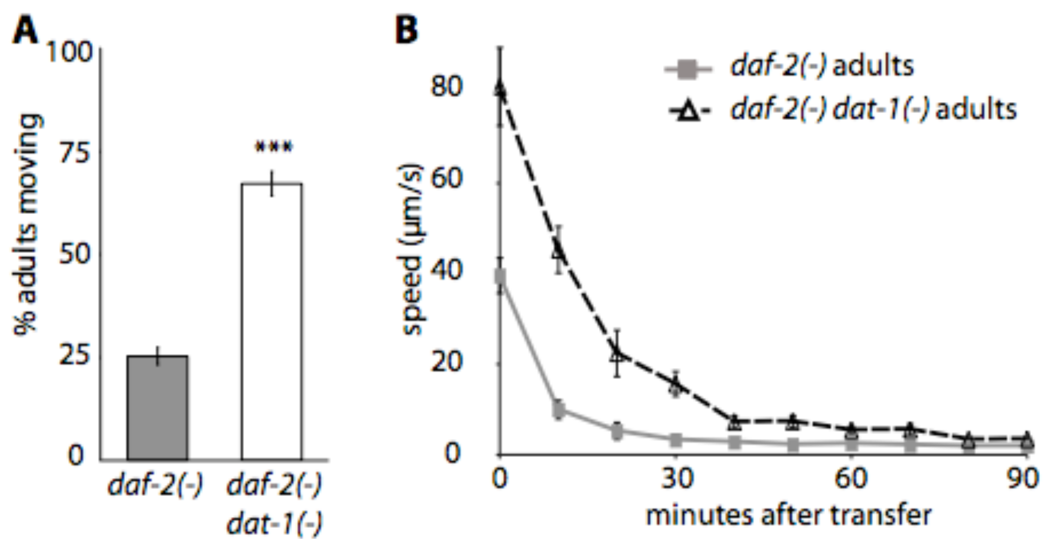


Figure 2.4. Long-term exogenous addition of dopamine suppresses quiescence in *daf-2*/InsR mutant animals.

daf-2(e1370) (**A**, **C**) and wild-type (wt) (**B**, **D**) animals were incubated on plates containing 0 mM and 30 mM dopamine for 3.5 hours (2.5 hours of pre-incubation and 1 hour of recording). Exogenous dopamine increased the average speed [**A**, $p < 0.05$ (Two-way ANOVA)] and the percentage of animals moving [**C**, $p < 0.001$ (Two-way ANOVA)] in *daf-2*/InsR mutant animals but had no effect on the speed [**B**, $p = 0.8$ (Two-way ANOVA)] and percentage of animals moving [**D**, $p = 0.9$ (Two-way ANOVA)] in wild-type animals.

All animals were grown at 15°C, shifted to 25°C at the L4 stage and tested at day 3 of adulthood on food. Error bars represent s.e.m.

Figure 2.4

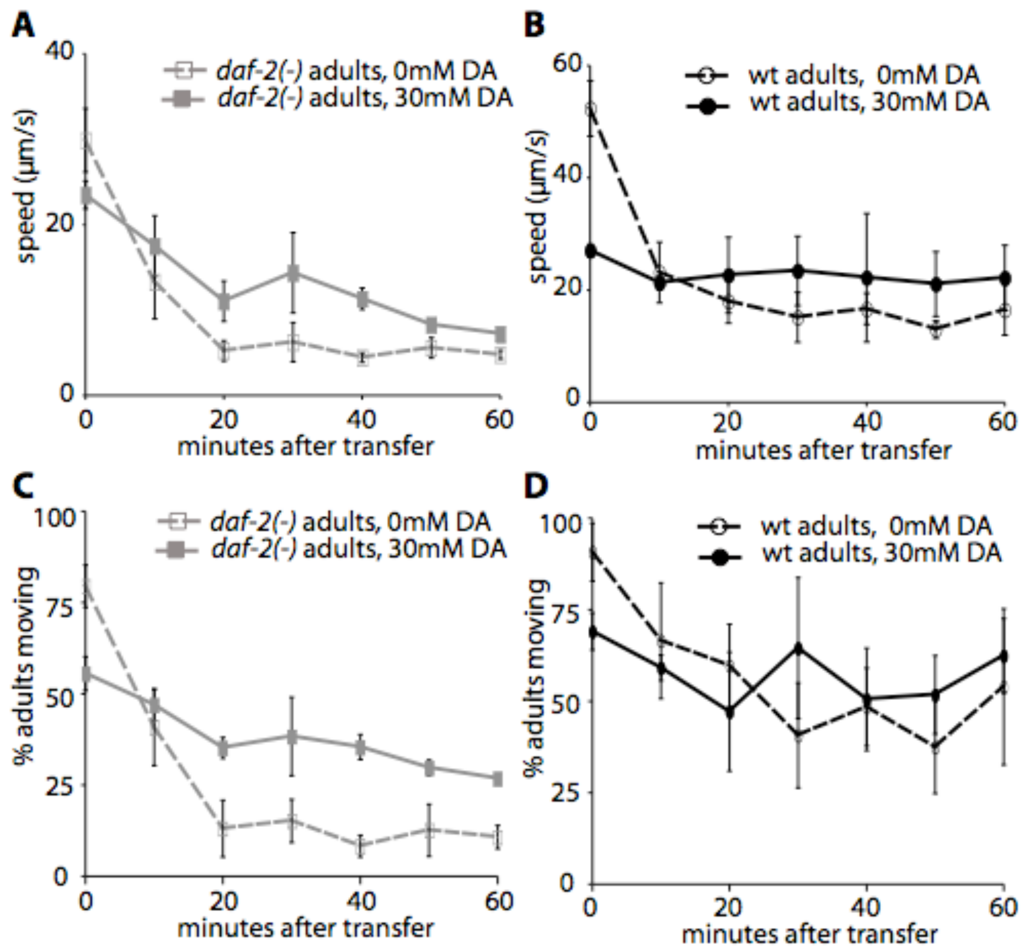


Figure 2.5. Mutations in the well-studied suppressor of *daf*-2/InsR dauer formation and longevity, daf-*16*/FOXO, also suppress the quiescence of *daf-2*/InsR mutant adults.

A-B) *daf-16(mu86); daf-2(e1370)* double mutant animals were more active than *daf-2(e1370)* mutants, both in the visual (**A**) and the tracker (**B**) assays, but were still less active than wild-type (wt) animals.

A) Percentage moving ± s.e.m: wild-type = 77% ± 3%, *daf-2(-)* = 25% ± 2%, *daf-16(-); daf-2(-)* = 57% ± 3%. ***$p < 0.0001$ (Dunn-Sidak's corrected *t*-test) vs. wild-type (wt) control, ††† $p < 0.0001$ (Dunn-Sidak's corrected *t*-test) vs. *daf-2(-)*.

B) The average speed of *daf-16(mu86); daf-2(e1370)* animals was higher than that of *daf-2(e1370)* but lower than that of wild-type animals, suggesting that *daf-16*/FOXO can only partially rescue *daf-2(e1370)* quiescence. $p < 0.0001$ (Two-way ANOVA) for *daf-16(-); daf-2(-)* vs *daf-2(-)* and *daf-16(-); daf-2(-)* vs wt. Error bars represent s.e.m.

C) Neuronal expression of *daf-16*/FOXO was sufficient for *daf-2*/InsR mutant worms to become inactive. *daf-16(-); daf-2(-)* animals carrying transgenes expressing *daf-16* cDNA under different promoters were tested against *daf-2(-)* and *daf-16(-); daf-2(-)* strains carrying a transgene expressing the co-injection marker *rol-6*. Expression of *daf-16*/FOXO under the control of its own promoter or the neuronal *punc-119* promoter led to quiescence, whereas intestinal (*pges-1)* or muscle (*pmyo-3*) expression had no effect. Percentage moving ± s.e.m: *daf-16(+); daf-2(-)* =28% ± 5%, *daf-16(-); daf-2(-)* = 62% ± 3%, *daf-16(-); daf-2(-); pdaf-16::GFP::daf-16* = 35% ± 6%, *daf-16(-); daf-2(-); punc-*

119::GFP::daf-16 = 43% ± 4%, *daf-16(-); daf-2(-); pges-1::GFP::daf-16* = 76% ± 3%, *daf-16(-); daf-2(-); pmyo-3::GFP::daf-16* = 76% ± 3%. $^{**}p < 0.01$ (Dunnet's corrected *t*-test) vs *daf-16(-); daf-2(-)*.

All animals were grown at 15°C, shifted to 25°C at the L4 stage and tested at day 3 of adulthood on food.

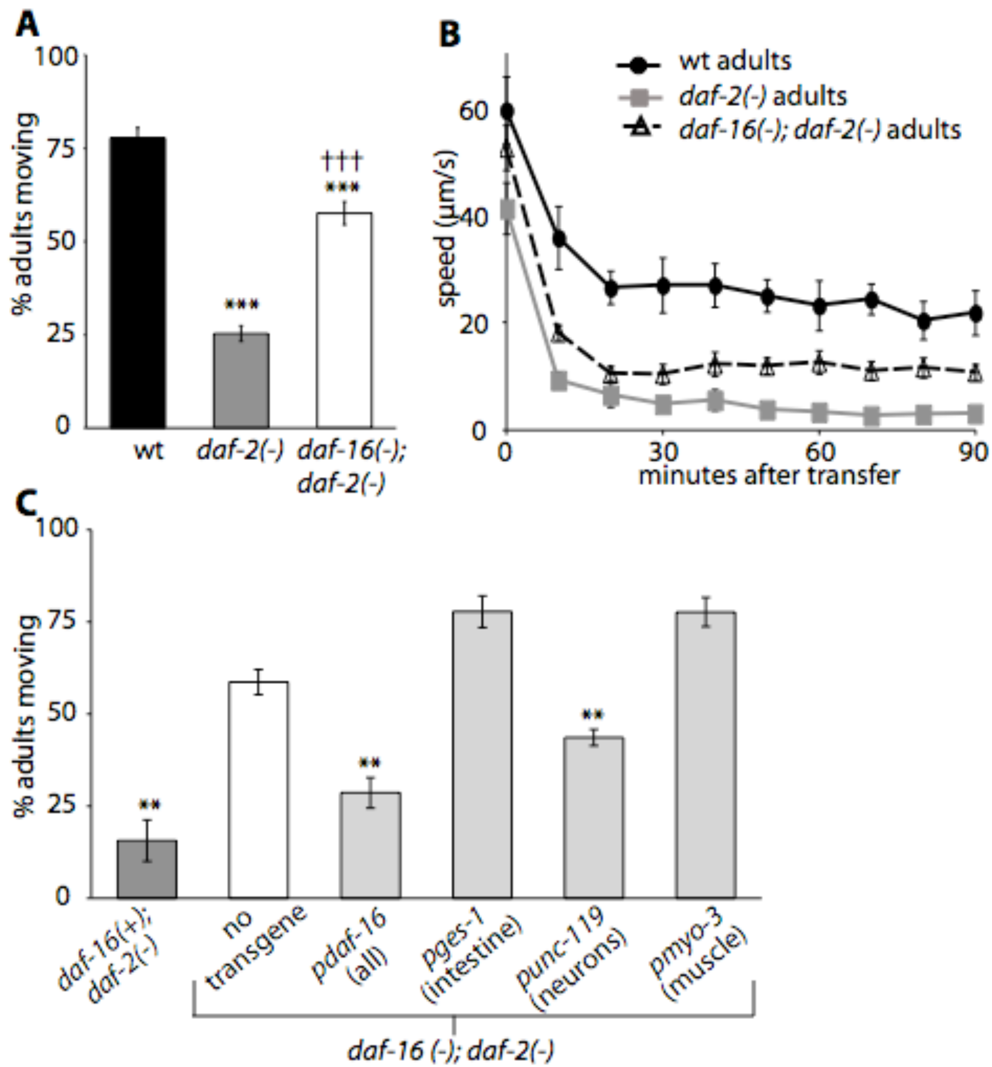


Figure 2.6. *dat-1* mutations have a relatively minor effect on the movement *daf-16(-); daf-2(-)* double mutants.

dat-1(ok157) mutations had a small effect on the speed (**A**) of *daf-16(mu86); daf-2(e1370)* animals but increased the percentage of animals moving (**B**). This is similar to the effect that *dat-1(ok157)* mutations had on wild type. Compare Figure 2.6A with Figure 2.3B and 2.9A, and Figure 2.6B with Figure 2.10A and 2.8C. **A**) $p < 0.001$ (Two-way ANOVA). **B**) $p < 0.0001$ (Two-way ANOVA).

All animals were grown at 15°C, shifted to 25°C at the L4 stage and tested at day 3 of adulthood on food. Error bars represent s.e.m.

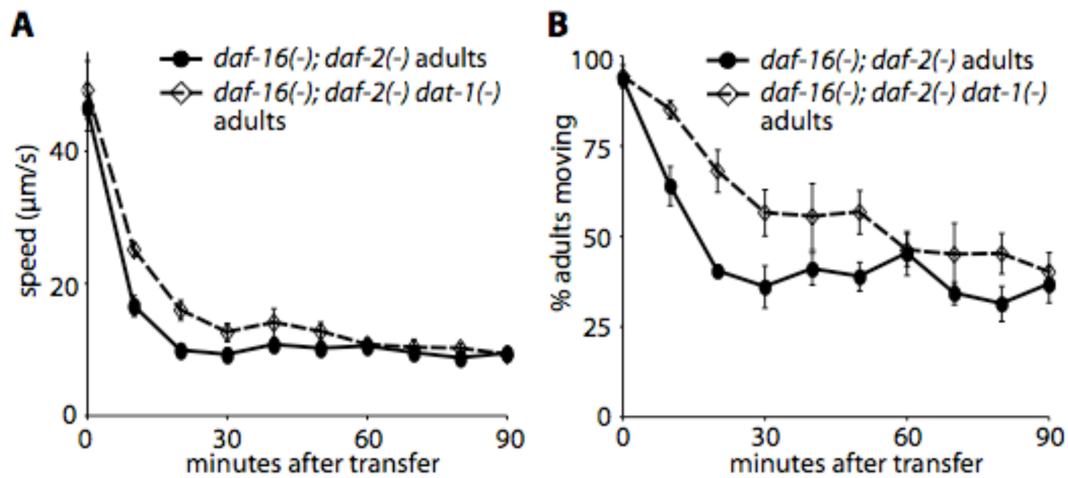


Figure 2.7. ***daf-2(e1370)*** **animals display a basal slowing response and are stimulated by mutations that mimic lack of food.**

A) Ablation of the dopamine-producing neurons by expression of a human interleukin converting enzyme (-DA neurons) (Hills et al., 2004) in *daf-2(e1370)* animals increased their movement as adults compared to control *daf-2(e1370)* animals expressing only GFP in the dopamine neurons (+DA neurons). Percentage moving ± s.e.m: *daf-2(-)* +DA neurons = 39% ± 10%, *daf-2 (-)* -DA neurons = 80% ± 3%. $^*p < 0.05$ (Student's *t*-test).

B-C) *daf-2(e1370)* animals and wild-type (wt) were transferred to plates with or without food and observed for 90 minutes. The average speed of the worms is plotted as a function of time after transfer for *daf-2*/InsR mutants (B) and wild type (wt, C). As previously reported for younger wild-type animals (Sawin et al., 2000), the speed of wild-type animals was higher in the absence of food. In *daf-2(-)* animals, the average speed was also higher right after stimulation, but decreased over time. $p < 0.0001$ (Two-way ANOVA) for wild-type on food vs. off food and *daf-2(-)* on food vs off food. Error bars represent s.e.m.

D) *daf-2(e1370); dop-3(vs106)* dauers moved more than *daf-2(e1370)* dauers, but their increased movement was suppressed by *dop-1(vs100)* mutations. Percentage moving ± s.e.m: *daf-2(-)* = 14% ± 2%, *daf-2(-); dop-3(-)* = 27% ± 3%, *daf-2(-); dop-1(-)* = 8% ± 2%, *daf-2(-); dop-1(-) dop-3(-)* =13% ± 2%. $^{***}p < 0.0001$ and $^{**}p < 0.01$ (Dunn-Sidak's corrected *t*-test).

E) Mutations in the dopamine receptor *dop-3* caused *daf-2(e1370)* adults to move more.

Conversely, mutations in the dopamine receptor *dop-1* caused them to move even less. *daf-2(e1370); dop1(vs100) dop-3(vs106)* animals moved similarly to *daf-2(e1370); dop-1(vs100)* animals, suggesting that *dop-1* acts downstream of *dop-3* in the regulation of *daf-2(e1370)* adult quiescence. Percentage moving ± s.e.m: *daf-2(-)* = 25% ± 2%, *daf-2(-); dop-3(-)* = 53% ± 4%, *daf-2(-); dop-1(-)* = 2% ± 1%, *daf-2(-); dop-1(-) dop-3(-)* = 6% ± 1%. ***$p < 0.0001$ (Dunn-Sidak's corrected *t*-test) vs *daf-2(-)*. ††† $p < 0.0001$ (Dunn-Sidak's corrected *t*-test) for *daf-2(-); dop-3(-)* vs *daf-2(-); dop1(-) dop-3(-)*.

F) Mutating all the four known *C. elegans* dopamine receptors increased the movement of *daf-2(e1370)* adults, but did not recapitulate the effects of the ablation of dopamine-producing neurons. Percentage moving ± s.e.m: *daf-2(-)* = 25% ± 2%, *daf-2(-); dop-2(-); dop-4(-) dop-1(-) dop-3(-)* = 40% ± 4%. **$p < 0.01$ (Student's *t*-test).

All adult animals were grown at 15°C, shifted to 25°C at the L4 stage and tested at day 3 of adulthood.

Figure 2.7

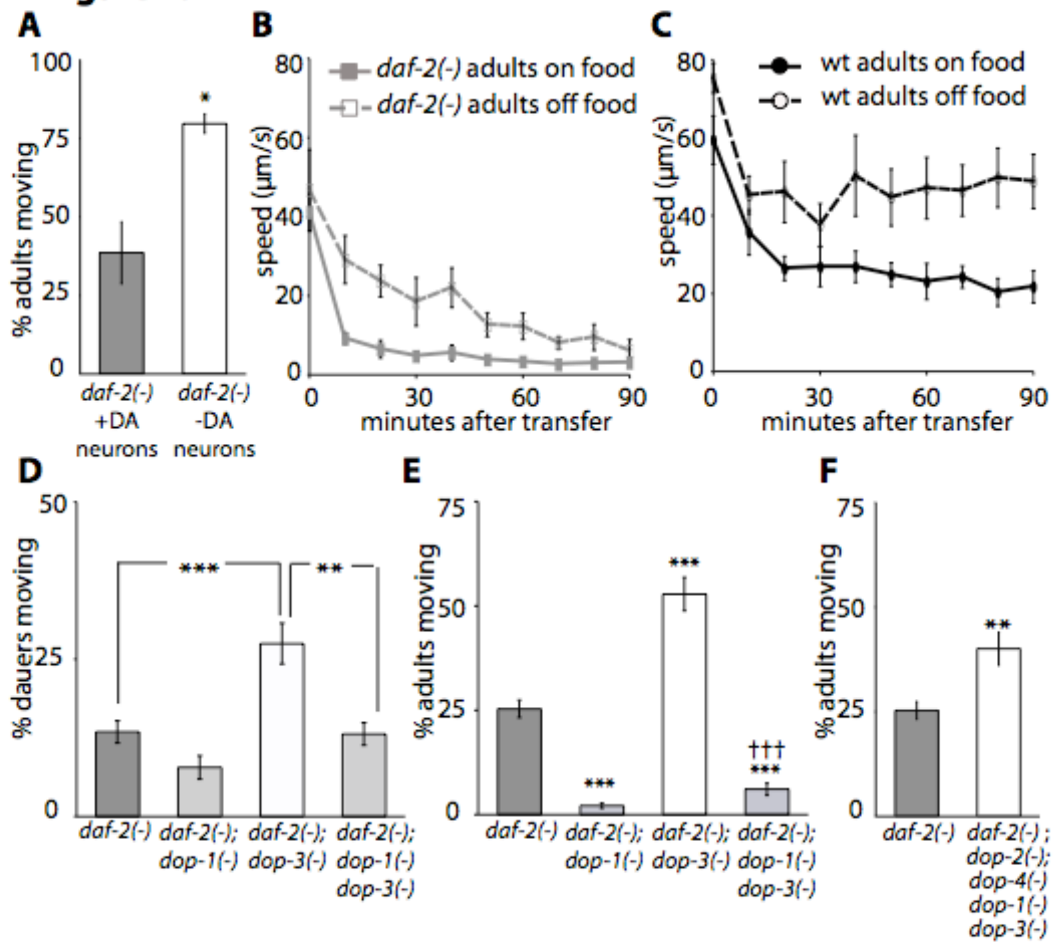


Figure 2.8. Shifting *daf-2(e1370)* animals to 25° C causes a reversible decrease in movement and a decrease in pumping rates.

A) *daf-2(e1370)* L4 larvae were shifted from 15°C to 25°C and the percentage of worms moving was scored every day for 3 days. Unlike wild-type animals (wt), *daf-2(e1370)* mutant animals progressively stopped moving (Gems et al., 1998). Error bars represent s.e.m.

B) *daf-2(e1370)* adults were shifted back to 15ºC at day 3 of adulthood (day 3 in Figure 2.8A). When shifted back to 15°C, *daf-2(e1370)* animals resumed movement (Gems et al., 1998). ***$p < 0.0001$ (Dunn-Sidak's corrected *t*-test) for wt vs *daf-2(-)*. Error bars represent s.e.m.

C) The pumping rate of adult *daf-7(e1372)* and *daf-11(sa195)* worms was not significantly different from that of wild type (wt), whereas the pumping rate of *daf-2(e1370)* worms was much lower. Pumps/second ± st. dev.: wt = 3.7 ± 0.5, *daf-2(-)* = 0.4 ± 0.6, *daf-7(-)* = 3.0 ± 0.6, *daf-11(-)* = 3.3 ± 1.0. 20% of *daf-2(-)* animals showed no detectable pumping in 30 seconds. n = 15 per strain. **$p < 0.01$ (Kruskal-Wallis and Dunn's test) vs wt.

D) The percentage of animals moving was computed from the tracker data using a speed cut-off. When moved to a fresh plate, *daf-2(e1370)* mutant adults initially moved in the same proportion as wild-type adult worms, but within 30 minutes they returned to a state of inactivity. $p < 0.0001$ (two-way ANOVA).

Figure 2.8

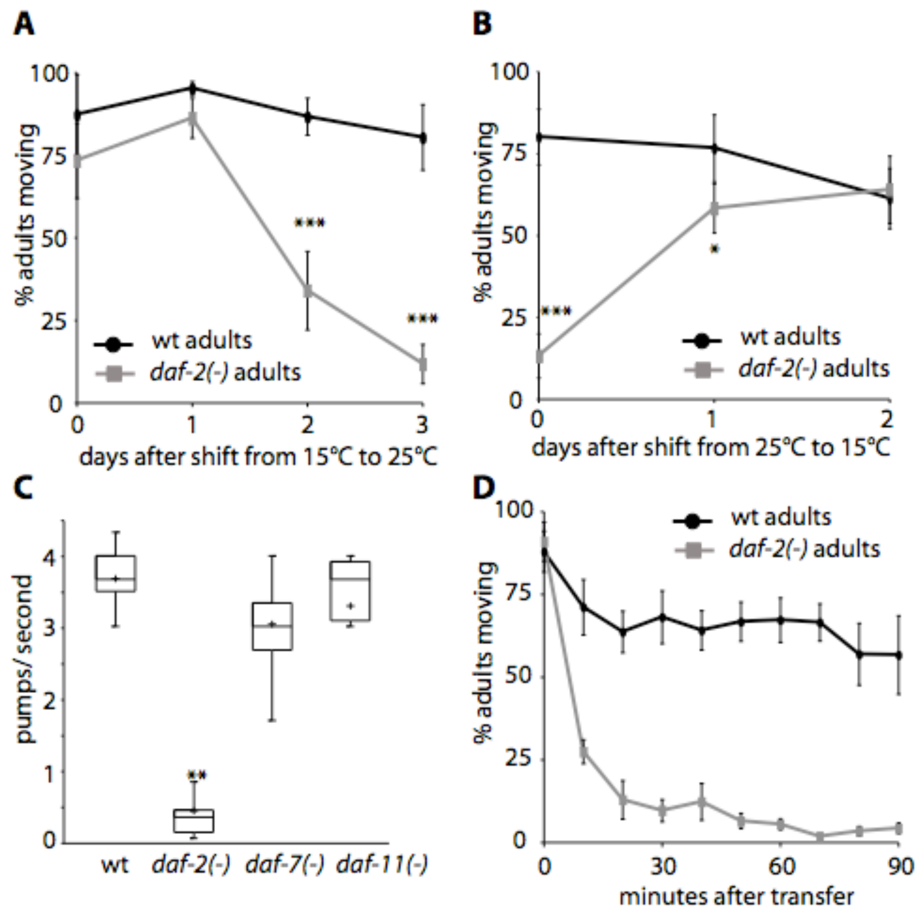


Figure 2.9. *dat-1* mutations have no effect on speed but change the percentage of *daf-2(+)* animals moving.

A-B) Wild-type (wt) and *dat-1(ok157)* animals had similar average speed when tested on food (**A**) or off food (**B**).

C) Despite having similar average speed to wild-type (wt) animals, a greater percentage of *dat-1(ok157)* adult animals moved after stimulation and possibly longer term. $p < 0.0001$ (Two-way ANOVA).

Animals were grown at 15°C, shifted to 25°C at the L4 stage and tested at day 3 of adulthood.

Figure 2.9

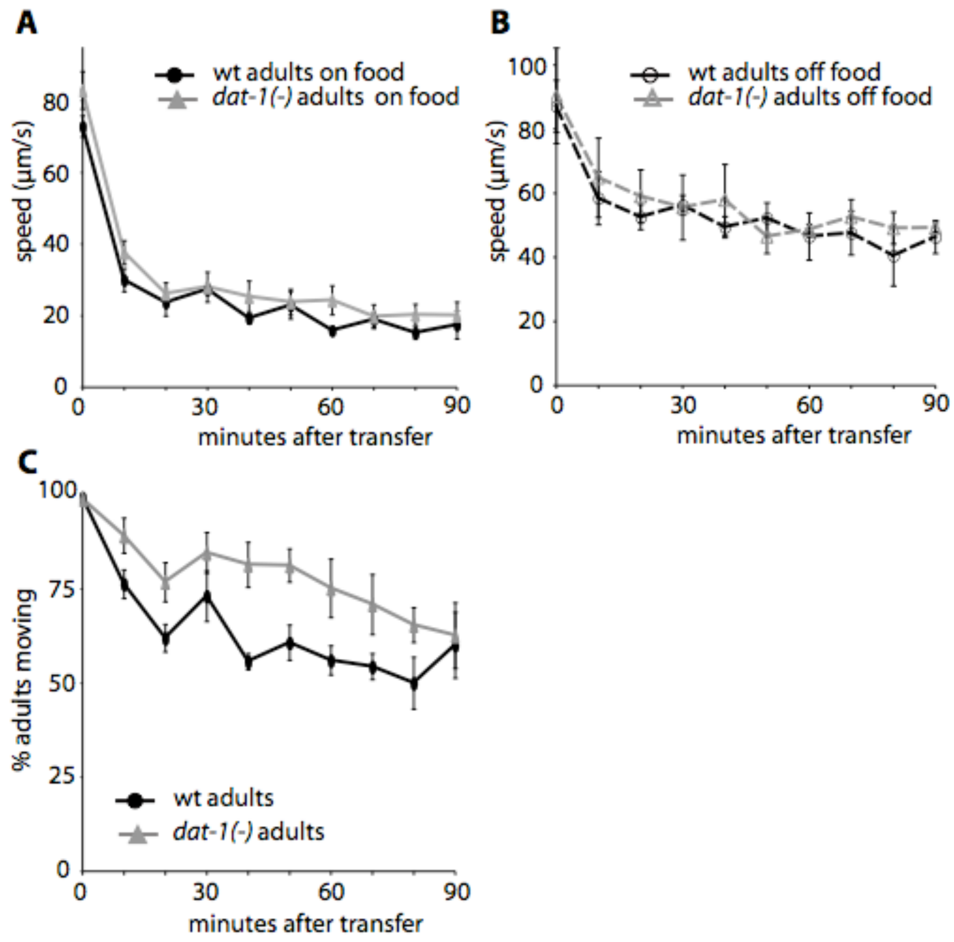


Figure 2.10. Characterization of *daf-2(-) dat-1(-)* and *daf-2(-); dop-3(-)* adults using the worm tracker.

A) The percentage of *daf-2(e1370)* and *daf-2(e1370) dat-1(ok157)* animals moving was computed from the tracking data by applying a speed cutoff. Similarly to what seen when plotting speed, *dat-1* mutations appeared to dramatically increase the percentage of animals moving at early time points, but not later, suggesting an effect of these mutations on the ability of the animals to respond to stimulation. Interestingly, the percentage of animals moving at t = 0 minute, right after stimulation, was very similar, but the average speed was very different (compare to Figure 2.3B). $p < 0.0001$ (Two-way ANOVA).

B-C) *dop-3* mutations also affected *daf-2*/InsR mutant adult quiescence by affecting the response to stimulation. This is evident when the percentage of animals moving (**B**) or the average speed (**C**) of the population is plotted. $p < 0.0001$ (Two-way ANOVA).

All animals were grown at 15°C, shifted to 25°C at the L4 stage and tested at day 3 of adulthood on food. Error bars represent s.e.m.

Figure 2.10

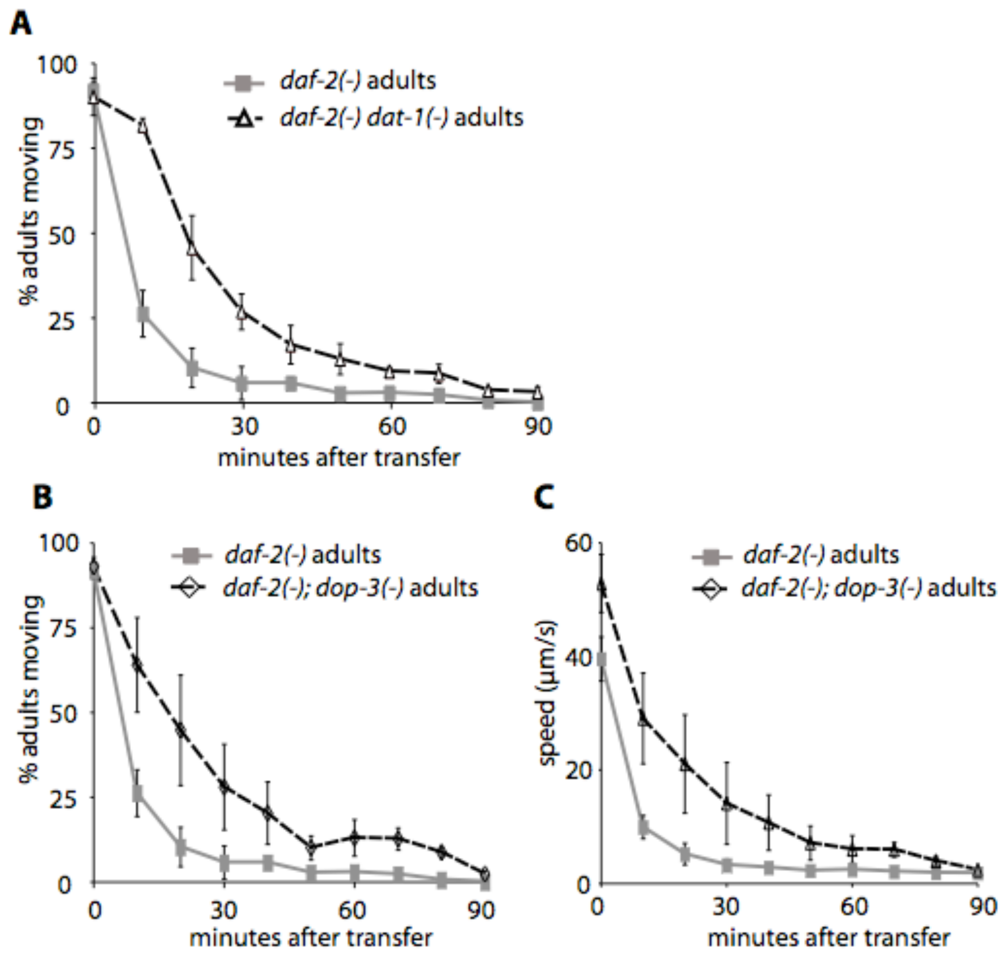


Figure 2.11. Mutations in the four known dopamine receptors do not prevent the paralysis induced by acute dopamine treatment.

dop-2(-); dop-4(-) dop-1(-) dop-3(-) animals became paralyzed as much as wild-type (wt) animals after acute dopamine treatment. Wild-type and *dop-2(-); dop-4(-) dop-1(-) dop-3(-)* animals were treated with increasing concentrations of dopamine for one hour. As previously reported for wild-type animals (Schafer and Kenyon, 1995), this led to dose-dependent paralysis.

All animals were grown at 15°C, shifted to 25°C at the L4 stage and tested at day 1 of adulthood in the absence of food. Error bars represent s.e.m.

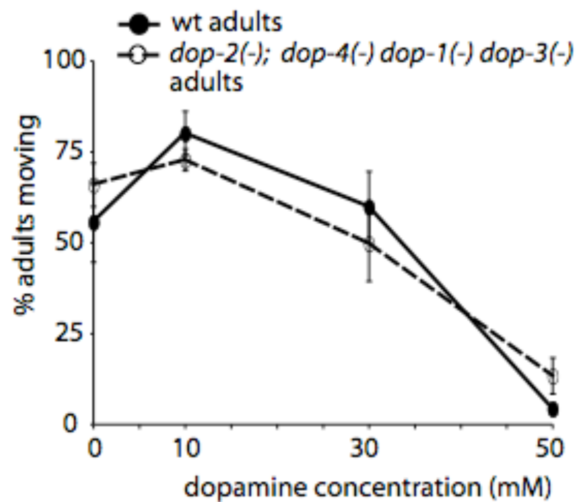
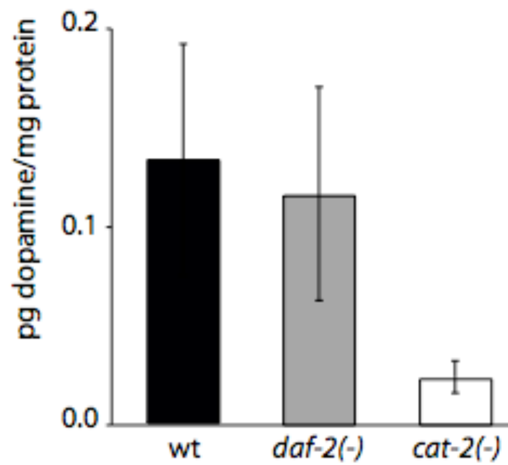


Figure 2.12. The dopamine content of *daf-2(e1370)* worms is similar to that of wild-type animals.

The total content of dopamine in wild-type and *daf-2(e1370)* adult worms, measured by HPLC, was similar. *cat-2/*tyrosine hydroxylase mutants were tested as a control, and shown to have much lower levels of dopamine, as previously reported (Sanyal et al., 2004). All animals were grown at 15°C, shifted to 25°C at the L4 stage and collected at day 3 of adulthood.



Chapter 3:

Sensory mutations affect longevity, dauer formation and resistance to pathogens in *C. elegans* by directing specific transcriptional changes.

Abstract

The sensory system of multicellular organisms is designed to provide information about the environment, which can be translated into appropriate changes in physiology as well as behavior. In the nematode *C. elegans*, sensory neurons affect the decision to arrest in a hibernation-like state, the dauer larva, during development, and also modulate the lifespan of the animals in adulthood. However, the molecular mechanisms underlying these effects are still poorly understood. Using whole-genome microarray analysis, we examined the genes that are transcriptionally regulated by the sensory system. We found that the transcription factors DAF-16/FOXO and DAF-12/nuclear hormone receptor appeared to be activated by perturbations of the sensory system, and are responsible for the extended lifespan and dauer formation phenotype of sensory mutant animals. In addition, we found that immune response genes were strikingly repressed in sensory

mutant animals, leading to increased susceptibility to bacterial pathogens. Thus, sensory input seems to coordinately regulate an extensive transcriptional network to modulate basic biological processes of the worm. This is similar to the complex regulation of physiology by the mammalian hypothalamus, which also receives innervation from sensory systems, most notably the visual and olfactory systems.

Introduction

Living organisms are constantly interacting with their environment. Behavioral responses as well as physiological processes such as energy homeostasis, development, immune homeostasis need to be modulated depending on the environmental situation. For example, an animal's feeding and immune response are crucial to survival in general, but may need to be suppressed in the presence of danger. This allows the most efficient allocation of resources for survival. To achieve such modulation, animals have developed complex sensory systems to acquire and integrate various sorts of information about their environment and their intern al state. It is now clear that the nervous system extensively controls peripheral functions and has a role in many physiological processes ranging from immune responses to reproduction. However, these processes are regulated and how they are affected by the environment are still incompletely understood.

The nematode *C. elegans* has been used extensively as a model organism to dissect the molecular mechanisms regulating sensory control of behavior because of its simple nervous system and genetic tractability. A small number of ciliated sensory neurons, located mostly near the head and tail of the animal, detect various environmental

signals including soluble and volatile compounds, gases, osmolarity changes, mechanosensory and noxious stimuli [reviewed in Inglis et al. (2007)]. As in other organisms, it is now clear that the sensory system of *C. elegans* regulates physiological functions of the animals as well as behavior. When the sensory neurons are compromised, for example, worms increasingly arrest in the alternative hibernation-like developmental stage called dauer at high temperatures (Bargmann and Horvitz, 1991; Ailion and Thomas, 2000). In addition, defects in the sensory system extend adult lifespan (Apfeld and Kenyon, 1999; Alcedo and Kenyon, 2004). How the disruption of a few neuronal cells results in changes in the physiology of the whole organism is still poorly understood.

The regulation of transcriptional programs in distal tissues could be responsible for the extensive physiological effects of sensory mutations. Indeed, both dauer formation and longevity in the sensory mutants are in part due to transcriptional changes mediated by the activation of the FOXO transcription factor *daf-16* (Apfeld and Kenyon, 1999; Ailion and Thomas, 2000). However, loss of *daf-16*/FOXO does not completely abolish either of those physiological outputs in some sensory mutants (Apfeld and Kenyon, 1999; Ailion and Thomas, 2000). Therefore, it is possible that the sensory system may require other transcription factors to exert its effects on dauer formation, longevity and potentially other physiological processes.

In this study, we examined the transcriptional profile of sensory mutant animals to identify the transcriptional network activated by the sensory system to regulate the physiology of the whole animal. As a good validation of our microarray data, we found

that sensory mutations affected the expression of many target genes of the *daf-16*/FOXO transcription factor. In addition, we found evidence that the sensory system regulates another transcription factor *daf-12*/nuclear hormone receptor (NHR), which influences normal lifespan (Larsen et al., 1995; Hsin and Kenyon, 1999; Gerisch et al., 2001) and dauer formation (Riddle et al., 1981; Antebi et al., 1998) in other contexts. These data suggest that de-regulated expression of DAF-16/FOXO and/or DAF-12/NHR target genes in sensory mutant animals may result, during development, in an increase propensity to form dauer larvae and, during adulthood, in an extension of the animals' lifespan. Interestingly, we found that many *C. elegans* pathogen-inducible genes (Shapira et al., 2006; Troemel et al., 2006) were downregulated in the sensory mutant animals compared to wild-type animals under normal non-pathogenic conditions, suggesting that sensory mutants may have defects in their immune responses. Consistent with this idea, we found that the long-lived sensory mutants were actually more susceptible to the pathogen *Pseudomonas aeruginosa* than wild-type animals. These data indicate that transcriptional regulation by the sensory system is required for the normal immune response. Taken together, our data suggest that the sensory system modulates various transcriptional programs to exert its effects on lifespan, dauer formation and immune reactions in response to the environmental changes.

Results

Mutations in *daf-10* cause differential expression of a set of genes

To characterize the mechanisms underlying the physiological changes of sensory mutant *C. elegans* strains, we compared the gene expression profile of young adult wild-type worms with that of worms carrying mutation in *daf-10*, which encodes an intraflagellar transport (IFT) protein (Bell et al., 2006). *daf-10(-)* animals have structural defects in their sensory cilia leading to defects in odor sensation (Perkins et al., 1986). In addition to the sensory defects, *daf-10* mutant animals show a significant increase in longevity (Apfeld and Kenyon, 1999), as well as increased dauer formation at high temperature (27ºC) (Apfeld and Kenyon, 1999; Ailion and Thomas, 2000).

Using whole-genome oligonucleotide-based microarrays, we identified 14 genes that were upregulated in the *daf-10(-)* sensory mutants, and 56 genes that were downregulated (Table 3.1). Based on comparison with published microarray data on tissue-specific transcripts (Pauli et al., 2006; Von Stetina et al., 2007), we expect 8 of the 70 genes to be expressed in neurons and 14 in the intestine (Table 3.1). None of the genes we identified was specifically enriched in the muscle or the germline.

Two categories of genes appeared very prominently downregulated: the P-glycoproteins (*pgp-5, -6* and *-7*: P value < 0.0001, hypergeometric probability) and proteins containing a CUB-like domain (formerly known as DUF141) (C32H11.4, C17H12.6, C17H12.8, C29F3.7, *dod-24, dod-17*: P value < 0.0001, hypergeometric probability). P-glycoproteins are members of the ABC transporter family and function to

extrude large hydrophobic molecules from cells (Schinkel, 1997; Sheps et al., 2004). *pgp-5* is thought to have a role in immune responses in *C. elegans* (Kurz et al., 2007). CUB-like domains are *C. elegans*-specific protein domains that resemble CUB domains, found on extracellular and membrane proteins, such as complement. Proteins with a CUB-like domain, which are probably secreted, may have important, though unknown, roles in longevity. *dod-24* and *dod-17* are downregulated in the long-lived *daf-2*/insulin-IGF-1 receptor (InsR) mutant animals and knockdown of these genes by RNAi leads to increased lifespan (Murphy et al., 2003). CUB-like domain proteins were also identified among the transcriptional targets of the nuclear hormone receptor (NHR) *daf-12* (Fisher and Lithgow, 2006) and among genes that are activated by exposure to the pathogen *Pseudomonas aeruginosa* (Shapira et al., 2006; Troemel et al., 2006).

To validate our results, we measured the mRNA levels of 22 selected genes from our microarray list by using quantitative RT-PCR and we also examined their expression in another sensory mutant strain, *osm-5(-)*. The *osm-5* gene encodes another component of the intraflagellar transport complex, which is also required for correct formation of the cilia (Haycraft et al., 2001; Qin et al., 2001), and *osm-5(-)* animals are very long-lived (Apfeld and Kenyon, 1999). Of these 22 genes, 19 were regulated as expected based on microarray results in at least one of the two mutants. Among them, 10 genes were regulated in a similar fashion in both mutant strains (Figure 3.1A and 3.1B), whereas the expression of another 11 was only different from wild type in *daf-10* mutants (Figure 3.1B and 3.1C). Quantitative RT-PCR results for 3 genes did not recapitulate the microarray results (Figure 3.1D). Together, these data suggest that *daf-10* and *osm-5*

mutations may affect the expression of a common set of targets that are regulated by the sensory system in general.

Some of the genes regulated by *daf-10* mutations are targets of *daf-16*/FOXO and/or *daf-12*/NHR

Both sensory mutants and *daf-2*/InsR(-) mutants live long and exhibit dauer-formation phenotypes. Thus, we wondered whether the transcriptional profile of *daf-10* mutant animals was similar to that of *daf-2*/InsR mutant animals. Several groups have carried out microarray analysis of *daf-2*/InsR mutant animals compared to *daf-16(-); daf-2(-)* double mutants, defining a set of genes that are putative DAF-16/FOXO targets. We compared our list to the microarray results in Murphy et al. (2003) and McElwee et al. (2004), as well as with results generated during the course of this study (Lee and Kenyon, unpublished data). We found a significant overlap between our regulated gene sets and all three DAF-16/FOXO -target lists [Figure 3.2, out of 70 differentially regulated genes in our array list, 9 were in common with the results from Murphy et al. (2003), 20 with those of McElwee et al. (2004) and 9 with the data generated by Lee and Kenyon. $p < 0.0001$].

CUB-like domain genes, which were overrepresented in our study, were previously reported to be targets of the nuclear hormone receptor DAF-12 based on a microarray study of *daf-12* gain-of-function and loss-of-function mutant animals (Fisher and Lithgow, 2006). Therefore, we compared our results to those reported by Fisher and Lithgow (2006) and found a very significant overlap (Figure 3.3A, 13 out of 70

differentially regulated genes, $p < 0.0001$) [Table 3.1 also marks those genes that were reported in another study of DAF-12/NHR targets (Shostak et al., 2004)]. Since there is a small but statistically significant overlap between the genes regulated by DAF-16/FOXO and DAF-12/NHR (Fisher and Lithgow, 2006), it seemed possible that all conditions that extend longevity regulate a common set of genes, independently of the specific pathway. We therefore tested whether our gene list also overlapped with the set of genes regulated by addition of resveratrol (Viswanathan et al., 2005). We chose this gene set because the lifespan extension due to resveratrol addition does not depend on the *daf-16*/FOXO transcription factor (Wood et al., 2004). We found that none of the genes regulated by resveratrol addition was differentially regulated in the *daf-10(-)* sensory mutants (data not shown). We conclude that the overlap between the genes whose expression is altered by *daf-10* mutations and those regulated by *daf-16*/FOXO or *daf-12*/NHR is not simply a result of all longevity pathways regulating a similar set of genes. Together, these data suggest that sensory mutations affect the activity of two transcription factors, *daf-16/*FOXO and *daf-12/*NHR, both of which have been previously implicated in the regulation of lifespan and dauer formation.

To confirm that the putative DAF-12/NHR-target genes we identified were regulated by DAF-12/NHR in the context of sensory mutants, we performed qRT-PCR to compare their expression in the presence and absence of *daf-12*/NHR, both in sensory mutants and in otherwise wild-type animals. We examined the mRNA levels of 9 genes identified in our arrays that were previously shown to be regulated by *daf-12*/NHR according to Fisher and Lithgow (2006) and found that at least 7 of them are also

regulated in a *daf-12*/NHR-dependent fashion in *daf-10(-)* sensory mutants (Figure 3.3B and 3.6A). K10C2.3, and perhaps *dod-17*, were the only exceptions. In *osm-5(-)* mutant animals, 7 of these genes were also clearly downregulated and the expression of four of these required *daf-12*/NHR (Figure 3.6B). Interestingly, the expression of only two of the nine genes (Y49AG5A.1 and *dod-17*) was altered in *daf-12(-)* mutant versus wild-type animals (Figure 3.3B and 3.6A), and in one case the effect was opposite to the one observed in the sensory mutant background (*dod-17*). We conclude that *daf-10* and *osm-5* mutations both influence the expression of a number of DAF-12/NHR-target genes. Furthermore, our finding that loss of *daf-12*/NHR alone does not lead to changes in the basal expression of most genes suggests that in the sensory mutant background DAF-12/NHR is more active than in wild-type animals.

Some all DAF-16/FOXO-target genes are not differentially expressed in sensory mutants

Some well-known targets of DAF-16/FOXO, such as *sod-3* and *mtl*-1 (Honda and Honda, 1999; Libina et al., 2003; Murphy et al., 2003), were clearly missing from our results. When we measured the levels of these genes by qRT-PCR, we found that *sod-3* mRNA levels were actually decreased in *daf-10(-)* mutant animals, and unchanged in *osm-5(-)* mutant animals. *mtl-1* mRNA levels, on the other hand, were clearly upregulated in *osm-5(-)* mutant animals, but only slightly changed in *daf-10(-)* mutant animals (Figure 3.4). This result indicates that not all DAF-16/FOXO targets regulated by the *daf-2*/InsR pathway are also consistently regulated by the sensory system.

daf-12/NHR is required for high-temperature dauer formation, but not for lifespan extension, in _daf-10(-)_ sensory mutant animals

daf-16/FOXO has previously been shown to be required, at least partially, for two phenotypes of the *daf-10(-)* mutants: extended lifespan and increased dauer formation at high temperatures (Apfeld and Kenyon, 1999; Ailion and Thomas, 2000). *daf-12*/NHR is required for dauer formation in a number of contexts (Vowels and Thomas, 1992), and in some cases also for lifespan extension (Larsen et al., 1995; Hsin and Kenyon, 1999; Gerisch et al., 2001). We therefore tested whether *daf-12*/NHR was also required for these phenotypes in sensory mutants. We found that despite the differential expression of DAF-12/NHR-target genes in the sensory mutants, *daf-12*/NHR was not required for the lifespan extension caused by *daf-10(-)* or *osm-5(-)* mutations (Figure 3.3C and 3.6C). However, *daf-12*/NHR was required for the increased dauer formation at high temperatures due to *daf-10* mutations (Figure 3.3D, percentage dauers, mean ± s.e.m: wild type = 25.9% ± 7.5%, *daf-12(-)*= 0.8% ± 0.8%, *daf-10(-)* = 96.0% ± 2.0%, *daf-10(-); daf-12(-)* = 13.9% ± 3.7). This is consistent with studies of *tax-4* mutations, which eliminate the function of a cyclic-nucleotide-gated channel required for sensory transduction (Komatsu et al., 1996). *tax-4(-)* mutant animals form dauers at 27ºC in a *daf-12*/NHR-dependent manner (Ailion and Thomas, 2000). These results indicate that the *daf-10(-)* mutant animals increasingly form dauers at high temperature because they have basally altered expression of DAF-16/FOXO and DAF-12/NHR-target genes.

Sensory mutations lead to downregulation of pathogen-response genes

The two classes of genes that were overrepresented our list of downregulated genes, encoding CUB-like domain-containing proteins and P-glycoprotein, are upregulated in response to a bacteria pathogen, *Pseudomonas aeruginosa* (Shapira et al., 2006; Troemel et al., 2006). In addition, many of the other gene classes generally associated with pathogen responses in *C. elegans*, such as C-type lectins and DUF274 proteins, are also represented in our gene list. We therefore compared our list of differentially regulated genes with transcriptional profiles of *C. elegans* exposed to pathogens (Shapira et al., 2006; Troemel et al., 2006). We found that a significant number of the genes that were downregulated by *daf-10(-)* mutations were upregulated in response to *P. aeruginosa* in wild-type animals (15/56 compared to the data in Shapira et al. (2006) or 21/56 compared to Troemel et al. (2006), Figure 3.5A). Some of these genes were reported in Shapira et al. (2006) to be required for resistance to *P. aeruginosa.* Our finding that pathogen response genes are downregulated in sensory mutants is surprising, because one might expect long-lived sensory mutant animals to be more resistant to infection. In fact, it has been shown that mutants that have increased *daf-16*/FOXO activity, such as *daf-2*/InsR mutants, are more resistant to bacterial pathogens (Garsin et al., 2003). We therefore tested how long *daf-10(-)* animals lived when cultured on the pathogenic *Pseudomonas aeruginosa* strain PA14. Consistently with the expression data and despite their extended longevity, we found that there was a significant decrease in the survival of *daf-10(-)* animals on PA14 (Figure 3.5B). This

result suggests that the decrease in immune-responsive genes causes *daf-10(-)* animals to be more susceptible to PA14 infection. The sensory system may thus be involved in modulating immune responses by affecting the basal expression of genes involved in pathogen clearance. It is also possible that cues from pathogenic bacteria may need to be sensed to activate immune responses.

Discussion

The sensory system in *C. elegans* has profound effects on the physiology of the animals: it influences development by affecting sensitivity to dauer-inducing cues (Albert et al., 1981; Vowels and Thomas, 1992; Ailion and Thomas, 2000) and modulating starvation arrest at the L1 stage (Lee and Ashrafi, 2008), regulates adult lifespan (Apfeld and Kenyon, 1999), and causes changes in fat storage (Mukhopadhyay et al., 2005; Mak et al., 2006). In this study we examined the transcriptional regulatory network that lies downstream of the sensory system. By performing a whole-genome DNA microarray analysis that compared transcriptome of well-defined sensory *daf-10(-)* mutants and that of wild-type animals, we found that the output of several transcription factors is under the control of the sensory system. Consistent with the previous finding that the sensory system regulates the activity of DAF-16/FOXO transcription factor (Apfeld and Kenyon, 1999; Ailion and Thomas, 2000; Lin et al., 2001), we found a significant overlap between the genes whose expression is changed in *daf-10(-)* sensory mutants and those previously reported as DAF-16/FOXO targets (Murphy et al., 2003; McElwee et al., 2004). We also

found many genes that were putative targets of the nuclear hormone receptor DAF-12 (Fisher and Lithgow, 2006). Consistent with this finding, we observed that *daf-12*/NHR is required for the high-temperature dauer arrest phenotype in the sensory *daf-10(-)* mutants. Intriguingly, we found that normal expression of a number of immune-responsive genes (Shapira et al., 2006; Troemel et al., 2006) required the sensory system. The abnormal repression of these immune-responsive genes in the sensory *daf-10(-)* animals may be responsible for the increased susceptibility of *daf-10(-)* animals to the pathogenic bacteria *P. aeruginosa*. Thus, we have found that the sensory system of *C. elegans* can enact substantial transcriptional changes, presumably in multiple tissues. This effect is mediated by a number of transcription factors, and has profound impacts on basic physiological processes such as development, aging and immune response.

There is a shared set of target genes in sensory mutant animals

We tested the mRNA levels of a number of genes whose expression was altered in *daf-10(-)* animals based on our microarray results in a second sensory mutant strain, *osm-5(-)*. We found that about half of the genes tested were also differentially expressed in the *osm-5* mutants. Our data suggest that there is a shared set of genes whose expression is affected by different sensory mutations. However, not all genes are regulated in the same manner in the two mutants. This may be due to the fact that the molecular lesions in the two mutants are different (Qin et al., 2001; Bell et al., 2006), and thus may have different impact on cellular activity. Moreover, as the expression pattern of *daf-10* has not been characterized in detail, it is possible that *daf-10* and *osm-5* mutations

compromise the function of different neurons. As we discuss below, it is likely that the activity of multiple neurons contributes to longevity regulation, and therefore each sensory mutant strain may have a different set of inputs to its longevity regulating pathways as well.

Sensory mutations cause differential expression of some, but not all, *daf-16*/FOXO target genes

Because sensory mutant animals live long largely in a *daf-16*/FOXO-dependent fashion, sensory mutations could, in principle, lead to altered release of insulin-like ligands (Pierce et al., 2001), which would result in changes in DAF-16/FOXO activity. However, our results suggest that this simple picture may not be true. As we expected from the genetic data, we observed differential expression of many DAF-16/FOXO targets in the sensory mutant animals. However, we noticed the conspicuous absence from our array results of genes that are routinely used as reporters of DAF-16/FOXO activation, such as *sod-3* and *mtl-1*. This was not due to insufficient sensitivity of the arrays, because we could not detect upregulation of *sod-3* and *mtl-1* mRNA in *daf-10(-)* animals using qRT-PCR either. *mtl-1* mRNA, however, was upregulated in another sensory mutant, *osm-5*.

One explanation could be that sensory mutations may provide an input into DAF-16/FOXO activation that does not go through *daf-2*/InsR signaling. There are other treatments that extend lifespan in a *daf-16*/FOXO-dependent manner, but may do so without necessarily affecting *daf-2*/InsR signaling, such as germline ablation (Arantes-

Oliveira et al., 2003), overexpression of the histone deacetylase *sir-2.1* (Tissenbaum and Guarente, 2001), the JUN-kinase *jnk-1* (Oh et al., 2005), and the MST1 kinase *cst-1* (Lehtinen et al., 2006). Overexpression of *jnk-1* or *cst-1*/MST1 may mimic the input that sensory mutations have on lifespan, and, if so, one might expect the effect of sensory mutations to require the presence of that gene. We do not think *sir-2.1* would be a candidate, because mutations of this gene does not preclude *osm-5(-)* sensory mutants from living long (Jeff Farrell and C.K. unpublished).

An alternatively explanation for our expression results is that disruption of multiple sensory neurons at the same time could provide a number of different signals, which both positively and negatively regulate DAF-16/FOXO. Even though the net outcome is the extended longevity of sensory mutants, the transcriptional output may be subtly different from that of the insulin/IGF-1-like pathway. We know, for example, that at least some sensory mutations and ablation of particular sensory neurons actually shorten lifespan of long-lived *daf-2*/InsR mutant animals (Apfeld and Kenyon, 1999; Alcedo and Kenyon, 2004), which points at the existence of negative inputs into longevity.

Sensory mutations probably lead to increased dauer formation by activating DAF-12/NHR transcriptional activity

The activity of DAF-12/NHR is necessary for worms to go into dauer both in response to natural cues such as dauer pheromone and food scarcity and in response to mutations in all the major pathways that control dauer entry (Riddle et al., 1981; Vowels

and Thomas, 1992). Therefore, it was not surprising that sensory mutant animals could not go into dauer at high temperature in the absence of *daf-12*/NHR. However, it is interesting to note that DAF-12/NHR-target genes are differentially expressed in sensory mutants, which may indicate that at least part of the reason sensory mutants arrest in the dauer stage easily at high temperature is that they have a basal over-activation of DAF-12/NHR and/or DAF-16/FOXO. Therefore, at least in the case of sensory mutants, DAF-12/NHR may have instructive role in dauer formation, rather than its basal activity being permissive for it. This is consistent with the fact that certain mutations in DAF-12/NHR can cause constitutive arrest in the dauer stage in a ligand-independent way. It is possible that DAF-16 and DAF-12 are acting in separate tissues to control dauer formation. When we knocked down *daf-12*/NHR using RNAi in *daf-10(-)* animals, we were able to reproduce the suppression of dauer formation at 27ºC seen with the genetic mutants. However, we had no suppression when we inactivated *daf-16*/FOXO by RNAi (Figure 3.7). Since neurons are well known to be refractory to RNAi (Timmons et al., 2001), these data suggest that DAF-16/FOXO may be acting in the neurons, whereas DAF-12/NHR may be acting in peripheral tissues. This may be similar to what happens in *daf-2*/InsR mutant animals, as DAF-16/FOXO has also been shown to act in the neurons of these animals to regulate dauer formation (Libina et al., 2003).

***daf-10* mutations alter transcription of pathogen response**

We found that many pathogen-inducible genes were abnormally repressed in the sensory *daf-10(-)* mutants. As a result, *daf-10(-)* animals have reduced survival

compared to wild-type animals when exposed to the bacterial pathogen *P. aeruginosa*. This result suggests that sensory input may be used by *C. elegans* to modulate immune responses. While this study was in progress, Styer et al. (2008) reported that *npr-1(-)* animals were sensitive to *P. aeruginosa* and had altered transcription of pathogen-response genes. *npr-1* mutations are thought to hyperactivate a set of ciliated neurons that sense oxygen concentration in the body cavity of the worm, the pseudocoelom (Gray et al., 2004). Because *daf-10* mutations affects ciliated sensory neurons (Perkins et al., 1986), it is possible that *daf-10* mutations also affect the activity of the AQR, PQR and URX neurons, which are responsible for the effect of *npr-1* mutations on immune responses (Styer et al., 2008). It will be interesting to test whether *gcy-35*, the guanylate cyclase that acts as the oxygen sensor (Gray et al., 2004), suppresses the limited survival phenotype of *daf-10(-)* on *P. aeruginosa*, as it rescues that of *npr-1(-)* animals (Styer et al., 2008). It is also possible that the effect of *daf-10* mutations is distinct, and that multiple sensory cues may be analyzed by worms to modulate their immune response. If some of the cues are olfactory or gustatory in nature, then other sensory mutant strains, which only compromise the main olfactory/gustatory organ of the worm, the amphid (Perkins et al., 1986), might also show compromised survival on *P. aeruginosa.*

Evans et al. (2008) reported that *P. aeruginosa* exposure induces inactivation of DAF-16/FOXO. Indeed, the downregulated genes that match the *P. aeruginosa*-induced genes are also targets of DAF-16/FOXO, which could mean that the overlap is fortuitous. However, this does not explain why *daf-10(-)* animals are more susceptible to killing by *P. aeruginosa*. Also, Evans et al. (2008) reported that the genes whose expression was

affected by *P. aeruginosa* exposure were mainly, though not only, those that are normally induced by DAF-16/FOXO activation. In contrast, we found that a large number of the genes that overlap were downregulated by DAF-16/FOXO activation. Moreover, the genes in our set that were tested in Evans et al. (2008) appeared to be regulated by *P. aeruginosa* independently of *daf-2*/InsR. Shapira et al. (2006) identified another transcription factor, the GATA factor *elt-2*, as being responsible for the expression of immune response genes during a *P. aeruginosa* infection. Interestingly, a possible GATA factor binding site was identified as being overrepresented in the promoters of putative DAF-16/FOXO targets (Murphy et al., 2003; Budovskaya et al., 2008). It is possible that *elt-2*/GATA may control transcription of immune response genes in response to sensory cues. It will be interesting to test whether *elt-2*/GATA levels or localization is altered in sensory mutant animals. A complex interaction between these two transcription factors may explain how different sets of DAF-16/FOXO targets can be regulated independently in situations such as *P. aeruginosa* infection or impairment of the sensory system.

Implications for other organisms

The importance of the neuronal regulation of many basic physiological processes has been fully appreciated only recently. In mammals, hormonal release regulated by the hypothalamus has important role for energy homeostasis, fat storage, immune responses (reviewed in Purves, 2001) and possibly aging (Purves, 2001; Kappeler et al., 2008). For example, recent studies have shown that loss of the IGF-1 receptor specifically in the

brain has profound effects on energy homeostasis and results in increased lifespan by changing the growth hormone axis (Kappeler et al., 2008). Interestingly, the hypothalamus receives direct innervation from many cortical areas devoted to sensory processing (Risold et al., 1997), including the olfactory cortex and the insular cortex, which processes gustatory information, pointing at a clear role for environmental input in the regulation of physiology. Although the *C. elegans* circuitry is much simpler and more reduced than that of mammals, studying the signal relay from the nervous system to peripheral tissues in this simple, genetically tractable organism, may give insights into what pathways may be used in higher organisms to mediate similar signals.

Materials and Methods

Strains

Nematodes were raised under standard laboratory conditions on agar plates containing a lawn of *Escherichia coli* strain OP50, as described previously (Brenner, 1974). "Wild type" was the *C. elegans* strain N2. The mutant and transgenic strains used were as follows: CF1983 *rrf-3(pk1426); daf-10(m79)*, CF2100 *daf-10(m79),* CF2479 *daf-12(rh61rh411)*, CF2555 *osm-5(p813)*, CF3295 *daf-10(m79); daf-12(rh61rh411)*, CF3284 *osm-5(p813) daf-12(rh61rh411)*. All strains were backcrossed to our laboratory N2 strain at least 3 times with the exception of CF1983.

Microarray analysis

To make sure that background differences did not affect our results, prior to doing

microarray analysis we backcrossed *daf-10(-)* animals four times to our N2 strain. We confirmed that *daf-10(-)* animals lived about 30% longer than wild-type animals and that this increase was partially dependent on *daf-16*/FOXO (data not shown). Wild-type (N2) and *daf-10(-)* worms were synchrnonized by arresting at L1 overnight in M9 buffer, then grown at 20ºC collected as young adults. Total RNA was purified using TriZol™ reagent (Invitrogen), and mRNA was purified using Oligotex kit (Qiagen). cDNA was generated, coupled to Cy3/Cy5 dyes and hybridized using standard techniques to single-stranded DNA nucleotide arrays printed in-house. The oligonucleotides were purchased from Illumina® and represented 20,374 unique *C. elegans* genes. Three repeats of a direct comparison between wild-type and *daf-10(-)* animals were carried out. Chips were scanned using a GenePix 4000B scanner, and initial quality check and identification of spots was done using Genepix 6.0 software. Linear normalization was carried out with the Acuity 4.0 software and significance analysis using the Cyber T-test program. Genes with a *p* value lower than 0.02 were considered significant. The significant gene lists were compared to known gene lists using the hypergeometric probability.

Quantitative RT-PCR

Extraction using TriZol™ reagent (Invitrogen), purification using RNeasy kit (Quiagen) and reverse transcription using Proto-Script 1st strand cDNA synthesis kit (NEB) of RNA were performed as described (Taubert et al., 2006). Quantitative RT-PCR was performed using a 7300 Real Time PCR System (Applied Biosystems) and analyzed by the Ct method (Applied Biosystems Prism 7700 Users Bulletin No. 2 http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). mRNA levels of *act-1*,

nhr-23 and/or *ama-1* were used for normalization. Average of at least 2 technical repeats was used for each biological data point for data generation. Primer sequences are available upon request.

Survival analysis

Lifespan analysis was conducted as previously described (Hansen et al., 2005). All assays were carried out at 20ºC in the presence of 75 µM 5'-fluorodeoxyuridine (Sigma). Stata 8.0 statistics package was used to analyze the data, and *p* values were calculated using the log-rank test (Mantel-Cox method).

Pathogen resistance analysis

Assays were carried out as described with minor modifications (Tan et al., 1999). *Pseudomonas aeruginosa* strain PA14 plates were made by seeding 10-20 µl of an overnight culture of PA14 in Terrific broth and incubating at 37ºC overnight. Plates were subsequently kept at room temperature. Worms were grown at 20ºC on OP50, then were placed on PA14 plates and kept at 20ºC. Plates were scored every day, and live animals were scored by assessing movement in response to mechanical stimulation. Animals that crawled onto the sides of the plates were excluded from the analysis. Stata 8.0 statistics package was used to analyze the data, and *p* values were calculated using the log-rank test (Mantel-Cox method).

Dauer assays

Eggs were placed at 27ºC for 2 days, and plates were then scored for presence of dauer larvae. For the RNAi experiment in Figure 3.7, *rrf-3(-); daf-10(-)* mutant eggs were placed on plates seeded with control bacteria containing empty vector, or bacteria

expressing double-stranded RNA against *daf-16*/FOXO or *daf-12*/NHR. The progeny of these animals was transferred to 27ºC as eggs, and scored for the presence of dauer larvae two days later. The experiments were repeated 5 times.

Table 3.1. Genes differentially regulated in *daf-10(-)* mutant versus wild-type animals

A. Genes upregulated in *daf-10* mutants

Gene	Brief description	*P* value	Fold change	qRT-PCR data	DAF-16 target?	DAF-12 target?	Pathogen-repressed?	Tissue expression?
F56A4.3	Glutathione-S-tranferase	8.20E-05	18.42	Not repeated				
Y19D10A.7	EGF receptor, L-domain	5.73E-04	6.44			D, E		Neuronal (enriched, H)
W02D7.8	Unknown	0.003	4.47					Neuronal (expressed, H)
F14D7.7	Unknown	0.003	4.41					
K12G11.3/ *sodh-1*	Alcohol dehydrogenase	0.006	4.09	Up in *osm-5(-)*	A, C		F	Intestinal (expressed, I)
ZC84.3	Cytoplasmic-linker associating protein	0.006	3.64					Neuronal (enriched, H)
Y19D10A.12	Monocarboxylate transporter	0.007	3.55	Up in *daf-10(-)*, down in *osm-5(-)*		E		
ZK105.5	Unknown	0.011	3.26	Not repeated	C			
F53A3.1	Unknown	0.016	3.21					
F45D11.14	Unknown, DUF684	0.015	3.08					
F21C10.11	Unknown	0.016	3.03		C	D		
C09H5.2	Trasporter ATPase	0.016	2.99	Up in *osm-5(-)*				
F53B2.2/ *tsp-4*	Tetraspanin family integral membrane protein	0.018	2.98					
R08E5.4	Unknown	0.020	2.96					

B. Genes downregulated in *daf-10(-)* mutant versus wild-type animals

Gene	Brief description	*P* value	Fold change	qRT-PCR data	DAF-16 target?	DAF-12 target?	Pathogen-induced?	Tissue expression?
T21E8.2/ *pgp-7*	P-glycoprotein, ABC transporter	7.97 E-05	0.08	Down in *daf-10(-)*			G	
C05A9.1/ *pgp-5*	P-glycoprotein, ABC transporter	2.44 E-04	0.10	Down in *daf-10(-)*	B		G	
K01A2.3	Unknown	0.004	0.14					
C07G3.2	Riboflavin synthesis protein	0.003	0.15				G F	Intestinal (enriched, I)
B0024.1/ *col-149*	Collagen	0.001	0.17					
Y49G5A.1	Serine protease inhibitor	0.002	0.18	Down in *daf-10(-), osm-5(-)* Regulated by *daf-12*	B	D	G (repressed)	
W06G6.12	Unknown	0.004	0.20					
T21E8.1/ *pgp-6*	P-glycoprotein, ABC transporter	0.002	0.21				G	Neuronal (expressed, H)
C32H11.12 / *dod-24*	Unknown, DUF141, Age	0.002	0.21	Down in *daf-10(-), osm-5(-)* Regulated by *daf-12*	A,B	D	G F	
T24C4.4	Unknown, DUF1164	0.003	0.23	Down in *daf-10(-)*	B,C		G F	Intestinal (expressed, I)
C32H11.4	Unknown, DUF141	0.003	0.25	Down in *daf-10(-), osm-5(-)* Regulated by *daf-12*	A,B, C	D	G F	
C10H11.6/ *ugt-26*	UDP-glucuronoyl transferase	0.005	0.25		B		G (only 8 hours)	

C03H5.1/ *clec-10*	C-type lectin	0.004	0.26		B		G (repressed) F (repressed)	Intestinal (expressed, I)
F55G11.2	Unknown, DUF141	0.004	0.26	Down in *daf-10(-), osm-5(-)* Regulated by *daf-12*	B,C	D	G F	
F38A5.9/ *nspb-5*	Unknown, worm specific, DUF1459	0.018	0.27					
K10D11.1/ *dod-17*	Unknown, DUF141, Age	0.007	0.27	Down in *daf-10(-), osm-5(-)* Regulated by *daf-12*	A,B, C	D	G F	Intestinal (expressed, I)
T20D4.3	Unknown, worm specific, DUF750	0.009	0.28				G (repressed)	
C17H12.6	Unknown, DUF141	0.007	0.29	Down in *daf-10(-), osm-5(-), daf-12-* dependent	B	D	G F	Intestinal (enriched, I)
Y22D7AR.10	Histone acetyltransferase	0.006	0.29					
T20D4.5	Peptide N-glycanase, DUF750	0.008	0.30					
F42A9.6	Unknown, possible function in germline	0.006	0.30					Commonly expressed in germline, muscle and intestine (I) Neuronal (expressed, H)
Y53F4B.11	Unknown	0.011	0.31					
T02E1.7	SURF4 family	0.015	0.31					
Y45F10C.2	Unknown, DUF1505	0.007	0.31					
F35E12.5	Unknown, DUF141	0.008	0.31		A,B, C	D	G (only 8 hours)	
Y69A2AR.25	Unknown	0.011	0.32	Up in *osm-5*		D		
C29F9.14	PROBLEM	0.009	0.32					
ZK6.11	Unknown, DUF274, regulates fat accumulation	0.011	0.32	Down in *daf-10(-)*	A,B, C		G	Intestinal (enriched, I)

F19B2.5	Helicase-like transcription factor	0.013	0.33				F, G	Intestinal (expressed, I)
C29F9.3	Unknown	0.010	0.33					Intestinal (enriched, I)
F55G11.3	Pseudogene	0.014	0.34	Down in *daf-10(-), osm-5(-)* Regulated by *daf-12*	B	D	G	
F31F4.15/ *fbxa-27*	F-box protein	0.017	0.34	Down in *daf-10(-), osm-5(-)* Regulated by *daf-12*	B	D	G (repressed)	Intestinal (enriched, I)
C08F11.12	Unknown, DUF1505	0.015	0.35					
K11H12.4	Unknown, DUF274, predicted GPI-anchor	0.017	0.35	Not repeated	B,C		G	
C17H12.8	Unknown, DUF141	0.016	0.37		A		G	Intestinal (enriched, I)
C14C6.5	ShK toxin domain	0.015	0.38				F	Intestinal (expressed, I)
D1025.6/ *nspc-16*	Unknown, worm specific	0.015	0.38					
C33C12.4	Unknown, worm specific, Emb	0.020	0.38					Intestinal (expressed, I)
Y58A7A.3	Zn-finger protein	0.018	0.39		B		G	
T19D12.4	Unknown, Esp	0.017	0.40	Down in *daf-10(-)*	A.B		F, G (8hrs)	Intestinal (enriched, I)
Y69A2AR. 13	Unknown	0.019	0.40					
C29F3.7	Unknown, DUF141, Gro	0.020	0.44		B		F, G	Intestinal (enriched, I)
T24B8.5	ShK toxin domain	0.019	0.56	Down in *daf-10(-), osm-5(-)*	A,B		F	
R06C1.4	RNA binding protein	0.019	0.56		B			Intestinal (expressed, I)
C55B7.4/ *acdh-1*	Acyl CoA dehydrogenase, Esp, Age	0.018	0.56				F, G (both repressed)	Intestinal (expressed, I)

C36C5.5	Worm-specific Cys-rich secreted protein, DUF19	0.019	0.57					
F36D1.4	Unknown	0.019	0.57			D		
C06G3.12	Transposon	0.019	0.57					
Y69E1A.8	Unknown	0.017	0.57					
F25H5.8	Member of UPF0057, stress responsive protein	0.018	0.57					
F38E1.3	Protein kinase	0.020	0.57					
ZK1251.1	Histone protein	0.017	0.57			D		
T22A3.2/ *hsp-12.1*	Member of Hsp20 protein family	0.019	0.57					
K08E7.4	Unknown	0.019	0.57					
K10C2.3	Aspartyl protease	0.020	0.57	Down in *daf-10(-)*		D	F,G	
C54F6.13/ *nhx-3*	Sodium/ protomn exchanger	0.019	0.69			D		

A= Murphy et al., 2003
B= McElwee et al., 2004
C= Lee and Kenyon (unpublished)
D= Fisher and Lithgow, 2006
E= Shostak et al., 2004
F= Shapira et al., 2006
G= Troemel et al., 2006
H= Von Stetina et al., 2007 (Expressed: genes whose expression is detected in the neurons, Enriched: genes that are solely or more highly expressed in neurons)
I= Pauli et al., 2006 (Expressed: genes whose expression is detected in the intestine, Enriched: genes that are solely or more highly expressed in intestine)

Figures

Figure 3.1. *daf-10* and *osm-5* mutations result in the changes in the expression of a common set of genes.

qRT-PCR was used to test the levels of several genes from the microarray results in both *daf-10(-)* and *osm-5(-)* animals. **A.** Genes that were downregulated in both mutants. **B.** Genes that were upregulated in at least one of the two mutants. **C.** Genes that were downregulated in *daf-10(-)* but not *osm-5(-)* animals. **D.** Genes that were not regulated as predicted based on microarray results in either mutant.

Figure 3.1

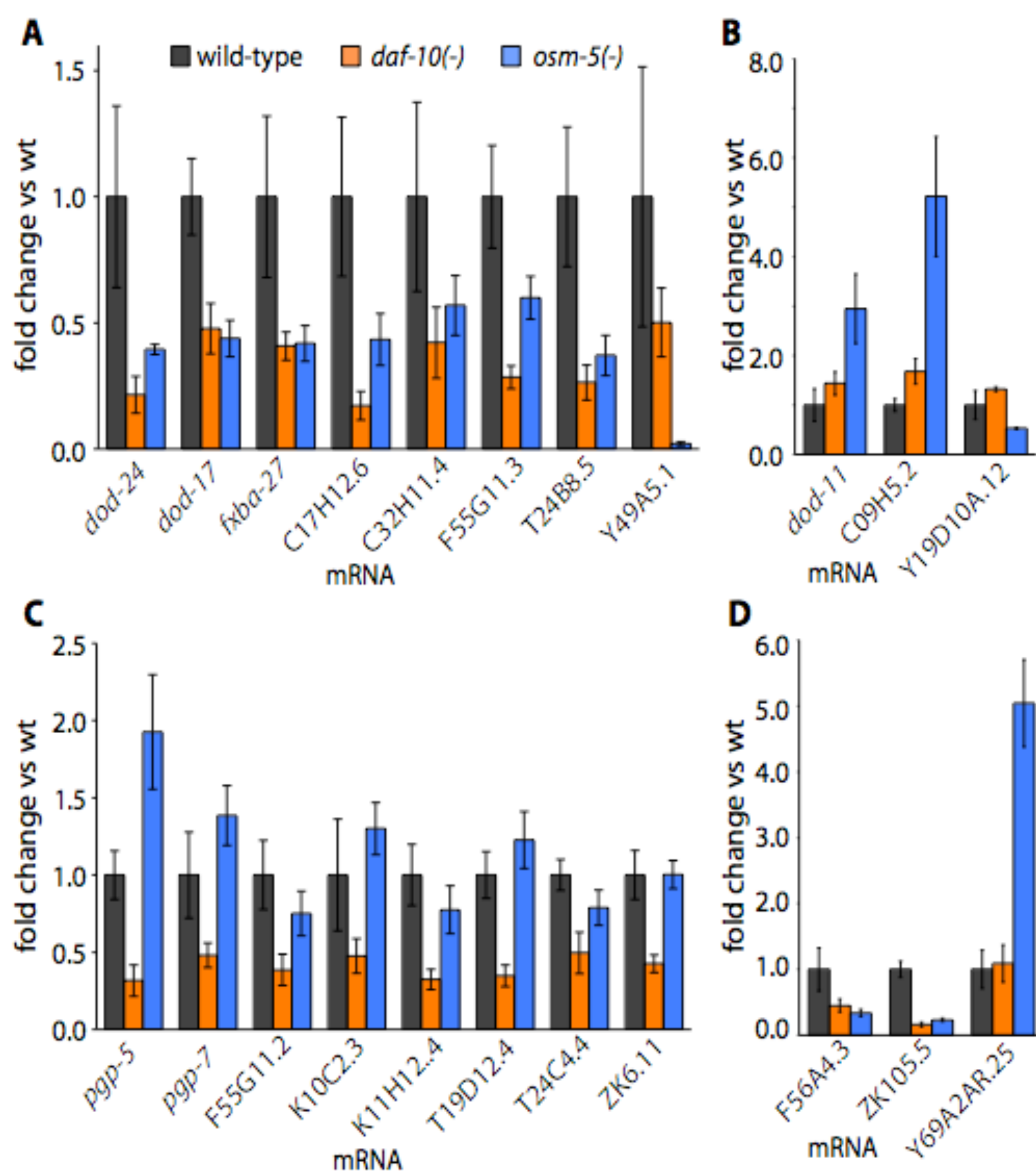


Figure 3.2. DAF-16/FOXO target genes are differentially expressed in the sensory *daf-10* mutants.

Venn diagrams showing overlap between the genes regulated in *daf-10(-)* mutants and the genes regulated by *daf-16*/FOXO in three separate studies (Murphy et al., 2003; McElwee et al., 2004, Lee and Kenyon, unpublished data). All three studies identified genes differentially regulated in *daf-2(-)* vs. *daf-16(-); daf-2(-)* animals. $p < 0.0001$ for all studies (hypergeometric distribution).

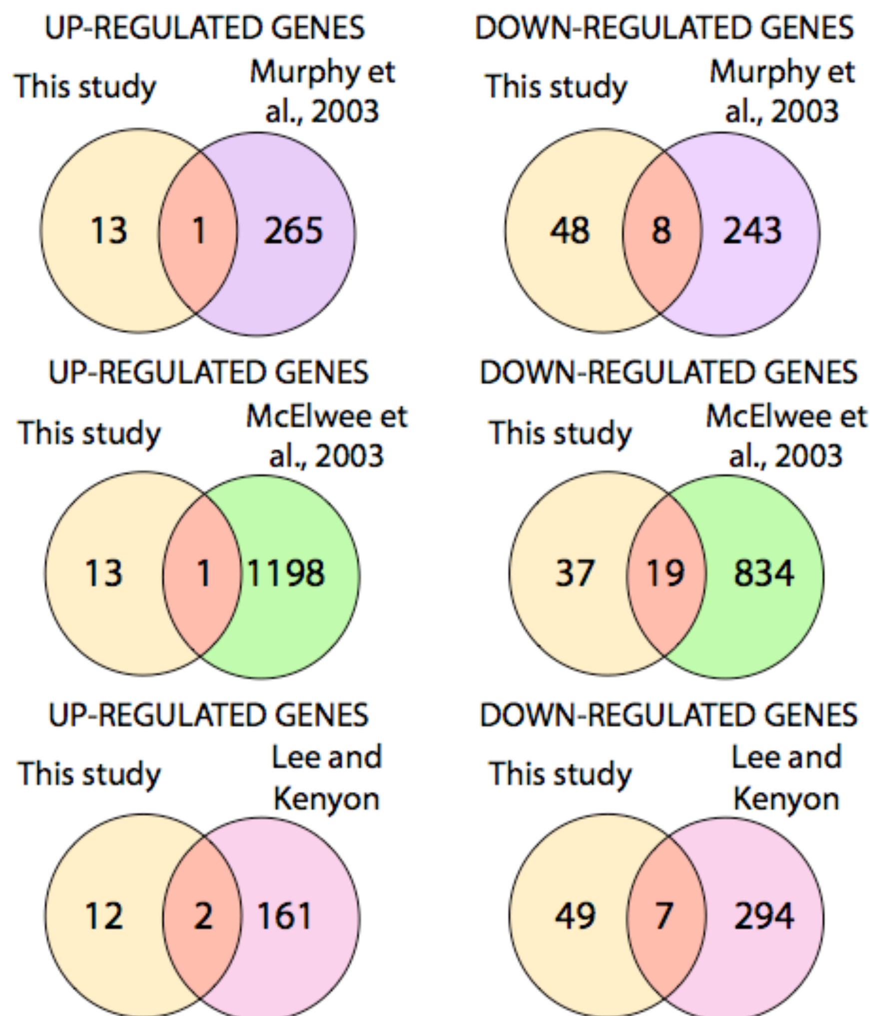


Figure 3.3. The sensory *daf-10* mutations influence the expression of DAF-12/NHR target genes.

A. Venn diagrams showing overlap between the genes regulated in *daf-10(-)* mutants and the genes regulated by *daf-12*/NHR in Fisher and Lithgow (2006). This study compared the gene expression profile of animals carrying gain-of-function *daf-12(rh273)* mutation with that of the null allele mutants *daf-12(rh61rh411)*. $p < 0.001$ (hypergeometric probability). **B.** qRT-PCR was used to confirm that the putative DAF-12-targets were regulated by *daf-12*/NHR in the *daf-10(-)* background. Only K10C2.3 and perhaps *dod-17* were not. (See also Figure 3.6). **C.** *daf-12*/NHR is not required for *daf-10(-)* mutant animals to live long. **B.** *daf-12*/NHR is required for *daf-10(-)* to constitutively arrest at the dauer stage when grown at 27ºC.

Figure 3.3

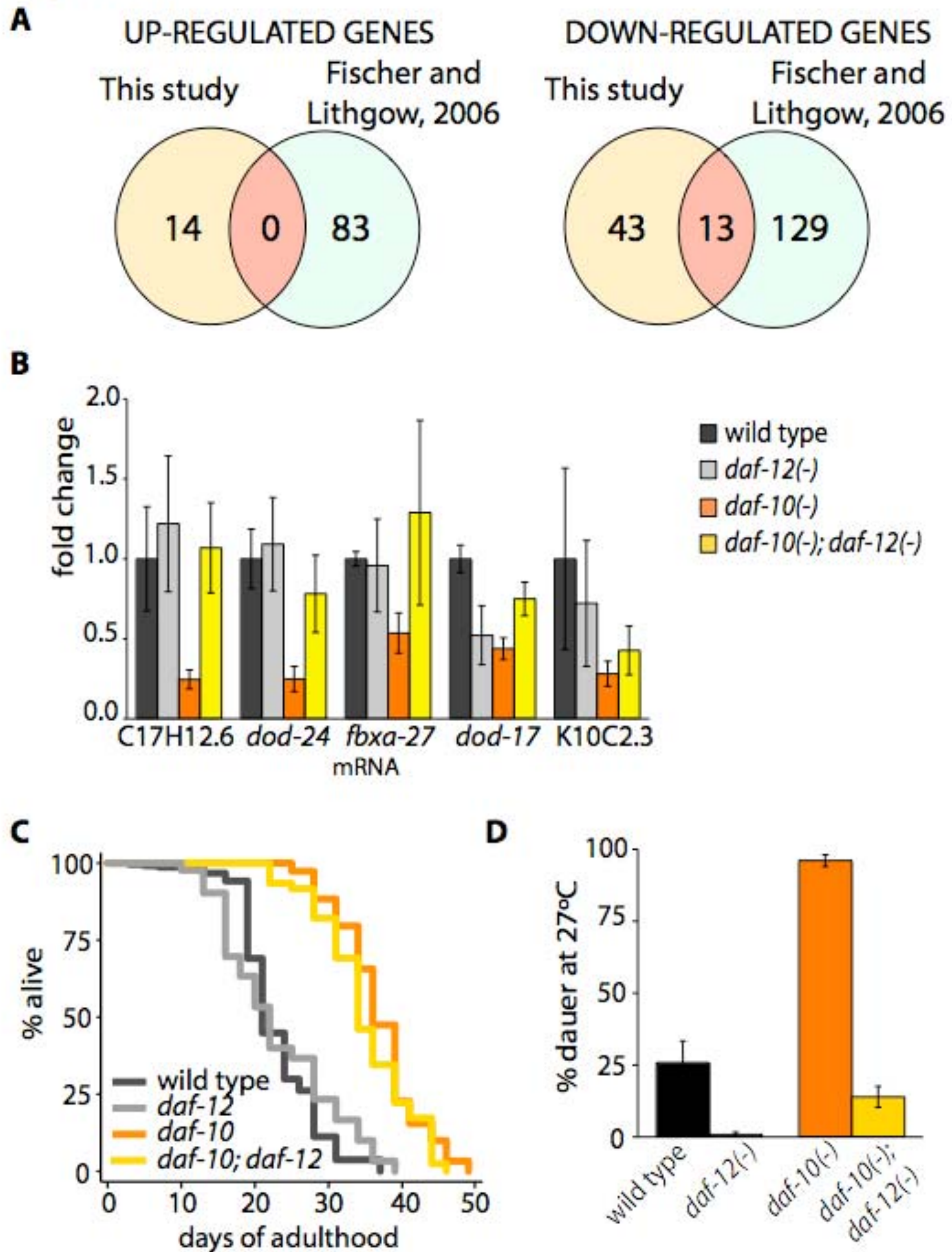


Figure 3.4. Some canonical DAF-16/FOXO target genes are not differentially regulated by *daf-10* mutations.

Two “canonical” targets of DAF-16/FOXO were not upregulated in *daf-10(-)* animals. One of them (*sod-3*) was downregulated in *daf-10(-)* animals. The other (*mtl-1*) is upregulated in *osm-5(-)* animals.

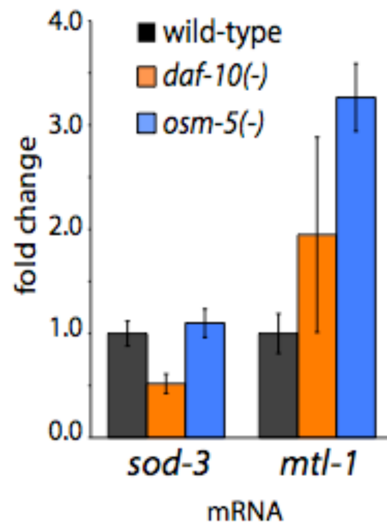


Figure 3.5. ***daf-10*** **mutations compromise the worm's response to pathogens.**

A. Venn diagrams showing overlap between the genes downregulated in *daf-10(-)* mutants and the genes induced by exposure to the pathogenic bacteria *Pseudomonas aeruginosa* in Shapira et al. (2006) and Troemel et al. (2006). $p < 0.001$ (hypergeometric probability). **B.** *daf-10(-)* animals died faster than wild-type animals when fed *P. aeruginosa*. Mean survival ± s.e.m.: wild type = 5.4 ± 0.2 days, *daf-10(-)* = 3.9 ± 0.1 days, $p < 0.0001$. A second trial gave similar results (mean survival ± s.e.m.: wild type = 5.7 ± 0.2 days, *daf-10(-)* = 4.5 ± 0.2 days, $p < 0.0001$).

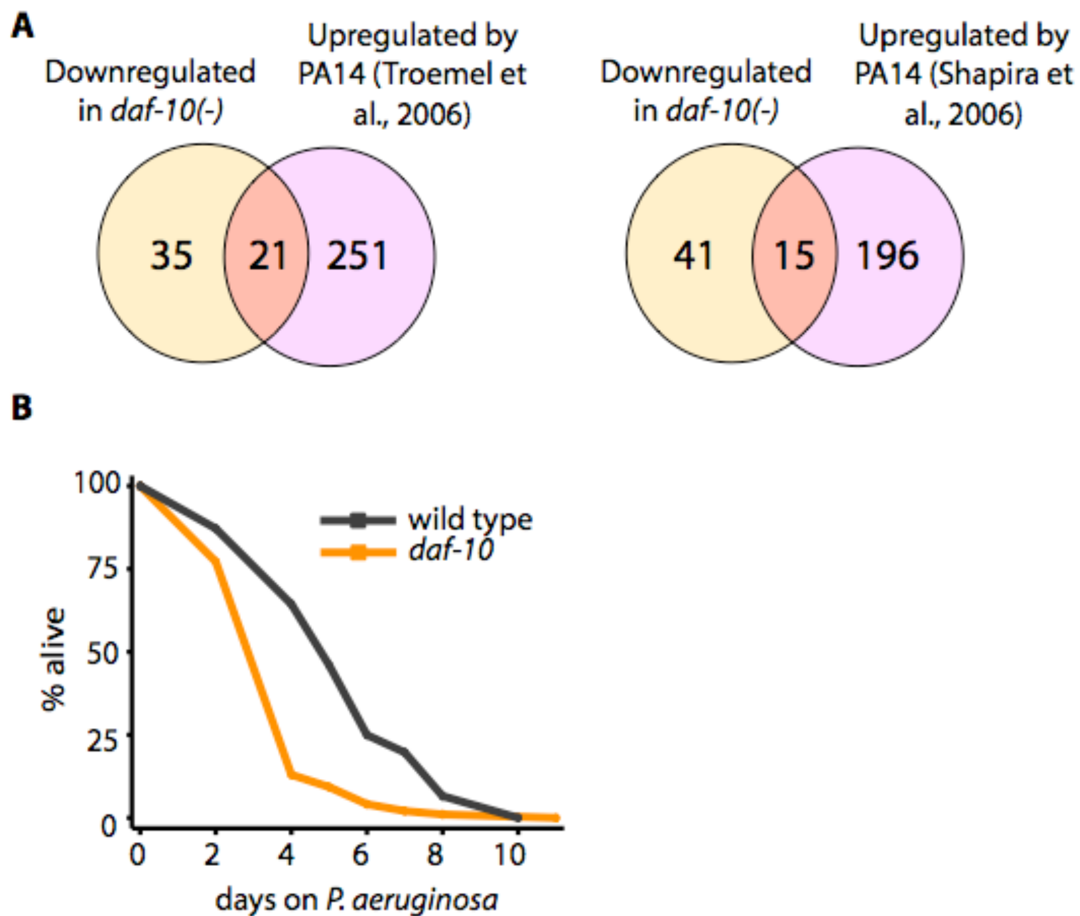


Figure 3.6. The sensory *daf-10* and *osm-5* mutations influence the expression of DAF-12/NHR target genes.

A-B. qRT PCR was used to confirm that expression of putative DAF-12/NHR targets was actually dependent on *daf-12*/NHR in the *daf-10(-)* background (**A**) and in the *osm-5(-)* background (**B**). **C**. *daf-12*/NHR mutations do not suppress the longevity of *osm-5(-)* sensory mutants.

Figure 3.6

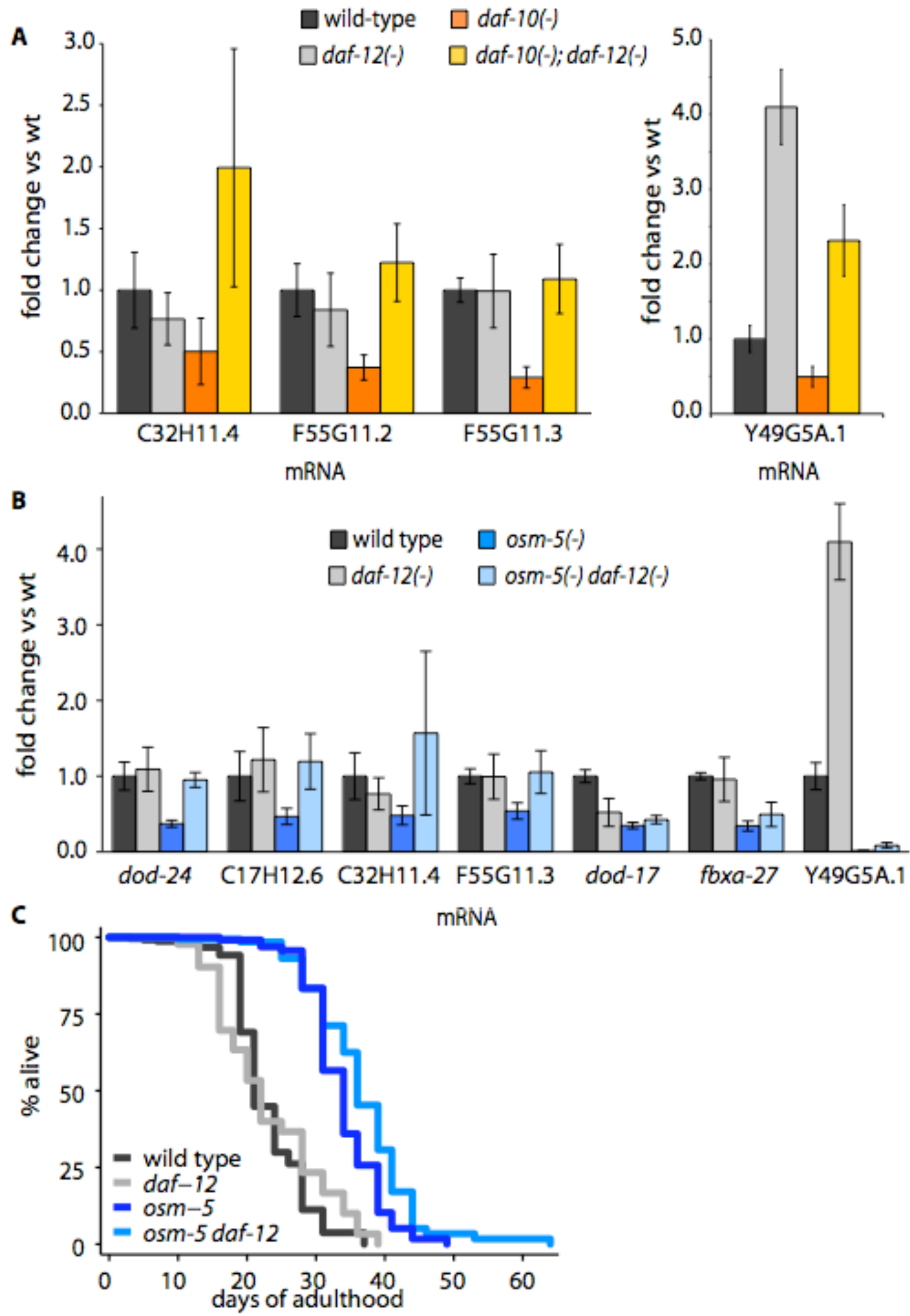
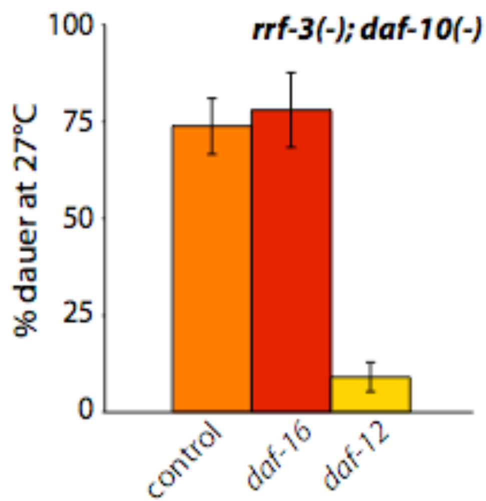


Figure 3.7. RNAi for *daf-12*/NHR, but not for *daf-16*/FOXO, suppresses dauer formation in *rrf-3(-); daf-10(-)* mutant animals.

RNAi-hypersensitive *rrf-3(-); daf-10(-)* double mutant animals were fed control bacteria (control) or bacteria containing double-stranded RNA against *daf-16*/FOXO or *daf-12*/NHR. The percentage of their progeny that arrested in the dauer stage at 27ºC was then tested. RNAi for *daf-16*/FOXO had no effect, whereas RNAi for *daf-12*/NHR suppressed the dauer formation significantly.



Chapter 4:

Effects of neurotransmitter signaling on dauer formation and lifespan in *C. elegans*

Introduction

The nervous system of *C. elegans* is involved in the regulation of whole-organism phenotypes such as fat metabolism (Mukhopadhyay et al., 2005; Mak et al., 2006; Greer et al., 2008), immune responses (Kawli and Tan, 2008; Styer et al., 2008)), dauer formation (Albert et al., 1981; Vowels and Thomas, 1992) and lifespan (Apfeld and Kenyon, 1998, 1999; Wolkow et al., 2000; Bishop and Guarente, 2007), and has been widely used as a model for similar regulation in higher organisms. In the case of lifespan, the nervous system is clearly involved in the lifespan regulation in response to sensory perception in both *C. elegans* (Apfeld and Kenyon, 1999) and *Drosophila melanogaster* (Libert et al., 2007). In addition, reports in both worms (Apfeld and Kenyon, 1998; Wolkow et al., 2000; Libina et al., 2003) and mice (Taguchi et al., 2007; Kappeler et al., 2008) have identified the nervous system as a site of action for lifespan regulation by the insulin/IGF-1 pathway, and in *C. elegans* dietary restriction appear to be regulated by a neuronal circuit involving the amphid neuron ASI (Bishop and Guarente, 2007). Although these data suggest that neurons signal to other tissues to regulate their aging, it

is currently not known what signals they use. Having a better grasp of the signaling events involved in these processes may help in dissecting the circuitry and identifying target tissues.

C. elegans neurons, like those of other organisms, signal through a variety of neuropeptides and neurotransmitters (Jorgensen, 2005; Chase and Koelle, 2007; Rand, 2007; Li and Kim, 2008). Most major neurotransmitters present in mammals are also used by worms, with the exception of noradrenaline. In addition, the invertebrate neurotransmitters tyramine and octopamine are also used; octopamine is considered the invertebrate equivalent of noradrenaline. Worms use acetylcholine and GABA to directly control excitation of the muscle, and mutants in the acetylcholinergic and GABAergic system have severely impaired locomotion [reviewed in (Jorgensen, 2005; Rand, 2007)]. In contrast, mutations that abolish dopamine (Lints and Emmons, 1999; Hills et al., 2004), serotonin (Sze et al., 2000), tyramine and/or octopamine (Alkema et al., 2005) have less obvious phenotypes, suggesting that these neurotransmitters have a modulatory role. Indeed, many of them have been shown to relay information on environmental cues such as food availability to the locomotory system, the pharyngeal pumping circuit or the egg laying circuit [reviewed in Chase and Koelle (2007)].

The components of neurotransmitter signaling systems are conserved from *C. elegans* to mammals. In *C. elegans*, receptors for various neurotransmitters have been identified either through functional genetic screening (e.g. the serotonin receptor (5HTR) *mod-1* (Ranganathan et al., 2000)) or through sequence homology (e.g. the dopamine receptor (DR) *dop-1* (Chase et al., 2004)). Functional roles have been described for some

but not all of the receptors found by sequence homology. Conserved synthesis enzymes are used to make the neurotransmitters and, at least in the case of dopamine and serotonin, conserved re-uptake transporters take up the neurotransmitters from the extracellular medium to terminate the signal and recycle the molecules [reviewed in Chase and Koelle (2007)].

When we carried out these studies, none of the neurotransmitters had been characterized for their role on lifespan. We therefore examined the effect of the "modulatory" neurotransmitters dopamine, serotonin, tyramine and octopamine on lifespan by examining the effects of mutations that specifically affect one of these systems. We also tested potential genetic interactions between the neurotransmitter systems and the insulin/IGF-1 pathway. Specifically, we chose to examine mutations in two key genes in the pathway: the insulin/IGF-1 receptor (InsR) homolog *daf-2* and the FOXO transcription factor *daf-16*. *daf-2*/InsR mutants are long-lived, but require *daf-16*/FOXO for their extended lifespan (Kenyon et al., 1993). In addition, the insulin/IGF-1 pathway also controls the process of arrest in the dauer stage, an alternative larval stage the worm develops into when conditions are unfavourable (Riddle et al., 1981; Gottlieb and Ruvkun, 1994; Larsen et al., 1995). Because this process is also extensively regulated by the nervous system, we also tested the effect of neurotransmitter signaling changes on *daf-2*/InsR-dependent dauer formation. We found that both dopamine and serotonin signaling had an interaction with the *daf-2*/InsR signaling pathway. Dopamine had significant effects on dauer formation and exit, and potentially some effects on lifespan too. Loss of serotonin appeared in several assays to have additive effects to loss

of insulin/IGF-1-like signaling, which is consistent with recent reports of serotonin inactivating DAF-16/FOXO (Liang et al., 2006).

In the case of tyramine and octopamine, we tested the interaction with a different pathway, the germline pathway. Loss of germ-line stem cells by laser ablation or genetic mutation of genes such as *glp-1* causes lifespan extension in worms (Hsin and Kenyon, 1999; Arantes-Oliveira et al., 2002). Studies suggest that there is a signal coming from the reproductive system to other tissues for the lifespan extension to be achieved, but the nature of this signal is currently unknown. We noticed that the enzymes required to synthesize tyramine and octopamine are expressed in uterine and gonadal sheath cells (Alkema et al., 2005) and we thus tested whether these neurotransmitters may be used to modulate lifespan in response to loss of a proliferative germline. We found that there is no specific interaction between tyramine/octopamine and germ cell ablation mediated longevity.

Results

Mutations in dopamine system components have complex effects on the lifespan and dauer formation of *daf-2*/InsR mutant animals

We wanted to investigate how altering the signaling in the dopamine systems in *C. elegans* would contribute to the regulation of lifespan. We initially measured the lifespan of animals carrying dopamine receptor mutations. Prior to outcross, we found that loss of the dopamine receptor (DR) *dop-2* (Chase et al., 2004) resulted in a small decrease in the lifespan of wild-type animals (data not shown). Even though the effect on

otherwise wild-type animals was not seen after backcrossing the strain, two separate alleles of *dop-2*/DR did shorten the lifespan of *daf-2(e1368)* mutants (Figure 4.1A and 4.1C, and Table 4.1), and one of them (*vs105*) shortened the lifespan of animals carrying a stronger loss-of-function allele, *daf-2(e1370)*(Figure 4.1B and Table 4.1). The lifespan shortening effect of *dop-2*/DR mutations was specific to *daf-2*/InsR mutant animals, as mutations in *dop-2* had no effect on the extended lifespan of animals lacking a germline (*glp-1(-)* mutants (Arantes-Oliveira et al., 2002)) (Figure 4.1E and Table 4.1) or dietary-restricted animals (*eat-2(-)* mutant animals (Lakowski and Hekimi, 1998)) (Figure 4.1F and Table 4.1). In addition, we found that *vs105* (but not *tm1062*) mutations in *dop-2*/DR cause a decrease in the percentage of *daf-2(e1370)* animals that arrest in the dauer stage when grown at 22.5ºC. (This temperature is intermediate between 25ºC, where *daf-2(e1370)* animals arrest in dauer very readily, and 20ºC, where *daf-2(e1370)* animals rarely arrest in the dauer stage). These results suggest that the dopamine receptor *dop-2* may play a specific role in the regulation of lifespan and dauer formation controlled by the insulin/IGF-1 signaling pathway. The *dop-2* receptor is expressed in a subset of neurons, which interestingly include the neurons that produce dopamine (Suo et al., 2003). It has therefore been proposed that *dop-2*/DR may act as an autoreceptor, perhaps exerting negative feedback on dopamine release (Suo et al., 2003). To determine whether the effect of *dop-2*/DR mutations is due to reduced dopamine signaling, or increased (de-regulated) dopamine production, we examined the effects of loss or increase of dopamine signaling directly. Loss of the dopamine re-uptake transporter *dat-1* (Jayanthi et al., 1998), which should lead to increase in dopamine signaling, slightly shortened the

lifespan of *daf-2(e1368)*, but had no effect on *daf-2(e1370)* animals (Figure 4.2A and Table 4.2). However, the loss of the tyrosine hydroxylase (TH) homolog *cat-2* (Sulston et al., 1975; Lints and Emmons, 1999), which decreases dopamine production (Sanyal et al., 2004), also slightly shortened the lifespan of both *daf-2*/InsR mutant strains (Figure 4.2B and Table 4.2), although the results were inconsistent between trials (Table 4.2). In addition, complete loss of dopaminergic neurons due to expression of a caspase-like enzyme under a specific promoter (Hills et al., 2004) did not appear to have an effect on the lifespan of either control or *daf-2(e1370)* mutant animals (Figure 4.2C). Together, these data suggest that roles of dopamine signaling in lifespan regulation in *daf-2*/InsR mutants are complex and not directly correlated with the levels of dopamine.

We also tested whether any mutations in the dopamine system would affect entry or exit from the dauer stage. For entry into dauer, we tested *daf-2(e1370)* double mutants at the intermediate temperature 22.5ºC and for dauer exit we tested *daf-2(e1368)* mutant animals at 25ºC. Loss or increase of dopamine had no effect on the ability of *daf-2(e1370)* animals to enter dauer. The same percentage of animals arrested as dauer larvae after 4 days at 22.5ºC (Figure 4.2D). However, loss of the dopamine transporter had a very striking effect on the motility of the dauer larvae, which became the cornerstone of a separate study detailed in chapter 2. In contrast, changes in dopamine levels had profound effects on the dauer exit of *daf-2(e1368)* mutant animals. Normally, *daf-2(e1368)* mutant animals grown at 25ºC form transient dauers within two days of hatching, which resume normal development as soon as a day later. When we scored for the presence of dauer larvae 5 days after hatching, we found that 42% of *daf-2(e1368)*

mutants had exited dauer, whereas 80% of the *daf-2(e1368) dat-1(-)* animals had (Figure 4.2E). Moreover, only 3% of the *cat-2(-); daf-2(e1368)* mutant animals had exited dauer (Figure 4.2E). These results suggest that levels of dopamine positively correlate with increasing exit from the dauer stage, at least as far as *daf-2(e1368)* mutant dauers are concerned.

We then tested mutations in the four known dopamine receptors for their effect on dauer exit in *daf-2(e1368)* mutants. We found that *dop-3*/DR (Chase et al., 2004; Sugiura et al., 2005) and *dop-4*/DR (Sugiura et al., 2005) may have an effect in the process of dauer exit, as 94% of *daf-2(e1368); dop-3(-)* and only 56% of *daf-2(e1368); dop-4(-)* mutant animals were still dauer at day 5 after hatching, compared to 79% of *daf-2(e1368)* in this set of experiments (Figure 4.2F). These results suggest that dopamine may be acting through these two receptors to modulate exit from the dauer stage.

Loss of serotonin potentiates dauer arrest and longevity in *daf-2*/InsR mutant animals

We wanted to investigate how altering the signaling in the serotonin systems in *C. elegans* would contribute to the regulation of lifespan in wild-type animals or in the long-lived *daf-2*/InsR mutant animals.

We found that reduction in serotonin through loss of the tryptophan hydrolase enzyme *tph-1* (Sze et al., 2000) had little or no effect on wild-type lifespan, but had significant and interestingly opposite effects on the lifespan of the two *daf-2*/InsR mutant animals. Loss of serotonin increased the lifespan of the weaker *daf-2(e1368)* mutants but

decreased the lifespan of the stronger *daf-2(e1370)* mutants (Figure 4.3A and Table 4.3). In addition, *tph-1; daf-2(e1370)* animals arrest at the L1 when grown at 25ºC, unlike *daf-2(e1370)* animals which arrest later as dauer larvae (Figure 4.3B). *tph-1; daf-2(e1368)* animals also formed stable dauers at 25ºC, unlike *daf-2(e1368)* which forms transient dauers. At a time (day 5 post-hatching) when *daf-2(e1368)* dauers have started to recover, *tph-1; daf-2(e1368)* animals still arrest at the dauer stage (Figure 4.3B and Table 4.3). These data indicate that reduction in serotonin signaling may lead to a hyperactivation of the insulin/IGF-1 pathway. Consistent with these data, Liang et al. (Liang et al., 2006) showed that loss of serotonin results in activation of the FOXO transcription factor *daf-16*, the major downstream effector of DAF-2/InsR. Thus, *daf-2*/InsR and *tph-1*/tryptophan hydroxylase may have additive effects on *daf-16*/FOXO regulation and mutations in these two genes may result in an increase in all *daf-16*/FOXO-related phenotypes. The reason *tph-1; daf-2(e1370)* animals live shorter may be that really high levels of DAF-16/FOXO activity could be detrimental for the organism.

We also tested whether mutations in serotonin receptors (5HTR) [reviewed in Chase and Koelle (2007)] and the serotonin re-uptake transporter (SERT) *mod-5* (Ranganathan et al., 2001), which lead to increases in serotonin signaling, influence lifespan. Two alleles of *mod-5*/SERT did not result in altered lifespan, nor did mutations in a serotonin-gated chloride channel *mod-1*/5HTR (Table 4.3) (Ranganathan et al., 2000). We found that prior to backcross *ser-1*/5HTR (Carnell et al., 2005; Xiao et al., 2006; Dernovici et al., 2007) and *ser-4*/5HTR mutant animals (Tsalik et al., 2003;

Dempsey et al., 2005), which carry mutations in two G-protein-coupled serotonin receptors, lived long (Table 4.3). However, neither strain lived longer after backcross (Figure 4.3C and Table 4.3). Together, these data suggest that these serotonin receptors may have no direct roles or redundant roles in the lifespan regulation.

Tyramine and octopamine do not regulate longevity in worms without a proliferating germline

Lastly, we also examined the tyramine and octopamine system in *C. elegans* by using loss-of-function mutants in the enzymes required for their synthesis. *tdc-1(-)* mutants lack tyrosine decarboxylase, which is required to make tyramine from tyrosine, and *tbh-1(-)* mutants lack tyramine beta-hydroxylase, which is required to make octopamine from tyramine (Alkema et al., 2005). Because tyramine is a precursor of octopamine, *tdc-1(-)* mutants lack both neurotransmitters, whereas *tbh-1(-)* animals lack only octopamine. Previous studies have focused on the behavioral roles of tyramine and octopamine (Alkema et al., 2005; Wragg et al., 2007) as well as their role in fat metabolism (Greer et al., 2008). However, we were intrigued to notice that the enzymes are expressed not only in the nervous system, but also in the somatic gonad (Alkema et al., 2005). The presence of the somatic gonad is required for loss of germline stem cells to result in extended longevity (Hsin and Kenyon, 1999), suggesting that a signal is generated from the somatic gonad to affect longevity. Therefore, we tested the effects of loss of tyramine and octopamine on the longevity of otherwise wild-type animals and germcell-deficient *glp-1(-)* mutant animals. We found that *tdc-1(-)* animals were short-

lived in two out of two trials, and *tbh-1(-)* animals were also short-lived in one out of two trials (Figure 4.4 and Table 4). These data suggest that the loss of tyramine and maybe also octopamine lead to either sickness or progeria in worms. *tdc-1* mutations also consistently shortened the lifespan of long-lived germcell-less *glp-1(-)* animals, but *tdc-1(-); glp-1(-)* animals still lived longer than *tdc-1(-)* mutants (Figure 4.4), suggesting that tyramine is not required for germ-cell loss to extend lifespan.

Discussion

In this study we attempted to examine the role of neurotransmitters in control of lifespan, both in normal animals and in long-lived mutant animals. At the time of this study, there were no reports of neurotransmitters' role in lifespan regulation. However, studies on the tissue-specific activity of *daf-2*/InsR (Apfeld and Kenyon, 1998; Wolkow et al., 2000; Libina et al., 2003), on sensory perception (Apfeld and Kenyon, 1999) and on dietary restriction (Bishop and Guarente, 2007) have implicated the nervous system in the regulation of lifespan, which suggested that some neurotransmitters might be involved too. We tested four neurotransmitter systems that are not required for the basic circuitry of locomotion or pumping, but rather modulate these processes in response to environmental changes, such as food withdrawal. We found that the dopamine and serotonin systems interact with the insulin/IGF-1 pathway to regulate longevity and dauer formation.

Signaling through *dop-2*/DR interacts with insulin/IGF-1 signaling to regulate

longevity and dauer formation

Mutations in a dopamine receptor, *dop-2*, shortened the lifespan of *daf-2*/InsR mutant animals, and decreased dauer formation. The effect appeared to be specific to the insulin/IGF-1 pathway because *dop-2* mutations did not affect the extended lifespan due to loss of germ cell proliferation or dietary restriction. We were intrigued by this finding, because a receptor of the same family, the D2-like dopamine receptor family, in mammals, regulates the activity of the protein kinase Akt, which is also a downstream effector of the insulin/IGF-1 pathway (Beaulieu et al., 2007). Because of its expression in the dopamine-producing neurons (Suo et al., 2003), DOP-2 has been proposed to provide feedback to the dopamine-producing cells and modulate or shut down dopamine release. However, we could not recapitulate the effects of *dop-2*/DR mutations by decreasing or increasing dopamine production, and analysis of a second allele of the *dop-2* receptor did not recapitulate all our findings. Therefore, it is possible that dopamine has a role in lifespan regulation, but at this time we are unable to clarify how this may work.

Dopamine increase may promote dauer exit

Loss of dopamine and increase in dopamine signaling (but not *dop-2*/DR mutations) did have a striking effect on dauer recovery. The percentage of *daf-2(e1368)* animals that spontaneously recovered from dauer was shifted in mutants with more or less dopamine. Increasing amounts of dopamine appears to promote recovery from the dauer stage. Preliminary analysis of the mutants of the four known dopamine receptors

suggests that *dop-3* and *dop-4* may be involved in the signaling. The *dop-3* receptor has previously been shown to work in the basal slowing response (Chase et al., 2004), a change in locomotory speed in the presence of food (Sawin et al., 2000). In this context, like in the dauer recovery of *daf-2(e1368)* animals, *dop-3*/DR mutations have a similar effect to loss of *cat-2*/TH (Chase et al., 2004). Based on these results, DOP-3/DR is postulated to detect dopamine release in response to mechanical contact with a potential food source, and affect the activity of motor neurons, thus changing the speed of locomotion. The presence of food is also one of the two main signals thought to regulate entry into and exit from the dauer stage, the other being dauer pheromone, which provides information regarding population density (Golden and Riddle, 1984). Therefore, these data indicate that dopamine signaling may work in the food-sensing pathway required to regulate exit from the dauer stage.

Serotonin and insulin/IGF-1 signaling synergistically regulate lifespan and dauer formation

We found that mutations in the synthesis enzyme for serotonin, *tph-1* (Sze et al., 2000), had additive effect with *daf-2*/InsR mutations. They increased lifespan and dauer arrest in *daf-2(e1368)* animals and caused an even earlier arrest, at the L1 larval stage, in *daf-2(e1370)* animals. Some strong alleles of *daf-2*/InsR have been previously reported to cause L1 larval arrest (Gems et al., 1998). Surprisingly, *tph-1* mutations shortened the lifespan of *daf-2(e1370)* animals. However, it is possible that excessive activation of DAF-16/FOXO may have deleterious effects. We were not able to identify a receptor

that would explain the effect of serotonin.

As mentioned above, our results are in keeping with a study by Liang et al. (Liang et al., 2006), where the authors show that a decrease in serotonin leads to DAF-16/FOXO nuclear localization and activation. Perhaps serotonin signaling and *daf-2*/InsR activity act in an additive or synergistic manner at the level of DAF-16/FOXO activation. Interestingly, a separate study has also shown that DAF-2/InsR regulates the expression of *tph-1*/tryptophan hydroxylase (Estevez et al., 2006), and we (Chapter 2) and others (McElwee et al., 2004) found that *bas-1*, an aromatic amino acid decarboxylase required for dopamine and serotonin synthesis is also transcriptionally regulated by DAF-2/InsR. While this study was in progress, two studies reported effects for serotonin signaling in lifespan extension in *C. elegans*. Murakami and Murakami (2007) reported that *ser-1(-)* animals are long-lived and that their lifespan extension is reduced by *daf-16*/FOXO and *mod-5*/SERT mutations, and is not additive with the effect of *daf-2*/InsR knockdown by RNA interference. Petrascheck et al. (2007) reported that mianserin, an antidepressant that acts as an antagonist of serotonin receptors, could extend lifespan in a manner dependent on the serotonin receptor *ser-4*, the octopamine receptor *ser-3*, *tph-1*/tryptophan hydroxylase and *mod-5*/SERT. This lifespan extension was also abrogated in the dietary-restricted *eat-2* mutants, suggesting a relationship between serotonin signaling and dietary restriction. In this study, none of the mutations that affected serotonin signaling, including *ser-1*/5HTR mutations, caused lifespan extension on their own. The discrepancies among the three results may be caused by unappreciated environmental factors. The study by Murakami and Murakami (2007) measured lifespan

on plates, like this study, but at 25ºC instead of 20ºC, whereas the study by Petrascheck et al. (2007) was carried out in liquid culture at 20ºC. Temperature is an important cue regulating lifespan. Worms live longer when grown at lower temperature (e.g. 15ºC) and shorter at higher temperatures (e.g. 25ºC) (Klass, 1977). In addition, a study by Lee and Kenyon (in preparation) suggests that temperature-sensation has a role in modulating lifespan at different temperatures. It is possible that serotonin signaling has a differential effect on lifespan at different temperatures.

Dopamine and serotonin signals could regulate release of insulin-like ligands or downstream components of the insulin/IGF-1 signaling pathway

Both of the mutations in *daf-2*/InsR we tested are reduction-of-function, rather than loss-of-function mutations, as loss-of-function mutations in *daf-2*/InsR cause constitutive and permanent arrest in the dauer stage at all temperatures (Gems et al., 1998). Therefore, we cannot determine from our data whether dopamine and serotonin signaling modulate the *daf-2*/InsR mutant phenotypes by regulating events upstream or downstream of *daf-2*/InsR. For example, dopamine and serotonin signaling could modulate release of a DAF-2/InsR ligand or alternatively they could independently regulate downstream effectors of the insulin/IGF-1 pathway such as DAF-16/FOXO. This latter mechanism has been proposed for serotonin signaling (Liang et al., 2006). In the case of dopamine, it is also possible that insulin/IGF-1 signaling may modulate dopamine signaling, as we propose in chapter 2.

Conclusions

Neurotransmitter signals in mammals are used to coordinate a number of physiological and behavioral responses to changing environmental conditions. It is possible that ageing may also be regulated in a similar manner, and in fact age-related changes in serotonin signals are risk factors for some age-related diseases (Mattson et al., 2004). In flies, quantitative trait locus mapping has uncovered a link between longevity variation and dopa decarboxylase, an enzyme required for dopamine and serotonin synthesis (De Luca et al., 2003). However, because of the pervasive effects of complete loss of neurotransmitters in higher animals, it is hard to separate their effects on other processes and aging. *C. elegans* may be a good model to study this link, because many nervous system mutants are viable and have little obvious phenotypes. We have found a potential link between the dopamine and serotonin systems and the insulin/IGF-1 pathway in *C. elegans.* Further studies are necessary to better tease apart what the nature and functional relevance of this interaction is. Also, we did not test whether these pathways may be involved in the regulation of lifespan by the sensory system. Because the sensory system and the insulin/IGF-1 pathway may be related (Apfeld and Kenyon, 1999), this could explain the link between neurotransmitters and the insulin/IGF-1 pathway.

Materials and Methods

Strains

Nematodes were raised under standard laboratory conditions on agar plates

containing a lawn of *Escherichia coli* strain OP50, as described previously (Brenner, 1974). "Wild type" was the *C. elegans* strain N2. The mutant and transgenic strains used were as follows: CF1037 *daf-16(mu86)*, CF1041 *daf-2(e1370),* CF1903 *glp-1(e2141),* CF1908 *eat-2(ad1116)*, CF2264 *daf-2(e1370); dop-2(vs105)*, CF2285 *eat-2(ad1116); dop-2(vs105)*, CF2314 *dop-2(vs105)*, CF2351 *daf-2(e1368); dop-2(vs105)*, CF2365 *ser-1(ok345)*, CF2445 *daf-16(mu86); ser-1(ok345)*, CF2452 *ser-4(ok512)*, CF2454 *glp-1(e2141); dop-2(vs105)* CF2456 *daf-16(mu86); ser-4(ok512),* CF2470 *dat-1(ok157)*, CF2532 *daf-2(e1370) dat-1(ok157)*, CF2536 *daf-2(e1368) dat-1(ok157),* CF2555 *daf-2(e1370); ser-1(ok345)*, CF2563 *dop-2(tm1062)*, CF2564 *tph-1(mg280)*, CF2568 *tph-1(mg280); daf-2(e1368),* CF2590 *cat-2(e1112); daf-2(e1368),* CF2591 *cat-2(e1112); daf-2(e1370),* CF2592 *tph-1(mg280); daf-2(e1370),* CF2593 *cat-2(e1112),* CF2619 *daf-2(e1370); dop-2(tm1062)* CF2620 *daf-2(e1368); dop-2(tm1062),* CF2625 *daf-2(e1370); lin-15(n765ts); akEx248[pdat-1::GFP, lin-15(+)],* CF2626 *daf-2(e1370); lin-15(n765ts); akEx387[pdat-1::GFP, pdat-1::hICE, lin-15(+)],* CF2645 *daf-2(e1368); dop-3(vs106)*, CF2689 *daf-2(e1368); dop-1(vs100)*, CF2724 *daf-2(e1368); dop-4(ok1321)*, CF3231 *tdc-1(ok914)*, CF3233 *tdc-1(ok914); glp-1(e2141)* CF3234 *tbh-1(ok1196),* CF3236 *glp-1(e2141); tbh-1(ok1196)*, DA1814 *ser-1(ok345)*, DR1572 *daf-2(e1368)*, GR1321 *tph-1(mg280)*, LX702 *dop-2(vs105)*, MT8944 *mod-5(n822)*, MT9668 *mod-1(ok103)*, MT9972 *mod-5(n3314)*, RB745 *ser-4(ok512).*

Survival analysis

Lifespan analysis was conducted as previously described (Hansen et al., 2005).

All assays were carried out at 20ºC. For assays involving *glp-1(-)* mutant animals, worms were transferred to 25ºC approximately 16 hours after hatching and then back to 20ºC at the young adult stage. Because the *glp-1(e2141)* mutation is temperature sensitive, this should ensure correct development of the worms, but prevent formation of proliferating germ-line stem cells, thus causing lifespan extension. In some assays, 75µM 5'-fluorodeoxyuridine was used to prevent the development of progeny. Stata 8.0 statistics package was used to analyze the data, and *p* values were calculated using the logrank test (Mantel-Cox method).

Dauer assays

Assays for dauer entry were carried out using *daf-2(e1370)* single and double mutants grown at 22.5ºC. Animals were scored four days after hatching for the presence of dauer larvae.

Assays for dauer exit were carried out using *daf-2(e1368)* single and double mutants grown at 25ºC. At this temperature *daf-2(e1368)* single mutant animals constitutively enter the dauer stage, but start exiting it spontaneously as soon as a day later. Animals were scored five days after hatching for the presence of dauer larvae and recovered adults.

Figures

Figure 4.1. Mutations in the dopamine receptor *dop-2* specifically shorten lifespan and reduce dauer formation of *daf-2*/InsR mutants.

A-C. *vs105* mutations that delete part of the dopamine receptor *dop-2* shorten the lifespan of *daf-2(e1368)* (**A**) and *daf-2(e1370)* (**B**) animals, but *tm1062* mutations in the same receptor only shorten the lifespan of *daf-2(e1368)* animals (**C**). **D.** *vs105* but not *tm1062* mutations reduce the percentage of *daf-2(e1370)* animals that arrests as dauer larvae at 22.5ºC. All strains are *daf-2(e1370)* (Percentage dauer, mean ± s.e.m.: *daf-2(e1370)* = 63.3% ± 7.6%, *daf-2(e1370); dop-2(vs105)* = 26.5% ± 6.1%, $p < 0.01$, *daf-2(e1370); dop-2(tm1062)* = 75.0% ± 11.8%, $p = 0.5$). **E-F.** *dop-2(vs105)* mutations do not shorten the lifespan of *glp-1* animals (**E**), which live long due to lack of germ cells, or *eat-2(-)* animals (**F**), which are a genetic mimic of dietary restriction.

For mean lifespans and *p* values, refer to Table 4.1 (shaded cells represent data plotted in the figure).

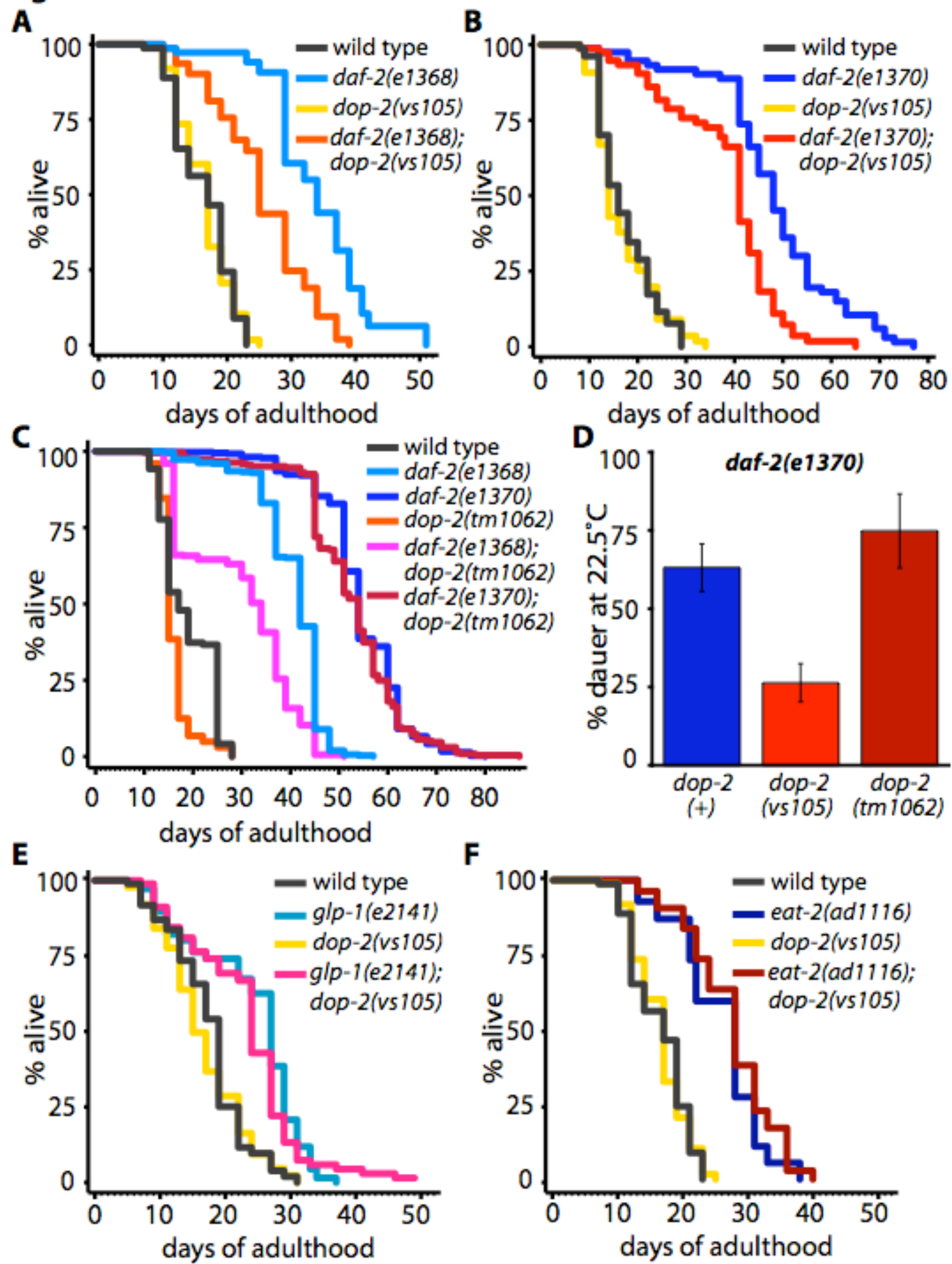


Figure 4.1
A
wild type
daf-2(e1368)
dop-2(vs105)
daf-2(e1368); dop-2(vs105)
% alive
days of adulthood
B
wild type
daf-2(e1370)
dop-2(vs105)
daf-2(e1370); dop-2(vs105)
% alive
days of adulthood
C
wild type
daf-2(e1368)
daf-2(e1370)
dop-2(tm1062)
daf-2(e1368); dop-2(tm1062)
daf-2(e1370); dop-2(tm1062)
% alive
days of adulthood
D
daf-2(e1370)
% dauer at 22.5°C
dop-2 (+)
dop-2 (vs105)
dop-2 (tm1062)
E
wild type
glp-1(e2141)
dop-2(vs105)
glp-1(e2141); dop-2(vs105)
% alive
days of adulthood
F
wild type
eat-2(ad1116)
dop-2(vs105)
eat-2(ad1116); dop-2(vs105)
% alive
days of adulthood

Table 4.1

Repeat	Strain	Mean lifespan	Std. error	% of wild type	% of long-lived mutant
1	Wild type	14.5			
	daf-2(e1370)	44.5		+ 207%	
	dop-2(vs105)	16.2		+ 12% n.s	
	daf-2(e1370); dop-2(vs105)	37.5			- 16% n.s
2	Wild type	17.3			
	daf-2(e1370)	48.2		+ 179% ***	
	dop-2(vs105)	16.7		- 3% n.s	
	daf-2(e1370); dop-2(vs105)	38.3			- 20% ***
3	Wild type	16.3	±0.6		
	daf-2(e1368)	34.1	±1.0	+ 109% ***	
	eat-2(ad1116)	25.8	±1.2	+ 58% ***	
	dop-2(vs105)	16.4	±0.5	+ 1% n.s	
	daf-2(e1368); dop-2(vs105)	25.5	±1.0		- 25% ***
	eat-2(ad1116); dop-2(vs105)	27.4	±1.0		+ 7% n.s.
4	Wild type	16.9	±0.5		
	daf-2(e1368)	33.3	±1.0	+ 97% ***	
	eat-2(ad1116)	26.0	±1.0	+ 54% ***	
	dop-2(vs105)	16.8	±0.6	- 1% n.s	
	daf-2(e1368); dop-2(vs105)	25.5	±1.4		- 23% **
	eat-2(ad1116); dop-2(vs105)	23.8	±1.1		- 8% n.s.
5	Wild type	17.5	±0.7		
	glp-1(e2141)	23.9	±0.8	+ 37% ***	
	dop-2(vs105)	16.6	±0.8	- 5% n.s.	
	glp-1(e2141); dop-2(vs105)	23.3	±0.9		- 2.5% n.s.
6	Wild type	19.8	±0.9		
	glp-1(e2141)	27.2	±1.0	+ 37%***	
	dop-2(vs105)	17.7	±0.8	- 11%*	
	glp-1(e2141); dop-2(vs105)	28.9	±1.1		+ 6% n.s.
7	Wild type	18.6	± 0.7		

	daf-2(e1368)	38.9	± 1.2	+ 109% ***	
	daf-2(e1370)	53.8	± 1.5	+ 189% ***	
	dop-2(tm1062)	17.3	± 0.5	- 7% n.s.	
	daf-2(e1368); dop-2(tm1062)	31.9	± 1.3		- 18% ***
	daf-2(e1370); dop-2(tm1062)	52.5	± 1.7		- 2% n.s.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, n.s. $p > 0.05$

Figure 4.2. Mutations that affect dopamine levels have little effect on the lifespan of *daf-2*/InsR mutant, but may affect their recovery from dauer arrest.

A-C. Mutations in the dopamine transporter *dat-1* (**A**) or in the tyrosine hydroxylase *cat-2* (**B**) have little or no effect on the lifespan of wild-type animals, *daf-2(e1368)* or *daf-2(e1370)* mutants. Complete loss of dopamine (DA) neurons (**C**), obtained by expressing a caspase-like enzyme under the *dat-1* promoter, also has no effect on the lifespan of wild-type or *daf-2(e1370)* animals.

For mean lifespans and *p* values, refer to Table 4.2a (shaded cells represent data plotted in the figure).

D. Mutations in the dopamine transporter *dat-1* or in the tyrosine hydroxylase *cat-2* have no effect on dauer formation in *daf-2(e1370)* mutants at 22.5ºC (Percentage dauer, mean ± s.e.m.: *daf-2(e1370)* = 68.3% ± 4.2%, *daf-2(e1370) dat-1(ok157)* = 48.8% ± 12.0%, p = 0.2, *cat-2(e1112); daf-2(e1370)* = 73.3% ± 6.4%, p =0.5).

E. Mutations in the dopamine transporter *dat-1* or in the tyrosine hydroxylase *cat-2* have opposite effects on dauer recovery in *daf-2(e1368)* mutants at 25ºC. See Table 4.2b for mean values.

F. Mutations in the dopamine receptor *dop-3* and *dop-4* have opposite effects on dauer recovery in *daf-2(e1368)* mutants at 25ºC, whereas mutations in the dopamine receptors *dop-1* and *dop-2* have no effect. See Table 4.2b for mean values.

Figure 4.2

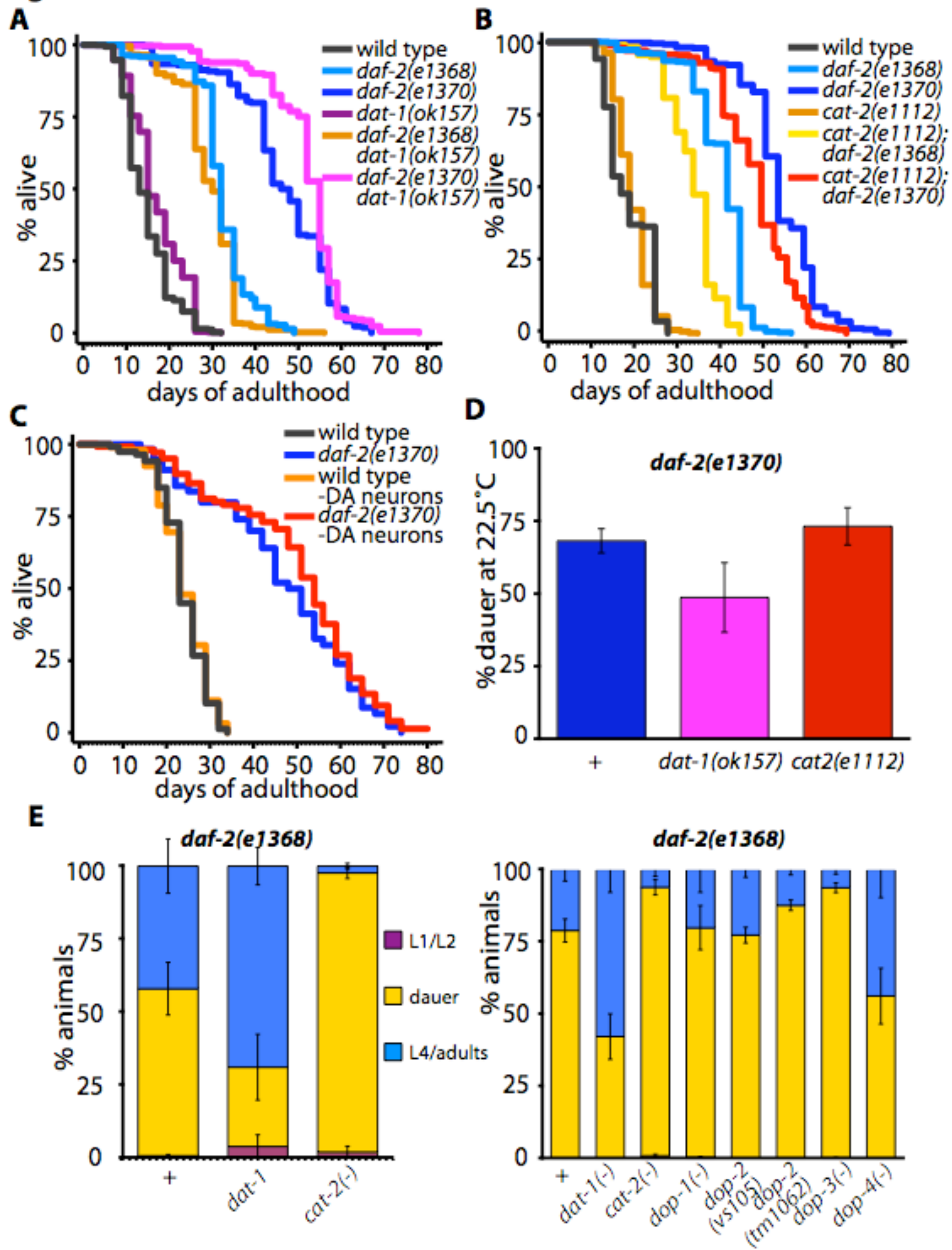


Table 4.2a

Repeat	Strain	Mean lifespan	Std. error	% of wild type	% of *daf-2*
1	Wild type	16.1			
	daf-2(e1368)	32.3	±1.1	+ 119% ***	
	daf-2(e1370)	45.8	±1.6	+ 184% ***	
	dat-1(ok157)	17.3	±0.7	+ 5% n.s	
	daf-2(e1368) dat-1(ok157)	28.6	±1.1		- 10% ***
	daf-2(e1370) dat-1(ok157)	50.9	±1.5		+ 11% n.s.
2	Wild type	23.2	±0.4		
	daf-2(e1368)	30.5	±0.6	+ 31% ***	
	daf-2(e1370)	47.4	±0.7	+ 104% ***	
	cat-2(e1112)	24.5	±0.3	+ 6% n.s	
	cat-2(e1112); daf-2(e1368)	32.3	±0.4		+ 6% *
	cat-2(e1112); daf-2(e1370)	41.1	±1.2		- 13% ***
3	Wild type	18.6	±0.7		
	daf-2(e1368)	38.9	±1.2	+ 109% ***	
	daf-2(e1370)	53.8	±1.5	+ 189% ***	
	cat-2(e1112)	20.4	±0.6	+ 10% n.s.	
	cat-2(e1112); daf-2(e1368)	32.7	±0.9		- 16% ***
	cat-2(e1112); daf-2(e1370)	46.7	±1.4		- 7% **
4	Control	24.0	±0.6		
	daf-2(e1370) control	46.8	±2.2	+ 95%***	
	No dopamine neurons	24.0	±0.5	+ 0% n.s.	
	daf-2(e1370), no dopamine neurons	50.1	±1.7		+ 7% n.s.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, n.s. $p > 0.05$

Table 4.2b

Set	Strain	% L1/L2 (mean ± s.e.m.)	%dauer (mean ± s.e.m.)	% L4/adults (mean ± s.e.m.)
1	*daf-2(e1368)*	0.7% ± 0.4%	57.3% ± 9.0%	42% ± 9.3%
	daf-2(e1368) dat-1(ok157)	4.6% ± 4.6%	31.5% ± 13.1%	79.9% ± 7.4%
	cat-2(e1112); daf-2(e1368)	2.0% ± 2.0%	95.7% ± 1.9%	2.3% ± 1.0%
2	*daf-2(e1368)*	0%	78.9% ± 3.9%	21.1% ± 3.9%
	daf-2(e1368) dat-1(ok157)	0%	42.2% ± 7.8%	57.8% ± 7.8%
	cat-2(e1112); daf-2(e1368)	0.9% ± 0.6%	92.9% ± 2.7%	6.1% ± 2.3%
	daf-2(e1368); dop-1(vs100)	0.3% ± 0.3%	79.5% ± 7.6%	20.1% ± 7.8%
	daf-2(e1368); dop-2(vs105)	0%	77.3% ± 2.7%	22.7% ± 2.7%
	daf-2(e1368); dop-2(tm1062)	0%	87.6% ± 1.8%	12.4% ± 1.8%
	daf-2(e1368); dop-3(vs106)	0.2% ± 0.2%	93.6% ± 1.6%	6.3% ± 1.6%
	daf-2(e1368); dop-4(ok1321)	0%	56.2% ± 9.7%	43.8% ± 9.7%

Figure 4.3. Mutations that impair synthesis of serotonin have an effect on lifespan.

A. Mutations in the tryptophan hydroxylase *tph-1* increase the lifespan of *daf-2(e1368)* mutant animals, but decrease the lifespan of *daf-2(e1370)* animals. **B.** Mutations in *tph-1* also cause L1 arrest in *daf-2(e1370)* mutants at 25ºC (Percentage of animals ± s.e.m.: *daf-2(e1370)*: L1/L2 = 0.9% ± 0.9%, dauer = 98.6% ± 1.2%, L4/adults = 0.6% ± 0.3%; *tph-1(mg280); daf-2(e1370)*: L1/L2 = 45.8% ± 11.4%, dauer = 46.5% ± 8.5%, L4/adults = 1.0% ± 1.0%) and increased stability of dauer formation in *daf-2(e1368)* mutants (Percentage of animals ± s.e.m.: *daf-2(e1368)*: L1/L2= 0.7% ± 0.4%, dauer= 57.3% ± 9.0%, L4/adults= 42.0% ± 9.3%; *tph-1(mg280); daf-2(e1368)*: L1/L2= 17.4% , dauer= 82.3% ± 4.0%, L4/adults= 0.3% ± 0.3%). **C.** Mutations in the serotonin receptor *ser-1* have no effect on the lifespan of wild-type or *daf-2(e1370)* mutant animals.

For mean lifespans and *p* values, refer to Table 4.3 (shaded cells represent data plotted in the figure).

Figure 4.3

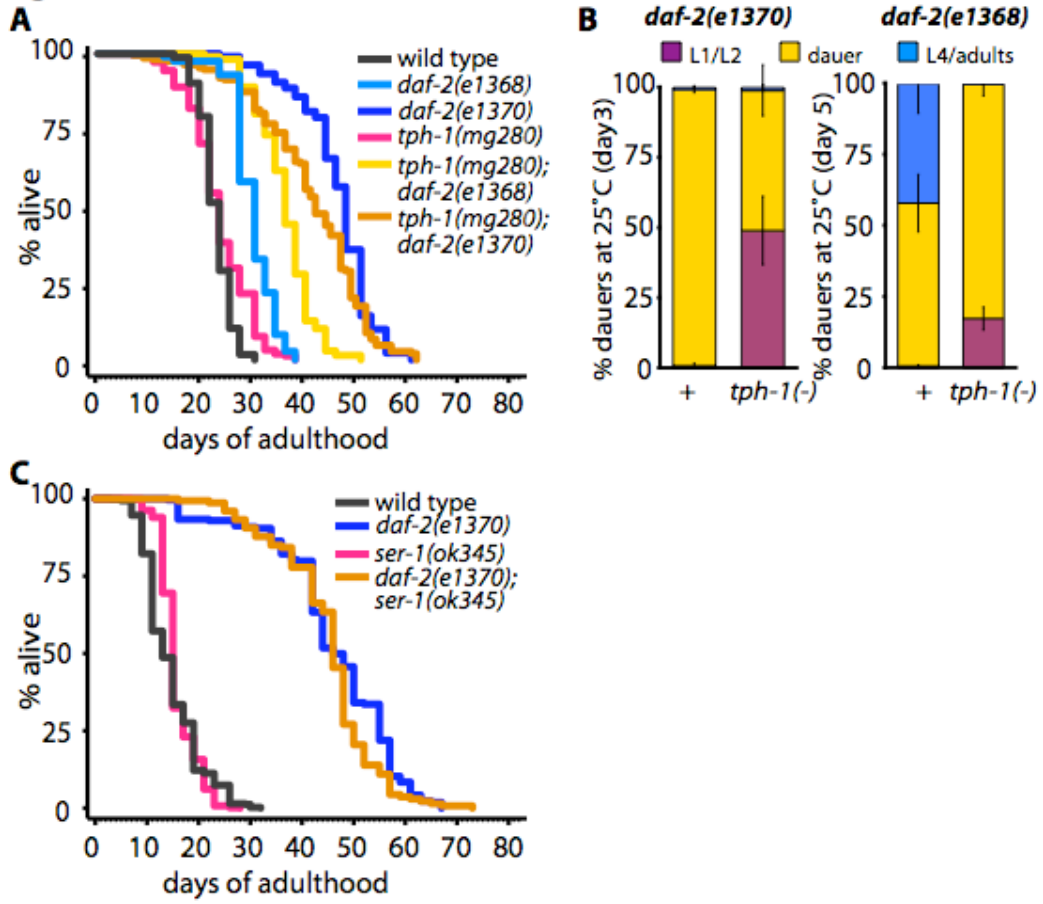


Table 4.3

Repeat	Strain	Mean lifespan	Std. error	% of wild type	% of *daf-2(-)* or *daf-16(-)*
1	Wild type	18.5	± 0.6		
	ser-1(ok345)^	22.8	± 0.5	+ 23% **	
	mod-5(n822)^	18.5	± 1.0	+ 0% n.s.	
	mod-5(n3314)^	18.0	± 0.6	- 3% n.s.	
2	Wild type	13.1	± 0.6		
	ser-4(ok512)^	17.9	± 0.6	+ 36% ***	
	mod-1(ok103)^	14.9	± 0.7	+ 14% *	
	tph-1(mg280)^	15.5	± 0.8	+ 15% *	
3	Wild type	23.2	± 0.4		
	daf-2(e1368)	30.5	± 0.6	+ 31% ***	
	daf-2(e1370)	47.4	± 0.7	+ 104% ***	
	tph-1(mg280)	23.9	± 0.4	+ 3% n.s.	
	tph-1(mg280); daf-2(e1368)	36.9	± 0.6		+ 21% ***
	tph-1(mg280); daf-2(e1370)	42.0	± 0.8		- 11% **
4	Wild type	16.1	± 0.8		
	daf-2(e1370)	45.8	± 1.6	+ 184% ***	
	ser-1(ok345)	16.6	± 0.5	+ 3% n.s.	
	daf-2(e1370); ser-1(ok345)	45.3	± 1.7		- 1% n.s.
5	Wild type	17.3	±0.7		
	daf-16(mu86)	15.0	±0.6	- 13% n.s.	
	ser-1(ok345)	19.8	±0.8	+ 14% **	
	ser-4(ok512)	18.6	±0.8	+ 7%	
	daf-16(mu86); ser-1(ok345)	14.0	±0.6		- 7% n.s.
	daf-16(mu86); ser-4(ok512)	13.7	±0.5		- 9% n.s.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, n.s. $p > 0.05$

^= not outcrossed

Figure 4.4. Tyramine and octopamine are not involved in the lifespan extension of germ cell-less mutant animals. A. Mutations in the enzymes that abolish the synthesis of tyramine and octopamine (*tdc-1(-)*) shorten the lifespan of both wild-type and *glp-1(-)* animals. **B.** Mutations that abolish the synthesis of octopamine alone (*tbh-1(-)*) have little or no effect on the lifespan of either strain.

For mean lifespans and *p* values, refer to Table 4.4 (shaded cells represent data plotted in the figure).

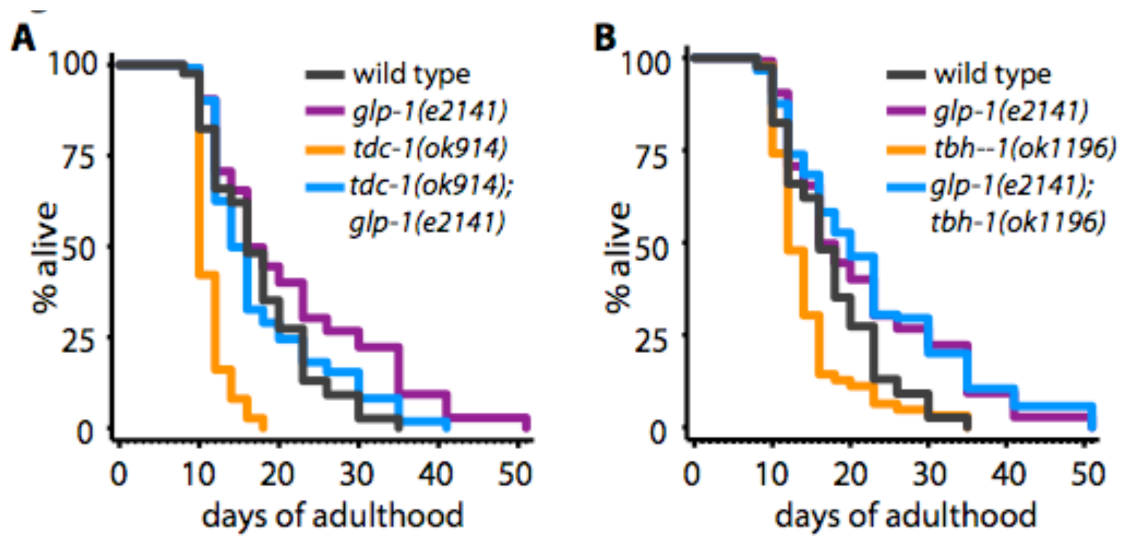


Table 4.4

Repeat	Strain	Mean lifespan	Std. error	% of wild type	% *glp-1(-)*
1	Wild type	21.1	± 0.8		
	glp-1(e2141)	26.3	± 1.1	+ 25% ***	
	tdc-1(ok914)	18.2	± 0.7	- 14% *	
	tdc-1(ok914); glp-1(e2141)	21.2	± 0.9		- 19% **
	tbh-1(ok1196)	22.6	± 1.2	+ 7% n.s.	
	glp-1(e2141); tbh-1(ok1196)	28.6	± 1.2		+ 9% n.s.
2	Wild type	17.5	± 0.7		
	glp-1(e2141)	21.5	± 1.0	+ 17% **	
	tdc-1(ok914)	11.4	± 0.3	- 35% ***	
	tdc-1(ok914); glp-1(e2141)	17.7	± 0.7		- 13% **
	tbh-1(ok1196)	14.4	± 0.7	- 18% **	
	glp-1(e2141); tbh-1(ok1196)	22.4	± 1.1		+ 4% n.s.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, n.s. $p > 0.05$

Chapter 5:

Perspectives

Further Discussion

In this thesis, we have examined the interplay between the nervous system and basic physiological processes in the free-living soil nematode *C. elegans*. We examined both how behavior is regulated in response to changes in the developmental stage of the animals and conversely how sensory input and neuronal pathways that generally control behavior also influence development, aging and pathogen resistance.

We found that at least one neurotransmitter pathway, the dopamine pathway, may be altered in the hibernation-like dauer larval stage, perhaps to dampen the animal's response to environmental stimuli. Our results suggest that the insulin/IGF-1 pathway, one of the pathways that controls entry into the dauer stage in response to food and pheromone cues (Riddle et al., 1981; Gottlieb and Ruvkun, 1994; Kimura et al., 1997), may direct changes in dopamine signaling in animals in the dauer stage, and that this is one of the reasons dauer larvae become behaviorally quiescent and unresponsive to stimulation. As dopamine signaling, however, cannot fully explain the locomotory quiescence, another pathway must also be altered in the dauer larval stage. We think it is likely to be a neuronal pathway, as the activity of the major downstream effector of the insulin/IGF-1 pathway, the DAF-16/FOXO transcription factor, in the neurons is

sufficient to regulate dauer locomotory quiescence.

It is interesting that the dauer stage physiology affects nervous system pathways, as inputs from the nervous system control the process of dauer formation itself. We studied how mutations that extensively disrupt perception of environmental cues lead to increased dauer formation in sub-threshold conditions, such as temperatures around 27ºC (Ailion and Thomas, 2000), and found that this effect is probably mediated by inappropriate activation of several transcription factors, including DAF-16/FOXO and DAF-12/NHR. Based on RNA interference data, it is possible that DAF-16/FOXO and DAF-12/NHR have different sites of action for dauer formation: DAF-16/FOXO is likely to act in the neurons and DAF-12/NHR in peripheral tissues. DAF-12/NHR may either be downstream of DAF-16/FOXO, which is consistent with the extensive overlap in their target genes, or independently. In the course of this study we also made an interesting and unexpected finding. Genes that have been associated with pathogen responses (Shapira et al., 2006; Troemel et al., 2006) are downregulated in *daf-10(-)* mutant animals, and this appears to result in increased sensitivity of these mutants to pathogens. Thus, sensory mutations could also regulate another transcription factor, which in turn affects pathogen responses.

For the neurons to communicate with other tissues, they must release signals. Knowing identity of these signals could help better understand how the system functions. We took a candidate approach and examine the role of neurotransmitter signaling in the regulation of both dauer formation and longevity, especially in the context of the insulin/IGF-1 pathway. We found that dopamine signaling may have a role in the exit

from the dauer stage, and possibly in lifespan regulation. Even though we found that changes in dopamine may change the ability of dauer larvae to respond to stimuli, it is possible that stimuli that are strong enough to elicit a response may be carried by the dopamine system to direct dauer exit. Serotonin also appeared to have a clear effect on both dauer formation and lifespan. The results suggest that serotonin inactivates DAF-16/FOXO, and that loss of serotonin and of *daf-2*/InsR act additively to regulate the transcription factor. It will be interesting to know whether either of these pathways is involved in the sensory regulation of lifespan.

The nervous system of *C. elegans* is much simpler than that of other organisms, and some of the processes we analyzed, such as dauer formation, are not shared by higher organisms. However, recent studies have found extensive similarities in the regulation of aging by the central nervous system in other organisms. Studies in flies have shown that mutations that affect sensory perception (Libert et al., 2007) and dFOXO activation in the brain (Hwangbo et al., 2004) can extend lifespan, and recently it was found that loss of one copy of the IGF-1 receptor in the mouse brain can extend lifespan too (Kappeler et al., 2008). In this study, the authors suggest the hypothalamus was probably responsible for altering the activation of the HPA axis, and thus for regulating aging. The hypothalamus receives inputs from many sensory systems and controls many basic physiological process (Risold et al., 1997). Interestingly, dopamine is one of the signals released by the hypothalamus. In addition, the hypothalamus receives serotonergic input (Purves, 2001). Therefore, it is possible that despite the reduced complexity of its nervous system, the circuitry that we find in the worm may be very similar to that of

higher organisms.

Future Directions

The studies outlined in this thesis offer some interesting novel observations, but also raise a number of questions.

As mentioned above, we still do not know what actually regulates quiescence in dauer and *daf-2*/InsR(-) dauer-like adults. Nor do we fully understand why both increasing and decreasing dopamine can stimulate the movement of the quiescent animals. Thus studies aimed at understanding these questions may greatly increase our understanding of dauer quiescence, and may perhaps tell us something about the regulation of movement in general, in the worm and in higher organisms.

Although we have found that the sensory system affects the activity of several transcription factors, we still do not know how the signals works and where to the signals are relayed. We could not determine from our study whether the sensory system signals through the insulin/IGF-1 receptor, and we have not established at this point which transcription factor is used to regulate the pathogen resistance genes. Also, some of the genes identified in our microarray study could be used as reporters to screen potential novel environmental factors that influence longevity and dauer formation.

We have surveyed some of the neurotransmitter pathways in the worms, and found somewhat inconclusive results. However, it is possible that focusing on a relationship to the insulin/IGF-1 pathway was too restrictive. Also, it is possible that

neuropeptides are the signals that are used by the neurons in regulating lifespan. There are at least three families of neuropeptides in the worm [reviewed in Li and Kim (Li and Kim, 2008)], including the *ins* genes, the putative insulin ligands. Now that many mutants for neuropeptides are becoming available, their role in the regulation of dauer formation, lifespan, pathogen resistance and other systemic phenotypes could be explored.

Concluding Remarks

From our results we can build a tentative picture of how environmental cues are translated into behavioral and physiological changes in the dauer state and during aging. During dauer formation, environmental signals may regulate DAF-16/FOXO activity in the neurons, which in turns regulate downstream pathways, including, perhaps, the dopamine pathway and the steroid signaling pathway involving DAF-12, to direct behavioral, morphological and metabolic changes that are typical of dauer larvae. Alternatively, DAF-12/NHR may be directly regulated by the sensory system. Despite their reduced behavioral responses to environmental stimuli, dauer larvae do respond to food cues by resuming development, and, based on our data, dopamine signaling may mediate some of the environmental signals directing dauer recovery.

During aging, an analogous process may occur. However, based on studies by Libina et al. (2003) and Murphy et al. (2007), it is likely that in this case DAF-16/FOXO is probably acting both in the neurons and in peripheral tissues, regulating its own

activity by a sort of feed-forward pathway.

Despite its relative simplicity of the worm's nervous system deals with a lot of the inputs and outputs as those of higher organisms, and there are surprisingly similarities in the molecular pathways used by worms, flies and mammals. Thus, *because* of its simplicity, stereotypy and ease of manipulation, studying how the nervous system of *C. elegans* regulates basic, whole-organism physiological process may give us valuable clues to how similar systems function in higher organisms.

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