FOOD-DEPENDENT SWIMMING-INDUCED PARALYSIS IN *C. ELEGANS*: A NOVEL SEROTONIN TRANSPORTER DEPENDENT PHENOTYPE

By

Leda L Ramoz

Thesis

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

In

Interdisciplinary Studies: Molecular Neuropharmacology

August, 2010

Nashville, Tennessee

Approved:

Professor Randy D. Blakely

Professor Aurelio Galli

ACKNOWLEDGEMENTS

I would like to thank my mentor, Randy Blakely, for his support and for the opportunity to work with him in his lab. His guidance has helped me achieve a great success and imparted invaluable knowledge and training that I will carry with me to all my future endeavors. I would also like to thank Aurelio Galli for his direction and counsel during my graduate career. These individuals have effortlessly imparted indispensible lessons and opportunities, both at and away from the bench, for which I am grateful.

I would also like to thank the Blakely and Galli lab members, especially Dawn Matthies and Sabrina Robertson for their scientific feedback and camaraderie. They have made my time at Vanderbilt more enjoyable than any other, and given me some often times much needed perspective. I would like to thank all my friends and family for their endless encouragement. I would like to acknowledge my funding source, NIH T32NS061201, without which this work would not be possible.

Finally, I would like to thank Morgan Sammons for his unwavering support. He has been my champion throughout my graduate career, whom without graduate school would have been hardly as enjoyable.

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CHAPTER I

INTRODUCTION

5-HT and SERT

Since its debut in 1986, the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Prozac™) and its successors have taken society and mental illness by storm, becoming one of the most widely prescribed medications in America for the treatment of depression, obsessive-compulsive-disorder, bulimia nervosa, and anxiety[1]. Despite their pervasive use in society, the exact mechanism of action of these and other antidepressants, as well as their effects on endogenous regulation of their target protein, the serotonin transporter (SERT)[2] are largely unknown. Synaptic serotonergic activity is primarily regulated by recycling of serotonin (5-hydroxytryptamine, 5-HT) from the synaptic cleft through activity of the presynaptic serotonin transporter (SLC6A4)[3, 4], a transmembrane protein that is also a major target of psychostimulants such as MDMA ("ecstasy") and cocaine[5,2]. 5-HT is an important modulator of vertebrate cardiovascular and cognitive function regulating a wide range of physiological and behavioral processes including gut function, body temperature, sleep, appetite, aggression, and mood[6]. SERT deregulation has been linked to a variety of disease states, those listed above as well as alcoholism and autism[1,7-9], yet we are only beginning to understand the mechanisms behind endogenous regulation of SERT.

Current investigations of SERT regulation implicate several Ser/Thr kinases that modulate both activity and localization, possibly in part through presynaptic receptor activity [10-15]. Rodent models demonstrate the impact of a loss in SERT activity and SERT alleles on behavior[16, 17] and are critical for understanding the complex role of 5-HT in human disease states. However, there is a pressing need for identification of endogenous regulators of 5-HT signaling, particularly SERT, and these investigations can profit from tools drawn from the powerful model organism *Caenorhabditis elegans* (*C. elegans)*. Although debatable as a model for the study of most human disease states, the *C. elegans* model system offers approaches impractical with mammalian SERT to provide insight into the mechanism of action of antidepressants, potential drug targets for treatment of 5-HT-linked disorders, and genes responsible for the control of SERT modulation. This work assesses SERT (MOD-5) dependent phenotypes in *C. elegans* to investigate the mechanisms regulating SERT activity by examining the role of 5-HT and SERT in *C. elegans* behavior, with an eye to how these behaviors may serve as the basis for a forward genetic screen. These studies contribute new information on endogenous 5-HT signaling in the nematode using novel, automated approaches for monitoring *C. elegans* locomotion. With further investigation, these studies can contribute to the discovery of novel therapeutic treatments for 5-HT linked disorders and elucidation of molecular underpinnings of behavior and disease.

C. elegans and forward genetics

The nematode *C. elegans* is an ideal model system for neurogenetic research: animals are transparent and therefore ideal for fluorescent reporter imaging, there are many viable neuronal knockouts available where the cognate disruption in mammals is inviable, the core synaptic machinery is well conserved from invertebrates to man (Figure 1-1), and there are a plethora of well developed techniques for studying this organism spanning genetics, biochemistry, primary cell cultures, and RNAi technology. Animals are easily cultivated in the laboratory, withstand cryopreservation, in sub-optimal environmental conditions (such as prolonged starvation), maintain a metabolically inactive state known as dauer arrest for months. Each individual *C. elegans* hermaphrodite contains a numerically and morphologically invariant 959 cells, including 302 neurons, enabling lineage mapping for each cell[18, 19]. Reconstruction of the entire animal by serial electron micrograph[20] has provided an intimate knowledge of the structure and connectivity of the nematode nervous system. In particular, the easily monitored behaviors (egg-laying, locomotion) and short generation time of *C. elegans* (~3 days from egg to adult) make the worm an optimal organism for forward genetic approaches. Hermaphroditic reproduction permits line and mutation propagation without staged crosses and also simplifies isolation of homozygous mutants. Random mutagenesis of a parental group of animals yields F2 progeny, 25% of which are homozygous for any given mutation. These mutants are then assayed to identify a particular phenotype of interest. As a result, forward genetic screens have been the

technique of choice for nematode biologists for many years to impartially isolate any number of participants in a given genetic pathway. Screens isolating mutants that phenocopy a known mutant, such as abnormal egg-laying and touch sensitivity, have been utilized in the past to elucidate functional components of neuronal signaling such as neurotransmitter biosynthesis and packaging, as well as led to the discovery of programmed cell death[21-23], or apoptosis. The tedious prospect of screening tens of thousands of random mutants in search of the few mutants of interest stresses the importance of having a phenotype that is easily observable in the laboratory and optimally amenable to a high-throughput process. Only a subset of mutants isolated in a screen will contain defects in a particular pathway of interest, for example a screen for animals defective in egg-laying may yield mutations in the nervous system as well as vulval muscle development. Potentially interesting mutants therefore must undergo further genetic or pharmacological tests to determine the deficient pathway. In the case of a abnormal egg-laying screen, animals defective in vulval formation rather than malfunction in neural circuitry are distinguished by their egg-laying responses to exogenous 5-HT[22]. Thus, a phenotype for a forward genetic screen should not only be easily scored in the laboratory, but also sensitive to genetic and pharmacological tools with which to examine the integrity of supporting molecular and cellular circuits. In my work I have been guided by the hypothesis that the actions of 5-HT within *C. elegans* can provide insight into the potential phenotypes expressed by SERT-defective animals (which theoretically express

elevated synaptic 5-HT) and that may then be exploited in a screen for genes controlling SERT trafficking, localization, and activity.

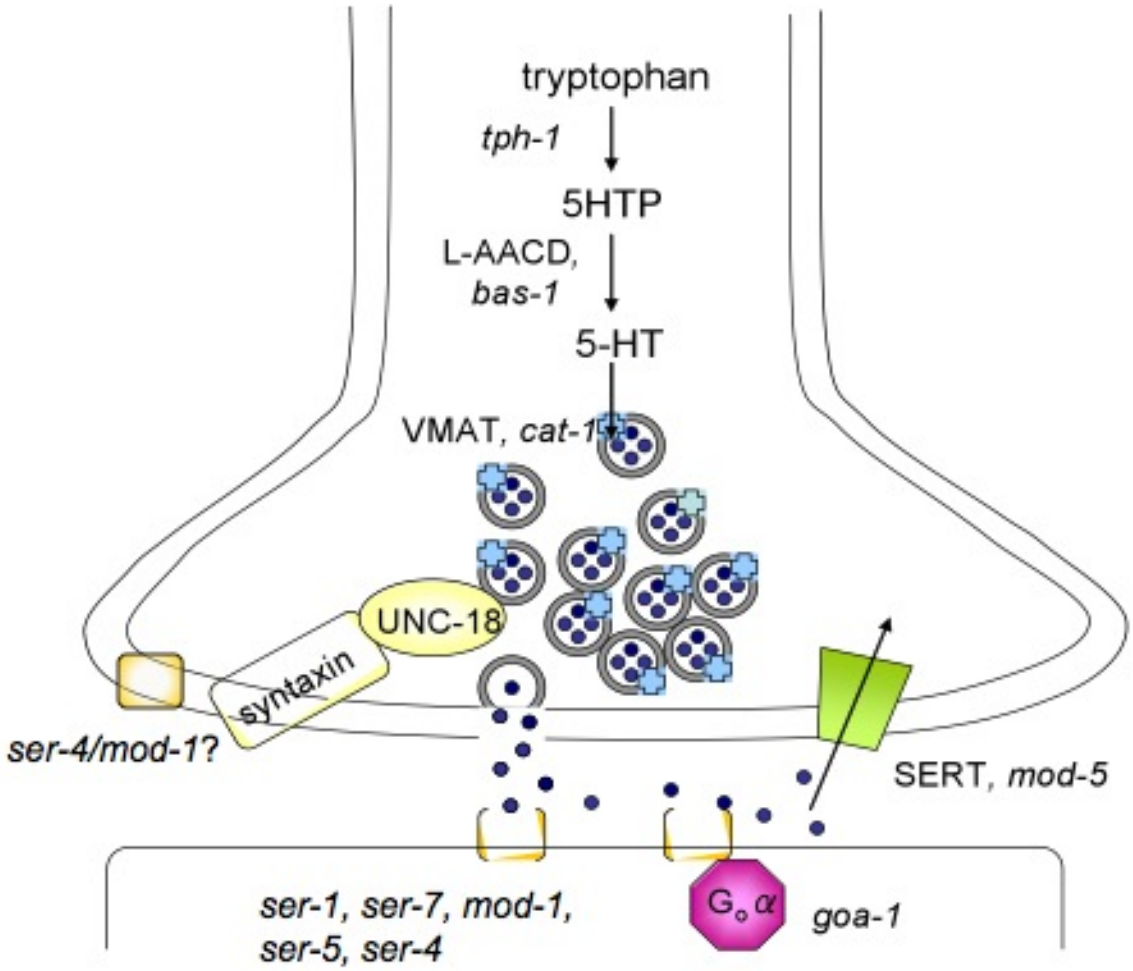

Figure 1-1. 5-HT biosynthesis is conserved from *C. elegans* to man[24]. 5-HT is packaged into vesicles (grey spheres) through the activity of a vesicular monoamine transporter (VMAT, *cat-1*[25], pale blue plus). Synaptic vesicle release is facilitated by the well conserved SNARE complex (yellow), many of the components of this complex include the two illustrated above (UNC-64/syntaxin, UNC-18/nSec-1) were originally identified in *C. elegans*[26, 27]. As in mammals *C. elegans* 5-HT receptors are divided into metabotropic (*ser-1*[28], *ser-4*[29], *ser*-5[30], and ser-*7*[31] coupled to Gαq, Gαo, Gαs and Gαs respectively), and ionotropic (*mod-1*[32]) categories. Putative presynaptic 5-HT receptors include *ser-4* or *mod-1*[31].

C. elegans and 5-HT

In *C. elegans,* 5-HT is an active participant in a variety of motor, metabolic, and reproductive behaviors. Application of exogenous 5-HT mimics the presence of food resulting in increased egg-laying and pharyngeal pumping (the nematode feeding mechanism) as well as decreased locomotion[33]. Animals deficient in 5-HT synthesis also display decreased male mating efficiency[34], increased reproductive lifespan, increased fat storage, increased dauer arrest, decreased egg-laying[24], and defective starvation-dependent slowing in response to food (known as "enhanced slowing")[35]. In addition, 5-HT modulates complex chemosensory[36] and olfactory learning[37] behaviors. These behaviors are thought to be regulated by eight classes of serotonergic neurons identified through anti-5-HT immunofluoresence (Fig. 1-2, Table 1-1), four of which are located in the head of the animal (see expanded view Fig. A-2). Cloning of the *tph-1* gene in *C. elegans* combined with GFP imaging has identified the NSMs, ADFs, HSNs, CPs, AIMs and RIH as 5-HT production sites[24]. Serotonergic neurons not expressing *tph-1* are presumed to obtain their 5-HT through activity of the *C. elegans* 5-HT transporter, *mod-5*[38], although this idea requires further investigation.

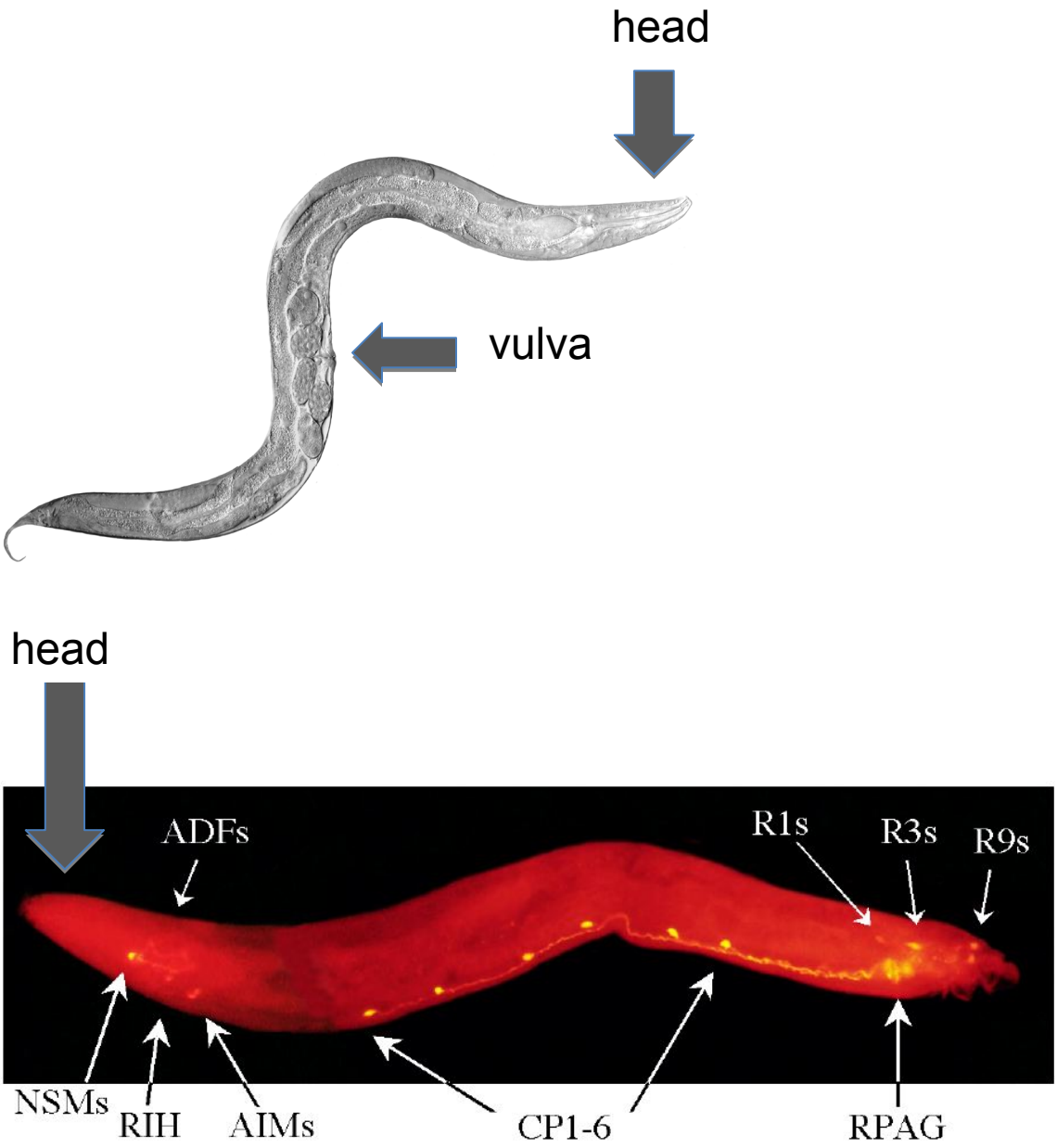

Figure 1-2. The nematode, *Caenorhabditis elegans*. *Top panel,* Nomarski image of adult *C. elegans*. Adapted from the Hardin Lab, worms.zoologly.wisc.edu. *Bottom panel,* Anti-5-HT immunofluoresence of adult male *C. elegans*. Adapted from the Loer Lab, sandiego.edu/~cloer/loerlab/5-HTcells.html.

Table 1: Serotonergic neurons in *C. elegans*			
Class	**Type**	**Location**	**Function**
NSMs[39] (2, bilaterally symmetric)	Neurosecretory Motor Neuron	Anterior bulb of pharynx	Pharyngeal pumping
ADFs[25] (2, bilaterally symmetric)	Amphid sensory neuron	Nerve ring	Dauer entry
AIMs[25] (2, bilaterally symmetric)	Interneuron	Nerve ring	Unknown
RIH[25] (unpaired)	Interneuron	Nerve ring	Unknown
HSNs[40] (2, bilaterally symmetric)	Motor neuron, Hermaphrodite Specific Neuron,	Vulva	Egg-laying
VC4, VC5[25] (unpaired)	Motor neuron, hermaphrodite specific	Vulva/ventral cord	Egg-laying
CP1-6[34] (unpaired)	Possible motor neuron, male specific	Ventral cord	Male mating
R1, R3, R9[34] (bilaterally symmetric)	Ray sensory neurons, male specific	Male tail/lumbar ganglia	Male mating

MOD-5

The *C. elegans* 5-HT transporter (*mod-5)* gene encodes a protein with 44% amino acid identity with mammalian SERT proteins and confers paroxetine-sensitive 5-HT transport on nonneuronal cells after heterologous expression[38]. MOD-5 activity within the HSNs, ADFs, and NSMs is inferred from the detection of 5-HT immunofluoresence in mutants that lack the ability to synthesize 5-HT (*tph-1*) after incubation with exogenous 5-HT and that can be blocked by selective serotonin reuptake inhibitor (SSRI) fluoxetine[38]. *mod*-5 null mutants are viable and healthy, and, consistent with the hypothesis that these animals express excess synaptic 5-HT, these animals exhibit hyperenhanced slowing, increased egg-laying in response to 5-HT, and reduced fat content[38, 41]. The effects of exogenous 5-HT, as well as behaviors in animals lacking of 5-HT synthesis, suggest that *mod-5* mutants might express dauer entry resistance and increased pharyngeal pumping, although this has not yet been demonstrated.

MOD-5 and locomotion

Abnormal locomotor activity is one of the most easily observed *C. elegans* behaviors in the laboratory. Paralyzed animals are easily identified within a population or in response to exogenous drug, and many mutations have been characterized that result in abnormal or uncoordinated movement (e.g. *unc* mutants). Application of exogenous 5-HT results in decreased locomotion[33] and animals starved for a brief period (30 min) display a normal locomotory rate that dramatically slows upon encountering a bacterial lawn (enhanced slowing[35]), a

trait evolved presumably to protect the animal from starvation. This behavior is quantified in the laboratory by manually assessing the locomotory potential and rate of starved animals as they move from an area without food to a bacterial lawn. Starved animals are not hypersensitive to inhibition of locomotion by exogenous 5-HT, suggesting this behavior is modulated presynaptically. 5-HT synthesis mutants completely lack an enhanced slowing response, a deficit that is rescued by the application of exogenous 5-HT[35]. Enhanced slowing is blocked by the 5-HT receptor antagonists mianserin and methiothepin,[35] further supporting a role of 5-HT in this behavior, and importantly can be potentiated by fluoxetine[38], suggesting the enhanced slowing response is a direct measure of *mod-5* activity. Although *mod-5* mutants display wildtype locomotory rates under standard laboratory conditions, they exhibit an exaggerated or "hyperenhanced" slowing response, distinct from the enhanced slowing response observed in the wildtype animal[38]. Enhanced slowing is partially mediated through the putative food sensing NSMs, as laser ablation of these neurons significantly, but not completely, impairs the enhanced slowing response[35]. Enhanced slowing in NSM ablated animals is not potentiated by fluoxetine[35], indicating *mod-5* influences locomotion at the NSMs. These data demonstrate the important regulatory role of *mod-5* within the *C. elegans* motor circuit as well as the utility of this phenotype in a screen to elucidate mechanisms of SERT function. However, observation of this phenotype in the laboratory is labor intensive and would be more effective in a screen if amenable to higher throughput methods. My work explores a novel 5-HT and *mod-5* dependent phenotype within the *C. elegans* system that may be of

use in both a forward genetic screen to identify endogenous regulators of SERT as well as to test candidate SERT interactors. Increased formaldehyde induced fluorescence (FIF) staining in *C. elegans* NSMs after preincubation with 5-HT suggested the presence of a mechanism capable of concentration extrasynaptic 5-HT[33,38]. This idea led to a genetic screen for mutants lacking FIF within the NSMs after exogenous 5-HT incubation, revealing the *C. elegans* SERT ortholog, *mod-5*[38]. Although a *mod-5::GFP* fluorescent reporter fusion protein has yet to be made, *tph-1::GFP* expression reveals 5-HT synthesis in NSMs, ADFs, HSNs, CPs, and intermittently within AIMs and RIH *C. elegans* serotonergic neurons[24]. This work implicates the activity of *mod-5* within at least the VCs and male ray sensory neurons, and also likely within the AIMs and RIH due to their robust staining of 5-HT[24]. Current investigations examining 5HT immunostaining in the presence of fluoxetine[42] combined with future fluorescent MOD-5 reporter analysis will aid in the clarification of the different types of serotonergic neurons within *C elegans*.

CHAPTER II

MATERIALS AND METHODS

C. elegans strains and husbandry

All strains utilized were derived from wild-type Bristol (N2) strain, grown on NGM plates seeded with the *Escherichia coli* strain OP50, and maintained at 12-20°C using standard methods[43]. N2, *mod-5*(n3314), and *ser-1*(ok345) strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN). The *dat-1*(ok157) loss of function allele removes the majority of the DAT-1 coding sequence and was a gift from J. Duerr and J. Rand (Oklahoma Medical Research Foundation, Oklahoma City, OK). The *tph-1*(n4622) strain was a generous gift from H.R. Horvitz (Massachusetts Institute of Technology, Cambridge, MA) and carries a ~2.2 kb deletion of the TPH-1 coding sequence, including the transcription start site. Double mutant lines were constructed using *mod-5*(n3314) males and *ser-1*(ok345), *tph-1*(n4622), or *mod-1*(ok103) hermaphrodites. Genomic DNA was isolated as described[44] and used at 1ng/µL to determine the genotype of double crossed lines first at the *mod-5* locus and then at the second locus of interest using single worm PCR[45,46].

C. elegans locomotory rate assay

Manual assessment of swimming behavior was conducting using groups if 3-10 L4 hermaphrodites in 20µL of M9 medium in a Pyrex Spot Plate (Fisher

catalog number 13–748B). Automated locomotory analysis of worm swimming behavior was executed using single L4 hermaphrodite animals in 20μL of M9 in a Pyrex Spot Plate as described in Matthies et al. 2006[47,48]. Briefly, 10 minute AVI movies were recorded (at least 10 animals per group) by VidCap32 AVI capture application (Microsoft, Redmond, CA). AVI movies were analyzed by a custom Matlab 7.0.1 script (Matlab, MathWorks, Natick, MA), selection of the worm centroid by pixel location allows worm position recognition per frame by motion tracking. Worm movement is calculated in Hz and average frequencies (at least 10 animals per group) for 6 second windows were plotted and analyzed across a 10 minute time period. Statistical analyses of swimming rates comparing strains and/or drug treatments were analyzed in Prism 5.0 software (vendor) as noted in Fig legends. Food for liquid food swimming assays was prepared as previously described in Avery and Horvitz 1990[49]. Briefly, OP50 was grown to saturation in LB, pellets were collected by centrifugation and then resuspended in a volume 1/40 of the original culture. An aliquot (35μL) of the 40X OP50 suspension was diluted in 400μL of M9 to a working density for liquid food swimming experiments. Preserving the 40x OP50 stock for several months at 4°C did not have deleterious effects on feeding assays.

For mixed population assays, worms exhibiting intermittent paralysis at the end of the 10-minute assay period were singled onto NGM plates, grown into clonal populations and genotyped for the presence of the *mod-5* allele. A total of 10-12 animals were analyzed in 6 trials, 30% of each trial group consisted of *mod-5* mutants, and all trials were conducted in a double-blind manner.

Enhanced slowing assays were executed as in Sawin et al. 2000[35], briefly, well fed L4 animals were washed free of bacteria three times by centrifugation (1500 rpm) and plated onto freshly poured unseeded NGM plates. The well fed group of animals were assayed immediately whereas starved animals were incubated without food for 30 min. Assay plates contained lawns freshly seeded with concentric rings (approximately 1.5cm inner diameter, 2cm outer diameter) of OP50 grown overnight at 37°C to obtain a uniformly thick lawn. Animals were placed in the center of the plate and assayed for locomotory rate after 5 minutes, a maximum of three animals were assayed per 60mm plate.

Drug treatments

The fluoxetine drug pretreatment protocol followed that of Sawin and coworkers[35]. For these assays, 5mg/ml fluoxetine hydrochloride (Sigma CAS #56296-78-7) stock was prepared in water and 300µL of this stock was diluted in 5mL M9 to obtain a 300µg/mL working stock, with 400µl of this solution added to OP50-seeded plates prior to drying plates with the lids ajar for 3-4 hours. Worms were cultured on these or control plates for 4 hours after which L4 animals were isolated for swimming assays. Incubation on fluoxetine did not appear to affect basal levels of locomotion on solid substrate in either N2 or *mod-5* strains.

Methiothepin (methiothepin mesylate salt Sigma CAS #74611-28-2) was added to assay buffer at a concentration of 175µg/mL. 5mg/mL methiothepin stock was prepared in water and diluted in M9 to obtain a 175µL/mL working stock. 20ul of this was used as the assay buffer for automated analysis, or 35ul

of 40X OP50 was added to 400ul of this stock to generate the liquid food assay buffer.

5-HT hypersensitivity assays were conducted as in Ranganathan et al. 2001[38]. Serotonin creatinine sulfate monohydrate (Sigma CAS # 971-74-4) was dissolved in water at 5mg/mL and diluted in M9 to a 35mM working concentration. 200ul of 35mM 5-HT was used per 20 L4 worms per assay. After 5 minutes animals were visually inspected and number of paralyzed worms manually recorded.

Quantum pumping

Animals and food for measuring pharyngeal pumping rates by the consumption of quantum dots was adapted from Avery and Horvitz 1990[49]. Briefly, OP50 was grown to saturation in LB and the pellet was collected by centrifugation and resuspended in 1/40 the volume of the original culture. A well-fed plate of worms was washed free of bacteria into 1mL of K-medium[50], the desired concentration of worms is 500-1000 worms/mL. 50ul of 40X OP50 was added to the worm mixture, and mixed on nutator for 10 minutes. A final concentration 16nM of160nM AMP coated (amphiphilic poly(acrylic acid) primary coat) quantum dots dissolved in calcium free PBS with a fluorescent maximum of 605nm was added to the worm mixture and allowed to mix for 60min, after which the solution was titrated o a final concentration of 7mM sodium azide. Worms were imaged using a Zeiss Axiovert S100 with a coolsnap camera and openlab software V 5.5.0.

CHAPTER III

RESULTS

mod-5 mutants display decreased locomotory activity in liquid

We previously observed that *mod-5* mutants exhibit an experience dependent and food dependent hyperenhanced slowing response[38] (Fig 3-1). Starved wildtype animals typically slow from a rate of 60 body bends per minute to 15 body bends per minute upon encountering food, whereas *mod-5* mutants become almost immobile[38] (Fig 3-1). This phenotype would be useful for a screen for serotonin transporter regulatory molecules in that it is both 5-HT and *mod-5* specific, however it is labor intensive and in our hands undergoes high variability in response to slight changes in development, bacterial growth, and other assay conditions. Our lab has successfully characterized a novel dopamine transporter (*dat-1*, DAT-1, DAT) *C. elegans* phenotype, swimming induced paralysis (SWIP[48]), and that has been used in a forward genetic screen for regulators of the DAT (Hardie, Hardaway, et al. personal communication). We reasoned the addition of food to this paradigm might elicit a more robust phenotype in the *mod-5* strain, similar to that observed in the hyperenhanced slowing response. To test this hypothesis, we placed well-fed wildtype and *mod-5* L4 hermaphrodite animals in a liquid environment where they execute sustained

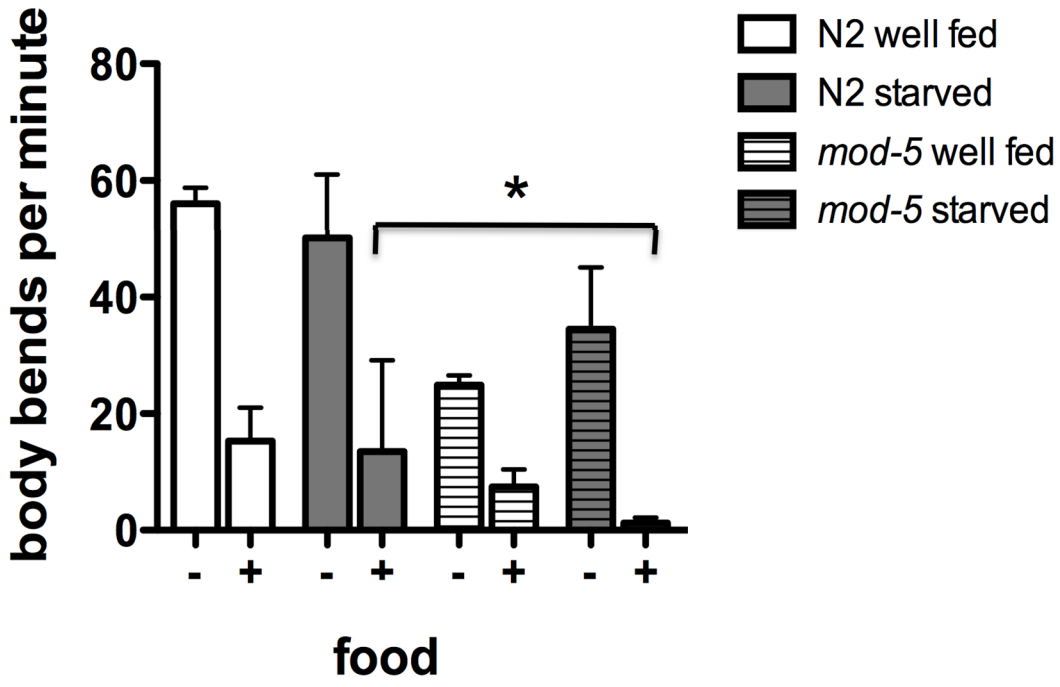

Figure 3-1. mod-5 mutants display a hyperenhanced slowing response compared to wildtype, as reported in Ranganathan et al. 2001. n = 80 * = *P* <0.01, two way ANOVA.

swimming behavior and analyzed their frequency of movement using an automated computer controlled system[47]. We observe wildtype L4 animals thrash vigorously without rest throughout the entire 10 minute assay period in either M9 or M9 plus the addition of liquid *E. coli* OP50. *mod-5* animals thrash significantly more slowly than N2 animals in M9 (P <0.0001 two way ANOVA, Fig. 3-2, A), and are likely to stop thrashing altogether for a brief period of time (15 - 60s) within the 10-minute assay period. The addition of OP50 to the assay medium increases the incidence and duration of *mod-5* immobilization within the 10-minute assay period (P <0.0001 two way ANOVA, Fig. 3-2, B). During this immobilization state the *mod-5* animal assumes a kinked body posture (Fig. 3-2, C, bottom panels), which appears to initiate in the tail region of the animal. This posture is quite distinct from the very smooth curvature observed in a wildtype animal undergoing typical thrashing behavior (Fig. 3-2, C, top panels). Total number of body bends executed by individual animals over the 10 minute assay period were calculated from the area under the curve (AUC) of the individual tracker plots and represented in Figure 3-3, demonstrating a significant decrease in *mod-5* bend rate in both M9 and M9 + OP50 (P < 0.05 Bonferroni's Multiple Comparison test, One-way ANOVA P <0.001). As observed in the hyperenhanced slowing response, exogenous OP50 may trigger endogenous 5-HT release that the *mod-5* strain is incapable of clearing from the synaptic cleft, resulting in decreased locomotion (immobilization, Swimming induced paralysis, SWIP).

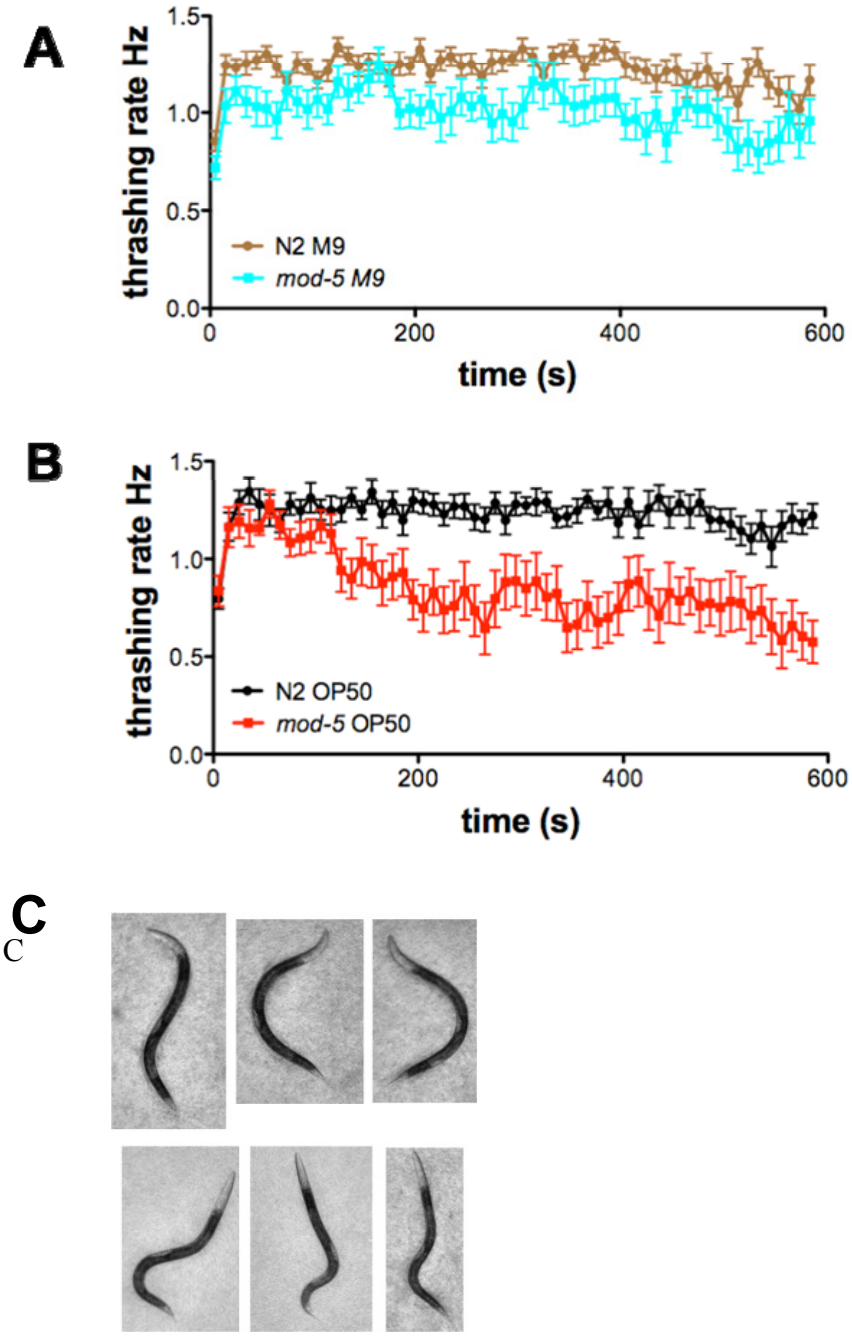

Figure 3-2. mod-5 mutants display decreased locomotory activity in liquid. *A, mod-5* animals display decreased average thrashing rate compared to wildtype in M9 buffer. N2 M9 n = 22, *mod-5* M9 n = 18, Two-way ANOVA *P* <0.0001. *B,* The incorporation of OP50 into the assay medium further decreases the *mod-5* thrashing rate compared to wildtype. N2 OP50 n = 16, *mod-5* OP50 n = 15, Two-way ANOVA *P* < 0.0001. *C,* Top panels, Curved body posture exhibited by thrashing N2 animals in OP50. Bottom panels, Kinked body posture exhibited by immobilized *mod-5* animals in OP50.

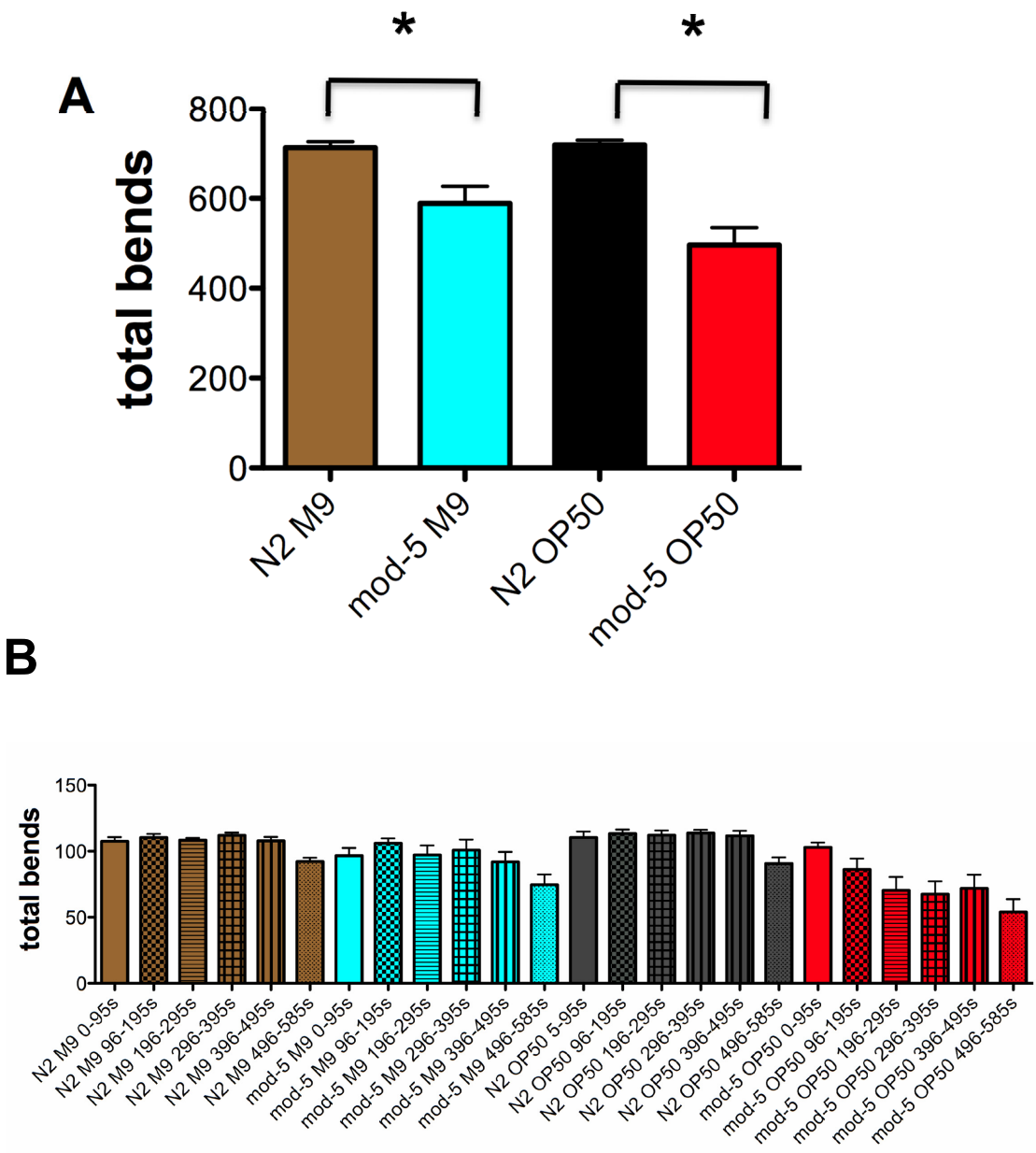

Figure 3-3. Total number of body bends executed by N2 and *mod-5* animals throughout the assay period in M9 or OP50. *A, mod-5* animals display decreased total number of body bends compared to wildtype in M9 and OP50. N2 M9 n = 22, *mod-5* M9 n = 18, N2 OP50 n = 16, *mod-5* OP50 n = 15. * = $P < 0.05$ Bonferroni's Multiple Comparison test, One-way ANOVA $P < 0.001$. *B,* Total number of body bends executed by N2 and *mod-5* animals in M9 and OP50, distributed by time.

The biogenic amine dopamine (DA) has also been shown to play a critical role in *C. elegans* locomotion, including the basal slowing response[35]. To examine whether DA plays a role in the food-dependent paralysis phenotype, we placed *dat-1* animals into our swimming assay. These animals are thought to express excess endogenous DA, and DA and *dat-1* dependent swimming induced paralysis (SWIP[48]). In contrast to *mod-5* animals, *dat-1* animals do not show a decreased thrashing rate in liquid OP50 compared to M9 (Fig 3-4). Additionally, the SWIP behavior of *dat-1* animals in water is characterized by a rod-like pattern of immobilization (S. Hardie and A. Hardaway, personal communication), further underscoring differences between 5-HT and DA elicited SWIP behavior.

Mixed population analysis of both *mod-5* mutant and wildtype animals in the same solution was performed to ascertain whether *mod-5* mutant animals could be efficiently separated from wildtype animals based on only visual observation of thrashing behavior. 100% of animals recovered from these experiments expressed the *mod-5* allele (n_{total} = 65, n_{mod-5} = 19, data not shown), demonstrating this phenotype is both readily observable and reliable and may serve as a basis for a forward genetic screen.

Presynaptic dependence of MOD-5 food-dependent SWIP

To determine if excess synaptic 5-HT is contributing to *mod-5* OP50 induced immobilization in liquid, we crossed the *mod-5* allele onto the *tph-1*

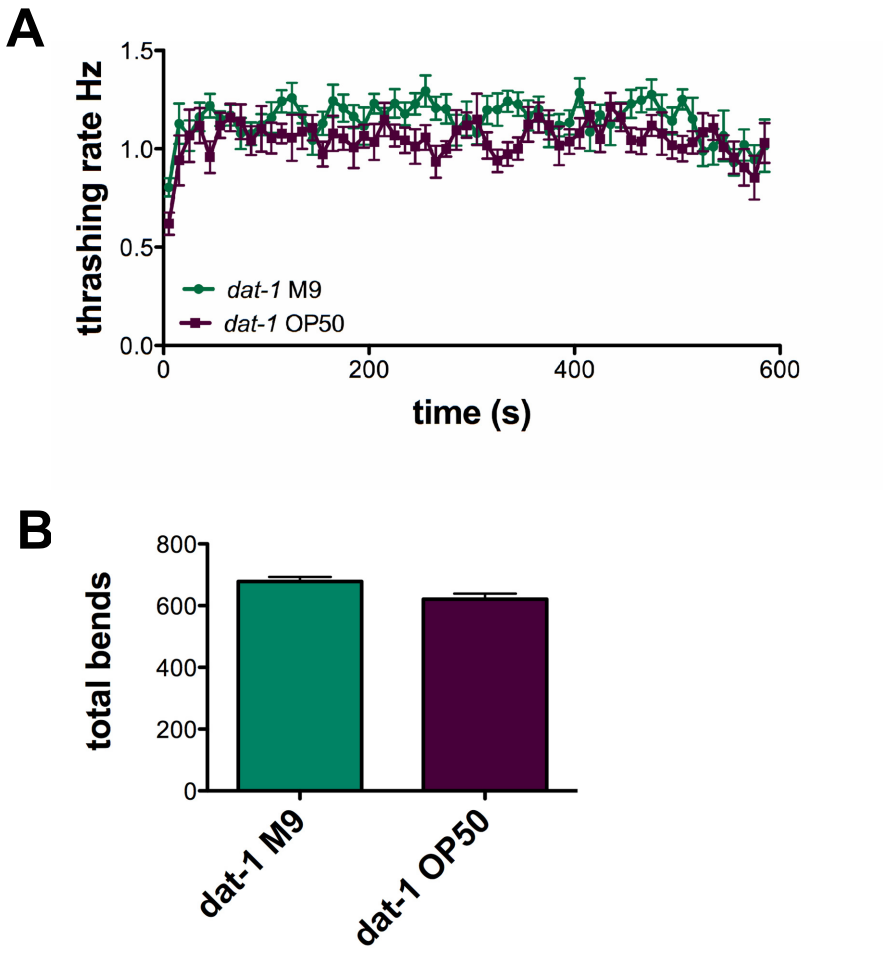

Figure 3-4. Excess endogenous dopamine does not result in *mod-5* food-dependent immobilization in liquid. *A,* Animals lacking the dopamine transporter (*dat-1*) do not show food induced SWIP in M9 or OP50. *dat-1* M9 n = 11, *dat-1* OP50 n = 10. *B,* Total bends executed by *dat-1* animals is marginally reduced by the addition of OP50. *dat-1* M9 n = 11, *dat-1* OP50 n = 10. *P* =0.0254 unpaired t-test.

background that lacks tryptophan hydroxylase, an enzyme responsible for catalyzing the first step in endogenous 5-HT synthesis. *mod-5;tph-1* double knockout homozygotes were found to be viable, healthy, and showed no obvious locomotory defects. *tph-1* animals show wildtype swimming rate in both M9 and OP50 (Fig. 3-5, A). *mod-5;tph-1* double mutants display a wildtype swimming rate that is unaffected by the addition of OP50 to the swimming medium (Fig 3-5, B). These data provide important evidence that the food-dependent SWIP phenotype is 5-HT dependent, inviting further investigation to substantiate this phenotype as also a 5-HT transporter specific phenotype to use in a screen for novel SERT regulatory genes.

To establish food-dependent SWIP phenotype as 5-HT transporter specific, we investigated whether mammalian SERT specific antidepressants could phenocopy food-dependent immobilization in a wildtype animal. The selective 5-HT reuptake inhibitor fluoxetine (Prozac^{TM}) is thought to increase synaptic 5-HT by preventing 5-HT transport by the transporter[2], and has been shown to phenocopy the hyperenhanced slowing response in wildtype animals at low concentrations[35]. Preincubation of wildtype animals on plates with fluoxetine for 3 hours at 300ug/mL results in significantly decreased swimming rates in response to OP50 with no change in basal swimming rate (Fig. 3-5, A, P <0.0001, two way ANOVA) or locomotory rate on solid substrate (unpublished observation). This change in thrashing rate corresponds to a significant decrease in total number of bends executed throughout the assay period (Fig. 3-6, B, P

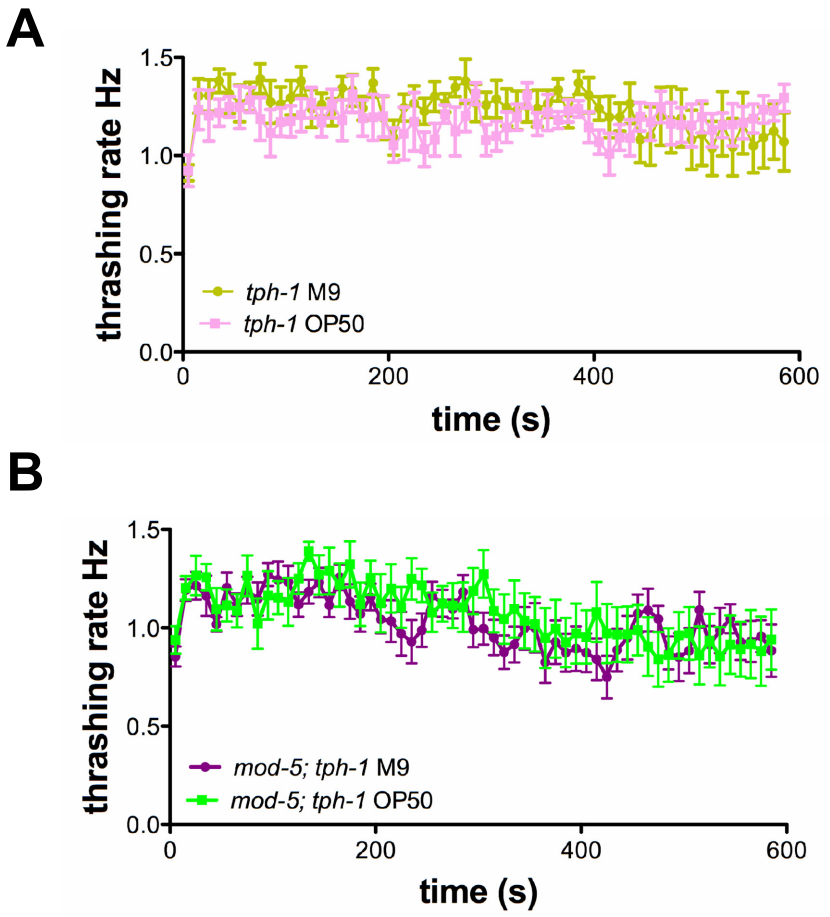

Figure 3-5. Presynaptic dependence of MOD-5 food-dependent SWIP. *A, tph-1* animals show wildtype swimming rate in M9 and OP50. n = 12 M9 n = 10. *P* < 0.4891, AUC unpaired t-test. *B,* Addition of the *tph-1* allele to the *mod-5* background suppresses the food-dependent intermittent paralysis phenotype of *mod-5*. *mod-5; tph-1* M9 n = 12 *mod-5;tph-1* OP50 n = 10 *P* = 0.8151, AUC unpaired t-test.

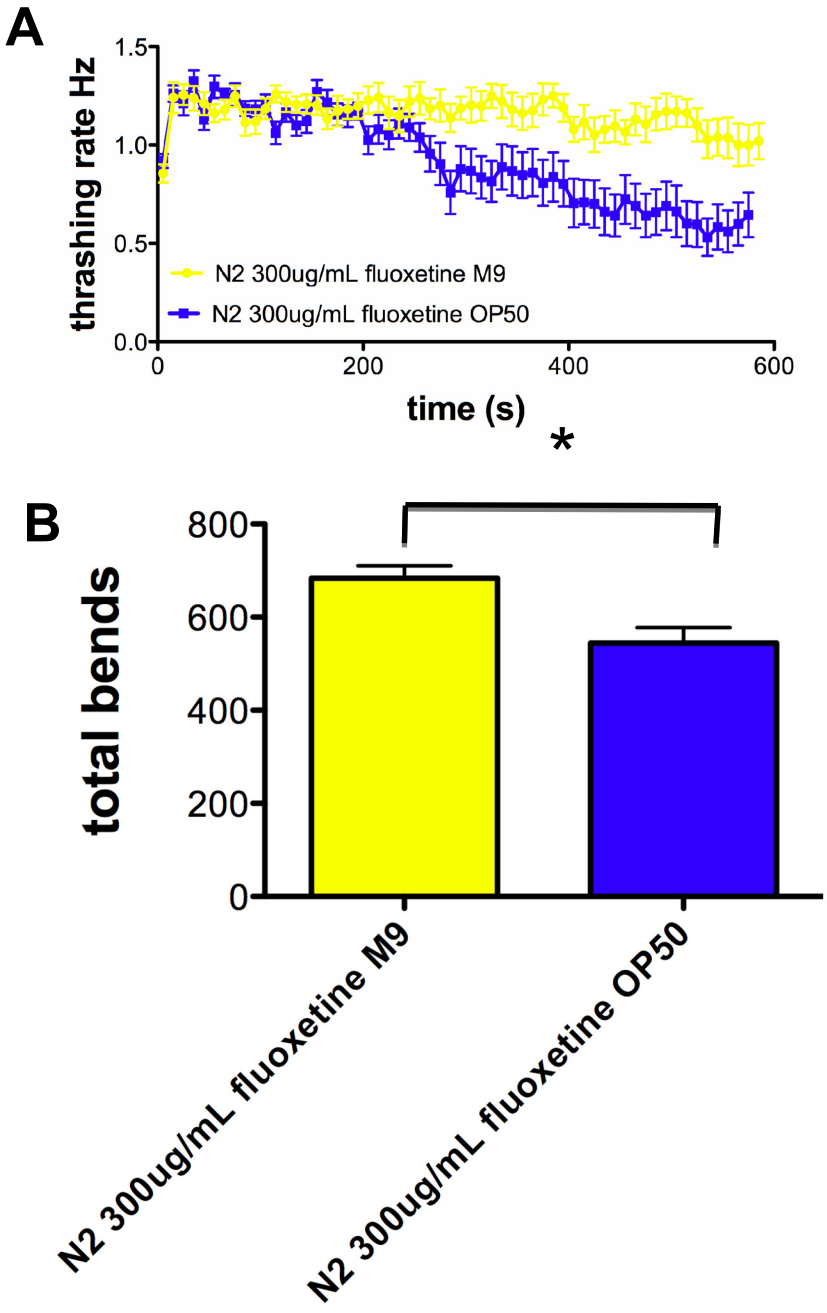

Figure 3-6. Pharmacological modulation of *mod-5* food-dependent immobilization. *A,* Fluoxetine phenocopies *mod-5* food-dependent intermittent paralysis in wildtype animals. N2 OP50 n = 22, N2 M9 n = 18, $P < 0.0001$ Two-way ANOVA. *B,* Fluoxetine treatment reduces total number of body bends executed by wildtype animals. N2 OP50 n = 22, N2 M9 n = 18. $P = 0.0030$ unpaired t-test.

=0.0030, unpaired t-test). These data support the notion of this phenotype as not only 5-HT but also *mod-5* dependent.

Postsynaptic dependence of MOD-5 food-dependent SWIP

We previously provided genetic evidence that our *mod-5* immobilization phenotype is 5-HT dependent. Here we examined the behavioral effects of pharmacologically blocking the action of 5-HT at the receptor using the metabotropic 5-HT receptor antagonist methiothepin. In this assay we treat with methiothepin during the assay period only and observed a slight decrease in average *mod-5* thrashing rate in the presence of OP50 and drug (Fig. 3-7, A). Notably, *mod-5* animals thrashed continuously in the presence of methiothepin and OP50, resulting in a reduced number and duration of immobilization events experienced by individual animals in a given 10 minute assay period. This significantly increasing the total number of body bends executed by *mod-5* animals in the OP50 condition, compared to the OP50 condition without methiothepin treatment (P <0.05 Bonferroni's Multiple Comparison's test, one way ANOVA P = 0.0085).

The *C. elegans* 5-HT-gated chloride channel MOD-1 has been implicated as a target for methiotheipen[32], and this drug has also been used to block the hyperenhanced slowing response in the *mod-5* strain[35]. Additionally, the *C. elegans* metabotropic 5-HT receptor SER-1 has also been implicated as a target for methiotheipen[29], suggesting there may be multiple levels of 5-HT regulation of locomotion. To examine the role of the postsynaptic metabotropic receptor SER-1 in this phenotype, we crossed the mod-5 strain onto the SER-1 deletion

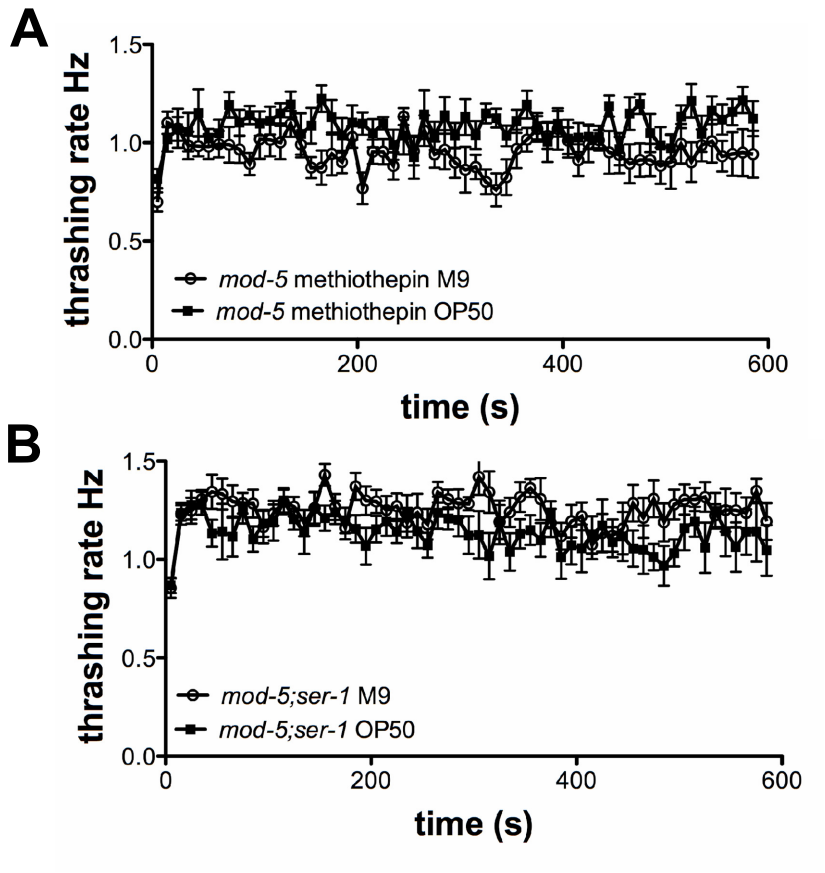

Figure 3-7. Postsynaptic dependence of MOD-5 food-dependent SWIP. *A,* Acute treatment with 175ug/mL 5HT receptor antagonist methiothepin blocks food-induced *mod*-5 immobilization, restoring *mod-5* behavior to basal conditions. *mod-5* M9 n = 11, *mod-5* OP50 n = 10. $P < 0.1100$, AUC unpaired t-test. *B, mod-5;ser-1* double homozygous animals do not display *mod-5* OP50 induced immobilization during swimming. *mod-5;ser-1* M9 n=10, *mod-5;ser-1* OP50 n=10 $P < 0.0337$, AUC unpaired t-test..

background. *mod-5;ser-1* double mutants are healthy, viable, and display no obvious outward or locomotory defects. When examined in our swimming assay they display no decrease in locomotory rate when exposed to liquid OP50 (Fig.3-7, B), consistent with the theory that increased immobilization in the *mod-5* strain in response to OP50 is due to increased endogenous 5-HT. Fluorescent reporter expression using SER-1 reveals expression on vulval and pharyngeal muscle, as well as head, vulval, nerve ring, and ventral cord motor neurons[51]. The prominent expression of SER-1 in the RIC and RIA integrating head neurons suggests this may be one point of indirect modulation for MOD-5 on speed and direction of movement in the locomotory circuit, as these neurons receive direct projections from the serotonergic RIH and ADF neurons. RIC and RIA then synapse onto the command interneurons, AVA, which control nematode backward movement.

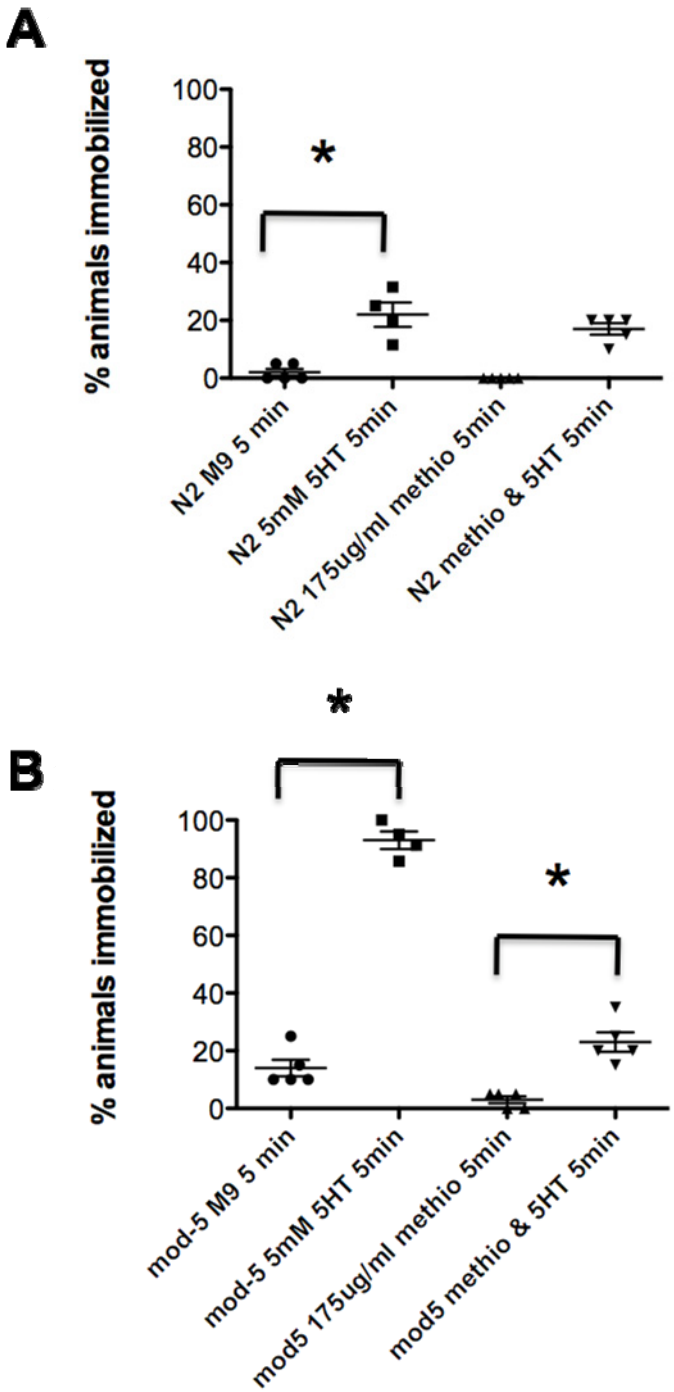

Figure 3-8. Postsynaptic modulation of acute *mod-5* paralysis as a result of 5-HT hypersensitivity. *A,* Less than 25% of wildtype animals paralyze in response to 35mM 5HT. n = 100 for each group. * = $P < 0.05$ Bonferroni's Multiple Comparison test, One-way ANOVA $P < 0.001$. *B,* methiothepin suppresses *mod-5* 5HT hypersensitivity in M9. n = 100 for each group. * = $P < 0.05$ Bonferroni's Multiple Comparison test, One-way ANOVA $P < 0.001$.

CHAPTER IV

DISCUSSION

The primary focus of these experiments was to identify and validate a 5-HT and *mod-5* dependent phenotype upon which to base a forward genetic screen for regulators of *mod-5* expression and activity, which may provide novel therapeutic targets, if conserved in man, for human disease states such as depression, anxiety, and obsessive compulsive disorder. The exogenous application of 5-HT has striking similarities to the application of food and affects the most obvious *C. elegans* phenotypes, resulting in increased pharyngeal pumping[52] and egg-laying[33], and decreased locomotion[33,35]. Our phenotype was based on our liquid swimming paradigm (SWIP[48]) and a previously characterized *mod-5* phenotype, the hyperenhanced slowing response[40]. The laborious process of screening thousands of animals underscores the importance of carefully selecting a phenotype and assembling genetic and pharmacological tools with which to screen potential mutants of interest identified from a screen. The following sections will discuss the role that *mod-5* activity plays in easily scored phenotypes and discuss their suitability as a basis for a forward genetic screen.

MOD-5 and food-dependent SWIP

We successfully characterized a novel, food-dependent phenotype in the *mod-5* strain of *C. elegans*, which our data suggests to be both 5-HT and *mod-5* dependent. This phenotype has been repeatedly identified by several individuals within our lab, and demonstrated to be easily discernable within a population of wildtype animals that supports its use within a forward genetic screen for regulators of *mod-5* transporter activity. This phenotype is rescued by genetic removal of the tryptophan hydroxylase gene, *tph-1*, and pharmacologically phenocopied in the wildtype animal by treatment with fluoxetine, supporting food-dependent immobilization as a *mod-5* dependent phenotype that relies on endogenous release of 5-HT. Animals lacking the dopamine transporter, *dat-1*, do not express this phenotype suggesting that elevated 5-HT, not DA, is essential for the food-dependent SWIP behavior. Supporting this contention, food-dependent SWIP can be blocked by application of the 5-HT antagonist methiothepin and by crossing *mod-5* mutant animals onto the *ser-1* metabotropic receptor null background, which further validates the role of serotonergic pathways within this phenotype. The ability to block food-dependent SWIP with an exogenous 5HT receptor blocker also provides a pharmacological paradigm with which to characterize mutants isolated from a screen for animals that immobilize in liquid food.

In a screen of 12,000 haploid genomes, within which an estimated six mutations in any given gene will be examined, mutagenized F2 animals displaying normal locomotory behavior on a solid substrate can be screened in

our liquid food swim assay in batches of at least 20 animals per assay. Mutants recovered in this screen can be propagated as clonal lines and rescreened using pharmacological agents blocking receptor activation (methiothepin) to validate the mutation is within *mod-5* or a *mod-5* regulatory gene. A mutant not displaying phenotypic reversal in the presence of methiothepin likely contains a mutation within a pathway outside of the 5-HT signaling network, or possibly could contain a post synaptic upregulation of a 5-HT receptor mutation for example, and would be discarded. All lines can then be outcrossed onto a wildtype background for at least 6 generations to rid the background of unwanted mutations, confirm the phenotype is the result of a mutation within a single gene and to characterize the transmission dynamics of the mutation. These mutants can also be examined for other phenotypes that would indicate a lack of *mod-5* function such as increased egg-laying, hypersensitivity to serotonin, and lack of sensitivity to fluoxetine[38].

Phenotypes that indicate a lack of mod-5 function may also be used as a tool to weed out confounding mutants isolated from our screen, further targeting our focus towards presynaptic mutations. *mod-5* null mutants display increased sensitivity to 5-HT induced immobilization[38] (see results, Fig 3-8, A, B, $P < 0.05$). This phenotype could be exploited by incubating a population of mutagenized animals on a plate containing 5-HT and isolating immobilized animals. However, isolated mutants may contain defects in 5-HT reuptake as well as body muscle formation and GABA and acetylcholine synthesis and release. To prevent isolation of animals with general mutations of the motor circuit, a locomotory-based screen should require animals to move to a particular area of the plate

before assessment of 5-HT induced immobilization, similar to the paradigm used to observe the enhanced slowing response. Methiothepin is also able to reverse this effect (Fig. A-1, B), providing additional support for this pharmacological tool to help identify novel regulators of 5-HT transporter trafficking and localization.

After secondary behavioral analyses, the *mod-5* region within each mutant can be sequenced and the effects of novel *mod-5* alleles can be examined on transporter localization and activity through *in vivo* fluorescent reporter imaging and expression in heterologous cell culture[44]. These studies could identify novel SERT regulatory sites enabling identification of novel SERT regulatory genes. Mutant lines determined to have a wildtype *mod-5* sequence should then be examined for mutations in potential *mod-5* regulatory genes. Complementation tests will identify lines with overlapping genetic disruptions; mutant lines will be crossed and wildtype behavior restored if the mutation is in a distinct gene. Crossing two lines with nonfunctioning alleles in the same gene will result in progeny expressing the same mutant phenotype as each of the founder lines. Construction of complementation groups will decrease the total number of crosses performed. Identification of disrupted genes within these mutant lines can then be performed using single-nucleotide polymorphism (SNP) mapping and whole genome sequencing approaches. These studies have the potential to help accelerate the identification of endogenous regulation of SERT expression and function supporting normal 5-HT reuptake, providing insight into the wiring of the nematode locomotory system and, ultimately, helping to treat many 5-HT linked disorders.

Conclusions

The unique in-depth knowledge of neuronal wiring and development in *C. elegans* paired with the elegant combination of genetic tractability and simplified behavior, makes the synaptically conserved nematode system amenable to many powerful approaches, particularly forward genetics. Until recently the effects of 5-HT in this system have been broadly examined through excessive exogenous application of 5-HT or a widespread loss of 5-HT synthesis. Recent characterizations of SERT-defective mutants provide phenotypes, particularly food-dependent immobilization, with which to investigate endogenous regulators of SERT and 5-HT signaling. Further characterization of these mutants may reveal additional phenotypes, including increased pharyngeal pumping, resistance to dauer entry and fat accumulation, to use in a screen which may reveal the impact of SERT alleles on 5-HT transport and turnover. These approaches may provide unbiased assessments of transporter regulatory molecules both in the worm and in man, potential novel drugable targets for the treatment of many 5-HT-related disorders, and help elucidate the genetic basis of behavior.

Although enhanced slowing has several characteristics making it amenable to a forward genetic screen including that it is 5-HT and *mod-5* dependent, and the behavior is pharmacologically mutable, the problems are the phenotype is not high-throughput, variable, and not inherently obvious (requires quantification of body bends executed per animal). Egg-laying is a 5-HT dependent phenotype frequently used in forward genetics because it is so easily

identifiable, however as noted in the appendix, our pharmacological data indicate cause us to question how MOD-5 activity is regulating egg-laying activity, and illustrate the often observed off-target effects of antidepressants in the nematode model system. Food-dependent slowing is both 5-HT and *mod-5* dependent, pharmacologically mutable, and also semi-high throughput and readily observed in the laboratory. This phenotype not only provides a mechanism to asses SERT regulatory molecules, but also can be used to characterize serotonergic signaling and locomotory pathways within the worm.

APPENDIX

MOD-5 and pharyngeal pumping

Nematodes feed by the peristaltic motion of the pharynx known as pharyngeal pumping, which serves to suck in and trap a slurry of bacteria within a bulbular extension of the pharynx which is then ground and pushed into the intestine[53]. Worms perform this motion about 40 times a minute in the absence of food and greater than 200 times a minute in the presence of food[52]. Traditional methods of measuring pharyngeal pumping involve manual quantification of pumping rates, hence this behavior is not frequently used in forward genetic screens. More sophisticated methods of quantifying pumping rates exploit the transparent nature of the worm, correlating pumping rate with an intake of a fluorescent reporter comparable in size to bacteria[50]. This paradigm is amenable to high-throughput methods but requires an initial investment in instrumentation capable of isolating and recording fluorescence from a single worm (Fig. A-1). Pharyngeal pumping is thought to be partly regulated by the two serotonergic neurosecretory motor neurons (NSMs) located in the anterior bulb of the pharynx (Fig. A-2). These are the most robustly stained serotonergic neurons within the animal that send processes to the region of the pharynx where bacteria accumulate, suggesting they are the “food sensing” neurons of the worm[53]. Exogenous 5-HT increases pharyngeal pumping[33], however laser ablation of the NSMs only modestly decreases pharyngeal pumping[52]. Further ablation of all neurons within the pharynx except M4 causes only minor deficits in pharyngeal

pumping[52], suggesting that an intrinsic pacemaker ability may exist within pharyngeal muscle cells and that most pharyngeal neurons are dispensable under standard laboratory conditions. Interestingly, *tph-1* mutants show wildtype pumping rates in the absence of food but deficient pumping in the presence of food[24] demonstrating serotonin is not required for basal pumping activity. *mod-5* mutants are expected to show increased pharyngeal pumping for which there is a much smaller potential pool of confounding mutants than other phenotypes. Further investigation will demonstrate the potency of this phenotype and role of *mod-5* in this behavior that has the potential to provide a basis for a screen to elucidate regulators of SERT expression and function.

MOD-5 and egg-laying

Egg-laying is one of the most popular phenotypes for genetic screens in *C. elegans* because abnormal egg-laying is easily observable in the laboratory with simple visual inspection. The effects of 5-HT and other pharmacological agents on egg-laying are readily examined by incubating a single animal in buffer containing drug and counting the number of eggs laid after a short period. Mutant animals incapable of egg-laying are easily identified within a large population as they become bloated with eggs retained in the uterus, a phenotype known as "*egl*" or more colorfully as "bag of worms," which describes the process of egg-hatching within the adult animal. Egg-laying is regulated by activity of the HSNs (Fig. A-3) and VCs, both which innervate the vulval muscle[25, 39, 40, 54]. Mutant hermaphroditic animals in which the HSNs undergo cell death display an *egl*

phenotype[21], and observation that this mutation confers resistance to fluoxetine and imipramine induced egg-laying[55] indicates a modulatory role for *mod-5* at the HSNs in egg-laying. Consistent with the hypothesis that *mod-5* mutants express increased synaptic 5-HT, *mod-5* mutants are hypersensitive to the presence of 5-HT and lay more eggs than wildtype at a given 5-HT concentration[38]. The biggest difference between the two groups lies at a modest concentration of 5-HT (~6mM) where a wildtype worm will lay between 0 and 14 eggs within an hour, (on average about 2.5 eggs) and a *mod-5* animal under the same conditions will lay between 0 and 17 eggs, with an average of 10 eggs (unpublished data, Fig. A-4). Based on the variability observed in individual egg-laying responses, screening a mutant population for the *mod-5* egg-laying phenotype requires either generating an average egg-laying profile for clonal populations of mutagenized F2 animals (instead of assaying single mutants), thereby increasing the number of total experiments by 10-fold or the number of false positives recovered. Alternatively a screen could be envisioned utilizing the effects of SSRIs on the egg-laying system, where wildtype animals would be expected to lay eggs in response to fluoxetine whereas fail to induce egg-laying in *mod-5* mutants. However application of the antidepressants fluoxetine, imipramine, and clomipramine to both *mod-5* and *tph-1* animals results in egg-laying similar to that observed in wildtype[56] indicating these antidepressants activate alternative targets within the worm, possibly the 5-HT receptors themselves[56]. Thus, although SSRI-induced egg-laying is HSN dependent, it is 5-HT and *mod-5* independent. Together these studies indicate the egg-laying circuitry as well as

the influence of *mod-5* on egg-laying is more complex than initially envisioned and not yet fully understood. There are multiple levels for modulation of egg-laying, from neurons in the head to the vulval muscle, and the level at which the action *mod-5* most significantly influences egg-laying is unclear. The off-target effects of SSRIs in *C. elegans*[57] limit the potential egg-laying phenotypes of *mod-5* mutants for use in forward genetic screens and the use of these drugs to examine the integrity of *mod-5* and HSN function. However, egg-laying remains an easily identifiable, semi-high throughput, and well characterized phenotype that may be utilized to examine candidate regulatory genes controlling SERT trafficking, localization, and activity.

A

B

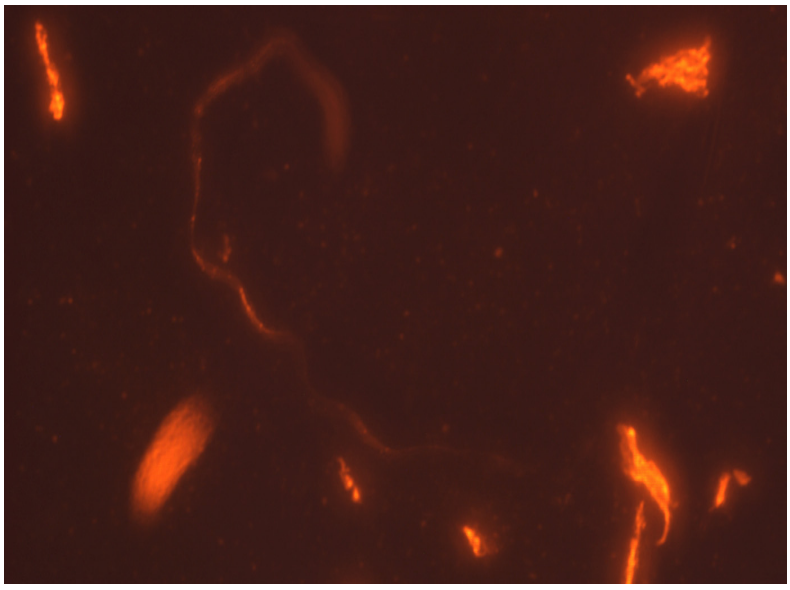

Figure A-1. Quantum dot labeling of the *C. elegans* digestive tract. *A*, White field image of *C. elegans*. *B*, Cy3 fluorescence image of *C. elegans* detecting quantum dots within digestive tract.

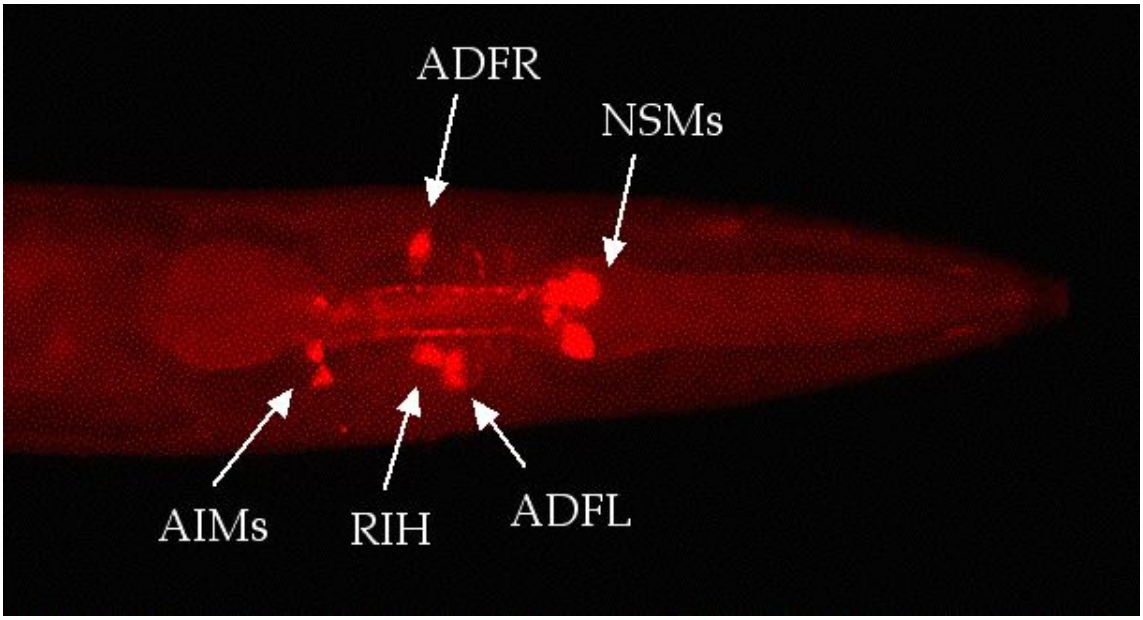

Figure A-2. Anti-5-HT immunofluoresence in the *C. elegans* head neurons. Photo courtesy of the Loer Lab: http://home.sandiego.edu/~cloer/loerlab/5-HTcells.html

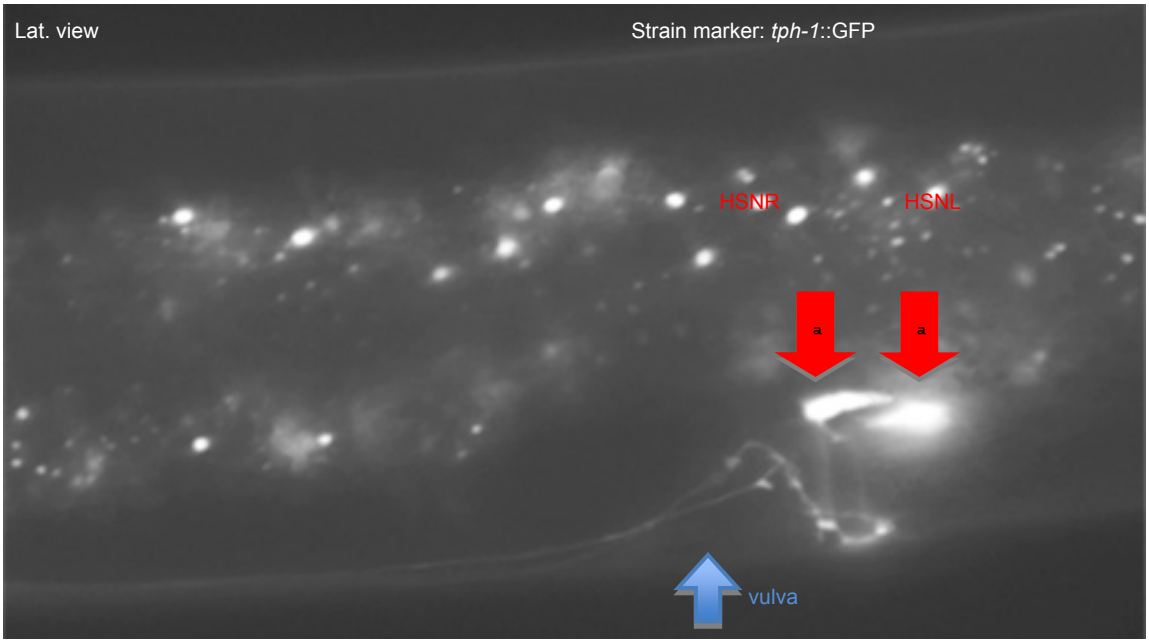

Figure A-3. GFP imaging of the *C. elegans* vulva revealing left and right HSN cell bodies with axons synapsing the vulval musculature. Anterior is left, ventral is down. Nonspecific fluorescence in anterior and dorsal areas is gut autofluoresence. Reprinted with permission from Wormatlas (www.wormatlas.org).

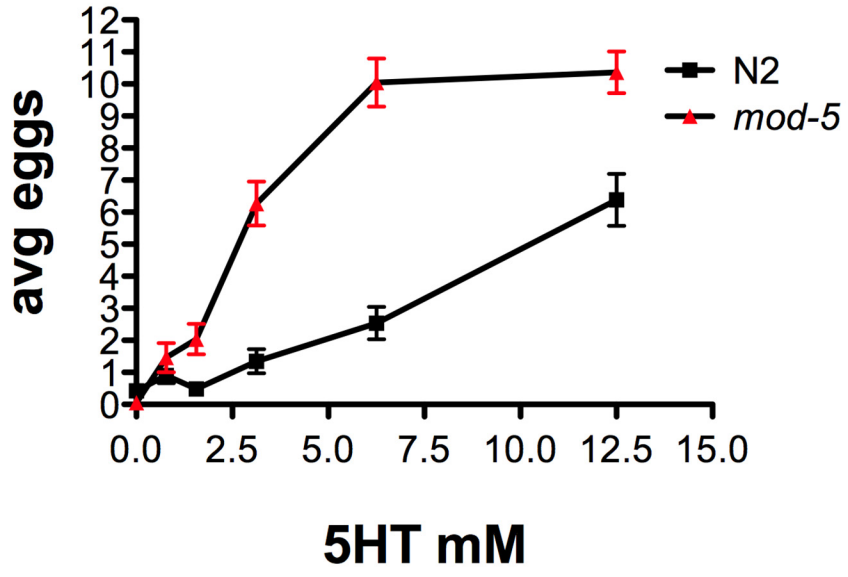

Figure A-4. Average egg-laying response of wildtype and SERT-defective mutants in increasing concentrations of 5-HT. n = 50 for each data point.

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