Getting beyond Prozac: A *C. elegans* approach

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Since its debut in 1986 the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Prozac[TM]) has 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 its pervasiveness 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[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 (SERT, 5-HTT, SLC6A4)[3, 4], a transmembrane protein that is a major target of psychostimulants such as MDMA ("ecstasy") as well as many antidepressants such as fluoxetine[5,2]. The monoamine neurotransmitter 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 is 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 in modulation of 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 behaviorally straightforward model organism *Caenorhabditis elegans* (*C. elegans*). Although unsuitable for modeling most human disease states, this model system offers approaches that are 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 identify genes responsible for behavior. This review describes the power of forward genetics in this model organism to investigate the mechanisms regulating 5-HT transporter activity by examining the role of 5-HT and SERT in *C. elegans* behavior, particularly how these behaviors may serve as the basis for a forward genetic screen.

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C. elegans AND FORWARD GENETICS

The nematode *C. elegans* is an excellent model system 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**). In addition, there are a plethora of well-developed techniques for studying this organism including genetics, biochemistry, primary cell cultures, and RNAi technology. *C. elegans* are easily cultivated in the laboratory, withstand cryopreservation, and in sub-optimal environmental conditions (such as prolonged starvation) maintain a metabolically inactive state known as dauer arrest for months. Each individual *C. elegans* contains a numerically and morphologically invariant 959 cells, including 302 neurons, enabling lineage mapping for each cell[27, 28] and reconstruction of the entire animal by serial electron micrograph[29]. These provide 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 it an optimal organism for forward genetic approaches. Hermaphroditic reproduction permits line and mutation propagation without staged crosses and also simplifies isolation of homozygous mutants, thus random mutagenesis of a parental group of animals yields 25% of F2 progeny that are homozygous for any given mutation. These mutants are then screened for 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 pathway. Screens isolating mutants that phenocopy a known mutant, such as abnormal egg-laying and touch
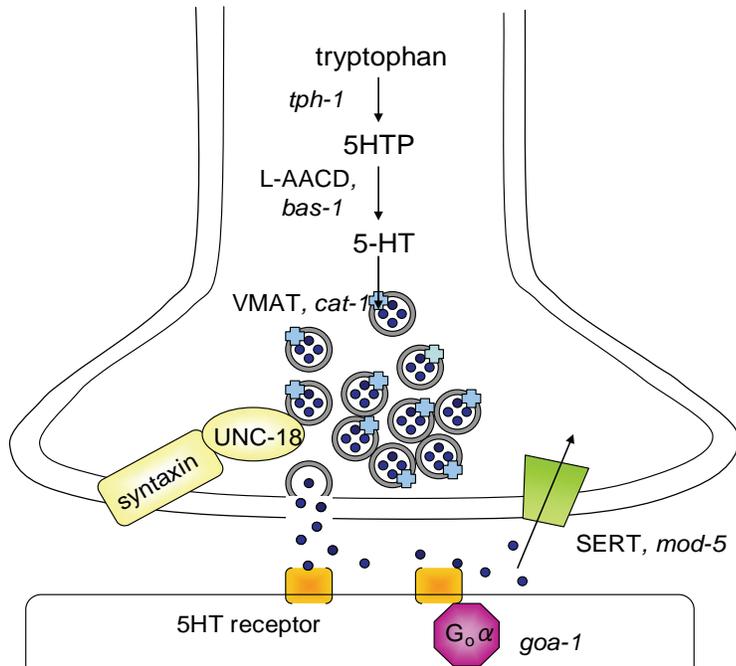
Figure 1 | 5-HT biosynthesis is conserved from *C. elegans* to man[18]. 5-HT is packaged into vesicles (grey spheres) through the activity of a vesicular monoamine transporter (VMAT, *cat-1*[19], pale blue plus). Synaptic vesicle release is facilitated by the well conserved SNARE complex (yellow), many of the components of this complex include the two illustrated above (UNC-64/syntaxin, UNC-18/nSec-1) were originally identified in *C. elegans*[20, 21]. As in mammals *C. elegans* 5-HT receptors are divided into metabotropic (*ser-1*[22], *ser-4*[23], *ser-5*[24], and *ser-7*[25] coupled to Gαq, Gαo, Gαs and Gαs respectively), and ionotropic (*mod-1*[26]) categories.

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[31]. 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 these circuits. The actions of 5-HT within *C. elegans* provides insight into the potential phenotypes expressed by SERT-defective animals (which theoretically express elevated synaptic 5-HT) which may then be exploited in a screen for genes controlling SERT trafficking, localization, and activity.

C. elegans AND 5-HT

In *C. elegans* (**Figure 2a**) 5-HT is an active participant in a variety of motor and autonomic behaviors. Application of exogenous 5-HT mimics the presence of food resulting in increased egg-laying and pharyngeal pumping (the nematode feeding mechanism) and decreased locomotion[33]. Animals deficient in 5-HT synthesis display decreased male mating efficiency[34], increased reproductive lifespan, increased fat storage, increased dauer arrest, decreased egg-laying[18], 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 (**Figure 2b**, **Table 1**), four of which are located in the head of the animal (see expanded view page 3). 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[18]. Serotonergic neurons not expressing *tph-1* are presumed to obtain their serotonin through activity of the *C. elegans* serotonin transporter, *mod-5*, although this requires further investigation.
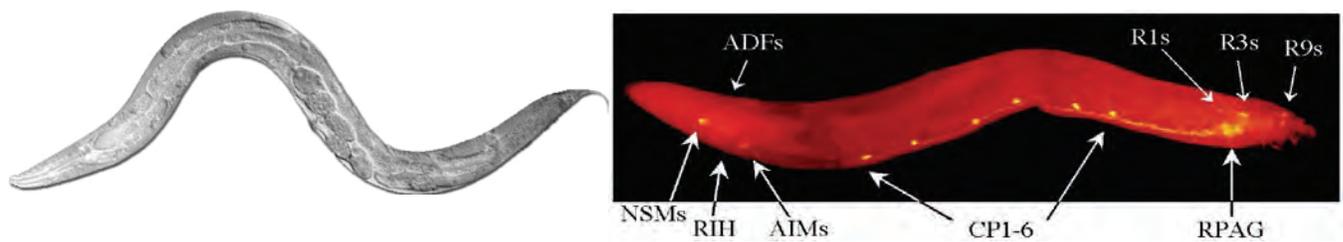
MOD-5

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[30-32]. 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

Figure 2 | *C. elegans*. a | Nomarski image of adult *C. elegans*. Image courtesy of the Hardin Lab. **b |** Anti-5-HT immunofluoresence of adult male *C. elegans*. Image courtesy of the Loer Lab.

Table 1 | **Serotonergic neurons in _C. elegans_.**

Class	Type	Location	Function
NSMs[38] (2, bilaterally symmetric)	Neurosecretory Motor Neuron	Anterior bulb of pharynx	Pharyngeal pumping
ADFs[19] (2, bilaterally symmetric)	Amphid sensory neuron	Nerve ring	Dauer entry
AIMs[19] (2, bilaterally symmetric)	Interneuron	Nerve ring	Unknown
RIH[19] (unpaired)	Interneuron	Nerve ring	Unknown
HSNs[39] (2, bilaterally symmetric)	Motor neuron, Hermaphrodite Specific	Vulva	Egg-laying
VC4, VC5[19] (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

The _C. elegans_ serotonin transporter (_mod-5_) gene encodes a protein with 44% amino acid identity with mammalian SERT proteins that confers paroxetine-sensitive 5-HT transport on nonneuronal cells after heterologous expression[40]. _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 which can be blocked by selective serotonin reuptake inhibitor (SSRI) fluoxetine[40]. _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[40, 41]. The effects of exogenous 5-HT and behaviors in animals lacking of 5-HT synthesis indicate _mod-5_ mutants might be expected to express dauer entry resistance and increased pharyngeal pumping, although this has not yet been characterized. In the following sections we will discuss the role of _mod-5_ activity within a selection of these phenotypes easily scored in the laboratory to ascertain their suitability as a basis for a forward genetic screen.

MOD-5 AND PHARYNGEAL PUMPING

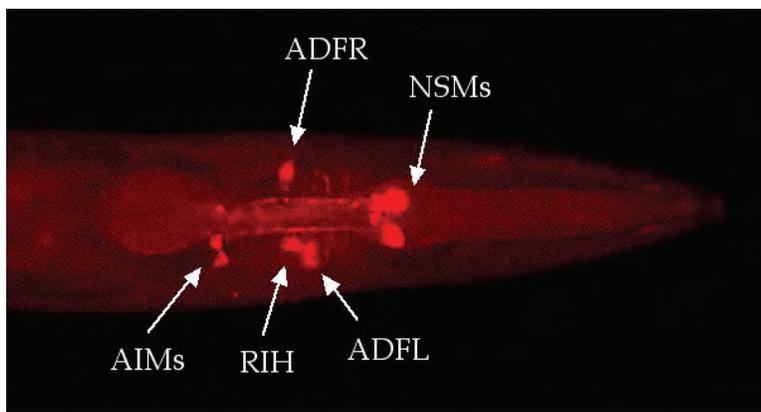Nematodes feed by the peristaltic motion of the pharynx known as pharyngeal pumping, which serves to suck in and trap a slurry of bacteria within a bulbular extension of the pharynx, which is then ground and pushed into the intestine[42]. Worms perform this motion about 40 times a minute in the absence of food and greater than 200 times a minute in the presence of food[43]. 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[44]. 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. Pharyngeal pumping is thought to be partly regulated by the two serotonergic neurosecretory motor neurons (NSMs) located in the anterior bulb of the pharynx (**Figure 3**). These are the most robustly stained serotonergic neurons within the animal and send processes to the region of the pharynx where bacteria accumulate, suggesting they are the "food sensing" neurons of the worm[42]. Exogenous 5-HT increases pharyngeal pumping[33], however laser ablation of the NSMs only modestly decreases pharyngeal pumping[43]. Further ablation of all neurons within the pharynx except M4 causes only minor deficits in pharyngeal pumping[43], 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[18] 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.
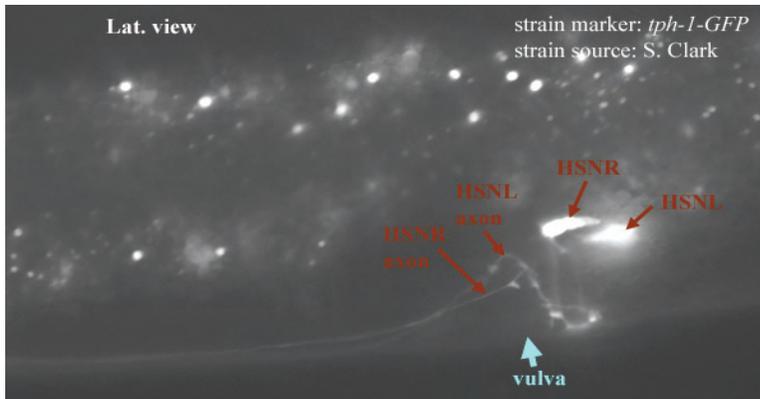

Figure 3 | **Anti-5-HT immunofluorescence in the _C. elegans_ head neurons.** Photo courtesy of the Loer Lab: http://home.sandiego.edu/~cloer/loerlab/5-HTcells.html

Figure 4 | **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. Photo courtesy of wormbase.org.

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 manual techniques. 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 (**Figure 4**) and VCs, both which innervate the vulval muscle[19, 38, 39, 45]. Mutant hermaphroditic animals in which the HSNs undergo cell death display an *egl* phenotype[30], and this mutation confers resistance to fluoxetine and imipramine induced egg-laying[46], which 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[40]. 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, **Figure 5**). 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, yet the drug would 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[47] indicating these antidepressants activate alternative targets within the worm, possibly the 5-HT receptors themselves[47]. Therefore, 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. There are multiple levels for modulation of egg-laying, from neurons in the head to the vulval muscle, thus the level at which the action *mod-5* most significantly influences egg-laying is unclear. The off-target effects of SSRIs in *C. elegans* 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 which may be utilized to examine regulatory genes controlling SERT transporter trafficking, localization, and activity.

MOD-5 AND LOCOMOTION

Abnormal locomotor activity is another *C. elegans* behavior easily observed 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. Application of exogenous 5-HT results in decreased locomotion[33] and *mod-5* null mutants display increased sensitivity to 5-HT induced immobilization[40]. This phenotype could be exploited by incubating a population of mutagenized animals on a plate containing 5-HT and isolating immobilized animals. However, isolated
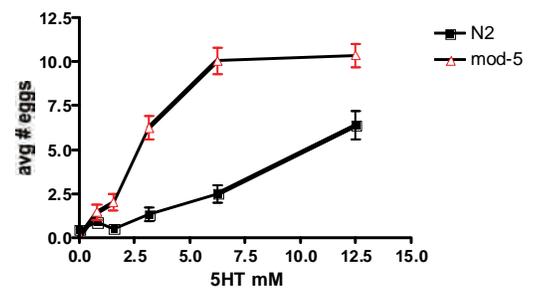

Figure 5 | **Average egg-laying response of wildtype and SERT-defective mutants in increasing concentrations of 5-HT.** n=50 for each data point.

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. Animals starved for a brief period (30 min) display a normal locomotor rate which dramatically slows upon encountering a bacterial lawn (enhanced slowing[35]), a trait evolved presumably to protect the animal from starvation. This is observed in the laboratory by manually quantifying the locomotor 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 5-HT, suggesting this behavior is modulated presynaptically. 5-HT synthesis mutants completely lack this response, a deficit that is rescued by the application of exogenous 5-HT[35]. Enhanced slowing is blocked by 5-HT receptor antagonists mianserin and methiothepin[35] further supporting the role of 5-HT in this behavior, and is potentiated by fluoxetine[40], suggesting this response is a direct measure of *mod-5* activity. *mod-5* mutants display wildtype locomotory rates under standard laboratory conditions and exhibit a hyper-enhanced slowing response. 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[40]. 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 and 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.

SUMMARY

The unique in-depth knowledge of neuronal wiring and development paired with the elegant combination of genetic tractability and simplified behavior makes the synaptically conserved *C. elegans* 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 pumping and locomotion, with which to investigate endogenous regulators of SERT and 5-HT signaling. Further characterization of these mutants may reveal additional phenotypes, including 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.

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FURTHER INFORMATION

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