

Receptors and Other Signaling Proteins Required for Serotonin Control of Locomotion in *Caenorhabditis elegans*

Güliz Gürel,^{*1} Megan A. Gustafson,^{*1} Judy S. Pepper,^{*1} H. Robert Horvitz,[†] and Michael R. Koelle^{*2}

^{*}Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06520, and [†]Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

ABSTRACT A better understanding of the molecular mechanisms of signaling by the neurotransmitter serotonin is required to assess the hypothesis that defects in serotonin signaling underlie depression in humans. *Caenorhabditis elegans* uses serotonin as a neurotransmitter to regulate locomotion, providing a genetic system to analyze serotonin signaling. From large-scale genetic screens we identified 36 mutants of *C. elegans* in which serotonin fails to have its normal effect of slowing locomotion, and we molecularly identified eight genes affected by 19 of the mutations. Two of the genes encode the serotonin-gated ion channel *MOD-1* and the G-protein-coupled serotonin receptor *SER-4*. *mod-1* is expressed in the neurons and muscles that directly control locomotion, while *ser-4* is expressed in an almost entirely non-overlapping set of sensory and interneurons. The cells expressing the two receptors are largely not direct postsynaptic targets of serotonergic neurons. We analyzed animals lacking or overexpressing the receptors in various combinations using several assays for serotonin response. We found that the two receptors act in parallel to affect locomotion. Our results show that serotonin functions as an extrasynaptic signal that independently activates multiple receptors at a distance from its release sites and identify at least six additional proteins that appear to act with serotonin receptors to mediate serotonin response.

DEPRESSION is hypothesized to involve dysfunction of the neurotransmitter serotonin (Cowen 2008). Understanding the molecular mechanism of serotonin signaling is complicated by the fact that the human brain expresses 14 types of serotonin receptors, one of which is a serotonin-gated ion channel, and the rest of which are G-protein-coupled receptors (Millan *et al.* 2008). An additional challenge to understanding serotonin signaling is the fact that serotonin can act locally at synapses where it is released or diffuse away and act at distant receptors. While classical neurotransmitters such as GABA and glutamate appear to function mainly locally at synapses, serotonin can diffuse several microns from its release sites at concentrations sufficient to activate its receptors (Bunin and Wightman 1998). Further-

more, serotonin receptors are often localized at nonsynaptic sites (Kia *et al.* 1996). These observations suggest that serotonin might act predominantly as an extrasynaptic signal to activate several receptor types on cells distant from its release sites and that the combined action of these several receptors somehow coordinates appropriate responses to serotonin. The details of how such action might occur remain unclear.

Caenorhabditis elegans uses serotonin as a neurotransmitter (Horvitz *et al.* 1982; Chase and Koelle 2007) and provides a model system with the potential to make important contributions to the study of serotonin signaling. First, *C. elegans* allows the use of forward genetic screens to identify the proteins beyond serotonin receptors that mediate serotonin response. Second, the known synaptic wiring of the *C. elegans* nervous system (White *et al.* 1986) provides the opportunity to directly test whether serotonin acts at synapses or extrasynaptically.

Previous studies of *C. elegans* have shown that, of the precisely 302 neurons found in an adult hermaphrodite, the *NSM*, *HSN*, *ADF*, *VC4/5*, *AIM*, and *RIH* neurons contain serotonin, although the *AIM*, *RIH*, and possibly also the *VC4/5* neurons do not synthesize serotonin themselves but

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¹These authors contributed equally to this work.

²Corresponding author: Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, SHM CE30, New Haven, CT 06520-8024.

E-mail: michael.koelle@yale.edu

rather take up serotonin made by the other neurons (Horvitz *et al.* 1982; Sawin *et al.* 2000; Jafari *et al.* 2011). Animals with mutations in the enzymes that synthesize serotonin or in which the NSMs have been ablated are defective for the strong reduction in locomotion behavior, known as enhanced slowing, that occurs when food-deprived animals encounter food (Sawin *et al.* 2000). This observation suggests that the NSMs release serotonin to slow locomotion. While enhanced slowing is only one of several behaviors known to be controlled by serotonin in *C. elegans* (Chase and Koelle 2007), it is amenable to genetic analysis, and direct genetic screens for mutants defective in enhanced slowing behavior have succeeded in isolating mutants defective for serotonin signaling (Sawin *et al.* 2000). However, assays for this behavior are relatively tedious. In a second approach, mutants carrying gene knockouts for all five *C. elegans* serotonin receptor homologs have been tested for defects in the ability of serotonin to slow locomotion (Hapiak *et al.* 2009). Several of the receptor knockouts cause defects in this behavior, but basic issues about the functions of these receptors have remained unresolved. How do the receptors act together to slow locomotion? Do they act at sites distant from serotonin release sites? Do the various receptors act in the same or different cells? What other signaling proteins act with the receptors to mediate serotonin response?

In this study, we developed genetic screens for *C. elegans* mutants defective for the effects of serotonin on locomotion. We identified 36 mutants, found the relevant molecular lesions in 19 of them, and thus identified eight proteins required for serotonin response. Among these proteins were two serotonin receptors, **MOD-1** and **SER-4**. Since understanding how the remaining signaling proteins affect serotonin response first requires understanding the function of the two receptors, we analyzed how **MOD-1** and **SER-4** affect locomotion. Our results suggest that the two receptors act in parallel in different cells to mediate different effects of extrasynaptic serotonin on locomotion.

Materials and Methods

Nematode culture

C. elegans strains were maintained at 20° under standard conditions, and double- and triple-mutant strains were generated using standard genetic techniques (Brenner 1974). All strains and transgenes used in this study are described in Supporting Information, File S5, Table S1, and Table S2.

Genetic screens

In a clonal screening strategy, fourth-stage larval (L4) wild-type hermaphrodites were mutagenized (Brenner 1974) using ethyl methanesulfonate (EMS), and F₁ progeny were grown from eggs harvested after bleaching the P₀ gravid adults. First larval-stage F₁ animals were diluted to 1/100 μl in S medium (Sulston and Hodgkin 1988) supplemented with penicillin and streptomycin (final concentrations 100 units/ml and 100 μg/ml,

respectively; Gibco 15140-122), nysatin (final concentration 100 units/ml; Sigma N1638), and *Escherichia coli* strain HB101 diluted to a final OD₅₅₀ = 2.4. A 12-channel pipette was used to distribute 50 μl/well into 96-well plates. Wells containing >1 F₁ were marked and not screened. Plates were placed in a plastic container with a loose-fitting lid containing wet paper towels and incubated for 6 days at 20° until mixed-stage F₂ progeny were present but the cultures were not yet starved. A total of 100 μl of 45 mM serotonin (5-hydroxytryptamine creatine sulfate complex; Sigma H-7752) was added to each well. The supersaturated 45-mM serotonin stock solution was prepared by adding serotonin powder to S medium lacking trace metals, heating in a water bath to 80° for 5 min, vortexing, cooling to room temperature, and using within 30 min.

For nonclonal genetic screens, *mod-5(n3314)* L4 hermaphrodites were mutagenized using EMS or the *MosI* transposon (Boulin and Bessereau 2007). Five P₀ animals were transferred to each of several 10-cm diameter NGM plates (Brenner 1974) and allowed to produce F₁ progeny for 3–5 days at 20°. F₁ progeny were bleached to collect eggs. Approximately 300 eggs from each 10-cm plate were spotted to 6-cm-diameter NGM plates and grown at 20° for 2 days. These F₂ progeny were transferred to 24-well cell culture plates containing 20 or 60 mM serotonin dissolved in M9 buffer (Sulston and Hodgkin 1988) and assayed for movement 5 min later. Moving animals were transferred from wells using a mouth pipette to individual 6-cm NGM plates, and their progeny were retested for serotonin resistance.

Measurement of the serotonin-resistance phenotype in liquid

Serotonin-resistance measurements in Table 1 and Figure 2A were performed in M9 buffer in microtiter wells containing serotonin. Serotonin was prepared as a 45-mM stock solution in M9 by heating to 80°, vortexing, and cooling as described above. Animals were isolated at the late L4 stage based on the morphology of the developing vulva and cultured for 24 hr at 20° to produce young adults. Animals (typically 25–33 animals per well) were transferred with a platinum-wire worm pick to microtiter wells containing 50 μl M9 buffer. A 12-channel pipette was used to add serotonin stock solution to the final desired concentration, and 20 min later wells were scored for the number of animals “moving,” defined as having smooth swimming movements of the entire body. Animals showing only movements of the head or only stiff or jerky movements of ≤50% of the body were scored as “not moving.”

Serotonin-resistance measurements shown in Figure 2, B and C were performed in an analogous fashion, except that the young adult animals were picked directly into microtiter wells containing various concentrations of serotonin dissolved in water and were scored for movement 20 min later. We found that ~30-fold less serotonin is sufficient to paralyze *C. elegans* when it is applied in water rather than in M9 buffer, analogous to the similarly large increase in effectiveness of

Table 1 Isolation and characterization of serotonin-resistant mutants

Serotonin resistance mutation ^a	Chromosome ^b	Genetic background ^c	Serotonin in screen (mM) ^d	Mutagen	Serotonin in assay (mM) ^e	% moving in assay ^f
None		Wild type			30	4
None		<i>mod-5</i>			5	4
None		<i>mod-5</i>			15	1
None		<i>mod-5</i>			30	0
<i>goa-1(vs115)</i>	I	Wild type	30	EMS	30	98
<i>goa-1(vs134)</i>	I	Wild type	30	EMS	30	74
<i>goa-1(n4093)</i>	I	<i>mod-5</i>	20	Mos1	5	58
<i>goa-1(n4402)</i>	I	<i>mod-5</i>	60	EMS	15	15
<i>goa-1(n4405)</i>	I	<i>mod-5</i>	60	EMS	15	88
<i>goa-1(n4439)</i>	I	<i>mod-5</i>	60	EMS	15	100
<i>goa-1(n4492)</i>	I	<i>mod-5</i>	60	EMS	15	100
<i>goa-1(n4493)</i>	I	<i>mod-5</i>	60	EMS	15	95
<i>goa-1(n4494)</i>	I	<i>mod-5</i>	60	EMS	15	83
<i>eat-16(n4403)</i>	I	<i>mod-5</i>	60	EMS	15	83
<i>abts-1(n4094)</i>	I	<i>mod-5</i>	20	Mos1	5	90
<i>n3498</i>	I	<i>mod-5</i>	20	EMS	5	69
<i>n3792</i>	I	<i>mod-5</i>	20	EMS	5	43
<i>n4404</i>	I	<i>mod-5</i>	60	EMS	15	72
<i>n3461</i>	II	<i>mod-5</i>	20	EMS	5	86
<i>n3488</i>	II	<i>mod-5</i>	20	EMS	5	40
<i>n3495</i>	II	<i>mod-5</i>	20	EMS	5	35
<i>n3775</i>	II	<i>mod-5</i>	20	EMS	5	80
<i>ser-4(vs122)</i>	III	Wild type	30	EMS	30	66
<i>emb-9(vs114)</i>	III	Wild type	30	EMS	30	20
<i>flp-1(n4491)</i>	IV	<i>mod-5</i>	60	EMS	15	83
<i>flp-1(n4495)</i>	IV	<i>mod-5</i>	60	EMS	15	100
<i>mod-1(vs107)</i>	V	Wild type	30	EMS	30	61
<i>mod-1(n3791)</i>	V	<i>mod-5</i>	20	EMS	30	100
<i>mod-1(n4054)</i>	V	<i>mod-5</i>	20	Mos1	5	86
<i>n3510</i>	V	<i>mod-5</i>	20	EMS	30	45
<i>n3774</i>	V	<i>mod-5</i>	20	EMS	30	28
<i>n3799</i>	V	<i>mod-5</i>	20	EMS	10	34
<i>elpc-3(vs119)</i>	V	wild type	30	EMS	30	70
<i>n3477</i>	X	<i>mod-5</i>	20	EMS	5	49
<i>vs113</i>	Unmapped	Wild type	30	EMS	30	12
<i>vs108</i>	Unmapped	Wild type	30	EMS	30	15
<i>vs111</i>	Unmapped	Wild type	30	EMS	30	16
<i>vs116</i>	Unmapped	Wild type	30	EMS	30	19
<i>vs120</i>	Unmapped	Wild type	30	EMS	30	20
<i>vs121</i>	Unmapped	Wild type	30	EMS	30	21

^a Allele designations without gene names are given for the 17 mutations not assigned to a gene.

^b Six weak mutations at bottom ("Unmapped") have not been genetically mapped and thus are not assigned to a chromosome.

^c *mod-5* denotes that the genetic screen and assays were performed in the *mod-5(n3314)* genetic background.

^d Concentration of serotonin in buffer used in the genetic screen that initially isolated the mutation.

^e Concentration of serotonin in M9 buffer in which mutant animals were assayed. The strains listed as "None" carried no serotonin resistance mutations and serve as controls.

^f Data averaged from two to three separate assays of 20–40 animals each.

exogenous dopamine on *C. elegans* when it is applied in water rather than M9 buffer (Schafer and Kenyon 1995). We thus performed quantitative assays of response to serotonin in water or on agar dissolved in pure water (see below) so that serotonin could be used to affect behavior at concentrations below its solubility limit, producing more reliable assays.

Measurement of serotonin resistance after optogenetic activation of endogenous serotonin release

B broth inoculated with OP50 bacteria (Sulston and Hodgkin 1988) was cultured overnight at 37° without shaking, and just before use 4 μl of 100 mM all-*trans* retinal (Spectrum Chemical, #R3041) in 100% ethanol was added per 1 ml.

Standard NGM plates were seeded with 200 μl of this mix. Plates were incubated in a light-tight box overnight at room temperature to allow a bacterial lawn to grow. Control plates were made with no *trans*-retinal. Twenty-four hours before the assay, late L4 animals were picked onto freshly prepared retinal or control plates and incubated at 20° overnight in the dark. Thirty minutes prior to assay, unseeded NGM assay plates were ringed with an osmotic barrier to prevent worms from leaving the agar surface by placing a 70-μl drop of 4M fructose on the edge of each plate and tilting the plate around in a circle. For the assay, individual worms not on the OP50 lawn were picked to separate fructose-ringed NGM plates using a bare pick. Ten minutes after transfer, each plate was

moved to a Zeiss M²BIO fluorescence dissecting scope, handling the plates gently and leaving the lids in place to avoid mechanically stimulating the animals. Animals were illuminated with 1.2 mW/mm² of 480 nm light, as measured with a Solar Meter model 9.4 blue light radiometer (Solartech Inc.). The number of body bends was counted during the first 30 sec of blue light exposure. A body bend was defined as “a maximum flexure of the body just behind the pharynx in the opposite direction as the previously scored bend.” ≥15 animals per condition were tested with the experimenter blinded to the genotype, and the exp was repeated three times. Figure 4 shows the results from one representative experiment.

Locomotion-slowness assays

Slowness assays were performed as described by Sawin *et al.* (2000). Measurements shown are averaged from assays repeated over 2–9 days with samples totaling at least 20 animals for the no-food assays and at least 40 animals for the + food assays.

Gene identification

Serotonin-resistance mutations were mapped to regions of individual chromosomes using a combination of conventional marker mutations and single-nucleotide polymorphisms (Fay 2006). For mutations that mapped to regions including genes known to mutate to the serotonin-resistant phenotype (*goa-1*, *eat-16*, *ser-4*, *mod-1*, and *flp-1*), we performed complementation tests with mutations in these genes and also amplified the candidate genes from the mutant strains and used DNA sequencing to identify mutant lesions (Table S1). The mutation *emb-9(vs114)*, in addition to causing weak serotonin resistance, caused a temperature-sensitive lethal phenotype that facilitated mapping and complementation tests. *elpc-3(vs119)* was mapped to an ~40-kb interval using single-nucleotide polymorphisms, and all genes within that interval were amplified and then sequences were determined from the mutant strain to identify the *elpc-3* lesion. For the three *Mos1* transposon insertion alleles, we used inverse PCR amplification of genomic DNA flanking the insert and DNA sequencing to determine that the insertions were in *goa-1*, *mod-1*, and *abts-1*.

Fluorescence microscopy

C. elegans was immobilized with 10 mM levamisole, and a Zeiss LSM 710 confocal microscope was used to obtain Z-stack images. The 3D reconstructions seen in Figure 3, Figure S1, and File S3 were created using Volocity software (Improvision).

Results

Genetic screens for mutants with defects in response to exogenous serotonin

When *C. elegans* is placed in liquid medium it continuously flexes its body (File S1), and we found that addition of

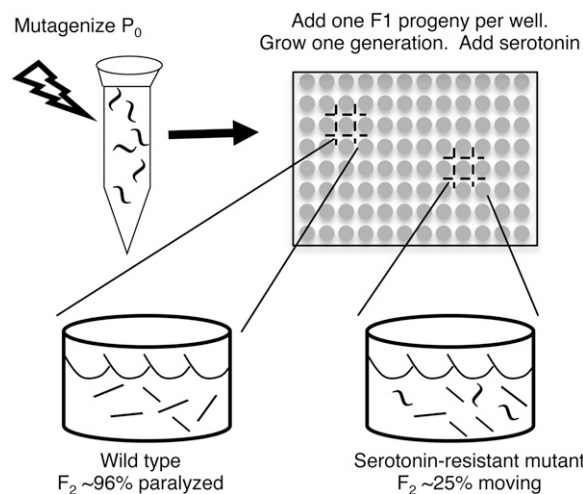


Figure 1 Schematic diagram of one of four genetic screens used to isolate serotonin-resistant mutants of *C. elegans*. Individual F₁ progeny of mutagenized animals, each heterozygous for different mutations, were cultured in microtiter wells. Each F₁ self-fertilized to produce a brood of F₂ progeny. Serotonin (30 mM) was added to paralyze ~96% of animals in most wells (bottom left). Rare wells (bottom right) had ~25% F₂ progeny that continued to move vigorously because they were homozygous for a mutation causing serotonin resistance.

serotonin reduces these movements in a dose-dependent manner such that high concentrations eliminate almost all body bends except for small movements of the head (File S2).

We performed several genetic screens for serotonin-resistant mutants that continue to move at high doses of exogenous serotonin. In one “clonal” strategy, schematized in Figure 1, individual F₁ progeny of mutagenized animals were cultured for one generation in separate microtiter wells so that each well contained a brood of F₂ animals sharing the same mutations. Serotonin (30 mM) was added to the cultures, and wells containing >20% serotonin-resistant animals were identified. We thus screened 6000 wells, representing 12,000 mutagenized haploid genomes, and identified 12 serotonin-resistant mutants.

We performed additional screens in which F₁ progeny were cultured in groups rather than individually, and their F₂ progeny were screened en masse for individuals that were not paralyzed by 20 or 60 mM serotonin. This “non-clonal” strategy allowed animals representing many more mutagenized genomes to be screened but was complicated by the fact that, since even high concentrations of serotonin do not paralyze 100% of wild-type animals, a large number of false positives might be initially isolated that would then have to be discarded during subsequent retesting. To minimize this problem, the nonclonal screens were performed using a strain mutant for the serotonin reuptake transporter (SERT) *MOD-5* (Ranganathan *et al.* 2001). SERT acts to terminate serotonin signaling by removing serotonin from the extracellular space, so a SERT-defective mutant has elevated serotonin signaling. We found that serotonin at concentrations >20 mM can paralyze nearly 100% of *mod-5* mutant animals. The nonclonal screens used either a chemical mutagen

(a total 42,000 mutagenized haploid genomes from screens performed at 20 or 60 mM serotonin) or *Mos1* transposon mutagenesis (63,000 mutagenized haploid genomes screened at 60 mM serotonin). We thus isolated 24 more mutants. Table 1 lists details of all 36 serotonin-resistant mutants identified.

Serotonin-resistance mutations identify eight proteins, including two serotonin receptors, that affect response to exogenous serotonin

To identify the genes affected by the serotonin-resistance mutations, we performed a combination of genetic mapping, complementation tests, inverse PCR amplification in the case of transposon-insertion mutations, and PCR amplification of candidate genes followed by DNA sequence determination in the case of chemically induced mutations. We thus mapped 30 of the 36 mutations to specific chromosomes and subsequently identified the molecular lesions causing serotonin resistance in 19 of the mutants. In each case of a gene identified by a single mutation from our serotonin-resistance screens, we obtained a deletion or other independently isolated mutation for that gene and verified that it induced a similar serotonin-resistance phenotype.

The 19 serotonin-resistance mutations identified eight different proteins required for serotonin response (Table 2). *MOD-1* is a serotonin-gated chloride channel (Ranganathan *et al.* 2000), while *SER-4* is a G-protein-coupled serotonin receptor (Olde and McCombie 1997). *GOA-1* is the *C. elegans* ortholog of the major neural G protein G_{α_o} (Lochrie *et al.* 1991) and is a member of the $G_{\alpha_{i/o}}$ class of G proteins thought to be activated by *SER-4* (Olde and McCombie 1997). Serotonin resistance of *MOD-1*, *SER-4*, and *GOA-1* mutants has been noted previously (Ségalat *et al.* 1995; Ranganathan *et al.* 2000; Hapiak *et al.* 2009) and suggests that serotonin acts directly on two different receptors to inhibit locomotion.

Five additional proteins that affect serotonin response were identified in our screens. *EAT-16* is a G-protein regulator known to function with *GOA-1* (Hajdu-Cronin *et al.* 1999) and thus could affect *SER-4/GOA-1* signaling. *ABTS-1* is a transmembrane chloride transporter that controls the chloride gradient across the plasma membrane (Bellemer *et al.* 2011) and thus could affect the function of the serotonin-gated chloride channel *MOD-1*. We note, however, that we isolated an *abts-1* mutant in a screen performed using the *mod-5* mutant background, and we have not detected any effect of *abts-1* mutations on serotonin response in a wild-type background (data not shown). *FLP-1* is a neuropeptide previously known to affect serotonin response (Nelson *et al.* 1998), *ELPC-3* is the catalytic subunit of a lysine acetylase protein complex (Chen *et al.* 2009; Solinger *et al.* 2010), and *EMB-9* is a collagen that is a component of basement membranes (Graham *et al.* 1997).

Our results suggest *MOD-1* and *SER-4* are serotonin receptors that inhibit *C. elegans* locomotion, and we propose that the other proteins detected in our genetic screens affect

Table 2 Genes identified by serotonin-resistance mutations

Gene	No. of mutations isolated	Protein encoded
<i>mod-1</i>	3	Serotonin-gated ion channel
<i>ser-4</i>	1	G-protein-coupled serotonin receptor
<i>goa-1</i>	9	Neural G protein α -subunit G_{α_o}
<i>eat-16</i>	1	Regulator of G protein signaling
<i>abts-1</i>	1	Bicarbonate chloride exchanger
<i>flp-1</i>	2	Neuropeptide
<i>elpc-3</i>	1	Lysine acetyl transferase
<i>emb-9</i>	1	Basement membrane collagen

signaling by *MOD-1* and/or *SER-4*. To lay the foundation for understanding of how all of the proteins mediate serotonin response, we first focused on how the two receptors affect locomotion.

***SER-4* and *MOD-1* act in parallel to mediate response to exogenous serotonin**

We quantitated the effects of *ser-4* and *mod-1* null mutations on the ability of exogenous serotonin to paralyze *C. elegans*. When assayed under conditions similar to those of our genetic screens, in M9 buffer, the receptor single mutants were strongly serotonin resistant, with ~80% of the mutant animals continuing to move at a serotonin concentration sufficient to paralyze all but 10% of the wild type (Figure 2A). While systematically varying the assay conditions, we found that the salts present in nematode growth media strongly inhibit serotonin response, so that ~30-fold less serotonin was sufficient to paralyze animals if the assay was performed in water instead. By assaying in water, for the first time we were able to observe responses of *C. elegans* to exogenous serotonin at concentrations of serotonin below its solubility limit, greatly improving the reliability our serotonin response assays. We generated serotonin dose-response curves by placing young adults into microtiter wells with various concentrations of serotonin in water and analyzed the ability of serotonin to paralyze mutants lacking the receptors individually or in combination. Wild-type animals were paralyzed in a dose-dependent fashion by serotonin in water (Figure 2B). In this assay condition, the *ser-4* and *mod-1* single null mutants showed only weak serotonin resistance, but the *ser-4; mod-1* double mutant showed much stronger serotonin resistance.

In a second approach to understanding the functional relationship between the two receptors, we generated a strain carrying a chromosomally integrated transgene with multiple copies of a *mod-1* genomic clone, which presumably overexpresses *MOD-1*. We found that these *mod-1(xs)* animals were hypersensitive to paralysis by exogenous serotonin in water, in that *mod-1(xs)* animals were paralyzed by serotonin at a concentration ~30-fold lower than that required to paralyze the wild type (Figure 2C). The *mod-1(xs)* transgene also caused a baseline paralysis of ~25% of the animals in this assay even when no exogenous serotonin was added, possibly

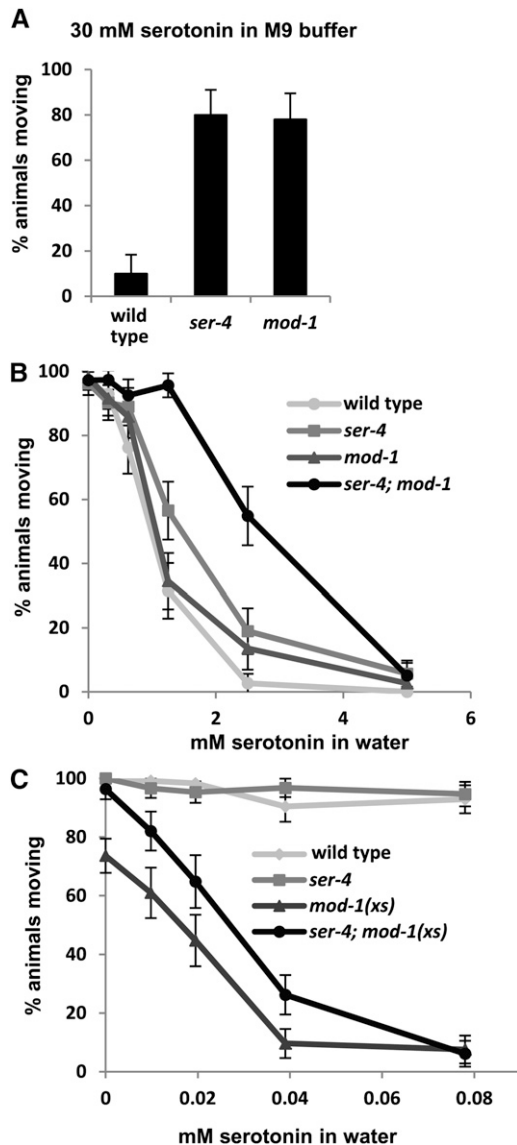


Figure 2 Effects of MOD-1 and SER-4 on the ability of serotonin to paralyze *C. elegans* in liquid. Young adult animals were placed in microtiter wells containing serotonin at the indicated concentrations and 20 min later were scored for movement. (A) Effects of *ser-4* or *mod-1* deletion mutations on serotonin response when assayed in M9 buffer. (B) Effects of these mutations on serotonin response, alone or in combination, when assayed in water. (C) A MOD-1 overexpressor transgene, *mod-1(xs)*, produces serotonin hypersensitivity in a wild-type or *ser-4* mutant background. Assays are in water as in B, but note the ~100-fold change in scale of the x-axis. Shown are the percentages of animals paralyzed from samples of $n = 50$ (A) or $n > 100$ (B and C) for each genotype and concentration, with error bars representing 95% confidence intervals.

because of endogenous serotonin signaling through overexpressed MOD-1. When the *mod-1(xs)* transgene was combined with the *ser-4(null)* mutation, this baseline paralysis was suppressed, but the *ser-4; mod-1(xs)* strain still showed hypersensitivity to exogenous serotonin similar to that of the *mod-1(xs)* strain, as the slopes of the dose-response curves for the two strains were similar. Thus MOD-1 can mediate the

ability of exogenous serotonin to paralyze animals even in the absence of SER-4.

Our results indicate that SER-4 and MOD-1 are the principal receptors that mediate the ability of serotonin to slow locomotion, as they are the only serotonin receptors so far identified by our genetic screen, and at least in high-salt buffer, null mutations for either receptor lead to strong serotonin resistance. When assayed in water, the *ser-4; mod-1* double null mutant can still be paralyzed by sufficiently high concentrations of exogenous serotonin, so additional receptor(s) must also mediate the ability of serotonin to slow of locomotion in this condition. SER-4 and MOD-1 appear to function in parallel to each other to mediate slowing of locomotion since null mutants for the individual receptors are not as serotonin resistant as is the *ser-4; mod-1* double mutant.

mod-1 and *ser-4* are expressed in almost completely non-overlapping sets of cells

To further clarify the relationship between SER-4 and MOD-1, we examined the expression patterns of the *mod-1* and *ser-4* genes. Because the specific muscles, motor neurons, and interneurons that control *C. elegans* locomotion have been well characterized (Von Stetina *et al.* 2006), understanding precisely which of these cells express the receptors serves as a starting point for understanding how each receptor affects locomotion. Previous studies have generated transgenic animals that express the green fluorescent protein (GFP) from the *mod-1* or *ser-4* promoters and have reported the identification of some cells that express each reporter (Ranganathan *et al.* 2000; Tsalik *et al.* 2003; Carnell *et al.* 2005; Harris *et al.* 2009). However, these studies do not entirely agree, probably because of the weak and variable expression from the extrachromosomal transgenes used. We used chromosomally integrated *mod-1::mCherry* and *ser-4::GFP* transgenes that gave strong stable expression and allowed us to directly compare the expression of *mod-1* and *ser-4* reporters in the same animals.

We found that the *mod-1* and *ser-4* reporter transgenes were expressed in almost non-overlapping sets of cells (Figure 3, A–F, and File S3). In agreement with previous work (Ranganathan *et al.* 2000; Tsalik *et al.* 2003; Carnell *et al.* 2005; Harris *et al.* 2009), we found that *mod-1* and *ser-4* are each expressed in a subset of head and tail neurons. Using double-transgenic animals, we found that there was only one type of cell that reliably coexpressed both reporter transgenes (Figure 3C and File S3). The coexpressing cells were a bilaterally symmetric pair of head neurons previously identified as either RIB (Tsalik *et al.* 2003) or AIB (Harris *et al.* 2009), two interneuron types that have very similar cell-body positions and process morphology. Harris *et al.* (2009) reported that *mod-1* functions in AIB, and our analysis of the cell-body position of the double-labeled cell is also most consistent with AIB. RIB and AIB are among the many head interneurons that directly or indirectly synapse onto the command interneurons to control whether

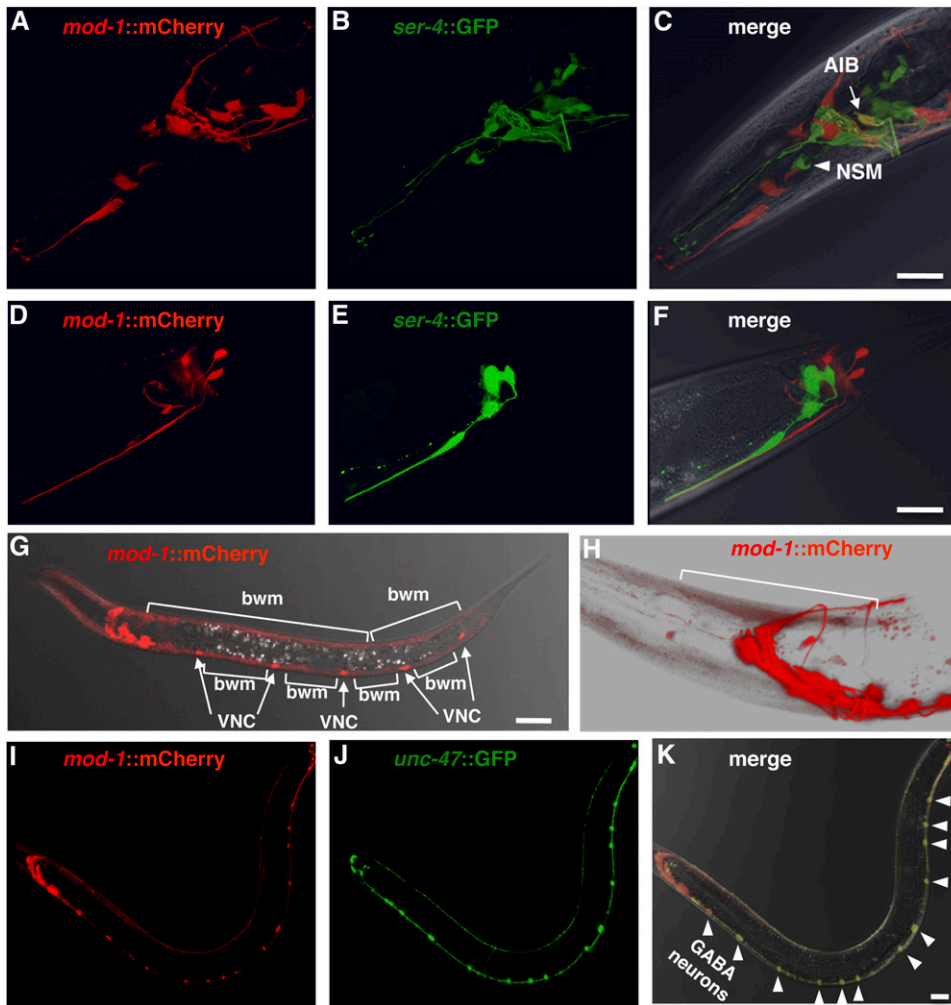


Figure 3 Expression patterns of *mod-1* and *ser-4* reporter transgenes are mostly distinct. Shown are confocal micrographs of transgenic animals in which the *mod-1* promoter drives expression of the red fluorescent protein mCherry and the *ser-4* promoter drives expression of green fluorescent protein (GFP). Red or green fluorescence and a merge of both superimposed on a brightfield image of the animal are shown, as indicated. (A–C) Adult head region. Arrow indicates AIB, the only cell type that reliably coexpressed both reporters. Arrowhead indicates the serotonergic NSM neurons. The double-labeling pattern seen in C is shown in more detail in File S3, a movie of a rotating three-dimensional confocal image of the head region. (D–F) Adult tail region. (G) *mod-1*::mCherry fluorescence in an L3 larva superimposed on a brightfield image of the animal. Arrows point to mCherry-labeled ventral cord neurons (VNC), while brackets indicate labeled body-wall muscles (bwm). (H) *mod-1*::mCherry expression in adult head. The white background used allows weak red fluorescence in body-wall muscle to be visualized. Bracket indicates an individual muscle cell. (I–K) Lower magnification images of a young adult expressing *mod-1*::mCherry and an *unc-47*::GFP transgene that labels GABAergic neurons. Arrowheads in K point to the double-labeled GABA neurons of the ventral nerve cord that are most visible in this individual. Bars, 20 μ m.

C. elegans moves forward or backward (White *et al.* 1986; Von Stetina *et al.* 2006). We identified some cells that had not previously been reported to express *mod-1* or *ser-4* reporters. Specifically, we saw *mod-1*::mCherry expression in RME neurons (Figure S1A) and *ser-4*::GFP expression in the serotonergic NSM neurons (Figure 3C) and in the *vm2* egg-laying muscles (Figure S1B).

The *mod-1*::mCherry reporter was expressed in muscles and neurons that directly control locomotion. By examining larvae at the L3 stage or younger, we saw that the *mod-1* reporter was expressed strongly in all body-wall muscles, the cells that directly execute body bends during *C. elegans* locomotion (Figure 3G). We then found that in L4 larvae and adults this body-wall muscle expression was much reduced, but still detectable (Figure 3H). It was previously reported (Ranganathan *et al.* 2000) that a *mod-1* reporter was expressed in unidentified cells of the ventral nerve cord, which contains motor neurons that synapse onto the body-wall muscles. We also saw ventral cord neurons expressing *mod-1*::mCherry (Figure 3I). To identify these cells, we generated strains that carried the *mod-1*::mCherry transgene plus an *unc-47*::GFP transgene to label the GABAergic motor neurons (Figure 3J) or an *unc-17*::GFP transgene to label the

cholinergic motor neurons (data not shown). MOD-1 expression overlapped perfectly in the ventral cord with the *unc-47*::GFP-expressing neurons, with *mod-1*::mCherry expressed in both VD and DD classes of GABAergic motor neurons (Figure 3K). These motor neurons release GABA directly onto the body-wall muscles to help generate body bends during locomotion (Von Stetina *et al.* 2006). We note that the *mod-1* reporter is not expressed in all 26 GABAergic neurons; in the head, the only overlap between the *mod-1* and *unc-47* reporter expression is in RME interneurons (Figure S1A).

In summary, the *ser-4* reporter transgene expression was restricted to cells, including several types of head interneurons, that likely affect locomotion only indirectly. In contrast, the *mod-1* expression pattern included the body-wall muscles and GABAergic ventral cord neurons that directly control locomotion. The only overlap in the *mod-1* and *ser-4* expression patterns appeared to be in the AIB head interneurons.

MOD-1 and SER-4 serotonin receptors together mediate effects of optogenetically released endogenous serotonin on locomotion

MOD-1 and SER-4 are required for exogenous serotonin to paralyze *C. elegans*. Do MOD-1 and SER-4 have a normal

physiological role in the control of locomotion by endogenously released serotonin? We initially addressed this question by optogenetically stimulating neurotransmitter release from the serotonergic neurons of *C. elegans* and measuring whether *MOD-1* and *SER-4* affect the resulting locomotion response.

Our optogenetic assays used a chromosomally integrated transgene, *ljs102* (Ezcurra *et al.* 2011), to express the blue-light-activated cation channel, channelrhodopsin-2 (ChR2) fused to the yellow fluorescent protein (YFP). A fragment of the promoter for the *tph-1* gene (Sze *et al.* 2000) was used to drive expression of ChR2::YFP specifically in serotonergic NSM and ADF neurons (Figure 4A). We performed all of our ChR2 assays in genetic backgrounds mutant for the *C. elegans* endogenous blue light receptor *LITE-1* (Edwards *et al.* 2008), so that any locomotion responses to blue light should be entirely caused by ChR2 activation.

Upon stimulation by blue light to activate ChR2 and depolarize NSM and ADF, animals were nearly paralyzed (File S4). To quantitate this locomotion response, we counted the number of body bends during the first 30 sec after turning on blue light illumination. To further ensure that the effects measured were caused by ChR2 activity, we assayed animals grown either in the presence or absence of all-*trans* retinal, a cofactor required for ChR2 activity. We found that ChR2 activation of NSM and ADF caused a 5.7-fold decrease in body bends (Figure 4B). This slowing of locomotion appeared to depend absolutely on release of serotonin because it was absent in a mutant for *bas-1*, which encodes an enzyme required for serotonin and dopamine biosynthesis. We note that in this experiment we were unable to use a *tph-1* mutation, which would block only serotonin and not dopamine biosynthesis, because the ChR2 transgene lies on chromosome II too close to the *tph-1* gene to allow the necessary strain to be constructed. However, since the NSM and ADF neurons make serotonin and not dopamine, the *bas-1* result strongly suggests that ChR2 stimulation caused slowing via release of serotonin.

Our optogenetic assays showed that *mod-1* and *ser-4* together are required for the effects of endogenous serotonin on locomotion (Figure 4B). In the *mod-1* or *ser-4* mutant backgrounds, ChR2 activation caused only a 1.8- or 3.1-fold decrease in body bends, respectively. In a genetic background mutant for both *mod-1* and *ser-4*, ChR2 activation caused little if any reduction in body bends ($P > 0.05$).

MOD-1 and SER-4 serotonin receptors together mediate hyper-enhanced slowing, an effect of endogenous serotonin on locomotion

The optogenetic assay showed that *MOD-1* and *SER-4* can mediate effects of endogenously released serotonin on locomotion. For a more physiological assay of endogenous serotonin signaling, we analyzed how *SER-4* and *MOD-1* mutations affect a locomotion-slowing behavior known to depend on natural release of endogenous serotonin. *C. elegans* slows when it encounters food in the form of a lawn of *E. coli*

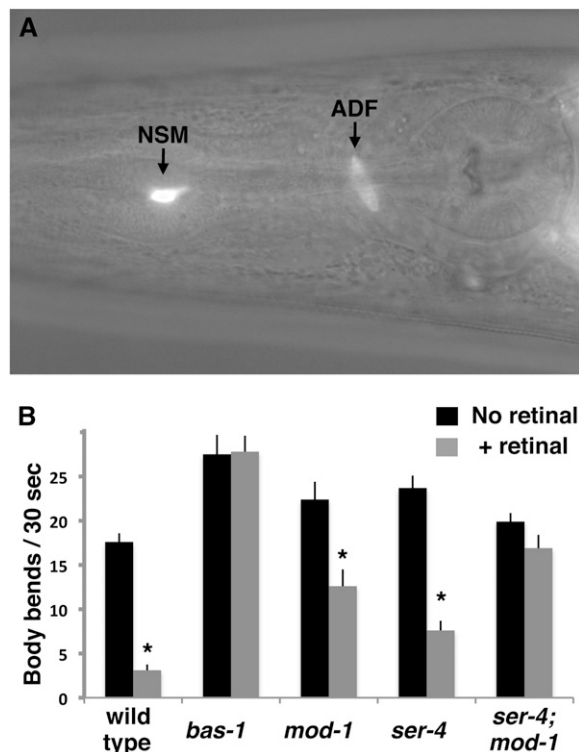


Figure 4 *SER-4* and *MOD-1* receptors are required for control of locomotion by optogenetically released endogenous serotonin. (A) Image of the head region of an animal carrying the *ljs102* transgene, which expresses ChR2::YFP in serotonergic neurons. A Nomarski optics image showing the anatomy of the head is superimposed on an image of yellow fluorescence. Labeled arrows point to YFP fluorescence in the NSM and ADF neurons. Each neuron type is a bilaterally symmetric pair of cells, of which only the left member is visible in this micrograph. (B) Optogenetically stimulated serotonin release slows locomotion dependent on the *MOD-1* and *SER-4* serotonin receptors. Young adults of the indicated genotypes grown either with or without the all-*trans* retinal cofactor required for ChR2 function were illuminated with blue light, and the number of body bends during the first 30 sec of illumination was counted. In addition to the mutations indicated, all animals in this experiment carried a mutation in *lite-1* (Edwards *et al.* 2008) to block the endogenous response to blue light of *C. elegans*. Asterisks indicate significant slowing ($P < 0.05$, one-tailed *t*-test) in all-*trans* retinal-treated animals relative to the controls without all-*trans* retinal.

bacteria on an agar plate. If the animals are well fed, they show a modest “basal slowing,” which depends on the release of the neurotransmitter dopamine (Sawin *et al.* 2000). If the animals assayed are food deprived for 30 min prior to being presented with food, they show a stronger “enhanced slowing” response, in which the additional slowing depends on release of serotonin from the NSM and other serotonergic neurons (Sawin *et al.* 2000). Finally, an even more dramatic hyper-enhanced slowing response occurs in a null mutant for the serotonin reuptake transporter *MOD-5*: since *MOD-5* activity terminates serotonin signaling, exaggerated responses to serotonin are seen in *mod-5* mutants (Ranganathan *et al.* 2001). As shown in Figure 5B, food-deprived wild-type animals slowed their locomotion by ~75% when they encountered food, demonstrating enhanced slowing, while food-deprived

mod-5 animals were almost completely paralyzed when they encountered food, demonstrating hyper-enhanced slowing.

mod-1 was originally defined by Ranganathan *et al.* (2000) based on a mutant strain that was both serotonin resistant and defective in the enhanced slowing response. Using the serotonin-resistant phenotype, these investigators mapped and cloned the *mod-1* gene and found it to encode a serotonin-gated chloride channel. Subsequent work has indicated that *mod-1* mutants are not defective in the enhanced slowing response. First, the enhanced-slowing defect of the original *mod-1* strain carrying the *n3034* mutation proved to be conferred by a locus genetically separable from the *mod-1* mutation as defined by the serotonin-resistant phenotype (D. Omura, personal communication). This observation indicated that the enhanced-slowing defect in this *mod-1* strain was caused by a background mutation, now named *n4954*. Second, we have been unable to reproduce the enhanced-slowing defects reported by Ranganathan *et al.* (2000) for the *mod-1(ok103)* and *mod-1(nr2043)* strains. For example, Figure 5A shows our data for *mod-1(ok103)* animals. We do not know why the observations of Ranganathan *et al.* (2000) concerning the *mod-1(ok103)* and *mod-1(nr2043)* strains differ from our more recent observations.

We saw a very weak enhanced-slowing defect for *ser-4* (Figure 5A) and also, at most, a weak enhanced-slowing defect in the *mod-1; ser-4* double mutant (data not shown). To help understand if the enhanced-slowing response truly depends on serotonin, we measured enhanced slowing in a null allele for the serotonin biosynthesis gene *tph-1*, mutations of which were not available at the time of the previously published studies of enhanced slowing (Figure 5A, right). In the absence of food, this *tph-1* deletion mutant moved somewhat more slowly than did the wild type. Despite this complication, the *tph-1* mutant failed to slow in the presence of food to the low rate of body bends seen in the wild type ($P = 0.002$) and thus did show the expected enhanced-slowing defect.

Since the hyper-enhanced slowing response shows more dramatic effects of endogenous serotonin on locomotion than does the enhanced-slowing response, we tested how *ser-4* and *mod-1* null mutations affected hyper-enhanced slowing. Whereas food-deprived *mod-5* single mutants were essentially paralyzed when they encountered food, two independent deletion alleles of *mod-1* were significantly defective in this hyper-enhanced slowing response (Figure 5B). The *ser-4* deletion mutant was also defective in hyper-enhanced slowing, and combining *ser-4* and *mod-1* deletions together produced yet a stronger defect (Figure 5C). Since we did not have a second independent deletion allele of *ser-4*, we used a *ser-4* rescuing transgene to check if the effect of this deletion on enhanced slowing was indeed caused by the loss of *ser-4* function. We found that the *ser-4* transgene rescued the *mod-5; ser-4; mod-1* triple-mutant phenotype in the slowing assay to the phenotype of the *mod-5; mod-1* double mutant. A mock *ser-4* transgene, with a frameshift

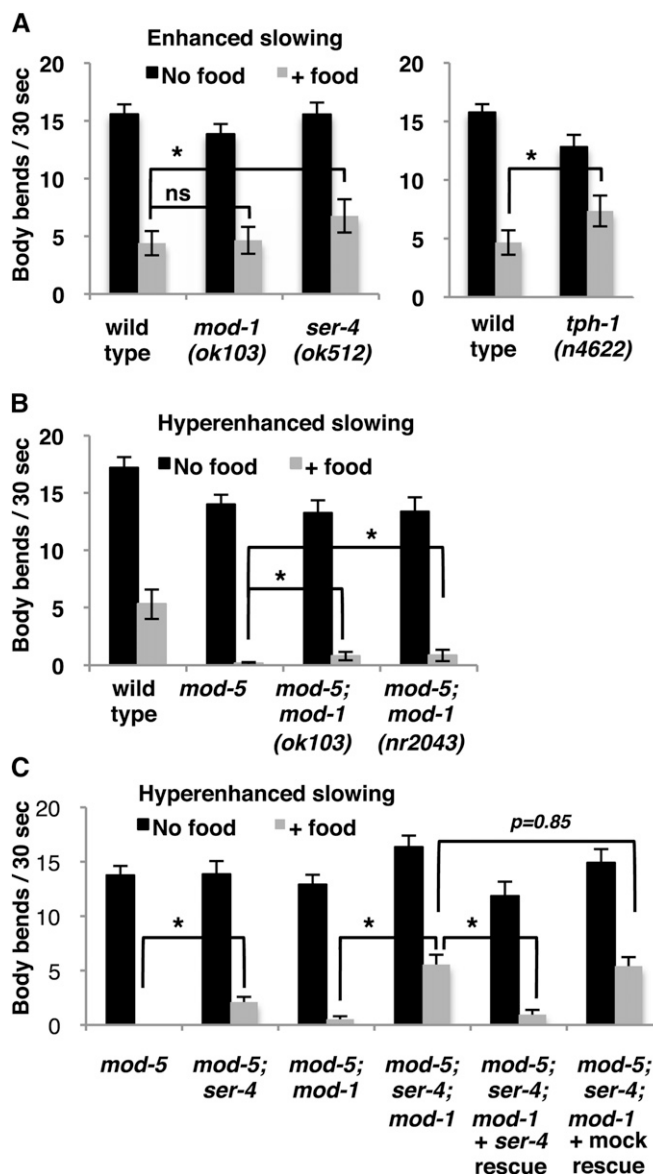


Figure 5 Effects of SER-4 and MOD-1 receptors on enhanced and hyper-enhanced slowing behavioral responses dependent on endogenous serotonin. (A) Enhanced slowing was measured by first food-depriving young adults for 30 min, transferring the animals to agar plates with or without food (a lawn of bacteria), and scoring locomotion by measuring the rate of body bends. *mod-1(ok103)* (shown) had no significant defect ($P = 0.76$, Student's *t*-test), and two other *mod-1* alleles gave similar results (data not shown). The *ser-4* deletion mutation caused a modest but significant ($P = 0.01$) defect in enhanced slowing. As a positive control, the serotonin-deficient mutant *tph-1(n4622)* was assayed and failed to slow as much as did the wild type ($P = 0.02$). (B and C) Hyper-enhanced slowing was measured by using the same assay on animals carrying mutations in the MOD-5 serotonin reuptake transporter. (B) Upon exposure to food, wild-type animals slowed, while *mod-5* mutants demonstrated hyper-enhanced slowing by almost completely stopping all body bends. Two independent *mod-1* deletion mutants were partially defective for hyper-enhanced slowing. (C) The *ser-4* deletion mutation caused defective hyper-enhanced slowing and added to the defect of the *mod-1(ok103)* mutant. This additional defect was rescued by a *ser-4* transgene but not by a mock *ser-4* transgene containing a frameshift mutation that blocks SER-4 expression. Brackets with asterisks indicate significant differences ($P < 0.05$, Student's *t*-test). ns, not significant.

mutation in the *ser-4* coding sequences, showed no such rescue (Figure 5C).

Our results show that *MOD-1* and *SER-4* together mediate hyper-enhanced slowing, a behavioral effect of endogenous serotonin release.

Discussion

A genetic system for discovery and functional analysis of serotonin-signaling proteins

We developed large-scale genetic screens to isolate mutants with defects in serotonin response. Many past studies of serotonin signaling in *C. elegans* have analyzed gene knockouts for worm homologs of serotonin-signaling proteins already known from studies in vertebrates. For example, mutants defective in enzymes required for serotonin biosynthesis and for serotonin receptor homologs have revealed a number of behaviors that depend on serotonin (Sze *et al.* 2000; Carnell *et al.* 2005; Dempsey *et al.* 2005; Hobson *et al.* 2006; Dernovici *et al.* 2007; Hapiak *et al.* 2009; Harris *et al.* 2009). However, the greatest potential for *C. elegans* genetics to illuminate mechanisms of serotonin signaling likely lies in genetic screens to discover new serotonin-signaling proteins not previously known from vertebrate studies.

We chose locomotion as a serotonin-regulated behavior in *C. elegans* suitable for analysis by large-scale genetic screens. The simplest approach to such screens, applying serotonin to descendants of mutagenized animals and screening for animals that were not paralyzed, would have produced an overwhelming number of false-positive isolates that did not carry serotonin-resistance mutations because even high concentrations of serotonin do not paralyze 100% of wild-type animals. We developed two alternative approaches: clonal genetic screens and nonclonal screens using a mutation in the *MOD-5* serotonin reuptake transporter in the genetic background. These approaches allowed us to screen animals representing 117,000 mutagenized haploid genomes and to isolate 36 mutants resistant to the paralytic effects of exogenous serotonin. Although serotonin is used as a neurotransmitter in perhaps all organisms with a nervous system, to our knowledge no other large-scale systematic screens for mutants with serotonin-signaling defects have been performed.

We developed new assays for measuring the effects of serotonin on locomotion to assess how specific mutations affect serotonin response. We discovered that *C. elegans* responds to exogenous serotonin at ~30-fold lower concentrations when it is dissolved in media lacking any salts instead of in the *C. elegans* media that have previously been used for serotonin response assays. This finding allowed us to perform more reliable assays in which serotonin was used at concentrations below its solubility limit. Even when applied in water, exogenous serotonin must be presented at concentrations >100 μ M to affect wild-type *C. elegans* locomotion, raising concern that the responses seen might not be related to the physiological effects of endogenous serotonin.

For this reason, we developed a new serotonin response assay based on channelrhodopsin-2 stimulation of endogenous serotonin release. We also used hyper-enhanced slowing behavior as a further assay of endogenous serotonin signaling. *mod-1* and *ser-4* mutations caused defects in all four assays that we used: response to exogenous serotonin in high or low salt liquid, the optogenetic assay, and the hyper-enhanced slowing assay. The strongest defects were seen when both receptors were mutated, and in some assays mutants lacking only a single receptor failed to show significant defects. Our results suggest mutations in other genes isolated based on defects for response to exogenous serotonin likely also affect endogenous serotonin signaling. However, any mutation that affects only one receptor-signaling pathway might need to be combined with a second mutation affecting the other receptor-signaling pathway to reveal effects on endogenous serotonin signaling.

Eight proteins required for serotonin control of locomotion in C. elegans

Our genetic screens identified eight genes required for serotonin response. Three of these genes encode the G-protein-coupled serotonin receptor *SER-4*, its potential target $G\alpha_o$ protein *GOA-1*, and the G-protein regulator *EAT-16*, previously known to indirectly affect $G\alpha_o$ signaling (Hajdu-Cronin *et al.* 1999). Two more genes encode the serotonin-gated chloride channel *MOD-1* and the chloride transporter *ABTS-1*, previously known to control chloride channel function by affecting the chloride gradient across the plasma membrane (Bellemer *et al.* 2011).

How the remaining three proteins identified in our screens might affect serotonin response is less clear. *EMB-9* is a basement membrane collagen (Graham *et al.* 1997). The NSM neurosecretory neurons release serotonin directly onto a basement membrane surrounding the pharynx, so the serotonin must traverse the basement membrane to reach target cells outside the pharynx (Axäng *et al.* 2008). Perhaps exogenous serotonin also enters *C. elegans* through its pharynx by traversing this same basement membrane, and *EMB-9* affects diffusion of serotonin through the basement membrane.

The *flp-1* gene encodes several FMRFamide-related neuropeptides. Previous studies have shown that *flp-1* mutations decrease the ability of exogenous serotonin to slow locomotion (Nelson *et al.* 1998) and that *flp-1* appears to potentiate the ability of both endogenous and exogenous serotonin to stimulate egg-laying behavior (Waggoner *et al.* 2000). *flp-1* is expressed in a subset of head interneurons (Nelson *et al.* 1998), but the mechanism by which *FLP-1* released from these cells directly or indirectly affects serotonin response in the locomotion and egg-laying systems of *C. elegans* remains to be determined.

The final protein identified by our genetic screens is *ELPC-3*, a subunit of a multisubunit complex known as elongator based on its initial discovery in yeast as a factor involved in transcriptional elongation (Otero *et al.* 1999). The *ELPC-3* subunit has lysine acetylase activity and is highly

evolutionarily conserved among all eukaryotes, with >65% identity among orthologs in various species from yeast to humans (Wittschieben *et al.* 1999). In animals, elongator is found in the nervous system, and mutations in the human elongator subunit Elp1 cause the neurodegenerative disease familial dysautonomia (Slaugenhaupt and Gusella 2002). Elongator is found in both the nucleus and the cytosol and has been shown to acetylate tubulin in *C. elegans* (Solinger *et al.* 2010). Elongator has not previously been implicated in serotonin signaling. However, a genome-wide association study found an association of a polymorphism upstream of the *Elp3* gene with suicide ideation during treatment with an antidepressant that acts as a selective serotonin reuptake inhibitor (Perroud *et al.* 2010). We suggest that elongator positively regulates serotonin signaling by acetylating and thus activating another protein that directly mediates serotonin signaling. Alternatively, elongator might very indirectly affect serotonin signaling since, for example, it is required for proper modification of transfer RNA in *C. elegans* (Chen *et al.* 2009) and might cause subtle defects in protein translation in neurons.

Our genetic screens have not yet reached a saturation scale. Nevertheless, as shown in Table 2, we recovered nine alleles of the $G\alpha_o$ gene *goa-1*, despite the fact that strong loss-of-function *goa-1* mutants are relatively sick and slow growing. The relatively high rate of isolating *goa-1* mutants is likely because of their strong serotonin-resistance phenotype. Five genes were identified in our screens by only single alleles. Null mutants for these five genes are relatively healthy and show serotonin resistance similar to that seen in the screen isolates, and the genes are not particularly small, so there is no obvious reason why alleles for these genes should be rare. Of the 36 serotonin-resistant mutants that we isolated, 17 have not yet been assigned to genes, and at least 11 of the 17 show a serotonin-resistance phenotype sufficiently strong to allow genetic mapping. We expect that further analysis of these mutants will identify additional serotonin-signaling genes.

Two receptors act in parallel to allow extrasynaptic serotonin to inhibit locomotion

We have initiated analysis of the serotonin-signaling proteins identified in our genetic screens with studies of the serotonin receptors. Because the other serotonin-signaling proteins presumably affect signaling by one or more serotonin receptor, understanding how serotonin receptors control locomotion should provide the basis for assigning the remaining signaling proteins to the receptors that they regulate or to which they respond.

MOD-1 and *SER-4* are the principal receptors that slow locomotion in response to exogenous serotonin and to the presumably high concentrations of endogenous serotonin released by optogenetic stimulation of the *NSM* and *ADF* neurons or in the hyper-enhanced slowing assay. *MOD-1* and *SER-4* were the only serotonin receptors identified in our genetic screens for defects in response to exogenous

serotonin, and knocking out both of these receptors together eliminated slowing in response to optogenetic release of endogenous serotonin (Figure 4). The *ser-4; mod-1* double knockout might also completely eliminate the serotonin-induced hyper-enhanced slowing response: the residual slowing that occurs in the double knockout in this assay (Figure 5) might be entirely caused by the dopamine-induced basal slowing response. We note, however, that *mod-1* and *ser-4* knockouts had little or no effect on enhanced slowing, a more natural behavioral effect of presumably lower levels of endogenous serotonin. Furthermore, application of high concentrations of exogenous serotonin caused residual slowing of *ser-4; mod-1* double null mutants (Figure 2), again suggesting that other serotonin receptors also contribute to the effects of serotonin on locomotion. In addition to *SER-4* and *MOD-1*, there are three other serotonin receptor homologs in *C. elegans*, and one report suggests that the *SER-1* receptor has a significant effect on slowing in response to exogenous serotonin (Dernovici *et al.* 2007). However, another study did not find a strong effect of *SER-1* (Hapiak *et al.* 2009), and we found that a *ser-1* null mutation individually or in combination with *mod-1* or *ser-4* mutations had little effect in our assays for response to exogenous serotonin (data not shown). Thus existing assays show little effect of other serotonin receptors on locomotion compared to the strong effects of *MOD-1* and *SER-4*.

Our genetic and expression-pattern studies suggest *SER-4* and *MOD-1* act independently of each other to slow locomotion. In one assay of response to exogenous serotonin and two assays of response to endogenously released serotonin, we found that *ser-4; mod-1* double null mutants had more severe defects in serotonin response than did the single mutants lacking only one receptor. These results are consistent with the hypothesis that *SER-4* and *MOD-1* act independently and in parallel to mediate slowing in response to serotonin. In agreement with this hypothesis, *ser-4* and *mod-1* appear to be expressed in almost entirely non-overlapping sets of cells, with *ser-4* expressed in many head interneurons and *mod-1* expressed in different interneurons as well as in the GABAergic motor neurons and body-wall muscles that directly cause locomotion. Either *SER-4* and *MOD-1* act in parallel in these different cells, or they act in parallel in the only two cells in which they are coexpressed, the *AIB* interneurons, which synapse onto command interneurons and thus could mediate the effects of serotonin on locomotion. The precise sites of action and mechanism by which the two receptors act to slow locomotion remain to be resolved.

Expression of the *SER-4* and *MOD-1* serotonin receptors does not appear to be limited to cells that receive synapses from serotonergic neurons. There are six pairs of neurons that contain serotonin—*NSM*, *ADF*, *HSN*, *RIH*, *AIM*, and *VC4/5* (Sawin *et al.* 2000; Duerr *et al.* 2001; Jafari *et al.* 2011)—and they form significant synapses onto 20 other types of neurons and onto the *vm2* egg-laying muscle cells (White *et al.* 1986). We found that the *vm2* muscles express

ser-4::GFP (Figure S1B). While we have not identified all of the neurons that express our *ser-4* and *mod-1* reporters, the neurons that we have identified do not correspond to any of the 20 neuron types postsynaptic to serotonergic neurons. For example, the GABAergic motor neurons of the ventral cord and the body-wall muscles that express *mod-1* do not receive synapses from serotonin neurons and are quite distant from any serotonin neurons. Thus, serotonin might diffuse from its release sites to act on distant cells. Consistent with this idea, previous work has shown that serotonin from the NSM and ADF neurons travels extrasynaptically to the RIH and AIM neurons, which use the serotonin uptake transporter MOD-5 to accumulate serotonin (Jafari *et al.* 2011). Furthermore, the neurons with by far the largest serotonin content in *C. elegans* are the NSM neurons, which are neurosecretory cells with no postsynaptic partners. The NSMs appear to be the principal neurons that release serotonin to cause slowing of locomotion (Sawin *et al.* 2000). Studies of vertebrate brain are also consistent with the idea that serotonin diffuses and signals extrasynaptically (Bunin and Wightman 1998).

Our results lead to a model in which the NSM neurosecretory cells release serotonin extrasynaptically to diffuse across a basement membrane with an EMB-9 collagen component that facilitates diffusion across the membrane. The serotonin continues to diffuse to distant target cells and acts in parallel on different serotonin receptors, likely found on different cells. Signaling through the G-protein-coupled receptor SER-4 and its target G protein GOA-1, potentially in head interneurons, would provide one mechanism to slow locomotion. Signaling via the serotonin-gated chloride channel MOD-1, potentially on the GABAergic motor neurons and/or body-wall muscles, would provide a second independent mechanism to slow locomotion. The remaining serotonin response proteins identified in our genetic screens could act to facilitate either the MOD-1- or SER-4-signaling pathways. It should be possible using the assays that we describe to determine for each new *C. elegans* serotonin-signaling protein which receptor pathway is affected. It would then be possible to determine if the mammalian homologs of these proteins play corresponding roles in serotonin signaling in the brain and if deficits in any of these proteins are involved in human neuropsychiatric disorders, such as depression.

Acknowledgments

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GENETICS

Supporting Information

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Receptors and Other Signaling Proteins Required for Serotonin Control of Locomotion in *Caenorhabditis elegans*

Güliz Gürel, Megan A. Gustafson, Judy S. Pepper, H. Robert Horvitz, and Michael R. Koelle

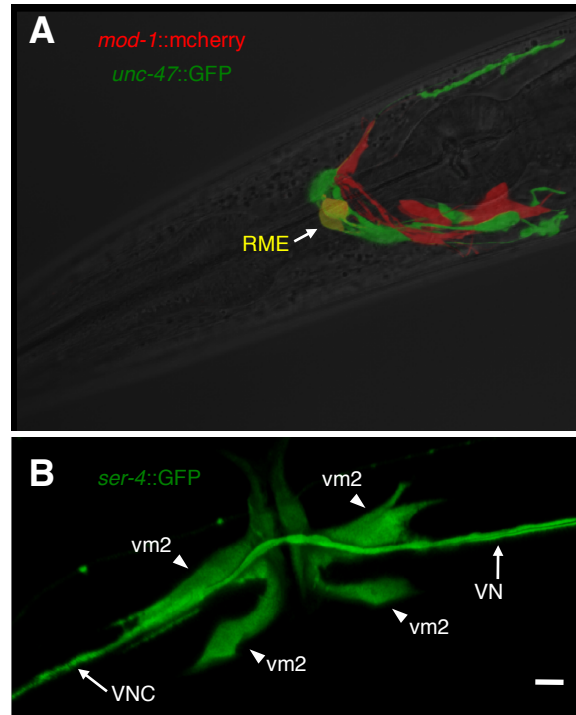


Figure S1 Further details of *mod-1* and *ser-4* reporter expression patterns.

(A) Adult head region showing *mod-1::mCherry* and *unc-47::GFP* fluorescence superimposed on a brightfield image of the animal. The *unc-47* reporter labels GABAergic neurons. This image demonstrates that not all GABAergic neurons expressed *mod-1::mCherry*. The two doubled-labeled GABAergic cells (arrow) that did express *mod-1::mCherry* were RME interneurons.

(B) Ventral view of the vulval region of a young adult expressing *ser-4::GFP*. Arrows, the fluorescent ventral nerve cord (VNC) running the length of the animal. Arrowheads, the four *vm2* muscle cells also labeled by *ser-4::GFP*. Bar=40 μ m.

File S1

Wild-type animals moving in water

A 30-second video clip showing wild-type animals in a microtiter well containing 50 μ l of water and no serotonin. These animals were all scored as “moving,” in that they showed continuous full-body thrashing movements.

File S1 is available for download at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.142125/-/DC1> as a Quicktime movie.

File S2

Wild-type animals paralyzed by exogenous serotonin

A 30-second video showing wild-type animals in a microtiter well containing 50 μ l water with a serotonin concentration (5 mM) sufficient to fully paralyze the animals, prepared as for the assays shown in Figure 2B. These animals were all scored as not “moving,” in that they did not show the continuous full-body thrashing movements. One animal briefly made body bends halfway through the video.

File S2 is available for download at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.142125/-/DC1> as a Quicktime movie.

File S3

Head neurons expressing *mod-1* and *ser-4* reporter transgenes

Three-dimensional rotation of the head region of a double-transgenic animal carrying the *mod-1::mCherry* and *ser-4::GFP* transgenes showing a merge of the red and green fluorescence. The bilaterally symmetric double-labeled neurons visualized in yellow are the left and right AIB neurons.

File S3 is available for download at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.142125/-/DC1> as a

Quicktime movie.

File S4

Optogenetic stimulation of endogenous serotonin release

Two 15-second videos showing slowing of locomotion during optogenetic stimulation of serotonin release. Animals shown carried the *ljls102* transgene, which expresses Chr2::YFP in the NSM and ADF serotonergic neurons, as well as a mutation in *lite-1*, to block the endogenous blue light response of *C. elegans*. Animals are shown prior to, during, and after a several second illumination with blue light.

File S4 is available for download at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.142125/-/DC1> as a Quicktime movie.

File S5

Extended Materials and Methods

C. elegans strains

The wild-type strain was Bristol N2. Additional *C. elegans* strains used in this work are listed below, followed by a table listing the transgenes shown within the genotypes and a description of the plasmids used to generate the transgenes.

Figure 2: N2, AQ866 *ser-4(ok512)* III, MT9668 *mod-1(ok103)* V, LX1834 *ser-4(ok512)* III; *mod-1(ok103)* V, LX1166 *lin-15(n765ts)* X; *vsIs123*, LX1835 *ser-4(ok512)* III; *lin-15(n765ts)* X; *vsIs123*

Figure 3: LX1851 *lin-15(n765ts)* *vsIs163* X, LX1858 *lin-15(n765ts)* X; *vsIs154*; *ljIs570*, LX1857 *oxIs12 lin-15(n765ts)* X; *vsIs163*

Figure 4: AQ2050 *lite-1(ce314)* X; *ljIs102*, LX1841 *bas-1(ad446)* III; *lite-1(ce314)* X; *ljIs102*, LX1838 *mod-1(ok103)* V; *lite-1(ce314)* X; *ljIs102*, LX1839 *ser-4(ok512)* III; *lite-1(ce314)* X; *ljIs102*, LX1842 *ser-4(ok512)* III; *mod-1(ok103)*; *lite-1(ce314)* X; *ljIs102*

Figure 5: N2, MT9668 *mod-1(ok103)* V, MT9667 *mod-1(nr2043)* V, MT9772 *mod-5(n3314)* I, MT14121 *mod-5(n3314)* I; *ser-4(ok512)* III, MT9849 *mod-5(n3314)* I; *mod-1(ok103)* V, MT10143 *mod-5(n3314)* I; *mod-1(nr2043)* V, MT14126 *mod-5(n3314)* I; *ser-4(ok512)* III; *mod-1(ok103)* V, MT17972 *mod-5(n3314)* I; *ser-4(ok512)* III; *mod-1(ok103)* V; *nEx1403*, MT17973 *mod-5(n3314)* I; *ser-4(ok512)* III; *mod-1(ok103)* V; *nEx1404*, MT14984 *tph-1(n4622)* II

Figure S1: LX1857 *oxIs12 lin-15(n765ts)* X; *vsIs163*, LX1858 *lin-15(n765ts)* X; *vsIs154*; *ljIs570*

File S1: N2

File S2: N2

File S3: LX1858 *lin-15(n765ts)* X; *vsIs154*; *ljIs570*

File S4: AQ2050 *lite-1(ce314)* X; *ljIs102*

Construction of transgenes

The *ser-4::GFP* reporter transgene *adEx1616* developed by Tsalik *et al.* (2003) was used to produce the chromosomally-integrated transgene *ljls570* by S. Shyn and W. Schafer and kindly provided to us for these studies. The *mod-1::mCherry* reporter plasmid pGG17 was constructed by inserting a 1645 bp *mod-1* promoter fragment upstream and the 1172 bp 3' untranslated region (UTR) of *mod-1* downstream of the mCherry coding sequences to generate plasmid pGG17. The primers used to amplify the promoter were GACTCTGCAGGCGTTCGTCACATTCTGCCG and CTGAGGTACCAATTTCTTTCACCGCATTGGC. The primers used to amplify the 3' UTR were GACTGAGCTCTGAAGTTTATCCCTT and GACTGGGCCCTAATCACAGGTGCATCGG. Injection of pGG17 into *C. elegans* gave transgenes showing very weak mCherry expression, but following the method of Etchberger and Hobert (2008) we found that PCR amplification of the promoter::mCherry::3' UTR cassette from the plasmid and injection of the linear amplified DNA gave much stronger expression. An extrachromosomal transgene generated in this manner was chromosomally integrated using psoralen/UV mutagenesis to produce two independent integrated transgenes, *vsIs154* and *vsIs163*. For double labeling, animals carrying these mCherry transgenes were crossed with animals carrying the *unc-47::GFP* transgene *oslx12* (McIntire *et al.* 1997), which labels GABAergic neurons or the *unc-17::GFP* transgene *vsIs48* (Chase *et al.* 2004), which labels cholinergic neurons.

The *mod-1* overexpressing transgene *vsIs123* was generated by directly microinjecting a long-range PCR product containing the entire *mod-1* gene into a *lin-15(n765ts)* strain of *C. elegans* at 20 ng/μl with the *lin-15* rescuing plasmid pL15EK at 50 ng/μl, selecting non-Lin progeny, and subsequently using psoralen/UV mutagenesis to chromosomally integrate the transgene. The *mod-1* PCR product was amplified from *C. elegans* genomic DNA using the primers CTAATCACAGGTGCATCGG and GCGTTCGTCACATTCTGCCG.

The *ser-4* rescuing plasmid pMG12 contained a 5 kb fragment of the *ser-4* promoter region followed by a *ser-4* cDNA and the 3' untranslated region from the *unc-54* gene. The *ser-4* promoter fragment was PCR amplified using the primers GCGGCGATGCCAGAGGAGTTCGCCACACAACACGTAC and GCGGCGATGCGTGGAGTTGCACACAACACCGGAAGC containing the restriction sites *SphI* and *BamHI*, respectively. We amplified the *ser-4* cDNA yk1731h09 (kindly provided by Y. Kohara) using the primers GCGGGTACCATGATCGACGAGACGCTTCJCAATC and GCGGATACTAGTCTAGCGCCGCGACCTGCAGC containing the restriction sites *KpnI* and *EcoRV*, respectively. These restriction sites were used to ligate the two fragments into the vector pPD49.25 (kindly provided by A. Fire), which supplied the *unc-54* 3' untranslated region. A negative control plasmid, pMG13, was identical to pMG12 but carried a frameshift mutation in the *ser-4* cDNA: we inserted two G residues after nucleotide 91 of *ser-4* exon 1. The transgenes *nEx1403* and *nEx1404* were generated by microinjecting pMG12 or pMG13, respectively, at 10 ng/μl, along with the *lin-15* rescuing plasmid pL15EK at 20 ng/μl, into a *ser-4(ok512); lin-15(n765ts)* strain and selecting non-Lin progeny.

Additional References for Extended Materials and Methods

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Nat. Neurosci. 7: 1096-103.

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3.

Table S1 Molecular Lesions of Mutations Identified in This Work

Serotonin resistance mutation	Nucleotide sequence of the wild type ^a	Nucleotide sequence of the mutant ^a	Affected amino acid in the wild-type protein	Amino acid in the mutant protein
<i>goa-1(vs115)</i>	tgcgtatatt C aagcacaatt	tgcgtatatt T aagcacaatt	Q305	Stop
<i>goa-1(vs134)</i>	agacggcatg C aagcggcaaa	agacggcatg T aagcggcaaa	Q29	Stop
<i>goa-1(n4093)</i>	tgcaccacatacagtgagtca	tgcaccacat[Mos1]acagtgagtca		
<i>goa-1(n4402)</i>	cttcgtggat G cggttgat	cttcgtggat A cggttgat	C351	Y
<i>goa-1(n4405)</i>	ctagcgccat G ggttgacca	ctagcgccat A ggttgacca	M1	N/A
<i>goa-1(n4439)</i>	catattttca G aaccgaatgc	catattttca A aaccgaatgc	intron 5 splice acceptor	N/A
<i>goa-1(n4492)</i>	ttttcagaac C gaatgcacga	ttttcagaac T gaatgcacga	R243	Stop
<i>goa-1(n4493)</i>	aacggtgtg G ggagacgcag	aacggtgtg A ggagacgcag	W132	Stop
<i>goa-1(n4494)</i>	agcgaataag G taagaaaaaa	agcgaataag A taagaaaaaa	intron 7 splice donor	N/A
<i>eat-16(n4403)</i>	aacctcgat C agacattgga	aacctcgat T agacattgga		
<i>abts-1(n4094)</i>	actttcatcgattatacagct	actttcatcga[Mos1]ttatacagct	N/A	N/A
<i>ser-4(vs122)</i>	atTTTTgcag G ttacctaaac	atTTTTgcag A ttacctaaac	G410	D
<i>emb-9(vs114)</i>	ggtcagccag G ttatccagga	ggtcagccag A ttatccagga	G1197	D
<i>flp-1(n4491)</i>	ttattattca G gtgtcggcag	ttattattca A gtgtcggcag	intron 1 splice acceptor	N/A
<i>flp-1(n4495)</i>	atcattttca G tcgaagtga	atcattttca A tcgaagtga	intron 4 splice acceptor	N/A
<i>mod-1(vs107)</i>	atggatgtgt G gatgcttgga	atggatgtgt A gatgcttgga	W305	Stop
<i>mod-1(n3791)</i>	ttgatcttg G gtttcattct	ttgatcttg A gtttcattct	W258	Stop
<i>mod-1(n4054)</i>	ctacgtcttactttccagttt	ctacgtcttac[Mos1]ttccagttt	N/A	N/A
<i>elpc-3(vs119)</i>	catgtatacg G atccgtcgtt	catgtatacg A atccgtcgtt	G475	E

^aThe affected nucleotide is capitalized and 10 nucleotides on either side are shown.

Table S2 C. elegans Transgenes Used in This Work

Transgene	Purpose	Used in	Source
<i>vsIs123</i>	Overexpression of MOD-1. Carries multiple copies of wild-type <i>mod-1</i> genomic DNA.	Figure 2	This work. Microinjection of a <i>mod-1</i> long-range PCR product at 20 ng/ μ l and pL15EK (a <i>lin-15</i> rescuing plasmid used as a coinjection marker) at 50 ng/ μ l. Chromosomal integration via UV/psoralen mutagenesis
<i>vsIs154</i> <i>vsIs163</i>	Expression of mCherry from the <i>mod-1</i> promoter. These two alleles are independent chromosomal integrants.	Figures 3, S1, File S3	This work. Microinjection of the <i>mod-1::mCherry</i> plasmid pGG17 at 65 ng/ μ l and pL15EK at 50 ng/ μ l. Chromosomal integration via UV/psoralen mutagenesis
<i>oxIs12</i>	Expression of GFP in GABAergic neurons from the <i>unc-47</i> promoter.	Figures 3, S1	McIntire <i>et al.</i> 1997 ^a
<i>ljIs102</i>	Expression of Channelrhodopsin2::YFP in the serotonergic NSM and ADF neurons.	Figure 4 File S4	Ezcurra <i>et al.</i> 2011
<i>nEx1403</i>	Rescue of the <i>ser-4</i> mutant phenotype by expression of a <i>ser-4</i> cDNA from the <i>ser-4</i> promoter.	Figure 5	This work. Microinjection of the plasmid pMG12 at 10 ng/ μ l and pL15EK at 20 ng/ μ l
<i>nEx1404</i>	Negative control for <i>ser-4</i> rescue. Similar to <i>nEx1403</i> except that the <i>ser-4</i> cDNA used carries a frame shift mutation that prevents expression of SER-4 protein.	Figure 5	This work. Microinjection of the plasmid pMG13 at 10 ng/ μ l and pL15EK at 20 ng/ μ l
<i>ljIs570</i>	Expression of GFP from the <i>ser-4</i> promoter.	Figure 3 File S3	A gift of S. Shyn and W. Schafer. Chromosomal integrant of the extrachromosomal transgene <i>adEx1616</i> from Tsalik <i>et al.</i> (2003)
<i>vsIs48</i>	Expression of GFP in cholinergic neurons from the <i>unc-17</i> promoter.	Data not shown	Chase <i>et al.</i> 2004

^a McIntire, S. L., R. J. Reimer, K. Schuske, R. H. Edwards, and E. M. Jorgensen, 1997 Identification and characterization of the vesicular GABA transporter. *Nature* 389: 870-876.