Serotonin Signaling in *C. elegans*

by
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ABSTRACT

Wild-type animals that have been acutely food deprived slow their locomotory rate upon encountering bacteria more than do well-fed animals. This behavior, called the enhanced slowing response, is partly serotonin (5-HT) dependent. Animals mutant for the 5-HT reuptake transporter gene *mod-5* slow even more than wild-type animals because endogenous 5-HT activity is potentiated. This behavior, called the hyperenhanced slowing response, can be suppressed by mutations in genes that encode proteins important for 5-HT signaling, like the 5-HT receptor encoded by *mod-1* and the G_α subunit of a G protein encoded by *goa-1*. This ability to suppress indicates that these genes likely act downstream of or in parallel to one or more 5-HT synapse(s) that mediate(s) the enhanced slowing response. To find genes that play a role in 5-HT signaling, we screened for suppressors of the 5-HT hypersensitivity of *mod-5*. We found at least seven alleles of *goa-1* and at least two alleles of *mod-1*. This shows that our screen is able to target genes that play a role in endogenous 5-HT signaling. We identified two alleles of the FMRFamide-encoding gene *flp-1*, which was known to mediate paralysis in exogenous 5-HT. We showed that loss-of-function mutations in *flp-1* confer an enhanced slowing response defect. We also identified an allele of *abts-1*, which encodes a bicarbonate transporter, and showed that it has defects in cholinergic signaling. We identified three mutants that show linkage to LG I, four to II, three to V and one to X, most of which display defects consistent with a role in 5-HT signaling. We used a candidate gene approach to find that deletions in *ser-4*, which encodes a metabotropic 5-HT receptor, confer 5-HT resistance. *ser-4* acts redundantly with the ionotropic 5-HT receptor *mod-1* to suppress the hyperenhanced slowing response of *mod-5*. Our genetic analysis suggests that *ser-4* acts in a pathway with *goa-1*, in parallel to *mod-1*. We found that the enhanced slowing response defect of *flp-1* is primarily due to its defect in transmitting a 5-HT signal and that *flp-1* likely acts downstream of *ser-4* and *mod-1*.

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TABLE OF CONTENTS

Chapter 1

Introduction

Serotonin signaling in *C. elegans*

Introduction

Discovery of serotonin

Serotonin was initially identified and subsequently isolated in two independent lines of investigation (Whitaker-Azmitia, 1999). In the 1930s in Italy, Vittorio Erspamer identified a substance he called enteramine in the enterochromaffin cells of the gut that caused smooth muscle contraction. Meanwhile, in the United States, Irvine Page was attempting to isolate factors involved in hypertension from blood serum (Rapport et al., 1948a). However, he first had to identify and eliminate a contaminating substance with vasoconstrictor activity that appeared upon coagulation of the blood. Maurice Rapport and Arda Green, scientists in Page's lab, purified this substance and showed that it was a potent vasoconstrictor (Rapport et al., 1948a). The name serotonin was chosen because it "indicates that its source is serum and its activity is one of causing constriction" (tone) (Rapport et al., 1948b) and its structure was determined (Rapport, 1949). When Erspamer and Asero (Erspamer and Asero, 1952) determined the structure of enteramine, they realized that enteramine and serotonin were the same molecule, 5-hydroxytryptamine (5-HT).

Establishment of 5-HT as a neurotransmitter

The discovery that 5-HT was present in the mammalian brain (Twarog and Page, 1953) and the first description of neurons that contained 5-HT in the mammalian brain (Dahlstrom and Fuxe, 1964) cited in (Pineyro and Blier, 1999) provided the first evidence that 5-HT might act as a neurotransmitter. In addition, the discovery that the hallucinogen

LSD (d-lysergic acid diethylamide) shared structural similarities and could antagonize 5-HT in peripheral tissues led to the independent proposals by (Gaddum and Hameed, 1954) and (Woolley and Shaw, 1954) that 5-HT might normally play a role in maintaining normal brain function, and that LSD might cause hallucinations by antagonizing those actions of 5-HT in the brain (Aghajanian and Marek, 1999).

5-HT is an important signaling molecule in mammals

5-HT plays an important role in a variety of biological processes in many animals. While 5-HT is most commonly studied as a neurotransmitter, an estimated 95% of 5-HT in the human body is present in the gut (Gershon and Tack, 2007), primarily in enterochromaffin cells (Costedio et al., 2007). These cells respond to mechanical pressure or the presence of nutrients by releasing 5-HT, which contributes to coordinated smooth muscle contractions (peristalsis) and secretory reflexes necessary for the function of the gut (De Ponti, 2004). Excess 5-HT is taken back up into epithelial cells and also into platelets by the *se*rotonin *r*euptake *t*ransporter (SERT), discussed in more detail below. Platelet 5-HT, which accounts for about 2% of total 5-HT (Gershon and Tack, 2007), has been shown to contribute to α-granule release, a crucial step in hemostasis (the process through which bleeding is stopped), through a novel receptor-independent mechanism involving the transamidation of 5-HT to small GTPases, a process called serotonylation (Walther et al., 2003).

Despite the prevalence of 5-HT in the gut and platelets, most studies have focused on the neurotransmitter function of 5-HT in the brain (Figure 1). Brain 5-HT accounts for about

3% of the total amount of 5-HT in the body (Gershon and Tack, 2007). In neurons, 5-HT is synthesized in two steps from tryptophan (Frazer and Hensler, 1999; Veenstra-VanderWeele et al., 2000). First, in the rate-limiting step of 5-HT synthesis, tryptophan hydroxylase converts tryptophan into 5-hydroxytryptophan (5-HTP). Second, the aromatic L-amino acid decarboxylase (AADC) converts 5-HTP into 5-HT. 5-HT is transported into and stored in vesicles by the *v*esicular *m*ono*a*mine *t*ransporter VMAT2. Release occurs by exocytosis in response to an increase in Ca^{2+} in the nerve terminal. 5-HT released into the synapse can bind to 5-HT receptors on the postsynaptic membrane. Fifteen different mammalian 5-HT receptors belonging to seven families ($5\text{-}HT_1$-$5\text{-}HT_7$) have been cloned to date (Bockaert et al., 2006). One class of receptor, $5\text{-}HT_3$, is a ligand-gated cation channel (Derkach et al., 1989; Maricq et al., 1991). The other receptors are G protein-coupled receptors that activate or inhibit signal transduction pathways (Barnes and Sharp, 1999; Bockaert et al., 2006). Termination of 5-HT signaling occurs primarily by reuptake of 5-HT into the presynaptic terminal by the *se*rotonin *r*euptake *t*ransporter (SERT), a member of the Na^+/Cl^- family of transporters (Blakely et al., 1991; Hoffman et al., 1991; Nelson, 1998). Following reuptake into the cell, 5-HT can be degraded by *m*ono*a*mine *o*xidase (MAO) (Frazer and Hensler, 1999).

In the brain, most 5-HT-containing cells have cell bodies in the raphe nuclei of the brainstem and project to many areas of the brain (Kandel et al., 2000). 5-HT has been proposed to generally regulate brain function by modulating the tone of the nervous system, affecting many different aspects of brain functions including mood, appetite, sleep and motor function (Frazer and Hensler, 1999). One example of the effect of 5-HT

on motor activity is its involvement in mediating the activity of central pattern generators, circuits responsible for mediating repetitive movements like swimming, walking or whisking (whisker movement in rodents) (Arshavsky et al., 1997; Cramer and Keller, 2006). In addition, 5-HT plays a role in neuroendocrine function, regulating release of hormones from the anterior pituitary gland (Frazer and Hensler, 1999).

Abnormal 5-HT signaling is implicated in many diseases

Abnormal 5-HT signaling has been implicated in many diseases that affect the tissues in which 5-HT has been detected. There is evidence for abnormal 5-HT signaling in diseases of the gut, *u*lcerative *c*olitis (UC) and *i*rritable *b*owel *s*yndrome (IBS). Patients with UC and IBS show lower levels of serotonin, tryptophan hydroxylase mRNA and SERT mRNA in the colon than healthy patients, indicating misregulation of several different aspects of 5-HT signaling (Coates et al., 2004). While 5-HT has not been implicated in any disease directly related to platelet function, there have been reports of abnormal bleeding associated with the use of selective serotonin reuptake inhibitors (SSRIs), consistent with a role for platelet 5-HT in hemostasis (Turner et al., 2007). Many human psychiatric conditions, including depression, anxiety disorders, and obsessive-compulsive disorder have been associated with mutations in or abnormal expression of genes that function in 5-HT signaling (Veenstra-VanderWeele et al., 2000). Finally, many of the most frequently used neuropsychiatric drugs are believed to have serotonergic neurons as their principal site of action. The widely-used selective serotonin reuptake inhibitors (SSRIs) and monoamine oxidase inhibitors (MAOs) are used to treat depression and anxiety. Atypical antipsychotic drugs (*e.g.* clozapine), defined as atypical

because they act on multiple types of receptors, have been shown to potently antagonize $5HT_{2A}$ receptors as well as weakly antagonize dopamine receptors (De Oliveira and Juruena, 2006).

Studies of 5-HT signaling in the nematode *Caenorhabditis elegans* can facilitate an understanding of mammalian 5-HT signaling

Identifying components of 5-HT pathways and determining how they generate or modulate a behavior will provide insight into the interesting biological processes of neurotransmission and behavior. As discussed above, the role of 5-HT signaling in mammals is complex and involves many different organs, tissues and signaling mechanisms. Identification of new 5-HT signaling molecules and pathways can be facilitated by use of a simpler organism. The roundworm *C. elegans* is well suited to this purpose. *C. elegans* exists as a 1 mm long, self-fertilizing hermaphrodite, making it ideal for use in genetic studies. The nervous system of *C. elegans* is relatively simple and well defined; the connectivity of all 302 neurons in the hermaphrodite has been determined by reconstruction from serial electron micrographs (White et al., 1986). These few neurons mediate a variety of behaviors, including locomotion, feeding, egg laying, and detecting and responding to temperature, mechanical stimuli, osmolarity and soluble chemicals and odorants (Wood, 1988). 5-HT is present in *C. elegans* (Horvitz et al., 1982), as are many other neurotransmitters known to play an important role in mammals, including acetylcholine (Culotti et al., 1981; Johnson et al., 1981), GABA (Hedgecock, 1976), dopamine (Sulston et al., 1975), glutamate (Cully et al., 1994) and neuropeptides

(Schinkmann and Li, 1992) and the invertebrate-specific neurotransmitters octopamine (Horvitz et al., 1982) and tyramine (Alkema et al., 2005).

5-HT is present in 13 neurons in *C. elegans*

5-HT can be visualized in neurons using formaldehyde-induced fluorescence (FIF) or anti-5-HT antibodies (Caspersson et al., 1966). Horvitz et al. (1982) used FIF to show that 5-HT is present in the neurosecretory motor neurons (NSMs) in the pharynx of *C. elegans*. Use of anti-5-HT antibodies in analyses of the genetic pathway controlling the development (Desai et al., 1988) and outgrowth (McIntire et al., 1992) of the HSN neurons, which regulate egg laying, showed that they also contain 5-HT. Sawin (1996) found that, along with the NSMs and HSNs, the chemosensory ADFs and the RIH and AIM interneurons stained with anti-5-HT antibodies. In addition, the chemosensory PHB neurons in the tail and the I5 neuron in the pharynx stained with anti-5-HT antibodies following pre-incubation with 5-HT, which might indicate that these cells are capable of taking up 5-HT from the surrounding environment rather than synthesize 5-HT. Duerr *et al.* (Duerr et al., 1999) used anti-5-HT antibodies to confirm that 5-HT was present in the NSMs, HSNs, ADFs, RIH and AIMs and also found light and variable staining of the VC4 and VC5 neurons. Six VC class motor neurons innervate the vulva, and VC4 and VC5 are closest to the vulva (White et al., 1986). Subsequent analysis with a GFP reporter for the gene *tph-1,* which encodes the 5-HT biosynthetic enzyme *t*ryptop*h*an *h*ydroxylase, expresses in the NSMs, HSNs, ADFs and rarely in the AIMs and RIH (Sze et al., 2000). Because *tph-1* is required for the synthesis of 5-HT (Sze et al., 2000), cells that synthesize 5-HT must express *tph-1*. This suggests that cells that contain 5-HT but

do not express *tph-1*, the VCs, PHBs and I5, have a way of taking 5-HT up from the surrounding environment. However, we will present results in Chapter 3 that suggest that these neurons do not express *mod-5*, the only SERT in *C. elegans* (Ranganathan et al., 2001) Alternatively, the immunoreactive material in the VCs, PHBs and I5 might be a compound structurally related to 5-HT, rather than 5-HT itself. To summarize, two classes of neuron with sensory endings in the the pharynx (NSMs and I5), two classes that innervate the egg-laying musculature (HSNs and VCs), two classes of chemosensory neurons (ADF and PHB) and two classes of interneurons (AIM and RIH) are reported to contain 5-HT in the *C. elegans* hermaphrodite. Further investigation is needed to determine which of these cells synthesizes 5-HT and which, if any, only take it up from the surrounding environment.

Conserved 5-HT signaling molecules exist in *C. elegans*

The genome of *C. elegans* encodes many molecules known to function in 5-HT signaling (Figure 2). In addition to *tph-1*, described above, the genome encodes other biosynthetic enzymes necessary to synthesize 5-HT: *bas-1* (*b*iogenic *a*mine *s*ynthesis related), which encodes an AADC required for the synthesis of 5-HT and dopamine (Sze et al., 2000; Hare and Loer, 2004) and *cat-4*, abnormal *cat*echolamine distribution, which encodes GTP cyclohydrolase I, an enzyme required to make a cofactor used by *tph-1* and other enzymes (Cronin et al., 2005). *cat-1* encodes a VMAT homolog, which is required to concentrate 5-HT and other biogenic amines into vesicles for release (Duerr et al., 1999). Four 5-HT receptors are known: the 5-HT-gated chloride channel *mod-1* (Ranganathan et al., 2000), and three G protein coupled receptors, *ser-1*, *ser-4* and *ser-7* (Olde and

McCombie, 1997; Hamdan et al., 1999; Hobson et al., 2003). Six other genes, *T02E9.3, C24A8.1, ser-3, M03F4.3, F14D12.6,* and *Y54G2A.35,* are similar to G protein-coupled biogenic amine receptors and might bind 5-HT, but their ligand-binding properties have not yet been tested (see Chapter 3). Members of a G protein-signaling pathway that mediates 5-HT signaling are known and include *goa-1* (Gαo) and *dgk-1* (*d*iacylglycerol *k*inase) (Mendel et al., 1995; Segalat et al., 1995; Hajdu-Cronin et al., 1999). Two proteins responsible for termination of 5-HT signaling are the serotonin reuptake transporter (SERT) and monoamine oxidase (MAO). The *C. elegans* genome encodes one SERT, *mod-5* (Ranganathan et al., 2001), and three genes with similarity to MAO, *amx-1, amx-2* and *amx-3* (Wormbase website, http://www.wormbase.org, release WS176, 7/4/07). The existence of many conserved 5-HT signaling molecules suggests that studying 5-HT signaling in *C. elegans* will contribute to our understanding of human biology.

5-HT might act to signal food availability

Detecting and responding appropriately to food is necessary for an organism's survival. 5-HT might act to signal the presence of bacteria (food) in *C. elegans*. The profile of behaviors exhibited by animals exposed to exogenous 5-HT is similar to that of animals in the presence of bacteria. Both 5-HT and bacteria stimulate egg laying and pharyngeal pumping (feeding) and inhibit locomotion (Horvitz et al., 1982; Segalat et al., 1995; Riddle, 1997). Animals that lack *tph-1,* and thus 5-HT, are egg-laying defective (Egl) and have lower rates of pharyngeal pumping in the presence of food (Sze et al., 2000), consistent with the hypothesis that 5-HT mediates a food signal.

How might 5-HT release occur in response to food? Based on NSM morphology and ultrastructure (Albertson and Thomson, 1976), the NSMs were proposed to function by detecting food and then secreting 5-HT (Horvitz et al., 1982) humorally into the pseudocoelom. The NSMs have small processes that extend to just under the lumen of the pharynx and major processes that run along the length of the pharynx in close proximity to the pseudocoelom. Thus, upon detection of food in the lumen, these cells might release 5-HT into the pseudocoelom where it could act humorally. Humoral dispersal of 5-HT could allow the coordination of distant tissues necessary to respond to detection of food. These behavioral changes likely serve an adaptive purpose. Inhibiting locomotion upon detection of food allows animals to stay in the vicinity of a food source longer. Increasing pharyngeal pumping when food is present allows animals to maximize food intake and a reduced rate of pumping in the absence of food allows animals to conserve energy. Laying eggs in response to food allows animals to leave offspring near a food source, which is also likely to benefit survival.

The neuroanatomy of the locomotory circuit of *C. elegans* is simple

Understanding how 5-HT might inhibit the locomotion of an animal requires an understanding of the locomotory circuit (Figure 3). *C. elegans* locomotion is characterized by sinusoidal movements in which coordinated contraction and relaxation of ventral and dorsal body wall muscles propagates a wave that propels the animal forward or backward (Chalfie and White, 1988). Three classes of motorneurons (A, B, and D) innervate these body wall muscles. Motorneurons that innervate dorsal muscles

are called DA, DB and DD and those that innervate ventral muscles are called VA, VB and VD. The A and B class motorneurons are innervated by command interneurons: AVB and PVC drive forward locomotion by activating the cholinergic B motor neurons and AVA, AVD and AVE drive backward locomotion by activating the cholinergic A neurons.

To generate coordinated locomotion, dorsal muscles must relax while opposing ventral muscles contract (Chalfie and White, 1988). Relaxation of opposing muscles is proposed to occur by means of the D class of motorneurons. Unlike the A and B classes, which are innervated by interneurons, the D class motorneurons are innervated by the opposing motorneurons: VD neurons are innervated by DA and DB, while the DD neurons are innervated by VA and VB motorneurons. D motorneurons are GABAergic and likely act to inhibit contraction of the opposite body wall muscle. Thus, DA and DB cause contraction of dorsal body wall muscle and simultaneous activation of VD, which inhibits the contraction of opposing body wall muscle.

It is not yet clear whether the circuit has an intrinsic oscillatory nature, like central pattern generators that have been described to regulate locomotory behaviors in other organisms. In *Ascaris*, a larger nematode with similar anatomy to *C. elegans,* the D-like GABAergic motor neurons display intrinsic oscillatory activity (cited in (Chalfie and White, 1988)). However, this is likely not the case for *C. elegans,* because mutants lacking functional D motorneurons can still move rhythmically (Chalfie et al., 1985). One model that has been proposed asserts that the command interneurons (AVA, AVB, AVD and PVC) function

as a central pattern generator by maintaining rhythmic activity through the interconnections present between the cells (Niebur and Erdos, 1993).

How might the speed of movement be regulated through this locomotory circuit? Korta *et al.* (Korta et al., 2007) propose a model in which the speed of movement is regulated through presynaptic inputs to the command interneurons (PVC, AVB, AVA, AVD, and AVE). Ablating the ALM neurons, which are presynaptic to the command interneurons, causes an animal to slow its rate of locomotion. ALM is a touch receptor neuron that mediates reversals in response to head touch by stimulating the command interneurons that mediate backward movement (AVB and PVC) and inhibiting the command interneurons that mediate forward movement (AVA and AVD). Korta *et al.* (Korta et al., 2007) propose that ALM might also be able to regulate the intensity of command interneuron firing to modulate locomotion rate; *i. e.* at lower firing intensity, instead of completely inhibiting forward movement and initiating backward movement, a partial inhibition of forward movement might simply cause a reduction in the rate of forward movement.

5-HT inhibits *C. elegans* locomotion, allowing animals to remain near a food source

Both exogenous 5-HT and bacteria inhibit locomotion, but does endogenous 5-HT signaling inhibit locomotion in response to bacteria? Under what circumstances might this occur? Sawin *et al.* (2000) showed that when a wild-type animal encounters a bacterial lawn, it slows its rate of locomotion (Figure 4). A combination of genetic and laser-ablation experiments showed that this response, called the basal slowing response,

is mediated by dopamine. When a wild-type animal is food-deprived for 30 minutes prior to encountering a bacterial lawn, the animal slows its rate of locomotion even more than when it is well-fed. This behavior, called the enhanced slowing response, was shown to be mediated by a distinct neurotransmitter, 5-HT, because animals mutant for the 5-HT biosynthetic enzymes *bas-1* or *cat-4* exhibit reduced slowing that is rescued by supplying exogenous 5-HT. In addition, ablating the serotonergic NSMs or ADFs causes animals to exhibit reduced slowing. Later studies showed that *tph-1* mutants, which should specifically lack 5-HT, show reduced slowing and that this can be rescued by expressing a *tph-1* transgene in the NSMs, further supporting a role for 5-HT in this behavior (Zhang et al., 2005). Therefore, endogenous 5-HT signaling does appear to slow the rate of an animal's locomotion in response to bacteria, but only when the animal has been food deprived. Thus, the increased slowing of an animal that has recently experienced an unfavorable environment (food deprivation) is not simply increased basal slowing, but a distinct mechanism that occurs as a result of changes in an animal's internal state (Sawin et al., 2000). These results suggest the following model: well-fed animals that are in contact with a food source stay in the proximity of that food source by releasing dopamine, which inhibits locomotion. When animals have lost contact with a food source for a brief period, an internal state change occurs such that when they re-encounter a food source, 5-HT is released. 5-HT inhibits their rate of locomotion to an even greater degree, ensuring that they stay in the proximity of that food source.

Mutations in the *C. elegans* SERT gene *mod-5* cause greater inhibition of locomotion in response to exogenous 5-HT and in the enhanced slowing response

As previously discussed, 5-HT can be detected in the NSMs of *C. elegans* using FIF (Horvitz et al., 1982). This staining is much brighter if animals are preincubated in 5-HT, indicating that animals can actively take up 5-HT into neurons. In a screen for mutants that could not take up 5-HT into the NSMs, two non-complementing mutations, *n822* and *n823,* were isolated. In these mutants, FIF was seen in the NSM axonal processes but not in cell bodies following pre-incubation in 5-HT (Ranganathan et al., 2001), indicating that these mutants had endogenous 5-HT but were defective in exogenous 5-HT uptake. These mutants were also abnormal in the enhanced slowing response: when food-deprived *n822* or *n823* mutants encounter a bacterial lawn, they slow to an even greater extent than wild-type animals. Because 5-HT mediates the enhanced slowing response, it is likely that this hyperenhanced slowing response is caused by hypersensitivity to endogenous 5-HT. To test whether *n822* and *n823* mutants are hypersensitive to exogenous 5-HT, they were assayed for mobility in a 5-HT solution. Wild-type animals will swim for a given period of time depending upon 5-HT concentration, and then become immobilized. *n822* and *n823* animals become immobilized sooner and at lower concentrations than wild-type animals, indicating a hypersensitivity to 5-HT (Sawin, 1996; Ranganathan et al., 2001).

The gene mutated in *n822* and *n823* was named *mod-5* (*mod*ulation of locomotion defective) and cloned (Ranganathan et al., 2001). *mod-5* encodes a SERT similar in sequence to the protein on which SSRIs like Prozac act. *n822* and *n823* were determined

to be nonsense and missense alleles, respectively. A deletion allele, *mod-5(n3314),* was then isolated by screening a chemical deletion library. *mod-5(n3314)* was also defective in the uptake of exogenous 5-HT into the NSMs, and exhibited a more severe 5-HT hypersensitivity than *n822* and *n823* in both the enhanced slowing response (Figure 5) and the exogenous liquid assay. Like other SERTs, MOD-5 is thought to remove 5-HT from synapses by transporting it back into the presynaptic terminal. Presumably, 5-HT cannot be efficiently cleared from the synapse in *mod-5* mutants, resulting in a greater concentration of 5-HT in the synapse. More 5-HT might cause more downstream signaling, resulting in a greater degree of inhibition of locomotion. Loss-of-function mutations in genes downstream of *mod-5*, *i.e.* those involved in the signaling pathway in which *mod-5* acts, should suppress the *mod-5* phenotype.

Mutations known to suppress the 5-HT hypersensitivity of *mod-5* are components of 5-HT signaling transduction pathways

Two genes that can suppress the 5-HT hypersensitivity and hyperenhanced slowing response of *mod-5* (Ranganathan et al., 2001) have been cloned and characterized: *mod-1* and *goa-1*. Mutations in these genes also cause 5-HT resistance in a wild-type background (Mendel et al., 1995; Segalat et al., 1995; Ranganathan et al., 2000), and are reported to exhibit defects in the enhanced slowing response in a wild-type background (Ranganathan et al., 2000; Sawin et al., 2000) (Figure 5). In addition, *dgk-1* confers a defect in the enhanced slowing response (Sawin et al., 2000) (Figure 5) and is resistant to exogenous 5-HT (Nurrish et al., 1999). *mod-1* encodes a 5-HT-gated Cl^- channel (Ranganathan et al., 2000), and *goa-1* encodes the Gαo subunit of a G protein required

for the transduction of a signal from a metabotropic receptor (Mendel et al., 1995; Segalat et al., 1995). *dgk-1* encodes diacylglyerol (DAG) kinase and is thought to act downstream of *goa-1* (Miller et al., 1999).

mod-1 is required for another 5-HT-mediated behavior

mod-1 has also been shown to play a role in another experience-dependent behavior, learned aversion to pathogenic bacteria (Zhang et al., 2005). Naïve animals show no preference for either pathogenic or non-pathogenic bacteria; they are equally likely to move toward and feed on either strain. However, animals that have been exposed to pathogenic bacteria for four hours will prefer the non-pathogenic bacteria. This behavior was shown to be mediated by 5-HT acting through the serotonergic ADF neurons and to require *mod-1*. While this report shows no role for *tph-1* in the ADFs to mediate the enhanced slowing response, previous studies showed that ablating the ADFs caused a small defect in the enhanced slowing response (Sawin, 1996). So, while the contribution of the ADFs to enhanced slowing remains unclear, these studies show that 5-HT acting through *mod-1* modulates two experience-dependent behaviors: 5-HT in the ADFs and *mod-1* are required for learned aversion to pathogenic bacteria, and 5-HT in the NSMs and *mod-1* are required for animals to slow in response to bacteria following food-deprivation (Sawin et al., 2000; Zhang et al., 2005).

goa-1 and *dgk-1* act in many tissues to mediate different behaviors

The signaling pathway containing *goa-1* and *dgk-1* is thought to regulate 5-HT signaling to control both locomotion and egg laying. *goa-1* and *dgk-1* loss-of-function mutants

show many of the same defects, including hyperactive locomotion that is resistant to the paralyzing effects of exogenous 5-HT and hyperactive egg laying. *goa-1* and *dgk-1* mutants are also hypersensitive to paralysis by the acetylcholinesterase inhibitor aldicarb, an indication of excessive levels of acetylcholine at the synapse (Mendel et al., 1995; Segalat et al., 1995; Hajdu-Cronin et al., 1999). The neuromuscular junctions of *C. elegans* use acetylcholine as an excitatory neurotransmitter. Thus, the hyperactivity and aldicarb resistance of *goa-1* and *dgk-1* mutants could both occur as a result of an increased rate of acetylcholine release (see below for further discussion). This *goa-1*-containing pathway opposes the action of another G protein pathway containing a Gαq subunit encoded by *egl-30* and its downstream effector, the PLCβ gene *egl-8* (Brundage et al., 1996; Miller et al., 1999). *egl-30* and *egl-8* mutants, in contrast to *goa-1* and *dgk-1* mutants, are sluggish, egg-laying defective and resistant to aldicarb. The genes in these opposing pathways are expressed throughout the nervous system and in other tissues, which suggests they might mediate many different types of signals in addition to 5-HT to mediate different behaviors. For example, 5-HT-stimulated egg laying appears not to require *egl-8* function, indicating that non-overlapping sets of signaling molecules might be required for different functions or in different tissues (Bastiani et al., 2003). In addition, many of the genes in this pathway have also been shown to play a role in dopamine-mediated slowing (Chase et al., 2004), suggesting that these pathways might act downstream of both 5-HT and dopamine and represent the convergence of multiple signals that cause an animal to decrease its rate of locomotion.

The *goa-1* and *egl-30* second messenger pathways mediate the speed of locomotion by regulating acetylcholine release

Genetic and pharmacological evidence, as well as evidence drawn from homologous proteins in mammalian studies, indicate that these conserved proteins mediate 5-HT-induced slowing by regulating acetylcholine release in motorneurons: EGL-30 Gαq activates EGL-8 PLCβ, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into the lipid second messengers DAG and inositol triphosphate (IP_3). DAG then binds UNC-13, a highly conserved molecule that alters the conformation of syntaxin, allowing vesicles to fuse with the presynaptic membrane and release acetylcholine (Lackner et al., 1999). DGK-1, a putative downstream effector of *goa-1* Gαo, acts oppositely, phosphorylating DAG to form phosphatidic acid (PA), decreasing the amount of DAG available to stimulate UNC-13 and thus promote acetylcholine release (Nurrish et al., 1999). The aldicarb hypersensitivity of *goa-1* mutants can be rescued by expressing *goa-1* under the *acr-2* (a nicotinic acetylcholine receptor (Squire et al., 1995)) promoter, which expresses in the cholinergic A and B motorneurons (and in IL1, RMD and PVQ), indicating that these signaling pathways directly regulate acetylcholine release (Nurrish et al., 1999). However, the cellular location of 5-HT action has yet to be determined.

Conclusion

As discussed in this introduction, identifying new components of 5-HT signaling pathways will facilitate our understanding of how 5-HT mediates a variety of interesting behaviors and other processes. Additionally, newly discovered components of a 5-HT pathway might help identify human counterparts that can be tested for therapeutic

applications. Only a few genes are known that act downstream of *mod-5* to mediate response to exogenous 5-HT and the enhanced slowing response. The studies that follow represent our efforts to find and characterize additional genes that play a role in 5-HT signaling by screening for mutants that suppress the 5-HT hypersensitivity of *mod-5(n3314)* SERT animals. Many of the mutants that we identified in this way also suppressed the hyperenhanced slowing response of *mod-5* animals. We characterized the interactions among genes that play a role in the enhanced slowing response, as well as the cellular expression patterns of these genes and propose a genetic and cellular model for how a food-deprived animal uses 5-HT to decrease its rate of locomotion in the presence of food.

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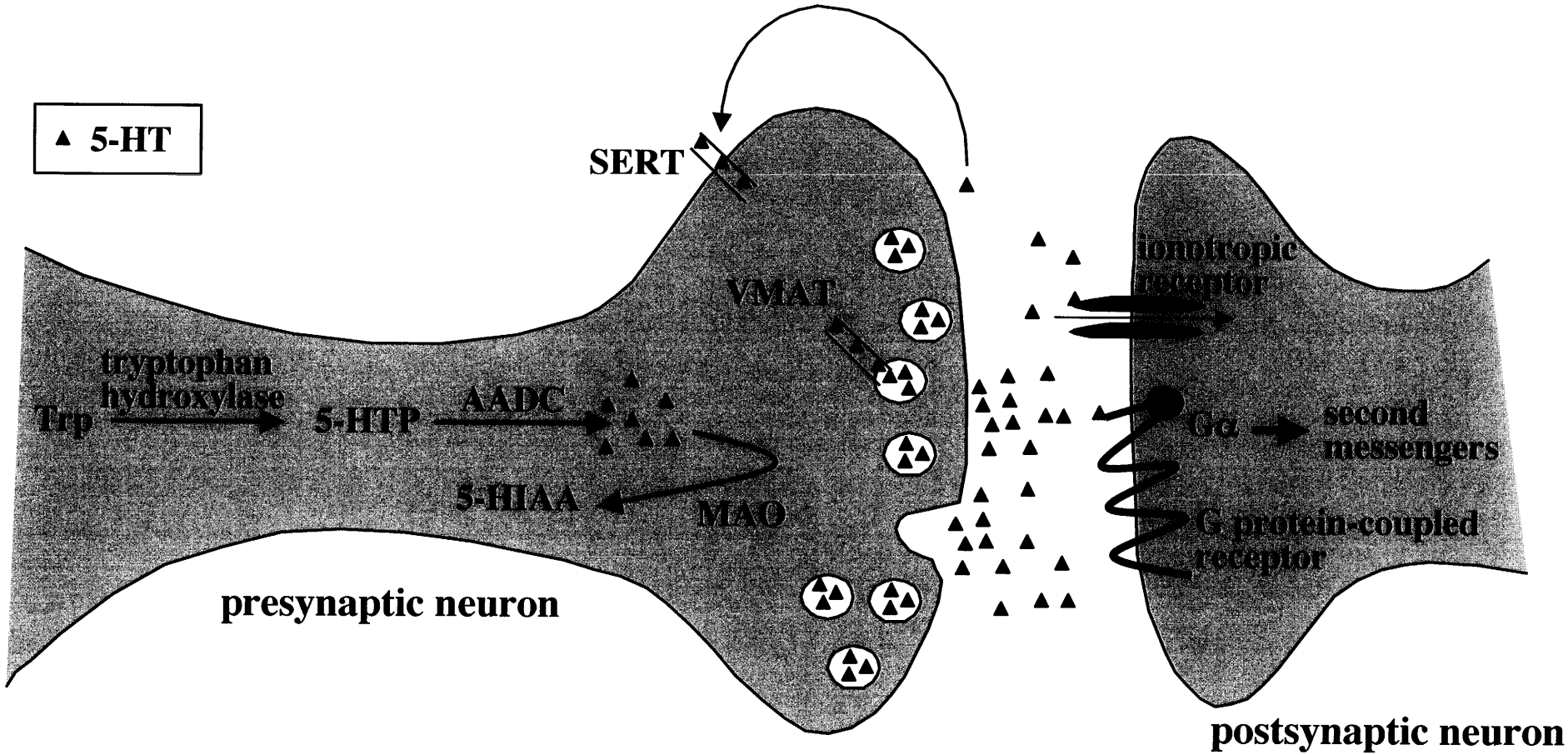
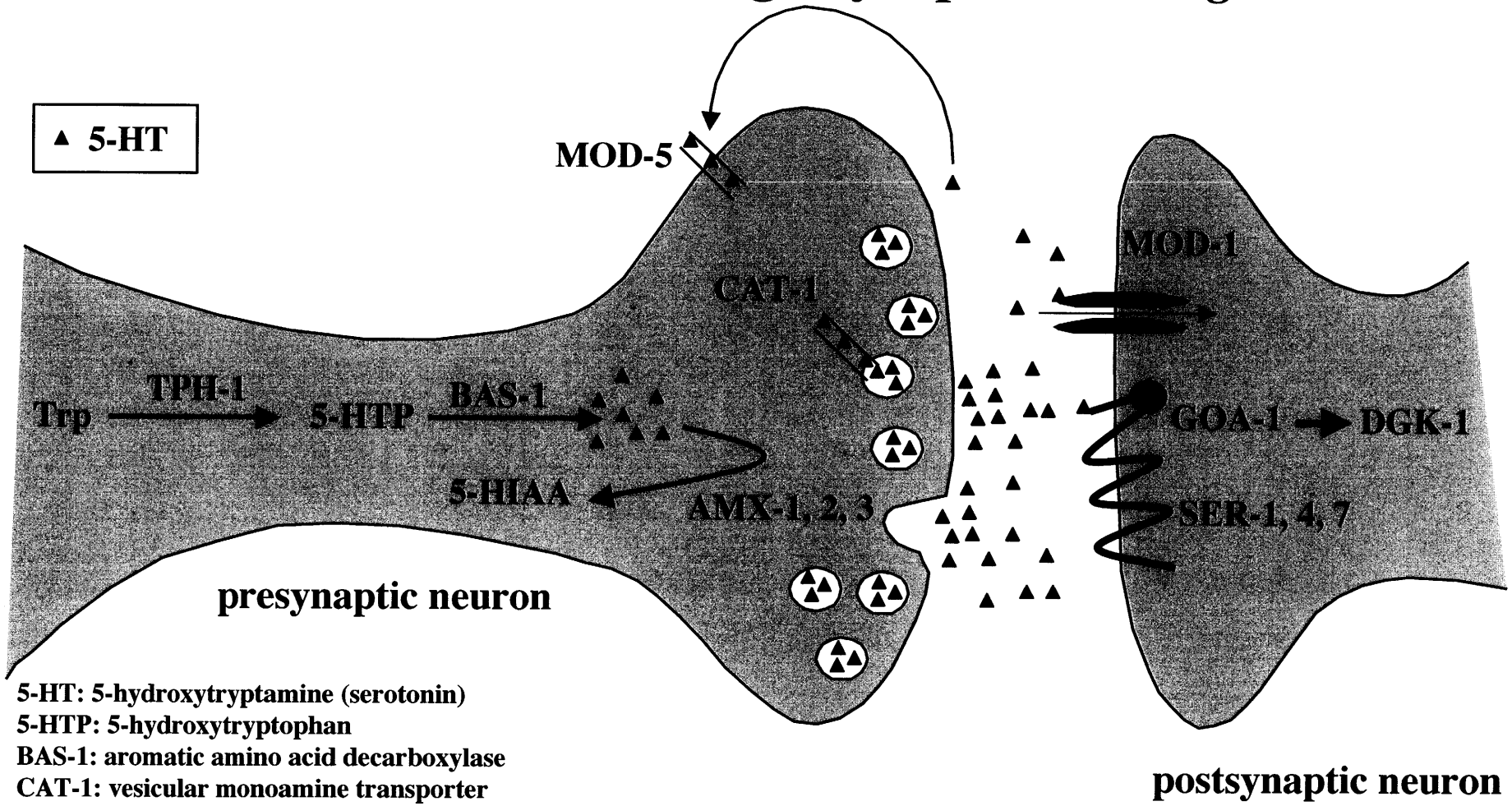
Model of a serotonergic synapse



5-HT: 5-hydroxytryptamine (serotonin)
5-HTP: 5-hydroxytryptophan
AADC: aromatic amino acid decarboxylase
VMAT: vesicular monoamine transporter
SERT: serotonin reuptake transporter
MAO: monoamine oxidase
5-HIAA: 5-hydroxyindoleacetic acid

Figure 1: Model of a serotonergic synapse. 5-HT is synthesized in neurons from the amino acid tryptophan (Trp) by tryptophan hydroxylase and AADC. 5-HT is packaged into vesicles by the vesicular monoamine transporter (VMAT). In response to an increase in intracellular Ca^{+}, vesicles fuse with the presynaptic terminal and 5-HT is released into the synapse. Synaptic 5-HT can act on postsynaptic ionotropic or metabotropic receptors. Termination of 5-HT signaling occurs by reuptake into the presynaptic membrane by the serotonin reuptake transporter (SERT) and can be degraded by monoamine oxidase (MAO) (reviewed in Frazer and Hensler, 1999 and Veenstra-VanderWeele *et al.*, 2000).

Model of a serotonergic synapse in *C. elegans*



5-HT: 5-hydroxytryptamine (serotonin)
5-HTP: 5-hydroxytryptophan
BAS-1: aromatic amino acid decarboxylase
CAT-1: vesicular monoamine transporter
MOD-5: serotonin reuptake transporter
AMX-1, 2, 3: monoamine oxidases
5-HIAA: 5-hydroxyindoleacetic acid
MOD-1: 5-HT-gated Cl^- channel
GOA-1: alpha subunit of a G protein
DGK-1: diacylglycerol kinase
SER-1, 4, 7: G protein-coupled receptors

Figure 2: Model of a serotonergic synapse in *C. elegans*. Proteins involved in 5-HT signaling are conserved in *C. elegans*.

The neural circuit for *C. elegans* locomotion

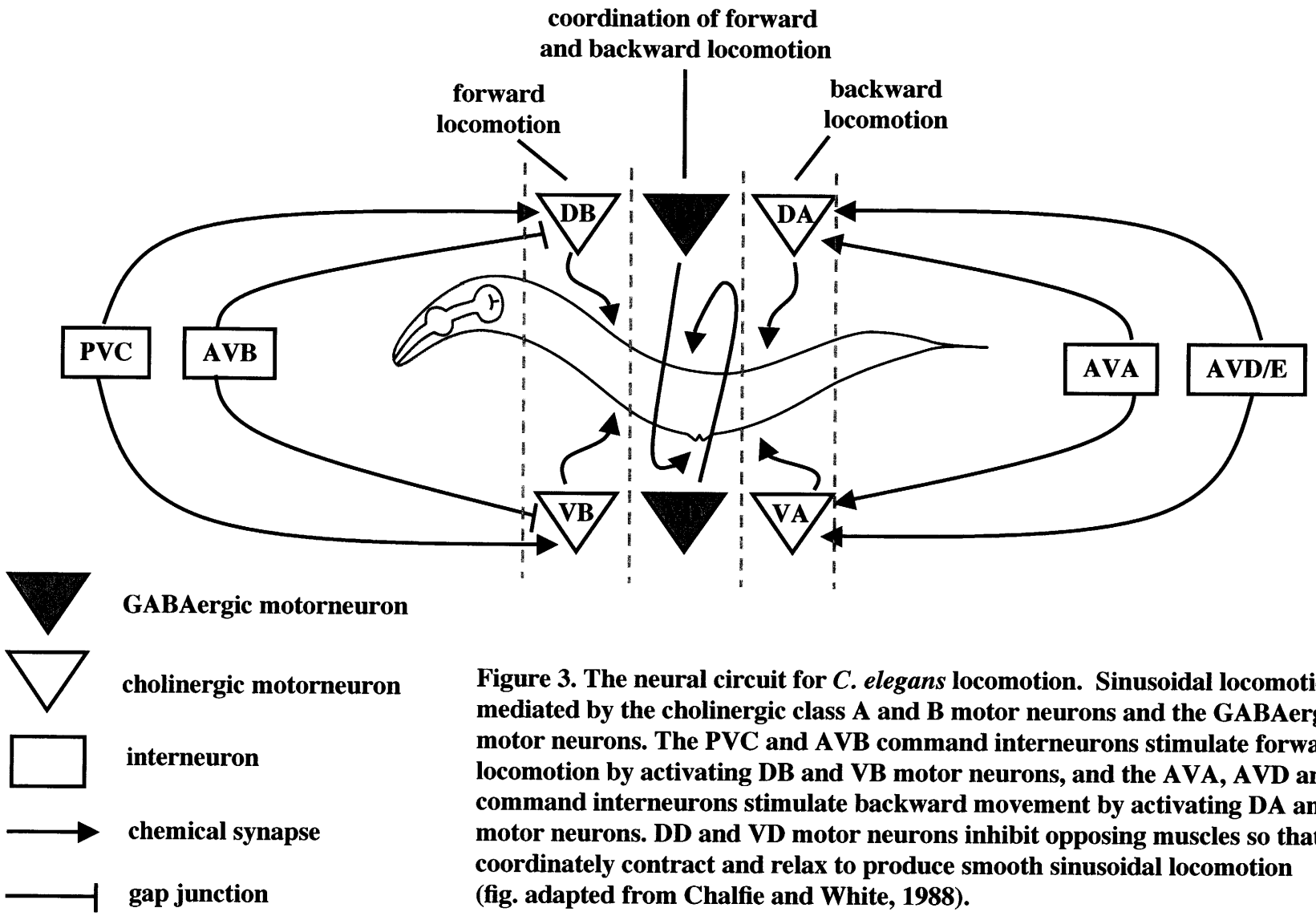


Figure 3. The neural circuit for *C. elegans* locomotion. Sinusoidal locomotion is mediated by the cholinergic class A and B motor neurons and the GABAergic class D motor neurons. The PVC and AVB command interneurons stimulate forward locomotion by activating DB and VB motor neurons, and the AVA, AVD and AVE command interneurons stimulate backward movement by activating DA and VA motor neurons. DD and VD motor neurons inhibit opposing muscles so that muscles coordinately contract and relax to produce smooth sinusoidal locomotion (fig. adapted from Chalfie and White, 1988).

C. elegans modulates its behavior based on experience and environment

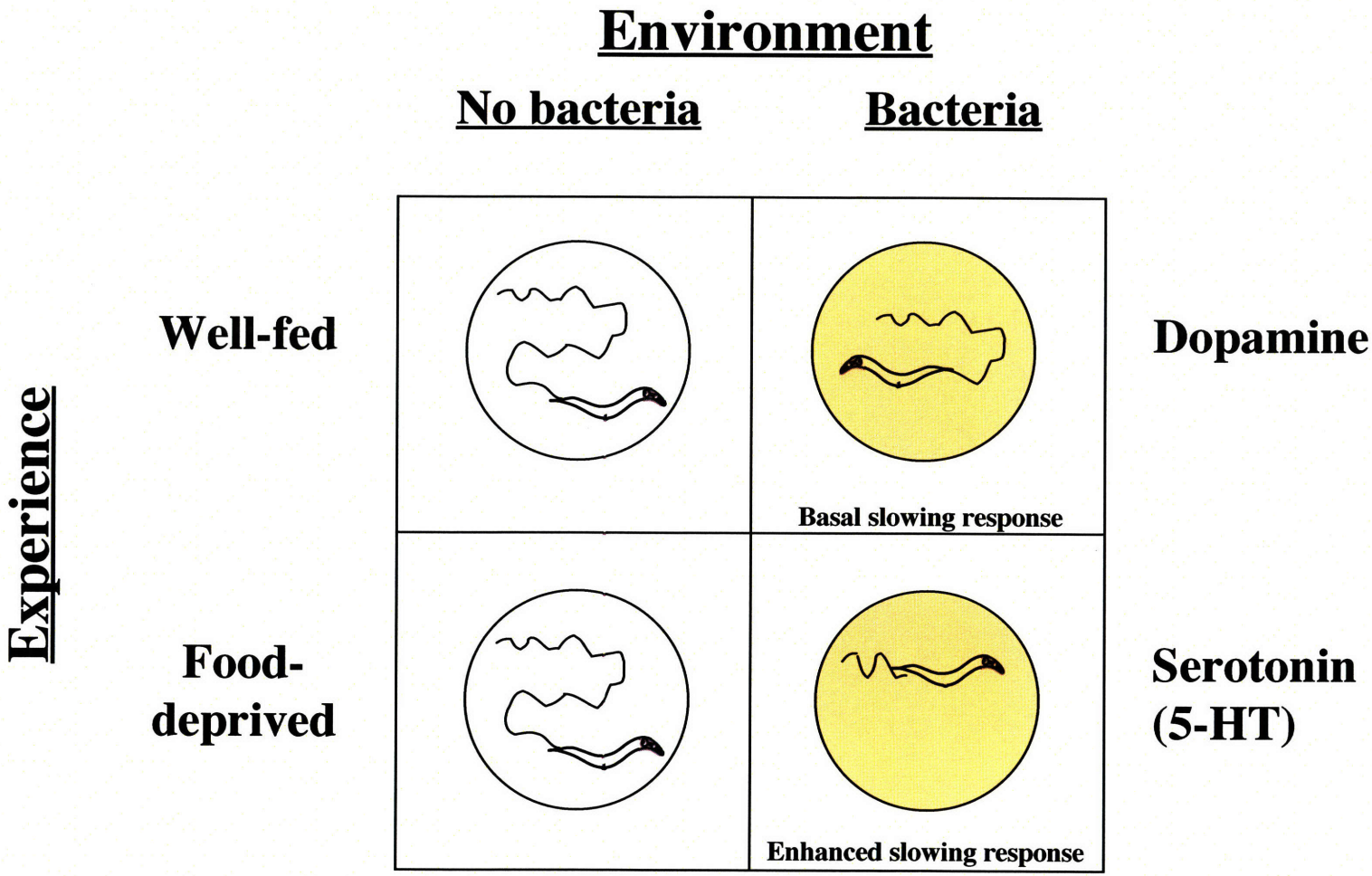


Figure 4. ***C. elegans*** **modulates its behavior based on the environment and experience. Well-fed animals exhibit a slower rate of locomotion on plates with food (bacteria) than on plates without food. This basal slowing response is mediated by dopamine. Animals that have been food deprived slow even more than do well-fed animals on food. This enhanced slowing response is mediated by 5-HT (Sawin et al., 2000).**

Mutations in 5-HT signaling genes cause defects in the enhanced slowing response

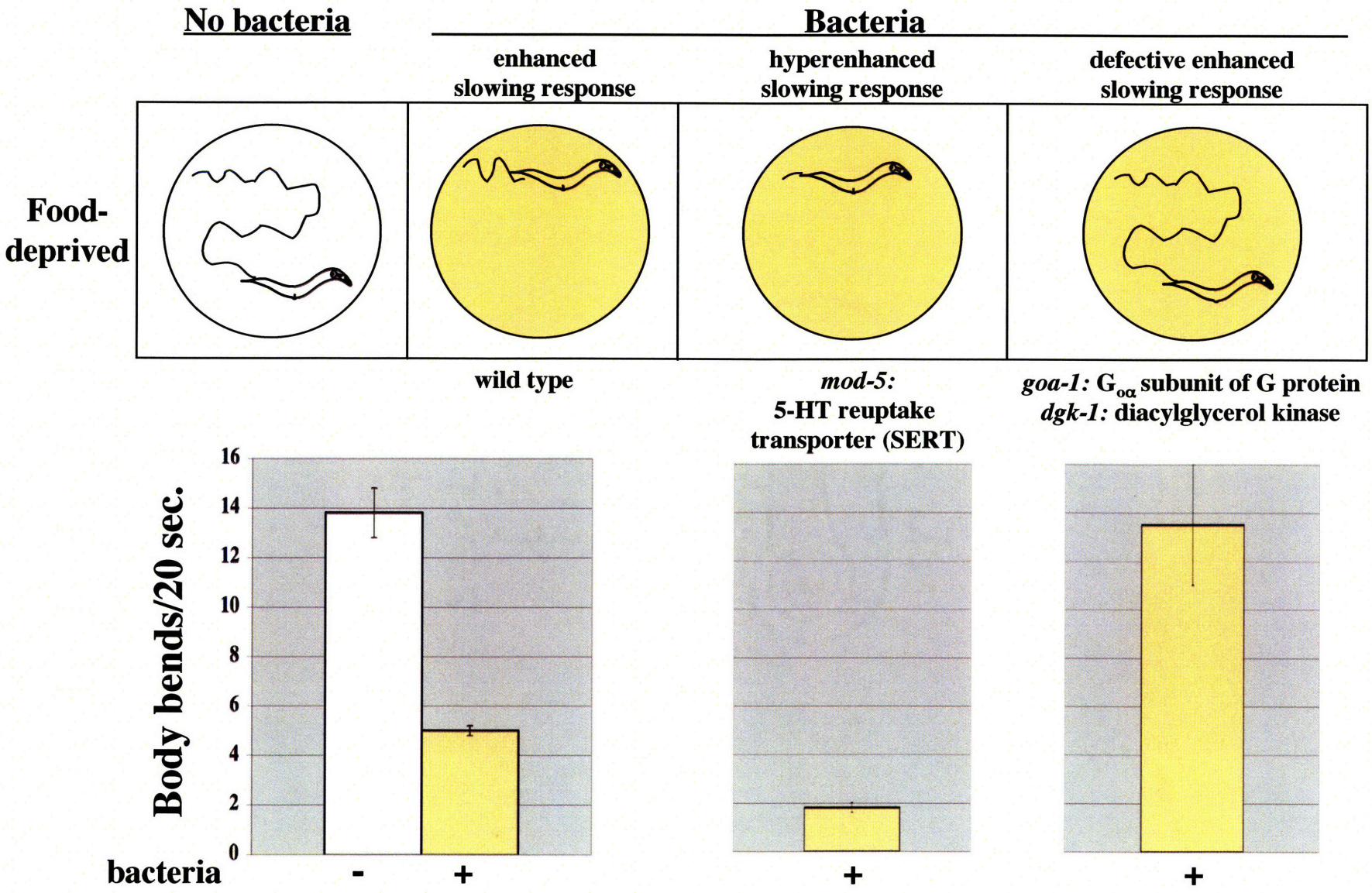


Figure 5. Mutations in 5-HT signaling genes cause defects in the enhanced slowing response. Mutating the SERT gene *mod-5* causes animals to exhibit more slowing on food following food deprivation, likely as a result of increased 5-HT signaling (Ranganathan *et al.*, 2001). Mutating members of a G protein signaling cascade (*goa-1 and dgk-1*) causes animals to exhibit less slowing, likely as a result of defective 5-HT signaling (Sawin *et al.*, 2000). Data shown are unpublished observations (M.A.G. and H.R.H).

Chapter 2

Suppressors of the serotonin hypersensitivity of *mod-5* SERT mutants define new genes involved in serotonin signaling in *C. elegans*

Megan A. Gustafson, Rajesh Ranganathan and H. Robert Horvitz

This chapter is written for submission to *Genetics*. Rajesh Ranganathan conceived, planned and helped perform the first screen described here. Daniel Omura helped generate Mos1-mutagenized *mod-5(n3314)* animals for the second screen described here.

ABSTRACT

The neurotransmitter serotonin (5-HT) is involved in many behaviors of *C. elegans*, including egg laying, pharyngeal pumping, and mediating the rate of locomotion on food following food deprivation ("the enhanced slowing response"). Loss-of-function mutations in *mod-5*, which encodes the 5-HT reuptake transporter, cause hypersensitivity to exogenous 5-HT and increased ("hyperenhanced") slowing in the enhanced slowing response. We used animals lacking *mod-5* as a sensitized genetic background to identify mutant animals defective in the response to 5-HT. We recovered at least 11 alleles of genes known to play a role in endogenous 5-HT signaling: seven alleles of the $G_{\alpha o}$ subunit gene *goa-1*; two alleles of the 5-HT-gated Cl^- channel gene *mod-1*; one allele of *eat-16,* which encodes an RGS protein; and two alleles of *flp-1,* which encodes eight FMRFamide neuropeptides. We identified 11 other mutants representing at least four new genes; most of these mutants display defects consistent with their being abnormal in endogenous 5-HT signaling. We also identified a mutation in the bicarbonate transporter gene *abts-1* and showed that loss of *abts-1* function causes altered responses to drugs that affect cholinergic signaling, which is known to function downstream of 5-HT signaling.

INTRODUCTION

Serotonin (5-HT) plays an important role in a variety of biological processes in many animals. The role of 5-HT as a neurotransmitter has been widely studied. Abnormalities in 5-HT function have been implicated in many human neuropsychiatric conditions, including depression, anxiety disorders, and obsessive-compulsive disorder (GERSHON and TACK 2007). Many of the most frequently used psychiatric drugs, such as the 5-HT reuptake inhibitor Prozac (fluoxetine), are believed to have serotonergic neurons as their principal site of action (VEENSTRA-VANDERWEELE *et al.* 2000). In addition to its roles as a neurotransmitter, 5-HT is found outside the nervous system and alterations in 5-HT signaling are associated with a variety of diseases and conditions including cardiac arrhythmias, migraine, and possibly irritable bowel syndrome (YUSUF *et al.* 2003; BUZZI and MOSKOWITZ 2005). A better understanding of the pathways in which 5-HT can act could lead to improved treatments for patients suffering from a variety of diseases.

5-HT is involved in a number of behaviors of the nematode *C. elegans*: 5-HT stimulates egg laying and pharyngeal pumping, inhibits locomotion and defecation, and is required for male mating behavior (HORVITZ *et al.* 1982; LOER and KENYON 1993; SEGALAT *et al.* 1995). 5-HT also mediates the enhanced slowing response of food-deprived animals: when a wild-type animal is food-deprived for 30 minutes prior to encountering a bacterial lawn, its food source, the animal slows its rate of locomotion to a greater extent than when it is well-fed. Animals with loss-of-function mutations in the 5-HT biosynthetic enzymes *bas-1* or *cat-4* exhibit reduced slowing, which can be restored by supplying exogenous 5-HT (SAWIN *et al.* 2000).

mod-5, which encodes the only 5-HT reuptake transporter in *C. elegans,* mediates the uptake of exogenous 5-HT into the serotonergic *n*euro*s*ecretory *m*otor neurons (NSMs) (RANGANATHAN *et al.* 2001). Loss of *mod-5* function causes hypersensitivity to the paralyzing

effects of 5-HT and increases the enhanced slowing response, causing a hyperenhanced slowing response. Presumably in *mod-5* mutants 5-HT cannot be efficiently cleared from the synapse, resulting in a higher concentration of 5-HT in the synapse. More 5-HT in the synapse would then cause increased downstream signaling, resulting in a further inhibition of locomotion. Loss-of-function mutations in genes acting downstream of the serotonergic synapse, *i.e.* genes involved in the signaling pathway that *mod-5* modifies, should suppress the *mod-5* phenotype of hypersensitivity to exogenous serotonin. Two genes required for the 5-HT hypersensitivity and hyperenhanced slowing response of *mod-5* mutants have been cloned and characterized: *mod-1* and *goa-1* (RANGANATHAN *et al.* 2001). Mutations in these genes also cause 5-HT resistance in animals with normal *mod-5* function, and *goa-1* animals with normal *mod-5* function exhibit a defect in the enhanced slowing response (RANGANATHAN *et al.* 2001). *mod-1* encodes a 5-HT-gated chloride channel (RANGANATHAN *et al.* 2000), and *goa-1* encodes the $G_o\alpha$ subunit of a G-protein required for the transduction of a signal from a metabotropic receptor (MENDEL *et al.* 1995; SEGALAT *et al.* 1995).

To find additional genes involved in signaling downstream of one or more 5-HT synapses, we screened for suppressors of the 5-HT hypersensitivity of *mod-5* mutants. We have identified new alleles of genes known to mediate 5-HT neurotransmission, indicating that our screen has successfully recovered mutants defective in 5-HT signaling. We have identified an allele of the bicarbonate transporter gene *abts-1* (SHERMAN *et al.* 2005) which has not been previously shown to play a role in 5-HT signaling. We identified mutations that define new genes that might encode components of signal transduction pathways that regulate response to 5-HT. Identifying components of 5-HT pathways in *C. elegans* and determining how they generate or modulate behavior should provide insight into the relationship between neuromodulation and behavior. The

identification of new proteins that mediate serotonin signaling in *C. elegans* suggests that the counterparts of these proteins in humans might be promising targets for therapeutic intervention.

MATERIALS AND METHODS

Strains and strain constructions: Nematodes were grown on NGM (nematode growth media) at 20°C as described by Brenner (1974), except that the *E. coli* strain HB101 instead of OP50 was used as the food source. The wild-type strain was *C. elegans* N2 (Bristol), except for some multifactor mapping experiments in which the wild-type strain CB4856 (Hawaii) was used. The following mutations were used and are described in Riddle (1997) unless otherwise indicated: LGI: *dpy-5(e61), mod-5(n3314)* (RANGANATHAN *et al.* 2001), *goa-1(n1134), goa-1(n4402, n4405, n4439. n4492. n4493. n4494. (n4093)* (this work), *eat-16(sa609), eat-16(n4403)* (this work), *abts-1(n4094, n4401, ok1566)* (this work), *n3498* (this work), *n3792* (this work), *n4404* (this work); LGII: *dpy-10(e128), unc-4(e120), rol-6(e187), n3461* (this work), *n3488* (this work), *n3495* (this work), *n3775* (this work); LGIII: *unc-32(e189);* LGIV: *unc-5(e53), flp-1(n4762, n4491, n4495)* (this work), *flp-1(yn2, yn4)* (NELSON *et al.* 1998); LGV: *unc-46(e177), dpy-11(e224), mod-1(n3791, n4054)* (this work), *n3510* (this work), *n3799* (this work), *n3774* (this work); LGX: *dpy-3(e27), lon-2(e678), dgk-1(nu62)* (NURRISH *et al.* 1999), *n3477* (this work). The extrachromosomal arrays *oxEx166* and *oxEx229* (BESSEREAU *et al.* 2001) were used to perform Mos1 transposon mutagenesis.

Isolation of *mod-5* suppressors: Fourth-stage larval (L4) *mod-5(n3314)* hermaphrodites were mutagenized using EMS or the Mos1 transposon as described (BRENNER 1974; BESSEREAU *et al.* 2001; BOULIN and BESSEREAU 2007) and allowed to recover for one hour. Five P_0 animals were transferred to 10 cm diameter NGM Petri plates and allowed to produce F_1 progeny for 3-5 days at 20°C. F_1 progeny were bleached to collect eggs. Approximately 300 eggs from each 10 cm plate were spotted to each 6 cm diameter NGM plate and grown at 20°C for two days. These F_2

progeny were then assayed in 24-well cell culture plates in 20 mM(screens one and two) or 60 mM (screen 3) 5-HT (serotonin creatinine complex, Sigma, St. Louis, MO) dissolved in M9 buffer (BRENNER 1974) for swimming after five minutes of exposure. Swimming animals were transferred from wells using a mouth pipette individual 6 cm Petri plates, and their progeny were retested for suppression of the 5-HT hypersensitivity of *mod-5(n3314)* animals.

Linkage group assignment: Newly isolated *mod-5* suppressors were mapped to linkage groups using standard methods (BRENNER 1974), except for *flp-1* mutations, which were mapped using Single Nucleotide Polymorphisms (SNPs) present in the CB4856 wild-type strain as described (WICKS *et al.* 2001).

Complementation tests: To test for complementation with *flp-1, n4495* males were mated with *unc-73(e936); flp-1(yn2)* or *unc-73(e936); flp-1(yn4)* strains and non-Unc progeny were assessed for 5-HT resistance. To test for complementation with *goa-1* or *eat-16,* wild-type males were mated into *mod-5(n3314) goa-1(n1134)* or *mod-5(n3314) eat-16(sa609)* hermaphrodites and the heterozygous male offspring were then crossed to suppressor strains marked with a visible phenotype, such as a Dpy or an Unc, to distinguish self- from cross-progeny. For the next generation, 20 cross-progeny hermaphrodites were assessed for 5-HT resistance; for screen isolates that failed to complement the mutation introduced by heterozygous males, roughly 50% of cross-progeny should have been resistant to exogenous 5-HT.

***flp-1* mapping and identification:** *n4491* and *n4495* were mapped to linkage group IV (LG IV) using standard mapping techniques (BRENNER 1974). *n4491* and *n4495* failed to complement for suppression of the 5-HT hypersensitivity of *mod-5(n3314)* (data not shown). *n4495* was crossed out of a *mod-5* background by following 5-HT resistance. Using three-point and SNP mapping (WICKS *et al.* 2001), we mapped the *n4495* mutation to an interval on LG IV

between the SNPs *pk4040* and *pk4041*, close to and left of *pk4041*. Because *flp-1*, a gene reported to cause 5-HT resistance when mutated (NELSON *et al.* 1998), was located close to and left of *pk4041*, we performed a complementation test between *n4495* and the two existing *flp-1* alleles *yn2* and *yn4*; both failed to complement *n4495* for insensitivity to exogenous 5-HT (data not shown).

Mapping *n3510* V to an interval: *n3510* males were crossed with *dpy-11 unc-42* hermaphrodites, and cross-progeny L4 hermaphrodites were transferred to Petri plates. Fifteen Unc-non-Dpys and 15 Dpy-non-Uncs were picked to individual Petri plates in the next generation, crossed with *n3510* males, and assessed for 5-HT resistance. 15/15 Unc-non-Dpys carried *n3510* and 15/15 Dpy-non-Uncs did not carry the suppressor, indicating that *n3510* is to the left of or close to the right of *dpy-11*. A similar experiment was performed using *unc-46 dpy-11*, and 13 Unc-non-Dpys were picked. Of these, five carried *n3510*, indicating that *n3510* is located between *dpy-11* and *unc-46*.

Southern blot analysis: Genomic DNA from various backcrossed *mod-5(n3314); n4094* isolates was examined by Southern blot analysis using probes with sequences complementary to the Mos1 transposon.

Deletion allele isolation: *abts-1(n4401)* was isolated from a library of animals mutagenized with EMS. The deletion library was constructed and screened essentially as described (JANSEN *et al.* 1997). *n4401* is a 2691 bp deletion that removes the fifth, sixth and seventh exons; the sequences flanking the deletion are TAGGCACGTT and AAAATTTGCC.

Drug sensitivity assays: 5-HT sensitivity assays were performed as described, except that animals were assayed at 10 or 20 minutes, as specified (RANGANATHAN *et al.* 2000).

Aldicarb (aldicarb hydrochloride, Sigma, St. Louis, MO) assays were performed as

described (MAHONEY *et al.* 2006), with the following exceptions: aldicarb plates were made the day of the assay by adding a 100 mM stock of aldicarb in 70% ethanol to molten NGM agar that had been cooled to 50°C, to reach a final concentration of 1 mM aldicarb. Two ml of molten aldicarb-NGM media was added to each well of a 24-well tissue culture plate. HB101 was used instead of OP50. Animals that burst at their vulvae were excluded from analysis.

Levamisole (Chem Service, West Chester, PA) sensitivity assays were performed identically to aldicarb sensitivity assays, except that a 30 mM stock of levamisole in water was added to NGM agar to reach a final concentration of 300 μM.

Behavioral Assays: The enhanced slowing response was assessed as described (SAWIN *et al.* 2000).

The stage of newly laid eggs was assessed as described (KOELLE and HORVITZ 1996), except that assays were performed 36 hours post-L4, and eggs were classified into the following categories: 1-2 cell; 3-8 cell; 9 cell-comma; post-comma.

RESULTS

Isolation of *mod-5* suppressors: We performed three screens for *mod-5* suppressors. We mutagenized *mod-5(n3314)* animals with either EMS or Mos1 transposons and resistant (moving) animals were selected after 5 min. in either 20 or 60 mM 5-HT and retested in either 5 or 60 mM 5-HT (see Materials and Methods). We isolated a total of 24 *mod-5* suppressors from these three screens (Table 1).

In the first screen, we screened F_2 progeny of EMS-mutagenized *mod-5(n3314)* animals corresponding to 22,000 mutagenized haploid genomes for resistance to 20 mM 5-HT. We retested the progeny of resistant isolates and retained strains in which at least 50% of the animals continue to thrash after 5 min. of exposure to 5 mM 5-HT. From this screen, we identified 11 *mod-5* suppressors (Table 1, "EMS, 20 mM 5-HT"). 5-HT dose-response curves for those suppressor strains are shown in Figure 1A.

We performed a second *mod-5* suppression screen using transposon mutagenesis. The *Drosophila* mariner element Mos1 can be mobilized in *C. elegans*, and genes disrupted by transposons can be easily cloned using inverse PCR and DNA sequence analysis to identify genomic sequence adjacent to the site of transposon insertion (BESSEREAU *et al.* 2001). We screened F_2 progeny of EMS-mutagenized *mod-5(n3314)* animals corresponding to 63,000 mutagenized haploid genomes for resistance to 20 mM 5-HT. We retested the progeny of resistant isolates and retained strains in which at least 50% of the animals continue to thrash after 5 min. of exposure to 5 mM 5-HT. From this screen, we identified 3 *mod-5* suppressors (Table 1, "Mos1, 20 mM 5-HT"). 5-HT dose-response curves for those suppressor strains are shown in Figure 1B.

We performed a third *mod-5* suppressor screen at a higher (60 mM) concentration of 5-HT to

isolate mutants that are more strongly resistant to 5-HT. We screened the F_2 progeny of EMS-mutagenized *mod-5(n3314)* animals corresponding to 20,000 homozygosed mutagenized haploid genomes for resistance to 60 mM 5-HT. We retested the progeny of resistant isolates and retained strains in which at least 50% of animals continued to thrash after 5 minutes of exposure to 60 mM 5-HT. From this screen, we identified 10 suppressors (Table 1, "EMS/60 mM 5-HT"). 5-HT dose-response curves for those suppressor strains are shown in Figure 1C.

Identification of new alleles of genes known to mediate 5-HT signaling: When we began screening, loss-of-function mutations in two genes were known to suppress both the 5-HT hypersensitivity and the hyperenhanced slowing response of *mod-5(n3314)* mutants: *mod-1* and *goa-1* (RANGANATHAN *et al.* 2001). *mod-1* encodes a 5-HT-gated chloride channel (RANGANATHAN *et al.* 2000), and *goa-1* encodes the $G_o\alpha$ subunit of a G-protein required for the transduction of a signal from a metabotropic receptor (MENDEL *et al.* 1995; SEGALAT *et al.* 1995). Several other members of the *goa-1* signal transduction pathway have been identified, including *eat-16*, which encodes a regulator of G protein-signaling (RGS) protein (HAJDU-CRONIN *et al.* 1999). Also acting in this pathway is the diacylglycerol kinase gene, *dgk-1* (MILLER *et al.* 1999; NURRISH *et al.* 1999), which phosphorylates the lipid second messenger diacylglycerol (DAG), converting it to phosphatidic acid and thereby terminating its function (TOPHAM 2006). A loss-of-function allele of *dgk-1(n2949)* was found in a screen for mutants with defects in the enhanced slowing response (SAWIN 1996) and the loss-of-function allele *dgk-1(nu199)* confers resistance to exogenous 5-HT (NURRISH *et al.* 1999). We tested the loss-of-function allele *dgk-1(nu62)* and found that it suppressed the 5-HT hypersensitivity and hyperenhanced slowing response of *mod-5(n3314)* animals (data not shown). In addition, because *eat-16*, *goa-1*, and *dgk-1* mutants share many of the same defects (hyperactive movement, constitutive egg laying and increased

pharyngeal pumping), we hypothesized that *eat-16* might also play a role in mediating exogenous 5-HT sensitivity and in the enhanced slowing response. We found that *eat-16(sa609)* suppressed the hyperenhanced slowing response of *mod-5* mutants and caused a defect in the enhanced slowing response in animals normal for *mod-5* function (data not shown).

We mapped five suppressors to linkage group V (LGV), where *mod-1* is located. We determined the sequence of the coding regions of *mod-1* in these four isolates and found that one isolate, *n3791,* contained a mutation in *mod-1* (Figure 2). The fifth LGV suppressor, *n4054,* was isolated in our Mos1 screen, so we used inverse PCR to determine that it was also an allele of *mod-1* (Figure 2). We mapped 12 suppressors to LGI, where *goa-1* and *eat-16* are located. Using either inverse PCR (for Mos1 isolates) or complementation tests (for EMS isolates) we determined that seven were alleles of *goa-1* and one was an allele of *eat-16.* We identified corresponding mutations in each strain, confirming the molecular identities of these suppressors (Figure 2).

We mapped two suppressors, *n4491* and *n4495*, to LGIV and found that they failed to complement for suppression of the 5-HT hypersensitivity of *mod-5(n3314)* animals (data not shown). We determined that *n4495* was located close to and left of *pk4041* on LGIV (see Materials and Methods). Deletions of *flp-1,* located in the interval we identified as containing *n4495*, have been reported to cause 5-HT resistance (NELSON *et al.* 1998). *flp-1* encodes eight FMRFamide-like neuropeptides that differ by only 1 to 4 amino acids at their N-termini. We performed complementation tests and found that the deletion alleles *flp-1(yn2)* and *flp-1(yn4)* fail to complement *n4495* for 5-HT resistance (data not shown). We then determined the sequence of the *flp-1* locus and found that *n4491* contains a mutation in the splice acceptor for the second exon and *n4495* contains a mutation in the splice acceptor for the fifth exon (Figure 2). For further discussion of the role of *flp-1* in 5-HT signaling, see Chapter 3. These observations confirm that

our screen targeted genes known to affect both exogenous 5-HT sensitivity and the enhanced slowing response.

***n4094* is an allele of the bicarbonate transporter *abts-1*:** To determine the identity of *n4094* I, a Mos1 screen isolate, we first outcrossed it five times to separate unlinked transposon insertions. A Southern blot indicated that at least five insertions remained on LGI in this strain (data not shown), so we obtained a recombinant between the suppressor and *dpy-5* to remove transposons on LGI distal to *dpy-5* from the mutation causing the suppression phenotype. Using Southern blot analyses, we examined the genomic DNA of this strain for the presence of transposon sequences. We observed two bands (data not shown), indicating that only two transposons remained in this strain. We performed inverse PCR and found that one of the two bands corresponded to an insertion in the gene *F52B5.1*. We isolated a deletion allele of *F52B5.1* by screening a deletion library (see Materials and Methods). This allele, *n4401,* suppressed the 5-HT hypersensitivity of *mod-5(n3314)* animals to the same degree as *n4094,* (Figure 3A). *n4401* and *n4094* share other defects described below. Therefore, *n4094* is likely an allele of *F52B5.1* (Figure 2). *F52B5.1* has been named *abts-1* (*a*nion/*b*icarbonate *t*ran*s*porter family) by Sherman *et al.*, (2005) who showed that *abts-1* has Cl^-/HCO_3^- exchange activity.

Mutations in *abts-1* do not cause resistance to 5-HT nor defects in the enhanced slowing response: While the Mos1 insertion allele *abts-1(n4094)* and the deletion allele *abts-1(n4401)* suppressed the hypersensitivity of *mod-5* animals to exogenous 5-HT (Figure 7A), we found that in a wild-type background, *abts-1(n4094)* and the two deletion alleles *abts-1(n4401)* and *abts-1(ok1566)* did not cause a defect in 5-HT sensitivity (Figure 3B). We found that *abts-1(n4094)* and *abts-1(n4401)* did not suppress the hyperenhanced slowing response of *mod-5(n3314)* mutants, and *abts-1(n4401)* did not confer an enhanced slowing response defect on

its own (data not shown) which indicates that *abts-1* does not play a role in the enhanced slowing response.

Mutations in *abts-1* cause defects in cholinergic signaling: 5-HT inhibits locomotion by acting through *goa-1, eat-16* and *dgk-1* to decrease acetylcholine release from the motor neurons (NURRISH *et al.* 1999; CHASE *et al.* 2004). Increases or decreases in acetylcholine release at the neuromuscular junction can be evaluated by measuring response to the acetylcholinesterase inhibitor aldicarb (MAHONEY *et al.* 2006). Mutants with increased acetylcholine release are hypersensitive to aldicarb and mutants with reduced acetylcholine release are resistant. To distinguish between a pre- and postsynaptic defect, a mutant must also be tested for response to levamisole, a nicotinic acetylcholine receptor agonist. Nicotinic acetylcholine receptors are located postsynaptically to acetylcholine release, in body wall muscle. Mutants that show hypersensitivity or resistance to aldicarb, but normal sensitivity to levamisole, most likely have a presynaptic defect.

To test if *abts-1* mutants had defects in cholinergic signaling, we tested *abts-1* mutants for sensitivity to aldicarb and levamisole. We found that *abts-1(n4094)* animals were hypersensitive to both levamisole and aldicarb, indicating that a bicarbonate transporter may play a role in cholinergic signaling (Figure 3C, D). However, a pre- or postsynaptic site of action for this gene cannot be determined from these data. Mutations in *abts-1* might act to increase acetylcholine release or to make body wall muscles more responsive to acetylcholine, or both. This is consistent with the ability of *abts-1* mutations to suppress the 5-HT hypersensitivity of *mod-5(n3314)* animals: 5-HT inhibits the release of acetylcholine, so mutations that increase 5-HT signaling, like those in *mod-5,* might be suppressed by mutations that increase cholinergic signaling, like those in *abts-1.*

Our eleven remaining suppressors likely represent at least four new *mod-5* suppressor genes: Three of our remaining suppressors map to LGI, four to LGII, three to LGV and one weakly to LGX (data not shown). We determined the sequences of the coding regions of *goa-1* and *eat-16* in LGI mutants, but found no mutations. The remaining LGI suppressors are unlikely to be alleles *abts-1* because they do not exhibit a reduced body bend amplitude characteristic of *abts-1* mutants (data not shown). No known *mod-5* suppressors are located on LGII. As mentioned above, we determined the sequences of the coding regions of *mod-1* in LGV mutants, but found no mutations. In addition, we mapped *n3510* V to an interval between *dpy-11* and *unc-46,* outside of the region where *mod-1* is located. Our LGX suppressor, *n3477,* is unlikely to be an allele of *dgk-1* because: (1) *n3477* X showed weak linkage (about 30% recombination frequency) to *dpy-3,* which is about 1 mu from *dgk-1* X (data not shown) and (2) *dgk-1* and *n3477* do not display the same defects in 5-HT-mediated behaviors (see below). Therefore, we have likely identified at least four additional new genes involved in 5-HT signaling in *C. elegans.*

Some suppressors of 5-HT hypersensitivity also suppress the hyperenhanced slowing response: As discussed above, *mod-1, goa-1* and *dgk-1* mutants are each resistant to exogenous 5-HT and have defects in the enhanced slowing response (NURRISH *et al.* 1999; RANGANATHAN *et al.* 2000; SAWIN *et al.* 2000). We found that *eat-16(sa609)* also has an enhanced slowing response defect (data not shown).

To determine whether our remaining 11 suppressors showed defects in a behavior mediated by endogenous 5-HT signaling, we tested them to find which suppress the hyperenhanced slowing response of *mod-5(n3314)* animals. We found that five (*n3498* I, *n4404* I, *n3461* II, *n3488* II, and *n3775* II) could also suppress the hyperenhanced slowing response of *mod-5(n3314)* animals (Figure 4A). To determine if the ability of a mutation to strongly suppress 5-HT hypersensitivity

would correlate with its ability to suppress the hyperenhanced slowing response, we compared the strength of suppression of the 5-HT hypersensitivity to the strength of suppression of the hyperenhanced slowing response of *mod-5(n3314)* for each mutant (Figure 4B). We saw no correlation between the ability to strongly suppress 5-HT hypersensitivity and the ability to suppress the hyperenhanced slowing response. Thus, only a subset of genes involved in paralyzing an animal in exogenous 5-HT are required for the enhanced slowing response.

Some suppressors show cholinergic signaling defects: To determine whether our suppressors caused defects in cholinergic signaling, we tested their sensitivity to aldicarb and levamisole. As previously mentioned, mutations in *goa-1, eat-16* and *dgk-1* cause hypersensitivity to aldicarb, but normal sensitivity to levamisole, consistent with a presynaptic site of action. Similar responses might indicate that a given suppressor acts in the same pathway with *goa-1, eat-16* and *dgk-1.*

We found that none of our suppressors was resistant to either aldicarb or levamisole (data not shown), indicating that none of our suppressors is generally resistant to paralyzing drugs. All *mod-5(n3314) goa-1* and *mod-5(n3314) eat-16* mutants were hypersensitive to aldicarb (Figure 5A), as were *mod-5(n3314) n4404* I (Figure 5A), *mod-5(n3314) n3498* I (Figure 5B), and *mod-5(n3314); n3477* X mutants (Figure 5B). These mutants were normally sensitive to levamisole (data not shown), indicating that these mutants likely have a presynaptic cholinergic signaling defect. These data suggest that *n3498* I, *n4404* I and *n3477* X might act in a signaling pathway that includes *goa-1, eat-16* and *dgk-1,* and thus might define new genes in this pathway. *mod-5(n3314) n3792* I and all LGII and LGV mutants showed normal sensitivity to aldicarb and levamisole (data not shown).

Some suppressors regulate egg-laying behavior: *C. elegans* oocytes are fertilized and retained in the uterus while the first few embryonic cell divisions occur; in general, eggs are laid by the time of onset of morphogenesis, which is at the comma stage of embryogenesis (KOELLE and HORVITZ 1996). Most eggs are therefore laid at between the 9-cell and comma stages of development. Mutations in genes functioning in the 5-HT signaling cascade that includes *goa-1, eat-16* and *dgk-1* cause early egg laying. We examined 10 of our 11 remaining suppressors for their egg-laying behaviors to determine whether any had similar defects. We found that, like *goa-1* mutants, *n3498* I, *n3792* I, *n3775* II, *n3488* II, *n3495* II and *n3477* X mutants laid eggs at an early stage (Figure 6). *n3498* I, *n3775* I and *n3477* X showed stronger defects: more than 30% of the total number of eggs were laid at an early stage. *n3792* I, *n3488* II, and *n3495* II showed weaker defects: between 5 and 30% of the total number of eggs were laid at an early stage (compared to 0% for wild-type and *mod-5(n3314)* animals).

In summary, all LGI, LGII and LGX mutants showed some or all of the defects displayed by *goa-1, eat-16* and *dgk-1* mutants, which include 5-HT resistance, aldicarb hypersensitivity, ability to suppress the hyperenhanced slowing response of *mod-5(n3314)* and constitutive egg laying. These results suggest that these screen isolates may act in some or all of the serotonergic pathway(s) with *goa-1, eat-16* and *dgk-1* to control locomotion and egg laying. LGV mutants only displayed defects in response to exogenous 5-HT, but were not generally resistant to drugs, indicating some level of specificity to 5-HT signaling.

DISCUSSION

To identify genes that function downstream of or in parallel to the *se*rotonin-*r*euptake *t*ransporter (SERT) MOD-5, we screened for mutations that suppressed the 5-HT hypersensitivity of *mod-5* mutants. We isolated at least seven alleles of the $G_o\alpha$ gene *goa-1* and at least two alleles of the 5-HT-gate chloride channel gene *mod-1*. Both of these genes are known to mediate the paralytic response of *C. elegans* to exogenous 5-HT (SEGALAT *et al.* 1995; RANGANATHAN *et al.* 2000). *goa-1* and *mod-1* and act in parallel pathways downstream of *mod-5* in the enhanced slowing response (RANGANATHAN *et al.* 2001), a behavior partially dependent on endogenous 5-HT (SAWIN *et al.* 2000). We identified two alleles of the FMRFamide-encoding gene *flp-1*, deletions of which are reported to mediate the paralytic response to exogenous 5-HT and to act downstream of *goa-1* in controlling locomotion rate (NELSON *et al.* 1998). These observations confirm that our screen targeted genes that function in endogenous 5-HT signaling. We identified 11 additional mutants that could not be readily identified as alleles of known genes: *n4404* I, *n3498* I, *n3792* I, *n3461* II, *n3488* II, *n3495* II, *n3775* II, *n3510* V, *n3774* V, *n3799* V and *n3477* X. Our mapping results and phenotypic characterizations indicate that these mutations define at least four new genes with defects consistent with a role in 5-HT signaling. We also identified *abts-1*, which encodes a Na^+-dependent Cl^-/HCO_3^- transporter. Bicarbonate transporters are known to regulate cell volume and pH, but a specific neuronal function for Na^+-dependent Cl^-/HCO_3^- transporters has not been examined. We showed that animals lacking *abts-1* have abnormal responses to drugs that affect serotonergic and cholinergic signaling.

Our 11 uncloned suppressors show defects in 5-HT-mediated behaviors: We have characterized our remaining 11 suppressors, and most show some of the same defects as do members of a G protein signal transduction pathway containing the known *mod-5* suppressor

goa-1 (Table 2). Members of the *goa-1* pathway are expressed in many different neurons and muscles and have been proposed to interact with different receptors and downstream effectors to mediate different behaviors (MENDEL *et al.* 1995; SEGALAT *et al.* 1995; HAJDU-CRONIN *et al.* 1999; NURRISH *et al.* 1999; BASTIANI *et al.* 2003). Therefore, *goa-1* may act in one circuit with one set of effectors to control locomotion, and in another circuit with a different set of effectors to control egg laying.

We found that two LGI mutants, *n3498* and *n4404*, show all of the same defects as do *goa-1* mutants: suppression of the hyperenhanced slowing response of *mod-5(n3314),* aldicarb hypersensitivity and early egg laying. (While the egg laying of *n4404* mutants was not quantified, we saw 2-cell stage embryos on *n4404* culture plates, indicating that these mutants also lay eggs at an early stage.) On the basis of these shared defects, we propose that *n3498* and *n4404* are mutations in a new gene or genes that act in a pathway with *goa-1*. *n3792* I, *n3461* II, *n3488* II, *n3495* II, *n3775* II, and *n3477* X each showed a subset of *goa-1*-like defects. We propose that these suppressors may act in one or a subset of the circuits in which *goa-1* pathway members act. The three unidentified mutants linked to LGV showed no additional defects consistent with a role in endogenous 5-HT signaling. However, LGV mutants are not resistant to any other drugs or neurotransmitters we tested, and so show some degree of specificity to 5-HT. Further characterization of these suppressors, as well as the molecular identification of the genes disrupted in these suppressors, will provide insight into how the enhanced slowing response, cholinergic signaling and egg laying are regulated by 5-HT.

A bicarbonate transporter acts in serotonergic and cholinergic neurotransmission: We identified a Mos1 insertion allele of the bicarbonate transporter *abts-1* in our screen. *abts-1* is most closely related in sequence to members of the SLC4 (*sol*ute *c*arrier) family of HCO_3^-

transporters, which are involved in the regulation of pH and cell volume (ALPER 2006). SLC4 family members have been classified into three types: (1) Na^+-independent Cl^-/HCO_3^- exchangers, (2) Na^+-HCO_3^- cotransporters and (3) Na^+-driven Cl^-/HCO_3^- exchangers (PUSHKIN and KURTZ 2006). ABTS-1 is most similar to the human Na^+-driven Cl^-/HCO_3^- exchangers SLC4A8 (50% identity, 66% similarity) and SLC4A10 (48% identity, 65% similarity). *abts-1* was shown to confer Cl^-/HCO_3^- transport when expressed in *Xenopus* oocytes, consistent with the activity of SLC4A8 and SLC4A10 (GRICHTCHENKO *et al.* 2001; WANG *et al.* 2001; SHERMAN *et al.* 2005).

To determine the function of *abts-1*, we identified a deletion allele. We found that, like the screen isolate *abts-1(n4094)*, the deletion allele *abts-1(n4401)* suppressed the 5-HT hypersensitivity of *mod-5(n3314)* animals. We found that animals lacking *abts-1* function have a reduced body bend amplitude and are hypersensitive to two drugs that potentiate cholinergic signaling, aldicarb and levamisole. The neuromuscular junctions of *C. elegans* use the neurotransmitter acetylcholine. Aldicarb, an acetylcholinesterase inhibitor, and levamisole, a nicotinic receptor agonist, both induce paralysis by causing hypercontraction. The hypersensitivity of *abts-1* mutants to aldicarb and levamisole indicates that these mutants are not generally impermeable to exogenously added drugs and reveals that *abts-1* mutants have defects in cholinergic transmission. We propose that *abts-1* functions in neurons that express a 5-HT receptor (perhaps *mod-1,* a Cl^- channel). When *abts-1* is mutated, the pH or concentration of other ions, like Cl^-, is altered, causing a decrease in the excitability of the neuron, which decreases the efficiency of a stimulatory 5-HT signal. Because 5-HT inhibits ACh release, in *abts-1* mutants there might be increased ACh release, causing hypersensitivity to both aldicarb and levamisole. A neuronal function for *abts-1* is consistent with a report that an *abts-1::gfp* transgene is expressed strongly in neurons and hypodermis but weakly in body wall muscle, anal gland and intestine

(SHERMAN *et al.* 2005).

Because *abts-1* is expressed weakly in body wall muscle (SHERMAN *et al.* 2005), an alternative model consistent with our results is that mutations in *abts-1* cause a slight depolarization in the resting state of body wall muscles, so that less acetylcholine is needed to cause the muscle to contract. This would cause *abts-1* mutants to be hypersensitive to aldicarb and levamisole. This model would also account for the suppression of the 5-HT hypersensitivity of *mod-5* mutants: in a *mod-5* mutant, there could be excess synaptic 5-HT, which inhibits acetylcholine release. *abts-1* might suppress *mod-5* by making any acetylcholine that is released more effective in inducing muscle contraction, counteracting the effects of excess 5-HT.

Human genetic analyses and studies of mouse strains deleted for other SLC4 family members have provided some insight into the functions of bicarbonate transporters. Loss- and gain-of-function mutations in SLC4 family member genes have been associated with human diseases affecting red blood cells, as well as eye, kidney and brain. In mice, deletions of SLC4A2 are associated with embryonic lethality, and deletions of SLC4A7 are associated with retinal degeneration and mild auditory impairment (PUSHKIN and KURTZ 2006). In mammals, SLC4A8 and SLC4A10 transcripts are expressed primarily in brain (WANG *et al.* 2000; GRICHTCHENKO *et al.* 2001), consistent with a neuronal function, but no mouse model for SLC4A8 exists to examine its function in an intact animal. We suggest that our analysis of *abts-1*, which also shows neuronal expression (SHERMAN *et al.* 2005), might provide insight into the endogenous roles for SLC4A8 and/or SLC4A10. Future studies using the alleles isolated in our study could help to address whether ABTS-1 acts to regulate pH or cell volume in neurons, and how such regulation affects neuronal function and behavior.

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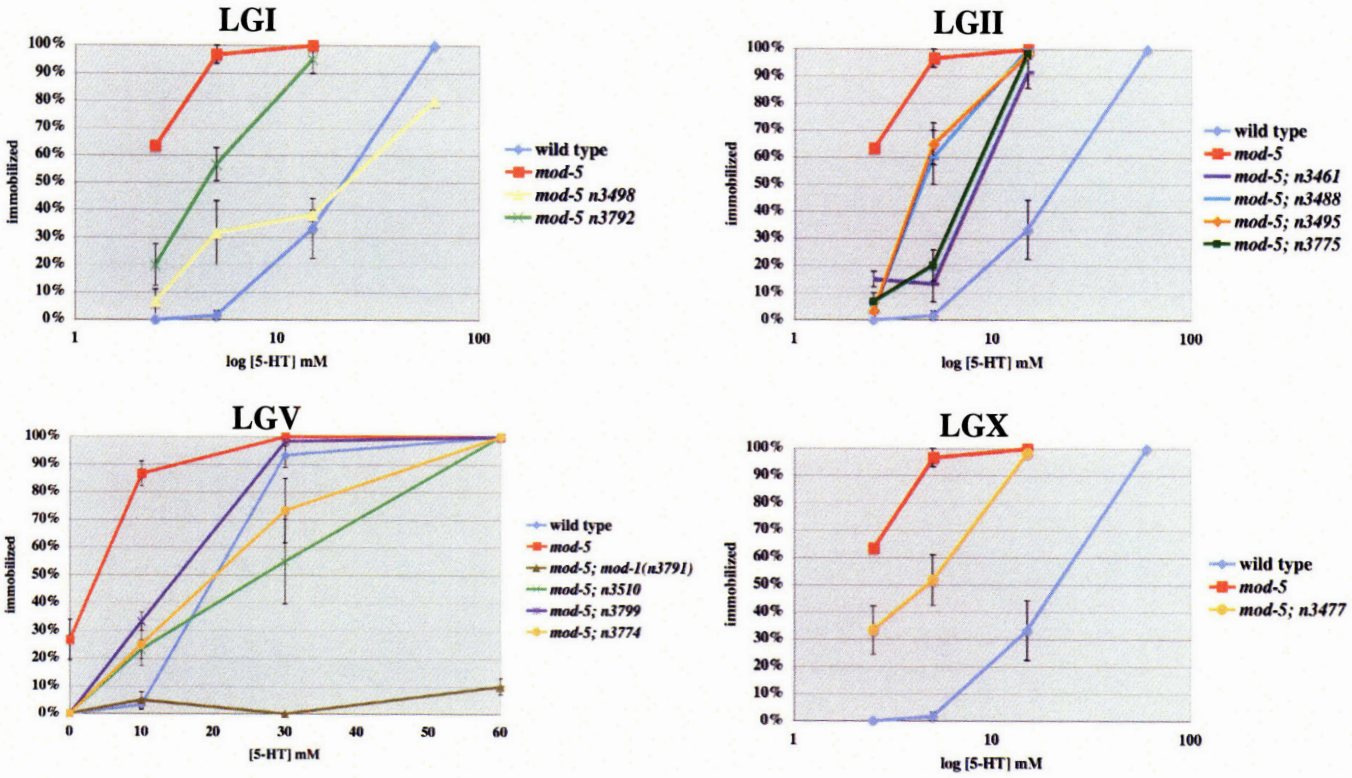
TABLE 1
Isolates from three *mod-5* suppressor screens

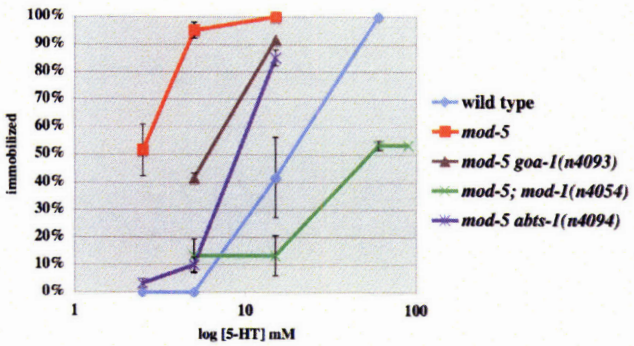
Isolate	Linkage Group
EMS, 20 mM 5-HT	
n3498	I
n3792	I
n3461	II
n3488	II
n3495	II
n3775	II
mod-1(n3791)	V
n3510	V
n3774	V
n3799	V
n3477	X
Mos1, 20 mM 5-HT	
goa-1(n4093)	I
abts-1(n4094)	I
mod-1(n4054)	V
EMS, 60 mM 5-HT	
goa-1(n4402)	I
goa-1(n4405)	I
goa-1(n4439)	I
goa-1(n4492)	I
goa-1(n4493)	I
goa-1(n4494)	I
eat-16(n4403)	I
n4404	I
flp-1(n4491)	IV
flp-1(n4495)	IV

Three screens for *mod-5* suppressors were performed. *mod-5(n3314)* animals were mutagenized with either EMS or Mos1 transposons and resistant (moving) animals were selected after 5 min. at either 20 or 60 mM 5-HT. A total of 24 stably mutant strains were recovered from all three screens.

A. Dose-response curves for EMS, 20 mM 5-HT screen isolates



B. Mos1, 20 mM 5-HT screen isolates



C. EMS, 60 mM 5-HT screen isolates

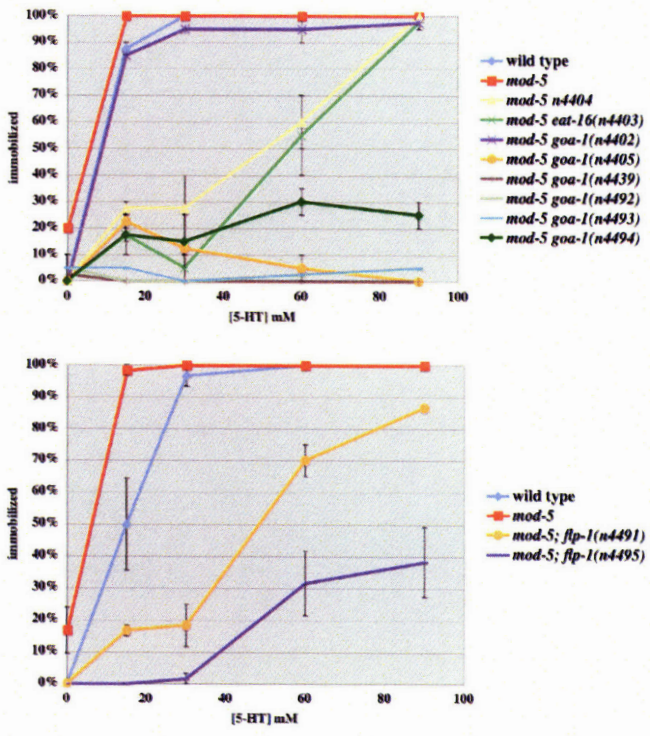


Figure 1. 5-HT dose-response curves for each screen isolate. Percentage of animals immobilized after 20 min. for each [5-HT]. n≥2 sets of 20 animals. In some cases the x-axis is presented in log scale. A. EMS, 20 mM screen isolates. We tested LGI, LGII and LGX isolates in 2.5, 5, 15 and 60 mM 5-HT; all were tested on the same day with the same controls. We tested LGV isolates in 0, 10, 30 and 60 mM 5-HT. B. We tested Mos1, 20 mM screen isolates in 2.5, 5, 15 and 60 mM 5-HT. C. We tested EMS, 60 mM screen isolates at 0, 15, 30, 60 and 90 mM 5-HT.
***mod-5* = *mod-5(n3314)*. Error bars, S.E.M.**

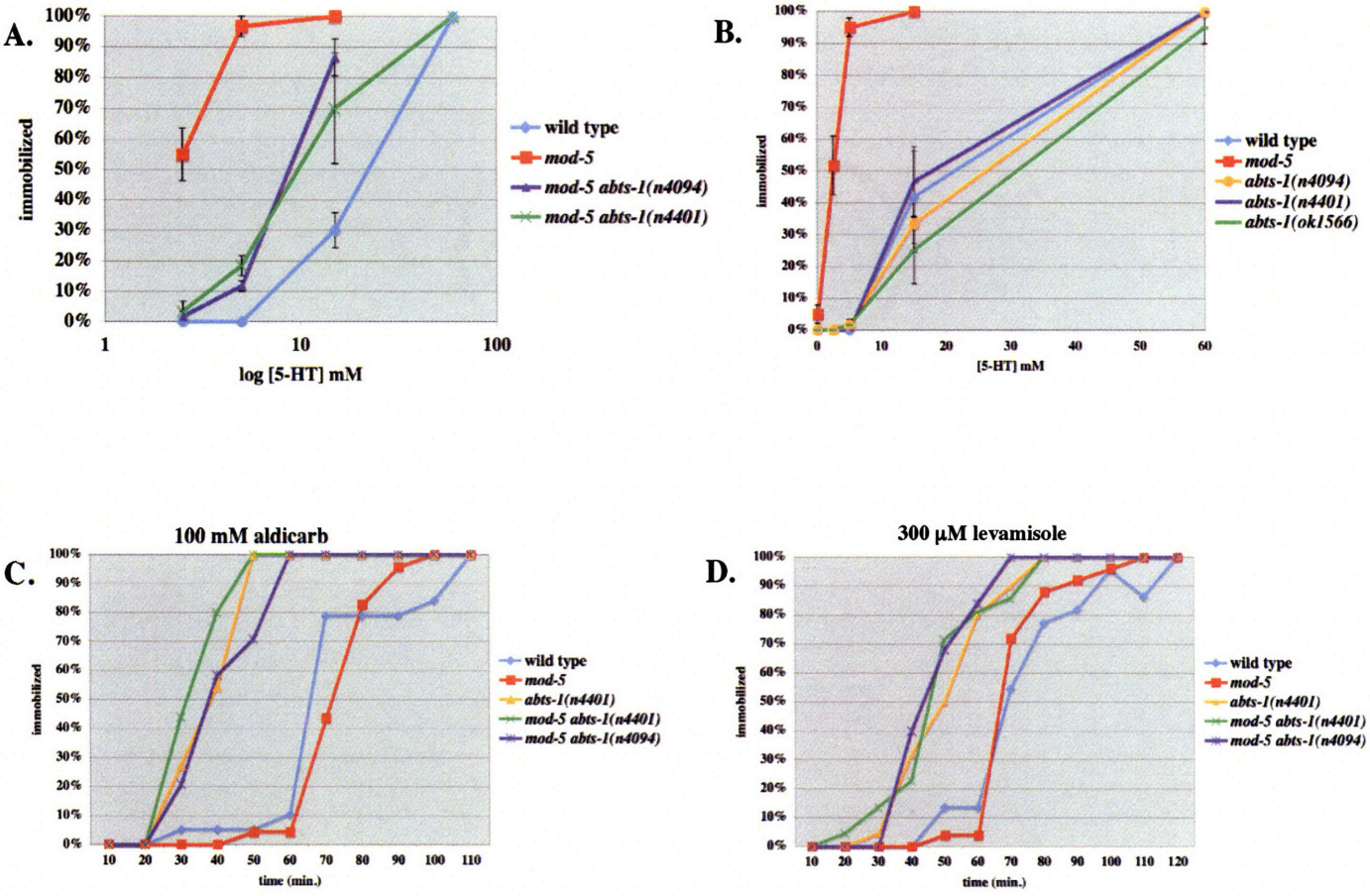


Figure 3. Phenotypic characterization of *abts-1*. A. The deletion allele *abts-1(n4401)* and the Mos1 allele *abts-1(n4094)* show identical [5-HT] dose-response curves. Percentage of animals immobilized after 10 min. for each [5-HT] (2.5, 5, 15 and 60 mM). n≥2 sets of 20 animals. The x-axis is presented as a log scale. B. *abts-1(n4094)* and the deletion alleles *abts-1(n4401)* and *abts-1(ok1566)* do not confer 5-HT resistance. Percentage of animals immobilized after 20 min. for each [5-HT] (2.5, 5, 15 and 60 mM). n≥2 sets of 20 animals. C. *abts-1* mutants are aldicarb hypersensitive. Percentage of animals immobilized over time on 100 mM aldicarb. D. *abts-1* mutants are levamisole hypersensitive. Percentage of animals immobilized on 300 µM levamisole. *mod-5* = *mod-5(n3314)*. Error bars, S.E.M.

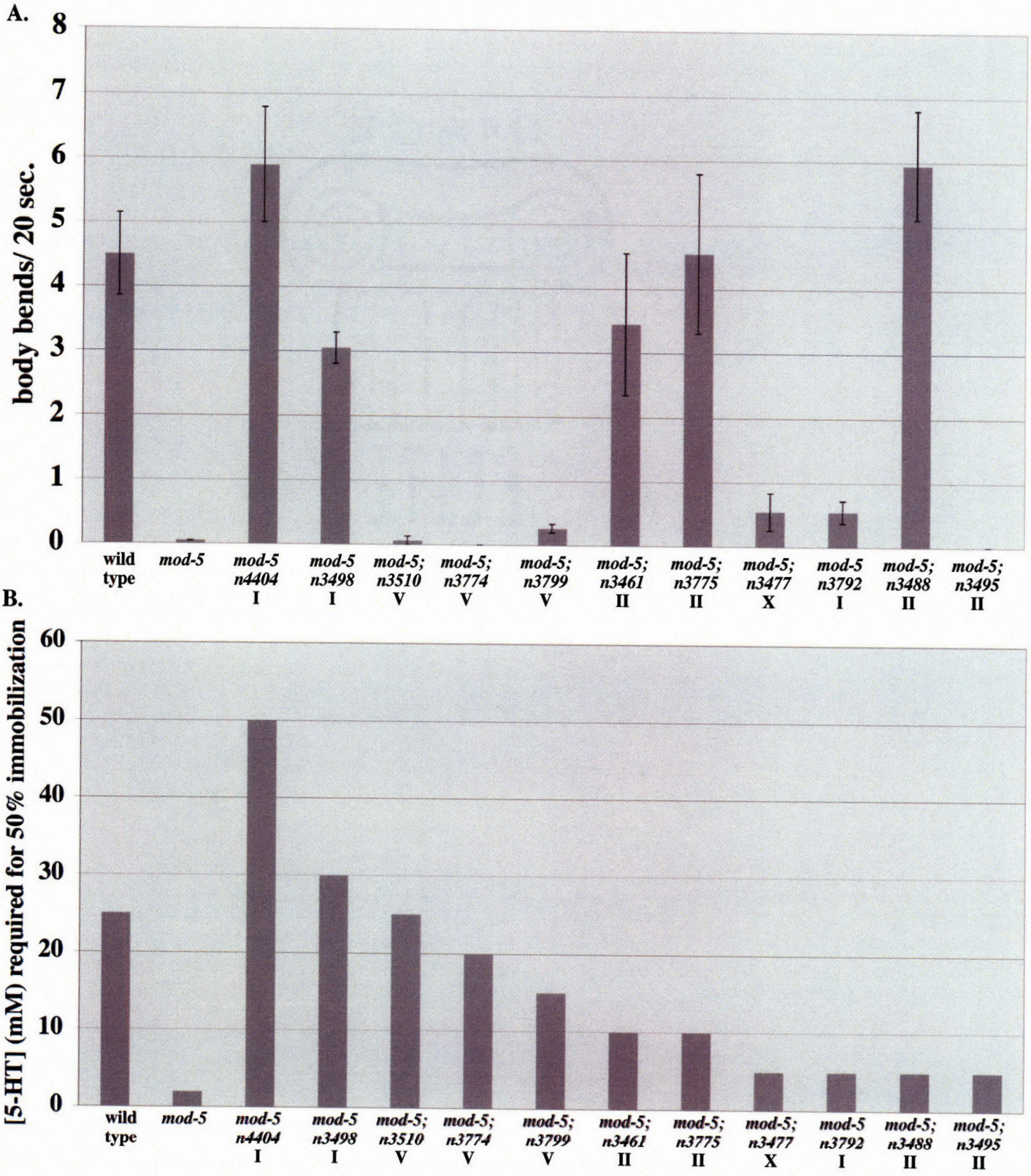
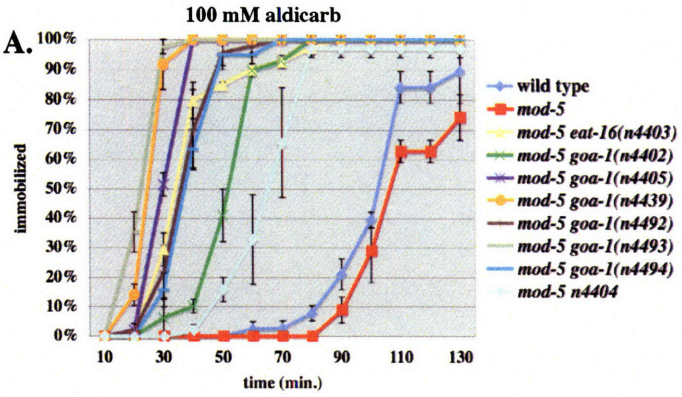


Figure 4. Enhanced slowing response and 5-HT resistance of the remaining 11 screen isolates. A. Five suppress the hyperenhanced slowing response of *mod-5(n3314)* animals. Body bend of food-deprived animals on food were counted. B. The [5-HT] at which 50% of animals were immobilized at 20 min was determined from 5-HT dose-response curves for each suppressor for comparison. *mod-5* = *mod-5(n3314)*. Error bars, S.E.M.



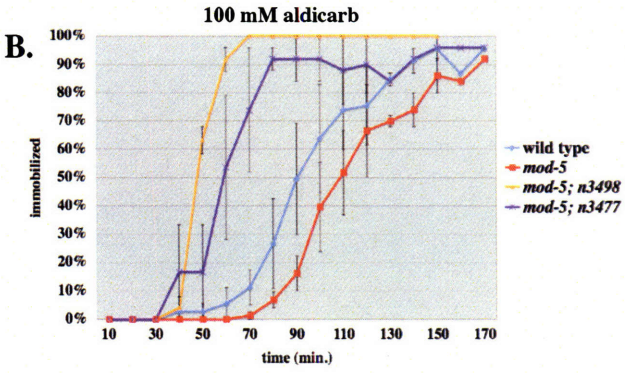


Figure 5. Aldicarb sensitivity of suppressors. Percentage of animals immobilized over time on 100 mM aldicarb. A. All *mod-5; goa-1* and *mod-5; eat-16* mutants, as well as *mod-5; n4404* I, are hypersensitive to aldicarb. B. Two mutants from the EMS, 20 mM 5-HT screen, *mod-5 n3498* I and *mod-5; n3477* X are hypersensitive to aldicarb. *mod-5* = *mod-5(n3314)*. Error bars, S.E.M.

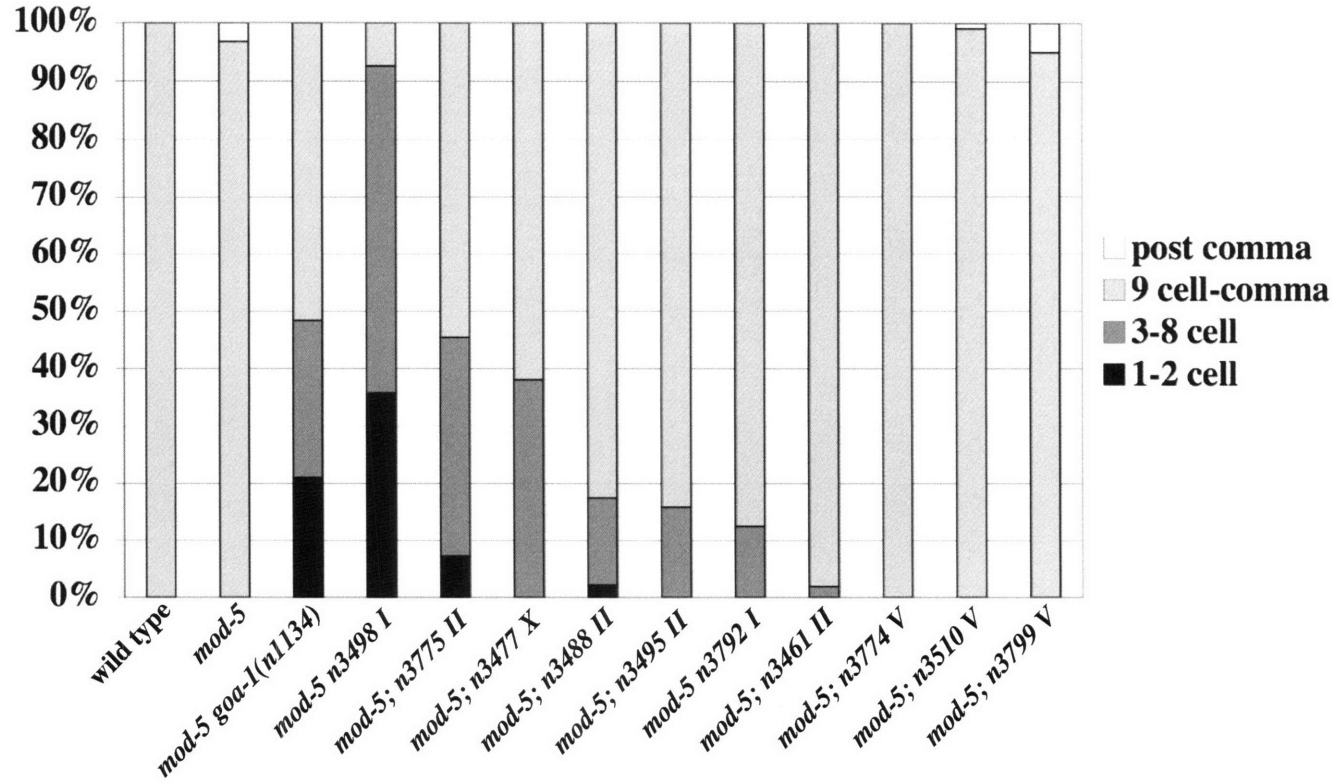


Figure 6. Some suppressors cause early egg laying. 20 staged animals per genotype were placed on a plate and left for 30 min. to lay eggs. Embryos were examined for stage and categorized as 1-2 cell, 3-8 cell, 9 cell-comma or post comma. *n3498, n3775* and *n3477* confer strong Egl-c defects, *n3792, n3488* and *n3495* confer weak Egl-c defects. *mod-5* = *mod-5(n3314)*.

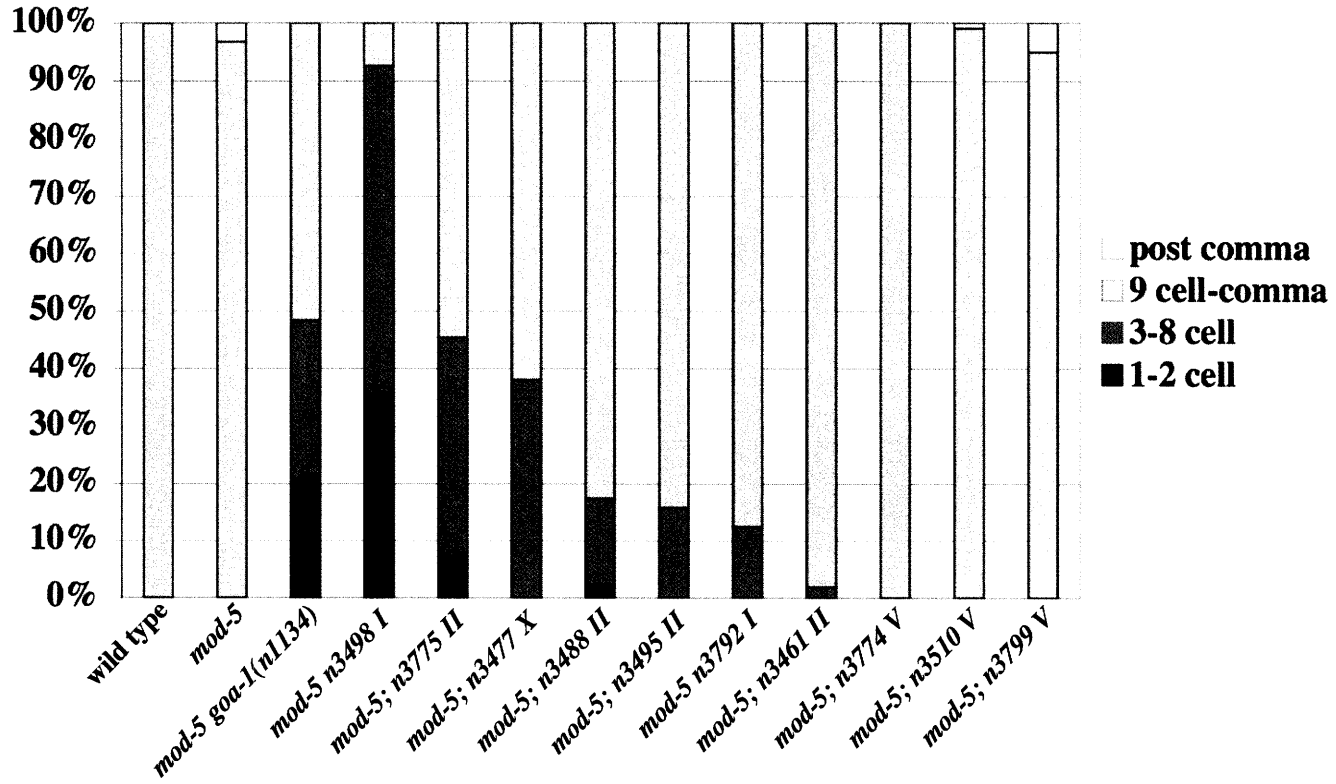


Figure 6. Some suppressors cause early egg laying. 20 staged animals per genotype were placed on a plate and left for 30 min. to lay eggs. Embryos were examined for stage and categorized as 1-2 cell, 3-8 cell, 9 cell-comma or post comma. *n3498, n3775* and *n3477* confer strong Egl-c defects, *n3792, n3488* and *n3495* confer weak Egl-c defects. *mod-5* = *mod-5(n3314)*.

allele	[5-HT] mM at which 50% are immobilized in 20 min.	suppresses hyperenhanced slowing response of *mod-5*?	aldicarb	egg laying
goa-1(n1134)	≥ 10 to > 90	**YES**	**hypersensitive**	**early**
n4404 I	50	**YES**	**hypersensitive**	**early**
n3498 I	30	**YES**	**hypersensitive**	**early**
n3792 I	5	no	wt	**early**
n3477 X	5	no	**hypersensitive**	**early**
n3461 II	10	**YES**	wt	wt
n3488 II	5	**YES**	wt	**early**
n3495 II	5	no	wt	**early**
n3775 II	10	**YES**	wt	**early**
n3510 V	25	no	wt	wt
n3774 V	20	no	wt	wt
n3799 V	15	no	wt	wt

Table 2: Summary of defects shown by our remaining 11 suppressors. Defects shown by *goa-1(n1134)* mutants listed for comparison.

Chapter 3

The Serotonin Receptors MOD-1 and SER-4 and FMRFamide-like Peptides Interact to Modulate *C. elegans* Locomotion

Megan Gustafson, Eric Miska and H. Robert Horvitz

This chapter is written for submission to *J. Neurosci.* Erik Miska made the *mod-1::dsRed* construct described here and identified the neurons in which it is expressed.

Abstract

Upon encountering a bacterial food source, wild-type *C. elegans* that have been acutely food deprived slow more than do well-fed animals upon encountering a bacterial food source. This behavior, called the enhanced slowing response, is partly serotonin (5-HT) dependent. Animals mutant for the 5-HT reuptake transporter gene *mod-5* slow even more than wild-type animals, because endogenous 5-HT signaling is potentiated. This behavior, called the hyperenhanced slowing response, can be suppressed by mutations in genes that encode proteins important for 5-HT signaling, including the MOD-1 ionotropic 5-HT receptor and the GOA-1 $G_o\alpha$ G protein subunit. MOD-1 and GOA-1 likely act downstream of or in parallel to one or more 5-HT synapse(s) that mediate(s) the enhanced slowing response. We sought new genes that function in 5-HT signaling and specifically in the enhanced slowing response. We found that the metabotropic receptor SER-4 and one or more FMRFamide-like neuropeptides encoded by *flp-1* mediate paralysis in exogenous 5-HT and play a role in the enhanced slowing response, a behavior mediated by endogenous 5-HT signaling. We propose that MOD-1 and SER-4 act in parallel in the RIB interneurons and that one or more FLP-1 neuropeptides act downstream of MOD-1 and SER-4 to mediate the enhanced slowing response.

Introduction

Serotonin (5-HT) plays an important role in a variety of biological processes in many organisms. In neurons, the effects of 5-HT are mediated by both fast-acting ionotropic (Maricq et al., 1991; Ranganathan et al., 2000) and by slow-acting metabotropic receptors that activate or inhibit signal transduction pathways (Barnes and Sharp, 1999; Bockaert et al., 2006). Termination of 5-HT signaling occurs primarily by reuptake of 5-HT into the presynaptic terminal by the *se*rotonin *r*euptake *t*ransporter (SERT), a member of the Na^+/Cl^- family of transporters (Blakely et al., 1991; Hoffman et al., 1991; Nelson, 1998).

In the nematode *C. elegans,* 5-HT modulates several behaviors including pharyngeal pumping, egg laying, and locomotion (Horvitz et al., 1982). Locomotion is modulated in response to the environment and experience: animals slow their rate of locomotion in response to the presence of food, and animals that have been acutely food-deprived slow their rate of locomotion even more than do well-fed animals (Sawin et al., 2000). This latter behavior, the enhanced slowing response, is mediated in part by 5-HT: animals lacking genes that encode 5-HT biosynthetic enzymes, *bas-1* and *cat-4,* exhibit a reduced enhanced slowing response (Sawin et al., 2000).

Animals with mutations in *mod-5,* which encodes the only SERT in *C. elegans*, exhibit even greater slowing than do wild-type animals after food deprivation; this *mod-5* response is called the hyperenhanced slowing response (Ranganathan et al., 2001). The 5-HT-gated chloride channel MOD-1 (Ranganathan et al., 2000) and the Gα protein GOA-1 (Mendel et al., 1995; Segalat et al., 1995) act in parallel to mediate the effects of the elevated serotonin signal in *mod-5* mutants, leading to the hyperenhanced slowing

response (Ranganathan et al., 2001). MOD-1 and GOA-1 also mediate the paralyzing effects of exogenously supplied 5-HT (Segalat et al., 1995; Ranganathan et al., 2001). In short, the current model is that upon encountering food, food-deprived animals release 5-HT, which causes the animal to slow its rate of locomotion, and that at least some proteins that act downstream of 5-HT release in the enhanced slowing response also act downstream of exogenous 5-HT to cause paralysis.

We sought new genes that function in 5-HT signaling and specifically in the enhanced slowing response. We found that the metabotropic 5-HT receptor SER-4 and one or more FMRFamide peptides encoded by *flp-1* mediate sensitivity to exogenous 5-HT and modulate the enhanced slowing response in *C. elegans*. We looked for enhancement, or lack of enhancement, of the enhanced slowing response defects of animals lacking different combinations of the genes *mod-1, goa-1, ser-4* and *flp-1* to determine whether these genes act in the same or in different pathways to mediate the enhanced slowing response. We determined the sites of *mod-5* and *mod-1* expression and use the known sites of expression of *ser-4* and *flp-1* (Nelson et al., 1998; Tsalik et al., 2003) to generate a model for the neural circuit controlling the 5-HT-mediated enhanced slowing response.

Materials and Methods

Strains and strain constructions

Nematodes were grown at 20°C as described (Brenner, 1974), except that the *E. coli* strain HB101 instead of OP50 was used as the food source because HB101 has been used in previous studies of the enhanced slowing response (Sawin et al., 2000; Ranganathan et al., 2001). The wild-type strain was *C. elegans* N2 (Bristol), except for some multifactor mapping experiments in which the wild-type strain CB4856 was used. The following mutations were used and are described in Riddle (1997) unless otherwise indicated: LGI: *mod-5(n3314)* (Ranganathan et al., 2001), *goa-1(n1134), ser-3(ad1774)* (Carre-Pierrat et al., 2006); LGII: *tph-1(n4622)* (this work); LGIII: *ser-4(n4577)* (this work), *ser-4(ok512)* (Komuniecki et al., 2004); LGIV: *Y54G2A.35(n4921)* (this work), *flp-1(n4762)* (this work), *flp-1(n4491)* (this work), *flp-1(n4495)* (this work); LGV: *mod-1(ok103)* (Ranganathan et al., 2000), *T02E9.3(ok568)* (this work); LGX: *ser-1(ok345)* (Carnell et al., 2005), *ser-7(n4542)* (this work), *ser-2(pk1357)* (Tsalik et al., 2003), *lin-15AB(n765ts), tyra-3(ok325)* (this work), *tag-24(ok371)* (this work).

Behavioral assays

5-HT sensitivity assays were performed as described, except that animals were assayed at 10 or 20 min., as specified (Ranganathan et al., 2000). The enhanced slowing response assay was performed as described (Sawin et al., 2000).

Deletion alleles

Y54G2A.35(n4921), ser-4(n4577), and *flp-1(n4762)* were isolated from a library of animals mutagenized with diepoxybutane (DEB) (*n4921)* or EMS (*n4577* and *n4762).* The deletion library was constructed and screened as described (Jansen et al., 1997; Liu et al., 1999). We determined the extent of each deletion by PCR amplifying the region containing the deletion and analyzing the sequence of the PCR product. *n4921* is a 1592 bp deletion, beginning 268 bp before the Y54G2A.35 start site and deleting the first three exons. The sequences flanking the deletion are TCCAAGTGAT and TTTTTTTGGA. *n4577* is a 2485 bp deletion, beginning 1350 bp before the *ser-4* start site and deleting the first two exons. The sequences flanking the deletion are TGCTCGGAGA and CGAAAAATTG. The *n4762* deletion is a 684 bp deletions, beginning 695 bp after the start site deleting the remainder of the gene and 115 bp of 3' sequence. The sequences flanking the deletion are GAGAAAGCCA and TTTGTTTTTG.

The deletion alleles *T02E9.3(ok568), tyra-3(ok325),* and *tag-24(ok371)* were isolated by The *C. elegans* Knockout Consortium (http://celeganskoconsortium.omrf.org/). We obtained these strains from the *C. elegans* Genetics Center (University of Minnesota).

Generation of rescuing and reporter constructs

To create pMG12, we PCR amplified 5 kb of sequence upstream of *ser-4* using forward (GCGCGCATGCCAGAGGAGTTCGCCACACAACACGTCAC) and reverse primers (GCGCGCATGCGTGGAGTTGCACACAACACCGGAAGC) containing the restriction

sites *Sph*I and *BamH*I, respectively. We amplified the *ser-4* cDNA yk1731h09 (kindly provided by Y. Kohara and colleagues) using forward (GCGCGGTACCATGATCGACGAGACGCTTCTCAATC) and reverse (GCGCGATATCACTAGTCTAGCGGCCGCGACCTGCAGC) primers containing the restriction sites *Kpn*I and *EcoR*V, respectively. We ligated the resulting products into pPD49.26 (kindly provided by A. Fire).

To create pMG13, we used the QuickChange Site-Directed Mutagenesis Kit (Stratagene) to insert two G residues after nucleotide 91 of *ser-4* exon 1.

To create pMG14, we used forward (GGACCACCGAGAACCTAAAAGTTTGT) and reverse (GCTGATTTCTTGATTGGTTTCTTGACG) primers to PCR amplify a 6 kb sequence from N2 genomic DNA consisting of the 1.4 kb *flp-1* genomic region with 3 kb of upstream sequence and 1.6 kb of downstream sequence. We used the TOPO-TA Cloning Kit (Invitrogen) to clone this PCR product into the pCRII-TOPO vector.

To create pRM43 we used pRR06, the minimal rescuing fragment of *mod-1,* a SacI/ClaI fragment of the cosmid K06C4, subcloned into pBluescript SK+. We inserted a PmeI and NotI site after the *mod-1* ATG and inserted the dsRed1.1 cDNA into these sites. The cDNA is followed by a stop codon. Therefore, it is unlikely that any DSRED::MOD-1 fusion protein is produced.

Transformation rescue

We performed transformation rescue as described (Mello et al., 1991), injecting 20 ng/µl pL15EK (which contains the wild-type *lin-15* gene) and 10 ng/µl pMG12 (*ser-4* cDNA under native promoter) or pMG13 (frameshifted pMG12) into a *ser-4(ok512); lin-15(n765ts)* strain and scoring for 5-HT sensitivity in transgenic lines that produced non-Lin progeny at 22.5°C.

We injected 20 ng/µl pL15EK and 10 ng/µl pMG14 (*flp-1* genomic region) into a *flp-1(n4762); lin-15(n765ts)* strain and scored for 5-HT sensitivity and the enhanced slowing response in transgenic lines that produced non-Lin progeny at 22.5°C.

We injected 20 ng/µl *sur-5::gfp* (pTG96, (Yochem et al., 1998)) and 10 ng/µl pMG12 (*ser-4* cDNA under native promoter) or pMG13 (frameshifted pMG12) into a *mod-5(n3314); ser-4(ok512); mod-1(ok103)* strain and assayed the enhanced slowing response in animals that expressed nuclear *gfp*.

***mod-5(n3314)* suppression screen**

mod-5(n3314) hermaphrodites (L4) were mutagenized with EMS (Brenner, 1974), and their F2 progeny were assayed for swimming in 60 mM 5-HT at 5 minutes. Swimming animals were transferred by mouth pipette to individual plates, and their progeny were retested.

Antibody preparation

Anti-MOD-5 antiserum was generated by immunizing rabbits with purified MBP-MOD-5 (amino acids 43-100) fusion protein. The antiserum was affinity purified against GST-MOD-5 (amino acids 43-100) as described by (Koelle and Horvitz, 1996). Animals for immunostaining were fixed as described (Finney and Ruvkun, 1990). Purified antibodies were used at a 1:25 dilution for immunocytochemistry. Rabbits were maintained by Covance (Denver, PA).

Identification of RIB neurons

Animals carrying a *mod-1::dsRed* transgene were incubated in the green fluorescent dye, 3,3'-dioctacdecyloxacarbocyanine perchlorate (DiO)(Invitrogen), which stains the amphid neurons ASI, ADL, ASK, AWB, ASH and ASJ (Hedgecock et al., 1985; Herman and Hedgecock, 1990). Likewise, animals carrying a *ser-4::gfp* transgene were incubated in the red-orange fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, (DiI)(Invitrogen), which stains the same amphid neurons (Koga et al., 1999). Dye-filling incubations were performed as described (Herman and Hedgecock, 1990).

Results

SER-4 is a metabotropic 5-HT receptor that mediates paralysis in exogenous 5-HT

MOD-1, a 5-HT-gated Cl^- channel, acts in a pathway parallel to GOA-1, a Gα homolog, to mediate the enhanced slowing response (Ranganathan et al., 2001). The involvement of a subunit of a G protein in the 5-HT-mediated enhanced slowing response suggests that a metabotropic 5-HT receptor might also be involved. To identify such a receptor, we searched the genome of *C. elegans* for genes with similarity to metabotropic biogenic amine receptors. The *C. elegans* genome encodes three genes highly related to metabotropic 5-HT receptors. These genes, *ser-1, ser-4* and *ser-7,* had already been studied and found to bind to and be activated by 5-HT when expressed in heterologous systems (Olde and McCombie, 1997; Hamdan et al., 1999; Hobson et al., 2003). Using the sequences of these three 5-HT receptors, we performed a reiterative BLAST search (Tsalik et al., 2003) and found 12 additional genes with significant similarity. Four of these genes encode known dopamine receptors (*dop-1, dop-2, dop-3* and *dop-4*) (Suo et al., 2002, 2003; Sugiura et al., 2005) and two encode known tyramine receptors (*ser-2* and *tyra-2*) (Rex and Komuniecki, 2002; Rex et al., 2005). Six had not been tested for their ligand–binding properties (*T02E9.3, C24A8.1, ser-3, tyra-3, F14D12.6,* and *Y54G2A.35*). We obtained or constructed deletion strains (see Materials and Methods) for all of the characterized 5-HT receptor genes and the receptor genes with unknown ligands, except *C24A8.1.* We tested these strains for rates of paralysis in exogenous 5-HT. Two independently isolated deletion alleles of *ser-4 (ok103* and *n4577,* Fig. 1*A)*

conferred 5-HT resistance (data not shown for *ser-4(n4577)*, Fig. 1*B*). All other strains displayed wild-type sensitivity to 5-HT (data not shown).

This resistance was rescued by a transgene (*nEx1371*) carrying a *ser-4* cDNA under the native *ser-4* promoter but not by the same transgene with two bases inserted in the first exon (*nEx1372*) causing a nonsense mutation and subsequent frameshift (Fig. 1*B*). These results indicate that the activity of the *ser-4* open reading frame is necessary to mediate the paralytic effects of exogenous 5-HT.

No single 5-HT receptor is required for the enhanced slowing response

The experiments described above indicate that SER-4 is required for paralysis in response to exogenous 5-HT. To determine if the 5-HT receptors SER-1, SER-4 or SER-7 are involved in the enhanced slowing response, a behavior mediated by endogenous 5-HT, we examined *ser-1(ok345)*, *ser-7(n4542)*, and *ser-4(ok512)* mutants for defects in this response. We found no reproducible defects in the enhanced slowing response (data not shown).

mod-1 and *ser-1* single mutants are reported to have enhanced slowing response defects (Ranganathan et al., 2000; Dernovici et al., 2007). We could not repeat these results. In our studies of *mod-1* mutants, we have searched extensively for differences between the conditions of our assay and those used previously, but we were unable to find any condition that gave the published result that *mod-1(ok103)* or *mod-1(nr2043)* deletion mutants show enhanced slowing response defects (data not shown). By contrast, we have

reproduced results for other mutants reported to be defective in the enhanced slowing response, including *goa-1* and *dgk-1* mutants (Sawin, 1996)(data not shown). In our studies of *ser-1* mutants, we used the *E. coli* strain HB101, whereas Dernovici *et al.* (2007) used the *E. coli* strain OP50. Animals have different locomotory responses to different bacterial strains (Shtonda and Avery, 2006), and it is possible that the dependence of the enhanced slowing response on *ser-1* varies with the bacterial strain used. Also, unlike Dernovici *et al.* (2007), we did not find *ser-1* mutants to be 5-HT resistant, but our assay was performed in liquid while theirs used 5-HT-containing plates. Therefore, we could not detect a role for any single 5-HT receptor in the enhanced slowing response under the conditions used in this study.

Roles for SER-4 and MOD-1 in the enhanced slowing response are revealed in a *mod-5* SERT mutant animal

mod-5 SERT mutants exhibit increased slowing (a hyperenhanced slowing response), on food following food deprivation (Ranganathan et al., 2001). One explanation for this defect is that *mod-5(n3314)* animals cannot appropriately terminate 5-HT signaling, and therefore have increased 5-HT signaling that leads to increased slowing. We reasoned that smaller contributions to enhanced slowing could be detected in a *mod-5(n3314)* background because these mutants are essentially paralyzed on food following food deprivation. Although we did not find that *mod-1* mutants are defective in the enhanced slowing response (see above), we tested a *mod-5(n3314); mod-1(ok103)* double mutant for enhanced slowing defects and found that *mod-1(ok103)* could also partially suppress the paralysis of *mod-5(n3314)* animals, consistent with published results (Ranganathan et

al., 2001) (Fig 1*C*). Because the 5-HT receptors SER-4 and MOD-1 both mediate paralysis in exogenous 5-HT, we tested whether a role for SER-4 in the enhanced slowing response might also be detected in a *mod-5* background. We tested a *mod-5(n3314); ser-4(ok512)* double mutant for enhanced slowing defects and found that *ser-4(ok512)* could partially suppress the hyperenhanced slowing response of *mod-5(n3314)* animals (Fig. 1*C*). Therefore, SER-4 is required to mediate the effects of increased 5-HT signaling in a *mod-5* SERT mutant. Animals carrying a *mod-5(n3314)* mutation are slower in both the "off food" and "on food" condition (Fig. 1*C*), suggesting a role for 5-HT in regulating speed of locomotion off of food as well as in the enhanced slowing response. However, when we normalized the speed of locomotion on food to the animals' rate of locomotion off food, we found that *mod-5(n3314)* animals moved even more slowly than did wild-type animals (Fig. 1*D*). We normalized speed on food to speed off of food for all strains shown, including *mod-5; mod-1* and *mod-5; ser-4* (Fig. 1*D*). We found that in each case, strains' relative speeds on food compared closely with their absolute speeds on food, so we chose to display the absolute speeds rather than the normalized values. In conclusion, we determined that MOD-1 and SER-4 can function in the enhanced slowing response when 5-HT signaling is potentiated. While we favor the hypothesis that MOD-1 and SER-4 normally play roles in the enhanced slowing response that cannot be detected in a wild-type background, we cannot rule out the possibility that MOD-1 and SER-4 only function to slow the animal in cases where 5-HT signaling is potentiated.

SER-4 acts in a pathway with GOA-1 and in parallel to MOD-1 to regulate the enhanced slowing response

Removing the function of both MOD-1 and GOA-1, a $G_o\alpha$ homolog, can suppress the hyperenhanced slowing response of *mod-5(n3314)* animals to a greater extent than removing MOD-1 or GOA-1 individually, indicating that MOD-1 and GOA-1 act at least in part independently, such as in two distinct signaling pathways, to mediate the enhanced slowing response (Ranganathan et al., 2001). We hypothesized that the metabotropic 5-HT receptor SER-4 could act in a pathway with GOA-1, given their molecular identities. If SER-4 and GOA-1 act independently to mediate the enhanced slowing response, then animals lacking both proteins should have a greater defect (move faster) than animals lacking only GOA-1 or only SER-4. Instead, we found that mutants lacking SER-4 and GOA-1 (in a sensitized *mod-5* SERT mutant background) moved at the same rate as mutants lacking only GOA-1, (Fig. 2*A*), consistent with the hypothesis that GOA-1 and SER-4 act in one pathway. *goa-1(n1134)* mutants have a more severe defect than do *ser-4(ok512)* mutants (Fig. 2*A*), indicating that GOA-1 also has a SER-4-independent role in enhanced slowing. Because SER-4 acts in the same pathway with GOA-1, which acts in a parallel pathway to MOD-1, we hypothesized that SER-4 and MOD-1 also act in parallel pathways. We found that mutants lacking both SER-4 and MOD-1 (in a sensitized *mod-5* SERT mutant background) moved even faster than do mutants lacking either SER-4 or MOD-1 alone (Fig. 2*B*), indicating that the two act in parallel pathways. We could rescue this enhancement with the transgene used to rescue the exogenous 5-HT resistance of *ser-4(ok512)* animals, but not with the frameshifted version of that transgene (Fig. 2*B*). We conclude that the ionotropic receptor MOD-1 acts

in parallel to a pathway containing the metabotropic receptor SER-4 and the G protein subunit GOA-1. We favor a model in which these pathways normally act to control the enhanced slowing response, but because we can detect these interactions only in a *mod-5(n3314)* mutant background, and not in a wild-type background, we cannot rule out the possibility that these interactions occur only when 5-HT signaling is potentiated.

FLP-1 mediates paralysis in exogenous 5-HT

Previously, we isolated two mutant alleles (*n4491, n4495)* of *flp-1,* which encodes eight FMRFamide-like neuropeptides, in a screen for mutations that suppress the 5-HT hypersensitivity of *mod-5(n3314)* animals (Chapter 2) (Fig. 3*A*). We found that in a wild-type background, each allele confers resistance to exogenous 5-HT (Figure 3*B*). These mutant alleles disrupt splice acceptor sites in exons two and five, respectively (Chapter 2). *flp-1* is located on the opposite strand of the first intron of another gene, *daf-10* (Bell et al., 2006). Previously isolated deletion alleles (*yn2, yn4)* (Nelson et al., 1998) of *flp-1* also removed coding sequences of *daf-10* (Ailion and Thomas, 2003). We wanted to isolate a deletion allele of *flp-1* that did not disrupt *daf-10* coding sequences, so we screened a library of EMS-mutagenized worms for a deletion that removed only *flp-1* coding sequences. We found a deletion, *n4762* (see Materials and Methods), which removes most of the first and all of the seven remaining encoded neuropeptides. Therefore, animals carrying the *flp-1(n4762)* deletion have no FLP-1 FMRFamide-like peptides. *flp-1(n4762)* also confers 5-HT resistance (Figure 3*B*). *flp-1(n4762)* mutants are slightly less resistant to 5-HT than are *flp-1(n4491)* and *flp-1(n4495)* mutants, which might be caused by background mutations in the *flp-1(n4762)* strain or might indicate

that the deletion allele has some effect on the expression of *daf-10* which causes animals to be more sensitive to 5-HT or generally unhealthy. While *flp-1(n4762)* does not remove *daf-10* coding sequences, any mutation in *flp-1* will also alter the sequence of the first intron of *daf-10,* which could affect the expression of *daf-10.* To address the possibility that *daf-10* disruption might cause the phenotypes we observe in these mutants, we found that a point mutation in *daf-10(e1387),* which does not affect *flp-1* sequences does not confer resistance to exogenous 5-HT (data not shown). This result indicates that it is the *flp-1* locus and not the *daf-10* locus that mediates sensitivity to 5-HT. In addition, the 5-HT resistance of *flp-1(n4762); lin-15AB(n765ts)* animals is rescued by a transgene containing the *flp-1* genomic region and a *lin-15AB* coinjection marker (*nEx1369)* but not by a transgene containing the coinjection marker only *(nEx1370)* (Fig. 3*C*), indicating that the *flp-1* genomic region is required to mediate sensitivity to exogenous 5-HT in *C. elegans.*

flp-1 acts in the enhanced slowing response

To examine whether FLP-1 plays a role in endogenous 5-HT signaling, we tested the enhanced slowing responses of our *flp-1* strains. We found that all three, *flp-1(n4491), flp-1(n4495)* and *flp-1(n4762),* conferred a partial defect in the enhanced slowing response (Figure 3*D*). The transgene (*nEx1370)* that rescued the 5-HT resistance of *flp-1(n4762)* animals also rescued the partial defect in the enhanced slowing response, but the transgene containing the coinjection marker alone, *nEx1370,* did not. (Fig. 3*E*). These results indicate that a neuropeptide signal is required for the enhanced slowing response, a behavior mediated by endogenous 5-HT signaling.

The function of FLP-1 in enhanced slowing is primarily 5-HT-dependent

We showed that FLP-1 is required for the enhanced slowing response, a behavior mediated by endogenous 5-HT. FLP-1 also mediates the paralysis of an animal in exogenous 5-HT. Therefore, we propose that the role of FLP-1 in the enhanced slowing response is to mediate the 5-HT signal required for the enhanced slowing response. We reasoned that if FLP-1 acted in a 5-HT-independent (parallel) manner, animals lacking both FLP-1 and 5-HT would have a greater defect than animals lacking either one individually. Tryptophan hydroxylase (TPH) performs the rate-limiting step of 5-HT synthesis (Frazer and Hensler, 1999), and animals deleted for the probable *C. elegans* homolog *tph-1* lack 5-HT (Sze et al., 2000). We compared the enhanced slowing response defect of *tph-1(n4622)* mutants, *flp-1(n4762)* mutants, and *tph-1(n4622); flp-1(n4762)* double mutants. We saw no enhancement by removing both *flp-1* and 5-HT (Fig. 4*A*). Because *flp-1* mutants are 5-HT resistant, and because *flp-1(n4762)* mutants do not greatly enhance the defect of *tph-1(n4622)* (5-HT-deficient) mutants, *flp-1* likely acts in a 5-HT-dependent fashion to regulate the enhanced slowing response. However, *flp-1(n4762)* mutants are slightly more defective than are *tph-1(n4622)* mutants, indicating that FLP-1 plays a small 5-HT-independent role in the enhanced slowing response.

FLP-1 likely acts postsynaptically to MOD-5 SERT to mediate the enhanced slowing response:

Animals lacking FLP-1 are resistant to paralysis in exogenous 5-HT, indicating that FLP-1 likely acts postsynaptically to 5-HT release. To test whether FLP-1 acts pre- or

postsynaptically to 5-HT release in the enhanced slowing response, we tested *flp-1* mutants to see whether they could suppress the hyperenhanced slowing response of *mod-5* animals. *flp-1(n4491)*, *flp-1(n4495)*, and *flp-1(n4762)* each suppressed the hyperenhanced slowing response of *mod-5* animals (Fig 4*B* and *C*), indicating that FLP-1 acts downstream of MOD-5 SERT, and thus downstream of 5-HT release, to mediate the enhanced slowing response.

FLP-1 likely acts downstream of the two parallel pathways defined by MOD-1 and SER-4

We showed that FLP-1 likely acts downstream of 5-HT release to mediate the enhanced slowing response. To determine whether FLP-1 acts downstream of SER-4 or MOD-1, which act in parallel pathways, we made double mutants between *flp-1* and *mod-1* and between *flp-1* and *ser-4* and looked for increased enhanced slowing. As above, strains were sensitized using a *mod-5(n3314)* mutation so that enhancement could be easily measured. We found that animals lacking both MOD-1 and FLP-1 (Fig. 5*A*) or animals lacking both SER-4 and FLP-1 move at the same rate as animals lacking only FLP-1 (Fig. 5*B*). We note that slight enhancement of the defect exhibited by animals lacking FLP-1 by removing MOD-1 or SER-4 might not be detectable because animals lacking MOD-1 or SER-4 (in a sensitized *mod-5* SERT background) move relatively little on their own. However, even removing both MOD-1 and SER-4 in animals lacking FLP-1 does not cause an increase in movement (Fig. 5*B*), indicating that FLP-1 likely acts downstream of the two parallel pathways defined by MOD-1 and SER-4 in the enhanced slowing response.

Expression of *mod-5* and *mod-1*

To identify cells that might be involved in the enhanced slowing response, we raised antibodies against MOD-5. Anti-MOD-5 antibodies stained two pairs of neurons in the wild type (Fig. 5) but not in *mod-5(n3314)* animals (data not shown), indicating specific recognition of the MOD-5 protein. The brightest staining neurons were the two serotonergic Neurosecretory Motor Neurons (NSMs) (Horvitz et al., 1982)(Fig. 6*A*). Based on morphology and position, the NSMs have been proposed to act as a food sensor, detecting when bacteria are present in the lumen and releasing 5-HT in response (Albertson and Thomson, 1976; Horvitz et al., 1982). This model is consistent with a role for the NSMs in the enhanced slowing response (see Discussion). Anti-MOD-5 antibodies stained another pair of neurons in the head dimly. These neurons had nuclear positions and process morphologies similar to those of the AIM or AIY interneurons. The AIMs, but not the AIYs, are recognized by anti-5-HT antibodies (Sawin, 1996; Duerr et al., 1999). We used a *ttx-3::gfp* reporter to mark the AIYs (Hobert et al., 1997) and found that the *ttx-3::gfp*-expressing cells were adjacent to rather than identical to the MOD-5 immunopositive cells (data not shown). We conclude that *mod-5* is most likely expressed in the AIMs (fig. 6*B*); the function of the AIM interneurons is largely unknown.

We made a *mod-1::dsRed* reporter and found that it was expressed in several neurons in the head. We identified these neurons as the AIB, AIM, AIY, AIZ, AVE, RIB, RIC, RIP, and RME neurons (Fig. 7*A* shows RIB identification) based on nuclear positions and process morphologies. MOD-1 and SER-4 act in parallel (see above). Do MOD-1 and SER-4 act in parallel in the same cell(s) or act in parallel by functioning in different

cells? A *ser-4::gfp* reporter is expressed primarily in the two RIB neurons and the RIS neuron, as well as in sublateral neurons, a pharyngeal neuron, retrovesicular neurons and a tail neuron (Tsalik et al., 2003). We confirmed that this *ser-4::gfp* reporter is expressed in the RIBs (see Materials and Methods) (Fig. 7*B*). By examining animals carrying both *mod-1::dsRed* and *ser-4::gfp* reporters, we found that two head neurons expressed both *dsRed* and *gfp,* in a position consistent with the two RIB neurons (data not shown). We conclude that both *mod-1* and *ser-4* are coexpressed, probably in the RIBs.

Discussion

We identified two genes, *ser-4* and *flp-1,* required for paralysis in exogenous 5-HT and that play a role in the enhanced slowing response, a behavior mediated by endogenous 5-HT signaling. *ser-4* encodes a metabotropic 5-HT receptor, and *flp-1* encodes eight FMRFamide peptides. The metabotropic 5-HT receptor SER-4 and the ionotropic receptor MOD-1 act in parallel to mediate the enhanced slowing response. We determined that FLP-1 likely acts downstream of the two parallel pathways defined by MOD-1 and SER-4 (Fig 8*A*). We propose that in the enhanced slowing response, recent acute food deprivation and the stimulus of bacteria cause the serotonergic NSM neurons to release 5-HT, which acts on both the MOD-1 ionotropic and SER-4 metabotropic receptor in the RIB neurons. One or more FLP-1 neuropeptides likely act downstream of the RIB neurons to modulate the animal's behavioral response.

The neural circuit that mediates the enhanced slowing response

The morphology and ultrastructure of the serotonergic NSMs (Neurosecretory Motor Neurons) led to the hypothesis that these neurons might function to detect food and then secrete 5-HT humorally into the pseudocoelom (Albertson and Thomson, 1976; Horvitz et al., 1982). Both the NSMs and the serotonergic ADF neurons have been implicated in the enhanced slowing response because ablating either class of neuron led to a partial defect in the enhanced slowing response (Sawin, 1996). Our finding that anti-MOD-5 antibodies brightly stain the NSMs also supports a role for the NSMs in the enhanced slowing response.

If the NSMs secrete 5-HT humorally, then any neuron or muscle tissue could act postsynaptically. Therefore, we attempted to identify the sites of expression of genes that act downstream of MOD-5. We found that the 5-HT receptors MOD-1 and SER-4 act in parallel downstream of MOD-5, and so could function redundantly to mediate the activity of a single cell or cell type, or might function in separate cells that act in parallel in the enhanced slowing response. We found that the expression of *mod-1* and *ser-4* overlap in only one pair of neurons, the RIBs. Thus, it is possible that by removing MOD-1 and SER-4 together, we are either removing or increasing the function of the RIBs. Ablation studies of the RIBs indicate that they function to increase the probability that an animal reverses direction and makes turns when initially removed from a food source, presumably in a search for food (Gray et al., 2005). Thus, the RIBs are likely involved in transmitting information about lack of available food into a locomotory output. We propose that the RIBs function to transmit information about the presence of food following food-deprivation into a different locomotory output: increased slowing (Fig. 8*B*). Because both MOD-1 and SER-4 are expressed in several other cells, we can not rule out the possibility that they function in other cells to mediate the enhanced slowing response.

Why might an ionotropic and metabotropic receptor be required to act together to produce a full response to 5-HT? Ionotropic receptors produce fast responses to neurotransmitters, and metabotropic receptors produce slower responses (Kandel et al., 2000). MOD-1 and SER-4 might act accordingly to mediate a 5-HT signal. Both fast and slow postsynaptic activity might be required for the proper rate and/or magnitude of the

postsynaptic potential in the RIB neurons. Because *C. elegans* only has 302 neurons, which show little variation in their connectivity (White et al., 1986), expressing different combinations of receptors with different properties in a given neuron might be a way to generate behavioral diversity and plasticity.

SER-4 and GOA-1 act in the same pathway

We found that the metabotropic 5-HT receptor SER-4 and the $G_o\alpha$ protein GOA-1 act in the same pathway. Our data are consistent with the possibility that SER-4 and GOA-1 interact directly to mediate the enhanced slowing response. *goa-1* is expressed in most if not all neurons (Mendel et al., 1995; Segalat et al., 1995), and so is likely expressed in the RIBs, and in the other neurons in which *ser-4* is expressed (Tsalik et al., 2003). Alternatively, GOA-1 might interact with an unidentified receptor. GOA-1 acts downstream of FLP-1 to control locomotion rate (Nelson et al., 1998), so one or more FLP-1-encoded peptides might activate a metabotropic receptor that also acts through GOA-1. Another possible model is that GOA-1 has a receptor-independent activity in the enhanced slowing response, as has been found for its role in positioning the mitotic spindle during early cell divisions in the embryo (Colombo et al., 2003; Gotta et al., 2003; Srinivasan et al., 2003).

FLP-1 likely acts downstream of two parallel pathways defined by SER-4 and MOD-1

flp-1 mutants are resistant to exogenous 5-HT. Thus, FLP-1 must act either downstream of or in parallel to 5-HT release. In other words, FLP-1 might propagate a 5-HT signal, or

FLP-1 might act to slow the animal independently of 5-HT. If FLP-1 acts in parallel to 5-HT signaling, then deleting *flp-1* and removing 5-HT should cause an even greater enhanced slowing response defect. We found that removing 5-HT did not increase the enhanced slowing response defect of animals lacking FLP-1. Therefore, we favor a model in which FLP-1 acts downstream of 5-HT. We also found that removing FLP-1 does not enhance the defect of animals lacking both MOD-1 and SER-4. We propose that FLP-1 also acts downstream of SER-4 and MOD-1 to mediate an endogenous 5-HT signal. Alternatively, *flp-1* might act in parallel to but not independently of 5-HT. In other words, *flp-1* and 5-HT might both be required for slowing to occur. Losing either kind of signaling will cause a defect, and losing both will not make the defect any worse. Our data cannot rule out this type of model.

The synaptic wiring diagram of *C. elegans* neurons (White et al., 1986) indicates that two of the major synaptic outputs from the RIBs are the *flp-1*-expressing neurons AVA and AVE (Nelson et al., 1998). The AVA and AVE are command interneurons required for normal backward locomotion and the cessation of forward locomotion (Chalfie et al., 1985). Release of *flp-1* from these neurons could inhibit the class A motor neurons, which are required for backward locomotion (Chalfie et al., 1985), although one might expect the animals to simply stop reversing instead of inhibiting forward locomotion. We propose that instead of completely inhibiting forward movement and initiating backward movement, a partial inhibition of forward movement might simply cause a reduction in the rate of forward movement. The identification of a *flp-1* receptor will help to further define the neurons required to mediate the enhanced slowing response.

Conclusion and future directions

In summary, we have discovered a role for a metabotropic 5-HT receptor SER-4 in mediating the enhanced slowing response in parallel to the 5-HT-gated chloride channel MOD-1. We found that SER-4 and MOD-1 are coexpressed only in the RIB neurons. Thus, the function of both an ionotropic and metabotropic receptor in the same cell might be required for the proper firing rate and/or controlling the magnitude of the postsynaptic potential in response to 5-HT. Additional investigation into the requirement for the RIBs in the enhanced slowing response could provide insight into how animals modulate their behavior in response to the environment and experience. Analysis of the electrophysiological properties of the RIBs could further our understanding of the general principles that govern strength and timing of postsynaptic potentials. Finally, we found that the FMRFamide-encoding gene *flp-1* likely acts downstream of the two parallel pathways defined by SER-4 and MOD-1 to mediate 5-HT-induced slowing. As mentioned above, the identification and analysis of a *flp-1* receptor that plays a role in the enhanced slowing response will help determine which cells mediate responses to *flp-1*-encoded peptides. Our findings are consistent with a model in which 5-HT released from the NSMs binds to SER-4 and MOD-1 on the RIB interneuron, which stimulates the release of FLP-1 peptides from the AVAs and AVEs. FLP-1 peptides could bind to postsynaptic receptors on the motorneurons and inhibit them, reducing the rate of the animal's locomotion.

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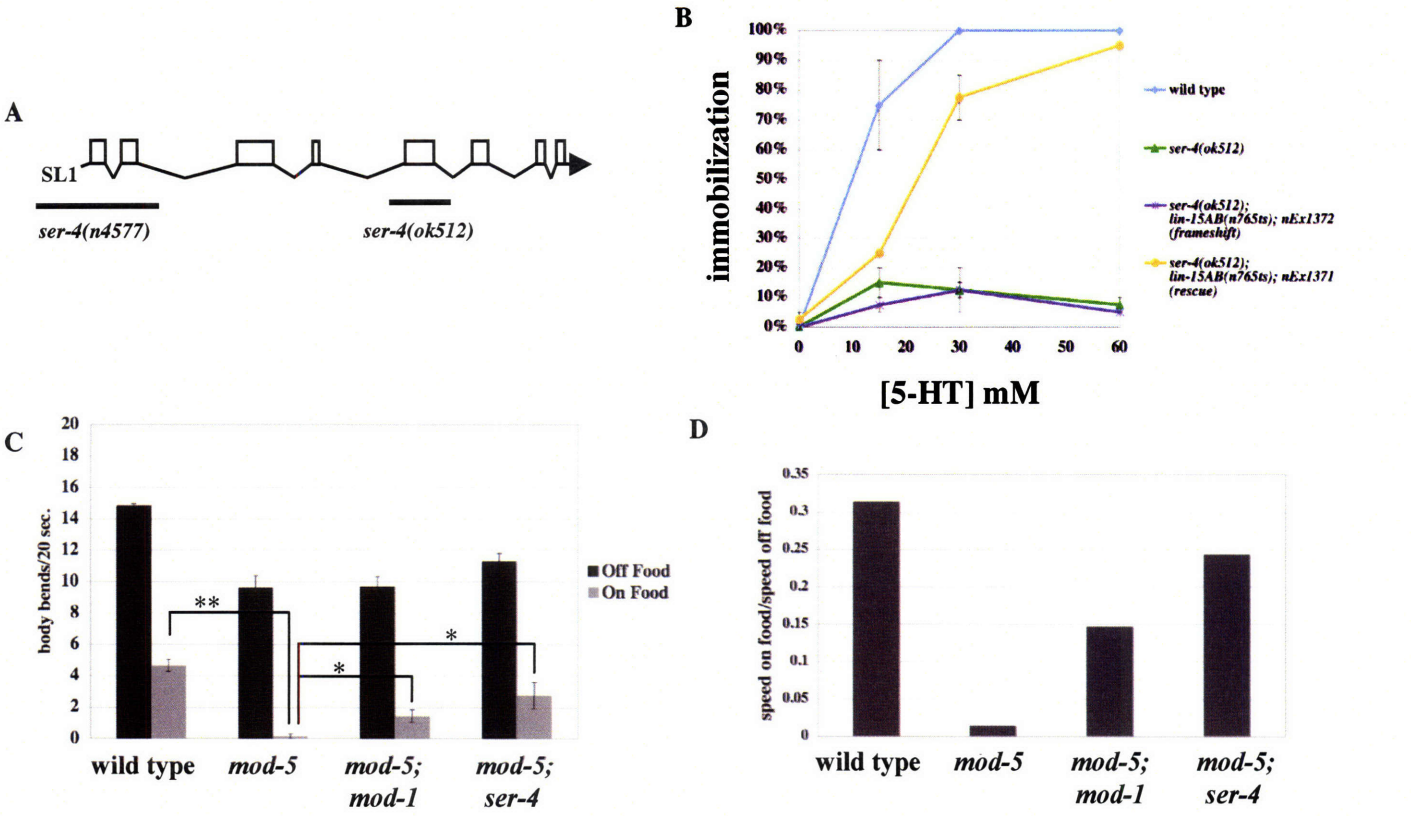

Figure 1. ***ser-4*** **mutants are resistant to exogenous 5-HT and suppress the hyperenhanced slowing response of** ***mod-5*** **animals.** ***A.*** **Deletions present in the** ***ser-4*** **strains used in our analysis are depicted.** ***B.*** **Dose-response curves for** ***ser-4(ok512)*****, we measured percent immobilization after 20 min. at given 5-HT concentrations.** ***ser-4(ok512)*** **confers resistance to paralysis in 5-HT which is rescued by a** ***ser-4*** **transgene (*nEx1371*) but not by a frameshifted transgene (*nEx1372*). n ≥ 2 sets of 20 animals.** ***C.*** **We food deprived animals for 30 min. and counted the number of body bends each animal made over 20 sec. off food and on food (the enhanced slowing response). The** ***mod-1(ok103)*** **deletion partially suppresses the hyperenhanced slowing response (paralysis) exhibited by** ***mod-5(n3314)*** **animals. The** ***ser-4(ok512)*** **deletion partially suppresses the hyperenhanced slowing response (paralysis) exhibited by** ***mod-5(n3314)*** **animals.** ***D.*** **Data from** ***C*** **normalized to speed off of food (speed off food/speed on food). Although animals carrying a** ***mod-5(n3314)*** **mutation are slower in both conditions, they move even more slowly on food. n ≥ 3 sets of 5 animals for each condition. Error bars, S.E.M. * p<0.03, ** p<0.001, Student's *t* test.**

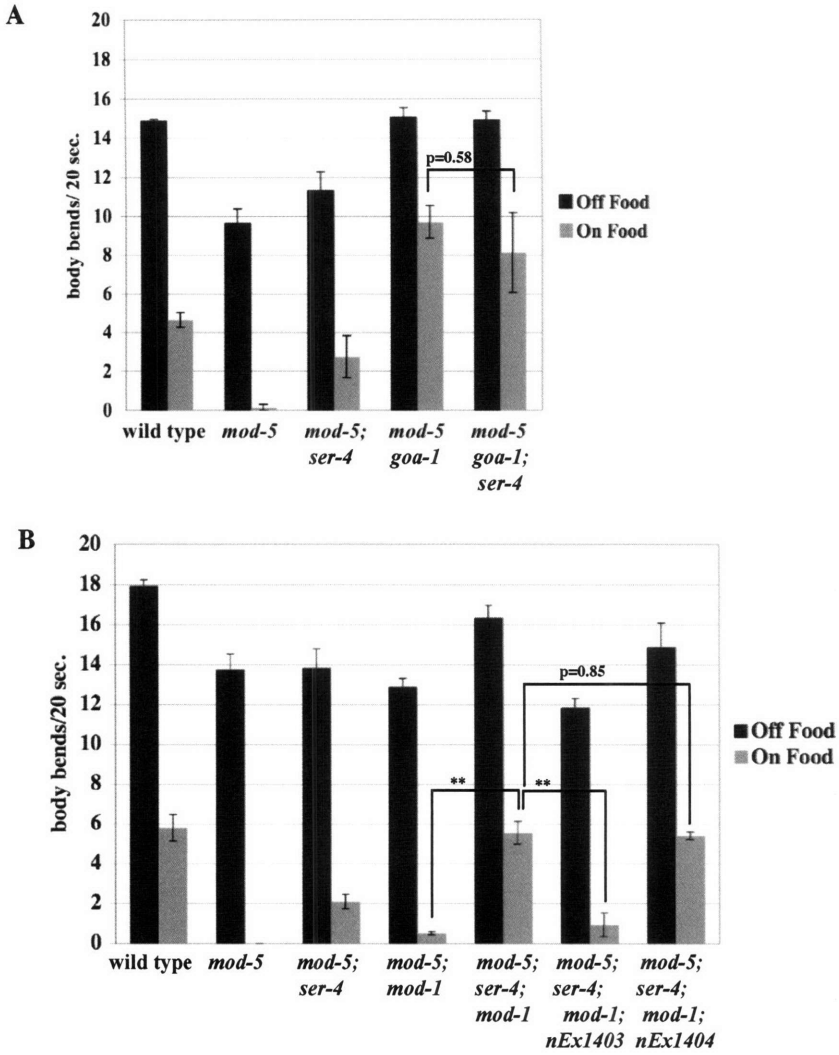

Figure 2. **SER-4 acts in a pathway with GOA-1 in parallel to MOD-1. We food deprived animals for 30 min. and counted the number of body bends each animal made over 20 sec. off food and on food (the enhanced slowing response). Alleles used are *mod-5(n3314)*, *mod-1(ok103)*, *goa-1(n1134)* and *ser-4(ok512)*. A. *ser-4* is not enhanced by *goa-1* in a *mod-5* background. *B. mod-1* is enhanced by *ser-4*, and this enhancement is rescued by *ser-4* transgene (*nEx1403*), but not by the same transgene with a frameshift mutation (*nEx1404*). (The first four sets of bars are identical to those shown in Fig. 1*C*.) n ≥ 3 sets of 5 animals for each condition. Error bars, S.E.M. **p<0.001, Student's *t* test.**

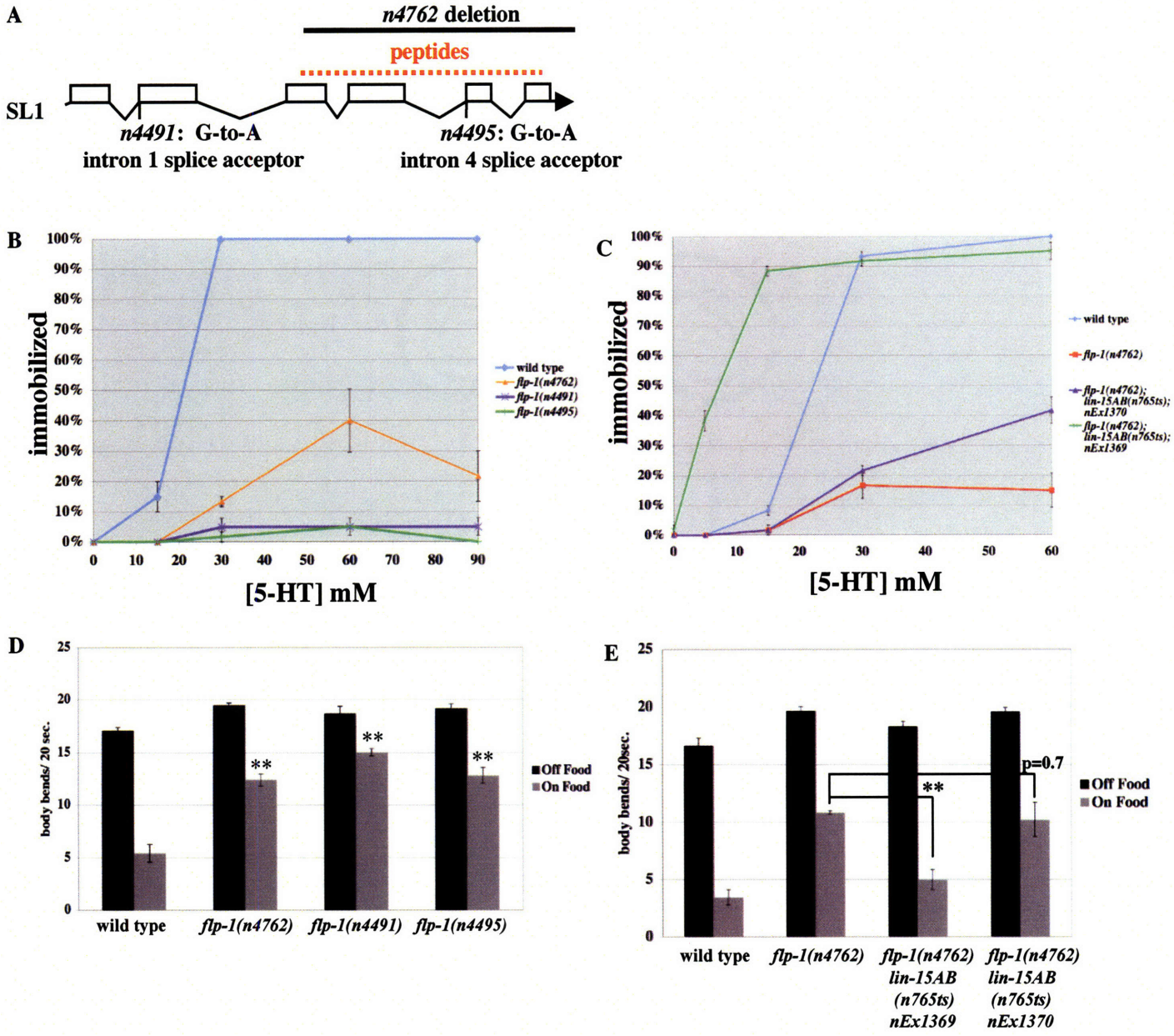

Figure 3. ***flp-1*** **mutants are 5-HT resistant and have enhanced slowing response defects.** ***A.*** **Mutations present in the** ***flp-1*** **strains used in our analysis are depicted. Approximate location of encoded peptides also depicted. B. Dose-response curves for** ***flp-1*** **mutants. Percent immobilization was measured after 10 min. and suppress the hypersensitivity of** ***mod-5(n3314)*** **animals.** ***flp-1*** **mutations confer resistance to paralysis in 5-HT.** ***C.*** **Dose-response curves for** ***flp-1*** **and** ***flp-1*** **rescue strains. Percent immobilization was measured after 10 min.** ***nEx1369*****, a transgene containing the** ***flp-1*** **genomic region and a** ***lin-15AB*** **coinjection marker, rescues the 5-HT resistance of** ***flp-1*** **animals.** ***nEx1370*****, a transgene containing only the** ***lin-15AB*** **coinjection marker, fails to rescue the 5-HT resistance of** ***flp-1*** **animals.** ***D.*** **We food deprived animals for 30 min. and counted the number of body bends each animal made over 20 sec. off food and on food (the enhanced slowing response). All** ***flp-1*****mutants exhibit decreased slowing.** ***E.*** **We food deprived animals for 30 min. and counted the number of body bends each animal made over 20 sec. off food and on food (the enhanced slowing response). The decreased slowing exhibited by** ***flp-1(n4762)*** **animals is rescued by a** ***flp-1*** **transgene (*nEx1369*) but not by the** ***lin-15*** **coinjection marker transgene (*nEx1370*). n ≥ 3 sets of 5 animals for each condition. Error bars, S.E.M. **p<0.001, Student's *t* test.**

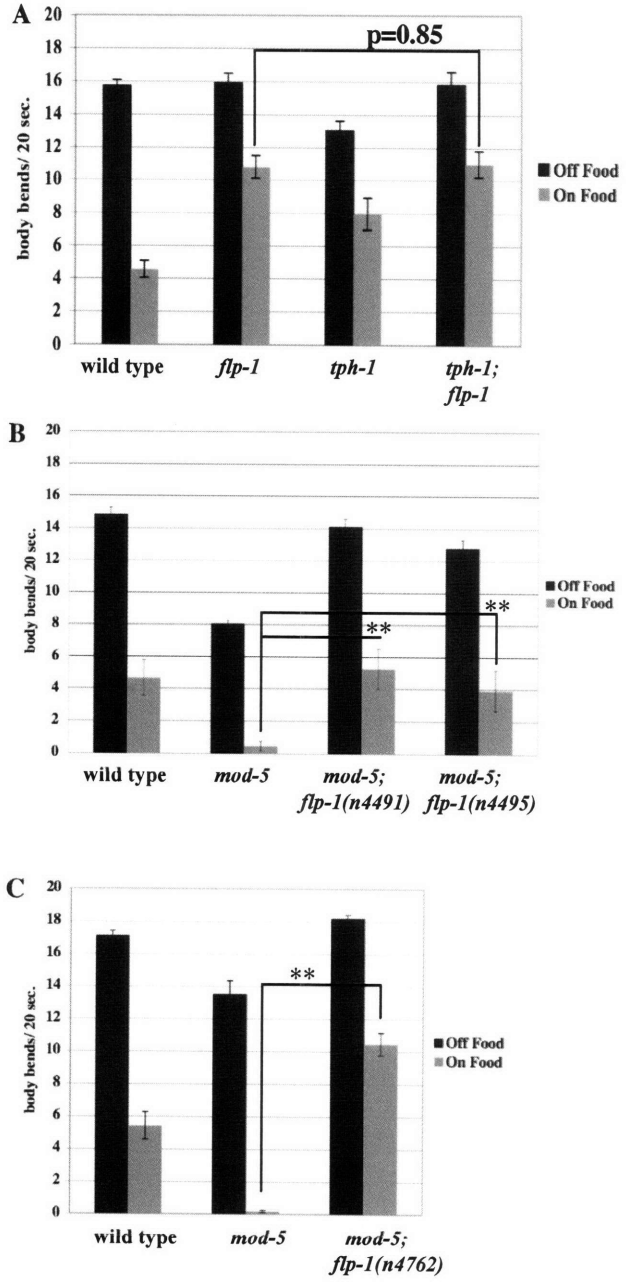

Figure 4. **We food deprived animals for 30 min. and counted the number of body bends each animal made over 20 sec. off food and on food (the enhanced slowing response). *mod-5 = mod-1(n3314). A.* The enhanced slowing response defect of *flp-1* is not enhanced by a mutation in *tph-1. B.* and *C. flp-1* mutations suppress the hyperenhanced slowing response of *mod-5(n3314)* animals. *flp-1* splice acceptor mutations *(B)* and a *flp-1* deletion mutation *(C)* suppress the hyperenhanced slowing response of *mod-5* animals. n ≥ 3 sets of 5 animals for each condition. Error bars, S.E.M. **p<0.001, Student's *t* test.**

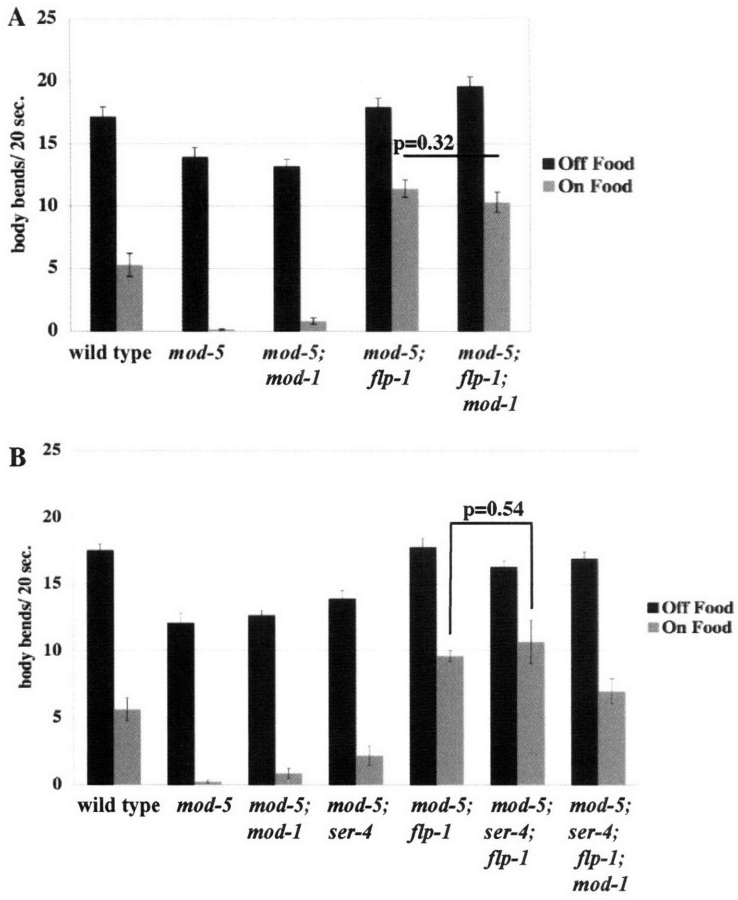

Figure 5: **The enhanced slowing response defect of *flp-1* animals is not enhanced by *mod-1* or *ser-4* deletions. We food deprived animals for 30 min. and counted the number of body bends each animal made over 20 sec. off food and on food (the enhanced slowing response). Alleles used are *mod-5(n3314)*, *mod-1(ok103)* and *ser-4(ok512)* *A.* The enhanced slowing response defect of *flp-1* is not enhanced by a mutation in *mod-1*. *B.* The enhanced slowing response defect of *flp-1* is not enhanced by a mutation in *ser-4*, or by both *mod-1* and *ser-4* together. n ≥ 3 sets of 5 animals for each condition. Error bars, S.E.M.**

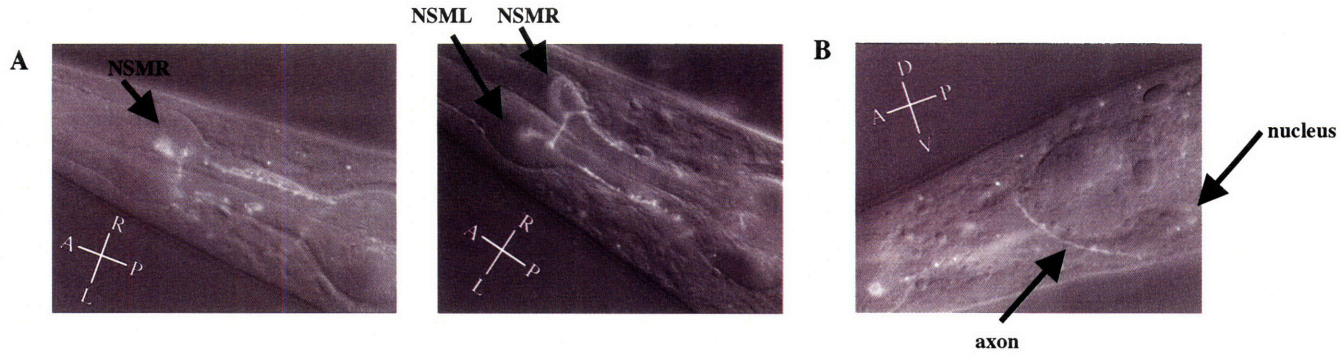

Figure 6: **We stained wild-type animals with anti-MOD-5 antibodies. *A.* We see strong fluorescence in the NSMs. *B.* We see weaker, more variable fluorescence in the AIMs.**

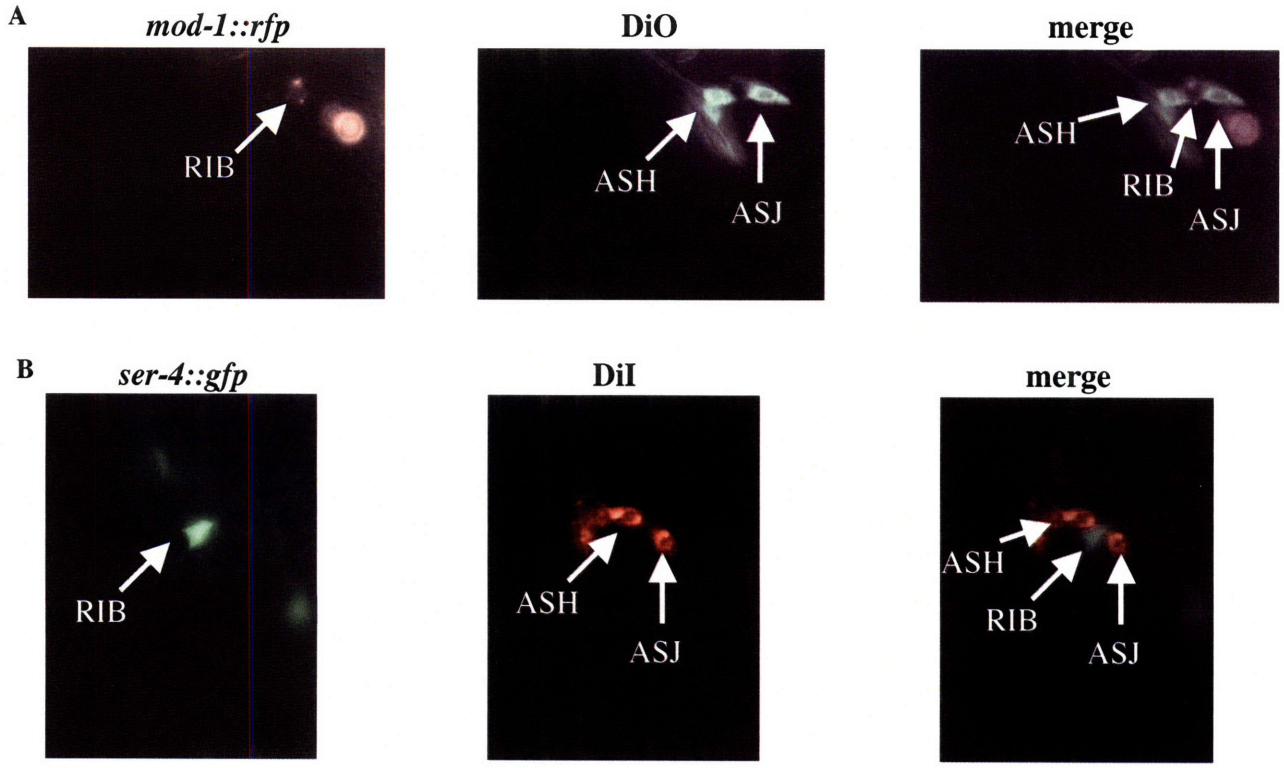

Figure 7: **Identification of the RIB neuron using the positions of nuclei that fill with the fluorescent dyes, DiO and DiI. *A. mod-1::dsRed* expression in an RIB neuron. We exposed animals carrying a *mod-1::dsRed* transgene to the green fluorescent dye, DiO (see Materials and Methods). The nucleus of an RIB neuron is between the dye-filling ASH and ASJ neuronal nuclei, a position characteristic of the RIBs. *B. ser-4::gfp* expression in an RIB neuron. We exposed animals carrying a *ser-4::gfp* transgene to the red fluorescent dye, DiI (see Materials and Methods). The nucleus of an RIB neuron is between the dye-filling ASH and ASJ neuronal nuclei.**

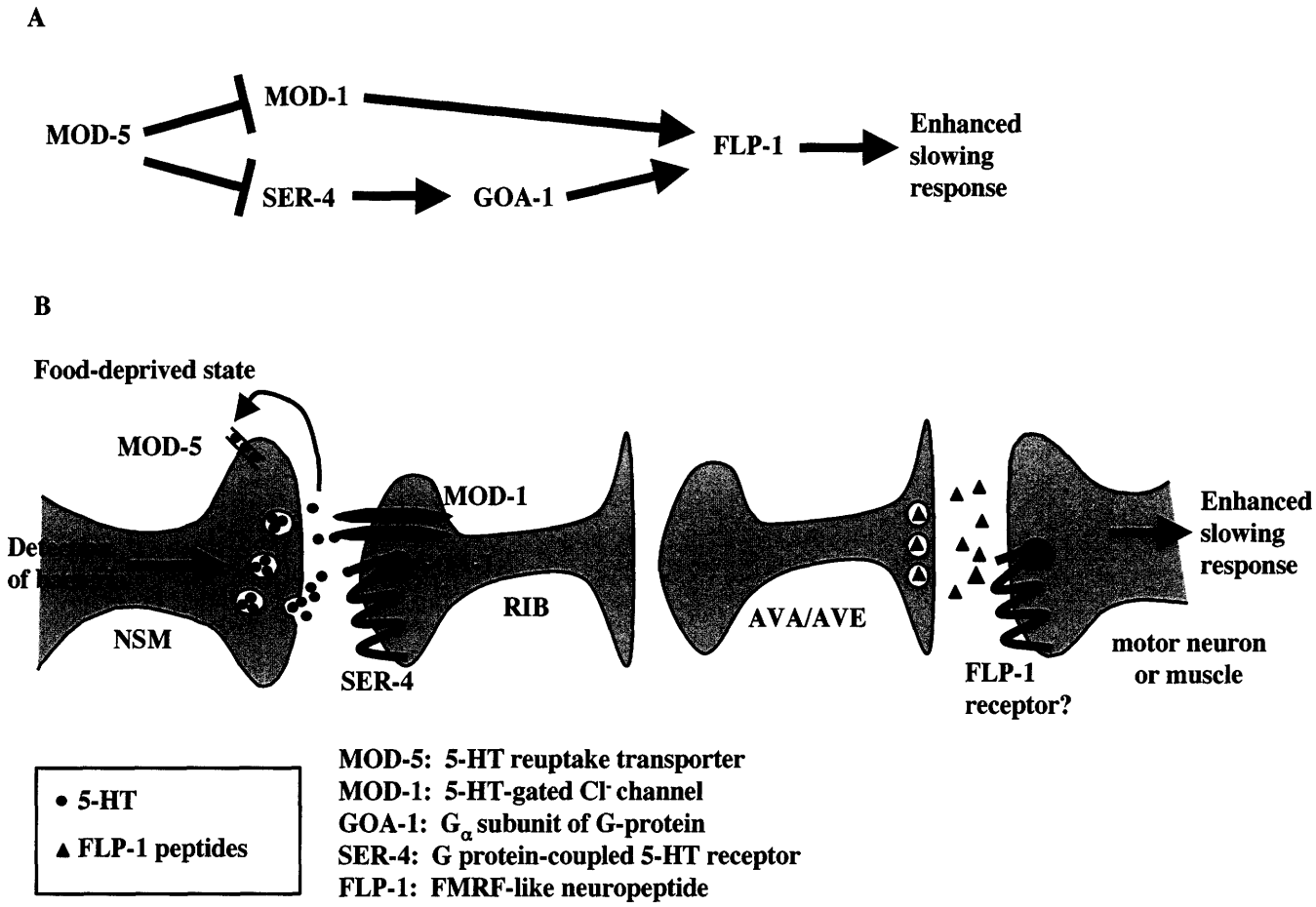

Figure 8: *A.* Genetic model for the pathways regulating locomotion in *C. elegans*. *B.* Cellular model for the action of 5-HT receptors and FLP-1 neuropeptide(s) in regulating locomotion in *C. elegans*. See text for details.

Appendix I

Additional Results and Future Directions

Included here are some promising preliminary results and information that should be useful to anyone continuing this work.

Additional Results and Future Directions

Progress toward finding a *flp-1* receptor

As discussed in Chapter 3, the discovery and characterization of a *flp-1* receptor may provide the information necessary to distinguish different circuit models for the enhanced slowing response. We searched for a *flp-1* receptor by screening candidate receptor genes by RNAi and looking for those that, like *flp-1*, conferred 5-HT resistance. We performed BLAST searches with the four known *C. elegans* G protein-coupled FMRFamide receptors, the *flp-2* receptor T19F4.1 (Mertens et al., 2005), the *flp-7/11* receptor C26F1.6 (Mertens et al., 2004), the *npr-1* receptor (Rogers et al., 2003) and the *egl-6* receptor (Niels Ringstad, personal communication). We combined the top 100 hits for each, removing any overlap. The final list contained 193 clones, 147 of which have RNAi clones publicly available (Table 1). Genes highlighted in purple (Lowery et al., 2007) or blue (Mertens et al., 2006) are genes identified as weakly activated by *flp-1* peptides. We then used an RNAi hypersensitive strain, *eri-1(mg366); lin-15B(n744),* in which RNAi-mediated gene knockdown in neurons has been shown to occur (Sieburth et al., 2005), and found that animals that were fed the same two control RNAi clones, *goa-1* and *flp-1*, did become resistant to 5-HT (Figure 1A). RNAi-mediated knockdown in neurons is not thought to occur in wild-type animals. When we fed wild-type animals two control RNAi clones, *goa-1* and *flp-1*, they failed to become resistant to 5-HT (Figure 1B). Therefore, we fed *eri-1(mg366); lin-15B(n744)* animals 136 of the 147 available clones, but have not found one that confers 5-HT resistance (data not shown). It is possible that this approach may not reveal a *flp-1* receptor because there may be more that one relevant

receptor (genetic redundancy) or the relevant receptor may be an ionotropic receptor, like the FMRFamide-gated sodium channel found in the snail *Helix aspersa* (Lingueglia et al., 1995). However, the remaining clones should certainly be tested.

The discovery that *eri-1(mg366); lin-15B(n744)* animals can be used to examine RNAi clones for a role in 5-HT signaling should make the search for new genes involved in 5-HT signaling much easier and more rapid. A *mod-5(n3314); eri-1(mg366); lin-15B(n744)* strain should be constructed and tested to see whether smaller contributions to 5-HT signaling can be detected in this background. An *abts-1* RNAi clone could be used as a positive control for this, because its contribution to 5-HT signaling can only be seen in a *mod-5* background. Testing LGII and LGX clones for suppression of the 5-HT hypersensitivity of *mod-5(n3314)* mutants may also provide an easier way to determine what gene or genes are mutated in the LGII and LGX isolates from the first *mod-5* suppression screen (EMS, 20 mM 5-HT), as these isolates have somewhat weaker defects.

Remaining questions regarding *mod-1*

n3033 and *n3034* were isolated in a screen for animals with reduced enhanced slowing responses (Sawin, 1996; Sawin et al., 2000). *n3033* and *n3034* were each resistant to exogenous 5-HT and failed to complement for the enhanced slowing response defect. The gene defined by *n3033* and *n3034* was named *mod-1* and *mod-1(n3034)* was backcrossed two times. *n3034* was mapped to LGIII following the enhanced slowing response defect (Sawin, 1996).

In a subsequent study, *mod-1(n3034)* was backcrossed three times following its 5-HT resistance phenotype and the animals retained the enhanced slowing response defect, indicating that *mod-1(n3034)* was likely responsible for both the 5-HT resistance and enhanced slowing response defects. *mod-1(n3034)* was mapped and cloned following its 5-HT resistance phenotype and a C to T mutation was found in the 5-HT-gated chloride channel gene K06C4.6. Subsequent analysis of two deletion alleles *mod-1(n3034)* and *mod-1(ok103)* revealed that they confer a reduced enhanced slowing response; *i.e.* animals with mutations in the 5-HT-gated chloride channel *mod-1* fail to slow as much as wild-type animals on food following food deprivation (Ranganathan et al., 2000). Although Sawin initially mapped *n3034* to LGIII (Sawin, 1996), K06C4.6 is located on chromosome V. Ranganathan thus repeated the complementation analysis but found that *n3033* and *n3034* fail to complement for the enhanced slowing response. Ranganathan also sequenced the K06C4.6 genomic locus in *n3033* and found no mutations (R. Ranganathan, personal communication).

I was unable to repeat the finding that the deletions *mod-1(ok103)* and *mod-1(nr2043)* conferred an enhanced slowing response (Figure 2A), so I thawed strains containing each of the mutations above, including the *mod-1(n3034)* allele backcrossed twice by Sawin following the enhanced slowing response defect (referred to hereafter as *mod-1(n3034)* 2x) and the *mod-1(n3034)* allele backcrossed an additional three times by Ranganathan following the 5-HT resistance phenotype (referred to hereafter as *mod-1(n3034)* 5x). While I was able to repeat the finding that *n3033* and *mod-1(n3034)* 2x exhibit reduced

enhanced slowing responses, I found an intermediate, inconclusive defect exhibited by *mod-1(n3034)* 5x and still no defect exhibited by *mod-1(ok103)* (Figure 2B). I sequenced the *mod-1* alleles in the strains *mod-1(n3034)* 2x, *mod-1(n3034)* 5x, *mod-1(ok103)* and *mod-1(nr2043)* and found the mutations indicated in Ranganthan *et al.*, 2000. I also tested responses to exogenous 5-HT and found that *mod-1(n3034)* 2x, *mod-1(n3034)* 5x, *mod-1(ok103)* and *mod-1(nr2043)* mutants were all resistant (data not shown). I then suspected that there might be two mutations in the original *n3034* strain: one causing 5-HT resistance and one causing an enhanced slowing response defect. The mutation causing the enhanced slowing response defect is present in the *n3034* 2x strain, but may be absent or not homozygous in the *n3034* 5x strain. Preliminary results from Daniel Omura suggest that the 5-HT resistance and enhanced slowing response defects of *n3034* 2x can be separated. The C to T mutation in the 5-HT-gated chloride channel K06C4.6 co-segregated with 5-HT resistance. A backcrossed strain in which the enhanced slowing response defect was reisolated did not carry the mutation C to T mutation in the 5-HT-gated chloride channel K06C4.6 (D. O., personal communication). This experiment should be repeated, and the strains saved for future analysis.

Based on these results, I revisited *n3033*. Both *n3033* and *n3034* have 5-HT resistance and enhanced slowing response defects. Sawin found that *n3033* and *n3034* are allelic. If this is true, it would be unlikely that both would have mutations in the 5-HT-gated chloride channel K06C4.6 and in a separate gene that mediates the enhanced slowing response. I assessed both strains (*n3033* and *n3034* 2x) for response to exogenous 5-HT. I found that the 5-HT resistance phenotype of *n3033* is much weaker than that of *n3034* 2x

(and any of the other K06C4.6 alleles, including two recovered in my screens, *mod-1(n3791)* and *mod-1(n4054)*) (Figure 3 and see Chapter 2) and I also sequenced 2.7 kb of the promoter and the coding sequences of K06C4.6 in the *n3033* strain and found no mutations. Taken together, these results suggest that *n3033* is not an allele of K06C4.6 but are not conclusive. The question remains as to whether the mutations in *n3033* and *n3034* that cause enhanced slowing response defects are allelic.

These results suggest a number of hypotheses to be tested. To show definitively that *n3034* contains a mutation in another gene that is responsible for the enhanced slowing response defect, *n3034* will have to be mapped and cloned following the enhanced slowing response defect. In addition, identification of the mutation in the *n3034* strain responsible for the enhanced slowing response defect will help determine whether the mutations in *n3033* and *n3034* that cause enhanced slowing response defects are allelic, because the *n3033* strain can be sequenced for mutations in the same gene or rescued with a transgene.

Testing enhanced slowing response assay conditions

I performed many troubleshooting experiments to ensure that I performed the enhanced slowing response assay under the exact conditions reported in Ranganathan *et al.* (2000). In the enhanced slowing response assay, animals to be tested are staged and placed at 20°C overnight, and a ring-shaped lawn of HB101 bacteria is spread on small (6 cm) Petri plates and incubated overnight at 37°C. In the morning, animals and plates are left for several hours to equilibrate to room temperature. The assay is then performed by picking

a few animals (see below) to a well of buffer, washing the animals free from bacteria and mouth pipetting them to a plate with no food and leaving them for 30 min. Then the animal is mouth-pipetted in a small drop of buffer to an assay plate, the buffer is removed with a Kimwipe, and after 5 min. the animals are assayed for body bends. Following several conversations with Ranganathan detailing the exact conditions used and a thorough reading of the description of the enhanced slowing response assay in Sawin's dissertation (2006), I found five differences between our methods (Table 2). First, the bacterial culture I used to make the ring lawns was younger (less than a month old compared to more than a month old (Ranganthan)). Second, the animals I used were slightly younger (animals were staged between 15 and 16 hours prior to the assay compared to 16 or more hours (Ranganathan and Sawin)). I may have used thicker bacterial lawns (I incubated plates overnight between 14 and 15 hours compared to 13 and 15 hours (Sawin) or 12 and 13 hours (Ranganathan)) After plates incubated overnight at 37°C, I would spread the plates out on a benchtop in a single layer and place the lids slightly ajar to allow them to cool, whereas Ranganathan left lids on and inverted the plates to cool. Thus, my plates were likely drier, because condensation could evaporate because the lids were ajar. Finally, when performing the assay, I typically picked six animals to plates, and counted body bends of five animals, whereas Sawin picked five and counted five. Ranganathan picked eight to ten and only counted those that moved forward within 5 seconds of observing them, up to five animals total. I tested wild type, *mod-1(n3034)* 5x, *mod-1(ok103)* and *mod-1(nr2043)* under the exact conditions communicated by Ranganathan (Figure 4A-C). When I used a bacterial culture that was more than a month old, I found a defect similar to what I initially observed for

mod-1(n3034) 5x (Figure 4A). However, *mod-1(n3034)* 5x showed a wild-type enhanced slowing response under all other conditions tested (Figure 4B, C). Both *mod-1* deletion strains showed a wild-type enhanced slowing response under all conditions (Figure 4A-C). It will be crucial for anyone continuing this work to determine the necessary conditions to repeat the results in Ranganthan *et al.*, 2000.

Determining the identity of LGI, LGII, LGV and LGX mutants

As discussed in Chapter 2, several mutants remain to be mapped and cloned. Based on the strength of these mutants in suppressing the 5-HT hypersensitivity of *mod-5(n3314)* animals, *n4404* I will be the easiest to map and clone. *n4404* and *mod-5* are both on LGI, so depending on how closely linked the two genes are, it may be difficult to reisolate *n4404* I in a wild-type background. However, reisolating *n4404* in a wild-type background is likely not necessary for mapping, because *n4404* confers strong 5-HT resistance. Therefore, the Hawaiian strain CB4856 can probably be used to map *n4404* I using snps. Once the molecular identity of *n4404* I is established, *n3498* I and *n3792* I should be sequenced for mutations in the same gene.

n3510 V and *n3461* II should also be mapped and cloned. Although *n3510* V has be reisolated in a wild-type background, it might be easier to map and clone in a *mod-5(n3314)* background, because the difference between *mod-5(n3314)* and *mod-5(n3314); n3510* V is greater than the difference between *n3510* V and wild type. Thus, mapping and cloning in a *mod-5(n3314)* background will make it easier to distinguish whether a recombinant strain is carrying *n3510* V. However, this requires that

all mapping strains be put in a *mod-5(n3314)* background. The same is true for mapping LGII suppressors. One way to avoid this would be to build a strain containing *mod-5(n3314)* in a CB4856 background. Although snps from the wild type N2 (Bristol) would likely remain around the *mod-5(n3314)* genomic locus, this should not interfere with mapping genes on LGII and LGV. Once the molecular identity of *n3461* II and *n3510* V are established, the other isolates that map to the respective linkage group should be sequenced for mutations in the same gene.

n3477 shows weak linkage to X. I initially mapped *n3477* using markers at or near the center of each chromosome. *n3477* should be examined for linkage to markers on the arms of each chromosome, because it is possible that *n3477* is located on a different chromosome entirely. *n3477* displays a weak suppression of the 5-HT hypersensitivity of *mod-5(n3314)*. If mapping using this suppression phenotype proves too difficult, one could also use the Egl-c defect to map *n3477*. I was able to reisolate the Egl-c defect in a wild-type background, which may represent a strain carrying *n3477* alone. However, it is possible that two mutations are present in the original *mod-5(n3314); n3477* strain: one causing suppression of the 5-HT hypersensitivity and one causing the Egl-c defect. Thus, if the Egl-c defect is used to map and clone, it must be shown that *n3477* causes both defects. Three of four LGII isolates also show Egl-c defects, which could also be used to map and clone these isolates, with the above caveat.

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Table 1: RNAi clones of genes that are similar to neuropeptide receptors

Ahringer RNAi feeding library: all tested

Gene	Plate	Well	Deletion allele available
C30F12.6	10	G07	*ok1381*
T23B3.4	10	B06	
F16D3.7	13	E07	
F16C3.1	17	D05	
M01G12.4	21	C04	
T07D10.2	22	F05	
T06G6.7	23	A01	
W05B5.2	23	E06	
F32A7.2	26	E10	
D1069.4	31	G09	
K10B4.4	31	D01	
K07E8.5	32	D09	
Y57G7A.4	34	C06	
ZC204.15	35	A05	
B0454.2	38	F10	
B0454.4	38	F12	
T11F1.1	38	D12	
R11F4.2	41	G04	
C54A12.2	44	C09	
T07D4.1	52	G10	
ZK1307.7	54	F07	
E04D5.2	56	C05	
Y48C3A.11	61	H02	
Y54G11B.1	63	F11	
Y54E2A.1	64	B08	*tm1665*
Y39A3B.5	68	A04	
T27D1.3	69	D07	*tm2048*
F59B2.13	81	D12	
Y48A6B.1	85	D04	
K03H6.1	94	B09	
AC7.1	98	F11	
H06H21.1	98	A09	
H06H21.2	98	A10	
C49A9.7	100	F09	
R13H7.2	101	E11	*ok1167*
C50F7.1	103	F01	
T05A1.1	107	H02	
C10C6.2	112	E10	*tm1583*
Y37A1B.10	117	F07	
H25K10.7	119	E05	
Y105C5A.23	119	D04	
T02D1.4	121	D04	
T02D1.6	121	D06	
Y41D4B.24	122	D02	
Y69A2AR.15	123	D08	
R11G11.13	126	A07	
ZK488.9	126	C12	
K04F1.2	128	C04	
K04F1.3	128	C05	
Y46H3C.1	128	D12	tm allele in progress
C44C3.1	131	G05	
C44C3.2	131	G06	
C44C3.3	131	G07	
C44C3.5	131	G09	
C44C3.6	131	G10	
C44C3.7	131	G11	
F37B4.8	131	E03	
H05B21.3	131	G03	
H27D07.2	131	F10	
H27D07.3	131	F11	
H27D07.4	131	F12	
H27D07.5	131	G01	
K12D9.10	131	H09	
K12D9.3	131	H02	
T24A6.14	133	G04	
T05B4.5	135	C05	
T05B4.6	135	C06	
T05B4.7	135	C07	

Ahringer RNAi feeding library: all tested, cont.

Gene	Plate	Well	Deletion allele available
H22D07.1	136	A02	
C04E6.2	138	H11	
H34P18.1	140	H07	
ZC404.10	140	H05	
ZC404.11	140	H06	
C33G8.1	141	D01	
T15B7.11	141	A06	
T15B7.12	141	A07	
C03G6.16	142	D04	
F18E3.4	142	E09	
F38E1.8	145	A08	
F57A8.4	149	A07	
C35A5.7	150	B10	
H09F14.1	150	E01	
F32D8.10	151	A09	
F57B7.1	152	B08	
T02E9.1	152	A02	
T02E9.3	152	A04	*ok568*
T11F9.1	152	B12	
C54D10.5	154	D07	
K10C8.2	154	H05	
C56A3.3	156	G06	
C15H11.2	158	H09	
T26H5.5	161	G08	
F36G9.1	163	D06	
H24D24.1	163	D04	
H24D24.2	163	D05	
T05G11.3	163	C12	
T05G11.6	163	D02	
T05G11.7	163	D03	
F57G8.4	164	G01	
F14F8.5	165	G03	
F14F8.6	165	G04	
F14F8.7	165	G05	
F26D2.11	165	A06	
T03E6.6	165	E03	
T08G3.10	165	B08	
Y32B12C.2	165	E07	
F40D4.8	167	A08	
K03D7.2	167	H09	
C52B11.3	177	E03	*tm1392, ok1321*
F02E8.2	182	G08	
M03F4.3	183	E09	*ok325*
F14D12.6	184	E09	*ok371*
F01E11.5	186	H03	*tm1815, tm1846*
C56G3.1	187	E01	*tm1553, ok1439, ok1446*
C02B8.5	188	G03	
C17H11.1	188	G09	
F53A9.5	189	G07	
C48C5.1	190	B10	*ok1387*
F35G8.1	190	G07	*ok527*
C43C3.2	191	C07	*ok1388*
C39B10.1	192	E01	
F41E7.3	192	C03	*tm1497*
F42C5.2	192	C03	
F13D2.3	193	E12	
ZK455.3	193	H04	*tm1652*
C16D6.2	195	H12	*tm1782*
C05E7.4	196	E02	
C53C7.1	196	E11	*tm1568, ok1442*
F42D1.3	199	E07	
Y70D2A.1	199	G02	
F59D12.1	200	G06	
R03A10.6	200	E07	
F31B9.1	201	B04	
T23C6.5	202	H07	
F39B3.2	203	E04	
R106.2	203	D07	

Missing from Ahringer library: not tested

Gene	Plate	Well	Deletion allele available
K12D9.7	131	H06	
F59D6.5	132	A04	
F18E3.2	142	E07	
F18E3.5	142	E10	
ZK105.2	143	E07	
F56B6.5	181	C03	*ok1541*

Clones not available: not tested

B0563.6
C03A7.3
C06G4.5
C09F12.3
C24A8.1
C25G6.5 *ok594*--not resistant
C30B5.5
C35A11.1
C44C3.11
F14F8.10
F14F8.11
F19B2.3
F21C10.12
F36D3.13
F36G9.16
F53B7.2
F57H12.4 *ok1504*
H23L24.4
K04F1.4
K06C4.17
K06C4.9
K12D9.4
K12D9.5
T15B7.13
T21B4.4
T26E4.11
Y116F11B.5
Y41D4A.8
Y43F8A.4
Y46H3C.2
Y46H3C.3
Y52E8A.5
Y54G2A.35 *tm2104, tm2146*
Y58G8A.4 *ok1583, tm1491*
Y75B7AL.1
ZC404.13
ZK1037.9
ZK697.12
ZK697.13
ZK697.5

Other

Y58G8a.1 *ok1770*--not resistant

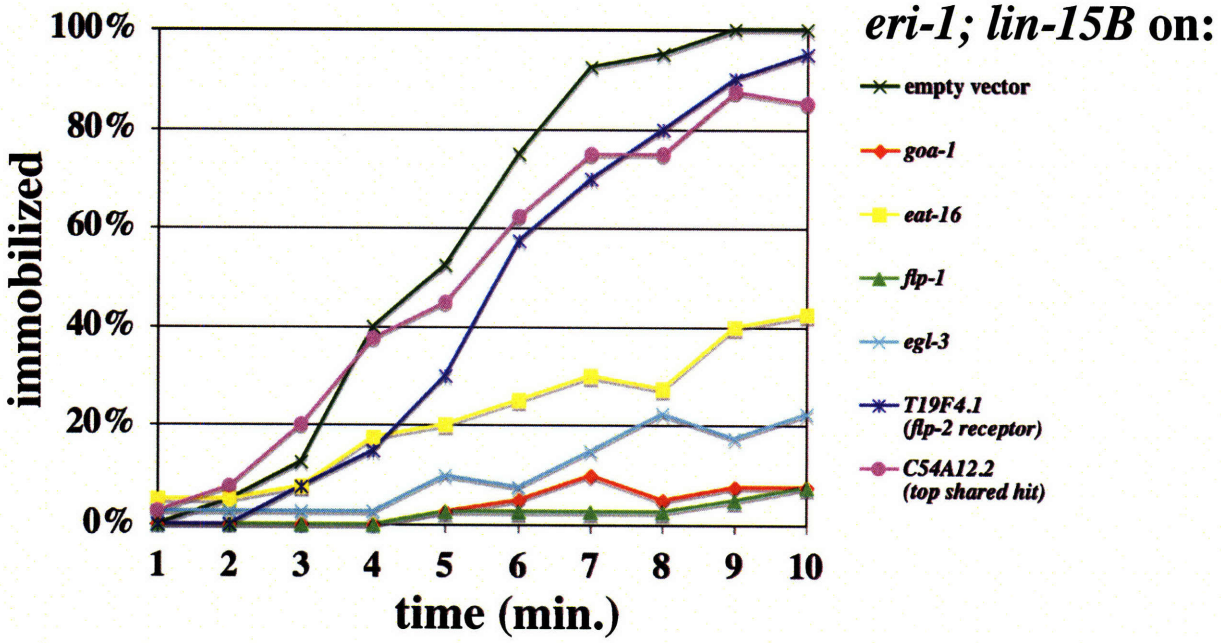
Vidal Unique library clones: not tested

AH9.1	18	C9	
B0334.6	10	B10	
F14F4.1	18	B6	*tm2243*
F54D7.3	19	B12	*ok238*
K06C4.8	15	H1	
R12C12.3	7	D1	
T22D1.12	12	G2	*tm1498*
Y23H5B.4	8	F3	
Y37E11AL.1	12	F12	
Y59H11AL.1	19	D11	
ZC412.1	19	F12	*tm1504*

Each of the three columns contains neuropeptide receptor-like genes and, if one exists, the location of its RNAi clone in a given library. Also shown are deletion alleles if available. Clones in the first two columns have been tested and none confer resistance. C26G6.5*(ok594)* and Y58G8a.1*(ok1770)* have been tested and found not to be 5-HT resistant. Highlighted in yellow are clones available in the Vidal Unique Library that should be tested. Genes highlighted in purple or blue are genes identified as weakly activated by *flp-1* peptides (see text).

Figure 1: RNAi of 5-HT signaling genes causes 5-HT resistance in *eri-1; lin-15B* animals but not in wild-type animals

A.



B.

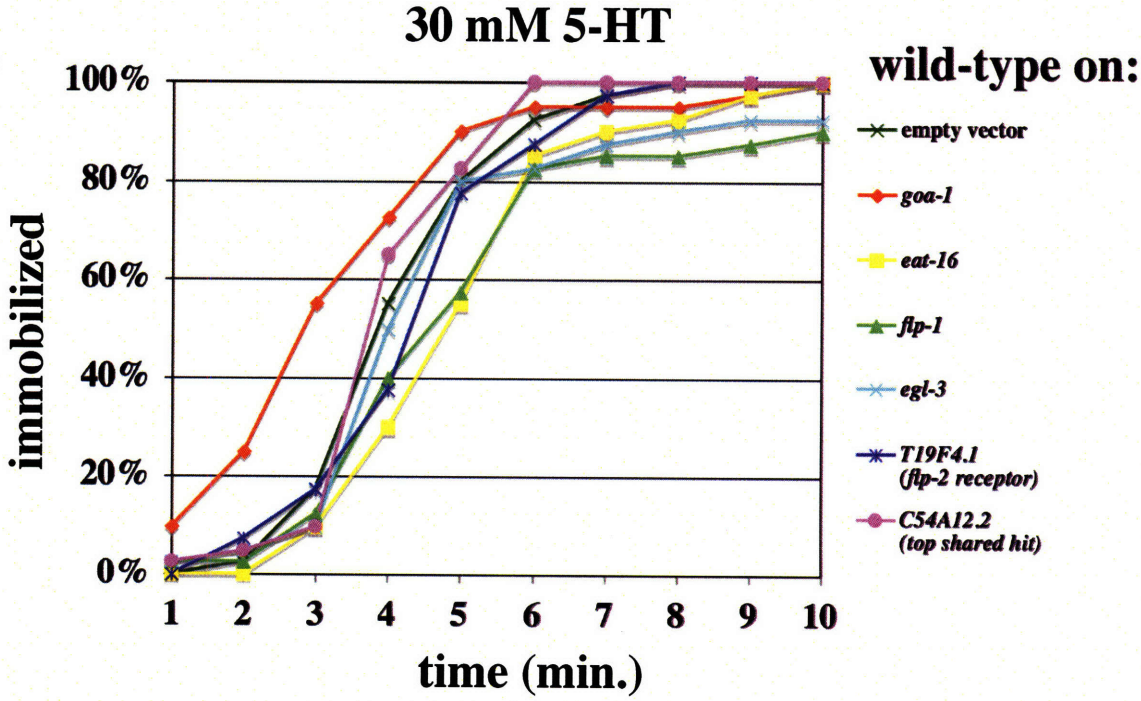
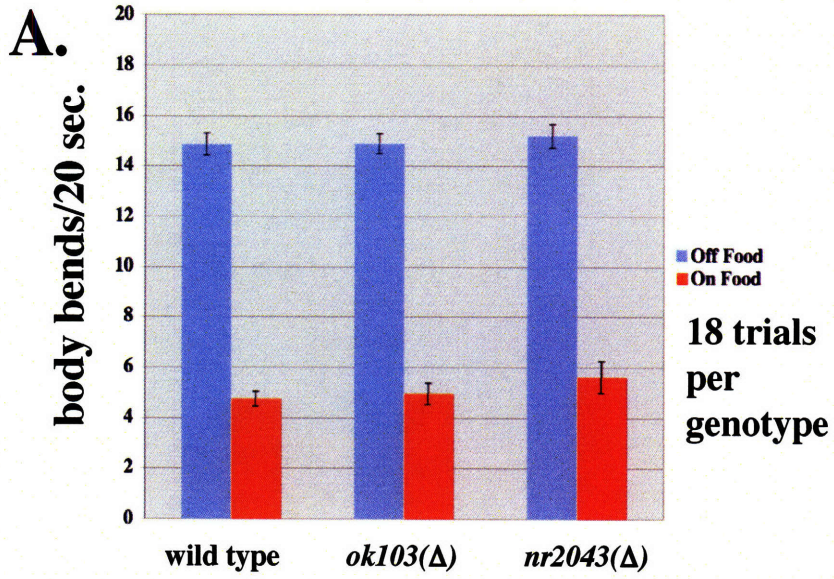
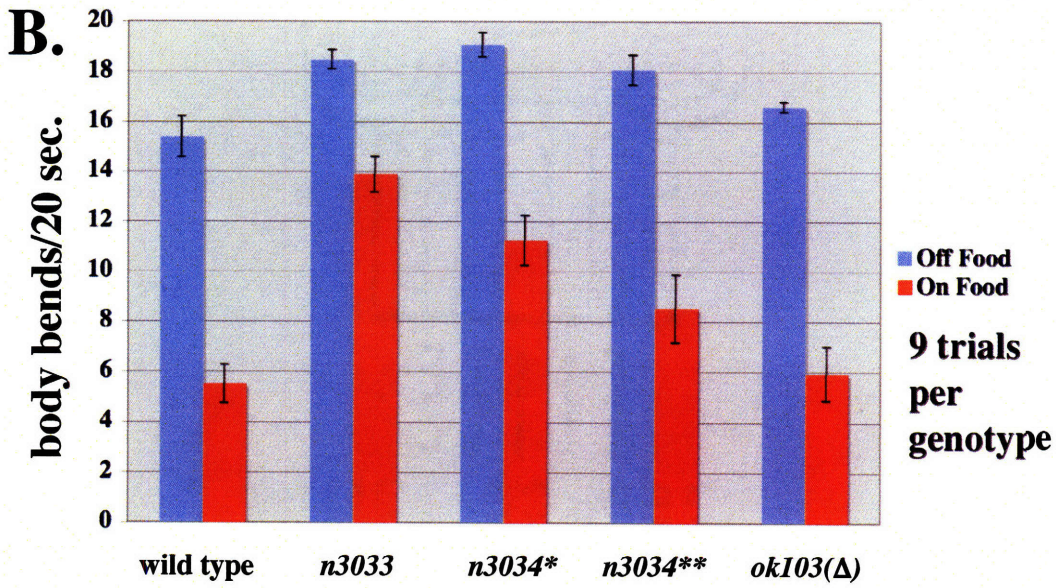


Figure 2: *mod-1* deletion mutants show a wild-type enhanced slowing response but the original *n3033* and *n3034* strains show a reduced enhanced slowing response





*the *n3034* isolate from Sawin's screen, backcrossed twice by Sawin following the enhanced slowing response defect
** the above *n3034* isolate backcrossed an additional three times by Ranganathan following 5-HT resistance

Figure 3: The 5-HT resistance of *n3033* animals is much weaker than that of *n3034* animals

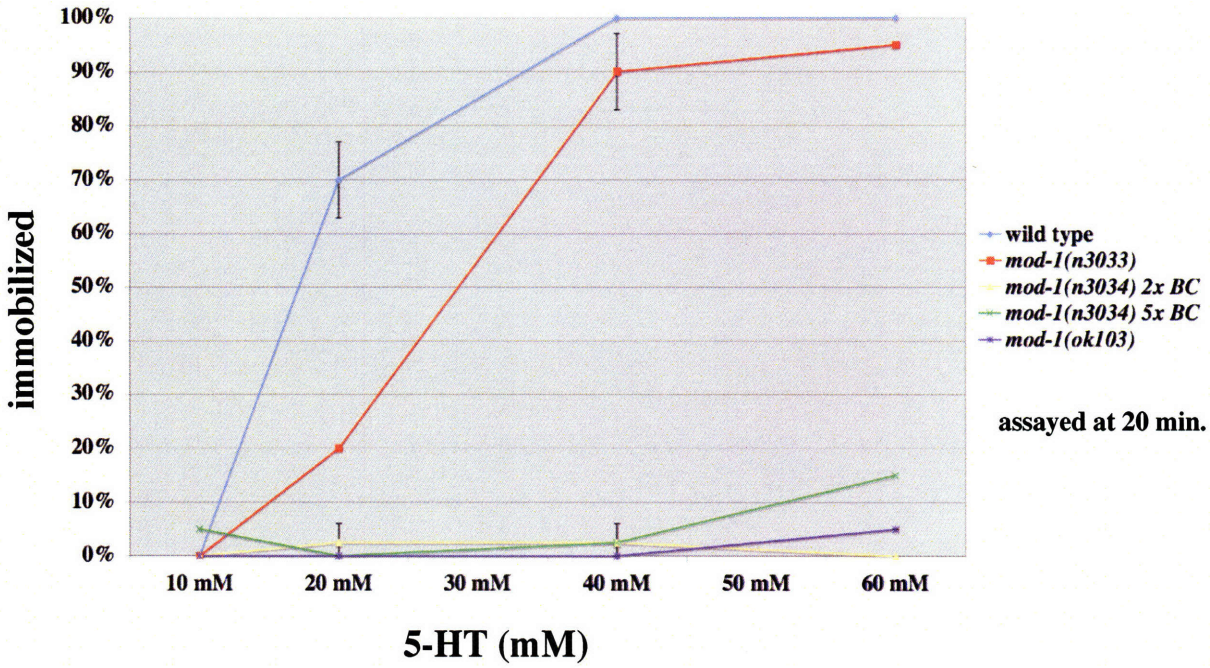
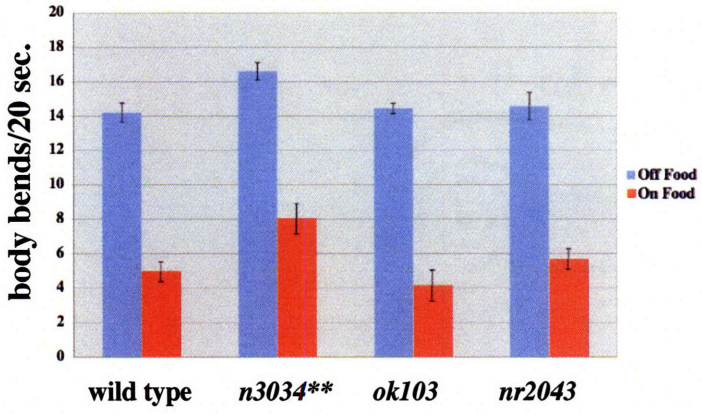
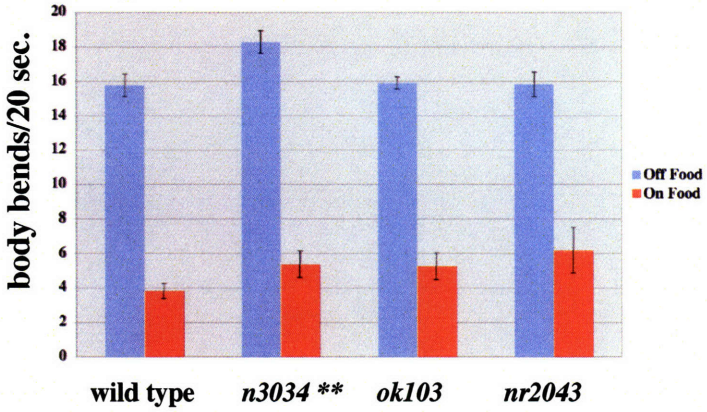


Figure 4: Analysis of *mod-1* alleles in various enhanced slowing response assay conditions

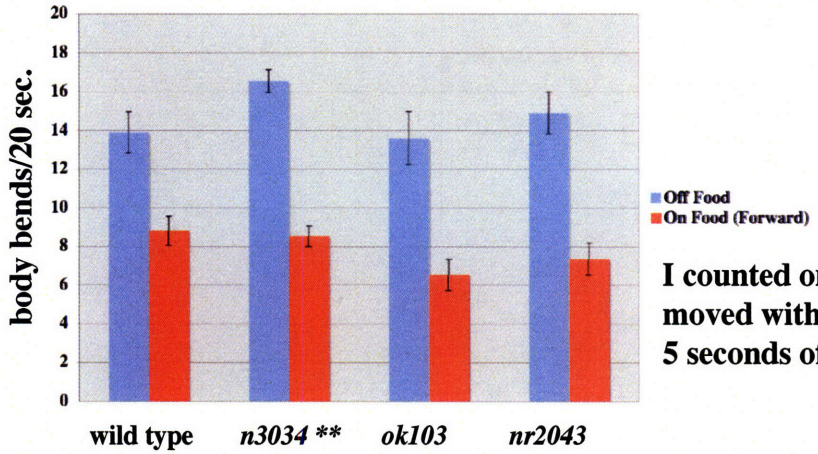
A. newly thawed *mod-1* alleles on bacteria from >1 month old culture



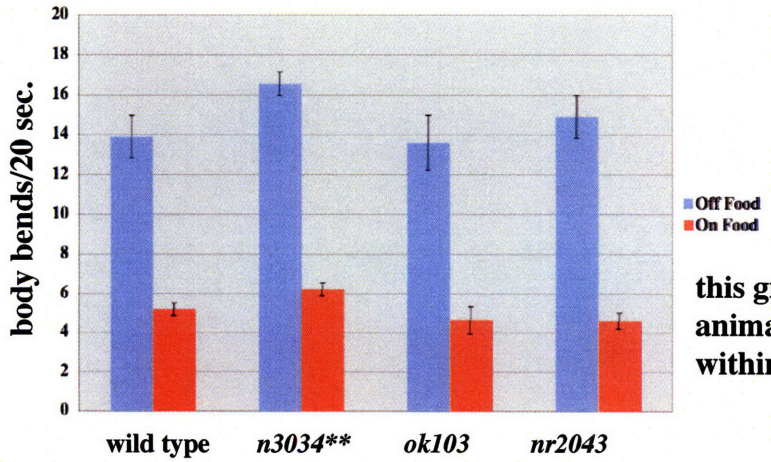
B. stage and spot plates at exact times as Ranganathan and leave lids on to cool



C. only count animals moving forward (at least one body bend in 5 sec.)



I counted only animals that moved within the first 5 seconds of observing them



this graph includes the animals that did not move within 5 seconds

***n3034* was backcrossed twice by Sawin following the enhanced slowing response defect, then backcrossed three times by Ranganathan following 5-HT resistance defect**