Serotonin-dependent modulation of Caenorhabditis elegans behaviors

by

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ABSTRACT

Understanding the genetic and molecular basis of behavioral plasticity is an important question in neurobiology. One such paradigm for behavioral plasticity involves the ability of *C. elegans* hermaphrodites to modulate their locomotory rate in response to the presence of a bacterial lawn, their food source, via a serotonin-mediated signaling pathway. Mutations in the genes *mod-1* and *mod-5* affect both the modulatory behavior and serotonergic neurotransmission.

To further understand how this modulation of locomotory behavior is achieved we have cloned *mod-1* and *mod-5*. Protein sequence comparisons revealed that MOD-1 is a ligand-gated ion channel and MOD-5 is a protein similar to mammalian serotonin reuptake transporters (SERTs).

We have performed electrophysiological analysis of the MOD-1 channel in *Xenopus* oocytes and mammalian cells to show that this channel is specifically gated by serotonin and that it conducts chloride ions, thereby defining a new type of ionotropic serotonin receptor, a serotonin-gated chloride channel. We have performed neurotransmitter uptake assays of MOD-5 in mammalian cells to show that MOD-5-mediated uptake is specific for serotonin and that selective serotonin reuptake inhibitors such as fluoxetine (Prozac) inhibit such uptake.

We have performed genetic epistasis analysis with *mod-1*, *mod-5*, and *goa-1* (a G-protein involved in serotonin signaling) to determine the genetic pathway involved in this modulatory behavior. We have also analyzed the various behavioral effects of fluoxetine treatment on *mod-5* and *tph-1* (serotonin-minus) mutants and determined that fluoxetine can act in *C. elegans* via both SERT- and serotonin-independent and -independent pathways.

Thesis Supervisor: H. Robert Horvitz
Title: Professor of Biology
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An environment that fosters as much scientific rigor and excellence as Bob's laboratory would be hard to duplicate. I am grateful to have been a part of it and can only hope to strive towards the same standards in my future endeavors.

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# Table of Contents

Title Page .................................................................................................................. 1

ABSTRACT .................................................................................................................... 2

Acknowledgments ........................................................................................................ 3

Table of Contents ........................................................................................................ 4

Chapter 1: Serotonin in Behavior .............................................................................. 11

- Introduction ............................................................................................................. 12
- Serotonin Synthesis, Metabolism, and Expression .................................................. 13
- Serotonin in Memory and Learning ......................................................................... 14
- Serotonin in *C. elegans* ....................................................................................... 17
- Serotonin Is Involved in the Responses of *C. elegans* to Food ......................... 18

Mammalian Serotonin Receptors ............................................................................ 19

* C. elegans* Serotonin Receptors ........................................................................ 22

Chloride Channels ........................................................................................................ 22

- Structure-function of the Cys-loop ligand-gated ion channel superfamily ... 23
- Determinants of agonist specificity ........................................................................ 24
- Determinants of ion selectivity ................................................................................ 25

Serotonin Reuptake Transporters ........................................................................... 27

- *in vivo* SERT function ......................................................................................... 30
- SERTs in Human Disease ....................................................................................... 31

Concluding Remarks .................................................................................................. 32

Acknowledgments ........................................................................................................ 32

References ................................................................................................................... 33

- Table 1. Mammalian Serotonin Receptor Subtype Localization and Function... 50

Chapter 2: *C. elegans* Locomotory Rate is Modulated by the Environment through a Dopaminergic Pathway and by Experience through a Serotonergic Pathway ........................................................................................................ 52

- Summary .................................................................................................................. 53

- Introduction ............................................................................................................. 54

- Results ....................................................................................................................... 56

- Locomotory Rate Decreases in the Presence of Bacteria ...................................... 56
- Food-deprived Animals Show More Pronounced Slowing in Response to Bacteria ........................................................................................................ 56
- The Basal Slowing Response Requires Dopamine ................................................ 57
- The Enhanced Slowing Response Requires Serotonin .......................................... 58
Exogenous Dopamine Restores the Basal Slowing Response of *cat-2* and *bas-1; cat-4* Mutants .......................................................... 59

The CEP, ADE, and PDE Dopaminergic Neurons Sense Mechanosensory Stimuli Required for the Basal Slowing Response ........................................... 60

Exogenous Serotonin Restores the Enhanced Slowing Response of *bas-1; cat-4* Mutants ................................................................................. 61

The Serotonergic NSMs Are Required for a Full Enhanced Slowing Response ................................................................................................. 62

The Enhanced Slowing Response Is Not Triggered by Mechanosensory Stimuli that Trigger the Basal Slowing Response ........................................ 64

Serotonin Antagonists Blocked the Enhanced Slowing Response .......... 64

Fluoxetine Potentiates the Enhanced Slowing Response ......................... 65

Food Deprivation Does Not Lead to Serotonin Hypersensitivity .............. 66

Isolation of Modulation-Defective Mutants ............................................. 66

**Discussion** .......................................................................................... 67

The Basal Slowing Response Involves a Mechanosensory Stimulus Mediated by Dopamine ................................................................. 68

The Enhanced Slowing Response Is Modulated by Serotonin and Is Distinct from the Basal Slowing Response ...................................................... 69

The Food-deprivation Signal Is Likely to Act Upstream of the Serotonin-responsi Service Circuit ................................................................. 70

Adaptive Significance of the Basal and Enhanced Slowing Responses .... 71

**Experimental Procedures** .................................................................. 72

Strains and Strain Constructions ............................................................ 72

Locomotory Rate Assays ........................................................................ 73

Laser Ablations ....................................................................................... 75

Neurotransmitter and Drug Pre-treatment .............................................. 76

Mutant Screen ....................................................................................... 77

**Acknowledgments** ............................................................................ 77

**References** .......................................................................................... 78

*Figures* .................................................................................................. 85

Figure 1. Modulation of Locomotory Rate of Wild-type Animals and Dopamine-and/or Serotonin-deficient Mutants ................................................ 85

Figure 2. Dopamine Pre-treatment Rescues the Defect in the Basal Slowing Response of Dopamine-deficient Mutants and Serotonin Pretreatment
Rescues the Defect in the Enhanced Slowing Response of Serotonin-
deficient Mutants .............................................................................. 87
Figure 3. Dopaminergic Neurons Function Redundantly to Sense
Mechanosensory Stimuli ..................................................................... 89
Figure 4. The Basal Slowing Response Involves a Mechanosensory
Stimulus, while the Enhanced Slowing Response Does Not............. 91
Figure 5. The Enhanced Slowing Response Involves Serotonin, Is
Potentiated by Fluoxetine, and Is Not a Result of Serotonin Hypersensitivity
.............................................................................................................. 93
Figure 6. Mutants Defective in the Enhanced Slowing Response....... 95
Figure 7. Models for the Basal and Enhanced Slowing Responses...... 97

Chapter 3: MOD-1 is a serotonin-gated chloride channel that modulates
C. elegans locomotory behaviour .......................................................... 99

Summary ............................................................................................. 100
Results and Discussion ....................................................................... 101

Methods .............................................................................................. 106
Mapping and cloning of mod-1 ............................................................ 106
Behavioral assays ................................................................................. 106
Electrophysiological studies of MOD-1 ................................................. 106

Acknowledgements ............................................................................. 108

References .......................................................................................... 109

Supplementary Information .................................................................. 112
A281V may block channel pore ........................................................... 112
mod-1 deletion mutants ....................................................................... 112

Methods .............................................................................................. 112
Mapping and cloning of mod-1 ............................................................ 112

References .......................................................................................... 113

Figures .................................................................................................. 114
Figure 1. Phenotypic characterization and cloning of mod-1 ............... 114
Figure 2. Sequence analysis of mod-1 .................................................. 116
Figure 3. mod-1 deletion alleles caused 5-HT resistance and defects in the
enhanced slowing response ................................................................. 118
Figure 4. MOD-1 is a 5-HT-gated ion channel distinct from the 5-HT3a
channel ............................................................................................... 120
Figure 5. MOD-1 is a 5-HT-gated chloride channel ................................ 122
Chapter 4: Mutations in the C. elegans Serotonin Reuptake Transporter MOD-5 Reveal Serotonin-Dependent and -Independent Activities of Fluoxetine .... 124

Summary ........................................................................................................................................... 125

Introduction ....................................................................................................................................... 126

Results ................................................................................................................................................. 127

Isolation of Mutants Defective in 5-HT Uptake .............................................................................. 127
5-HT-Uptake Mutants Exhibit a Hyperenhanced Slowing Response .................................... 128
Ablation of the Serotonergic NSMs or Decrease in Endogenous 5-HT Suppresses mod-5 Mutations ................................................................. 129
mod-5 Mutants Are Hypersensitive to Exogenous 5-HT ........................................................... 130
MOD-5 Is Similar to SERTs .............................................................................................................. 130
mod-5(n3314) Is a Null Allele ......................................................................................................... 131
MOD-5 Functions as a SERT in Mammalian Cells .................................................................... 132
MOD-5 Is Likely the Only SERT in C. elegans .............................................................................. 134
mod-5 Interacts Genetically with mod-1 and goa-1 .................................................................... 135
Fluoxetine Blocks 5-HT Uptake in vivo ......................................................................................... 136
Fluoxetine Is Likely the Only SERT in C. elegans .................................................................... 134
mod-5 Interacts Genetically with mod-1 and goa-1 .................................................................... 135
mod-5(n3314) Is a Null Allele ......................................................................................................... 131
MOD-5 Functions as a SERT in Mammalian Cells .................................................................... 132
MOD-5 Is Likely the Only SERT in C. elegans .............................................................................. 134
mod-5 Interacts Genetically with mod-1 and goa-1 .................................................................... 135
Fluoxetine Blocks 5-HT Uptake in vivo ......................................................................................... 136
Fluoxetine Induces Nose Contraction and Paralysis in mod-5 and tph-1 Mutants ................ 138
Fluoxetine Stimulates Egg Laying in mod-5 and tph-1 Mutants .................................................. 140

Discussion .......................................................................................................................................... 141

MOD-5 Probably Acts Upstream of MOD-1 and GOA-1 ............................................................ 141
Fluoxetine-Induced Egg Laying Is Only Partially 5-HT-Dependent ........................................... 142
Potentiation of the Enhanced Slowing Response by Fluoxetine Requires MOD-5 CeSERT and 5-HT ................................................................. 137
Fluoxetine Induces Nose Contraction and Paralysis in mod-5 and tph-1 Mutants ................ 138
Fluoxetine Stimulates Egg Laying in mod-5 and tph-1 Mutants .................................................. 140

Acknowledgments .............................................................................................................................. 144

Experimental Procedures ................................................................................................................. 145
mod-5 Mapping, Cloning, and cDNA .............................................................................................. 145
Laser Microsurgery ............................................................................................................................ 146
Neurotransmitter and Drug Pretreatment and Behavioral Assays ............................................ 146
5-HT-uptake Assays in vivo .............................................................................................................. 147
MOD-5-mediated Uptake in Mammalian Cells .......................................................................... 147

References ......................................................................................................................................... 149

Tables ................................................................................................................................................ 156

Table 1. Fluoxetine phenocopies mod-5 in 5-HT uptake assays in vivo ... 156
Figures ........................................................................................................... 157
Figure 1. The NSMs of mod-5 Mutants Are Defective in 5-HT Uptake...... 157
Figure 2. Phenotypic Characterization and Cloning of mod-5................... 159
Figure 3. mod-5 Encodes a Protein Similar to the Human and Drosophila 5-HT Reuptake Transporters ................................................................. 161
Figure 4. Physiological Characterization of MOD-5 CeSERT .................. 163
Figure 5. mod-5 Genetic Interactions and 5-HT- and MOD-5 CeSERT-Dependence of the Potentiating Effect of Fluoxetine ................................ 165
Figure 6. 5-HT- and MOD-5 CeSERT-independence of Fluoxetine-induced Egg Laying.............................................................................................. 167
Figure 7. mod-5(n3314) mutants, but Not tph-1 Mutants, Are Hypensensitive to Stimulation of Egg Laying by 5-HT................................. 169
Figure 8. Models for the Effects of Fluoxetine on C. elegans Behaviors... 171

Chapter 5: Supplementary Data for Chapters 2, 3, and 4 .................................. 173

Results Pertaining to Chapter 2 ................................................................. 174
Serotonin-Pretreated Food-Deprived bas-1; cat-4 Mutants Exhibit Normal Locomotion in the Absence of Bacteria................................................................. 174
The Enhanced Slowing Response of cat-2 mutants Is Potentiated by Fluoxetine ........................................................................................................... 174
Food Deprivation Does Not Lead to a Change in the Kinetics of Serotonin Sensitivity................................................................................................. 175
The Enhanced Slowing Response Does Not Depend on the Presence of Fructose During Food-deprivation................................................................. 175

Results Pertaining to Chapter 3 ................................................................. 176
High Concentrations of Dopamine, Octopamine, and Tyramine Activate MOD-1 ........................................................................................................ 176
Ondansetron Does Not Block 5-HT-Gated MOD-1 Currents....................... 176
Mianserin and Methiothepin Block 5-HT-Gated MOD-1 Currents .......... 176
MOD-1 Channel Pore Is Similar to that of GABA- and Glycine-Gated Chloride Channels .............................................................................................. 177
mod-1::gfp Transgene Generates Functional MOD-1 Protein................. 178

Results Pertaining to Chapter 4 ................................................................. 178
mod-5 Transgenes Render Animals Serotonin-Resistant.......................... 178
mod-5 Complementation Tests................................................................. 179
MOD-5-Mediated [3H]5-HT-Uptake Is not Inhibited by Other Neurotransmitters................................................................................................. 179
Potentiation of The Hyperenhanced Slowing Response of mod-5 Mutants Cannot be Assayed

Acknowledgments

Figures

Figure 1. 5-HT-Pretreated Food-deprived bas-1; cat-4 Mutants Exhibit Normal Locomotory Rate in the Absence of Bacteria
Figure 2. Fluoxetine Potentiates the Enhanced Slowing Response of cat-2 Mutants
Figure 3. Food-deprived Animals Are Not Hypersensitive to Serotonin
Figure 4. The Enhanced Slowing Response Is Not Dependent on High Osmolarity Ring on Food-Deprivation Plates
Figure 5. MOD-1 Channel is Responsive to High Concentrations of Dopamine, Octopamine, and Tyramine
Figure 6. The 5-HT₃α-Specific Blocker Ondansetron Did Not Block MOD-1
Figure 7. Mianserin and Methiothepin Block the MOD-1 Channel in Xenopus Oocytes and in vivo
Figure 8. Anion Selectivity of the MOD-1 channel
Figure 9. mod-1::gfp Transgene Rescues the Serotonin Resistance of mod-1(n3034) Mutants
Figure 10. Rescue of the Serotonin Hypersensitivity of mod-5(n823) Mutants
Figure 11. A mod-5 Minigene Rescues the Serotonin Hypersensitivity of mod-5(n3314) Mutants
Figure 12. mod-5 Complementation Tests
Figure 13. Antagonism of MOD-5 CeSERT-Mediated 5-HT Uptake
Figure 14. The Effect of Fluoxetine on the Enhanced Slowing Response of mod-5(n3314) Mutants Cannot be Measured

Chapter 6: Preliminary Results and Future Directions

Preliminary Results

Pharmacological Profile of mod Mutants
mod-6(n3076) Is a Special mod Mutant
Octopamine and the mod Mutants
A Genetic Screen forSuppressors of mod-5(n3314)
The Neural Circuit for the Enhanced Slowing Response

Future Directions
Chapter 1

Serothonin in Behavior
Introduction

Behavior is the output that ultimately characterizes an individual, be it a worm or a human. From a reductionist viewpoint, this output is the end product of two inputs, genes and the environment. The intimate interplay between these two inputs is the basis for a worm's backing movement and a person's mood. In humans, the impact of the environment is most relevant to the process of learning and memory. Elucidating the mechanisms of learning and memory at the level of molecules, cells, organisms, and particular circumstances is central to our understanding of both normal behavior as well as disorders of the mind.

The manifestations of learning and memory vary enormously from non-associative forms of learning such as habituation and sensitization of simple reflexes to extremely complex processes such as explicit or declarative long-term memory that allows for the recall of facts and events from decades past. Nevertheless, there appears to be remarkable conservation in the fundamental mechanisms that mediate this vast range of outputs from neural circuits. It is this conservation that validates the study of such higher-order processes in model organisms, where we may manipulate the input parameters of both genes and environment and record the output.

This dissertation focuses on the analysis of behavior in the nematode *Caenorhabditis elegans*. Specifically, we describe that *C. elegans* hermaphrodites are capable of a behavior with features similar to processes of learning in other organisms. This behavior involves one aspect of the response of the animal to the presence of food, a lawn of the bacterium *Escherichia coli*. If a well-fed animal is transferred to an environment where it encounters food, it slightly slows its locomotory rate, a response that we have termed "the basal slowing response." Additionally, if the animal is deprived of food for 30 minutes prior to the transfer, the animal slows down dramatically when it encounters food. We have termed this response of food-deprived animals "the enhanced slowing response." Although this behavioral paradigm was established primarily by prior work (Sawin, 1996), I helped complete the initial characterization, and hence a detailed description of this behavior will be presented in Chapter 2. For the purposes of this introductory chapter, the reader need only know that the enhanced slowing response is mediated by the neurotransmitter serotonin and is distinct from the basal slowing response that is mediated by the neurotransmitter dopamine.

The central finding of this dissertation is that the enhanced slowing response, an experience-dependent modulatory behavior in *C. elegans*, requires a novel type of serotonin receptor — a serotonin-gated chloride channel — and a serotonin reuptake
transporter. In this chapter, I will begin by discussing the role of serotonin in learning and memory in the sea slug *Aplysia californica* and then discuss what is already known about serotonin function in *C. elegans*. I will then dedicate a substantial portion of this chapter to a discussion of three classes of molecules relevant to the central findings of this dissertation: serotonin receptors, chloride channels, and serotonin reuptake transporters.

**Serotonin Synthesis, Metabolism, and Expression**

Serotonin was first discovered in the circulation system of vertebrates as a potent regulator of smooth muscle organs and was thought to act primarily as a hormone. Subsequently, serotonin was found in the central nervous systems of many vertebrates and invertebrates, where it acts as a neurotransmitter (see special supplement issue of Neuropsychopharmacology 21 (2S), 1999 for a historical overview on serotonin biology). This chapter will concern itself primarily with the neuronal functions of serotonin.

Serotonin is synthesized in a two-step process: The amino acid tryptophan is first hydroxylated by a specific tryptophan hydroxylase to the intermediate 5-hydroxytryptophan and then decarboxylated by a less-specific aromatic amino acid decarboxylase to generate 5-hydroxytryptamine (5-HT; serotonin) (Cooper et al., 1996). Degradation of serotonin occurs via the action of the enzyme monoamine oxidase found within either the cells that release serotonin or nearby cells (Cooper et al., 1996). For this metabolism to take place, the serotonin must be cleared from the synaptic cleft by Na⁺/Cl⁻-dependent reuptake transporters (Cooper et al., 1996).

Serotonergic cell bodies in the mammalian brain are restricted to clusters of cells mainly in the raphe (or midline) regions of the brainstem (Jacobs and Fornal, 1999). The cells can be divided into two major groups. The rostral group contains the nucleus centralis superior and the dorsal raphe nucleus and provides serotonin to the forebrain while the caudal group supplies serotonin to the spinal cord (Jacobs and Fornal, 1999). Despite this very small localized cluster of cell bodies, the serotonergic neurons appear to innervate extensive and far-reaching areas of the brain including the hippocampus, amygdala, medulla, cerebellum, and the cerebral cortex (Martin, 1996).
Serotonin in Memory and Learning

The best studied experimental model for the role of serotonin in learning is the gill-withdrawal paradigm in Aplysia. In response to a tactile stimulus, sensory neurons that innervate the siphon of the animal generate excitatory post-synaptic potentials in interneurons and motor cells that then lead to the withdrawal of the gill (Castellucci et al., 1970). If numerous such stimuli are applied repeatedly, the withdrawal response will lessen, or habituate (Castellucci et al., 1970). This process of habituation is mediated primarily by a decrease in the production of excitatory output by the sensory neurons (Castellucci and Kandel, 1974). Massed training, in which such repeated stimuli are presented without rest between training sessions, will result in short-term habituation, lasting for only a few minutes. Spaced training, in which the repetitive stimuli are appropriately spaced over time, can produce long-term habituation lasting several days to few weeks. Long-term habituation is characterized by a "pruning" of synaptic connections such that sensory and interneurons come to have fewer than normal presynaptic terminals (Bailey and Chen, 1983). Serotonin does not play a role in either short- or long-term habituation.

If the animal is challenged with a noxious stimulus, the reflexive defensive response to the tactile stimulus is heightened, or sensitized. A shock to the tail sensitizes the gill-withdrawal reflex (Castellucci and Kandel, 1974). Just as with habituation, sensitization can be either short- or long-term (Pinsker et al., 1973). While a single shock results in heightened responses for only five minutes, repeated shocks can result in sensitization that lasts days to weeks. The interneurons that perceive the tail shock make axo-axonal (axon-to-axon) connections with the sensory neurons that mediate the gill-withdrawal reflex (Hawkins and Schacher, 1989). These interneurons release serotonin that enhances neurotransmitter release from the sensory neurons. External application of serotonin to the axonal terminal of the sensory neuron mimics the shock to the tail in its ability to sensitize the gill-withdrawal reflex (Bernier et al., 1982). In short-term sensitization, serotonin that is released acts via two different G protein-coupled serotonin receptors, one coupled to adenylyl cyclase (Bernier et al., 1982) and the other to phospholipase C (PLC) (Byrne et al., 1991). The adenylyl cyclase generates cAMP, thereby activating protein kinase A (PKA). PKA enhances neurotransmitter release in two ways: direct activation of presynaptic vesicles (Byrne et al., 1991) and amplification of neurotransmitter release via an increase in Ca$^{2+}$ influx (Eliot et al., 1993). The Ca$^{2+}$ influx is the indirect result of prolonged action potentials caused by the phosphorylation and subsequent closure of a K$^+$ channel by PKA (Klein...
et al., 1982) and to a lesser extent by activation of L-type Ca$^{2+}$ channels by PKA phosphorylation (Braha et al., 1993). The activation of the second receptor coupled to PLC stimulates diacylglycerol to activate protein kinase C (Byrne et al., 1991), which then directly activates vesicles and also phosphorylates L-type Ca$^{2+}$ channels (Braha et al., 1993). There are indications that the PKC-mediated phosphorylation of L-type Ca$^{2+}$ channels might have slower kinetics than the PKA-mediated pathway (Braha et al., 1993). However, the physiological relevance of these differential kinetics has not been determined.

Long-term sensitization also uses the same serotonin-signaling pathway but requires protein synthesis (Schacher et al., 1988). In this case, the repeated activation of adenylyl cyclase leads to persistence in the activity of PKA. PKA recruits the mitogen-activated protein kinase (MAPK) and they both translocate to the nucleus of the neuron. Activity of both kinases is required for long-term sensitization to occur. PKA activates cAMP response element binding (CREB-1) protein (Kaang et al., 1993) and MAPK inactivates CREB-2, an inhibitor of CREB-1. CREB-1 binds to cAMP response element (CRE) sites and activates gene expression, but inactivation of CREB-2 is a requisite step for CREB-1 to function as a transcriptional activator (Bartsch et al., 1995). One of the early genes thus activated is an ubiquitin hydrolase that targets the regulatory inhibitory subunit of PKA for degradation (Hegde et al., 1997; Chain et al., 1999). Loss of this inhibitory subunit renders PKA autonomous, i.e., it no longer requires activation by cAMP. Another early gene activated by CREB-1 is the transcriptional activator C/EBP, which binds to CCAAT elements and activates genes required for the structural changes accompanying long-term sensitization (Alberini et al., 1994). Specifically, in contrast to long-term habituation, neurons display an increase in the number of synaptic connections in long-term sensitization, often via an increase in the area of the axonal terminal and the production of additional axonal branches (Bailey and Chen, 1988). There is also a concomitant increase in the arborization of the dendrites of postsynaptic neurons to accommodate the additional inputs (Kandel et al., 2000).

The gill-withdrawal reflex can also undergo classical conditioning (Carew et al., 1981). Classical conditioning requires the association of two types of stimuli. After a training period, that involves providing the unconditioned stimulus (the shock to the tail) immediately after the conditioned stimulus (the stimulation of the siphon), a large increase in the postsynaptic response is observed (Carew et al., 1981). There are two components to the increase, one presynaptic (Hawkins et al., 1983) and the other postsynaptic (Murphy and Glanzman, 1997). The presynaptic component involves the influx of Ca$^{2+}$ into the presynaptic sensory neuron in response to activation of the
conditioned stimulus pathway, thereby activating the Ca\textsuperscript{2+}-binding protein calmodulin. Calmodulin binds to adenylyl cyclase and potentiates its ability to respond to serotonin from the unconditioned stimulus pathway, thus producing more cAMP than normal (Eliot et al., 1989). Increase in cAMP levels then results in enhanced activation of PKA leading to increased neurotransmitter release by the process described above. The postsynaptic component involves a retrograde signal from the motor neuron to the sensory neuron, enhancing transmitter release even further (Bao et al., 1998). The motor neuron has two types of receptors that respond to the neurotransmitter glutamate released by the sensory neurons: N-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors (Kandel et al., 2000). The NMDA receptor is usually blocked by Mg\textsuperscript{2+} at resting membrane potentials while the non-NMDA receptors are not (Mayer et al., 1984). However, the appropriate pairing of the conditioned and unconditioned stimuli results in a train of action potentials in the motor neuron, expelling the Mg\textsuperscript{2+} from the mouth of the NMDA receptor and allowing Ca\textsuperscript{2+} to flow into the cell. This influx of Ca\textsuperscript{2+} has been implicated in the generation of the retrograde signal (Bao et al., 1998).

This dissertation describes a behavior in *C. elegans* where the same stimulus leads to two different responses, depending on whether or not the animal experienced a brief "training" regimen — 30 minutes of food-deprivation. There are two major differences between the enhanced slowing response paradigm in *C. elegans* and the sensitization and classical conditioning of the gill-withdrawal reflex in *Aplysia*. First, no explicit noxious stimulus is provided to elicit the enhanced slowing response. Rather, an attractive stimulus, namely food, is removed. Second, the basal and enhanced slowing responses use two different neural circuits in the animal (Chapter 2), while the classical conditioning of the monosynaptic pathway of the gill-withdrawal reflex in *Aplysia* is in part built upon the mechanisms utilized for sensitization. The enhanced slowing response does not involve two types of stimuli explicitly provided in close temporal juxtaposition. Therefore, we do not consider it a paradigm of classical conditioning. And to avoid confusion with the established definition of sensitization, we have chosen to not refer to the enhanced slowing response as a phenomenon of sensitization. Nevertheless, the enhanced slowing response involves the modulation of a response to the same stimulus after a specific experience and hence we refer to it as an experience-dependent modulatory behavior.
Serotonin in *C. elegans*

Serotonin was first detected in *C. elegans* (Horvitz et al., 1982), using the technique of formaldehyde-induced fluorescence (Sulston et al., 1975), in a single pair of neurons, the neurosecretory motor neurons (NSMs). Subsequently, by using anti-serotonin antisera, twelve additional serotonergic neurons were identified (Desai et al., 1988; Loer and Kenyon, 1993; and Chapter 2). The presence of serotonin in neurons in *C. elegans* suggested that serotonin is likely to function as a neurotransmitter in *C. elegans*. However, the NSMs by ultrastructure appear to be neurosecretory (Albertson and Thomson, 1975). Therefore, it is possible that serotonin released by the NSMs diffuses throughout the animal and acts as a hormone.

Application of exogenous serotonin elicits specific behavioral responses in *C. elegans*: stimulation of egg laying, inhibition of locomotion, and stimulation of pharyngeal pumping (Horvitz et al., 1982). The G protein GOA-1 is involved in mediating the effects of exogenous serotonin on egg laying and locomotion (Mendel et al., 1995; Segalat et al., 1995). Long-term exposure to serotonin leads to adaptation of the egg laying response; animals exposed to serotonin overnight no longer lay eggs in response to a fresh dose of serotonin (Schafer and Kenyon, 1995). A voltage-gated calcium channel, UNC-2, is required for this adaptation response (Schafer and Kenyon, 1995). It has been proposed that serotonin controls a switch between the on and off states of egg-laying behavior, in effect priming the egg-laying apparatus for the reception of stimulatory input (Waggoner et al., 1998).

Three genetic mutations exist that affect the levels of serotonin by perturbing its biosynthesis: *bas-1* (biogenic amine synthesis defective) (Loer and Kenyon, 1993), *cat-4* (catecholamine defective) (Desai and Horvitz, 1989; Weinshenker et al., 1995), and *tph-1* (tryptophan hydroxylase) (Sze et al., 2000). *cat-1* mutants also have reduced levels of serotonin expression, but this is likely not the result of defective serotonin biosynthesis but rather is caused by a defect in the loading of presynaptic vesicles with serotonin, since *cat-1* encodes a vesicle monoamine transporter (Duerr et al., 1999). The *cat-1* phenotype also suggests that extravesicular serotonin is rapidly degraded by monoamine oxidase.

Serotonin function in *C. elegans* has also been explored using drugs thought to interface with serotonin signaling, such as serotonin receptor antagonists and serotonin reuptake inhibitors. Treating animals with the serotonin antagonist gramine inhibits pharyngeal pumping, while the serotonin reuptake inhibitor imipramine stimulates pharyngeal pumping (Avery and Horvitz, 1990). Animals treated with the serotonin
receptor antagonist methiothepin and mutants defective in serotonin signaling are hypersensitive to the acetylcholinesterase inhibitor aldicarb (Nurrish et al., 1999). Increased sensitivity to aldicarb is indicative of higher than normal levels of acetylcholine release, suggesting that the normal function of serotonin is to inhibit acetylcholine release.

Inhibitors of serotonin reuptake also have various effects on C. elegans behavior. These effects are examined in greater detail in the context of the enhanced slowing response in Chapters 2 and 4 and in the context of egg-laying behavior in Chapter 4.

Serotonin Is Involved in the Responses of C. elegans to Food

Presence of food modulates several C. elegans behaviors: pharyngeal pumping (Avery and Horvitz, 1990), egg laying (Horvitz et al., 1982), and defecation (Liu and Thomas, 1994) are stimulated, but locomotion is inhibited (See Chapter 2). As mentioned above, exogenous serotonin has the same effect on these behaviors as does the sensation of food.

The effect of withdrawal from food on the olfactory ability of C. elegans and the ability of serotonin to mimic the presence of food has been described (Colbert and Bargmann, 1997). They assayed the ability of animals to chemotax toward benzaldehyde, as measured by the chemotaxis index (Bargmann and Horvitz, 1991). Chemotaxis towards benzaldehyde exhibits adaptation, i.e., after at least 60 minutes of prior exposure to a field of uniform benzaldehyde concentration, animals showed reduced chemotaxis towards a spot source of benzaldehyde indicating that the animals find the odorant less attractive (Colbert and Bargmann, 1995). If the animals have been starved for 60 minutes or longer prior to the adaptation regimen, the chemotaxis index is reduced even further (Colbert and Bargmann, 1997). In other words, starvation enhances adaptation. Normal levels of adaptation are observed if the animals are starved on plates containing serotonin, i.e., there is no longer any enhancement of adaptation (Colbert and Bargmann, 1997). Put simply, serotonin can mimic the presence of food; serotonin-treated starved animals behave as if they are well fed. By contrast, in the experiments presented in chapter 2, we did not observe a block of the enhanced slowing response when wild-type animals were deprived of food in the presence of serotonin. I believe that this apparent contradiction is rooted in the differences between the food-deprivation protocols used in this dissertation and the starvation protocol used by Colbert and Bargmann (1997). In Appendix 2, I describe these differences, propose a model that might account for these differences, and
suggest further experiments to explore the role of serotonin in the response of *C. elegans* to food.

*tph-1* mutants, which completely lack serotonin (Sze et al., 2000; and Chapter 4) have several defects in metabolic signaling: they accumulate abnormal stores of fat, have extended life spans, and exhibit the tendency to inappropriately enter the metabolically-inactive dauer stage (Sze et al., 2000). In addition, *tph-1* mutants also have reduced pharyngeal and egg laying rates (Sze et al., 2000) and have sluggish locomotion (Chapter 4). These observations suggest that *C. elegans* might use serotonin as a hormonal signal to generate a body-wide coordinated response to food availability. In other words, when food is abundant, serotonin levels in the animal are normal, the animal moves and forages actively, lays many eggs, and does not accumulate fat or enter the dauer state. In times of dearth, serotonin levels decrease and this allows the animal to generate an appropriate response on several fronts: feeding, locomotion, reproduction, and energy storage and use.

By contrast, acute food-deprivation, lasting only 30 minutes, is enough to elicit the enhanced slowing response but is not sufficient to generate the various changes seen in *tph-1* mutants (Chapter 2). Chapters 2-4 present evidence that serotonin signaling is required for the enhanced slowing response and that this signaling is likely to be neuronal. In Appendix 2, I discuss neuronal versus hormonal functions of serotonin in *C. elegans* with respect to food sensation. However, it has not been shown that the response to chronic food-deprivation does not require neuronal serotonin signaling, nor have we demonstrated in this dissertation that the enhanced slowing response does not involve hormonal effects of serotonin.

In this dissertation, I do not discuss the behavior of food-deprived *C. elegans* as a model for hunger and satiety as I feel that these concepts describe an internal state not easily accessible to evaluation by an external observer.

### Mammalian Serotonin Receptors

Since this dissertation describes the cloning of a new type of serotonin receptor (Chapter 2), I review here the various subtypes of serotonin receptors that have been previously characterized. There are seven major classes of vertebrate serotonin receptors, termed 5-HT₁ through 5-HT₇ (Hoyer and Martin, 1997). These seven classes can be grouped by their mechanisms of signaling: all of these classes except 5-HT₃ are metabotropic receptors, belonging to the superfamily of receptors with seven transmembrane regions coupled to G proteins; the 5-HT₃ receptor is a non-selective
cation channel belonging to the Cys-loop superfamily of ligand-gated ion channels. The second messenger cascades triggered by the metabotropic receptors include inhibition of adenylyl cyclase (5-HT₃), activation of phospholipase C and subsequent phosphatidylinositol turnover (5-HT₂), and activation of adenylyl cyclase (5-HT₄, 5-HT₆, 5-HT₇) (Hoyer and Martin, 1997). The coupling mechanisms for 5-HT₅ receptors are unknown.

The 5-HT₁ and 5-HT₂ classes have five (A, B, D, E, and F) and three (A, B, and C) subfamilies, respectively, while the 5-HT₃ and 5-HT₅ classes each have two (A and B) subfamilies (Hoyer and Martin, 1997). The 5-HT₄, 5-HT₆, and 5-HT₇ classes each have only one subtype (Baez et al., 1995), although 5-HT₄ may be present in two alternatively spliced forms, short (5-HT₄ₛ) and long (5-HT₄₇) (Gerald et al., 1995).

Table 1 summarizes the expression profiles of these serotonin receptor subtypes, the predicted function of the brain regions that the subtypes are found in, and the phenotype of available knockouts. In addition, I indicate below several additional properties of some of these receptor subtypes (refer to Table 1 for this section).

5-HT₁A receptors in the raphe nuclei are on the soma and dendrites of serotonergic neurons, where they are likely to function as autoreceptors that inhibit the firing of these serotonergic neurons. Desensitization of these 5-HT₁A autoreceptors may mediate the ability of chronic exposure to selective serotonin reuptake inhibitors (SSRIs) to raise levels of serotonin in the synaptic cleft (also see section below on serotonin reuptake transporters).

5-HT₁A knockout mice are less reactive, more anxious, and less aggressive than the wild type while 5-HT₁B knockout mice are more reactive, less anxious, and more aggressive (Zhuang et al., 1999; also see Table 1). Contradictory studies of the 5-HT₁B knockout mice argue for and against the 5-HT₁B receptor mediating the rate of alcohol consumption (Crabbe et al., 1996; Risinger et al., 1999). 5-HT₁B receptors may also act as inhibitory autoreceptors, but in a manner distinct from 5-HT₁A, since 5-HT₁B receptors are found at the nerve terminals of serotonergic neurons where they inhibit the synthesis and release of serotonin (Ghavami et al., 1999).

Stimulation of 5-HT₂A may mediate the effects of hallucinogens such as lysergic acid diethylamide (LSD) (Krebs-Thomson et al., 1998).

The 5-HT₃B subunit is a non-conducting subunit that modulates the properties of the 5-HT₃A channel (Davies et al., 1999). Stimulation of the 5-HT₃A channel can cause nausea and vomiting, induce vasodilation, and cause sensitization of nociceptive receptors. The 5-HT₃A-specific antagonists granisetron and ondansetron are potent antiemetics (Perez et al., 1998) that have been extremely helpful in the clinic in preventing the nauseous side-effects of chemotherapy.
5-HT$_{5A}$ receptors appear to be present mainly in glial cells in one study that uses a 5-HT$_{5A}$-specific antibody (Carson et al., 1996), while agonist binding studies label several neuronal areas of the brain not labeled in 5-HT$_{5A}$-knockout mice (Waeber et al., 1998). While immunoreactivity data are usually considered more reliable than data obtained by agonist binding, the knockout control for the agonist studies suggests that the antibody may not be sufficiently sensitive. Alternatively, the loss of 5-HT$_{5A}$ perturbs other targets that also bind the agonist.

Much of the expression data in Table 1 are derived from either in situ hybridization experiments and/or radiolabelled agonist binding data. Studies using specific antibodies are rare. It is important to note that nearly all of the inferences about the specificity of serotonin receptor function are based on the actions of various agonists and antagonists. The agonist and antagonist profiles of these receptors are usually derived from expression of the cloned receptors in heterologous systems or recordings from tissue-slice preparations and they may not reflect the in vivo situation. Therefore, while these functional generalizations may be correct in some cases, definitive evidence in each case will require the generation of animals with knockouts of these receptor genes, preferably spatially-restricted (McHugh et al., 1996; Tsien et al., 1996) knockouts, as well as temporally- and spatially-inducible (Mayford et al., 1996) and reversible (Mansuy et al., 1998) rescue of such knockouts. In fact, the 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor knockouts described in Table 1 were generated by disrupting the endogenous genes with a "knock-in" of a neomycin-containing cassette with a translational stop codon (Zhuang et al., 1999). This cassette was flanked by loxP sites (Tsien et al., 1996), and tissue-specific rescue of receptor expression was achieved for both 5-HT$_{1A}$ and 5-HT$_{1B}$ by expressing the Cre recombinase under the control of specific promoters (Zhuang et al., 1999). Behavioral analysis of these mice is in progress (Zhuang et al., 1999). These and other similar tissue-specific rescue experiments with knockout animals should allow the determination of brain regions necessary for the contribution of a particular receptor to a given behavior.

These recent studies notwithstanding, the current state of expression and functional data suggests that multiple cells could express one or more type of serotonin receptor, coupled to similar or different second messenger signaling mechanisms, leading to a hopelessly complicated array of possibilities for how behavioral responses are mediated by serotonin in the mammalian brain. In my opinion, the current reductionist approaches of molecular neuroscience cannot on their own clarify the picture completely. What is needed is an integrative approach that includes in vivo functional imaging of neural circuits with the ability to detect temporal and spatial
changes in the activity of the various signaling pathways. Promising advances such as PET imaging of receptor localization (Drevets et al., 1999; Meltzer et al., 1999) lend hope to the future possibility of a coherent picture of serotonin receptor expression and function.

**C. elegans Serotonin Receptors**

The *C. elegans* genomic sequence contains at least 18 receptors that show similarity to the class A amine receptors of the rhodopsin-like receptor family (Bargmann, 1998). However, in the absence of functional characterization in heterologous systems, these could well be receptors for acetylcholine, dopamine, tyramine, histamine, or octopamine. Nevertheless, a systematic sequence comparison seeking receptors more homologous to vertebrate and invertebrate serotonin receptors than to receptors activated by the other neurotransmitters has been performed and has identified 11 candidate metabotropic serotonin receptor homologs in *C. elegans* (T. Niacaris and L. Avery, personal communication; and my unpublished observations). Two of these respond to serotonin in heterologous expression systems and are tentatively classified as 5-HT$_{1A}$ (Olde and McCombie, 1997) and 5-HT$_{2}$ receptors (Hamdan et al., 1999) based on sequence and pharmacology. In addition, there are two genes with good homology to mammalian 5-HT$_{3}$ ionotropic receptors. If even a subset of these receptors prove to be responsive to serotonin, there is likely to be a complicated pattern of receptor expression and function even within the context of the mere 302 neurons of the *C. elegans* nervous system (White et al., 1986).

Recent advances in the ability to isolate *C. elegans* mutants carrying deletions in a given gene (Jansen et al., 1997; Liu et al., 1999) can now be coupled with cell-specific rescue of these receptor knockouts. The number of available cell-specific promoters is growing rapidly. These techniques should greatly facilitate the analysis of serotonergic neurotransmission within the context of the completely defined neural circuit of *C. elegans* (White et al., 1986).

**Chloride Channels**

The novel type of serotonin receptor (MOD-1) described in Chapter 2 is a serotonin-gated chloride channel. Therefore, I present here a discussion of the general properties of chloride channels followed by an analysis of determinants of the two properties of
MOD-1 that have never before been observed in a single channel — ligand specificity for serotonin and ion selectivity for chloride.

There are four major classes of chloride channels. The first three are mentioned here only briefly as they are not directly relevant to neuronal function. See Kozlowski (1999) for review.

1) CIC: These channels have 12 transmembrane regions, are expressed ubiquitously, and have roles in maintenance of cell volume and stabilization of skeletal muscle membrane potential. There are 7 subtypes within this family.

2) CFTR: There is only one member in this family — the product of the cystic fibrosis gene — and it is expressed in the heart and nearly all types of epithelial cells and is involved in transepithelial transport.

3) Ca\textsuperscript{2+} activated: These channels are activated by increases in intracellular Ca\textsuperscript{2+} concentrations typically triggered by neurotransmitters signaling through second messenger systems. These channels are expressed in smooth muscles and play a role in neurotransmitter-mediated smooth muscle contraction.

4) GABA/glycine: These are ligand-gated ion channels that belong to the same Cys-loop superfamily that includes the nicotinic acetylcholine receptor (nAChR) and 5-HT\textsubscript{3}. They are expressed exclusively in neuronal tissue, are gated by the neurotransmitters GABA or glycine, and are involved primarily in neuronal inhibition. These channels and the features that define them are discussed in more detail below.

**Structure-function of the Cys-loop ligand-gated ion channel superfamily**

The ligand-gated ion channels in the Cys-loop superfamily are all of very similar size (420-480 amino acids) and have the same deduced secondary structure and membrane topology — a large N-terminal extracellular domain, followed by three closely spaced transmembrane regions, followed by a large C-terminal globular domain, and ending with a fourth transmembrane domain and a very short extracellular tail. The N-terminal domains of these channels always have two cysteine residues separated by 13 amino acids that form the Cys-loop signature of this channel family (see Kozlowski, 1999 for review). This Cys-loop has been shown by mutagenesis studies to be involved in subunit assembly (Sumikawa and Gehle, 1992) and ligand binding (Rajendra et al., 1995; Gready et al., 1997).

The assembly of a functional channel in the membrane requires five subunits — this has been demonstrated for the GABA\textsubscript{A} (Nayeem et al., 1994) and glycine
(Langosch et al., 1988) chloride channels, and the nAChR cation channel (Cooper et al., 1991; Unwin, 1993; Karlin and Akabas, 1995).

For the nAChR, the second transmembrane region (M2) from each of the five subunits forms the lining of the pore of the channel (Unwin, 1993; Unwin, 1995). Numerous mutagenesis studies of M2 show involvement of this region in formation of a functional pore (reviewed in Karlin and Akabas, 1995). Similar mutagenesis studies have been conducted for the GABA\(_A\) (Tierney et al., 1996; Tierney et al., 1998) and glycine (Bormann et al., 1994) channels, and for the invertebrate glutamate-gated chloride channels (Etter et al., 1996; Etter et al., 1999). There is evidence that the amino-third of M1 contributes to the pore (Akabas and Karlin, 1995; Zhang and Karlin, 1997).

In Chapter 3, we describe the cloning of a novel serotonin-gated ionotropic receptor, a serotonin-gated chloride channel (MOD-1). MOD-1 is far more similar to GABA- and glycine-gated chloride channels than to 5-HT\(_3\) serotonin-gated non-selective cation channels (Chapter 3, Figure 2B). For this reason, I will now review the features important for a channel to be gated by serotonin and to conduct chloride ions.

**Determinants of agonist specificity**

The precise sequence and structural determinants for agonist specificity are poorly understood. Domain-swapping experiments with the nAChR and 5-HT\(_3\) receptors have shown that the N-terminal extracellular domain contains within it all the necessary determinants to bind acetylcholine (Eisele et al., 1993). By analogy, it is thought that the ligand-specificity of all channels in this family is contained in this N-terminal extracellular domain. As mentioned above, the Cys-loop has been implicated in ligand binding for some of the channels in this family (see above), while others do not appear to use the Cys-loop for ligand binding (Vandenberg et al., 1993). For the GABA\(_A\) receptor, several residues in the extra-cellular loop have been shown to be important for binding to GABA (Boileau et al., 1999; Westh-Hansen et al., 1999). Similarly, using alanine scan analysis, a single Arg residue in the N-terminal extracellular domain has been shown to be important for binding serotonin by 5-HT\(_3\) receptors (Yan et al., 1999). However, these few sequence motifs are not sufficient to allow the classification of members within this family according to their ligand specificities. The ligand that gates a particular channel must be determined empirically. For example, the MOD-1 channel (Chapter 2) is annotated in the *C. elegans* genome as a GABA-gated chloride channel, based on overall sequence homology (The *C. elegans* Sequencing Consortium, 1998).
Furthermore, MOD-1 has a Thr residue at the position corresponding to the Arg residue critical for serotonin binding in the 5-HT₃ receptor. But as we show in chapter 3, MOD-1 is gated by serotonin and not by GABA.

**Determinants of ion selectivity**

More precise information is available about the regions and specific residues that allow a channel to discriminate between anions and cations. While there are many studies that have mutated residues in the M2 region to determine the relevance of those residues to various intricacies of channel conductance, in my opinion there have been just two reports (Galzi et al., 1992; Keramidas et al., 2000) that address the selectivity question in a thorough manner. I will present the salient features from these two reports. (Figures 2B and 2C from Chapter 3 present the sequences relevant to the discussion below.)

Galzi et al. (1992) sought to convert the cation-selective nAChRα7 channel into an anion-selective channel. First, they tested a chimeric channel in which the segment linking M1 to M2 and a part or all of M2 of nAChRα7 was exchanged for the corresponding region from the glycine α1 chloride channel subunit. This exchange resulted in a channel that did not respond to acetylcholine. Then, instead of exchanging this entire stretch, they performed site-directed mutagenesis to convert six specific residues within this stretch to match the corresponding residues from the glycine α1 channel. In addition, they also inserted at the corresponding position a proline residue, located just before the beginning of the M2 region in GABA- and glycine-gated chloride channels but absent in nAChR and other cation channels. This mutant channel changed completely from cation-selective to anion-selective. To determine the minimum requirement for this change, the number of altered amino acids was progressively reduced until a minimum of three changes required to complete the transformation were found — the inclusion of the proline residue, the conversion of the very next amino acid from Glu to Ala, and conversion of a Val deep within the M2 region to Thr. Furthermore, in the absence of the proline insertion, the six other mutations do not affect the ion selectivity of the channel. The proline residue appears to be critical for the ion selectivity conversion since it brings about a conformational change to the constricted region of the pore (Corringer et al., 1999). The authors suggest that the reason the swap of the entire region failed is that determinants on the non-luminal face of M2 important for tertiary or quaternary structure might have been perturbed.
Keramidas et al. (2000) showed that the transformation was achievable in the other direction. Mutagenesis of the very same three residues in the glycine α1 chloride channel subunit to the corresponding residues from nAChRα7, i.e., deletion of the proline, conversion of the next Ala residue to Glu, and changing Thr within the M2 to Val, converted the channel from being anion-selective to cation-selective.

In the published version of Chapter 3, we made the conservative claim that we could not confidently predict the ion selectivity of the MOD-1 channel from sequence comparisons. However, MOD-1 has the key Pro and Ala residues at the corresponding positions (Chapter 3, Figure 2C), indicating that MOD-1 is quite likely to be a chloride channel. Our reason for not making this claim based merely on sequence rests on the fact that ligand-specificity for serotonin has never before been associated with a chloride channel.

The Chloride Switch

Unlike cation channels that are always excitatory in their actions, GABA- and glycine-gated chloride channels are not always inhibitory. For example, during the early development of the rat hippocampus, GABA- and glycine-gated chloride channels are initially excitatory (Cherubini et al., 1991). These GABA, chloride channels switch to their adult inhibitory state about a week after birth (Cherubini et al., 1991). The switch does not require any modification of the GABA, channel per se but rather is brought about a simple shift in the gradient of Cl- ions across the neuronal cell membrane (Rivera et al., 1999). Early on, Cl- ions are present at high concentrations inside the cell and therefore when the GABA, channel opens, Cl- ions exit the cell leading to depolarization. At about a week after birth, a K+/Cl- cotransporter is expressed in these GABAergic cells, and the activity of this cotransporter lowers the Cl- concentration inside the cell (Rivera et al., 1999). From this point onwards, as long as the K+/Cl- cotransporter is functional, whenever the GABA, channel is activated, it leads to neuronal inhibition.

What is the physiologic relevance of this dramatic shift during development in the neuronal activity of GABA, channels? In the developing cerebral cortex, GABA depolarizes postsynaptic neurons sufficiently to induce Ca2+ influx through NMDA receptors (Cherubini et al., 1991). This influx of Ca2+ has been shown to be important for control of gene expression and neurotrophin production in particular (Berninger et al., 1995), thus possibly playing a role in neuronal growth and the establishment of new connections. Furthermore, in the hippocampus GABA-mediated depolarization also
leads to synchronous cell firings (Serafini et al., 1995), which may play a role in activity-dependent reinforcement of nascent synaptic connections.

There are also instances where GABA<sub>A</sub> channels continue to exhibit excitatory actions even in the adult nervous system. I will not describe these cases as they are not directly relevant to the issue at hand, and furthermore the example just described illustrates the basic principle. This property of chloride channels to be either excitatory or inhibitory depending on the cellular environment is relevant to the in vivo function of the MOD-1 serotonin-gated chloride channel that we describe in Chapter 3. The electrophysiological characterization of MOD-1 in Chapter 3 does not allow us to predict how MOD-1 might function in vivo. Whether MOD-1 acts in an excitatory or inhibitory manner is of fundamental importance to understanding how the neural circuit in <i>C. elegans</i> brings about the enhanced slowing response. In Appendix 2, I discuss experiments that might help determine whether the MOD-1 serotonin-gated chloride channel activates or inhibits neuronal signaling in vivo.

**Serotonin Reuptake Transporters**

The ability to control precisely the circumstances that permit release of a particular neurotransmitter would be useless if it were not for the ability also to rapidly stop the said neurotransmitter from continuing to signal. The first mechanism for such termination of neuronal signaling was determined for acetylcholine — the degradative enzyme, acetylcholinesterase, destroys acetylcholine within milliseconds of its release (Cooper et al., 1996). This enzyme is abundant at cholinergic synapses and is always active. Therefore, the strength and duration of cholinergic signaling depends only on when and how much acetylcholine was released, ignoring for the moment the various postsynaptic mechanisms that also modulate cholinergic signaling. Surprisingly, the mechanism for acetylcholine removal is the exception rather than the rule (Cooper et al., 1996). All other neurotransmitters are cleared from the synaptic cleft by uptake into neighboring cells or reuptake into the neurons that release the neurotransmitter. Cloned vertebrate reuptake transporters exist for serotonin (Blakely et al., 1991), dopamine (Giros et al., 1991), epinephrine (Apparsundaram et al., 1997), norepinephrine (Pacholczyk et al., 1991), GABA (Guastella et al., 1990), glycine (Liu et al., 1992), and glutamate (Pines et al., 1992). Histamine is the only major neurotransmitter that does not have a cloned uptake transporter but histamine uptake has been observed in several cell preparations, including for example, photoreceptor cells (Stuart et al., 1996). The transporters for GABA, glycine, and glutamate belong to one family of transporters.
that bears little resemblance to the family of transporters for dopamine, serotonin, epinephrine, and norepinephrine.

The notoriety of the serotonin reuptake transporter (SERT) is primarily the result of the "biogenic amine hypothesis" of depression — which states that the underlying biological basis for depression is a deficiency of central noradrenergic or serotonergic systems — and the numerous inhibitors of serotonin reuptake that have proven to be effective antidepressants, such as imipramine (Tofranil), clomipramine (Anafranil), fluoxetine (Prozac), paroxetine (Paxil), and sertraline (Zoloft) (Baldessarini, 1996). We have now come to appreciate that the biogenic amine hypothesis is too simplistic and that the relationship between biogenic amines and depression is complex. Defects in various neurotransmitters systems can lead to depression. Depression is not just one disorder but a collection of disorders with distinct causes. Moreover, none of the neuromodulatory systems of the brain work in isolation but instead interact at various levels. Nevertheless, the remarkable efficacy of selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine, paroxetine, and sertraline, in alleviating depression suggests that enhancement of serotonergic neurotransmission must account for at least some of the therapeutic mechanism of these drugs.

In the nine years since the first mammalian SERT was cloned, nearly all of what we know about SERTs has come from studies in heterologous expression systems, primarily mammalian cell culture or *Xenopus* oocytes. The predicted secondary structure of SERTs places them within the family of Na*/Cl*-dependent transporters with 12 transmembrane domains (TMD) that includes transporters for dopamine and norepinephrine. This 12 TMD topology has been confirmed by measuring the reactivity of selected lysine and cysteine residues with extracellular reagents (Chen et al., 1998). These transporters until recently were thought to function as monomers in the plasma membrane. But coimmunoprecipitation studies with tagged SERT polypeptides suggests a tetrameric structure for SERTs (Kilic and Rudnick, 2000). Site-directed mutagenesis of putative N-glycosylation sites in the extracellular loop between the third and fourth transmembrane domains decreases serotonin uptake (Tate and Blakely, 1994). In these studies, the $K_m$ for transport was unaffected while the $B_m$ (substrate concentration for half-maximal binding) was vastly reduced, allowing the authors to conclude that N-glycosylation is likely to be important for either the expression or maintenance of expression of SERTs in the plasma membrane.

For each serotonin molecule transported, one Na* and one Cl⁻ ion is transported into the cell and one K⁺ ion is transported out (Rudnick and Clark, 1993). The Na* and Cl⁻ ions are transported along the same permeation pathway as serotonin (Galli et al.,
The substituted-cysteine accessibility method (Akabas et al., 1992) has been used to demonstrate that TMD3 has residues required for the binding and transport of serotonin (Chen et al., 1997). Studies of cross-species chimeras between the rat and human SERTs have implicated TMD12 in substrate recognition (Barker et al., 1994) and binding to tricyclic antidepressants (Barker and Blakely, 1996). Changing a specific Asp residue within TMD1 affects the binding of dopamine to the dopamine transporter (Kitayama et al., 1992). This Asp is absolutely conserved in serotonin, dopamine, and GABA transporters. Hence, based on a model for binding of catecholamines to β-adrenergic receptors (Strader et al., 1989), Kitayama et al. (1992) proposed that the Asp residue in these transporters might bind to the protonated NH₂ group on dopamine, serotonin, or GABA. Consistent with this hypothesis, mutating the corresponding Asp residue in rat SERT and human norepinephrine transporter leads to loss of uptake activity (Barker and Blakely, 1998); these authors confirmed that surface expression of these mutated transporters was unaffected. Nevertheless, binding studies with ligand derivatives are required to confirm that this Asp residue is indeed required for binding to the ligand.

Initially, it was thought that SERTs and other neurotransmitter transporters are always on the plasma membrane of the cell and ready to perform their uptake duties whenever substrate is present. But studies using HEK cells transfected with human SERT suggest that some of the canonical sites for protein kinases present on the cytoplasmic domains of SERTs are used by PKC to downregulate the expression of SERT on the plasma membrane (Qian et al., 1997). Similar modulation of expression has been reported for GABA (Quick et al., 1997) and norepinephrine (Apparsundaram et al., 1998) transporters. Furthermore, real-time visualization of GFP-tagged dopamine transporters has shown that this modulation of transporter activity occurs by transporter internalization rather than by reduced insertion of SERT molecules into the membrane (Daniels and Amara, 1999). Interestingly, SERT phosphorylation and sequestration is blocked by the addition of transportable substrates including serotonin (Ramamoorthy and Blakely, 1999). These observations suggest that activity-dependent modulation of transporter activity might occur.

Recently, SERTs have been shown to exhibit channel-like behavior with non-stoichiometric ion flow (Mager et al., 1994; Galli et al., 1997; Petersen and DeFelice, 1999) suggesting that at high serotonin concentrations (~0.3 mM) SERTs may function as serotonin channels, i.e., participate in an electrically non-neutral process where the primary charge carrier is the serotonin cation. The presence of such high serotonin concentrations at synapses may not be so unreasonable (Bunin and Wightman, 1998),
particularly when SERT inhibitors raise serotonin concentrations in the synaptic cleft, hence leading to the possibility that SERTs functioning as ion channels may have a physiological function.

_in vivo_ SERT function

SERT is abundantly expressed in the raphe nuclei in the midbrains of both humans (Austin et al., 1994) and rats (Lesch et al., 1993), where serotonergic cells are found (see above), and is coincident with the expression of tryptophan hydroxylase (McLaughlin et al., 1996), the enzyme specific for serotonin biosynthesis (see above). One study using $^{35}$S-labeled riboprobes has reported SERT expression in the substantia nigra, globus pallidus, and superior colliculi of the adult mouse brain (Bengel et al., 1997) and other studies have demonstrated transient expression of SERT in non-serotonergic cells during development in the rat (Hansson et al., 1998). Several studies report that chronic administration of certain drugs can affect the levels of SERT in the brain of animals. Specifically, in rats, clomipramine increases the level of SERT mRNA (Hansen and Mikkelsen, 1998) while fenfluramine and the tryptophan hydroxylase inhibitor p-Chlorphenylalanine decrease levels of SERT mRNA (Rattray et al., 1994; Rattray et al., 1996).

Recently, SERT knockout mice have been generated (Bengel et al., 1998). The authors found it surprising that these mice had few developmental defects, given prior reports of mothers treated with either serotonin or SSRIs such as fluoxetine or sertraline giving birth to pups with abnormal craniofacial or cardiac morphogenesis (as reported in Bengel et al., 1998). These findings underscore our ignorance about the spectrum of effects that any drug might have _in vivo_, even if the drug is termed "selective" based on _in vitro_ and _ex vivo_ assays. Chapter 4 describes our similar findings about the discrepancies between the phenotype of _C. elegans_ lacking SERT and the effects of the "selective" serotonin reuptake inhibitor fluoxetine on _C. elegans_ behavior.

These SERT$^{-}$ mice have an enhanced sensitivity to the effect of 3, 4-methylenedioxymethamphetamine ("Ecstasy") on locomotory behavior (Bengel et al., 1998). In addition, they also have vastly reduced levels of serotonin in their brains (Bengel et al., 1998), indicating that there are likely to be regulatory pathways that coordinate serotonin and SERT expression levels. Two other reports analyzing these mice have since emerged (Fabre et al., 2000; Li et al., 2000): they both demonstrate that 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor expression levels are dramatically reduced in these
SERT\(^{-}\) mice. Whether this decrease is a direct result of lack of SERT expression or the indirect effect of loss of serotonin expression remains to be determined. These SERT\(^{-}\) mice are the subject of intense investigations by numerous groups, and there will most certainly be a great deal more reported on the various other characteristics of these mice, including their performance in behavioral assays. In the meantime, the findings presented in chapter 4 constitute the only other report of in vivo consequences of mutations in a SERT.

**SERTs in Human Disease**

There are over 100 published reports that link polymorphisms at the human SERT locus to several human disorders ranging from late-onset Alzheimer's Disease and Parkinson's disease to numerous psychiatric disorders. In many of these reports, I found the data supporting the associations tenuous and unconvincing. The following study was a striking exception.

Three polymorphisms have been reported in the SERT locus to date. One of these is a 44-bp deletion within repeat elements in the regulatory sequence \(-1\) kb upstream of the gene transcription initiation site (Heils et al., 1996). The promoter with the deletion (the short allele) is less efficient than the long allele at driving the expression of a luciferase reporter gene in mammalian cells (Lesch et al., 1996). To discount the possibility that these results were artifacts of either variable transfection efficiencies or the lack of distant regulatory elements, Lesch et al., (1996) cultured lymphoblast cell lines from human subjects with either the short or the long SERT allele and found that the steady-state mRNA levels were 1.4- to 1.7-fold higher in cells containing the long allele. The authors then utilized three different personality trait assessment scales to evaluate 504 subjects from two independent groups and found that the shorter allele was strongly associated with various anxiety-related indices, such as neuroticism, tension, and harm-avoidance. The study also revealed many traits that showed no association, indicating that there was no overall systematic bias in the testing protocol. The authors had astutely chosen their 504 subjects to include 459 siblings from 210 independent families. Of these, 78 sib-pairs had different genotypes for the SERT locus. When these sib-pairs were analyzed for association of the SERT allele to the various traits examined, there was no statistically significant decrease in the index of association between the short allele and anxiety-related traits. Given that siblings are by definition genetically more homogenous, such differences between the siblings are strong indications of true genetic transmission.
What I find compelling about this case is that the "disorder" allele is only slightly different from the "normal" allele. I find it more plausible to consider subtle changes in neuromodulatory pathways as the basis for not only behavioral dysfunction but also perhaps individuality and personality. Furthermore, most genetically inherited behavioral variations in humans are likely to be polygenic traits and hence severe loss of function in a single molecule is unlikely to be the cause of complex traits.

Concluding Remarks

In this introductory chapter I have attempted to outline the topics pertinent to the chapters that follow and to provide a broader context for the work that I have done. The discussion presented here of learning and memory in *Aplysia* and serotonin- and food-related behaviors in *C. elegans* should be pertinent to Chapter 2, which describes the genetic, cellular, and physiological basis for the enhanced slowing response of *C. elegans*. The sections on serotonin receptors and chloride channels are directly relevant to Chapter 3, wherein the cloning and characterization of a serotonin-gated chloride channel is described. Finally, the section surveying the structure-function, *in vivo* roles, and connections to human disorders of SERTs should provide the framework for the findings described in Chapter 4, which presents the cloning of a *C. elegans* SERT and its characterization in the context of serotonin uptake, a genetic pathway for behavior, and fluoxetine pharmacology.

Acknowledgments

I would like to thank Ned Buttner and Brad Hersh for helpful comments concerning this chapter.
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<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Localizationa</th>
<th>Proposed functionb</th>
<th>Knockout phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT&lt;sub&gt;1A&lt;/sub&gt;</td>
<td>Hippocampus&lt;sup&gt;1&lt;/sup&gt; Amygdala&lt;sup&gt;1&lt;/sup&gt; Raphe Nuclei&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Anxiety Emotion&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Less reactive&lt;sup&gt;6&lt;/sup&gt; More anxious&lt;sup&gt;8&lt;/sup&gt; Less aggressive&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;1B&lt;/sub&gt;</td>
<td>Substantia Nigra&lt;sup&gt;1,5&lt;/sup&gt; Striatum&lt;sup&gt;1&lt;/sup&gt; Frontal Cortex&lt;sup&gt;1&lt;/sup&gt; Raphe Nuclei&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Regulation of motor function&lt;sup&gt;4,9&lt;/sup&gt;</td>
<td>More reactive&lt;sup&gt;6&lt;/sup&gt; Less anxious&lt;sup&gt;6&lt;/sup&gt; More aggressive&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>Motor control, Emotion&lt;sup&gt;9&lt;/sup&gt; Cognition&lt;sup&gt;9&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;2B&lt;/sub&gt;</td>
<td>Amygdala, Cerebellum also in smooth muscle</td>
<td>Smooth muscle contractions</td>
<td>Lethal; required for heart development&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;2C&lt;/sub&gt;</td>
<td>Choroid Plexus Cortex, Hippocampus, Substantia Nigra&lt;sup&gt;11&lt;/sup&gt;</td>
<td>Cerebrospinal fluid production&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Seizure-prone&lt;sup&gt;12&lt;/sup&gt; Obese&lt;sup&gt;13&lt;/sup&gt;</td>
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<td>Nausea Vasodilation Pain</td>
<td>Defective in pain perception</td>
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</tr>
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<td>Possible function&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Knockout phenotype</td>
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<td>Nausea Cognition enhancement</td>
<td>Increased exploratory behavior, Diminished response to LSD&lt;sup&gt;15&lt;/sup&gt;</td>
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<td>Smooth muscle relaxation Circadian rhythm</td>
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</tr>
</tbody>
</table>

<sup>a</sup>Localization data were obtained by either in situ hybrizidation experiments and/or radiolabelled agonist binding. In most cases, only the most prominent areas are listed. Many of these receptors have low level expression in other brain areas as well.

<sup>b</sup>Proposed function refers to the function of the brain region indicated by the expression data and the effects of agonists and antagonists. Only the most prominent effects are listed.

1Palacios et al., 1990; 2Frackowiak, 1994; 3Aggleton, 1993, Scott et al., 1997; 4Ramboz et al., 1996, Crocker, 1997; 5In patients with Parkinson's disease, the dopamine-containing neurons in the substantia nigra are lost (Damier et al., 1999); 6Zhuang et al., 1999; 7See text; 85-HT<sub>2A</sub> and 5-HT<sub>2B</sub> receptors are widely expressed in peripheral tissues where they mediate the contractile responses of various smooth muscle organs; 9Martin, 1996; 10Nebigil et al., 2000; 11Lower levels than in choroid plexus; 12Applegate and Tecott, 1998; 13Tecott et al., 1995; 14Gerald et al., 1995, Waebwr et al., 1996; 15Grailhe et al., 1999; 16Gerard et al., 1996, Gerard et al., 1997; 17Shen et al., 1993, Eglen et al., 1997
Chapter 2

C. elegans Locomotory Rate is Modulated by the Environment through a Dopaminergic Pathway and by Experience through a Serotonergic Pathway

Elizabeth R. Sawin, Rajesh Ranganathan, and H. Robert Horvitz

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My experimental contributions to this chapter are contained within Figures 4C, 4D, 5B, 5C (with Beth Sawin’s help), and 5D. I was the primary author of the text of this chapter.
Summary

*Caenorhabditis elegans* modulates its locomotory rate in response to its food, bacteria, in two ways. First, well-fed wild-type animals move more slowly in the presence of bacteria than in the absence of bacteria. This basal slowing response is mediated by a dopamine-containing neural circuit that senses a mechanical attribute of bacteria and may be an adaptive mechanism that increases the amount of time animals spend in the presence of food. Second, food-deprived wild-type animals when transferred to bacteria display a dramatically enhanced slowing response that ensures that the animals do not leave their newly encountered source of food. This experience-dependent response is mediated by serotonergic neurotransmission and is potentiated by fluoxetine (Prozac). The basal and enhanced slowing responses are distinct and separable neuromodulatory components of a genetically tractable paradigm of behavioral plasticity.
Introduction

An important function of the nervous system is to allow animals to respond flexibly to changing environmental stimuli. Such behavioral plasticity is manifested in numerous ways. In associative learning, associations between stimuli are recorded by the nervous system, and the stored information is used to alter behavioral outputs (Kupfermann and Kandel, 1995). In forms of non-associative learning, habituation and dishabituation alter the strength of reflexive responses based on an animal's past experience of repetitive or noxious stimuli, respectively, and sensitization enhances the response to a baseline stimulus after exposure to a novel or stronger stimulus. Another form of behavioral plasticity, also referred to as an alteration in behavioral state or as behavioral arousal, occurs when an animal's responsiveness to specific stimuli is altered as a result of a change in its internal state, a change that is often influenced by the animal's environment or past experience (Flicker et al., 1981). Understanding the mechanisms that produce such flexibility in behavioral output has been a major challenge in neurobiology.

Behavioral plasticity often involves the alteration of the properties of neurons and synapses. These alterations can be caused by the actions of neuromodulators, such as dopamine and serotonin. In both invertebrate and vertebrate nervous systems, dopamine plays a critical role as a neuromodulator by altering the intrinsic properties of neurons within circuits, both pre-synaptically (Cameron and Williams, 1993; Harris-Warrick et al., 1995) and post-synaptically (Barnes et al., 1994; Maguire and Werblin, 1994; Pereda et al., 1994). Genetic analyses of dopaminergic neurotransmission in Drosophila melanogaster (Tempel et al., 1984; Neckameyer, 1998) and mice (Drago et al., 1998, and references cited therein) have implicated dopamine in locomotory and spatial learning behaviors. Furthermore, the disruption of dopaminergic systems in human brains is involved in disorders such as Parkinson's disease (Marsden, 1992) and has been suggested to be involved in schizophrenia (Hietala and Syvalahti, 1996, and references cited therein), and dopaminergic neurons contribute to the circuitry involved in human motivation, reward, and drug addiction (Koob, 1992; Self and Nestler, 1995).

Serotonin is also an important neuromodulator in many animals. In the sensitization of defensive reflexes in the leech (Sahley, 1995, and references cited therein) and in Aplysia californica (Hawkins et al., 1993, and references cited therein).
cited therein), the application of the sensitizing stimulus releases serotonin and enhances the strength of reflex pathways. Serotonin mediates changes in behavioral state associated with food-deprivation in the leech (Groome et al., 1993; Brodfuehrer et al., 1995) and social dominance in the lobster (Kravitz, 1988). Serotonergic neurotransmission is implicated in numerous human processes and diseases (Osborne and Hamon, 1988; Takada and Curzon, 1995; Lucki, 1998, and references cited therein). Prozac (fluoxetine) and other selective serotonin re-uptake inhibitors used in the clinic to treat many of these human disorders are thought to work by primarily affecting serotonergic neurotransmission (Wong and Bymaster, 1995).

The simple anatomy, genetics, and behavior of the nematode *Caenorhabditis elegans* make this animal attractive for analyses of behavioral plasticity (Brenner, 1974; Wood et al., 1988). The chemotaxis response of *C. elegans* to volatile attractants adapts to the presence of high levels of certain attractants while animals maintain the ability to respond to other attractants (Colbert and Bargmann, 1995). *C. elegans* is capable of non-associative learning in the form of habituation when repeated mechanical stimulation is applied, dishabituation when the noxious stimulus of electric shock is administered, and sensitization when stronger mechanical stimulation is administered prior to the baseline stimulus (Rankin et al., 1990; Wicks and Rankin, 1997). Several hours of starvation affects *C. elegans* thermotaxis (Hedgecock and Russell, 1975; Mori, 1999), and olfaction (Colbert and Bargmann, 1997).

Several dopaminergic (Sulston et al., 1975) and serotonergic (Horvitz et al., 1982; Desai et al., 1988; Loer and Kenyon, 1993; E. R. S., and H. R. H, unpublished data) neurons have been identified in *C. elegans*. Locomotion and egg laying in *C. elegans* involves dopaminergic signaling (Schafer and Kenyon, 1995; Weinshenker et al., 1995), and serotonin affects several *C. elegans* behaviors, including locomotion, egg-laying, and pharyngeal pumping (Horvitz et al., 1982; Trent et al., 1983; Avery and Horvitz, 1990; Schafer and Kenyon, 1995). Furthermore, the selective serotonin re-uptake inhibitor fluoxetine (Prozac) affects *C. elegans* egg laying (C. Johnson, personal communication; Weinshenker et al., 1995), nose contraction (Choy and Thomas, 1999), and locomotory rate (Nurrish et al., 1999).

We have identified and analyzed a new paradigm for *C. elegans* behavioral plasticity. This paradigm provides a model for how behavioral state is controlled and how it affects the response to a specific stimulus. We observed
that well-fed C. elegans hermaphrodites when washed free of bacteria and later re-introduced to bacteria moved more slowly than when transferred to an environment without bacteria. Furthermore, this slowing response to bacteria was enhanced if the animals were not well fed but rather had been deprived of food for only 30 minutes. We note that in earlier experiments that have studied the effect of food withdrawal on C. elegans behavior, a brief 30 minutes absence from bacteria did not effect a behavioral change (Colbert and Bargmann, 1997; Mori, 1999), suggesting that our paradigm may involve a distinct mechanism of plasticity. We found that the responses of well-fed and food-deprived animals represent two distinct and separable modulatory behaviors and that dopamine and serotonin function in a non-overlapping manner to mediate the behaviors of well-fed and food-deprived animals, respectively.

Results

Locomotory Rate Decreases in the Presence of Bacteria

We quantified the locomotory rate of wild-type C. elegans hermaphrodites moving on an agar surface of a Petri plate by counting the number of bends in the anterior body region during a 20 second interval. Animals that had been continuously cultured on the bacterium Escherichia coli (well-fed) were washed free of bacteria and then transferred to assay plates containing or lacking a bacterial lawn. Well-fed animals that were transferred to assay plates containing bacteria moved more slowly upon re-entering the bacterial lawn than well-fed animals that were transferred to assay plates lacking bacteria (p<0.0001, Student's t-test) (Figure 1A, black bars). We refer to this response of well-fed animals after their re-entry into the bacterial lawn as the "basal slowing response."

Food-deprived Animals Show More Pronounced Slowing in Response to Bacteria

The strength of an animal's slowing response to bacteria depended on the animal's recent experience, in particular on whether the animal had been removed from bacteria 30 minutes before the behavioral assay (food-deprived). We compared the locomotory rate of well-fed animals to that of food-deprived
animals on assay plates containing or lacking a bacteria lawn. We found that food-deprived animals showed a more pronounced slowing response to the presence of bacteria than did well-fed animals \((p<0.0001, \text{ Student's } t\text{-test})\) (Figure 1A, gray bars). We refer to this response of food-deprived animals after their re-entry into the bacterial lawn as the "enhanced slowing response."

The enhanced slowing response was a specific modulatory response rather than physiological exhaustion resulting from food-deprivation, since food-deprived animals in the absence of bacteria moved as rapidly as well-fed animals in the absence of bacteria (Figure 1A). These results indicate that \textit{C. elegans} can use information about its prior exposure to bacteria to alter its behavior when it re-encounters bacteria.

\textbf{The Basal Slowing Response Requires Dopamine}

To explore the mechanisms underlying the basal and enhanced slowing responses, we examined several \textit{C. elegans} mutant strains with defects in neurotransmission. We first examined the behavior of animals bearing mutations in one or more of three genes known to affect biogenic amine synthesis: \textit{cat-2}, \textit{cat-4}, and \textit{bas-1}. \textit{cat-2(e1112)} (catecholamine defective) and \textit{cat-4(e1141)} mutants do not show the formaldehyde-induced fluorescence (FIF) indicative of the neurotransmitter dopamine (Sulston et al., 1975). \textit{cat-4} mutants also are reduced in serotonin expression (Desai et al., 1988; Weinshenker et al., 1995), whereas \textit{cat-2} mutants have normal levels of serotonin (G. Garriga, personal communication; E. R. S. and H. R. H., unpublished data). \textit{cat-2} encodes a tyrosine hydroxylase, an enzyme required for the biosynthesis of dopamine (Lints and Emmons, 1999). \textit{cat-4} encodes a GTP cyclohydrolase I (C. Loer, personal communication), an enzyme required for the synthesis of a co-factor needed for dopamine and serotonin biosynthesis (Kapatos et al., 1999). \textit{bas-1(ad446)} (biogenic amine synthesis defective) mutants have reduced serotonin levels (Loer and Kenyon, 1993) and also lack the FIF indicative of dopamine (E. R. S. and H. R. H., unpublished data). \textit{bas-1} encodes an aromatic amino acid decarboxylase, an enzyme needed for the biosynthesis of serotonin and dopamine (C. Loer, personal communication). Thus, \textit{cat-4} and \textit{bas-1} mutants appear to be deficient in both dopamine and serotonin, while \textit{cat-2} mutants are deficient in only dopamine.
We found that animals with mutations in any of these three genes were completely defective in the basal slowing response (Figures 1B-D, black bars). For example, in the presence of bacteria the locomotory rate of well-fed cat-2 mutants (Figure 1B, black bars) on bacteria was significantly faster (p<0.0001, Student's t-test) than that of well-fed wild-type animals that had been assayed in parallel. We generated a bas-1(ad446); cat-4(e1141) strain and found that the double mutant was indistinguishable from the single mutants with respect to the defect in the basal slowing response (Figure 1E, black bars). All of these mutants moved at the same rate as wild-type hermaphrodites in the absence of bacteria (Figures 1A-1E). Hence, the lack of the basal slowing response of these mutants was not a result of a general enhancement of locomotory rate or physical vigor but rather was likely to be a consequence of their common defect in dopamine expression. Moreover, the existence of mutants defective in the basal slowing response argues that the slowing of locomotory rate of well-fed animals on a bacterial lawn is a specific behavioral response to the presence of bacteria rather than a physical inability to move faster on a bacterial lawn.

The Enhanced Slowing Response Requires Serotonin

In addition to a defect in the basal slowing response, animals with mutations in bas-1 or cat-4, but not cat-2, were also defective in the enhanced slowing response (compare Figures 1B-D, gray bars, to Figure 1A, gray bars). That cat-2 mutants displayed a normal enhanced slowing response suggested that dopamine was not needed for this behavior and that the loss of serotonin biosynthesis in the bas-1 and cat-4 mutants was responsible for the defect in the enhanced slowing response. These results also suggested that this experience-dependent modulatory behavior was not just a stronger form of the basal slowing response. In support of this hypothesis, other treatments that affected the enhanced slowing response did not affect the basal slowing response (see below).

bas-1(ad446); cat-4(e1141) mutants were more defective in their enhanced slowing response (Figure 1E, gray bars) than was either of the single mutants (Figures 1C and 1D, gray bars). A plausible explanation for the increased defect is that the double mutants are more defective in serotonin biosynthesis than is either single mutant. It is unlikely, however, that the double mutants completely lack serotonin, since they do not have the developmental
defects seen in animals with a large deletion in the *tph-1* gene, which encodes a tryptophan hydroxylase, an enzyme essential for serotonin biosynthesis (Sze et al., 2000). Alternatively, it is conceivable that *bas-1* and *cat-4* are both involved in the biosynthesis of a signaling molecule other than dopamine or serotonin and the increased loss of that molecule led to the increased behavioral defect in the double mutant. We consider this second possibility unlikely, since we could restore wild-type basal and enhanced slowing responses to the double mutant by pre-incubating the animals in dopamine or serotonin, respectively (see below).

**Exogenous Dopamine Restores the Basal Slowing Response of *cat-2* and *bas-1; cat-4* Mutants**

We tested the ability of exogenous dopamine to restore the basal slowing response to *cat-2(e1112)* and *bas-1(ad446); cat-4(e1141)* mutants. Prior to the behavioral assay, we incubated wild-type, *cat-2(e1112)*, and *bas-1(ad446); cat-4(e1141)* animals on bacteria-containing plates with 2 mM dopamine and then examined the basal slowing response of these animals. Pre-incubation on plates containing dopamine caused *cat-2* and *bas-1; cat-4* mutants to exhibit a nearly normal basal slowing response (Figure 2). For example, control *cat-2* mutants pre-treated without dopamine had a significantly higher locomotory rate (Figure 2A, asterisks) than control wild-type animals pre-treated without dopamine, whereas *cat-2* mutants pre-treated with dopamine did not have a significantly different locomotory rate from wild-type animals pre-treated with dopamine (denoted by an "R" for Rescue; see Experimental Procedures for rescue criteria). *C. elegans* locomotion can be inhibited by high concentrations of dopamine (Schafer and Kenyon, 1995). Therefore, the concentration of dopamine used in this experiment was titrated so that it did not affect the locomotory rate of well-fed wild-type animals either in the absence or presence of bacteria (Figure 2), thereby allowing us to conclude that the rescue by dopamine in this assay was not the result of a general dopamine-induced locomotory slowing that bypassed the modulatory response to the stimulus of bacteria. Also, pre-incubation on dopamine-containing plates did not decrease the locomotory rate of either mutant class in the absence of bacteria (Figures 2A and 2B); thus, it is unlikely that the slowing of the dopamine-treated *cat-2* and *bas-1; cat-4* mutants in response to bacteria resulted from a hypersensitivity of the mutants to dopamine. Pre-treatment with exogenous serotonin did not rescue the defect in
the basal slowing response of well-fed cat-2 or bas-1; cat-4 mutants (Figure 2), suggesting that the effect of dopamine was specific and that exogenous serotonin cannot bypass the need for dopamine in the basal slowing response.

The CEP, ADE, and PDE Dopaminergic Neurons Sense Mechanosensory Stimuli Required for the Basal Slowing Response

The C. elegans hermaphrodite has eight dopaminergic neurons: the four CEPs, the two ADEs, and the two PDEs (Sulston et al., 1975). Ultrastructurally, the eight dopaminergic neurons have ciliated endings embedded in the cuticle, indicating likely mechanosensory functions (Ward, 1973; Perkins et al., 1986; White et al., 1986). The CEP sensory processes extend to the tip of the nose, the ADE sensory endings are located laterally in the anterior third of the body, and the PDE sensory endings are located laterally in the posterior body region.

To test if these dopaminergic sensory neurons were required for the basal slowing response, we ablated combinations of dopaminergic neurons in young larvae and examined the locomotory behavior of the resulting adults. Among the single cell-type ablations, only the ablation of the four CEPs resulted in a detectable defect in the basal slowing response (Figure 3A) and this defect was only modest. Among the double cell-type ablations, animals lacking the four CEPs and the two PDEs moved slightly faster than mock-ablated controls (Figure 3B). Animals lacking the four CEPs and the two ADEs did not show a defect in the basal slowing response; perhaps a larger number of experiments is necessary to obtain a statistically significant difference; alternatively the lack of a defect in these animals could have a biological basis. Ablating all three classes of dopaminergic neurons resulted in animals that were completely defective in the basal slowing response (Figure 3B). This result implied that these three classes of dopaminergic neurons function redundantly to mediate the basal slowing response.

If these dopaminergic neurons were directly involved in sensing the presence of bacteria, only those neurons with sensory endings in contact with bacteria might be required for the basal slowing response. Since C. elegans moves through bacteria lying on either its left or right side (White et al., 1986), we were able to test this hypothesis by abrating only the dopaminergic neurons having sensory endings on one side of the animal's body. Since the sensory endings of the CEP neurons are located in the tip of the animal's nose, the entire
circumference of which is in contact with bacteria, we ablated all four CEP neurons. We found that the ADE and PDE neurons were required only on the side of the animal’s body in contact with bacteria (Figure 3C). When animals lay on the unoperated side they responded normally to bacteria, but when these same animals lay on the opposite, operated side, they were defective in the basal slowing response.

To explore the nature of the stimulus detected by the dopaminergic neurons, we tested if substances other than bacteria could elicit the basal slowing response. We found that wild-type hermaphrodites slowed their rate of locomotion as they crawled through a three-dimensional matrix of sterile Sephadex G-200 beads (Figure 4A). G-200 Sephadex beads (20-50 μm) are much larger than bacteria and cannot be ingested by C. elegans. Therefore, any stimulus presented by these beads had to be sensed on the exterior of the animal. We found that cat-2(e1112) dopamine-deficient mutants did not exhibit the basal slowing response to the Sephadex beads (Figure 4A). Similarly, animals in which all the dopaminergic neurons had been ablated by laser ablation also did not exhibit the basal slowing response to Sephadex beads (Figure 4B).

These data argue that the slowing response to a non-bacterial stimulus was also mediated by dopaminergic neurotransmission. Since we added no bacteria in these experiments (and thus there were presumably no chemosensory cues associated with bacteria), these results taken together with the fact that the dopaminergic neurons are likely to be mechanosensory suggested that dopaminergic neurotransmission is required to respond to a mechanical attribute of bacteria to elicit the neuromodulatory basal slowing response.

**Exogenous Serotonin Restores the Enhanced Slowing Response of bas-1; cat-4 Mutants**

As discussed above, the enhanced slowing response appears to be independent of the dopamine-mediated basal slowing response and requires serotonin. We tested the ability of 2 mM exogenous serotonin to restore the enhanced slowing response to bas-1; cat-4 mutants. Pre-incubation on serotonin-containing plates caused bas-1; cat-4 mutants to exhibit a normal enhanced slowing response (Figure 2B). The concentration of serotonin used in this experiment was titrated.
so that it did not affect the locomotory rate of wild-type animals (Figure 2), or of \textit{bas-1; cat-4} mutants when well-fed (Figure 2B), or of food-deprived \textit{bas-1; cat-4} mutants in the absence of bacteria (data not shown), thereby allowing us to conclude that the slowing caused by serotonin in this assay is a restoration of the enhanced slowing response and not the result of a general serotonin-induced locomotory slowing that bypassed the modulatory response to the stimulus of bacteria. Exogenous dopamine did not rescue the defect in the enhanced slowing response of \textit{bas-1; cat-4} mutants (Figure 2B) but did rescue the basal slowing response of \textit{bas-1; cat-4} mutants (Figure 2B), indicating that the serotonin rescue of the defect in the enhanced slowing response is specific. Thus, endogenous dopamine was not required for the enhanced slowing response (\textit{cat-2} mutants; Figure 1B, gray bars), and exogenous dopamine could not bypass the need for serotonin in the enhanced slowing response.

**The Serotonergic NSMs Are Required for a Full Enhanced Slowing Response**

A number of serotonin-immunoreactive neurons have been reported to be present in the \textit{C. elegans} hermaphrodite: the most reproducibly staining neurons are the bilaterally symmetric pairs of NSMs (Horvitz et al., 1982), HSNs (Desai et al., 1988), ADFs, PHBs, AIMs, and the single I5 and RIH neurons (E. R. S. and H. R. H., unpublished data). Expression of a green fluorescent protein (GFP) reporter of the \textit{tph-1} gene is also observed in the NSMs, HSNs, ADFs, AIMs, and the RIH (Sze et al., 2000).

To test if these serotonergic sensory neurons were required for the enhanced slowing response, we ablated combinations of serotonergic neurons in young larvae and examined the locomotory behavior of the resulting adults. After ablating the NSMs using laser microsurgery, we found that well-fed animals lacking the NSMs (n=45) exhibited a normal basal slowing response (a change from 21.9±0.4 body bends/20 seconds (error represents the SEM) on plates with no bacteria to 14.8±0.5 body bends on plates with bacteria compared to mock ablated controls (n=45), which changed from 21.3±0.5 body bends to 14.6±0.6 body bends in the respective conditions). However, on plates with bacteria, food-deprived animals lacking the NSMs (n=45), moved slightly but significantly faster (7.3±0.6 body bends) than mock-ablated controls (5.1±0.4 body bends; n=45; \( p = 0.002 \), Student's \( t \)-test), suggesting that the NSMs contribute to the enhanced
slowing response. That the ablation of the NSMs did not cause a defect in the enhanced slowing response as strong as the one seen in *bas-1; cat-4* mutants (Figure 1E, gray bars) suggested that other cells, possibly including other serotonergic neurons, also contribute to the response.

We performed several double, triple, quadruple, quintuple, sextuple, and septuple ablations (all ablations included both members of the bilaterally symmetric classes), all of which included the ablation of the NSMs. In none of these multiple cell-type ablation experiments did we see a statistically significant defect in the enhanced slowing response beyond the defect seen in the NSM-ablated animals (Sawin, 1996).

It is possible that the NSMs are the only serotonergic neurons involved in the enhanced slowing response. For example, the NSMs might have continued to function even though their nuclei had been ablated; such residual function has been observed for the M4 neuron after its ablation in older animals (Avery and Horvitz, 1987). However, we consider this possibility unlikely, since we have found that the ablation of the NSMs leads to a complete loss of the potentiation of the enhanced slowing response by fluoxetine (see below). It is also conceivable that the NSMs provide both positive and negative inputs for the enhanced slowing response and that the ablation of the cell removes both classes of inputs leading to a weaker behavioral defect than would be seen if only the one or the other class of input was individually perturbed. The VC4 and VC5 neurons may also contain serotonin based on extremely weak immunoreactivity to anti-serotonin antibodies (G. Garriga, personal communication). We have observed such weak immunoreactivity in these neurons only very rarely (E. R. S. and H. R. H., unpublished data). Since tryptophan hydroxylase GFP reporter expression is not observed in the VC4 and VC5 neurons (Sze et al., 2000), it is possible that the variable and low level of serotonin immunoreactivity seen in these neurons is the result of these neurons taking up serotonin released by other serotonergic neurons (e.g., the HSNs, which are nearby). Nevertheless, it remains conceivable that the VC4 and VC5 neurons or other serotonergic cells not visualized by available anti-serotonin antibodies might function in the enhanced slowing response.
The Enhanced Slowing Response Is Not Triggered by Mechanosensory Stimuli that Trigger the Basal Slowing Response

We found that food-deprived wild-type hermaphrodites did not slow their locomotion rate as they crawled through a three-dimensional matrix of sterile Sephadex G-200 beads (Figure 4C). These data argue that in contrast to the basal slowing response, which is likely to be triggered by a mechanosensory stimulus to the surface of the animal, the enhanced slowing response appears not to be. Moreover, animals attained the food-deprived state even in the presence of Sephadex beads, since animals that were food-deprived in a three-dimensional matrix of Sephadex beads and then re-introduced to bacteria still exhibited the normal enhanced slowing response (Figure 4D). These observations demonstrate that the physical stimulus provided by Sephadex beads neither mimics the cues that food-deprived animals normally receive when re-introduced to bacteria nor prevents animals from attaining the food-deprived state.

Serotonin Antagonists Blocked the Enhanced Slowing Response

To test further the serotonin-dependence of the enhanced slowing response, we pre-incubated wild-type animals on Petri plates with two serotonin receptor antagonists, mianserin hydrochloride (Glennon, 1987) and methiothepin mesylate (Mylecharane, 1989), and tested these animals for the basal and enhanced slowing responses. While the specificities of these drugs on C. elegans behavior have not established, we found that both blocked the inhibition of locomotion induced by treatment with high concentrations of exogenous serotonin (data not shown), suggesting that these drugs interfere with serotonergic neurotransmission in C. elegans. Neither mianserin (20 μM) nor methiothepin (44 μM) had any effect on the basal slowing response (Figure 5A, black bars). However, food-deprived animals pre-treated with either drug were defective in the enhanced slowing response (Figure 5A, gray bars), providing further support for the role of serotonin in this modulatory behavior.
Fluoxetine Potentiates the Enhanced Slowing Response

We examined the effects of low concentrations of the selective serotonin re-uptake inhibitor fluoxetine (Prozac) on the locomotory rate of food-deprived animals. On plates without bacteria, there was no difference between the locomotory rates of animals that been food-deprived in the presence or absence of fluoxetine (Figure 5B). Since fluoxetine is a serotonin re-uptake blocker that most likely potentiates the concentration of serotonin at synapses, this observation suggested that in food-deprived animals, in the absence of bacteria, no serotonin is released and hence no potentiation by fluoxetine occurs. By contrast, on plates with bacteria, wild-type animals that had been food-deprived in the presence of fluoxetine moved much more slowly than wild-type animals that had been food-deprived in the absence of fluoxetine (Figure 5B). This observation suggests that fluoxetine amplifies the signaling process triggered when bacteria are encountered by food-deprived animals. We propose that bacteria trigger the release of serotonin, and fluoxetine amplifies the effect of the released serotonin by blocking its re-uptake. Furthermore, we postulate that fluoxetine affects the levels of only serotonin to potentiate the enhanced slowing response, since on plates containing bacteria, *bas-1; cat-4* mutants (which lack serotonin and dopamine) that had been food-deprived in the presence of fluoxetine had the same locomotory rate as did *bas-1; cat-4* mutants that had been food-deprived in the absence of fluoxetine (Figure 5B) while *cat-2* mutants (which lack dopamine but not serotonin) were no different from wild-type animals in this experiment (data not shown).

The NSM-ablated animals were resistant to the effects of fluoxetine. On plates containing bacteria, NSM-ablated animals that had been food-deprived in the presence of fluoxetine did not move any more slowly than NSM-ablated animals food-deprived in the absence of fluoxetine (Figure 5C). These data suggest that the potentiation of the enhanced slowing response by fluoxetine depends on the NSMs and that it was serotonin released by the NSMs that was specifically potentiated by fluoxetine. This result also indicates that if there is another, as yet unidentified, source of serotonin that modulates this behavior, the serotonin signaling from that source is not potentiated by the fluoxetine treatments used in these experiments.
Food Deprivation Does Not Lead to Serotonin Hypersensitivity

To determine whether the treatment of food-deprivation altered the sensitivity of the animals to serotonin, we compared the responsiveness of well-fed and food-deprived animals to exogenous serotonin. High concentrations of exogenous serotonin decrease the locomotory rate of well-fed *C. elegans* (Horvitz et al., 1982). We found that food-deprived worms were not hypersensitive to exogenous serotonin, since the locomotory rate of well-fed and food-deprived animals was inhibited by serotonin to the same extent over a range of concentrations (Figure 5D). This assay for serotonin sensitivity was done in the absence of food, since the presence of food would have led to the enhanced slowing response by food-deprived animals. The threshold concentration of serotonin required to affect well-fed and food-deprived animals was also very similar (Figure 5D). In addition, the well-fed and food-deprived animals did not show any significant difference in the kinetics of the response to any of the concentrations used in Figure 5D (data not shown). These results suggest that the increased responsiveness of food-deprived animals to bacteria is not a result of hypersensitivity to a food-induced serotonin signal.

Isolation of Modulation-Defective Mutants

We performed a genetic screen for mutants with defects in the enhanced slowing response. From a screen of approximately 16,000 haploid genomes, we isolated 17 mutant strains that when food-deprived and re-introduced to bacteria exhibit an abnormally fast locomotory rate (Figure 6). Our complementation studies indicated that four of these strains contain mutations in the known genes, *bas-1*, *goa-1* (*G-protein Q, alpha subunit*), and *dgk-1* (*diacylglycerol kinase*). *goa-1* and *dgk-1* have both been implicated by previous studies to be components of a serotonin signaling pathway (Mendel et al., 1995; Segalat et al., 1995; Nurrish et al., 1999). That we isolated alleles of these three genes in this genetic screen provides further evidence for the need for serotonin signaling in the enhanced slowing response.

All 17 mutant strains display the same locomotory rate as do the wild type on plates without bacteria, whether they were well-fed or food-deprived (Sawin, 1996). *bas-1(n2948)* and *bas-1(n3008)* mutants also showed a strong defect in the basal slowing response (Sawin, 1996), as expected given our
characterization of the bas-1(ad446) mutant (Figure 1C). Of the remaining 15 strains, 3 — n3007, n3033, and goa-1(n3055) — also have weak defects in the basal slowing response (Sawin, 1996). We assayed goa-1(n363) mutants (the n363 allele results in a complete loss-of-function of the gene; Segalat et al., 1995) and found that they have a more severe defect in both the basal and enhanced slowing responses than does our new goa-1(n3055) mutant (Sawin, 1996). This defect in basal slowing response of the goa-1 mutants suggests that the G-protein encoded by goa-1 might be involved not only in a serotonergic signaling pathway but also in a dopaminergic pathway.

Thus far, we have genetically mapped two of the mutations (n3034 and n3076) to small regions on chromosome V and I, respectively (see Chapter 3 and 5 respectively). These genetic positions are distinct from the location of all genes implicated to date in the basal and enhanced slowing responses (Sawin, 1996). These mutations define two new genes, mod-1 and mod-6 (modulation of locomotion defective).

Discussion

Food is a powerful stimulus that affects many C. elegans behaviors, including pharyngeal pumping (Avery and Horvitz, 1990), defecation (Liu and Thomas, 1994), and egg laying (Horvitz et al., 1982; Trent et al., 1983; Weinshenker et al., 1995). A prolonged withdrawal from food can affect C. elegans behavior (Colbert and Bargmann, 1997; Mori, 1999). We report that a brief 30 minute period of food-deprivation, a period insufficient to affect the other behaviors that have been studied (Colbert and Bargmann, 1997; Mori, 1999); and E.R. S. et al., unpublished data), elicits a robust change in behavior, namely the response to the presentation of the stimulus of food. In other words, 30 minutes of food deprivation can cause C. elegans to exist in an alternative behavioral state that allows it to respond appropriately to changes in the availability of food by modulating its locomotory rate. A dopamine-dependent process acts to reduce the locomotory rate of well-fed animals in response to a mechanosensory stimulus from bacteria. A serotonin-dependent process causes food-deprived animals to respond with an enhanced slowing of locomotory rate upon being transferred to bacteria. Both of these modulatory behaviors seem likely to be of adaptive significance (see below).
The ability of *C. elegans* to respond with differing intensities to the same environmental stimulus depending on the animal's state is similar to examples of behavioral plasticity seen in many animals. In the leech, a prolonged period without feeding results in an increase in the frequency of swimming and biting behaviors (O'Gara et al., 1991). Food-deprived leeches also exhibit other behavioral responses that are distinct from the responses of satiated leeches (Groome et al., 1993; Brodfuehrer et al., 1995). Food-induced arousal in *Aplysia* leads to an increase in the speed and strength of biting responses (Rosen et al., 1989). Similarly, a mammal's internal state can modulate the animal's responsiveness to various environmental stimuli (Kupfermann and Schwartz, 1995).

We have shown that our paradigm for behavioral plasticity in *C. elegans* involves dopamine and serotonin and is affected by fluoxetine. We propose that this experience-dependent modulatory behavior of *C. elegans* provides a simple model for the control of behavioral state and the related phenomena of motivation and mood.

**The Basal Slowing Response Involves a Mechanosensory Stimulus Mediated by Dopamine**

Recently, using the assay for locomotory rate we developed (Sawin, 1996) and used in the studies described in this paper, Duerr et al. (1999) observed that *cat-1* mutants, which are defective in loading pre-synaptic vesicles with monoamines, do not show the basal slowing response. This observation is consistent with our finding that dopamine-deficient mutants are defective in the basal slowing response. The apparent redundant function of the different dopaminergic neurons in the basal slowing response, established by our laser-ablation experiments, is similar to redundancies seen in other sensory systems in *C. elegans*. For example, three classes of mechanosensory neurons function together to sense touch to the nose (Kaplan and Horvitz, 1993), and four partially redundant cell types are required for direct chemotaxis to many attractants (Bargmann and Horvitz, 1991).

Since the dopaminergic neurons involved in the basal slowing response appear to sense a mechanical attribute of both bacteria and the Sephadex matrix (perhaps surface tension or pressure exerted on the cuticle), we suggest that in the basal slowing response the dopaminergic neurons transduce a
mechanosensory stimulus through interneurons and motor neurons to effect a slowing of locomotory rate (Figure 7A).

*C. elegans* is capable of discerning several attractive chemical cues in its search for food (Colbert and Bargmann, 1997). Why then might *C. elegans* sense bacteria mechanically? Perhaps while the chemosensory system is used to find new distant bacterial food sources, the mechanosensory system we have identified allows the animal to remain in the vicinity of the food source once that source has been reached.

The Enhanced Slowing Response Is Modulated by Serotonin and Is Distinct from the Basal Slowing Response

The process that allows food-deprived *C. elegans* to respond more strongly when transferred to bacteria is distinct from the process that well-fed *C. elegans* uses to respond to bacteria. The results of our rescue, ablation, and antagonist experiments strongly indicate that the experience-dependent enhanced slowing response is not simply a stronger form of the basal slowing response but rather is completely distinct. We therefore expect that complete loss of the enhanced slowing response would result in food-deprived animals having the same locomotory rate on plates with bacteria as they do on plates without bacteria. For this reason, we conclude that none of the mutants tested or the treatments performed completely abolished the enhanced slowing response.

If, as discussed above, serotonergic cells other than the NSMs are required for the enhanced slowing response, then the complete lack of potentiation of the enhanced slowing response by fluoxetine in NSM-ablated animals suggests that fluoxetine acts only on the NSMs and not on any of the other serotonergic cells involved. Given that the unique ultrastructure of the NSMs suggests that they might be neurosecretory (Albertson and Thomson, 1975), it is possible that fluoxetine acts on only neurosecretory cells in *C. elegans*. Alternatively, a non-serotonergic modulatory pathway insensitive to fluoxetine may act in parallel to the serotonergic pathway to transduce the signal(s) required for the enhanced slowing response.
The Food-deprivation Signal Is Likely to Act Upstream of the Serotonin-responsive Circuit

We sought to determine whether information about food-deprivation was stored in cells pre- or post-synaptic to the serotonergic synapses in the animal. If serotonin were released as an indicator of the presence of bacteria, then food-deprivation could result in diminished levels of serotonin release and perhaps a consequent sensitization of serotonin receptors by pathways well established for G-protein coupled receptors (Lefkowitz, 1993). If so, when animals are exposed to bacteria after food-deprivation, release of serotonin would resume and, because serotonin receptors had become sensitized, the same amount of released serotonin would have a greater behavioral effect. We do not believe that such a mechanism is responsible for the enhanced slowing response, since food-deprived and well-fed animals had similar sensitivities to exogenous serotonin. Rather, these findings suggest that the enhanced slowing response is regulated by a mechanism that does not depend either on altering the number of serotonin receptors on the cells that directly respond to serotonin or on enhancing the sensitivity of the serotonin receptors or any of the downstream components of the relevant signaling pathway(s).

One alternate model is that serotonin is released as an indicator of the absence of bacteria, i.e., during the 30 minute period of food-deprivation. Without such serotonin release, the enhanced slowing response could not occur. However, we observed that food-deprived animals did not move more slowly than well-fed animals on plates without bacteria and that the locomotory rate of food-deprived animals on plates without bacteria was unaffected by the fluoxetine treatment during food-deprivation. Given these observations, such a model would necessitate proposing that the re-introduction to food provides a permissive signal that allows the increased levels of serotonin in the synaptic cleft to effect a greater slowing of locomotory rate.

Instead, we favor a simpler model. We propose that the effect of food-deprivation is to increase the amount of serotonin released when food is re-encountered. Such an increase in serotonin release could be achieved by several possible molecular mechanisms, including an increase in the number of pre-synaptic vesicles that fuse to release serotonin or a faster fusion and exocytosis process of the normal number of pre-synaptic vesicles.
It is not obvious how the food-deprived state of an animal might lead to an increase of serotonin release from serotonergic neurons when bacteria are encountered. The information about food-deprivation could be stored in the serotonergic neurons themselves, through several possible molecular mechanisms, including transcriptional, translational, or post-translation changes of specific genes and proteins. One possible manifestation of such changes could be a depolarization in the resting membrane potential of the serotonergic neurons such that the neurons are primed to release serotonin when the next bacterial stimulus is encountered. Alternatively, the food-deprivation state could be stored anywhere in the nervous system upstream of or parallel to the serotonergic cells. Another possibility is that some non-neuronal physiological state, such as the extent to which the pharynx or the intestine is filled, is changed by food-deprivation, and such a change is detected and transduced by the nervous system. The serotonergic NSMs are plausible candidates to sense the presence of food, since these neurons have apparent mechanosensory endings (Albertson and Thomson, 1975), which could sense either bacteria in the pharynx or pharyngeal movements that occur during feeding.

In a simple model for the serotonergic regulation of the enhanced slowing response, serotonergic neurons (perhaps only the NSMs) might both detect the presence of food and store information about prior food-deprivation; these two inputs would then be integrated within these cells to regulate neural activity (Figure 7B). Alternatively, a serotonergic circuit, which may or may not include the NSMs, might mediate either the stimulus of bacteria or the food-deprivation signal, while another circuit responds to the other input, and the two inputs are integrated downstream of the serotonergic neurons. As discussed above, another modulatory pathway may act in parallel to the serotonergic pathway, and such redundancy could account for our failure to identify any pharmacological treatment, laser-ablation protocol, or mutation that completely abolished the enhanced slowing response. Such branched pathways are conceivable but are not the simplest interpretation of the data.

**Adaptive Significance of the Basal and Enhanced Slowing Responses**

Both the basal and the enhanced slowing responses could well be important for the survival of *C. elegans* in the wild. *C. elegans* eats bacteria, which in turn feed on decaying organic material in the soil, and the *C. elegans* population increases
until the food source is exhausted, at which point the population disperses (Nicholas, 1984). As discussed above, the basal slowing response could provide a mechanism to increase the likelihood that an animal will remain in the presence of a proximal bacterial food source. The enhanced slowing response would increase the certainty that an animal that had briefly wandered from or exhausted its food supply would, upon locating a fresh food supply, remain in the vicinity of that food source. In other words, the basal slowing response ensures that a well-fed animal will remain in the presence of food and continue feeding, while the enhanced slowing response ensures that an animal away from a food source for 30 minutes, for which food is likely to be even more important, stops when it encounters a new food source. Perhaps in its natural habitat a food-deprived animal is unwilling to forage further afield in search of a better or larger food source, or some alternative joy, since it risks going too far from a recently discovered and vital food source, whereas a well-fed animal may be willing to chance an exploration of its environment.

Our finding that *C. elegans* can modulate the rate of an ongoing motor program based on environmental conditions and its recent experience highlights the behavioral flexibility that is possible even with a nervous system comprising only 302 neurons of 118 distinct types (White et al., 1986). We have readily isolated a collection of mutations with defects in the enhanced slowing response and identified new genes involved in this paradigm of behavioral plasticity. We believe that the further dissection of this modulatory behavior at the level of defined cells, neurotransmitters, and genes will lead to an understanding of the complex mechanisms an animal uses to alter its behavioral output based on its prior experience.

**Experimental Procedures**

**Strains and Strain Constructions**

Nematodes were grown in non-crowded conditions at 20°C as described by Brenner (1974), except that *E. coli* strain HB101 instead of OP50 was used as the food source, since thin bacterial lawns of uniform thickness were more reliably generated with overnight growth of HB101 (data not shown); such uniformity was critical for reproducible results in the locomotory rate assays. Wild-type animals were *C. elegans* strain N2. Mutant strains used were CB1112
cat-2(e1112) II, CB1141 cat-4(e1141) V (Sulston et al., 1975), MT7988 bas-1(ad446) III (Loer and Kenyon, 1993), MT8943 bas-1(ad446) III; cat-4(e1141) V, MT363 goa-1(n363) I, MT7990 bas-1(n2948) III, MT7991 dgk-1(n2949) X, MT8201 mod(n3006), MT8203 mod(n3007), MT8202 bas-1(n3008), MT8539 mod(n3032), MT8630 mod(n3033), MT8541 mod-1(n3034) V, MT8623 mod(n3052), MT8624 mod(n3053), MT8625 mod(n3054), MT8626 goa-1(n3055) I, MT8627 mod(n3056), MT8628 mod(n3057), MT8689 mod(n3074), MT8690 mod(n3075), and MT8720 mod-6(n3076) I.

Locomotory Rate Assays

Assay plates were prepared by spreading the E. coli strain HB101 in a ring with an inner diameter of approximately 1 cm and an outer diameter of approximately 3.5 cm on NGM agar (Brenner, 1974) in 5 cm Petri plates. Assay plates were always freshly spread with bacteria, incubated overnight at 37°C, and allowed to cool to room temperature before use. Plates for measuring locomotory rate in the absence of bacteria were also incubated at 37°C. Only synchronized young adult hermaphrodites (16 hours after the late L4 larval stage) were tested. In all cases, plates were coded so that the experimenter was blind to the genotype or the laser-ablation state of the animal and to the neurotransmitter content of the pre-treatment plate.

For well-fed animals, locomotory rate was measured by removing five animals from plates with ample bacteria, washing the animals twice in S basal buffer (Brenner, 1974), and transferring them to an assay plate in a drop of buffer using a capillary pipette. If the assay plate contained a ring-shaped bacterial lawn, the animals were transferred to the clear zone at the center of the ring. The drop of buffer used to transfer the animals was absorbed with a Kimwipe. Five minutes after transfer, the number of body bends in 20 second intervals was sequentially recorded for each of the five animals on the assay plate. This slowing response of well-fed animals was not an artifact of the wash step, since animals transferred directly from the culture plates to assay plates also exhibited the basal slowing response (data not shown). Since food-deprived animals (see below) were washed, we washed all the animals to enable direct comparisons.

For food-deprived animals, 5-15 animals were washed free of bacteria in S basal buffer (two washes) and then transferred to 5 cm NGM agar plates that
had no bacteria but had a ring of high osmolarity fructose on the outer edge. The drop of buffer used to transfer the animals was absorbed with a Kimwipe. The animals were incubated on these plates for 30 minutes at room temperature. *C. elegans* avoids high osmolarity (Culotti and Russell, 1978), and this avoidance reflex prevented the animals from swimming off the agar and dying on the plastic edge of the Petri plate. The high osmolarity ring was created by adding approximately 100 µl of a 4 M fructose solution to the outer circumference of the agar surface and waiting approximately 5-10 minutes for this solution to dry into the plate. To make the high osmolarity ring visible, we added a small amount of bromophenol blue to the fructose solution. The presence of the high osmolarity ring or the bromophenol blue had no effect on the behavior of animals in the locomotory rate assays (data not shown). At the end of 30 minutes of food-deprivation, five worms were transferred in a drop of S basal buffer to assay plates and locomotory rate was measured as described above for well-fed animals.

Locomotory rate measurements for mock- and laser-ablated animals were done in a similar fashion, except that only one animal was transferred to each assay plate. Its locomotory rate was tested in the presence of bacteria, it was allowed to recover for 2-3 hours on plates containing bacteria, and then transferred, as described above, to food-deprivation plates. After five minutes on the food-deprivation plates, the locomotory rate of these well-fed animals in the absence of food was recorded; these locomotory rates were not different from locomotory rates on regular assay plates without bacteria that did not have the high osmolarity ring (data not shown). After 30 minutes the food-deprived animals were transferred to assay plates containing ring-shaped bacterial lawns, and the locomotory rate was again recorded. For the ablation and serotonin-antagonist experiments (Figure 5B and 5C), locomotory rate data for food-deprived animals on plates without bacteria were not collected, since we have never observed any difference between the behaviors of well-fed and food-deprived animals on plates without bacteria. For the unilateral ablations, there was no systematic bias to the order in which the sides were assayed, since the plates were coded prior to the locomotory assay and the experimenter did not know when assaying a given side whether or not the neurons had been ablated on that side.

Sephadex G-200 beads (Sigma Chemicals) were re-suspended in S basal buffer at 30 mg/ml and autoclaved for 45 minutes. Approximately 200 µl of this
mixture was transferred to an agar plate. Excess buffer that pooled at the edge of the Sephadex was removed with a Kimwipe. The rate of locomotion was measured two minutes after transferring the animals to the Sephadex matrix. The food-deprivations on Sephadex were performed by washing the animals twice in S basal buffer and transferring them to a 5 cm plate whose surface was completely covered by a layer of Sephadex beads. Thirty minutes later, animals were transferred to assay plates.

Statistical analysis was performed using the unpaired Student's t-test of the Statview program.

**Laser Ablations**

Neurons were ablated during the second larval stage using a laser microbeam, as previously described (Avery and Horvitz, 1987, 1989; Bargmann and Horvitz, 1991). Behavioral assays of young adult animals were performed two days later. Mock-ablated animals were transferred to agar pads and anesthetized in parallel to the animals that underwent laser ablation.

Successful identification and ablation of the dopaminergic neurons was confirmed using the FIF technique of Sulston et al. (1975), which generates fluorescence in the cell bodies of dopaminergic neurons, with modifications adapted from Jagdale and Gordon (1994). The eight dopaminergic cell bodies were routinely visible in animals of the first, second, and third larval stages.

To confirm that the behavioral phenotype we observed in our presumptive CEP-ablated ADE-ablated PDE-ablated animals was specifically caused by ablation of those neurons and not by accidental damage to neighboring cells, we ablated the nearest neighbors of the dopaminergic neurons in two animals. We ablated all cells adjacent to dopaminergic neurons (RID, ALA, URX, FLP, ADA, AQR, PVD, PVM, and SDQL) (n=2) except for the sheath cells of the dopaminergic neurons and cells that are post-synaptic to dopaminergic neurons. These animals showed a normal basal slowing response (Sawin, 1996).

Similarly, for the NSM-ablation experiments, we ablated I2 (n=3) and MC (n=4) and saw no effect on the enhanced slowing response (Sawin, 1996).
Neurotransmitter and Drug Pre-treatment

50 mM solutions of serotonin creatinine sulfate complex (Sigma) or dopamine hydrochloride (RBI) were prepared fresh in M9 buffer (Brenner, 1974). 400 µl of these solutions or of M9 buffer was added to each 5 cm plate containing approximately 10 ml of agar and a bacterial lawn to obtain an equilibrium concentration of approximately 2 mM of each neurotransmitter. The plates were allowed to dry at room temperature with their lids removed for 1 hour and then 40-50 animals of each genotype that had been picked as L4 animals 16-20 hours earlier were transferred to each plate. The animals were incubated on these plates for either 2 hours (bas-1; cat-4 rescue experiments) or 6-7 hours (cat-2 rescue experiments) at 20°C and then assayed according to our standard locomotory rate assay with the modification that the food-deprivation plates contained the appropriate neurotransmitter. The assay plates did not contain the neurotransmitters. For both cat-2 and bas-1; cat-4 mutants pre-treatment with dopamine resulted in the appearance of the FIF indicative of dopamine in the cell bodies of eight neurons in the positions of the dopaminergic neurons of the wild type (data not shown). For these drug experiments, pre-treatment was considered to rescue the mutant phenotype if the data from mutants were not statistically different (p>0.05) from the data from wild-type assayed in parallel under the same conditions; p=0.49 for rescue by dopamine of the basal slowing response of cat-2 mutants; p=0.28 for rescue by dopamine of the basal slowing response of bas-1; cat-4 mutants; p=0.1 for rescue by serotonin of the enhanced slowing response of bas-1; cat-4 mutants.

Plates containing Methiothepin mesylate (RBI) and mianserin hydrochloride (RBI) were prepared in a manner similar to the serotonin- and dopamine-containing plates. Animals were incubated for 30 minutes on either plates with bacteria and the antagonist or on plates without bacteria but containing antagonist and then assayed according to our standard locomotory rate assay. The assay plates did not contain the antagonists.

Fresh NGM agar plates, containing either no fluoxetine or 75 µg/ml fluoxetine (Sigma) were prepared the day before the assays and stored overnight in the dark. The fluoxetine stock (300 µg/ml) was dissolved in water and added after the agar solution had cooled to just above gelling temperature. Animals were incubated for 30 minutes on plates without bacteria and with or without fluoxetine and then tested on assay plates (without fluoxetine). For testing the
response of NSM-ablated animals, the locomotory rates of mock-ablated and the NSM-ablated animals were assayed, one animal at a time, first on plates with bacteria after food-deprivation in the absence of fluoxetine, then on plates with bacteria after food-deprivation in the presence of fluoxetine. Then all the animals were tested as a group, first on plates without bacteria after food-deprivation in the absence of fluoxetine, then on plates without bacteria after food-deprivation in the presence of fluoxetine. The animals were allowed to recover on plates with bacteria for 1-2 hours between each of the four conditions.

To examine the serotonin response of food-deprived versus well-fed animals, we prepared fresh NGM agar plates with the appropriate amounts of serotonin. The serotonin stock was dissolved in water and added after the agar solution had cooled to just above gelling temperature. After incubating the plates overnight at room temperature, we placed a single food-deprived or well-fed animal on a plate, and the locomotory rate of the animal was recorded at the start of every minute for five minutes.

Mutant Screen

N2 hermaphrodites (L4) were mutagenized with EMS (Brenner, 1974), and their F2 progeny were food-deprived for 30 minutes and then transferred to the center of large Petri plates spread with *E. coli* strain HB101 in the form of a ring-shaped lawn. After 5-10 minutes, the assay plates were examined for worms that had both reached the outer edge of the bacterial lawn and were moving rapidly. Such animals were picked to individual plates and their progeny were retested. Prior to detailed behavioral analysis, each strain was outcrossed at least twice.

Acknowledgments

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References


Figures

Figure 1. Modulation of Locomotory Rate of Wild-type Animals and Dopamine- and/or Serotonin-deficient Mutants

Well-fed and food-deprived animals were transferred to assay plates with or without a bacterial lawn, and five minutes later the locomotory rate of each animal was recorded. Food-deprived animals were transferred to plates without bacteria 30 minutes prior to the transfer to locomotory assay plates (see Experimental Procedures for details). In this and all subsequent figures, unless otherwise stated, each trial involved testing at least five animals in each of the conditions; a given animal was tested in only one of the conditions.

(A) The wild-type basal (black bars) and enhanced (gray bars) slowing responses. Eleven trials with wild-type animals. See Results for p values.

(B) - (E) Mutants defective in the basal and/or enhanced slowing responses. p values were calculated by comparing the combined data for the mutants from all the separate trials to the combined data for the wild-type animals assayed in parallel in each condition of each separate trial. Wild-type data from these parallel trials are not shown, since in no case were these data significantly different from the data in Figure 1A.

(B) Ten trials with cat-2(e1112) mutants.

(C) Four trials with bas-1(ad446) mutants.

(D) Five trials with cat-4(e1141) mutants.

(E) Six trials with bas-1(ad446); cat-4(e1141) mutants.

Error bars represent the SEM; **p=0.0002, ***p<0.0001, Student's t-test.
Figure 1

A. Wild type
B. cat-2(e1112)
C. bas-1(ad446)
D. cat-4(e1141)
E. bas-1(ad446); cat-4(e1141)

- Well-fed
- Food-deprived
Figure 2. Dopamine Pre-treatment Rescues the Defect in the Basal Slowing Response of Dopamine-deficient Mutants and Serotonin Pretreatment Rescues the Defect in the Enhanced Slowing Response of Serotonin-deficient Mutants

Following pre-incubation on bacteria-containing plates with no neurotransmitter (Buffer) or with dopamine or serotonin, animals were transferred directly to assay plates and locomotory rate was measured (A and B, black bars) or to food-deprivation plates with buffer, dopamine, or serotonin for 30 minutes and then to assay plates and locomotory rate was measured (B, gray bars); R = Rescued (see Experimental Procedures for details of rescue criteria).

(A) Rescue of the defect in the basal slowing response of *cat-2* mutants (four trials). Abbreviation: WT, wild type.

(B) Rescue of the defect in the basal and enhanced slowing responses of *bas-1; cat-4* mutants (four trials).

Error bars represent the SEM; ***p<0.0001, Student's t-test.
Figure 3. Dopaminergic Neurons Function Redundantly to Sense Mechanosensory Stimuli

(A) and (B) Basal slowing response of animals with dopaminergic neurons ablated. The dopaminergic neurons indicated were ablated in larvae two days before the behavioral experiment. In parallel, an equivalent number of mock ablated-animals were also tested. A given animal was tested first in the presence of bacteria and then in the absence of bacteria (see Experimental Procedures for details).

(A) Twenty-three CEP-ablated, 14 ADE-ablated, and 13 PDE-ablated animals were assayed.

(B) Fifteen CEP-ablated ADE-ablated, 21 CEP-ablated PDE-ablated, 20 ADE-ablated PDE-ablated, and 17 CEP-ablated ADE-ablated PDE-ablated animals were assayed.

(C) Only dopaminergic neurons in contact with bacteria were required for the basal slowing response. The locomotory rates of five animals in which CEPDL/R, CEPVL/R, ADEL, and PDEL had been ablated and six animals in which CEPDL/R, CEPVL/R, ADER, and PDER had been ablated were recorded in the presence and absence of bacteria with animals lying first on one side and then on the other (the order in which the sides were tested was random; see Experimental Procedures for details).

Error bars represent the SEM; *p=0.01; **p=0.0009; ***p<0.0001, Student's t-test.
Figure 3

A

Bacteria: 

Cells Ablated:
Mock CEPs Mock ADEs Mock PDEs

C

Bacteria: 

Cells Ablated:
Mock CEPs ADEs Mock CEPs PDEs

Unilateral ablations of ADE & PDE
Ablated side facing up
Ablated side facing down
CEPs ablated

** Mock CEPs Mock ADEs Mock PDEs

***
Figure 4. The Basal Slowing Response Involves a Mechanosensory Stimulus, while the Enhanced Slowing Response Does Not

(A) Response of wild-type and cat-2 animals to Sephadex beads. Thirty-five wild-type and 35 cat-2 animals were assayed in three trials.

(B) Animals with dopaminergic neurons ablated do not respond to Sephadex beads. Five animals in which the CEPs, ADEs, and PDEs had been ablated and five mock-ablated animals were tested in each of the two conditions. A given animal was tested first in the presence of Sephadex and then in the absence of Sephadex (see Experimental Procedures for details).

(C) Food-deprived wild-type animals do not slow in response to Sephadex beads. Twenty animals were tested in each condition in four trials.

(D) Animals food-deprived in the presence of Sephadex beads exhibit a normal enhanced slowing response. Twenty animals were tested in each condition in four trials.

Error bars represent the SEM; ***p<0.0001, Student's t-test.
Figure 4

A

<table>
<thead>
<tr>
<th>Sephadex</th>
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<tr>
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B

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<tr>
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<td>Mock</td>
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<td>+</td>
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C

- **Well-fed**
- **Food-deprived**
- **Food-deprived on Sephadex**

D

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>-</th>
<th>+</th>
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<tbody>
<tr>
<td>Body Bends/20 seconds</td>
<td>2</td>
<td>5</td>
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Significance: ***
Figure 5. The Enhanced Slowing Response Involves Serotonin, Is Potentiated by Fluoxetine, and Is Not a Result of Serotonin Hypersensitivity

(A) Serotonin antagonists disrupted the enhanced slowing response. Mianserin hydrochloride was at 20 μM, and methiothepin mesylate was at 44 μM (five trials).

(B) The potentiation of the enhanced slowing response by fluoxetine requires endogenous serotonin. Twenty wild-type animals and 20 bas-1; cat-4 mutants were tested in each condition in four trials.

(C) The potentiation of the enhanced slowing response by fluoxetine acts through the NSMs. Locomotory rates of seven mock-ablated and eight NSM-ablated animals were assayed in each of the four conditions (see Experimental Procedures for order of conditions tested). The effect of the NSM-ablations on the enhanced slowing response in the absence of fluoxetine was statistically significant (p=0.044). The p values denoted by the asterisks in Figure 5B and 5C were calculated by comparing the fluoxetine-treated animals to untreated animals of the same genotype or ablation state.

(D) Dose response of serotonin sensitivity. Twenty animals were tested at each concentration over 20 trials (one animal per condition per trial). Well-fed or food-deprived animals were transferred to plates (no bacteria) with the indicated concentration of serotonin, and locomotory rate after five minutes was plotted.

Error bars represent the SEM; ***p<0.0001, Student's t-test.
Figure 6. Mutants Defective in the Enhanced Slowing Response

At least twenty animals were tested for each genotype in at least four trials. 
$p$ values were calculated by comparing the combined data for the mutants from 
all the separate trials to the combined data for the wild-type animals assayed in 
parallel in each condition of each separate trial. Wild-type data from these 
parallel trials are not shown, since in no case were these data significantly 
different from the representative data for the wild type shown in the first column.

Error bars represent the SEM; ** $0.001 < p < 0.05$; ***$p < 0.0001$, Student's $t$-test.
Figure 6

Food-deprived Animals on Bacteria

Body Bends/20 seconds

Wild type
n3052
n3007
n3053
n3006
n3032
n3007
n3054
n3057
n3008
bas-1(n3008)
mod-6(n3076)
mod-1(n3034)
dgk-1(n2949)
bas-1(n2948)
mod-1(n3034)

Figure 7. Models for the Basal and Enhanced Slowing Responses

(A) In well-fed animals, the three classes of dopaminergic neurons (CEPs, ADEs, and PDEs) function redundantly to sense a mechanosensory stimulus from bacteria and then modulate the motor circuit to effect the basal slowing response.

(B) In food-deprived animals, serotonergic neurons sense the current level of food in the pharynx and integrate this information with information about the past experience of food-deprivation. The serotonergic neurons then release an increased amount of serotonin, which inhibits the motor circuit to a greater extent than in the basal slowing response to effect the enhanced slowing response (see Discussion for details).
Figure 7

A

Bacteria → ADEs, PDEs, CEPs → Dopamine → Motor Circuit → Basal Slowing Response

B

Bacteria → Past Experience of Food-deprivation → Serotonergic Neurons (NSMs?) → Serotonin → Motor Circuit → Enhanced Slowing Response
Chapter 3

MOD-1 is a serotonin-gated chloride channel that modulates C. elegans locomotory behaviour

Rajesh Ranganathan, Stephen C. Cannon, and H. Robert Horvitz

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Dr. Cannon performed the HEK cell recordings shown in figure 5. I performed the rest of the experiments.
Summary

The neurotransmitter and neuromodulator serotonin (5-HT) functions by binding either to metabotropic G-protein coupled receptors (for example, 5-HT₁, 5-HT₂, 5-HT₄ to 5-HT₇), which mediate "slow" modulatory responses through numerous second messenger pathways¹, or to the ionotropic 5-HT₃ receptor, a non-selective cation channel that mediates "fast" membrane depolarizations². Here we report that the gene mod-1 (for modulation of locomotion defective) from the nematode Caenorhabditis elegans encodes a new type of ionotropic 5-HT receptor, a 5-HT-gated chloride channel. The predicted MOD-1 protein is similar to members of the nicotinic acetylcholine receptor (nAChR) family of ligand-gated ion channels, in particular to GABA (γ-aminobutyric acid)- and glycine-gated chloride channels. The MOD-1 channel has distinctive ion selectivity and pharmacological properties. The reversal potential of the MOD-1 channel is dependent on the concentration of chloride ions but not of cations. The MOD-1 channel is not blocked by calcium ions or 5-HT₃a-specific antagonists but is inhibited by the metabotropic 5-HT receptor antagonists mianserin and methiothepin. mod-1 mutant animals are defective in a 5-HT-mediated experience-dependent behavior³ and are resistant to exogenous 5-HT, confirming that MOD-1 functions as a 5-HT receptor in vivo.
Results and Discussion

The locomotory rate of a well-fed C. elegans hermaphrodite slows when it encounters bacteria (the basal slowing response); this slowing response is enhanced markedly if the animal has been deprived of food for 30 minutes before the encounter (the enhanced slowing response) (Fig. 1a). A genetic screen for mutants defective in the enhanced slowing response led to the identification of the gene mod-1, which is defined by the mutation n3034. On Petri dishes with bacteria, the locomotory rate of food-deprived mod-1 mutants is significantly faster than that of the wild type (strain N2) (Fig. 1a, grey bars). On dishes without bacteria, the locomotory rate of food-deprived mod-1 mutants is not different from that of food-deprived wild-type animals (Fig. 1a, grey bars). Well-fed mod-1 mutants show no defect in their basal slowing response to bacteria (Fig. 1a, black bars).

The swimming behavior of mod-1(n3034) mutants was not affected by exogenous 5-HT treatment, whereas exogenous 5-HT inhibits wild-type C. elegans locomotion; and this phenotype was semi-dominant in a time-dependent manner (Fig. 1b), which aided our cloning of the mod-1 gene (see below). This 5-HT resistance of mod-1 mutants is consistent with the observation that the enhanced slowing response is mediated by 5-HT signaling and the hypothesis that mod-1 mutants are deficient in 5-HT signaling.

We used standard genetic three-factor mapping scoring the 5-HT resistance of mod-1 mutants to locate mod-1 to a ~400 kilobase (kb) region on chromosome V. Cosmid C38E12 from this region rescued the recessive aspect of the Mod-1 mutant phenotype of 5-HT resistance, as assayed by the response to exogenous 5-HT after 5 min (Fig. 1c). We narrowed the rescuing activity to a 5.5-kb fragment (Fig. 1c) and found that this fragment contained a single predicted gene. We isolated several complementary DNAs (cDNAs) with SL1 trans-spliced leaders, which are found at the 5' ends of many C. elegans transcripts, and thereby determined the complete mod-1 transcription unit (Fig. 2a). Genomic subclones lacking all or part of this transcription unit did not rescue the Mod-1 phenotype of 5-HT resistance (Fig. 1c).

MOD-1 is similar to members of the nicotinic acetylcholine receptor (nAChR) family of ligand-gated ion channels, in particular to GABA- and glycine-gated chloride channels (Fig. 2b). We found a single-base transition mutation in the mod-1 coding sequence in n3034 mutants (Fig. 2b, c), in which Ala 281 (codon GCT) is changed to a valine (GTT) within the predicted M2 transmembrane domain of the MOD-1 protein; the M2 domain is thought to be critical for channel function. We used
site-directed mutagenesis to introduce this C-to-T (A281V) mutation into the 5.5-kb minimal rescuing fragment and generated transgenic animals carrying extrachromosomal arrays of this fragment, Ex[MOD-1(A281V)]. These transgenic animals displayed resistance to exogenous 5-HT (Fig. 3a), confirming that the A281V mutation in MOD-1 is sufficient to cause 5-HT-resistance.

To determine the effect of eliminating mod-1 function, we analyzed two deletion alleles of mod-1, nr2043 (ref. 7) and ok103 (Fig. 2a), which were obtained by screening libraries of mutagenized animals to identify large deletions in the mod-1 genomic locus. Both mod-1(nr2043) and mod-1(ok103) mutants, when deprived of food, were defective in the enhanced slowing response (Fig. 3b), whereas neither was defective in the basal slowing response (data not shown). Both deletion mutants were resistant to exogenous 5-HT (Fig. 3a). The 5-HT-resistance caused by the two deletion alleles was completely recessive throughout the 20-min time course (Fig. 3a; and data not shown), consistent with our observation that animals heterozygous for large chromosomal deficiencies that uncover the mod-1 genomic locus are not 5-HT-resistant (data not shown). The molecular nature of the mod-1(nr2043) and mod-1(ok103) mutations suggests that they are null alleles. As null alleles confer the same phenotype as that conferred by the Ex[MOD-1(A281V)], mod-1(n3034) is likely to be a dominant-negative allele.

MOD-1 protein is predicted to contain a large extracellular amino terminus with two cysteine residues separated by 13 amino acids, a feature conserved in the nAChR family and that may have a role in subunit assembly and insertion into the membrane (Fig. 2b). There are four predicted transmembrane regions (M1-M4) and a large cytoplasmic domain between M3 and M4 (Fig. 2b). The M2 domain may participate in forming the pore of the channel after these nAChR family members assemble as multimeric complexes in the cell membrane. In other family members, the residues in and around the M2 domain are involved in establishing ion selectivity. We could not confidently predict the ion selectivity of the MOD-1 channel from sequence comparisons.

We examined the ability of MOD-1 to form functional channels in voltage-clamped Xenopus laevis oocytes. Oocytes injected with mod-1 complementary RNA (cRNA) and voltage-clamped at -70 mV responded to an application of 1 μM 5-HT with a large, rapidly developing, non-oscillatory inward current (Fig. 4a), whereas control oocytes injected with water showed no such response to application of any agonist (data not shown). Applications of 1,000 μM glycine, acetylcholine, GABA, glutamate, or histamine — all neurotransmitters that can activate ion channels —
did not elicit an inward current in *mod-1*-injected oocytes (Fig. 4a; and data not shown). Similarly, 10 μM ivermectin failed to elicit any current in *mod-1*-injected oocytes (data not shown). Ivermectin, an anthelmintic drug that activates glutamate-gated chloride channels in nematodes, can activate a rat α1β2γ2S- GABA-gated chloride channel and enhances acetylcholine-evoked responses of the human nAChRα7 receptor. Dopamine, octopamine or tyramine did not result in *mod-1*-dependent inward currents at 100 μM, but elicited small responses at millimolar concentrations (data not shown). *mod-1* mutants did not have defects associated with impaired dopamine or octopamine signaling (ref. 3; and M. Alkema, R.R., and H.R.H., unpublished observations). We conclude that 5-HT is most likely to be the native ligand for the MOD-1 channel. 5-HT dose-response experiments using oocytes showed that the MOD-1 channel has an effector concentration for half-maximal response (EC50) of 1.0 ± 0.1 μM (Fig. 4b), which is lower than that of the human 5-HT3a channel (2.9 ± 0.1 μM). Unlike the 5-HT3a channel, the MOD-1 channel was not blocked by calcium (Fig. 4c). High concentrations of granisetron (Fig. 4d) and ondansetron (data not shown), both of which are potent antagonists of the 5-HT3a channel, did not affect the action of 5-HT on MOD-1 channels. Taken together, these results indicate that the MOD-1 channel is very different from the 5-HT3a channel.

Pre-treatment of wild-type *C. elegans* with mianserin or methiothepin — 5-HT receptor antagonists — prevents food-deprived animals from exhibiting the wild-type enhanced slowing response after they encounter bacteria. Therefore, even though both compounds have so far been considered to be primarily antagonists of metabotropic 5-HT receptors, we tested their effects on MOD-1 in oocytes. The MOD-1 channel was inhibited by mianserin and methiothepin, with inhibition constants (Ki) of ~19 μM and ~32 μM, respectively (Fig. 4e; and data not shown). Pre-treatment of *mod-1* mutants with mianserin or methiothepin did not further affect the defective enhanced slowing response of these animals (data not shown). Mianserin and methiothepin may therefore interfere with the enhanced slowing response of *C. elegans* by antagonizing the MOD-1 5-HT-gated channel.

We tested the effect on channel function of the MOD-1(A281V) substitution in the *mod-1(n3034)* mutant. When cRNA encoding MOD-1(A281V) was injected into oocytes, no 5-HT-gated responses were observed (Fig. 4f). When the mutant cRNA was co-injected with roughly a fourfold excess of wild-type *mod-1* cRNA, the magnitude of the current through the wild-type channels was markedly reduced compared to that of oocytes that had been injected in parallel with the same amount
of only the wild-type cRNA (Fig. 4f). These findings suggest that the MOD-1 channel is multimeric, and that mutant MOD-1(A281V) channel subunits may interfere in a dominant manner with the function of wild-type MOD-1 channel subunits. Alternatively, MOD-1(A281V) channel subunits might interfere with wild-type MOD-1 channel function in a non-specific manner, for example, by affecting assembly, transport or stability of all membrane proteins. Our genetic data support the hypothesis that mod-1(n3034) encodes a dominant-negative form of MOD-1.

Current-voltage (I-V) relationships revealed that the 5-HT-dependent current from MOD-1-expressing oocytes reverses direction (in ND-96 bath solution; see Methods) near -20 mV (data not shown), significantly different from the near 0 mV reversal potential of the 5-HT₃ₐ non-selective cation channel (ref. 2; and our unpublished data). The chloride reversal potential (Eᵥ) in Xenopus oocytes is approximately -20 mV (ref. 21), consistent with the hypothesis that MOD-1 is a chloride channel. Moreover, when external sodium chloride was replaced with choline chloride, there was no change in the MOD-1 reversal potential (data not shown), indicating that sodium was not the primary charge carrier through the MOD-1 channel.

We directly tested whether changes in the concentration of chloride ions affected the reversal potential of the MOD-1 channel. To avoid activities of chloride channels endogenous to the Xenopus oocyte and to be able to manipulate the concentrations of ions on both sides of the membrane, we conducted these experiments using human embryonic kidney 293 (HEK293) cells transiently transfected with a mod-1 cDNA. We found that the peak I-V relationship showed no evidence of rectification (Fig. 5a), indicating that there was no voltage-dependent block by divalent cations or small organic molecules. Mock-transfected control HEK293 cells did not have any 5-HT-gated responses (data not shown). We also found that a 10-fold change in external chloride concentration shifted the reversal potential by 53 mV (Fig. 5b), in good agreement with the theoretical shift of 58 mV predicted by the Nernst equation if the MOD-1 channel were perfectly selective for chloride ions. These data establish that MOD-1 is a 5-HT-gated chloride channel.

As the M2 region of the MOD-1 channel is similar to those of GABA- and glycine-gated chloride-selective channels (Fig. 2c) and these show that these channels are permeant to anions other than chloride, we determined whether MOD-1 channels were also permeant to several anions. We measured the MOD-1 reversal potential in Xenopus oocytes, substituting chloride ions with an equivalent concentration of six other anions of various sizes. These measurements established
that the rank order of anion permeability for MOD-1 channels was thiocyanate \((E_{\text{rev}} < -40 \text{ mV})\) > bromide = iodide > chloride \((E_{\text{rev}} = -20 \text{ mV})\) > fluoride > aspartate >> gluconate \((E_{\text{rev}} > +50 \text{ mV})\) (data not shown). This rank order is in agreement with the rank order for GABA- and glycine-gated chloride channels\(^{22}\), suggesting that the permeation properties of the MOD-1 channel are similar to those of GABA- and glycine-gated chloride channels.

We generated a green fluorescent protein (GFP) reporter of \(\text{mod-1}\) by inserting the \(\text{gfp}\) gene\(^{23}\) into the \(\text{mod-1}\) minimal genomic rescuing fragment between the M3 and M4 regions (Fig. 2a). We obtained stable chromosomally integrated lines of this construct, as previously described\(^{24}\), and showed them to be rescued for the \(\text{mod-1}\) mutant phenotype (data not shown). \(\text{mod-1}::\text{GFP}\) reporter expression was observed in the cell bodies and axons (the latter presumably as a consequence of reporter overexpression) of several neurons in the head, ventral cord and tail of the animal (data not shown). No reporter expression was observed in any muscle cells. These observations indicate that the MOD-1 5-HT-gated chloride channel probably functions in neurons in \(\text{C. elegans}\).

Voltage-clamp recordings from isolated embryonic Retzius cells from the medicinal leech \(\text{Hirudo medicinalis}\) indicate that 5-HT applications can result in hyperpolarizing chloride currents\(^{25}\). Recordings from dialyzed whole cells and outside-out patches from these Retzius cells suggest that the 5-HT-gated chloride current is a consequence of a direct gating of a channel rather than the output of an indirect mechanism requiring second messenger cascades\(^{26}\). Our molecular identification and characterization of MOD-1 support this conjecture and demonstrate directly the existence of a 5-HT-gated chloride channel. We show that this channel acts as a 5-HT receptor \(\text{in vivo}\) and that its function is necessary for a 5-HT-mediated experience-dependent modulation of behavior. If mammalian counterparts of MOD-1 exist, their identification and characterization might provide insights concerning the myriad neurobiological effects of 5-HT and define new targets for the development of human pharmaceuticals.
Methods

Mapping and cloning of mod-1

We mapped mod-1 using standard procedures (see Supplementary Information for details). We performed germline transformation experiments by injecting the various constructs along with 80 μg ml⁻¹ pL15EK (which contains the wild-type lin-15 gene) into a mod-1(n3034); lin-15(n765ts) strain and scoring stable transgenic lines, which produced non-Lin progeny. Rescued lines had at least half the animals exhibiting normal sensitivity to 5-HT at 5 min in assays of 5-HT resistance.

Behavioral assays

Locomotory rate was assayed as previously described. In Figs 1a and 3b, each trial involved testing at least five animals for each of the conditions; a given animal was tested for only one condition. P values were calculated by comparing the combined data for the mutants from all the separate trials to the combined data for the wild-type animals assayed in parallel for each condition of each separate trial. To assay 5-HT resistance, we placed 20 animals in 200 μl of 33 mM 5-HT (creatinine sulphate salt, Sigma) dissolved in M9 buffer in 96-well microtiter wells and scored the swimming behavior of such animals as active or immobile every minute for 20 min for the time-course experiments, at 5 min for germline transformation rescue experiments, or at 20 min (Fig 3a). In this assay, an animal was scored as immobile if it did not exhibit any swimming motion for a period of 5 seconds.

Electrophysiological studies of MOD-1

An EcoRI/EcoRI fragment containing a full-length mod-1 cDNA, including the 5' and 3' UTRs, was cloned into an EcoRI site of the vector pGEMHE for oocyte expression and into the EcoRI site of vector GW1-CMV (British Biotech.) for transfection into HEK293 (ATCC # CRL-1533) cells. Capped cRNA for the oocytes was synthesized as described and dissolved in water. RNA concentrations were determined by agarose gel electrophoresis and absorption spectroscopy. Xenopus oocytes were collected and injected with 50 nl RNA as described, and two-electrode voltage-clamp recordings were performed 1-3 days later at room temperature (22-25°C) as described. All compounds and wash solutions were
applied to oocytes using a gravity-assisted perfusion system. The standard bath solution for the agonist, antagonist, and dose-response experiments was ND-96 (in mM): 96 NaCl, 2 KCl, 0.3 CaCl$_2$, 1 MgCl$_2$, 5 HEPES (pH 7.6). In Fig. 4b, each oocyte was sequentially subjected to 5-s treatments of increasing concentrations of 5-HT with 30 s of wash between applications. This protocol was continued from 10 nM to 1 µM and then only one concentration higher than 1 µM was tested on any particular oocyte, since concentrations greater than 1 µM 5-HT led to desensitization and a decrease in the maximal response elicited by subsequent 5-HT applications. Responses from separate oocytes were combined by normalizing the amplitudes of each oocyte's responses to the various 5-HT concentrations to that elicited by 1 µM 5-HT. The normalized data were fit to the function $y = V_{\text{max}} \left[ x^n / (k + x^n) \right]$ with a Hill coefficient ($n$) = 1 and then re-normalized to a maximal value of 1 by dividing by $V_{\text{max}}$.

In Fig. 4c, 5-HT$_3$-injected oocytes were first tested in buffer containing 0.3 mM Ca$^{2+}$ and then 10 mM Ca$^{2+}$, as the 5-HT$_3$-injected oocytes recovered very slowly from the 10 mM Ca$^{2+}$ treatment. mod-1-injected oocytes were tested in both orders. The concentration of Ca$^{2+}$ was varied in both the 5-HT and wash solutions. The 5-HT$_3$ traces were filtered digitally off-line at 5 Hz to reduce noise. For the experiments shown in Fig. 4d and 4e, the antagonists were present in both the 5-HT and wash solutions.

HEK293 cells were transiently transfected and whole-cell voltage-clamp recordings were performed the next day as described$^{30}$. The output was filtered at 10 kHz and sampled at 20 kHz using a Digidata 1200 interface. The patch pipette was coated with Sylgard, and the tip was heat-polished to a final tip resistance in bath solution of 0.5-2 MΩ. The HEK293 cell was lifted from the dish with the recording pipette, and 5-HT was applied from a pressure-controlled spritzer pipette in the presence of a continuously flowing bath solution. The internal (patch pipette) solution contained (in mM) 130 KAsparate, 10 KCl, 1 MgCl$_2$, 10 EGTA, 10 HEPES (pH 7.4). The external solution contained (in mM) 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES (pH 7.4), with varying proportions of sodium Gluconate and NaCl, resulting in a final concentration of 140 mM of both Na$^+$ cations and gluconate plus Cl$^-$ anions. Data analysis was performed off-line using Clampfit software (Axon), and curve fitting was accomplished using Origin (Microcal) or KaliedyGraph (Adelbeck) software.
Acknowledgements

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CORRESPONDENCE and requests for materials should be addressed to H. R. H. (e-mail: horvitz@mit.edu). The nucleotide sequence of the mod-1 cDNA has been deposited at GenBank under the accession number AF303088.
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Supplementary Information

A281V may block channel pore

When the alpha helix that forms the M2 domain of MOD-1 is viewed in a helical-wheel configuration\(^1\), Ala-281 is on the side of the alpha helix analogous to the pore-facing side of the nAChR\(^2\). Moreover, Ala-281 is in a position analogous to that of Ser-252 in nAChR\(\alpha\) and Thr-265 in GABA-A \(\beta1\), both of which have been predicted to face the lumen of the channel pore as a result of their accessibility to modification by polar sulfhydryl reagents\(^2\). We suggest that the addition of the two methyl side groups caused by the A281V substitution predicted in \(mod-1(n3034)\) mutants alters the pore of the channel and prevents ion flow.

\(mod-1\) deletion mutants

The \(ok103\) deletion removes 4135 bp, starting four bp after the first exon. The \(nr2043\) deletion removes 1209 bp, starting 146 bp 5' of exon 3, and adds the sequence ATAAAGTTTTTTT. If the end of exon 2 splices onto the next available splice acceptor site at the start of exon 6, an additional 15 unrelated amino acids would be present before the protein ends at a premature stop codon. The cDNA predicted by the \(C.\) \(elegans\) Genome Sequencing Project\(^3\) omits the first and seventh exons and does not include the first 54 bp of the second exon.

Methods

Mapping and cloning of \(mod-1\)

\(mod-1\) was mapped to LG V based upon its two-factor linkage to \(dpy-11\) \(unc-76\) \(V\). The following three-factor data were obtained: \(dpy-11\) (14/36) \(mod-1\) (22/36) \(unc-76\), \(dpy-11\) (27/40) \(mod-1\) (13/40) \(unc-42\), \(mod-1\) (0/32) \(unc-42\) (32/32) \(sma-1\), \(dpy-11\) (38/74) \(stP23\) (25/74) \(mod-1\) (11/74) \(unc-42\), \(dpy-11\) (38/74) \(pkP5056\) (25/74) \(mod-1\) (11/74) \(unc-42\), \(dpy-11\) (36/45) \(mod-1\) (6/45) \(pkP520\) (3/45) \(unc-42\), \(let-434\) (19/33) \(mod-1\) (14/33) \(unc-42\). \(stP23\), \(pkP5056\), and \(pkP520\) are Tc1-transposon polymorphisms present in strains RW7000 and KR1787, respectively\(^3\). All mapping experiments were performed by mating each homozygous recombinant with wild-type (N2) males and scoring the F1 cross progeny for semi-dominant 5-HT
resistance at 20 min. The presence of the Tc1 polymorphisms was assayed by performing PCRs with a Tc1-specific primer and a primer from genomic sequence adjacent to the Tc1 insert site on crude whole-worm lysates prepared from each homozygous recombinant strain.

References

Figures

Figure 1 Phenotypic characterization and cloning of mod-1

a, mod-1(n3034) mutants were defective in the enhanced slowing locomotory response exhibited by food-deprived wild-type animals. Ten trials were performed for each genotype. Error bars represent s.e.m.; asterisks: $P<0.0001$, Student’s $t$-test.

b, Time course of response to 33 mM 5-HT. The resistance to exogenous 5-HT of mod-1(n3034) mutants was recessive at early time-points and was semi-dominant at later time-points.

c, Genetic and physical maps of the relevant region of linkage group V. Cosmid C38E12 and subclones shown in bold rescued mod-1; +, rescue; -, no rescue. Numbers in parentheses represent the fraction of transgenic lines tested that rescued mod-1.
Figure 1

(a) Bar graph showing body bends per 20s for well-fed and food-deprived conditions. The graph compares bacterial genotypes and genotypes: - Wild type, + mod-1(n3034).

(b) Time course of animals immobilized. The graph compares wild type, mod-1/+, and mod-1.

(c) Genetic map of let-434, mod-1, and pkP520. The map indicates the mod-1 rescue for each condition: - (0/2), + (3/7).

Genotypes: - Wild type, + mod-1/+

Bacteria: - Wild type, + mod-1(n3034)

Genotype: - Wild type, + mod-1(n3034)

Time (min): 0, 5, 10, 15, 20

Animals Immobilized: 0, 20, 40, 60, 80, 100

Genetic Map:

- let-434
- mod-1
- pkP520

0.1 map unit

C31A10

C38E12

5.5 kb minimal rescuing fragment

Restriction Enzymes:

ClaI, SacI, ScaI, EcoRI, MscI

Clade Diagram:

1 kb

- (0/2)
- (0/5)
+ (9/9)
- (0/3)
+ (5/5)
- (0/4)
Figure 2 Sequence analysis of mod-1

a, mod-1 intron-exon structure. Open boxes, coding regions; lines, untranslated regions, arrow, direction of transcription; SL1, SL1 trans-spliced leader. The ok103 and nr2043 deletions are depicted (See Supplementary Information), and the positions of the n3034 mutation and insertion site of the gfp gene are indicated.

b, Amino-acid sequence alignment of MOD-1 with a C. elegans predicted protein (P41849) that is `most similar to MOD-1, human GABA_A receptor β1 subunit (A40336), human glycine receptor β subunit (NP_000815.1), and the human 5-HT3_a subunit (BAA08387). The putative signal sequence and M1-M4 regions are underlined, and the Cys-Cys loop is indicated (S-S). Amino acids conserved between MOD-1 and at least one other protein are shown in black boxes. mod-1(n3034) is a C-to-T mutation resulting in a A281V substitution.

c, Alignment of the M2 regions from MOD-1, human GABA_A α1 subunit (A60652), human GABA_A β1 subunit, human glycine α1 subunit (NP_000162), human glycine β1 subunit, 5-HT3_a, 5-HT3_b (AF080582), and human nAChR α subunit (P02708). Dots, residues likely to be facing the pore in the nAChR\textsuperscript{10}; arrows, residues likely to be facing the pore in the GABA_A receptor\textsuperscript{10}; circles, residues conserved in cation channels that are not conserved in MOD-1.
Figure 2
Figure 3 *mod-1* deletion alleles caused 5-HT resistance and defects in the enhanced slowing response

**a**, 5-HT sensitivity, scored after 20 min in 33 mM 5-HT. Ex[MOD-1(A281V)] is an extrachromosomal array containing the wild-type *lin-15* gene and the *mod-1* genomic locus encoding the A281V mutant form of the MOD-1 protein. Control transgenic lines with extrachromosomal arrays containing wild-type *lin-15* and *mod-1* genes were not resistant to 5-HT (data not shown).

**b**, *mod-1* deletion mutants were defective in the enhanced slowing response. Ten trials were performed for each genotype. Error bars represent s.e.m.; asterisks: \( P<0.0001 \), Student's \( t \)-test.
Figure 4 MOD-1 is a 5-HT-gated ion channel distinct from the 5-HT$_{3a}$ channel

Recordings from *Xenopus* oocytes voltage clamped at -70 mV in standard ND-96 bath solution, except in c where Ca$^{++}$ concentrations were varied. Bars, durations of agonist application.

a, 1 μM 5-HT elicited a rapidly developing inward current from oocytes injected with *mod-1* cRNA. Representative trace from an oocyte treated with 1 mM Gly, ACh, and GABA, and 1 μM 5-HT in ND-96 (n=5). The order of agonist application was not important (data not shown).

b, 5-HT dose response curve; EC$_{50}$ = 1.0 ± 0.1 μM. n ≥ 4 for each concentration; error bars represent s.e.m.

c, Extracellular calcium did not block the MOD-1 channel. Representative traces from oocytes injected with *mod-1* (n=3) or 5-HT$_{3a}$ (n=2) cRNA and treated with 1 μM (*mod-1*) or 10 μM (5-HT$_{3a}$) 5-HT.

d, The 5-HT$_{3a}$-specific blocker granisetron did not block MOD-1. Representative traces from oocytes injected with *mod-1* or 5-HT$_{3a}$ cRNA (n=2 for each) and treated with 1 μM (*mod-1*) or 10 μM (5-HT$_{3a}$) 5-HT in the presence or absence of 130 μM granisetron.

e, Mianserin blocked the MOD-1 channel. Representative traces from *mod-1*-injected oocytes (n=5) treated with 0.5 μM 5-HT in the presence or absence of 50 μM mianserin.

f, The MOD-1(A281V) mutant channel has a dominant-negative effect. Representative traces from oocytes injected with wild-type *mod-1* cRNA (WT), *mod-1* cRNA encoding the A281V mutant protein (A281V), or 4 parts WT to 1 part A281V (WT+A281V) (n ≥ 4 for each class). Wild-type oocytes were treated with 1 μM 5-HT; A281V and WT+A281V oocytes were treated with 10 μM 5-HT. 1 μM 5-HT did not elicit any response from either A281V or WT+A281V oocytes.
Figure 4

a

Gly  ACh  GABA  5-HT

0.5 μA  10 s

b

Relative Response

[5-HT] (μM)

1 10 10 10

0 0.2 0.4 0.6 0.8 1

c

MOD-1

5-HT

10 mM Ca++

0.3 mM Ca++

2 μA  10 s

5-HT3a

5-HT

10 mM Ca++

0.3 mM Ca++

250 nA  10 s

d

MOD-1

5-HT

granisetron

2 μA  10 s

5-HT3a

5-HT

granisetron

250 nA  10 s

e

5-HT

mianserin

250 nA  10 s

f

5-HT

A281V

WT+ A281V

0.5 μA  10 s
Figure 5 MOD-1 is a 5-HT-gated chloride channel

Whole-cell recordings from HEK293 cells transfected with a mod-1 cDNA.

a. The I-V relationship of the maximal responses from a representative cell when the external solution contained 120 mM NaGluconate and 20 mM NaCl, resulting in $[\text{Cl}^-]_{\text{out}} = 26 \text{ mM}$, $[\text{Na}^+]_{\text{in}} = 0$, and $[\text{Cl}^-]_{\text{in}} = 12 \text{ mM}$ (see Methods for complete salt compositions). $E_{\text{rev}} = -9.1 \text{ mV}$. Inset, raw traces for this cell at each of the nine voltage steps from -50 mV to +30 mV. Bar, duration of 100 nM 5-HT application.

b. The reversal potential varied linearly with log$[\text{Cl}^-]_{\text{out}}$. Each data point represents $E_{\text{rev}}$ of a single cell at one particular $[\text{Cl}^-]_{\text{out}}$ as in a.
Figure 5

a

$\begin{align*}
\text{I (pA)} &= 0 \\
\text{V (mV)} &= -60 -40 -20 20 40 \\
\text{time (msec)} &= 900 \\
\text{E}_{\text{rev}} &= -9.1 \text{ mV} \\
[\text{Cl}^-]_{\text{out}} &= 26 \text{ mM}
\end{align*}$

b

$slope = -53 \text{ mV/10-fold change in } [\text{Cl}^-]_{\text{out}}$
Mutations in the *C. elegans* Serotonin Reuptake Transporter MOD-5 Reveal Serotonin-Dependent and -Independent Activities of Fluoxetine

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Carol Trent characterized the initial 5-HT-uptake defect in *n822* and *n823* mutants (Figure 1A); Beth Sawin confirmed these uptake defects with anti-5-HT antibodies (Figure 1B), established that these mutants exhibited a hyperenhanced slowing response (parts of Figure 2A), performed the NSM ablations (Figure 2B), and analyzed the *mod-5; cat-4* double mutants (Figure 2D). I performed the rest of the experiments and wrote the text.
Summary

We isolated two mutants defective in the uptake of exogenous serotonin (5-HT) into the neurosecretory motor neurons (NSMs) of C. elegans. These mutants were hypersensitive to exogenous 5-HT and hyperresponsive in the experience-dependent enhanced slowing response to food modulated by 5-HT. The two allelic mutations defined the gene *mod-5* (*modulation of locomotion defective*), which encodes the only serotonin reuptake transporter (SERT) in C. elegans. The selective serotonin reuptake inhibitor (SSRI) fluoxetine (Prozac) potentiated the enhanced slowing response, and this potentiation required *mod-5*, establishing a 5-HT- and SERT-dependent behavioral effect of fluoxetine in C. elegans. By contrast, other responses of C. elegans to fluoxetine were independent of MOD-5 SERT and 5-HT. Further analysis of the MOD-5-independent behavioral effects of fluoxetine should lead to the identification of novel targets of fluoxetine and could facilitate the development of more specific human pharmaceuticals.
Introduction

The activity of serotonin (5-HT), a key neuromodulator, is mediated postsynaptically through metabotropic (Martin et al., 1998) and ionotropic (Maricq et al., 1991; Ranganathan et al., 2000) 5-HT receptors and their downstream signaling components (Hille, 1992). 5-HT modulates several behaviors of the nematode Caenorhabditis elegans, including egg laying and locomotion (Horvitz et al., 1982; Trent et al., 1983; Avery and Horvitz, 1990; Schafer and Kenyon, 1995). 5-HT also mediates the enhanced slowing response exhibited by food-deprived nematodes upon encountering bacteria (Sawin et al., 2000).

5-HT neurotransmission can be regulated by the removal of 5-HT from the synaptic cleft by a serotonin reuptake transporter (SERT) (Cooper et al., 1996). Na+/Cl−-dependent SERTs were cloned first from rats (Blakely et al., 1991; Hoffman et al., 1991) and subsequently from other species (Mortensen et al., 1999 and references cited therein), including humans and Drosophila melanogaster. SERT antagonists, such as the selective serotonin reuptake inhibitors (SSRIs) fluoxetine (Prozac), paroxetine (Paxil), and sertraline (Zoloft), are broadly used in the treatment of psychiatric disorders (Schloss and Williams, 1998). Therefore, in addition to the basic question of SERT function and regulation, it is of particular clinical importance to understand SERT function in vivo. SERT-deficient mice do not show gross developmental defects but have reduced 5-HT levels in the brain (Bengel et al., 1998), are insensitive to 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy")-induced hyperactivity (Bengel et al., 1998), and show a brain region- and gender-specific reduction in the density and expression of 5-HT1a receptors (Li et al., 2000). However, no obvious behavioral defects or abnormal responses to SSRIs in these SERT−/− mice have been reported.

In this paper, we report the isolation of two C. elegans SERT-deficient mutants and describe studies of these mutants revealing that the C. elegans SERT is required for the experience-dependent enhanced slowing response and that the SSRI fluoxetine can act on both 5-HT and SERT-dependent and 5-HT- and SERT-independent targets.
Results

Isolation of Mutants Defective in 5-HT Uptake

Formaldehyde-induced fluorescence (FIF) histochemistry indicates that the *C. elegans* neurosecretory motor neurons (NSMs), located in the pharynx, contain 5-HT in their cell bodies and axonal processes (Horvitz et al., 1982). FIF in the NSMs was more readily observed when animals were preincubated with exogenous 5-HT prior to the staining protocol (Horvitz et al., 1982; and our unpublished results). This observation suggested that the NSMs possess an active uptake system that can concentrate 5-HT from the extracellular environment.

We performed a genetic screen for mutants lacking FIF in the NSMs following preincubation with exogenous 5-HT (see Experimental Procedures). Two mutations that failed to complement each other, *n*822 and *n*823, were isolated. *n*822 and *n*823 mutants lacked FIF in the NSM cell bodies after 5-HT preincubation but retained FIF in the NSM axonal processes (Figure 1A). Nomarski optics (Ellis and Horvitz, 1986) revealed that these mutants had NSM cell bodies in their usual positions (data not shown).

5-HT can also be detected in *C. elegans* using anti-5-HT antisera, which have proven to be more sensitive than FIF and have allowed the reliable detection of endogenous 5-HT in the NSMs and other neurons without requiring preincubation with exogenous 5-HT (Desai et al., 1988; Loer and Kenyon, 1993; Sawin et al., 2000). We used anti-5-HT antisera to evaluate 5-HT reuptake in *n*822 and *n*823 mutants. Since endogenous 5-HT masks 5-HT reuptake in NSMs visualized using anti-5-HT antisera, we used a *cat-4(e1141)* (catecholamine defective) genetic background to reduce endogenous 5-HT levels (Desai et al., 1988; Weinshenker et al., 1995). *cat-4* encodes GTP cyclohydrolase I (C. Loer, personal communication) which is required for the synthesis of a biopterin co-factor needed for dopamine and 5-HT biosynthesis (Kapatos et al., 1999).

We counted the number of immunoreactive NSMs in *cat-4* single and *n*822; *cat-4* and *n*823; *cat-4* double mutants with or without exogenous 5-HT preincubation. While NSMs in the *cat-4* mutants were capable of 5-HT uptake, the NSMs in *n*822; *cat-4* and *n*823; *cat-4* double mutants were partially defective in 5-HT uptake (Figure 1B), confirming our FIF observations. For example, we observed no brightly-fluorescing NSMs in *n*823; *cat-4* mutants preincubated with 5-HT, while 43% of the NSMs in *cat-4* mutants had bright immunofluorescence (Figure 1B, black...
bars). That a slight increase in NSM immunoreactivity was observed in n822; cat-4 and n823; cat-4 mutants upon 5-HT preincubation suggests that the n822 and n823 mutations do not lead to a complete loss of 5-HT uptake. While previous reports (Desai et al., 1988; Loer and Kenyon, 1993; Weinshenker et al., 1995) did not detect 5-HT immunoreactivity in cat-4 mutants, we routinely observed a small percentage of 5-HT-positive NSMs in cat-4 mutants in the absence of 5-HT preincubation (Figure 1B). Our data argue that the cat-4 mutation does not lead to a complete loss of 5-HT, consistent with the conclusions of Desai et al. (1988) and Avery and Horvitz (1990) but not with those of Loer and Kenyon (1993) and Weinshenker et al. (1995). For this reason, previous conclusions about the actions of fluoxetine (Weinshenker et al., 1995; Choy and Thomas, 1999) based on the assumption that cat-4 mutants completely lack 5-HT need to be revisited (see below).

5-HT-Uptake Mutants Exhibit a Hyperenhanced Slowing Response

Since n822 and n823 mutants were defective in 5-HT uptake, we sought to determine if these mutants were abnormal in their responses to endogenous 5-HT release. We tested the 5-HT-dependent enhanced slowing response (Sawin et al., 2000) of these mutants. Whereas well-fed wild-type animals slow their locomotory rate slightly in response to bacteria (the basal slowing response) food-deprived wild-type animals display a greater degree of slowing of locomotory rate in response to bacteria (the enhanced slowing response) (Sawin et al., 2000; and Figure 2A). Strikingly, n822 and n823 mutants exhibited a hyperenhanced slowing response: on Petri plates with bacteria, the locomotory rates of food-deprived n822 and n823 mutants slowed significantly more than did those of food-deprived wild-type animals (Figure 2A, gray bars). On Petri plates without bacteria, the locomotory rates of food-deprived n822 and n823 mutants were not significantly different from those of food-deprived wild type animals (Figure 2A, gray bars). Well-fed n822 and n823 mutants exhibited no defect in the 5-HT-independent dopamine-dependent basal slowing response to bacteria (Figure 2A, black bars) (Sawin et al., 2000). Genes involved in the enhanced slowing response are called mod (modulation of locomotion defective) (Sawin et al., 2000), and we named the gene defined by the allelic mutations n822 and n823 mod-5. The hyperenhanced slowing responses of these mod-5 mutants were presumably a consequence of a defect in the clearing of 5-HT from the relevant synapses by uptake into serotonergic neurons, thereby leading to increased 5-HT signaling and a greater inhibition of locomotion.
Ablation of the Serotonergic NSMs or Decrease in Endogenous 5-HT Suppresses mod-5 Mutations

Ablation of the serotonergic NSMs with a laser microbeam leads to a defect in the enhanced slowing response (Sawin et al., 2000). Since the NSMs were defective in 5-HT uptake in mod-5 mutants (see above), we tested whether ablation of the NSMs affected the hyperenhanced slowing response of mod-5 mutants. On Petri plates with bacteria, food-deprived NSM-ablated mod-5(n823) mutants exhibited an enhanced slowing response that was significantly reduced in comparison to that of food-deprived mock-ablated mod-5(n823) mutants (Figure 2B, gray bars). Well-fed NSM-ablated mod-5(n823) mutants were not significantly affected in their basal slowing response to bacteria (Figure 2B, black bars). Ablation of the 15 neuron, another pharyngeal neuron, had no effect on the enhanced slowing response of mod-5 mutants (data not shown), indicating that the effect of the NSM-ablations was not a consequence of the ablation protocol per se.

We reasoned that the ablation of the NSMs probably led to a loss of 5-HT needed for the hyperenhanced slowing exhibited by mod-5 mutants. To test this hypothesis, we tested whether cat-4 mutants, which have decreased 5-HT levels (see above), could suppress the mod-5 phenotype. cat-4 mutants are defective in the enhanced slowing response (Sawin et al., 2000; and Figure 2C), and the reduced 5-HT in these mutants is the cause of this defect (Sawin et al., 2000).

On Petri plates with bacteria, the locomotory rate of food-deprived mod-5(n823); cat-4 double mutants was significantly faster than that of food-deprived mod-5(n823) mutants (compare Figure 2C to Figure 2A gray bars) but similar to that of NSM-ablated mod-5(n823) mutants (Figure 2B), suggesting that for the enhanced slowing response the ablation of the NSMs is equivalent to a reduction in 5-HT levels in the animal. That the locomotory rate of food-deprived mod-5(n823); cat-4 double mutants was significantly slower than that of cat-4 mutants (Figure 2C) is likely a consequence of the effect of residual 5-HT in cat-4 mutants (Figure 1B).

We propose that when food-deprived mod-5 mutants encounter bacteria, the NSMs release 5-HT and this 5-HT is inefficiently cleared, thus causing the hyperenhanced slowing response.
mod-5 Mutants Are Hypersensitive to Exogenous 5-HT

Exogenous 5-HT inhibits wild-type C. elegans locomotion (Horvitz et al., 1982). To determine if mod-5(n822) and mod-5(n823) mutants were abnormal in their response to exogenous 5-HT, we used a liquid swimming assay (Ranganathan et al., 2000). In this assay, mod-5(n822) and mod-5(n823) mutants were hypersensitive to exogenously added 5-HT (Figure 2D), presumably because this 5-HT was not efficiently cleared from the relevant synapses.

MOD-5 Is Similar to SERTs

We used the 5-HT hypersensitivity of mod-5 mutants in the liquid swimming assay to map and clone the gene. We mapped mod-5 to a ~2.0 map unit interval on chromosome I, between fog-1 and unc-11 (Figure 3A) (see Experimental Procedures). We noted an open reading frame, Y54E10A.154.A, in this region predicted to encode a protein with similarity to serotonin reuptake transporters (SERTs). As loss of SERT function would provide a simple explanation for both the defective 5-HT uptake and 5-HT hypersensitivity of mod-5 mutants, we decided to determine if Y54E10A.154.A was mod-5.

We generated an eight kilobase (kb) polymerase chain reaction (PCR) product from the genomic region spanning the first eight exons of Y54E10A.154.A (Figure 3A) and encoding the first 507 amino acids of the corresponding predicted protein. This construct robustly rescued the 5-HT hypersensitivity phenotype of mod-5(n823) mutants in 16 of 20 transgenic lines tested (data not shown). We obtained a partial cDNA clone of Y54E10A.154.A using reverse transcriptase-PCR (RT-PCR) and determined the 5' and 3' ends of the cDNA using 5'- and 3'-rapid amplification of cDNA ends (RACE), respectively. The 5' end of the cDNA contained an SL1 trans-spliced leader, which is found at the 5' ends of many C. elegans transcripts (Krause and Hirsh, 1987). The 3' end contained a poly-A stretch, indicating that we had determined the complete Y54E10A.154.A transcriptional unit (Figure 3A). This cDNA was capable of rescuing the 5-HT hypersensitivity of mod-5 mutants (see below).

Protein sequence comparisons revealed that the predicted protein encoded by our full-length cDNA is 44% identical to human SERT (hSERT; Ramamoorthy et al., 1993) and 45% identical to the other known invertebrate SERT, from Drosophila melanogaster (dSERT; Corey et al. 1994), which is itself 51% identical to hSERT
(Figure 3B). We identified single-base mutations in the Y54E10A_154.A coding sequence in mod-5(n822) and mod-5(n823) mutants (Figure 3B). The mutation in mod-5(n822) is predicted to change cysteine 225 (codon TGT) to an opal stop codon (TGA). The mutation in mod-5(n823) is predicted to change proline 569 (CCG) to serine (TCG) within a transmembrane region. We concluded that Y54E10A_154.A is mod-5.

Like SERTs from other species (Barker and Blakely, 1998 and references cited therein), the MOD-5 protein is predicted to contain 12 putative transmembrane regions (Figure 3B). Much of the sequence conservation is clustered in or around these transmembrane regions, suggesting that the membrane topology of the SERTs is important for their function. At position 119 (Figure 3B, diamond) within the first predicted transmembrane domain, MOD-5 has an aspartate residue that is conserved in 5-HT, dopamine, and norepinephrine (NE) reuptake transporters but not in gamma-aminobutyric acid (GABA) reuptake transporters (Barker and Blakely, 1998). This aspartate may be involved in binding to the amino group in 5-HT, dopamine, and NE (Kitayama et al., 1992). MOD-5 also has other similarities to hSERT and dSERT (Figure 3B legend).

**mod-5(n3314) is a Null Allele**

To determine the phenotypic consequence of completely eliminating mod-5 function, we screened libraries of mutagenized animals using PCR to identify large deletions (Jansen et al., 1997) in the mod-5 genomic locus. We isolated a deletion allele, n3314, that contains a 1688 base pair (bp) deletion in the mod-5 genomic locus (Figure 3A). The altered open reading frame (ORF) is predicted to encode the first 42 amino acids of MOD-5 and, if the end of exon 2 splices onto the next available splice-acceptor site at the start of exon 8, an additional 18 out-of-frame amino acids before ending at a premature stop codon.

n3314 displayed both a hyperenhanced slowing response and 5-HT hypersensitivity in the liquid swimming assay and failed to complement mod-5(n822) and mod-5(n823) for both these behaviors (data not shown), confirming that n3314 is an allele of mod-5. mod-5(n3314) mutants were more hypersensitive to 5-HT than were mod-5(n822) and mod-5(n823) mutants (Figure 2D). mod-5(n3314) mutants also exhibited a more severe hyperenhanced slowing response than did the nonsense mod-5(n822) and the missense mod-5(n823) mutants (Figure 2A). On Petri plates without bacteria, the locomotory rate of mod-5(n3314) mutants was not
different from that of the wild type (Figure 2A). Well-fed mod-5(n3314) mutants showed no defect in their basal slowing response to bacteria (Figure 2A). Given the stronger behavioral defects of mod-5(n3314) mutants and the molecular nature of the n3314 deletion, we believe that n3314 is a null allele of mod-5 and that both mod-5(n822) and mod-5(n823) are partial loss-of-function alleles. mod-5(n822), which is predicted to encode only the first 225 amino acids of MOD-5, is not a null allele, based on comparisons of the phenotypes of mod-5(n822), mod-5(n3314), and mod-5(n822)/mod-5(n3314) trans-heterozygous animals (Figures 2A and 2D; and data not shown). This activity of mod-5(n822) might be a consequence of the presence of full-length mod-5 RNA transcripts produced by read-through of the stop codon. Alternatively, it is conceivable that the first 225 amino acids of MOD-5 retain partial SERT function. This latter possibility is consistent with our rescue of the 5-HT hypersensitivity of mod-5(n823) mutants with a construct that encodes only the first 507 amino acids of MOD-5, suggesting that all 671 amino acids of MOD-5 are not essential for at least some aspects of SERT function.

Since we had isolated the mod-5 cDNA using RT-PCR and RACE, we sought to confirm that the protein encoded by this cDNA could function in vivo. We constructed a mini-gene in which the mod-5 cDNA was placed under the control of 2.7 kb of genomic DNA upstream to the first predicted methionine of mod-5. mod-5(n3314) animals transgenic for extrachromosomal arrays consisting of this mini-gene construct were no longer 5-HT hypersensitive (data not shown), confirming that we had defined a functional mod-5 gene and that the mod-5 cDNA could encode a functional SERT and was suitable for 5-HT-uptake assays in a heterologous system (see below).

MOD-5 Functions as a SERT in Mammalian Cells

Using retroviral-mediated gene transfer (see Experimental Procedures), we generated human embryonic kidney 293 (HEK293) cell lines that stably expressed MOD-5. Using these cell lines, we performed uptake assays similar to those previously done for other SERTs (Ramamoorthy et al., 1993; Demchyshyn et al., 1994). The uptake of [3H]5-HT by MOD-5-expressing cell lines was saturable, indicating that the accumulation of [3H]5-HT in the cells was facilitated by MOD-5 (Figure 4A). MOD-5-mediated [3H]5-HT transport was strictly dependent on Na+ ions (Figure 4B), as has been observed for 5-HT transport by hSERT (Ramamoorthy et al., 1993), rat SERT (rSERT; Blakely et al., 1991; Hoffman et al., 1991), and dSERT
(Demchyshyn et al., 1994). By contrast, MOD-5 did not display a strict dependence on Cl⁻ ions (Figure 4B), whereas both hSERT and rSERT, but not dSERT, do display such strict dependence (Blakely et al., 1991; Hoffman et al., 1991; Ramamoorthy et al., 1993; Demchyshyn et al., 1994).

MOD-5-mediated [³H]5-HT transport occurred in a concentration-dependent and saturable manner (Figure 4C), with a $K_m = 150 \pm 8$ nM, a value similar to those reported for other SERTs ($K_m$ range = 280 - 630 nM) (Blakely et al., 1991; Hoffman et al., 1991; Ramamoorthy et al., 1993; Corey et al., 1994; Demchyshyn et al., 1994; Chang et al., 1996; Padbury et al., 1997; Chen et al., 1998; Mortensen et al., 1999).

We tested the specificity of MOD-5 by assaying the ability of MOD-5 to transport various radiolabelled neurotransmitters besides 5-HT. MOD-5-mediated uptake was highly specific for [³H]5-HT and inefficient at translocating radiolabelled GABA, glutamate, glycine, NE, histamine, and dopamine (Figure 4D). We also tested the ability of these neurotransmitters to inhibit [³H]5-HT uptake via MOD-5. None of the six neurotransmitters tested, even when present at 100 µM, substantially inhibited the uptake of 50 nM 5-HT (data not shown). We also tested whether octopamine or tyramine, two invertebrate-specific neurotransmitters, could inhibit [³H]5-HT transport; we could not test MOD-5-mediated uptake of these neurotransmitters, as radiolabelled octopamine and tyramine are not available. Tyramine (100 µM) partially inhibited (54% ± 10% of control) the transport of [³H]5-HT (50 nM) by MOD-5; octopamine (100 µM) did not inhibit (86% ± 12% of control) MOD-5-mediated [³H]5-HT (50 nM) transport. By comparison, dSERT-mediated transport of 100 nM [³H]5-HT was reduced to 95% ± 15% of control by 200 µM tyramine and to 82% ± 15% of control by 200 µM octopamine (Corey et al., 1994). These data suggested that there are subtle differences in the properties of MOD-5 and dSERT.

We tested whether MOD-5-mediated [³H]5-HT transport was inhibited by tricyclic antidepressants, SSRIs, and non-specific monoamine transporter inhibitors (Blakely et al., 1991; Ramamoorthy et al., 1993; Demchyshyn et al., 1994). The rank order of potency for inhibition of MOD-5-mediated [³H]5-HT transport was imipramine ($K_i = 89 \pm 58$ nM) = fluoxetine ($K_i = 133 \pm 90$ nM) = paroxetine ($K_i = 179 \pm 64$ nM) > desipramine ($K_i = 334 \pm 115$ nM) > citalopram ($K_i = 994 \pm 298$ nM) >> cocaine ($K_i = 4076 \pm 349$ nM) (Figure 4E). This rank order is different from that of other SERTs (for example, the rank order of potency for inhibition of hSERT-mediated [³H]5-HT transport is paroxetine > fluoxetine > imipramine = citalopram >> cocaine; Ramamoorthy et al., 1993), and for some of the inhibitors the $K_i$ values

133
were higher than those reported for the other SERTs (Blakely et al., 1991; Hoffman et al., 1991; Ramamoorthy et al., 1993; Corey et al., 1994; Demchyshyn et al., 1994; Chang et al., 1996; Padbury et al., 1997; Chen et al., 1998; Mortensen et al., 1999).

Taken together, the specificity of MOD-5-mediated transport for 5-HT, the dependence of such transport on Na\(^+\) and Cl\(^-\) ions, and the inhibition of 5-HT transport by SSRIs establish that MOD-5 is a *C. elegans* SERT (CeSERT).

**MOD-5 Is Likely the Only SERT in *C. elegans***

To determine if MOD-5 is the only SERT in *C. elegans*, we analyzed the *C. elegans* genomic sequence for other potentials SERTs and performed *in vivo* assays of 5-HT uptake in *mod-5* mutants. We found 15 Na\(^+\)/Cl\(^-\)-dependent neurotransmitter transporter-like predicted ORFs in the completed *C. elegans* genomic sequence (The *C. elegans* Sequencing Consortium, 1998). Only two of these ORFs, T23G5.5 and T03F7.1, are nearly as similar (43% and 41% identity, respectively) to hSERT as is MOD-5 CeSERT, and only MOD-5 CeSERT and T23G5.5 have an aspartate corresponding to aspartate 119 in MOD-5 CeSERT, a conserved residue likely to be functionally important for amine transport (see above). T23G5.5 is a dopamine reuptake transporter and is very inefficient at transporting 5-HT (Jayanthi et al., 1998). Hence, from sequence analysis, it appeared likely that MOD-5 CeSERT is the only SERT in *C. elegans*. This finding is consistent with the observation that to date only one SERT gene per species has been identified.

If there existed a second SERT in *C. elegans*, serotonergic neurons in *mod-5(n3314)* mutants might be able to take up exogenously added 5-HT. It is also conceivable that non-serotonergic cells possess a SERT activity. We tested both these possibilities using anti-5-HT antisera to detect the uptake of 5-HT. To eliminate endogenous 5-HT, we used the *tph-1(mg280)* mutant, which contains a deletion in the tryptophan hydroxylase gene and is hence defective in an enzyme essential for 5-HT biosynthesis (Sze et al., 2000). *tph-1* mutants appear to completely lack 5-HT (Sze et al., 2000; Table 1), and are unlikely to be perturbed in the levels of other biogenic amines, since tryptophan hydroxylase functions in only 5-HT biosynthesis (Cooper et al., 1996; Sze et al., 2000).

To identify cells capable of 5-HT uptake, we examined the head, ventral cord, gut, and tail of *tph-1* mutants pretreated with 5-HT and observed 5-HT immunofluorescence in only the serotonergic neurons (see below; and data not shown). To examine more carefully the requirement of MOD-5 CeSERT for 5-HT
uptake by serotonergic neurons, we scored the NSMs for 5-HT uptake, since these neurons are the most brightly staining serotonergic neurons in the animal following incubation with exogenous 5-HT. Without 5-HT pretreatment, both tph-1 single and mod-5(n3314); tph-1 double mutants had no NSMs that were 5-HT positive (Table 1). By contrast, when pretreated with 5-HT, tph-1 mutants displayed robust 5-HT staining in the NSMs, while mod-5(n3314); tph-1 double mutants showed none (Table 1). We observed similar results for the serotonergic ADF neurons in the head and for the hermaphrodite-specific neurons (HSNs) in the mid-body (data not shown). Thus, no other transporter appeared to transport 5-HT into serotonergic neurons in the absence of the MOD-5 CeSERT. We also examined the head, ventral cord, gut, and tail of mod-5(n3314); tph-1 double mutants pretreated with 5-HT and observed no 5-HT immunofluorescence anywhere in the animal (data not shown), indicating that no other cells display 5-HT uptake activity in the absence of MOD-5 CeSERT.

These 5-HT uptake experiments taken together with the analysis of the C. elegans genomic sequence suggest that MOD-5 is the only SERT in C. elegans.

mod-5 Interacts Genetically with mod-1 and goa-1

Mutants defective in the 5-HT-mediated enhanced slowing response defined several mod genes (Sawin et al., 2000). One of these genes, mod-1, encodes a novel ionotropic 5-HT receptor, a 5-HT-gated chloride channel (Ranganathan et al., 2000). On Petri plates with bacteria, the locomotory rate of food-deprived mod-1 mutants is substantially faster than that of the wild type (Sawin et al., 2000; Ranganathan et al., 2000; also Figure 5A, gray bars). By contrast, mod-5 mutants exhibit a hyperenhanced slowing response (Figure 5A).

To define the genetic pathway in which mod-1 and mod-5 act in the enhanced slowing response, we characterized mod-5(n3314); mod-1(ok103) double mutants. mod-1(ok103) is a null allele by genetic and molecular criteria (Ranganathan et al., 2000). If the function of the MOD-1 5-HT receptor were essential for the effects of 5-HT not cleared from synapses in mod-5 mutants, then eliminating mod-5 function should have had no effect in a mutant that lacked mod-1 function, i.e., mod-5(n3314); mod-1(ok103) double mutants should exhibit the same phenotype as mod-1(ok103) single mutants. However, the enhanced slowing response of mod-5(n3314); mod-1(ok103) double mutants was intermediate to the responses of mod-1(ok103) and mod-5(n3314) single mutants (Figure 5A, gray bars). This observation suggests that the 5-HT signaling triggered by bacteria in the enhanced
slowly response acts via at least two parallel 5-HT signaling pathways, a MOD-1-dependent pathway and a MOD-1-independent pathway. This observation is also consistent with the observation that mod-1(ok103) single mutants were not completely defective in the enhanced slowing response (Figure 5A).

Animals carrying mutations in the G-protein gene goa-1 (Gαo, G-protein 0, alpha subunit) (Mendel et al., 1995; Segalat et al., 1995) are also defective in the enhanced slowing response (Sawin et al., 2000; also Figure 5A). Since GOA-1 animals are resistant to 5-HT in assays of locomotion (Segalat et al., 1995; and our unpublished observations), pharyngeal pumping (Segalat et al., 1995), and egg laying (Mendel et al., 1995; Segalat et al., 1995), we tested whether the MOD-1-independent pathway might involve goa-1. As with mod-5; mod-1 double mutants, the enhanced slowing response of mod-5(n3314) goa-1(n1134) double mutants was intermediate to the responses of mod-5(n3314) and goa-1(n1134) single mutants (Figure 5A, gray bars), indicating that 5-HT signaling triggered by bacteria in the enhanced slowing response does not act solely through goa-1. By contrast, food-deprived mod-5(n3314) goa-1(n1134); mod-1(ok103) triple mutants exhibited very little slowing in response to bacteria (Fig 5A). These observations suggested that MOD-1 and GOA-1 act in two parallel pathways that together mediate the response to the excess 5-HT signaling in mod-5(n3314) mutants.

**Fluoxetine Blocks 5-HT Uptake in vivo**

Since fluoxetine blocked [3H]5-HT transport in mammalian cells expressing MOD-5 CeSERT (see above), we tested whether fluoxetine could block 5-HT uptake in vivo in *C. elegans* (Table 1). We pretreated tph-1 mutants with fluoxetine, incubated the animals with 5-HT, and scored the number of 5-HT-positive NSMs. We observed, for example, few 5-HT-positive NSMs when tph-1 mutants were pretreated with 0.22 mM fluoxetine (Table 1), a concentration sufficient to potentiate the enhanced slowing response (see below). Furthermore, tph-1 mutants pretreated with as little as 0.44 mM fluoxetine, a concentration lower than that required for all the MOD-5 CeSERT-independent effects of fluoxetine (see below), were as defective in 5-HT uptake as were untreated mod-5(n3314); tph-1 double mutants (Table 1). These observations suggested that fluoxetine can block 5-HT uptake in *C. elegans in vivo* and does so by inhibiting MOD-5 CeSERT.
The Potentiation of the Enhanced Slowing Response by Fluoxetine Requires MOD-5 CeSERT and 5-HT

When wild-type animals that have been food-deprived in the presence of 0.22 mM fluoxetine encounter bacteria, they slow their locomotory rate more than if they had been food-deprived in the absence of fluoxetine (Sawin et al., 2000; see Figure 5A). This fluoxetine-mediated potentiation of the enhanced slowing response resembles the hyperenhanced slowing response exhibited by mod-5(n3314) mutants (Figure 5A), suggesting that fluoxetine causes this potentiation by blocking MOD-5 CeSERT function. If so, mod-5(n3314) mutants should be resistant to the potentiating effect of fluoxetine on the enhanced slowing response. Since food-deprived mod-5(n3314) mutants exhibit an extreme hyperenhanced slowing response that cannot be further potentiated by fluoxetine treatment (Figure 5A and data not shown), we used mod-5(n3314); mod-1(ok103) double mutants to test this hypothesis. These double mutants are partially suppressed for the hyperenhanced slowing response exhibited by mod-5(n3314) animals (Figure 5A, gray bars), and therefore a potentiation of the enhanced slowing response could be observed.

The enhanced slowing response of mod-1(ok103) mutants was potentiated by fluoxetine (Figure 5A, hatched bars), indicating that fluoxetine can potentiate the enhanced slowing response in the absence of MOD-1 5-HT receptor function. This observation was consistent with the phenotype of mod-5(n3314); mod-1(ok103) double mutants in this assay, which suggested that there are MOD-1-independent 5-HT pathways through which the enhanced slowing response is effected. By contrast, mod-5(n3314); mod-1(ok103) double mutants were completely resistant to the potentiating effect of fluoxetine on the enhanced slowing response (Figure 5A, hatched bars). Therefore, the MOD-5 CeSERT is likely the only in vivo target in C. elegans on which fluoxetine acts to potentiate the enhanced slowing response.

Since fluoxetine-mediated potentiation of the enhanced slowing response is MOD-5 CeSERT-dependent, it is also likely to be 5-HT-dependent, as we previously suggested (Sawin et al., 2000) based on the observation that enhanced slowing response bas-1(ad446); cat-4 double mutants (bas: biogenic amine synthesis defective) is resistant to such potentiation. However, our studies of egg laying by tph-1 mutants suggest that the resistance of cat-4 animals to the effects of high concentrations of fluoxetine is likely not to be caused by a deficiency in 5-HT in these animals (see below). Since tph-1 mutants display sluggish locomotion (data
not shown), they could not be assayed for resistance to the fluoxetine-mediated potentiation of the enhanced slowing response.

We sought to determine if it is the 5-HT-deficiency of bas-1; cat-4 double mutants that renders these animals resistant to the potentiating effect of fluoxetine. The defect in the enhanced slowing response of bas-1; cat-4 double mutants in the absence of fluoxetine treatment can be rescued by preincubating the animals on Petri plates containing 2 mM 5-HT (Sawin et al., 2000; and Figure 5B), a pretreatment sufficient for the detection of 5-HT in the NSMs of cat-4 (Figure 1B) and tph-1 (Table 1) mutants. When bas-1; cat-4 mutants were preincubated with 5-HT and then food-deprived in the presence of fluoxetine, they exhibited a potentiated enhanced slowing response (Figure 5B). Therefore, restoration of 5-HT to bas-1; cat-4 mutants is sufficient for fluoxetine to potentiate the enhanced slowing response of these mutants. We conclude that the effect of fluoxetine on the enhanced slowing response is dependent not only on MOD-5 CeSERT but also on 5-HT.

**Fluoxetine Induces Nose Contraction and Paralysis in mod-5 and tph-1 Mutants**

Treatment of *C. elegans* with high concentrations (0.25 - 1 mg/ml; 0.7 - 2.9 mM) of fluoxetine leads to paralysis (Choy and Thomas, 1999), contraction of nose muscles (Choy and Thomas, 1999), and stimulation of egg laying (Weinshenker et al., 1995). The concentrations of fluoxetine required for these effects are at least 2.5 fold higher than that required to detect a block of 5-HT uptake in vivo (see above) and for the potentiation of the enhanced slowing response (Figure 5B and Sawin et al., 2000). If the only in vivo target for fluoxetine were MOD-5 CeSERT, mod-5 mutants could be paralyzed, have contracted noses, and display excessive egg laying. mod-5 mutants exhibit none of these characteristics (data not shown), suggesting that either MOD-5 CeSERT is not the sole target of fluoxetine in *C. elegans* or that an altered developmental program compensates for the animals growing up without a SERT. In the latter case, mod-5(n3314) mutants would be expected to be resistant to the effects of fluoxetine. Therefore, we tested mod-5(n3314) mutants for their responses to high concentrations of fluoxetine.

*mod-5(n3314)* mutants retained wild-type sensitivity to fluoxetine in assays of paralysis induced by fluoxetine treatment (Figure 5C). There was no difference in the time-course of paralysis at any of the concentrations tested (data not shown). These
observations suggest that fluoxetine-induced paralysis in \textit{C. elegans} is not caused by the lack of 5-HT uptake from synapses. Fluoxetine-treated wild-type and \textit{mod-5(n3314)} mutant animals assumed a rigid body posture (data not shown), as observed by others (Choy and Thomas, 1999). By contrast, 5-HT-treated animals assumed a relaxed and flaccid body posture (data not shown), suggesting that the mechanisms of locomotory inhibition by 5-HT and fluoxetine are distinct. 5-HT has been proposed to decrease excitatory input to the locomotory muscles (Nurrish et al., 1999). Given the two distinct body postures, we suggest that fluoxetine may directly or indirectly increase excitatory input or decrease inhibitory input to the locomotory muscles.

When treated with fluoxetine for 20 minutes, a similar proportion of wild-type and \textit{mod-5(n3314)} mutant animals had contracted noses (100\% at 2.9 mM, and \(\sim\) 25\% at 1.5 mM). Thus, the effect of fluoxetine on nose contraction also appears to act via a \textit{MOD-5 CeSERT}-independent pathway. This conclusion is consistent with the conclusion by Choy and Thomas (1999) that fluoxetine-mediated nose contraction is 5-HT-independent, although their hypothesis was based on studies of \textit{cat-1(e1111)} mutants, which are defective in signaling by several biogenic amines (Duerr et al., 1999), and \textit{cat-4} mutants, which we found to not completely lack 5-HT (Figure 1B).

That the effects of high concentrations of fluoxetine on nose-contraction and paralysis were independent of \textit{MOD-5 CeSERT} suggested that fluoxetine acts either on another SERT or on a distinct non-SERT target(s). As discussed above, we think that \textit{MOD-5} is the only SERT in \textit{C. elegans}, making it likely that a non-SERT target(s) of fluoxetine mediates the \textit{MOD-5 CeSERT}-independent effects. Such non-SERT targets may or may not be part of a serotonergic signaling pathway. We decided to explore the requirement for 5-HT by testing whether fluoxetine can act in animals that lack 5-HT. The 5-HT-deficient mutants that have been used in numerous prior studies (e. g., Weinshenker et al., 1995; Choy and Thomas, 1999; Sawin et al., 2000), such as \textit{cat-1}, \textit{cat-4}, and \textit{bas-1} mutants, all affect multiple biogenic amines. None has been shown to cause a complete loss of 5-HT function. We therefore tested \textit{tph-1} mutants, which appear to completely lack 5-HT (see above), for their response to fluoxetine.

100\% of \textit{tph-1} animals displayed contracted noses after treatment with 2.9 mM fluoxetine for 20 minutes. These data confirm the hypothesis of Choy and Thomas (1999) that this effect of fluoxetine is 5-HT-independent. We found that fluoxetine treatment paralyzed \textit{tph-1} mutants to a similar extent as wild-type animals.
(data not shown), suggesting that paralysis by high concentrations of fluoxetine is also a 5-HT-independent process.

**Fluoxetine Stimulates Egg Laying in mod-5 and tph-1 Mutants**

In 1.5 mM fluoxetine, mod-5(n3314) mutants were stimulated to lay eggs to nearly the same extent as was the wild type (Figures 6A and 6B, black bars), suggesting that fluoxetine can stimulate egg laying via one or more MOD-5 CeSERT-independent pathways. Nevertheless, mod-5(n3314) mutants were hypersensitive to exogenous 5-HT in assays of egg laying (Figures 7A and 7B), suggesting that MOD-5 CeSERT can affect serotonergic synapses that regulate egg laying. mod-5(n3314) mutants and wild-type animals contain similar numbers of eggs (25.7 ± 2.8 and 26.3 ± 2.2, respectively), indicating that this hypersensitivity to exogenous 5-HT was not a consequence of differences in basal egg-laying rates between wild-type animals and mod-5 mutants but rather a result of excess 5-HT signaling in mod-5(n3314) mutants.

Given that the stimulation of egg laying by fluoxetine did not require the MOD-5 CeSERT, we wondered whether this stimulation required 5-HT. tph-1 mutants were partially resistant to the stimulation of egg-laying by fluoxetine (Figures 6A and 6C) indicating that 5-HT mediated some but not all of the egg-laying response to fluoxetine. By contrast, cat-4 mutants were completely resistant to fluoxetine-induced egg laying (Figures 6A and 6D), as reported by Weinshenker et al. (1995).

The reduction in egg laying by tph-1 mutants in response to fluoxetine (Figure 6C) is unlikely to be caused by a lower number of eggs within tph-1 mutants or the inability of egg-laying muscles in tph-1 mutants to respond to stimulatory input: tph-1 mutants contain more eggs than do wild-type animals (Sze et al., 2000; and data not shown) and tph-1 mutants laid about the same number of eggs in response to exogenous 5-HT as did wild-type animals (Figure 7C). That mod-5(n3314) and tph-1 mutants laid a significant numbers of eggs in response to fluoxetine argues that the mechanism(s) through which fluoxetine stimulates egg laying in C. elegans is not only MOD-5 CeSERT-independent but also, in part, 5-HT-independent.

The serotonergic HSN motor neurons innervate the egg-laying muscles and drive egg laying (Trent et al., 1983; Desai et al., 1988). egl-1(n1084) mutants, which lack the HSNs (Desai et al., 1988), released some eggs in the
absence of fluoxetine (Figure 6E, gray bars), presumably because these animals were severely bloated with eggs. However, treatment with fluoxetine had no effect on egg laying in egl-1 mutants (Figures 6E, black bars). These observations indicate that the HSNs are required for the stimulation of egg laying by fluoxetine.

Discussion

The *C. elegans* gene *mod-5* encodes a homolog of human SERT, a molecule of major importance in human neurobiology and psychiatric disease. MOD-5 CeSERT acts in a pathway that includes both a novel type of 5-HT receptor, the 5-HT-gated chloride channel MOD-1, and the G-protein GOA-1. We have identified a *C. elegans* modulatory behavior — the potentiation of the enhanced slowing response — in which fluoxetine (Prozac) affects 5-HT signaling by antagonizing MOD-5 CeSERT. We have also identified *C. elegans* behaviors in which fluoxetine acts independently of both SERT and 5-HT. These behaviors can now be analyzed to define SERT- and 5-HT-independent pathways affected by fluoxetine.

MOD-5 Probably Acts Upstream of MOD-1 and GOA-1

*mod-5* mutants, which exhibit a hyperenhanced slowing response, are opposite in phenotype to all other *mod* mutants, which were isolated on the basis of their failures to exhibit the enhanced slowing response (Sawin et al., 2000). These opposite phenotypes allow genetic epistasis experiments to be performed to help define a genetic pathway for this behavior.

We performed such epistasis analysis with the genes *mod-1*, *goa-1*, and *mod-5* (Figure 5A). The phenotypes of *mod-5* *goa-1* and *mod-5; mod-1* double mutants were intermediate between those of either *mod-5* or *goa-1* or *mod-5* or *mod-1* single mutants, respectively; *mod-5* *goa-1*; *mod-1* triple mutants were almost completely defective in the enhanced slowing response. The intermediate phenotypes of these double mutants with *mod-5* indicate that *mod-1* and *goa-1* act in parallel pathways for the enhanced slowing response (Figure 8A). *mod-1* and *goa-1* probably both act downstream of *mod-5* (Figure 8A), since in the absence of both *mod-1* and *goa-1*, the presence or absence of *mod-5* has little effect.

Since *mod-5* encodes a SERT and *mod-5* mutants are defective in the loading of 5-HT into serotonergic neurons, it is likely that MOD-5 CeSERT functions in neurons that synthesize and release 5-HT. That *mod-1* probably acts downstream...
of \textit{mod-5} and encodes a 5-HT receptor (Ranganathan et al., 2000), suggests that MOD-1 acts in cells that are postsynaptic to the serotonergic neurons in which MOD-5 functions (Figure 8B). Alternatively, MOD-1 could be a presynaptic 5-HT receptor, acting in the same serotonergic cells as MOD-5. We consider this latter possibility unlikely given that a \textit{mod-1::GFP} reporter that rescues the \textit{mod-1} mutant phenotype (Ranganathan et al., 2000) is not expressed in the NSM, HSN, and ADF serotonergic neurons (R.R. and H. R. H., unpublished data). Moreover, expression of this \textit{mod-1::GFP} reporter was observed in certain non-serotonergic neurons (data not shown) that are likely to be direct postsynaptic partners of some of the serotonergic neurons (White et al., 1986). Similarly, since \textit{goa-1} mutants are resistant to the effects of exogenous 5-HT (Mendel et al., 1995, Segalat et al., 1995; and our unpublished data), the functions of this G-protein required for responding to 5-HT are likely to be downstream of the serotonergic neurons in which MOD-5 functions (Figure 8B).

The molecular features of the MOD-5, MOD-1, and GOA-1 combined with our genetic epistasis analysis lead us to propose a simple model for the enhanced slowing response in which MOD-5 acts upstream of both MOD-1 and GOA-1, which in turn act in parallel to each other (Figure 8B). While this model places MOD-1 and GOA-1 within the same cells (Figure 8B), our data are also consistent with a model in which these proteins act in parallel in different neurons in the animal, possibly responding to distinct presynaptic serotonergic neurons.

\textbf{Fluoxetine-Induced Egg Laying Is Only Partially 5-HT-Dependent}

Weinshenker et al. (1995) observed that \textit{cat-4} mutants do not lay eggs in response to fluoxetine and argued that fluoxetine is likely to stimulate egg laying by affecting a 5-HT signaling pathway, possibly by inhibiting a SERT. However, we found that \textit{mod-5} null mutants are stimulated to lay eggs by fluoxetine. Moreover, we observed that \textit{tph-1} mutants, which appear to completely lack 5-HT, were only partially resistant to fluoxetine. Weinshenker et al. (1995) argued that the resistance of \textit{cat-4} mutants to fluoxetine is unlikely to be a consequence of their deficiency in dopamine. However, it is possible that the biopterin cofactor generated by the GTP cyclohydrolase I enzyme encoded by the \textit{cat-4} gene (C. Loer, personal communication) participates in the biosynthesis of small molecules in addition to dopamine and 5-HT. In fact, \textit{cat-4} animals have an altered cuticle (Loer and Kenyon, 1993), a characteristic not found in \textit{bas-1} mutants, which also have reduced levels of
both 5-HT and dopamine (Loer and Kenyon, 1993; and our unpublished results). Moreover, bas-1 mutants, unlike cat-4 mutants, were not resistant to fluoxetine-induced egg laying (data not shown). Hence, the resistance of cat-4 mutants to fluoxetine-induced egg-laying need not be a consequence of their deficiency in either 5-HT or dopamine. In addition, we have shown that cat-4 mutants retain some 5-HT immunoreactivity (Figure 1B). For these reasons, the cat-4(e1141) mutation cannot be considered as a null for 5-HT function.

*tph-1* mutants were partially resistant to fluoxetine-induced egg laying, while *mod-5(n3314)* mutants were not at all resistant. Since we have shown that MOD-5 is likely the only SERT in *C. elegans*, these observations suggest that fluoxetine-mediated stimulation of egg-laying through non-SERT pathways still involves 5-HT signaling. We propose that 5-HT signaling serves as a requisite permissive signal for the egg-laying circuitry to respond in full to a fluoxetine-stimulated non-5-HT signal. This model is consistent with the hypothesis that 5-HT promotes and maintains the active phase of egg laying (Waggoner et al., 1998). Alternatively, 5-HT could be required during development for the neuromusculature to be competent to respond fully to fluoxetine.

**Potentiation of the Enhanced Slowing Response by Fluoxetine Requires MOD-5 CeSERT and 5-HT**

The therapeutic effect of fluoxetine in human patients is thought to be mediated primarily by a block in 5-HT uptake by hSERT. However, fluoxetine causes a variety of adverse side effects, including headaches, sexual dysfunction, and sleep disorders (Baldessarini, 1996). It remains important to resolve whether these side effects result from the action of fluoxetine on hSERT or from the action of fluoxetine on non-hSERT target(s).

We have shown that high concentrations of fluoxetine have effects on both *mod-5* and *tph-1* mutants. Our results demonstrate that in *C. elegans* effects of fluoxetine previously thought to be 5-HT-dependent are MOD-5 CeSERT-independent and, to varying degrees, 5-HT-independent (Figure 8C). It has recently been proposed, based on the identification of fluoxetine-resistant mutants and the cloning of a novel family of *C. elegans* transmembrane proteins required for responses to fluoxetine, that some of these side-effects of fluoxetine could involve 5-HT-independent targets (Choy and Thomas, 1999; Schafer, 1999). Our demonstration in this study that there is a SERT- and 5-HT-dependent effect of
fluoxetine on *C. elegans* behavior (Figure 8B) — the potentiation of the enhanced slowing response — strengthens the argument that non-SERT and non-serotonergic targets of fluoxetine in *C. elegans* could well be relevant to the effects of fluoxetine in humans. Moreover, the potentiation of the enhanced slowing response by fluoxetine requires much lower doses of fluoxetine than do the other effects of fluoxetine on *C. elegans* behavior, arguing that fluoxetine is likely acting on its most sensitive target, i.e., MOD-5 CeSERT, to bring about the potentiation of the enhanced slowing response. By contrast, the non-SERT targets that mediate the other effects of fluoxetine in *C. elegans* are likely to have either lower binding affinities or lower bioavailability for fluoxetine.

We propose that the further genetic analysis of the *C. elegans* behaviors elicited by fluoxetine in *mod-5* and *tph-1* mutants will help define SERT- and 5-HT-independent targets of fluoxetine. Such studies could be instrumental in the development of pharmaceuticals that modulate 5-HT neurotransmission with fewer side-effects than those currently in use in the clinic.

**Acknowledgments**

We thank Brendan Galvin, Brad Hersh, Eric Miska, Ignacio Perez de la Cruz, Peter Reddien, and Hillel Schwartz for suggestions concerning this manuscript, Nancy Tsung for assistance in the isolation of the *n822* and *n823* alleles, Ron Ellis for directing our attention to the presence of a SERT gene in unfinished *C. elegans* genomic sequence, Subbu Apparsundaram for guidance concerning the SERT assays, Jay Schwartz for advice and assistance in generating the stable cell lines expressing MOD-5, and Beth Castor for DNA sequence determinations. This research was supported by United States Public Health Service grant GM24663 (H. R. H.). E. R. S. was supported by predoctoral fellowships from the National Science Foundation and the W. M. Keck Foundation. R. R. is supported by a Howard Hughes Medical Institute predoctoral fellowship. H. R. H. is an Investigator of the Howard Hughes Medical Institute.
Experimental Procedures

mod-5 Mapping, Cloning, and cDNA

Nematodes were grown at 20°C as previously described (Brenner, 1974), except that E. coli strain HB101 rather than OP50 was used as the food source (Sawin et al., 2000). Wild-type animals were C. elegans strain N2. mod-5(n822) and mod-5(n823) were isolated from a genetic screen in which clonal populations of F3 animals descended from P₀ animals mutagenized with ethyl methanesulphonate (Brenner, 1974) were pretreated with 5-HT (15 min incubation in 500 μl of 13 mM 5-HT followed by two washes with M9) and then examined for the presence of the NSMs using FIF (Sulston et al., 1975). mod-5(n3314) was isolated from a library of animals mutagenized with UV/trimethylpsoralen (Jansen et al., 1997). The deletion library was constructed essentially as described (Jansen et al., 1997; Liu et al., 1999; P. Reddien, R. R., and H. R. H., unpublished results). mod-5(n3314) was backcrossed to the wild type six times prior to behavioral assays. mod-5(n823) was mapped to LG I based upon two-factor linkage to dpy-5 unc-75 I. The following three-factor data were obtained: mod-5 (47/47) dpy-5 (0/47) unc-75, mod-5 (35/35) unc-73 (0/35) lin-44 dpy-5, lin-6 (27/27) lin-17 (0/27) mod-5, lin-17 (13/13) fog-1 (0/13) mod-5, and fog-1 (3/26) mod-5 (23/26) unc-11. All mapping experiments were performed by mating hermaphrodites homozygous for the recombinant chromosome with mod-5(n823) males and scoring the F1 cross progeny for 5-HT hypersensitivity at 5 min in 10 mM 5-HT. Germline transformation experiments (Mello et al., 1991) were performed by injecting various constructs with 80 μg/ml pL15EK (which contains the wild-type lin-15 gene) into a mod-5(n823); lin-15(n765ts) strain and scoring 5-HT sensitivity in transgenic lines that produced non-Lin progeny at 22.5°C. Long-range PCR was performed using the Advantage cDNA PCR kit (Clontech). DNA sequences were determined using an automated ABI 373A DNA sequencer (Applied Biosystems). RT-PCR was performed with primers corresponding to exons predicted by Genefinder (The C. elegans Sequencing Consortium, 1998). The 5' and 3' ends of the mod-5 cDNA were determined using 5'- and 3'-RACE kits (Gibco), respectively. To construct the mod-5 minigene, we used PCR and primers that contained restriction enzyme sites at their ends to amplify 2.7 kb of the mod-5 promoter region. A Pst I - Bam HI fragment of this PCR product was ligated into the pPD49.26 vector (A. Fire) digested with Pst I and Bam HI. This mod-5 promoter construct was then digested with Nco I and Sac I and ligated to an Nco I - Sac I
fragment of the *mod-5* coding region, PCR-amplified in a manner similar to that used for the *mod-5* promoter region.

**Laser Microsurgery**

Neurons were ablated during the second larval stage using a laser microbeam, as previously described (Avery and Horvitz, 1987; Bargmann and Horvitz, 1991). Behavioral assays of young adult animals were performed two days later. Mock-ablated animals were animals transferred to agar pads and anesthetized in parallel to the animals that underwent laser ablation. Sawin et al. (2000) describe details concerning how ablated animals were assayed sequentially in each of the different behavioral conditions.

**Neurotransmitter and Drug Pretreatment and Behavioral Assays**

Locomotory rate was assayed and 5-HT-containing plates were prepared as previously described (Sawin et al., 2000). Fluoxetine (HCl salt, Sigma) was dissolved in water, and 400 µl of a 25x stock solution were added to each 5 cm plate, containing ~10 ml of agar, to obtain the various final concentrations of fluoxetine. The plates were allowed to dry at room temperature with their lids removed for >2 hours.

To assay 5-HT hypersensitivity, we placed 20 animals in 200 µl of 5-HT solution (creatinine sulphate salt, Sigma, dissolved in M9 buffer; Wood et al., 1988) in 96-well microtiter wells and scored the swimming behavior of the animals as either active or immobile at 5 min; an animal was scored as immobile if it did not exhibit any swimming motion for a period of 5 seconds. Fluoxetine-induced paralysis was scored in a similar manner at 10 min.

Egg-laying assays were performed as previously described (Trent et al., 1983). Briefly, one day-old adult animals (staged by picking late L4 animals 36 hours prior to the assay) were placed in wells of microtiter dishes containing 100 µl of 12.5 mM 5-HT or 500 µg/ml fluoxetine, and the number of eggs laid was counted after 90 minutes.
5-HT-uptake Assays in vivo

FIF assays were performed as previously described (Sulston et al., 1975). For the anti-5-HT antisera experiments shown in Figure 1B, mod-5(n823); cat-4 and cat-4; lin-15(n765ts) double mutants were grown at 20°C, and animals of both genotypes were incubated separately on plates containing 2 mM 5-HT and bacteria (see Sawin et al., 2000 for details concerning how plates were prepared) for two hours and then incubated on plates with bacteria but without 5-HT for 30 min. Controls without exogenous 5-HT were similarly treated in parallel. Prior to fixation, mod-5(n823); cat-4 and cat-4; lin-15 double mutants preincubated on 5-HT-containing plates were combined, and mod-5(n823); cat-4 and cat-4; lin-15 double mutants preincubated on control plates were combined. 5-HT staining was performed as previously described (Desai et al., 1988) using affinity-purified rabbit polyclonal anti-5-HT antisera (H. Steinbusch, Maastrict University, the Netherlands). The cat-4; lin-15 mutants were not defective in the uptake of 5-HT (data not shown) and served as internal controls for each staining reaction. These animals could be distinguished from the test animals by the Multivulva phenotype caused by lin-15. Neuron with bright staining in cell bodies, axonal processes, and varicosities were termed "bright" and neurons with weak staining in just the cell bodies and axonal processes were termed "weak." For the results in Table 1, the procedure was essentially the same, except that the animals experienced an additional 1 hr incubation on control or fluoxetine-containing plates prior to the 2 hr incubation with 5-HT but did not experience the 30 min incubation on plates without drug after the 5-HT preincubation (see Table 1 legend for details). lin-15 adult animals, grown at 22.5°C were added to all plates at the first incubation step, and these animals served as internal controls for the staining reaction.

MOD-5-mediated Uptake in Mammalian Cells

We obtained a modified version of the MSCVpac vector (Hawley et al., 1994) in which the pac gene had been replaced with the gfp gene (MSCVGFP; a generous gift of J. Schwartz). We further modified MSCVGFP as follows: The ends of a Bgl II-Mfe I fragment containing the entire mod-5 cDNA were blunted using the Klenow fragment of DNA polymerase I and then ligated to the MSCVGFP vector digested with Hpa I, placing mod-5 under the control of the retroviral long terminal repeat promoter (MSCVGFPMOD-5). The Phoenix packaging cell line (ATCC) was used to
generate virus containing either MSCVGFPMOD-5 or MSCVGFP. HEK293 cells were infected with these viral stocks in the presence of 4 mg/ml polybrene, and clones expressing high levels of GFP were isolated using a fluorescence activated cell sorter (FACstar or FACSVantage; Becton Dickinson). The MSCVGFPMOD-5 clones were then screened for MOD-5 CeSERT-mediated [³H]5-HT uptake activity, and one clone was chosen for use in all further uptake experiments. Cells were plated at 10⁶ cells/well of a 6-well dish and allowed to grow overnight before being assayed. Cells were incubated in prewarmed wash buffer (120 mM NaCl, 10 mM HEPES pH 7.4, 4.7 mM KCl, 2.2 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.8 mg/ml glucose, 100 µM paraglyline, 100 µM ascorbic acid) for 10 min at 37°C and the buffer was then replaced with prewarmed wash buffer plus substrate. In Figure 4B, the NaCl was substituted with an equivalent amount of sodium gluconate or choline chloride. Except for the trials shown in Figures 4C and 4D, 50 nM [³H]5-HT was used as substrate (there was no dilution with non-radioactive substrate in these experiments). Since micromolar amounts of substrate were used in the trials shown in Figures 4C and 4D, radiolabelled substrates were diluted with non-radioactive substrate to maintain a specific activity of 0.1 Ci/mmol. Uptake was allowed to proceed at 37°C for varying times for the time course and for 10 min in all other experiments. Cells were then washed three times with ice-cold wash buffer, solubilized in 1% SDS and the radioactivity retained in the cells was determined by liquid scintillation. Cell numbers, quantified in parallel wells taken through all steps of the assay, were used to convert counts per minute (cpm) to nmoles per cell per min. The specific uptake of each substrate for each condition was obtained by subtracting the average value obtained from at least three trials with the MSCVGFP cell line from the average value obtained from at least six trials with the MSCVGFPMOD-5 cell line. Inhibitor Kᵣ values were determined from concentration vs. uptake profiles after adjustment for substrate concentrations (Cheng and Prusoff, 1973). Statistical significance was evaluated using the Student's t-test (Statview).
References


Cheng, Y., and Prusoff, W. H. (1973). Relationship between the inhibition constant \( (K_i) \) and the concentration of inhibitor which causes 50 per cent inhibition \( (I_{50}) \) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099-108.


Li, Q., Wichems, C., Heils, A., Lesch, K. P., and Murphy, D. L. (2000). Reduction in the density and expression, but not G-protein coupling, of serotonin receptors (5-


Table 1. Fluoxetine phenocopies *mod-5* in 5-HT uptake assays *in vivo*

<table>
<thead>
<tr>
<th>Genotype</th>
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<th></th>
<th>Pretreatment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Flouxetine (mM)</td>
<td></td>
<td>Flouxetine (mM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.22</td>
<td>0.29</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>No Drug</td>
<td>0</td>
<td>98</td>
<td>5-HT</td>
</tr>
<tr>
<td></td>
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<td>20</td>
<td>20</td>
<td>5-HT + Flouxetine</td>
</tr>
<tr>
<td></td>
<td>5-HT + Flouxetine</td>
<td>7</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>N/A</td>
</tr>
<tr>
<td><em>tph-1</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>mod-5; tph-1</em></td>
<td>0</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

In pretreatment 1, animals were incubated for 1 hr on plates containing no drug or the specified concentration of fluoxetine. In pretreatment 2, animals from plates with no drug in pretreatment 1 were transferred to plates containing no drug or 2 mM 5-HT, and animals from plates with fluoxetine were transferred to plates containing 2 mM 5-HT and the same specified concentration of fluoxetine as in pretreatment 1. After 2 hours, animals were fixed and stained with anti-5-HT antisera (see Experimental Procedures for controls included to ensure that lack of 5-HT staining did not result from a failure of the antibody staining reaction). N/A, not applicable. At least 100 animals were assayed in each condition for each genotype; > 200 *mod-5; tph-1* animals pretreated with 5-HT in pretreatment 2 were scored. *tph-1*(mg280) and *mod-5*(n3314) were the alleles used.
Figures

Figure 1. The NSMs of mod-5 Mutants Are Defective in 5-HT Uptake

(A) Wild-type and mod-5(n822) animals preincubated with exogenous 5-HT were stained using FIF to visualize 5-HT (Sulston et al., 1975). Arrow, NSM cell body. Arrowheads, NSM axonal processes. No NSM cell bodies were FIF-positive in mod-5(n822) mutants. The fluorescence seen in the axonal processes may be the consequence of partial 5-HT reuptake activity (see Figure 1B).

(B) NSMs in mod-5(n822) and mod-5(n823) mutants show reduced uptake of exogenous 5-HT. The mutation cat-4(e1141) was included to reduce the levels of endogenous 5-HT, which would otherwise obscure the detection of 5-HT uptake. At least 100 animals were tested for each genotype in each condition. See Experimental Procedures for details. Error bars, SEM.
Figure 1

A

Wild type

mod-5(n822)

B

<table>
<thead>
<tr>
<th>Preincubation:</th>
<th>Buffer</th>
<th>5-HT</th>
<th>Buffer</th>
<th>5-HT</th>
<th>Buffer</th>
<th>5-HT</th>
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<tbody>
<tr>
<td>Genotype:</td>
<td>Wild type</td>
<td>cat-4</td>
<td>mod-5(n822); cat-4</td>
<td>mod-5(n823); cat-4</td>
<td></td>
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<td></td>
<td></td>
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5-HT positive
- None
- Weak
- Bright

% NSMs
Figure 2. Phenotypic Characterization and Cloning of mod-5

(A) mod-5 mutants exhibited a hyperenhanced slowing response. Well-fed (black bars) and food-deprived (gray bars) animals were transferred to assay plates with or without a bacterial lawn, and the locomotory rate of each animal was recorded after 5 min; food-deprived animals were transferred to plates without bacteria 30 min prior to the transfer to locomotory assay plates (see Sawin et al., 2000 for details). At least 10 trials were performed for each genotype for each condition. For this and all subsequent panels, each trial involved testing at least five animals for each of the conditions; a given animal was tested in only one condition. p values were calculated by comparing the combined data for the mutants from all of the separate trials under one set of conditions to the combined data for the wild-type animals assayed in parallel under the same conditions.

(B) mod-5 mutants were hypersensitive to exogenous 5-HT. 5-HT dose-response curves for wild-type and mod-5 mutant animals were generated from averages of five trials with 20 animals of each genotype at each concentration in which animals were scored for movement after 5 min.

(C) The hyperenhanced slowing response exhibited by mod-5(n823) mutants was suppressed by ablation of the NSMs. No. animals tested: three of each ablation state when well-fed, seven mock-ablated food-deprived, 12 NSM-ablated food-deprived.

(D) Decrease in endogenous 5-HT partially suppressed the mod-5 phenotype. mod-5(n823); cat-4 double mutants displayed an enhanced slowing response intermediate to that of mod-5(n823) (see Figure 2A) and cat-4 mutants.

(A) - (D) Error bars, SEM. Asterisks, p<0.0001, Student's t-test.
Figure 2

A

Body Bends/20 s

Well-fed
Food-deprived

Bacteria:

Genotype:

Wild type
mod-5(n822)
mod-5(n823)
mod-5(n3314)

---

B

Body Bends/20 s

Well-fed
Food-deprived

Bacteria:

Genotype:

mod-5(n823)

---

C

Body Bends/20 s

Well-fed
Food-deprived

Genotype:

mod-5(n823); cat-4

---

D

% Animals Immobilized

Wild type
mod-5(n822)
mod-5(n823)
mod-5(n3314)

[5-HT], (mM)
Figure 3. *mod-5* Encodes a Protein Similar to the Human and *Drosophila* 5-HT Reuptake Transporters

(A) Top, Genetic map of the *mod-5* region of linkage group I (LG I). Bottom, intron-exon structure of *mod-5* (Y54E10A_154.A), inferred from cDNA sequences. Open boxes, coding regions; lines, untranslated regions, arrow, direction of transcription; SL1, SL1 trans-spliced leader. The length of the intron between the 8th and 9th exons is not known, since the sequence of this region has not been determined. Nonetheless, the cDNA we isolated by RT-PCR and RACE (see Experimental Procedures) and showed to be functional (see Results) suggests that no exons are missing in the predicted gene structure. The *mod-5* open reading frame is 2,016 bp within a 2,594 bp cDNA. The extent of the 1,688 bp *n3314* deletion is depicted.

(B) Amino-acid sequence alignment of MOD-5 CeSERT with human SERT (hSERT) and *Drosophila* SERT (dSERT). The 12 predicted transmembrane regions are underlined. Amino acids conserved between MOD-5 and at least one of the two other proteins are shown in black boxes, and the two *mod-5* point mutations are indicated. *mod-5*(n822) is a T-to-A transversion mutation resulting in a C225opal nonsense substitution, and *mod-5*(n823) is a C-to-T transition mutation resulting in a P569S missense substitution. ▼, potential PKA or PKC phosphorylation sites. Such sites have been implicated in SERT membrane distribution (Qian et al., 1997; Ramamoorthy et al., 1998a) and the rate of 5-HT transport (Miller and Hoffman, 1994). ★, potential N-linked glycosylation sites (consensus N-x-S/T). Such sites have been implicated in proper folding and insertion of SERTs into the membrane and/or protection from degradation (Tate and Blakely, 1994; Ramamoorthy et al., 1998b). ◇, the aspartate residue conserved in SERTs, NETs (norepinephrine transporters), and DATs (dopamine transporters).
Figure 3
Figure 4. Physiological Characterization of MOD-5 CeSERT

Experiments were performed using HEK293 cell lines stably transfected with either a mod-5::gfp (MSCVGFPMOD-5) or a gfp control (MSCVGFP) construct (see Experimental Procedures for details). Results indicate mean ± SEM from at least six replicate trials with a MOD-5 CeSERT-expressing cell line after subtracting the nonspecific 5-HT uptake from at least three replicate trials with a cell line expressing GFP from the parent vector lacking mod-5. Except in (A), all assays were performed for 10 min. In (A) and (B), 50 nM [3H]5-HT was used. In (C) and (D) the radiolabelled neurotransmitters were present at 0.1 Ci/mmol specific activity. Error bars, SEM.

(A) Time dependence of MOD-5 CeSERT-mediated [3H]5-HT transport.
(B) Dependence of MOD-5 CeSERT-mediated [3H]5-HT transport on Na+ and Cl− ions in the external buffer. Results are shown as a percentage of the normalized 5-HT uptake in standard NaCl-containing buffer. Transport is strictly dependent on Na+ but only partially dependent on Cl−.
(C) MOD-5 CeSERT-mediated [3H]5-HT transport as a function of 5-HT concentration. [3H]5-HT uptake was measured at various 5-HT concentrations. Inset, Eadie-Hofstee transformation (Stryer, 1995) of the data. Km = 150 ± 8 nM; Vmax = 8.31 × 10−9 nmoles per cell per min. This Vmax value cannot be compared in a meaningful way with values from other studies in the absence of information about relative SERT levels on the cell membrane.
(D) MOD-5 CeSERT-mediated transport was specific for [3H]5-HT. Assays were performed with 1 μM [3H]5-HT and 50 μM of each of the other radiolabelled neurotransmitters, and the results are presented as a percentage of normalized 1 μM [3H]5-HT uptake. There was no detectable transport when neurotransmitters other than 5-HT were added at 1 μM (data not shown).
(E) Antagonism of MOD-5 CeSERT-mediated 5-HT uptake. Inhibition curves for SSRIs and other transporter inhibitors. Assays were performed for 10 min with 50 nM [3H]5-HT in the presence of varying concentrations of each of the compounds. The extent of [3H]5-HT uptake is plotted as the percentage of [3H]5-HT uptake observed in the absence of antagonists vs. log [inhibitor]. See text for Ki values calculated from these data. Error bars, SEM.
Figure 4

A

\[ \text{[3H]5-HT Uptake (\times 10^{-6} \text{nmoles/cell})} \]

\begin{align*}
\text{Time (min)} & \quad 0 & \quad 10 & \quad 20 & \quad 30 & \quad 40 & \quad 50 & \quad 60 & \quad 70 \\
\text{[3H]5-HT Uptake} & \quad 0 & \quad 5 & \quad 10 & \quad 15 & \quad 20 & \quad 25 & \quad 30 & \quad 35 \\
\end{align*}

B

\[ \text{[3H]5-HT Uptake (% NaCl)} \]

\begin{align*}
\text{NaCl} & \quad 100 \\
\text{Na Gluconate} & \quad 80 \\
\text{Choline Cl} & \quad 60 \\
\end{align*}

C

\[ \text{[3H]5-HT Uptake Velocity (\times 10^{-6} \text{nmoles per cell per min})} \]

\[ K_m = 150 \pm 8 \text{nM} \]

D

\[ \text{Uptake (% 5-HT uptake)} \]

E

\[ \% \text{ uninhibited 5-HT uptake} \]

\begin{align*}
\text{Citalopram} & \quad 100 \\
\text{Desipramine} & \quad 90 \\
\text{Impiramine} & \quad 80 \\
\text{Cocaine} & \quad 70 \\
\text{Fluoxetine} & \quad 60 \\
\text{Paroxetine} & \quad 50 \\
\end{align*}
Figure 5. mod-5 Genetic Interactions and 5-HT- and MOD-5 CeSERT-Dependence of the Potentiating Effect of Fluoxetine

(A) Gray bars, the enhanced slowing response of mod-1, goa-1, mod-5 goa-1, goa-1; mod-1, mod-5; mod-1, and mod-5 goa-1; mod-1 mutants. At least five trials were performed for each genotype. Hatched bars, the effect of fluoxetine on the enhanced slowing response of wild-type animals (data reproduced from Sawin et al., 2000) and mod-1 and mod-5; mod-1 mutants (at least 10 trials for each genotype).

(B) Rescue by 5-HT preincubation (see Experimental Procedures) of the resistance of bas-1; cat-4 mutants to the potentiating effect of fluoxetine on the enhanced slowing response. At least 5 trials were performed with each genotype.

(C) mod-5(n3314) mutants retained normal sensitivity to fluoxetine-mediated paralysis. Five trials with 20 animals of each genotype at each concentration and the animals were scored for paralysis after 10 minutes.

(A) - (C) Error bars, SEM. Asterisks, p<0.0001, Student's t-test.
**Figure 5**

**A**

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<th>Genotype</th>
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<td>mod-5 goa-1</td>
<td>mod-5 goa-1; mod-1</td>
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**B**

<table>
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<th>Bacteria</th>
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</table>

**C**

After 10 minutes

% Animals Paralyzed

Fluoxetine Concentration (mM)
Figure 6. 5-HT- and MOD-5 CeSERT-independence of Fluoxetine-induced Egg Laying

Fluoxetine-induced egg laying (n=50 for each genotype).

(A) Wild-type animals.

(B) mod-5(n3314) mutants.

(C) tph-1 mutants.

(D) cat-4 mutants.

(E) egl-1 mutants.
Figure 6

A. Wild type
B. mod-5(n3314)
C. tph-1(mg280)
D. cat-4(e1141)
E. egl-1(n1084)

No. eggs laid in 90 min

- 1.45 mM Fluoxetine
- Buffer
Figure 7. *mod-5(n3314)* mutants, but Not *tph-1* Mutants, Are Hypersensitive to Stimulation of Egg Laying by 5-HT

5-HT-induced stimulation of egg laying (n=50 for each genotype).

(A) Wild-type animals.

(B) *mod-5(n3314)* mutants.

(C) *tph-1* mutants.
Figure 7

A  Wild type
B  mod-5(n3314)
C  tph-1(mg280)

No. eggs laid in 90 min
12.5 mM 5-HT

<table>
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<th>tph-1(mg280)</th>
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Figure 8. Models for the Effects of Fluoxetine on *C. elegans* Behaviors

(A) MOD-5 CeSERT acts upstream of the MOD-1 5-HT-gated chloride channel and the GOA-1 G-protein.

(B) The potentiating effect of low concentrations of fluoxetine on the enhanced slowing response is 5-HT- and MOD-5 CeSERT-dependent. The stimulus of food in a food-deprived animal results in the release of 5-HT that acts through the MOD-1 5-HT-gated chloride channel and through GOA-1-dependent 5-HT-signaling pathway that likely acts in parallel to the MOD-1 pathway and results in the slowing of locomotory rate. Mutations in *mod-5* or the application of fluoxetine leads to inefficient clearing of 5-HT and thus a hyperenhanced slowing response. 5-HTR, a metabotropic 5-HT receptor that GOA-1 might couple to. ▲, 5-HT. ●, chloride ions.

(C) The effects of high concentrations of fluoxetine on egg laying, nose contraction and paralysis are MOD-5 CeSERT-independent. High concentrations of fluoxetine act on non-SERT targets and on a non-5-HT pathway to paralyze *C. elegans* and to lead to the contraction of nose muscles. By contrast, stimulation of egg laying by fluoxetine is MOD-5 CeSERT-independent but still partially dependent on 5-HT (see Discussion for details).
Figure 8

A

MOD-5 CeSERT

MOD-1 5-HT-Gated Chloride Channel

GOA-1 G-protein

B

Fluoxetine

(Low concentrations)

MOD-5 CeSERT

Food Stimulus

Presynaptic neurons (NSMs & others)

5-HT

Cl⁻

Enhanced Slowing Response

Postsynaptic neurons

MOD-1

GOA-1

5-HT receptor?

C

Fluoxetine

(High concentrations)

Non-MOD-5 CeSERT targets

paralysis

egg laying

nose contraction
Appendix 1

Supplementary Data for Chapters 2, 3, and 4
In this chapter, I present additional data that were not included in the published or submitted versions of Chapters 2-4. These data substantiate the conclusions of Chapters 2-4 and are cited as data not shown therein.

Results Pertaining to Chapter 2

Serotonin-Pretreated Food-Deprived bas-1; cat-4 Mutants Exhibit Normal Locomotion in the Absence of Bacteria

Serotonin preincubation restores the enhanced slowing response of bas-1; cat-4 mutants, while dopamine preincubation does not (Chapter 2, Figure 2B, gray bars). Serotonin pretreatment does not affect the locomotory rate of these mutants when they were well-fed and on plates with no bacteria, suggesting that serotonin preincubation does not lead to non-specific slowing of locomotory rate (Chapter 2, Figure 2B, black bars). However, it remained possible that, when food-deprived, the serotonin-pretreated bas-1; cat-4 mutants slow their locomotory rate even in the absence of bacteria. Therefore, we assayed the locomotory rate of serotonin-pretreated food-deprived bas-1; cat-4 mutants on plates without bacteria and found that their locomotory rates were unaffected by the serotonin pretreatment (Figure 1). Therefore, the slowing of locomotory rate of serotonin-pretreated food-deprived bas-1; cat-4 mutants in response to bacteria (Figure 1 and Chapter 2, Figure 2B, gray bars) is not a non-specific slowing of locomotory rate caused by the serotonin preincubation but rather a specific restoration of the behavioral response.

The Enhanced Slowing Response of cat-2 mutants Is Potentiated by Fluoxetine

The potentiation of the enhanced slowing response by fluoxetine is dependent on endogenous serotonin (Chapter 2, Figure 5B and Chapter 4, Figure 5B). We sought to confirm that loss of endogenous dopamine does not affect the ability of fluoxetine to potentiate the enhanced slowing response using cat-2 mutants, which lack dopamine but have normal levels of endogenous serotonin (see Chapter 2). The enhanced slowing response of cat-2 mutants is still capable of being potentiated by fluoxetine (Figure 2), confirming that fluoxetine does not require endogenous dopamine for its potentiating effect.
Food Deprivation Does Not Lead to a Change in the Kinetics of Serotonin Sensitivity

The responsiveness of well-fed and food-deprived animals to exogenous serotonin is identical over a range of serotonin concentrations (Chapter 2, Figure 5D). We sought to determine if there was any difference in the onset of the response to exogenous serotonin by well-fed and food-deprived animals. We assayed the locomotory rate of animals at regular intervals during an exposure to 15 mM serotonin and found that there was no difference between well-fed and food-deprived animals in the kinetics of their responses to exogenous serotonin (Figure 3).

The Enhanced Slowing Response Does Not Depend on the Presence of Fructose During Food-deprivation

In the locomotory rate assay devised to measure the enhanced slowing response (Chapter 2), animals were incubated on a plate without bacteria for 30 minutes of food deprivation. These food-deprivation plates contained a ring of high osmolarity fructose on the outer edge so that the normal high osmolarity avoidance reflex of C. elegans would prevent the animals from swimming off the agar surface and dying on the plastic edge of the Petri plate. This food-deprivation protocol raised the formal possibility that the enhanced slowing response was a result of exposure to high osmolarity for 30 minutes or the combination of exposure for 30 minutes to both high osmolarity and food-deprivation. To test this possibility, we transferred a large number of wild-type animals to plates that had neither bacteria nor the high-osmolarity ring. After 30 minutes, several animals had wandered off the agar surface and died. The animals that remained alive on the agar surface were assayed and found to be capable of exhibiting a normal enhanced slowing response (Figure 4; compare with Chapter 2, Figure 1A). These observations confirm that the presence of the high osmolarity fructose ring does not cause the enhanced slowing response. This finding is further supported by the modified procedure that was used for the genetic screen to isolate mutants that did exhibit the enhanced slowing response (Chapter 2, Experimental Procedures), wherein the animals were deprived of food in liquid for 30 minutes rather than on plates with high osmolarity fructose rings.
Results Pertaining to Chapter 3

High Concentrations of Dopamine, Octopamine, and Tyramine Activate MOD-1

*Xenopus* oocytes expressing the MOD-1 ion channel responded specifically to serotonin application (Chapter 3). The effect of various neurotransmitters on MOD-1 was tested to support this finding. In the course of such studies, we observed that *mod-1*-injected eggs were capable of responding to high concentrations of dopamine, octopamine, and tyramine (Figure 5). Octopamine and tyramine are invertebrate-specific neurotransmitters. Such activation of receptors for one specific biogenic amine by high concentrations of other biogenic amines has been observed in other preparations (E. Kravitz, personal communication), and is likely to be a result of the highly related structures of these amines. The \( EC_{50} \) for dopamine, octopamine, and tyramine at MOD-1 receptors is at least 100-fold higher than that for serotonin (Figure 5); this is a conservative estimate since a plateau of maximal activation was not achieved with the concentrations tested for dopamine, octopamine, and tyramine. It is therefore unlikely that dopamine, octopamine, or tyramine function as ligands for the MOD-1 ion channel *in vivo*.

Ondansetron Does Not Block 5-HT-Gated MOD-1 Currents

Granisetron, an antagonist specific for the 5-HT\(_{3a}\) non-selective cation channel, had no effect on MOD-1 (Chapter 3, Figure 4D). Similar results were obtained with another such 5-HT\(_{3a}\)-specific antagonist, ondansetron (Figure 6). These observations suggested that it is unlikely to be a specific characteristic of granisetron that makes it ineffective at blocking MOD-1, but that MOD-1 lacks a common feature that is the target of these two drugs.

Mianserin and Methiothepin Block 5-HT-Gated MOD-1 Currents

Pretreatment of wild-type animals with mianserin and methiothepin, two serotonin receptor antagonists, prevented the animals from exhibiting a normal enhanced slowing response (Chapter 2, Figure 5A). These two drugs, even though they are usually thought of as antagonists of metabotropic serotonin receptors, blocked 5-HT-gated currents through MOD-1 (Chapter 3). The inhibition curves from which the
approximate $K_v$ values reported in Chapter 3 were derived are presented in Figure 7A.

That mianserin and methiothepin inhibited MOD-1 in *Xenopus* oocytes raised the possibility that these drugs were acting on MOD-1 *in vivo* to block the enhanced slowing response. If MOD-1 were the only *in vivo* target for these drugs to bring about the effect on the enhanced slowing response, animals lacking MOD-1 should be resistant to the effects of the drugs on the enhanced slowing response. There was no additional defect in the enhanced slowing response of *mod-1(ok103)* mutants when they were pretreated with methiothepin, but there was a small but significant increase in the defect when pretreated with mianserin (Figure 7B). These data suggested that methiothepin acts on an *in vivo* target(s) that is completely dependent on the MOD-1 ion channel, while a minor fraction of mianserin's effects are mediated via a target(s) that does not require MOD-1 for its function. Nevertheless, given that both of these antagonists inhibited MOD-1 in *Xenopus* oocytes (Figure 7A; and Chapter 3, Figure 4E), it is likely that mianserin and methiothepin directly inhibit MOD-1 *in vivo*.

**MOD-1 Channel Pore Is Similar to that of GABA- and Glycine-Gated Chloride Channels**

The rank order of anion permeability for the MOD-1 channel was described in Chapter 3. This rank order was determined by measuring the reversal potential of the 5-HT-gated current via the MOD-1 channel in *Xenopus* oocytes in the presence of external bath solutions containing the various anions tested. Figure 8 presents data from representative experiments that allowed us to measure the reversal potential ($E_{rev}$) under these different salt conditions ($E_{rev}$ is the voltage at which the current reverses direction from inward (traces below horizontal; see Nal trace) to outward (traces above horizontal; see Nal trace). Figure 8 demonstrates that the rank order of anion permeability is thiocyanate $>$ bromide $>$ iodide $>$ chloride $>$ fluoride $>$ aspartate $>$ gluconate. This rank order of anion permeability is exactly the same as that for the GABA- and glycine-gated chloride channels (Chapter 3). The $E_{rev}$ values cited in Chapter 3 were average values derived from at least five such recordings for each anion.
mod-1::gfp Transgene Generates Functional MOD-1 Protein

To determine the cells in which MOD-1 might function, a mod-1::gfp translational reporter construct was constructed and introduced into mod-1(n3034) mutants, and then a chromosomally integrated version of the transgene was generated (Chapter 3). These transgenic animals were no longer resistant to serotonin (Figure 9), confirming that the protein product made by the array had sufficient MOD-1 function to rescue the mutant phenotype. This rescue was more robust than that achieved by the extrachromosomal arrays used for transformation rescue experiments while cloning the gene (Chapter 3, Figure 1C), presumably because animals carrying a chromosomally integrated transgene have more consistent transgene expression and do not have transgene mosaicism.

Results Pertaining to Chapter 4

mod-5 Transgenes Render Animals Serotonin-Resistant

We cloned the gene mod-5 by rescuing the serotonin hypersensitivity of mod-5(n823) mutants (Chapter 4). The extent to which each of these transgenic lines, expressing the 8 kb fragment capable of encoding the first 507 amino acids of MOD-5 (671 amino acids at full length), rescued the serotonin hypersensitivity of mod-5(n823) mutants is depicted in Figure 10A. Three of the transgenic lines possessed sufficient serotonin reuptake transporter activity that they did not just restore the serotonin sensitivity to wild type but rendered the animals resistant to serotonin (Figure 10B). This result could be a consequence of overexpression of the transgene to levels beyond normal. Alternatively, it could be a consequence of inappropriate regulation of the truncated transporter expressed by this transgene; perhaps the C-terminal 164 amino acids contain key regulatory sites that modulate transporter activity.

To test the cDNA we had isolated by RT-PCR and RACE, we generated a mod-5 minigene (Chapter 4). This minigene is capable of rescuing the severe serotonin hypersensitivity phenotype of mod-5(n3314) mutants (Figure 11). The transgenic lines exhibited only partial rescue, probably caused by mosaic transgene expression from the extrachromosomal array.
mod-5 Complementation Tests

To confirm that the n3314 allele, which contained a large deletion in the mod-5 genomic locus is an allele of mod-5, we performed complementation tests with mod-5(n822) and mod-5(n823). n822/n3314 and n823/n3314 transheterozygote animals were hypersensitive to exogenous serotonin while n822/+ , n823/+ , and n3314/+ heterozygous animals were not, confirming that n3314 did not complement n822 or n823 (Figure 12). Moreover, the transheterozygote animals were more hypersensitive to serotonin than were mod-5(n822) or mod-5(n823) homozygotes (Figure 12), consistent with the conclusion (Chapter 4) that n3314 leads to a more severe loss of mod-5 function than either n822 or n823.

MOD-5-Mediated [3H]5-HT-Uptake Is not Inhibited by Other Neurotransmitters

MOD-5 did not mediate the uptake of radiolabelled GABA, glutamate, glycine, NE, histamine, or dopamine (Chapter 4, Figure 4D). We sought to determine if these neurotransmitters could inhibit MOD-5-mediated [3H]5-HT uptake. We found that none of the six neurotransmitters tested inhibited the 5-HT uptake (Figure 13), suggesting that the MOD-5 serotonin reuptake transporter is unlikely to have binding sites for any of these neurotransmitters that affect serotonin transport via MOD-5.

Potentiation of The Hyperenhanced Slowing Response of mod-5 Mutants Cannot be Assayed

If fluoxetine brings about the potentiation of the enhanced slowing response (Chapter 2, Figure 5B) by inhibiting MOD-5, then we reasoned that mutants lacking MOD-5 should be resistant to such potentiation. We tested this possibility by assaying mod-5 mutants after food-deprivation in the presence of fluoxetine. We were not able to discern a difference in the response of mod-5 mutants in the presence or absence of fluoxetine pretreatment because food-deprived mod-5 mutants were essentially immobile in response to bacteria (Figure 14). This observation prompted us to utilize mod-5; mod-1 double mutants to test whether the potentiation of the enhanced slowing response by fluoxetine requires MOD-5 (Chapter 4, Figure 5B).
Acknowledgments

I would like to thank Ho-Yon Hwang for helpful comments concerning this chapter.

Experimental Procedures relevant for all the data presented in this chapter except for Figure 8 can be found in Chapters 2-4. Details for Figure 8 have been included in the corresponding figure legend.
Figure 1. 5-HT-Pretreated Food-deprived bas-1; cat-4 Mutants Exhibit Normal Locomotory Rate in the Absence of Bacteria

Following pre-incubation on bacteria-containing plates with no neurotransmitter (Buffer) or with serotonin, bas-1; cat-4 mutants were transferred to food-deprivation plates with buffer or serotonin for 30 minutes and then to assay plates and locomotory rate was measured (two trials). In this and all subsequent figures, unless otherwise stated, each trial involved testing at least five animals in each of the conditions; a given animal was tested in only one of the conditions. Error bars, SEM.
Figure 2. Fluoxetine Potentiates the Enhanced Slowing Response of cat-2 Mutants

*cat*-2 mutants were not resistant to the potentiation of the enhanced slowing response by fluoxetine. Ten wild-type animals and 10 *cat*-2 mutants were tested in each condition in four trials. Error bars, SEM.
Figure 3. Food-deprived Animals Are Not Hypersensitive to Serotonin

Time course of serotonin sensitivity. A single well-fed or food-deprived animal was transferred to a plate (containing no bacteria) with 15 mM serotonin, and locomotory rate was recorded after every minute for five minutes (n=20 per condition). Error bars, SEM.
Figure 4. The Enhanced Slowing Response Is Not Dependent on High Osmolarity Ring on Food-Deprivation Plates

Animals were deprived of food for 30 minutes on plates that did not contain a ring of high-osmolarity fructose. The animals were then transferred to assay plates with or without a bacterial lawn, and five minutes later the locomotory rate of each animal was recorded (n=5 for each condition). Compare with Chapter 2, Figure 1A. Error bars, SEM.
Figure 5. MOD-1 Channel is Responsive to High Concentrations of Dopamine, Octopamine, and Tyramine

Each egg was first tested for its response to 50 nM serotonin, a concentration that does not desensitize the egg's response to subsequent treatments (Chapter 3, and data not shown) and then subjected to a single concentration of one of the other neurotransmitters. The response of each egg to dopamine, octopamine, or tyramine was normalized to the response of that egg to 50 nM serotonin. These normalized responses were then renormalized by equating the 50 nM response in this experiment to the relative response to 50 nM serotonin treatment on the serotonin dose-response curve (Chapter 3, Figure 4B). The serotonin dose-response curve (Chapter 3, Figure 4B) is included in the figure to illustrate that the responses of MOD-1-injected eggs to these three biogenic amines are much weaker than the response to serotonin. Error bars, SEM.
Figure 6. The 5-HT3a-Specific Blocker Ondansetron Did Not Block MOD-1

Representative traces from oocytes injected with *mod-1* cRNA (n=2) and treated with 1 μM serotonin in the presence or absence of 273 μM granisetron.
Figure 7. Mianserin and Methiothepin Block the MOD-1 Channel in Xenopus Oocytes and in vivo

(A) Inhibition curves for mianserin and methiothepin. mod-1-injected oocytes (n=5) first treated with 100 nM serotonin and then treated with 100 nM serotonin in the presence or absence of varying concentrations of mianserin and methiothepin. The maximal response in the presence of inhibitor is presented as a percentage of the uninhibited response. $K_i$ of ~19 μM (mianserin) and ~32 μM (methiothepin) were calculated from the resultant curves. Error bars, SEM.

(B) Methiothepin pretreatment did not further disrupt the enhanced slowing response of mod-1(ok103) mutants (3 trials). Mianserin pretreatment slightly exacerbated the defect in the enhanced slowing response of mod-1(ok103) mutants (3 trials). Also see Chapter 2, Figure 5A. Error bars, SEM.
Figure 7

A

% uninhibited response

[Inhibitor], (μM)

B

Food-deprived animals on bacteria

Body Bends/20 seconds

Preincubation:
- Mi Me

Genotype:
Wild type
mod-1

Mi: Mianserin
Me: Methiothepin
Figure 8. Anion Selectivity of the MOD-1 channel

*mod-1*-injected eggs were treated with 1 μM serotonin while the holding potential of the egg was ramped in 10 mV steps (each step took 5 msec) from -70 mV to + 50 mV and then back down to -70 mV (graphs at the bottom of the figure). This protocol was performed for each egg first in the presence of normal ND-96 external solution and then in the presence of solutions where the chloride ions normally present in ND-96 were substituted with an equivalent amount of one of the six other anions tested. The concentration of the various cations were identical in all these solutions. Representative traces from eggs in each of the salt conditions are shown. The NaCl condition is depicted in each column for comparison to the other solutions in that column. The vertical lines provide an approximate measure of the voltage in each situation at which the current reverses direction ($E_{rev}$). The scale of the y axis (current, in μA) for each egg at each condition is different and hence has been omitted for the sake of clarity. The magnitude of the current is not relevant to the dependence of $E_{rev}$ on the concentration of the anion present in the solution. A horizontal line depicting zero current flow has been included in one of the NaI traces to distinguish what is meant by inward vs. outward current.
mod-1(n3034); lin-15(n765ts) mutants were injected with 5 μg ml-1 of pRR16 (translational fusion of GFP within the mod-1 coding sequence; Chapter 3, Figure 2A) and 80 μg ml-1 of pL15EK (which contains the wild-type lin-15 gene). Stable transgenic lines, which produced a percentage of non-Lin progeny, were subjected to gamma-irradiation and two isolates were selected that produced only non-Lin progeny. One such isolate, nls126, harbored the transgene on chromosome II and this strain was no longer serotonin resistant, unlike mod-1(n3034) mutants. We confirmed that nls126 strain still contained the n3034 mutation by independent segregation of the GFP and the serotonin resistance in the F2 descended from nls126; mod-1(n3034) isolate mated with wild-type animals. The rescue was not complete, presumably because the dominant effect of the n3034 mutation was not completely quenched by the wild-type MOD-1 produced by the transgene. Error bars, SEM.
Figure 10. Rescue of the Serotonin Hypersensitivity of *mod-5*(n823) Mutants

(A) 10 μg ml⁻¹ of an eight kilobase PCR product capable of encoding the first 507 amino acids of MOD-5 was injected with 80 μg ml⁻¹ pL15EK (which contains the wild-type *lin-15* gene) into a *mod-5*(n823); *lin-15*(n765ts) strain and serotonin sensitivity was scored in 20 transgenic lines that produced non-Lin progeny at 22.5°C. Varying degrees of rescue was observed in 16/20 lines. Twenty animals were tested for each line. Zero; no animals were immobilized.

(B) Transgenic lines #1, #3, and #10 from (A) were resistant to serotonin. Twenty animals were tested for each line. Exposure to 33 mM serotonin immobilizes *mod-5* mutants faster than the wild type (data not shown).
Figure 10

A

% Animals immobilized 5 min in 10 mM serotonin

mod-5(n823); lin-15(n765ts); nEX[MOD-5(1-507)]

B

% Animals immobilized 20 min in 33 mM serotonin

Wild type

Transgenic line # 3

Transgenic line # 1

Transgenic line # 10
A *mod-5* minigene construct (see Chapter 4 for details) was injected at 36 µg ml-1 with 80 µg ml-1 pL15EK (which contains the wild-type *lin-15* gene) into a *mod-5(n3314); lin-15(n765ts)* strain and serotonin sensitivity was scored in transgenic lines that produced non-Lin progeny at 22.5°C. Data from one such transgenic line is presented. Error bars, SEM.
Animals of the following genotypes were tested for serotonin sensitivity by incubating 20 animals per trial (3 trials per genotype) in 5 mM serotonin for 5 min: mod-5(n822), mod-5(n823), mod-5(n3314), mod-5(n822)/mod-5(n3314), mod-5(n822)/+, mod-5(n823)/+, and mod-5(n3314)/+. The transheterozygotes exhibited a phenotype intermediate between the phenotypes of the n822 and n823 point mutants and the n3314 deletion mutant. All heretozygotes were generated by mating males of one genotype to hermaphrodites of the other genotype that were marked with the lon-2 mutation and selecting non-Lon cross progeny. The lon mutation does not affect serotonin sensitivity (data not shown). Zero; no animals were immobilized. Error bars, SEM.
Figure 13. Antagonism of MOD-5 CeSERT-Mediated 5-HT Uptake

The extent to which various neurotransmitters inhibited the ability of MOD-5 CeSERT to transport [3H]5-HT. Assays were performed for 10 min with 50 nM [3H]5-HT in the presence of 100 μM of each of the other neurotransmitters. Error bars, SEM.
mod-5(n3314) mutants were deprived of food for 30 minutes in the presence of fluoxetine and their locomotory rate assayed on plates with and without bacteria (3 trials). The severe hyperenhanced slowing response of mod-5(n3314) mutants rendered it impossible to detect any effect of fluoxetine treatment (also see Chapter 4). Error bars, SEM.
Appendix 2

Preliminary Results and Future Directions
In this chapter, I present the results of a preliminary pharmacological analysis of a panel of mod mutants, the characterization and genetic mapping of mod-6(n3076), and a screen for suppressors of mod-5(n3314). mod-6(n3076) is of particular interest because it exhibits an interesting combination of characteristics with respect to the enhanced slowing response and sensitivity to exogenous serotonin. In addition, I suggest in this chapter directions that I consider worthy of pursuit for the future.

Preliminary Results

Pharmacological Profile of mod Mutants

The first genetic screen for mutants with defects in the enhanced slowing response yielded 17 mod mutants (Chapter 2, Figure 6). However, it has proven prohibitively difficult to map and clone the mod genes on the basis of this defect in the enhanced slowing response. Specifically, visible mutations that are used for standard mapping purposes, for example unc and dpy mutations, affect aspects of general locomotion enough to render useless the locomotory assay for the enhanced slowing response. Even when recombinant chromosomes were made homozygous and the presence of a mod mutation was tested by complementation (Chapters 3 and 4), the laborious nature of the locomotory assay for the enhanced slowing response slowed analysis enormously. In fact, the focus of this dissertation, the cloning and characterization of the mod-1 and mod-5 genes, would not have been possible without the development of the liquid swimming assay (Chapters 3 and 4), which allowed us to rapidly score a pleiotropy in the mod-1 and mod-5 mutants.

One alternative to mapping with respect to visible mutations is to map with respect to single nucleotide polymorphisms or restriction length polymorphisms that are present in different naturally occurring isolates of wild type C. elegans. However, such strains are not always as healthy as the standard wild type strain used in the laboratory. Therefore, before such a strain can be used for mapping behavioral mutants, it must either exhibit normal responses in the behavioral assay of interest or the strain must be outcrossed enough to render it healthy while still continuing to carry the polymorphisms relevant for the mapping.

Given that assaying serotonin sensitivity proved useful for mapping and cloning mod-1 and mod-5, we tested a subset of 11 mod mutants that had not
been placed in a defined complementation group (Chapter 2, Figure 6) for their responses to serotonin, octopamine, and phentolamine, a putative octopamine-receptor antagonist (Howell and Evans, 1998). The results of these studies are summarized in Table 1. The strong serotonin-resistance of n3053 (which appeared to be a recessive phenotype; data not shown) and the strong phentolamine-resistance of n3057 and n3074 should make further mapping of these three mutants straightforward. As with any pleiotropy, it will be essential to verify that the pleiotropy cosegregates with the primary defect of interest, an impaired enhanced slowing response.

Such pharmacological analysis of all the existing mod mutants should be conducted in a broader manner, including chemicals that affect serotonergic, cholinergic, dopaminergic, histaminergic, and peptidergic neurotransmission. For example, n3032 mutants exhibit partial resistance (data not shown) to the paralysis induced by high concentrations of fluoxetine (Chapter 4, Figure 5C), a pleiotropy that was not robust enough to serve as a tool for mapping purposes (data not shown). Nevertheless, other chemicals with activities similar to fluoxetine, such as paroxetine or sertraline (Chapter 4), could be tested to determine if n3032 mutants exhibit a response that is robustly different from that of the wild type. While performing such preliminary analysis, several key parameters must be controlled and/or varied. Hence, to serve as a guide for such future analysis, we have described in detail in the Experimental Procedures how the analysis presented in Table 1 was performed.

**mod-6(n3076) Is a Special mod Mutant**

Using the pharmacological approach described in the section above, we determined that mod-6(n3076) mutants, which were defective in the enhanced slowing response (Chapter 2, Figure 6), were hypersensitive to exogenous serotonin (Figure 1). These findings contradicted the model we proposed in Chapter 2 (Figure 7): we expect mod mutants that have an impaired enhanced slowing response to either be defective in responding to serotonin or be defective in a non-serotonergic component that acts in parallel to the serotonin pathway (Chapter 4, Figure 8). Furthermore, we expect mod mutants that exhibit hypersensitivity to serotonin to exhibit a hyperenhanced slowing response, as do mod-5 mutants. For this reason, we found mod-6(n3076) interesting and chose to study it further.
The serotonin hypersensitivity was used to map *mod-6* to a 1.8 map unit interval on chromosome I (see Experimental Procedures for details). *mod-6(n3076)* has been subsequently mapped further by graduate student Daniel Omura in our laboratory and shown to be an allele of the gene *che-3* (data not shown), which encodes a dynein heavy chain (Wicks et al., 2000). *che-3* mutants are defective in the development of the chemosensory cilia (Wicks et al., 2000) that may be used to sense bacteria. An impairment in the ability to chemotactically perceive bacteria could explain the defect in the enhanced slowing response in *mod-6* mutants. In the next section, a hypothesis is presented for why *mod-6* mutants are hypersensitive to serotonin.

**Octopamine and the mod Mutants**

Octopamine has been proposed to act in opposition to serotonin in several *C. elegans* behaviors (Horvitz et al., 1982). Given that serotonin signaling is required for the enhanced slowing response, it is plausible that octopamine signaling modulates the enhanced slowing response and that *mod* mutants are defective in aspects of octopaminergic signaling. There are several preliminary observations that indirectly suggest a role for octopamine signaling in the enhanced slowing response.

A) *C. elegans* mutants containing deletions in the gene *tbh-1*, which encodes a tyramine beta hydroxylase enzyme that converts tyramine to octopamine, do not have detectable levels of octopamine (M. Alkema and H. R. Horvitz, personal communication). *tbh-1* mutants were hypersensitive to serotonin (M. Alkema, R. R., and H. R. H., unpublished data) suggesting that a lack of octopamine leads to a loss of an inhibitory influence on the ability to respond to serotonin. Given the phenotype of *mod-5* mutants, we expected *tbh-1* mutants to also have a hyperenhanced slowing response. However, *tbh-1* mutants move more slowly not only when food-deprived and on plates with bacteria, but under all conditions (M. Alkema, R. R., and H. R. H., unpublished data), making it difficult to interpret the abnormally slow locomotory rate of food-deprived *tbh-1* mutants on plates with bacteria as a hyperenhanced slowing response.

B) *che-3* mutants have reduced levels of octopamine (Horvitz et al., 1982). That *mod-6(n3076)* mutants were hypersensitive to exogenous serotonin might be explained by the deficiency in octopamine expected in *mod-6(n3076)* mutants (octopamine levels in this mutant have not been directly analyzed). The
confluence of an impaired enhanced slowing response and hypersensitivity to exogenous serotonin in the *mod-6* mutants might be serendipitous and unrelated. Nevertheless, it is intriguing to consider why *che-3* mutants lack octopamine. Might the inability to chemotax to bacterial stimuli affect the development of the nervous system in such a manner that octopamine biosynthesis is downregulated? Mutants such as *mod-6(n3076)* would have to be analyzed further to address this issue.

C) The preliminary pharmacological survey (Table 1) indicated that *n3057* and *n3074* were resistant to phentolamine, an octopamine-receptor antagonist (Howell and Evans, 1998). This suggests that at least indirectly *mod* mutants may be defective in aspects of octopaminergic signaling.

The class of mutants defined by *mod-6* (whose response to phentolamine has not been analyzed), *n3057*, and *n3074* may define a class of mutants that are incapable of chemotaxis to bacteria, and this inability leads to two effects: a defect in the enhanced slowing response and an unrelated yet intriguing defect in octopaminergic signaling.

D) Mianserin has been shown to act as an octopamine antagonist in other systems (Roeder, 1990). Perhaps some of its effects on the enhanced slowing response (Chapter 2, Figure 5A) act via the disruption of octopaminergic signaling.

**Hypothesis**

Might our analysis of the *mod* mutants have provided a serendipitous clue to the role of octopamine in the nervous system of *C. elegans*? Perhaps, it is octopamine that serves as a "mood regulator" in *C. elegans*. Specifically, certain internal states may be linked to levels of octopamine in the animal; animals that are food-deprived or are incapable of sensing food may have reduced levels of octopamine. This change in octopamine levels may in turn set the behavioral state or "mood" of the animal, such that there is a coordinated animal-wide behavioral response to the presentation of various stimuli.

This hypothesis could be tested in several ways. Levels of octopamine could be measured radio-enzymatically (Horvitz et al., 1982) in well-fed and food-deprived animals. Other mutants that have defects in sensory structures that might be used to sense food could be tested for levels of octopamine. *tbh-1* mutants could be analyzed for the expression levels of various neurotransmitters,
tested for behavioral responses related to food, such as egg laying (Horvitz et al., 1982), pharyngeal pumping (Horvitz et al., 1982), dauer formation (Golden and Riddle, 1984), and enhancement of olfactory adaptation (Colbert and Bargmann, 1997). Octopamine-treated wild-type animals could be assayed for global changes in behavioral responsivity in the above-mentioned assays. The effect of tbh-1 overexpression from extrachromosomal arrays in the various behavioral assays could also be explored. Octopamine receptor antagonists, such as phentolamine, promethazine, and epinastine (Roeder, 1990; Roeder et al., 1998), and receptor agonists such as NC-5Z (Nathanson and Kaugars, 1989) could be used to perturb octopaminergic signaling. Given that octopamine and serotonin have proposed antagonistic roles in C. elegans (Horvitz et al., 1982), mutants with defects in serotonin signaling could be tested for octopamine levels; especially tph-1 mutants, which completely lack serotonin (Sze et al., 2000), and mod-5 mutants, which have defective serotonin reuptake (Chapter 4).

A Genetic Screen for Suppressors of mod-5(n3314)

A key deterrent to the rapid analysis of mod mutants is the difficult nature of the locomotory assay for the enhanced slowing response. If a collection of mutants could be isolated on the basis of defects in serotonin signaling, which we know is required for the enhanced slowing response, then the analysis would be greatly facilitated. Therefore, we decided to screen for suppressors of the mod-5(n3314) mutation. There are two attributes of mod-5(n3314) mutants that lend themselves to easy screening - the severe hyperenhanced slowing response and the extreme hypersensitivity to exogenous serotonin. It is worth enumerating the merits and demerits of using one or the other of these characteristics as the basis for a genetic screen.

A screen that seeks suppressors of the hyperenhanced slowing response of mod-5(n3314) mutants remains firmly rooted in the behavioral response of interest - the enhanced slowing response. Therefore, the suppressors isolated are likely to be mod mutants in that they will affect the signaling pathway for the enhanced slowing response that involves mod-5. However, such suppressors might suffer from the same problem that has hampered the analysis of the original collection of mod mutants, i.e., their suppression of the hyperenhanced slowing response will be a difficult assay for mapping and cloning purposes.
Further analysis of such suppressors may require the identification of pleiotropies such as differential response to serotonin or some other chemical.

A screen that is designed to isolate suppressors of the hypersensitivity to exogenous serotonin exhibited by *mod-5(n3314)* mutants is extremely powerful in its efficiency and throughput. A large number of animals can be screened very rapidly and the screen can be performed to saturation. However, there is no *a priori* reason that the suppressors will be involved in the enhanced slowing response. They may non-specifically impair the ability of the animal to respond to serotonin, for example by making the cuticle of the animal more impermeable to serotonin, and not affect the response of food-deprived animals to bacteria. However, any suppressor mutation that robustly suppresses the hypersensitivity to exogenous serotonin exhibited by *mod-5(n3314)* mutants will be tractable, by the same strategy used to map and clone *mod-1* and *mod-5*. For this reason, we decided to perform the screen assaying the suppression of the hypersensitivity to serotonin of *mod-5(n3314)* mutants.

We performed an F2 non-clonal screen using ethyl methanesulphonate as the mutagen (Brenner, 1974). The general strategy is diagrammed in Figure 2. We screened 18,350 haploid genomes and isolated 98 suppressed isolates, of which 61 must be independent isolates (see Figure 2 legend for details of screen design). The serotonin sensitivity of these isolates varied enormously (Table 2; only the data for the independent isolates are presented). The 61 independent isolates were tested for suppression of the hyperenhanced slowing response conferred by *mod-5(n3314)* and in 18 isolates food-deprived animals had faster locomotory rates on plates with bacteria than did *mod-5(n3314)* mutants (Figure 3A). In this preliminary analysis we did not test whether these suppressed strains exhibited a faster baseline rate of locomotion. This remains an important test to perform as mutants with faster baseline rates of locomotion need not be true suppressors of the hyperenhanced slowing response of *mod-5(n3314)*. Interestingly, the strength of suppression of the hyperenhanced slowing response did not correlate with the strength of suppression of the hypersensitivity to exogenous serotonin (Figure 3B). This observation suggested that aspects of the serotonin signaling pathway that are affected by exogenous serotonin application differ from those utilized to bring about the enhanced slowing response.

Two of these suppressors, *n3461* and *n3488*, were mapped to chromosome 2 on the basis of weak two-factor linkage to *rol-6*. No existing *mod* mutations map to this chromosome. Both of these mutations exhibit dominant
characteristics at low serotonin concentrations while at high serotonin concentrations, they appear recessive (Figure 4). The reason for such dose-dependent differences requires further investigation.

The Neural Circuit for the Enhanced Slowing Response

One of the implicit goals of this dissertation was to understand how the enhanced slowing response is brought about at the level of a neural circuit within the animal. Sawin (1996) began such an analysis by attempting to define the subset of serotonergic neurons in the animal that are required for the enhanced slowing response. Extensive laser-ablation studies revealed a partial requirement for only the NSMs (Sawin, 1996; and Chapter 2). Our cloning of the mod-1 and mod-5 genes prompted us to identify the neurons in which these proteins were made as a first step towards defining the neural circuit that might be required for the behavior.

MOD-1

We generated two rabbit polyclonal antisera and three rat polyclonal antisera each against GST-fusion proteins of the predicted large N-terminal extracellular domain of MOD-1 (amino acids 18-232). We also generated two rabbit polyclonal antisera and two rat polyclonal antisera against GST-fusion proteins of the predicted intracellular domain (amino acids 329-456) between transmembrane regions M3 and M4 of MOD-1 (Chapter 3, Figure 2B). While the crude antisera were capable of recognizing the corresponding bacterial-expressed MOD-1 polypeptides (data not shown), none of them revealed specific immunoreactivity in either Western blots of total protein extracts from wild-type animals or whole mount preparations of fixed wild-type animals. Affinity purification of these antisera against 6xHistidine tagged versions of the two MOD-1 antigens did not result in any improvement in detection of specific immunoreactivity. It is possible that the regions chosen for generating antibodies were of poor antigenicity.

We generated two different reporters of the mod-1 gene by fusion with gfp (Chalfie et al., 1994): a transcriptional fusion in which ~1 kb of non-coding sequence upstream of the mod-1 translational start site served as the promoter for the gfp gene and an in-frame translational insertion of the gfp into the mod-1 genomic locus (Chapter 3, Figure 2A) which contains the 5' and 3' untranslated
regions required for rescue (Chapter 3, Figure 1C). We generated an integrated version of this \textit{mod-1::gfp::mod-1} translational reporter, \textit{nls126}, and found that it was capable of rescuing the serotonin resistance phenotype of \textit{mod-1} mutants (Chapter 5, Figure 9). This reporter was used to identify neurons that might express MOD-1. Twelve neurons within the head and retrovesicular ganglion, 17 neurons in the ventral cord, and 6 neurons in the tail of the animal exhibited reporter expression. From the position of the neuronal cell bodies with respect to their neighbors and the morphologies of the axons, we were able to make preliminary identification of several neurons (Table 3). Three of the neurons in the head were determined with good certainty to be AVE, AIB, and RIB by cell body position. The AIY and AIZ neurons were also identified as likely to be expressing the reporter. However, the position of AIZ is not always invariant from animal to animal, and we could not rule out that the neuron we identified as AIY was not AIM. In the young L1 larvae, we observed reporter expression in the DB3, 4, 5, 6, and 7 neurons in the ventral cord. In the adult, in addition to the DB neurons, several more ventral cord neurons displayed reporter expression. We think that these additional neurons are likely to be the VBs. We tentatively identified two other neurons in the head as RID and RIP or RMEL. Reporter expression in the RIP/RMEL neuron was observed only in the adult. All of these identifications must be verified using other reporters expressed in these cells, using antibodies that recognize epitopes expressed in the candidate neurons, and by a more careful analysis of cell position possibly combined with laser microsurgery, before a clear picture of the neural circuit can be derived.

The transcriptional GFP reporter was expressed in far fewer neurons than the translational \textit{mod-1::gfp::mod-1} reporter and also showed expressed in cells not seen in the translational GFP reporter. Therefore, the expression pattern of the transcriptional reporter was not examined in more detail as it was likely that this reporter lacked key regulatory sequences required for expression in the appropriate cells.

When the \textit{nls126}-carrying strain was stained with the various anti-MOD-1 antibodies described above, we observed that the two crude rat antisera raised against the predicted MOD-1 intracellular domain stained, albeit very weakly, the same set of cells that fluoresce in the \textit{nls126}-carrying strain (data not shown). It is possible that further affinity purification of at least these two antisera will yield a more sensitive tool for detecting endogenous MOD-1 protein.
MOD-5

We generated GST- and 6xHIS-tagged fusion constructs with the N-terminal portion (amino acids 67-273) of MOD-5. However, we did not detect any production of fusion protein when these constructs were introduced into bacteria (data not shown). It is possible that the three predicted transmembrane segments contained within this region (Chapter 4, Figure 3B) affected expression in bacteria. Therefore, we analyzed the MOD-5 protein sequence for a suitable short stretch that would serve as a antigenic peptide for raising MOD-5 specific antisera. The short peptide formed by amino acids 243-256 was chosen as this region was not part of any predicted transmembrane region, had few similarities to the entire set of predicted proteins in *C. elegans* (The *C. elegans* Sequencing Consortium, 1998) and was likely to be antigenic as per the PolyQuik computer algorithm (Zymed). We affinity-purified these antisera and tested them on both western blots of total protein extracts from wild-type animals and on whole-mount fixed preparations; we observed no detectable signal in either case.

The generation of *gfp* reporter constructs of *mod-5* has proven difficult as sequences from the *mod-5* genomic region do not appear to be tolerated by bacteria. Perhaps, this also explains why the *mod-5* genomic region is only contained within a yeast artificial chromosome and is not represented in any bacterially derived clones (The *C. elegans* Sequencing Consortium, 1998).

Given the *in vivo* serotonin reuptake results we obtained (Chapter 4, Table 1), we think that it is very likely that MOD-5-expressing cells will be the serotonergic neurons in the animal. Nevertheless, this possibility must be corroborated with direct evidence of protein or reporter expression. Two promising venues must be explored: (a) Baculovirus expression systems for making antigens for antibody generation and (b) making a *gfp* reporter construct by inserting the *gfp* gene within the functional *mod-5* minigene construct that we were able to generate (Chapter 4 and Chapter 5, Figure 11).

Future Directions

In this section, I briefly outline the broad spectrum of experiments that I consider most relevant to understanding the enhanced slowing response. This section is by no means an exhaustive list of all possible experiments and is only meant to serve as the starting point for further exploration.
Analysis of Existing mod Mutants

The further analysis of the 11 mutations that are yet to be placed in complementation groups is limited only by the absence of an exploration of the pleiotropies that these mutations might exhibit. Our preliminary pharmacological analysis (Table 1) has shown that at least three of these 11 mutations are immediately tractable. A thorough exploration of the pharmacological profile of these mutants should provide the requisite robust and easy assay for mapping and cloning purposes.

The pleiotropies in these mutants need not be limited to a difference in the liquid swimming assay described in this thesis and could well extend to other easily quantifiable behaviors, such as egg laying or pharyngeal pumping. Therefore, a survey of the responses of these mutants to various chemicals could also assay behaviors other than swimming in liquid.

Suppressors of mod-5

From the pilot screen performed thus far, there are numerous mutations that are amenable for further analysis (Table 2). However, I propose that it will be more efficient to perform the screen to saturation with more stringent criteria to isolate a collection of mutations such as n3461 and n3488, which exhibit strong suppression for both the hypersensitivity to exogenous serotonin and the hyperenhanced slowing response.

The large goal of this project is to analyze the enhanced slowing response at the level of genes and molecules in an attempt to delineate the processes that control the switch in the state of the animal from "well-fed" to "food-deprived." Such processes could well lie upstream of the serotonergic synapses in which MOD-1 and MOD-5 function (Chapter 4, Figure 8). If so, then the suppressor screen, based on the hypersensitivity to exogenous serotonin of mod-5(n3314) mutants, is unlikely to isolate mutations in upstream components, since it is mainly geared towards the targets downstream of mod-5 that are involved in responding to serotonin. Our preliminary observations suggested that the presence of bacteria in the microtiter wells renders animals more sensitive to exogenous serotonin (data not shown). It is possible that when bacteria are present, endogenous serotonin is released and hence results in a higher effective concentration at postsynaptic targets. Therefore, if this screen for
suppressors of mod-5(n3314) were performed in the presence of bacteria, it is possible that mutations will be isolated in the signaling pathway that triggers the release of endogenous serotonin. Such mutations might address the question of what is involved in establishing and changing the behavioral state of the animal.

**Further analysis of mod-1**

**Structure-Function**

MOD-1 has the unique property of being a chloride channel that is gated by serotonin. Structure-function analysis of the MOD-1 protein might reveal features that are important to these aspects of channel function. As discussed in more detail in the Introduction, merely three amino acids residues in the M2 region of a anion or cation channel determine the ion selectivity of the channel (Galzi et al., 1992; Keramidas et al., 2000). Can changing these three residues in MOD-1 convert MOD-1 into a cation channel? Answering this question is interesting, not because it provides yet another example for an established principle, but because it would allow us to understand how MOD-1 functions in vivo. As discussed in the Introduction, depending on the concentration of ions on the two sides of the neuronal membrane, activation of a chloride channel could lead to either depolarization or hyperpolarization of the neuron expressing that chloride channel. We do not know if activation of MOD-1 in vivo leads to neuronal activation or inhibition. However, if we were to generate a variant of MOD-1 that is a serotonin-gated non-selective cation channel, and express such a variant in mod-1(ok103) null mutants, we could explore whether such a transgene could rescue the Mod-1 mutant phenotype. If such rescue were observed, it would suggest that the wild-type MOD-1 chloride channel is likely to function to depolarize neurons. On the other hand, if the transgene-bearing animals were to exhibit a phenotype opposite to that of mod-1 mutants, for example they exhibited hypersensitivity to exogenous serotonin or a hyperenhanced slowing response, we would conclude that wild-type MOD-1 acts as an inhibitory chloride channel in vivo.
Direct in vivo Analysis

Recent advances have been made in performing in vivo electrophysiological recordings from \textit{C. elegans} (Richmond et al., 1999). Such techniques could be utilized to explore MOD-1 function \textit{in vivo}. For example, if the response of a MOD-1-expressing neuron to application of serotonin is measured, this would allow us to determine if activation of MOD-1 excites or inhibits the neuron it is expressed in. However, this experiment is subject to the drawback that the experimenter has to perfuse an external bath solution on the exposed neurons and therefore the MOD-1 activity recorded under these conditions may not reflect the \textit{in vivo} situation.

Another exciting alternative is the possibility of using FRET-based \textit{in vivo} measurement of chloride ion flux (Kuner and Augustine, 2000). Such measurements would allow us to determine whether upon activation of MOD-1, chloride ions enter the cell (leading to hyperpolarization) or they exit the cell (leading to depolarization).

Suppressors of \textit{mod-1}

A genetic screen for suppressors of \textit{mod-1} should define elements that function downstream of or in parallel to \textit{mod-1}. A screen seeking suppressors of the resistance to exogenous serotonin of \textit{mod-1} mutants is likely to be yield mutations that impair the ability of animals to swim, which would include a large number of non-specific mutations that lead to dysfunction of the locomotory neuromusculature. On the other hand, a screen for suppressors of the defective enhanced slowing response of \textit{mod-1} mutants should be feasible. The assay must be designed such that the animals have to move at a normal speed to reach the bacterial source within a time interval before they exhibit their response to bacteria. This requirement should eliminate the background of mutations that would plague the first screen.

The Role of Neuropeptides in Sensing Food

Neuropeptides are thought to act as co-neurotransmitters or neuromodulators in many systems and have been proposed to work in concert with serotonin in the nervous system of both invertebrates and vertebrates (Fuller, 1996). There are
approximately 30 neuropeptide genes in C. elegans (Bargmann, 1998) of which at least 15 belong to the FRMFamide (Phe-Met-Arg-Phe-amide)-related neuropeptide family (Nelson et al., 1998). These 15 FRMFamide-like genes are capable of encoding 53 distinct FRMFamide (Phe-Met-Arg-Phe-amide)-related neuropeptide (Nelson et al., 1998). Mutations in one such gene, flp-1, causes numerous behavioral defects, including an increased amplitude during the characteristically sinusoidal locomotion of C. elegans and a resistance to the inhibitory effects of serotonin on locomotion (Nelson et al., 1998). flp-1 also appears to act in concert with serotonin to control the states of egg laying in the animal (Waggoner et al., 2000). Specifically, flp-1 mutants were not as responsive as the wild type in assays of serotonin-mediated egg laying suggesting that the neuropeptides made by the flp-1 gene are required for serotonin to have its full effect on the egg-laying process (Waggoner et al., 2000).

Given the roles of the flp-1 gene in serotonin-mediated behaviors, I believe it will be worthwhile assaying flp-1 mutants and other mutations in neuropeptide genes for defects in the enhanced slowing response. Analysis of double-mutants between mutations in neuropeptide genes and cloned mod genes may also be informative since some of the contributions of neuropeptides might only be revealed in a sensitized background.

**Fluoxetine Targets**

We showed in Chapter 4 that fluoxetine can act on MOD-5- and serotonin-independent targets to mediate nose contraction and paralysis. Fluoxetine also stimulates egg laying in a MOD-5-independent and partially serotonin-independent pathway. We suggested that targets of fluoxetine other than human SERT might well be important for understanding the side-effects of fluoxetine in humans. We can use the power of C. elegans genetics to screen for non-MOD-5 targets in the hope that human homologs of such targets are involved in mediating the effects of fluoxetine in humans.

I suggest that future experiments be based on fluoxetine-induced egg-laying behavior, for several reasons. First, other groups (Choy and Thomas, 1999) are actively studying the nose contraction response to fluoxetine. Second, the paralysis phenotype is variable and unlikely to be robust enough to be useful in genetic screens (Choy and Thomas, 1999; my unpublished observations).
Third, the egg laying phenotype lends itself to robust quantitation, which should prove helpful during further analysis.

Before performing a genetic screen seeking mutants resistant to fluoxetine in the egg-laying behavior, a general survey should be performed of *C. elegans* mutants defective in neurotransmitter signaling pathways for their response to fluoxetine treatment. We tested *tph-1* mutants, that are defective in serotonin biosynthesis (Sze et al., 2000), and found them to be partially resistant to fluoxetine-mediated egg laying (Chapter 4). Preliminary analysis suggests that *tbh-1* mutants that are defective in octopamine biosynthesis (M. Alkema and H. R. H., unpublished observations) and *adc-1* mutants that are probably defective in histamine biosynthesis (M. Hunter-Ensor and H. R. H., unpublished observations) are no different from the wild type in their responses to fluoxetine in assays of egg laying, nose contraction, and paralysis (data not shown). *cat-2* mutants appeared to be resistant to fluoxetine-induced egg laying (data not shown). However, *cat-2* mutants have fewer eggs in the gonad than the wild type (data not shown). Therefore, these assays with *cat-2* must be repeated: the number of eggs laid in response to fluoxetine and the number of eggs retained in the animals after the fluoxetine treatment should be quantified to obtain a measure of the percentage of available eggs that are laid in response to fluoxetine treatment. Mutants that lack neuropeptides, such as *flp-1*, should be tested.

Fluoxetine has been shown to directly inhibit nicotinic acetylcholine receptors in heterologous expression systems (Maggi et al., 1998; Fryer and Lukas, 1999; Garcia-Colunga and Miledi, 1999). Since acetylcholine stimulates egg laying (Weinshenker et al., 1995; Waggoner et al., 2000), inhibition of acetylcholine function in *C. elegans* is unlikely to stimulate egg laying. There is no a priori reason to negate the possibility that fluoxetine could still affect cholinergic function in *C. elegans*. Therefore, I think that mutants defective in cholinergic neurotransmission should be tested for their response to fluoxetine treatment, since fluoxetine may well act differently in vivo. Such mutants should include *cha-1*, which encodes the choline acetyltransferase (Alfonso et al., 1994); *lev-1*, *unc-29*, and *unc-38*, which encode nicotinic acetylcholine receptor (nAChR) subunits (Fleming et al., 1997); and *unc-17*, *unc-18*, *unc-41*, *unc-13*, *unc-63*, *unc-11*, and *unc-64*, all of which have defects in acetylcholine release (Hosono and Kamiya, 1991). *unc-25* mutants deficient in GABA (Jin et al., 1999) should also be tested.
The design of any genetic screen for mutants resistant to stimulation of egg laying by fluoxetine will be shaped by the results of the general survey outlined above. Nevertheless, such a screen should be done in a *mod-5* genetic background, so that only targets other than *mod-5* are isolated.

**A Comparison of the Effect of Food and Serotonin on Olfaction and the Enhanced Slowing Response**

In Chapter 1, I discussed the olfaction studies by Colbert and Bargmann (1997) wherein they observed that if animals had been starved for 60 minutes or longer prior to the olfactory adaptation regimen (usually 60 minutes in the presence of a field of uniform odorant concentration), the adaptation was enhanced. They also noted that the presence of serotonin during the starvation prevented the starvation-mediated enhancement of adaptation. By contrast, we did not observe a block of the enhanced slowing response when wild-type animals were deprived of food in the presence of serotonin. (Chapter 2, Figure 2B). In this section, I discuss the possible reasons for this apparent difference, propose a model to explain both sets of observations, and outline further tests that could be performed.

Throughout this dissertation, we have refrained from referring to the removal of animals for 30 minutes from food as starvation primarily because we did not want to make assumptions about the motivational drive of the animal. Furthermore, this also allows us to maintain a distinction between the treatment used to elicit the enhanced slowing response and the longer periods of removal from food used and referred to as starvation by others (Colbert and Bargmann, 1997; Mori, 1999). The differences in the protocols used in this dissertation and by Colbert and Bargmann (1997) are as follows:

(1) The duration away from food

The enhanced slowing response is assayed after just 30 minutes of food-deprivation (Chapter 2), whereas at least 60 minutes of starvation are required to enhance olfactory adaptation; a two- to three-hour starvation period is the standard treatment used by Colbert and Bargmann (1997). Moreover, it is important to note that while the animals are adapting to the odorant (usually for 60-90 minutes) they are in the absence of food (Colbert and Bargmann, 1995;
Colbert and Bargmann, 1997). Therefore, all animals that experience the adaptation protocol (with or without the odorant) were starved for at least 60 minutes. And animals that were starved before the adaptation were away from food for at least 3 hours before the chemotaxis assay. Thus, Colbert and Bargmann (1997) are assaying the effect of the additional duration of time away from food (two- to three-hour starvation period) prior to the duration of adaptation.

(2) The differential effect of serotonin treatment

The experiments in Chapter 2 use a pretreatment to serotonin in the presence of food for two hours prior to the 30 minutes of food deprivation. The animals are also exposed to serotonin during the 30 minutes of food deprivation. In Colbert and Bargmann (1997), the animals are exposed to serotonin in the presence (well-fed) or absence (starved) of food for two hours before they undergo the adaptation regimen which, as noted above, involves another 60 minutes of further food-deprivation, but now in the absence of serotonin in all cases.

These differences in the assay may account for the discrepancy we noted above between our results and those of Colbert and Bargmann (1997).

Hormonal and Neuronal Roles for Serotonin

The following is a highly speculative model to explain the effect of the differences in the protocols in the two studies:

Animals when deprived of food for 30 minutes have an altered internal state that allows them to respond to newly-found sources of food by slowing their locomotory rate (the enhanced slowing response). This altered state does not change their olfactory sensibilities. When the animals are starved for 2 hours or greater, they are now in a different internal state that enhances their proclivity to ignore olfactory stimuli that have been present during the period of starvation. Suspending our disinclination to divine the motivational state of the worm under these situations, these two different internal states may be better understood from the perspective of the animal: When food has been scarce for only 30 minutes, there is no need yet to activate dramatically different sensory modalities to find a new food source, but it would still be prudent to slow down when a food source is perchance encountered. However, when food has been scarce for
longer periods of time, there is now the need to ignore the sensory stimuli that are present in the nearby locale where there has been no food and make the effort to sense olfactory stimuli further afield that might lead to new sources of food. Serotonin plays a role in both these internal states but in mechanistically distinct ways. During the first 30 minutes of food deprivation, no serotonin is released as no bacterial stimulus is present. And when a food source is then encountered, there is an increased release of serotonin that leads to the enhanced slowing response. This brief absence of serotonin signaling does not affect olfactory circuits. But starvation, i.e., 120 or more minutes away from food, results in a prolonged absence of serotonin release which allows the olfactory circuit to either lose certain negative feedback mechanisms or gain some new positive input involved in establishing normal olfactory adaptation. The end result is enhanced adaptation.

Furthermore, I propose that the mechanisms of serotonin action on the locomotory and olfactory circuit are distinct — I suggest that it is a hormonal effect of serotonin that leads to the enhancement of olfactory adaptation and that this can occur at the 2-5 mM exogenous serotonin used (Colbert and Bargmann, 1997) during the two or more hours of starvation. By contrast, I suggest that serotonin acting in the enhanced slowing response is neuronal. The 2 mM exogenous serotonin pretreatment does not lead to the stimulation of locomotory circuit, possibly because either a higher effective concentration of serotonin is required at the relevant synapses to achieve locomotory inhibition or the active serotonin reuptake mechanism in serotonergic neurons (Chapter 4) ensures that not enough serotonin lingers in the relevant synaptic cleft to have an effect. However, when food is detected again, the sudden burst of serotonin release from serotonergic neurons is sufficient to bring about the enhanced slowing response.

Outlined below are experiments that might begin to test the hypothesis presented above: tph-1, mod-1, and mod-5 mutants should be tested for their starvation-mediated enhancement of olfactory adaptation. mod-1 and mod-5 mutants exhibit normal chemotaxis to several chemicals (E. Miska and H. R. H., unpublished observations). For the sake of the model proposed here, these mutants need to be normal in the standard adaptation assay and in the starvation-mediated enhancement of olfactory adaptation. This may not be the case if absence of these genes during development affected the olfactory circuit in a manner not discernable in standard assays of chemotaxis or adaptation of
chemotaxis. The key question then would be whether the presence of serotonin during starvation can now inhibit the starvation-mediated enhancement of olfactory adaptation of any of these mutants.

If serotonin is acting as a hormone in the olfactory adaptation paradigm, \textit{tph-1}, \textit{mod-5}, and \textit{mod-1} mutants should exhibit normal inhibition since these gene products should not interfere with the hormonal effects of exogenously added serotonin on the olfactory circuit. However, it is conceivable that \textit{mod-1} mutants may be defective if the MOD-1 ion channel happens to be required in the olfactory circuit to respond to the effect of hormonal serotonin.

If serotonin is acting as a neurotransmitter in the olfactory adaptation paradigm, serotonergic neurons should be involved in the signaling process, and at least \textit{mod-5} mutants would be defective: they would be expected to show inhibition of starvation-mediated enhancement of olfactory adaptation at much lower concentrations of serotonin. \textit{mod-1} mutants may also be defective if the MOD-1 ion channel is required for this neuronal effect of serotonin on the olfactory circuit.

In sum, I propose that serotonin acts differently on different neural circuits; as a neurotransmitter while modulating locomotion, but as a hormone while modulating olfaction. Furthermore, I suggest that in response to the duration of time that the animal has been away from food, the animal can exist in different internal states and that these different states utilize serotonin in distinct ways.
Experimental Procedures

mod-6 Mapping

mod-6(n3076) was mapped to LG I based upon two-factor linkage to dpy-51. The following three-factor data were obtained: unc-11 (28/28) dpy-5 (5/28*) mod-6, mod-6 (2/22*) dpy-24 (22/22) unc-101, dpy-5 (43/62) mod-6 (19/62) unc-29, dpy-5 (37/37) unc-13 (2/37*) mod-6, dpy-14 (6/14) mod-6 (8/14) unc-29. The asterisks represent the instances where we inferred from all of the mapping experiments that the isolates must have had A-non B chromosomes with two crossover events. All mapping experiments were performed by mating hermaphrodites homozygous for the recombinant chromosome with mod-5(n3076) males and scoring the F1 cross progeny for 5-HT hypersensitivity.

Pharmacological Analysis

The data presented in Table 1 were generated following the scheme outlined here. Approximate synchrony of the animals was be achieved by picking L4 larval-stage animals the day before the assay. Assays of swimming behavior were performed in 96-well microtiter plates with 100 or 200 μl of M9 solution containing the drug. Dissolving chemicals in water appeared to dramatically enhance the bioavailability; this could be used in the case of compounds that do not dissolve easily or no response is seen at the highest concentrations that dissolve in M9. However, water is not a isotonic solution for C. elegans and hence extended exposure could result in non-specific effects. S-Basal solution is not recommended as a solvent for these assays as the cholesterol in S Basal interferes with solubility of many compounds. Fresh solutions were made for use the same day. Reusing frozen stocks solutions of neuroactive chemicals should be avoided, especially in the absence of information about stability. For each chemical, the dependence of the response of wild-type animals on both time (5-120 minute range) and concentration (at least 2 orders of magnitude) was determined. At least 10 animals were tested in each microtiter well at each concentration and their response over time was recorded at regular intervals. Controls with no drugs were performed for mutant animals to ensure that there were no non-specific defects in the swimming ability of the mutants. Once the relevant concentration and time range for wild-type animals had been
established, mutant and wild-type animals were tested in parallel using the same batch of solution. Tests were designed to reveal both resistance (using high concentrations and long times) and hypersensitivity (using low concentrations and short times) to the chemical. Often, a difference was be detected only within a specific time window within a certain concentration range. Once the assay was established, we determined whether the response to a chemical was recessive or dominant. If the mutant heterozygotes cannot be easily separated from either wild-type animals or homozygous mutants, using that particular assay for mapping purposes will be difficult.

Protein Production

PCR fragments were generated with the following primers using template plasmid pRR18 (full length mod-1 cDNA): GCTAGCAGGCATGGCGGATCCACA CAGGCTAAAGGAAAACG and GTAGCCAGCTAGCGGGATCCAACTTGAAG CTGATCCAG (GST fusion of MOD-1 N-terminus), GCTAGCAGGCATGGCGG ATCCTGTCAGAACAGCGTAAG and GTAGCCAGCTAGCGGGATCCAACTTGAAG CTGATCCAG (GST fusion of MOD-1 N-terminus), GCTAGCAGGCATGGCGG ATCCTGTCAGAACAGCGTAAG and GTAGCCAGCTAGCGGGATCCAACTTGAAG CTGATCCAG (GST fusion of MOD-1 intracellular domain), GCTAGCAGGCATGGCGG ATCCTGTCAGAACAGCGTAAG and GTAGCCAGCTAGCGGGATCCAACTTGAAG CTGATCCAG (GST fusion of MOD-1 intracellular domain), GCTAGCAGGCATGGCGG ATCCTGTCAGAACAGCGTAAG and GTAGCCAGCTAGCGGGATCCAACTTGAAG CTGATCCAG (GST fusion of MOD-1 intracellular domain), GCTAGCAGGCATGGCGG ATCCTGTCAGAACAGCGTAAG and GTAGCCAGCTAGCGGGATCCAACTTGAAG CTGATCCAG (GST fusion of MOD-1 intracellular domain). The PCR products for the GST fusions were digested with Bam HI and ligated into plasmid pGEX-2T (Pharmacia) digested with Bam HI. The PCR products for the 6xHIS fusions were digested with Xho I and ligated into plasmid pET19b (Novagen) digested with Xho I. In all cases, directionality of insert was checked by diagnostic restriction digests and the insert was sequenced to ensure that no errors were introduced during the PCR. The protein was produced in E. coli and purified by polyacrylamide gel electrophoresis according to standard methods (Harlow and Lane, 1988). Polyclonal antibodies were prepared by immunizing rabbits and rats with the purified fusion proteins. Crude antisera were used at dilutions ranging from 1:100 to 1:2000 on both western blots and whole mounts. Antisera were purified by binding to a nitrocellulose filter strip containing the 6xHIS fusions of the corresponding antigens and eluting the specifically bound antibodies with 100 mM glycine (pH 2.5). Affinity purified antisera were used at dilutions of ranging from 1:200 to
1:5000 on both western blots and whole mounts. For western blots, the immunoreactivity was visualized by using horse-radish peroxidase-conjugated secondary antibodies (Bio-Rad) and chemiluminescent detection reagents (Amersham). Whole-mount immunofluorescence staining was performed as described previously (Finney and Ruvkun, 1990; McIntire et al., 1992; Nonet et al., 1993; Nonet et al., 1997).

Acknowledgments

I would like to thank Mark Alkema, Melissa Hunter-Ensor, Eric Miska, and Daniel Omura for allowing me to discuss their unpublished findings; Megan Higginbotham for assistance in performing the mod-5 suppressor screen and for generating Figure 2; Cori Bargmann for her preliminary identification of neurons in the head that express the mod-1::gfp::mod-1 reporter; and Mark Alkema, Brad Hersh, Ho-Yon Hwang, Megan Higginbotham, and Ignacio Perez de la Cruz for helpful comments concerning this chapter.
References


Table 1. Preliminary Pharmacological Analysis of a Subset of *mod* Mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Serotonin (33 mM)</th>
<th>Serotonin (20 mM)</th>
<th>Serotonin(^a) (14 mM)</th>
<th>Octopamine (35 mg/ml)</th>
<th>Phentolamine (4 mg/ml)</th>
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</thead>
<tbody>
<tr>
<td>n3052</td>
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<td>N</td>
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<td>40% R</td>
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<td>inc.</td>
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</table>

N; Normal, R; Resistant, inc.; inconclusive. In all cases, the data presented are the responses after 20 minutes of exposure. There were instances where the difference between mutant and wild-type responses was greater at shorter time intervals (data not shown).

\(^a\)Since wild-type animals were not completely immobilized after 20 minutes at this serotonin concentration, we subtracted the percentage of wild-type animals not immobilized from the percentage of resistant mutant animals.
Table 2. Serotonin Sensitivity of Suppressors of *mod-5(n3314)*

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<table>
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</table>

20 animals were placed in 200 ml of 5 mM serotonin and swimming activity was recorded 5 minutes later.
Table 3. Preliminary Identification of Neurons Expressing *mod-1::gfp* Reporter.

**Certain**

AVE
AIB
RIB

**Need confirmation**

AIY — use *ttx-3* reporter (Hobert et al., 1997) to confirm; need to rule out AIM
AIZ — use *lin-11* reporter (Hobert et al., 1998) to confirm
DB neurons in the ventral cord — use *acr-5* reporter (Winnier et al., 1999) to confirm

A note of caution: Relying on other reporters for colocalization data can be misleading if the cell identifications using the other reporters were not performed rigorously.

**Likely**

RID
RIP or RMEL (adult-specific)
Figure 1. *mod-6(n3076)* Mutants Are Hypersensitive to Exogenous Serotonin

Animals were placed in 200 µl of 10 mM serotonin and the number of animals immobilized recorded after 9 minutes (3 trials with 20 animals each). *mod-6(n3076)/+* heterozygotes were obtained by crossing *mod-6(n3076)* males to *lon-2* hermaphrodites. *lon-2* does not affect serotonin sensitivity (data not shown). Error bars, SEM.
Figure 2. Genetic Screen for Isolating Suppressors of *mod-5(n3314)*

Gravid *mod-5(n3314)* hermaphrodites were treated with alkaline bleach (Wood et al., 1988) to release eggs contained within the animals. These animals were synchronized by starvation-arrest as L1 larvae. They were then seeded onto plates with food and allowed to grow. Standard ethyl methanesulphonate mutagenesis (Brenner, 1974) was performed on late L4 larvae. These mutagenized *P₀*ₕs were plated at low density onto large plates so that the F1s would become adults before they exhausted the food on the plates. These F1s were then subjected to the alkaline bleach treatment to harvest the F2 eggs and these F2s were once again synchronized by starvation-arrest as L1s. The F2 eggs from each plate containing *P₀*ₕs were kept separate. The F2s were then plated at ~300 animals/plate (one plate per F2 population) as this was the maximum number that could be optimally screened in one well of serotonin. Given the large number of F1s that generated the large collection of F2s from which the 300 animals were plated, each of the 300 animals has a high probability of harboring an independent mutation and hence each F2 animal tested was considered to represent 0.5 haploid genomes. The ~300 F2s were rinsed off the plate with 1 ml of M9 and added to 1 ml of 35 mM serotonin dissolved in M9, resulting in a final concentration of 17.5 mM serotonin. After 5 minutes, animals that were still swimming were removed and plated 1 animal per plate.
Mutagenize *mod-5(n3314)*

Place 5-6 P₀s/plate

bleach F₁s and synchronize F₂s

plate ~300 F₂s/plate, 1 plate per F₁ batch

rinse ~300 F₂s into well with serotonin

pick animals swimming after five minutes

retest ~20 F₃ animals