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Genetic Analysis of Egg-Laying in *Caenorhabditis elegans*: Insights into the Molecular Basis of Behavior

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biological Sciences

By

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1999

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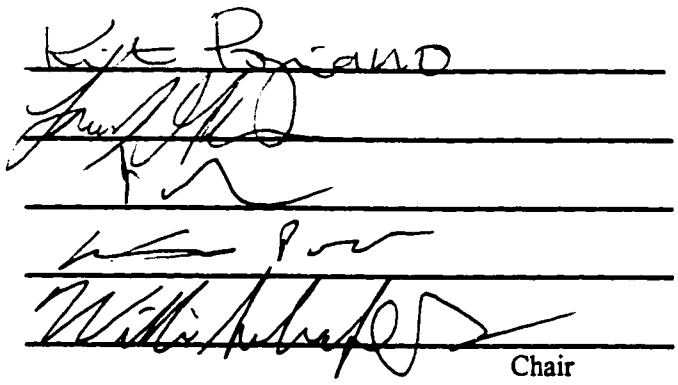

Chair

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1999

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ABSTRACT OF THE DISSERTATION

Genetic Analysis of Egg-Laying in *Caenorhabditis elegans*:

Insights into the Molecular Basis of Behavior

by

Laura E. Waggoner

Doctor of Philosophy in Biology

University of California, San Diego, 1999

Professor William R. Schafer, Chair

In order to understand the genetic, molecular, and cellular basis of behavioral regulation, I have analyzed egg-laying behavior in the nematode, *Caenorhabditis elegans*. The pattern of egg-laying events in *C. elegans* can be modeled as a novel random point process, in which animals fluctuate between an active egg-laying state, where eggs are laid in clusters, and an inactive state, where eggs are retained. Serotonin, released from two sets of neurons, HSNR/L and VC4/5, induces the active state. The effects of serotonin are mediated by a protein kinase C homolog, *tpa-1*, and by the $G_q\alpha$ homolog, *egl-30*. In addition, a FMRFamide like peptide, encoded by *flp-1*, also induces entry into the active state. Acting in a distinctly different capacity, acetylcholine, found in the HSNs and VCs, regulates egg-laying within the active state itself. Finally, experiments suggest that the behavioral states observed in *C. elegans* egg-laying might correspond to functional states of the muscles,

which involves modulation of voltage–gated calcium channels and negative regulation through a pathway including the $G_o\alpha$ homolog, *goa-1*.

Studies also indicate neuronal function is important for producing the egg-laying pattern. Part of the effects of *goa-1* on egg-laying are dependent on the HSNs, and thus, *goa-1* might modulate HSN activity to negatively regulate egg-laying. In addition, because the *flp-1* peptides are found only in head neurons, their effects might involve modulation of the HSNs, which make synapses with head neurons.

Lastly, I used the behavioral model of egg-laying to investigate the mechanisms that underlie long term adaptation of behavior. In *C. elegans*, nicotinic acetylcholine receptors (nAChRs) undergo adaptation to prolonged exposure to nicotine. In the egg-laying circuit, these nAChRs are expressed in both the VC neurons and the vulval muscles, and while acute exposure to nicotine stimulates egg-laying, long term exposure renders the animals insensitive to stimulation of egg-laying by nicotinic agonists. This adaptation is dependent on *tpa-1*, a homolog of PKC, and treatment of animals with phorbol esters mimics nicotine adaptation in a *tpa-1* dependent manner. Thus, these experiments suggest nicotine adaptation involves long term modulation of nAChRs by PKC.

CHAPTER I INTRODUCTION

One of the main goals in modern neurobiology is understanding how genes affect behavior. However, the extreme complexity of the human brain makes it nearly impossible to determine precisely how particular molecules, acting in a defined set of neurons, affect a specific behavior. In contrast, the relatively simplistic nervous systems in other organisms, such as the invertebrate *Caenorhabditis elegans*, can be utilized to address these questions. In addition, the simplicity of the *C. elegans* nervous system, which contains only 302 neurons of precisely determined position and cell lineage, allows for genetic analysis of these signaling pathways, and thus, a more in depth analysis of the molecules that function to regulate behavior. Through these studies, one can gain a reductionist understanding of the molecular basis of behavior. Furthermore, the ability to determine the role of particular molecules in a behavior makes it possible to use behavioral defects of mutants to genetically investigate the molecular events underlying poorly understood mechanisms such as drug addiction.

In this dissertation, I have studied the molecular mechanisms governing one behavior in *C. elegans*: egg-laying. In this introduction, I will first give a basic overview of nervous system structure in the nematode *C. elegans*. I will then describe how genetics has been used to analyze the molecular basis of three behaviors in the worm: locomotion, feeding and defecation. Lastly, I will present what was known about egg-laying behavior before these studies began.

AN OVERVIEW OF THE *C. ELEGANS* NERVOUS SYSTEM

C. elegans is a soil nematode of approximately 1 mm in length. There are both males and hermaphrodites in this species, but for the purposes of this introduction and the work presented herein, the description of nervous system structure and relevant behaviors will be based on the hermaphrodite. The nervous system of the hermaphrodite, which contains 302 neurons of 118 cell types, has been fully characterized and distinct cell lineages have been shown to create reproducible patterns of neurons and connectivity during development [1]. Many of these sublineages are reiterated along the axes of the animal. One example is the neuroblast precursor P cells, which give rise to motor neurons and are reiterated 13 times along the anterior-posterior axis [2]. Synaptic structures have also been fully characterized, and there are approximately 2000 neuromuscular junctions, 5000 chemical synapses, and 700 gap junctions [3]. Neuron-muscle and neuron-neuron connections are not as distinctly different as they are in vertebrates, and while pre-synaptic terminals can be observed to contain electron-dense specializations, the post-synaptic morphology is difficult to visualize and does not contain junctional folds as seen in mammals [1].

Despite the simplicity of this nervous system, a wide variety of classical neurotransmitters are utilized. Acetylcholine appears to be the primary excitatory neurotransmitter and is the only transmitter thus far that has been shown to be essential for viability of the worm. Animals completely lacking ACh, through mutations in *cha-1*, the gene that encodes choline acetyltransferase (ChAT), are able to hatch but can barely move or feed and die as L1 larvae [4] [5] [6]. The genes responsible for synthesis and vesicle loading of ACh are grouped into a single transcriptional unit, much the same way as in mammals [6] [7]. Staining with antibodies to ChAT or the synaptic vesicle acetylcholine transporter, UNC-17, shows that almost all cells containing ACh are motor

neurons, including the ventral cord motor neurons, pharyngeal motor neurons, and sublateral motor neurons [3].

The effects of ACh at neuromuscular junctions are mediated by receptors of the nicotinic acetylcholine receptor (nAChR) family and also of the muscarinic receptor family [8] [5] [9]. nAChRs in *C. elegans* differ slightly from vertebrate receptors, in that they do not bind α-bungarotoxin but do bind the nicotinic agonist levamisole, an anti-helminthic drug [10] [8]. At least 16 nAChR subunit genes have been identified, many of which have been characterized. Of those characterized, three subunits, *unc-29*, *unc-38* and *lev-1*, which comprise the receptor found in body wall muscle, encode both non-α and α type subunits [11]. Two receptor subunits expressed in neurons have also been characterized: *deg-3*, which encodes an α-7 like nAChR subunit and is expressed in touch receptors [12] and *acr-2*, which is expressed in multiple classes of motor neurons [13]. Finally, while pharmacological evidence suggests a role for muscarinic receptors in the worm, as of yet, muscarinic receptor genes have not been well characterized [5] [9].

GABA is another neurotransmitter found in *C. elegans*, mostly in inhibitory motor neurons, although use in excitatory motor neurons is also observed [14]. Loss of GABAergic transmission, through mutations in *unc-25*, the gene encoding glutamic acid decarboxylase (GAD), results in viable worms with motor defects such as a shrinking response to touch, which represents simultaneous contraction of both ventral and dorsal body wall muscle, as compared to a wild-type sinusoidal reversal [14].

The aminergic neurotransmitter serotonin was identified by both formaldehyde-induced flourescence (FIF) and serotonin immunostaining, and at least ten cells contain serotonin in the hermaphrodite [15] [16]. Exogenous serotonin has a variety of effects on worm behavior, from stimulation of egg-laying and pharyngeal pumping, to inhibition of movement and defecation [17] [18]. FIF also identified another aminergic neurotransmitter- dopamine. Exogenous dopamine inhibits locomotion and egg-laying,

although it has not been proven in vivo [19] [18]. However, dopamine is present in eight sensory neurons, and it is possible that it is required for their function [20].

Several other neurotransmitters have been identified in *C. elegans*, although their role in behavior and method of action is not as well understood. Glutamate acts in both an inhibitory and excitatory capacity, and multiple glutamate receptor genes have been identified [3]. One subunit gene, *glr-1*, encodes a protein homologous to the vertebrate AMPA receptor subunit and is expressed in at least 17 classes of neurons, including sensory neurons and mechanosensory neurons [21] [22]. Octopomine has been detected in extracts of *C. elegans*, but little is known about the expression pattern and biological action in vivo [17].

Lastly, *C. elegans* has been shown to contain multiple peptide neuromodulators, of which one class of FMRFamide like peptides has been extensively studied [23] [24]. The *flp-1* gene encodes a peptide-precursor that can be spliced to give eight related FLRFamide peptides [23]. Antibodies recognizing all of the FMRFamide and FLRFamide peptides show expression of the peptides in the VC motor neurons and 25 other cells including interneurons and motor neurons [24].

LOCOMOTION

Neuromusculature

Locomotion in the worm involves contraction of one side of body wall muscle and the concurrent relaxation of the opposing side body wall muscle, which creates a sinusoidal pattern of movement [25]. The body wall muscles are organized into two ventral and two dorsal rows, each consisting of 23 to 24 diploid mononucleate cells [26]. These muscles

are innervated by sets of motor neurons; specifically, the ventral body wall muscles receive input from motor neurons VA, VB, VC, and VD, while the dorsal body wall muscles are innervated by classes AS, DA, DB and DD [1]. For the sinusoidal pattern to be generated, these motor neurons have to stimulate one set of muscles to contract and simultaneously relax or inhibit the muscles on the opposing side; thus, generation of the pattern must involve of cooperation of excitatory and inhibitory neurons. The A, B, and AS neurons have been shown to contain acetylcholine and are most likely excitatory [25], while the class D motor neurons contain GABA and are inhibitory [14].

One possibility is that the precise contractions are coordinated by the D neurons acting as cross-inhibitors, which allow contraction of one side of body wall muscle and prevent contraction on the opposing side [1]. This is supported by the connectivity pattern observed. The A and B type neuromuscular junctions involve an A or B terminus apposed to two separate postsynaptic elements: a body wall muscle and a D dendrite [1]. For example, the ventral D neurons (VD) receive input from the dorsal excitatory motor neurons (DA and DB)) and synapse on the ventral muscles to inhibit their activity during dorsal excitation. This model for locomotion is supported by work in *Ascaris suum*, which contains very similar patterns of connectivity and development to *C. elegans*, yet is amenable to electrophysiological studies [27].

As is seen in vertebrates, this circuit of muscles and excitatory/inhibitory motor neurons is capable of creating a rhythmic pattern of behavior, in this case, of movement. Vertebrates have specific cells that regulate or control oscillating activity that is intrinsic to a particular behavior, and these cells are termed pattern generators [28]. Pattern generators have not been identified so far in *C. elegans*, in respect to movement. However, electrophysiological advances may soon aid in their identification.

Signaling pathways: the role of G proteins and diacylglycerol kinase

Having established the neuromusculature of the locomotion circuit, regulation of the pattern could be analyzed to study the neurotransmitters and signal transduction pathways acting in the motor neurons and muscles. Studies had shown that several neurotransmitters have effects on movement. Acetylcholine and cholinergic agonists were shown to stimulate body wall muscle [10] [8]. GABA was found to be necessary for the inhibition of simultaneous ventral/dorsal body wall contraction [29] [14], and exogenous application of serotonin or dopamine inhibits movement in general [19] [18] [17].

However, the most interesting advances on this subject were made more recently, and involve the identification of signaling pathway members. Both Segalat et al. (1995) and Mendel et al. (1995) identified the gene *goa-1*, which encodes a homolog of an alpha subunit of the heterotrimeric guanosine triphosphate-binding protein G_o. *goa-1* loss of function mutants exhibit a hyperactive movement phenotype, consisting of deeper than normal sinusoidal bends at a more rapid rate than wild-type, while overexpression of the gene confers a reduction in movement. In addition, the expression pattern of *goa-1::GFP* showed localization in the VC neurons, which synapse onto body wall muscle [18] [30]. This suggests that the endogenous level of *goa-1*, through function in the motor neurons, is important for regulation of the speed of movement as well as the amplitude [18] [30]. One possibility is that $G_o\alpha$ might function downstream of serotonin in inhibiting movement. Interestingly, *goa-1* mutants are resistant to the effects of serotonin on movement, which supports the idea that *goa-1* is regulating serotonin signaling in the ventral cord neurons [18].

More recently, Nurrish et al. (1999) discovered that another gene, *dgk-1*, which encodes diacylglycerol (DAG) kinase, is functioning downstream of serotonin in the VC neurons to inhibit movement. *dgk-1* mutants exhibit hyperactive locomotion, similar to

goa-1 animals, and DGK-1 is also expressed in the VC motor neurons [31]. The similarity of *goa-1* and *dgk-1* phenotypes suggests that they function in a similar pathway, but further analysis has not determined whether they function in parallel or if *dgk-1* acts downstream of *goa-1*. However, Nurrish et al. [31] did determine that serotonin was inhibiting release of acetylcholine from the motor neurons, and that this inhibition was dependent on *goa-1* and *dgk-1*. Observing the neuromuscular junction, Nurrish et al. [31] discovered that the DAG binding protein UNC-13, which stimulates neurotransmitter release, was increased in *goa-1* mutants, supporting a model where serotonin and $G_o\alpha$ function in the VC motor neurons to regulate DAG and UNC-13 at sites of acetylcholine release. Because all these pathway components are conserved in mammals, this signaling cascade might represent a conserved mechanism for modulation of synaptic transmission [31].

In addition to identification of the $G_o\alpha$ pathway in motor neurons of the locomotion circuit, another heterotrimeric G protein was shown to play a role in regulation of movement. Brundage et al. (1996) identified *egl-30*, which encodes a $G_q\alpha$ subunit with more than 80% homology to the mammalian family of $G_q\alpha$ proteins. Mutations in *egl-30* confer flaccid paralysis, most consistent with a loss in body wall muscle function (as compared to a nervous system defect, which would result in slow uncoordinated movement without flaccidity) [32]. Although direct expression of *egl-30* in the body wall muscles has not been demonstrated, several double mutant studies, between *egl-30* and mutations in genes involved in neurotransmitter release or synapse formation, support the hypothesis that *egl-30* functions in the muscles themselves [32]. Interestingly, this is paralleled in mammals, where G_q functions in smooth muscle to directly stimulate contraction through activation of the phosphoinositide pathway [33].

However, recent studies suggest a role for $G_q\alpha$ in the nervous system in regulation of movement. Lackner et al. (1999) and Miller et al. (1999) investigated the positive

regulation of movement by the *egl-30* pathway. Both groups identified the gene *egl-8*, which encodes a phospholipase Cβ protein that functions downstream of *egl-30* in this pathway, and expression studies show that *egl-8* is present throughout the nervous system. In addition, Lackner et al. (1999) showed expression of *egl-30*::GFP in the ventral cord neurons. Vertebrate studies have shown that $G_q\alpha$ activates PLCβ [34], which suggests that *egl-30* activates *egl-8* in neurons to positively regulate movement.

Interestingly, the two G protein pathways can be linked in the regulation of movement. Nurrish et al. (1999) showed the negative regulation of movement by *goa-1* and *dgk-1*, and double mutant studies between these genes and *egl-30/egl-8* indicate *goa-1* negatively regulates the *egl-30/egl-8* pathway [35]. This negative regulation might occur via an intermediate protein, EGL-16, which encodes an RGS protein shown to negatively regulate *egl-30* signaling and also shown to be downstream of *goa-1* [36]. Thus, the antagonistic interactions of G protein pathways give multiple levels for regulation of acetylcholine release at neuromuscular junctions in the locomotion circuit.

FEEDING

The *C. elegans* pharynx

The pharynx is the muscle responsible for feeding in *C. elegans*. It consists of eight muscles that surround the lumen, where bacteria and fluid pass; within the folds of these muscles lie the motor neurons, and all this is surrounded by a basal lamina [37]. When the muscle contracts, the pharyngeal lumen opens, allowing food to flow into the pharynx. The pharynx is further broken down into three functional parts: the corpus, the isthmus, and the terminal bulb [38]. The corpus comprises the anterior half of the pharynx, and

takes in and traps bacteria. The isthmus regulates the flow of bacteria from the corpus to the terminal bulb, which is then responsible for grinding it up [37].

The feeding process consists of two major events: pumping and isthmus paristalsis [37, 39]. Pumping involves the simultaneous contraction of the corpus, anterior isthmus and terminal bulb, followed by a simultaneous relaxation [38]. After the relaxation step, a peristaltic wave of contraction in the posterior isthmus brings the bacteria from the anterior pharynx to the grinder in the terminal bulb, and this occurs approximately once for every four pumps [40]. However, because of the lack of high speed video and computer/physical models, there are still many questions as to how the bacteria remains trapped in the lumen, while the liquid medium is expelled back through the mouth.

Although electrophysiology in *C. elegans* has been difficult, the pharynx was the first cell type from which intracellular recordings were possible. This allowed for detailed characterization of contraction of the pharynx. The action potentials recorded were very similar to those of *Ascaris* pharyngeal muscle, and also to the vertebrate heart muscle ventrical cells [38]. Raizen and Avery (1994) determined that there are three phases to this action potential: excitation, plateau, and repolarization. A visible contraction can be seen approximately 30 milliseconds after the excitation phase. Although the ionic basis of the action potential has not been clearly defined, it most likely involves voltage-activated calcium channels [38]. Studies on *unc-68*, which encodes a ryanodine receptor responsible for releasing intracellular stores of calcium, indicate the intracellular release of calcium through this receptor is not necessary for pharyngeal contraction. This suggests that enough calcium must enter through the plasma membrane [38, 41].

Regulation of pumping by signaling pathways in the nervous system

Suprisingly, the nervous system is not necessary for the pharynx to contract. Pumping continues even after the entire pharyngeal nervous system is killed [39], and Raizen and

Avery [42] showed that the quality of the different phases of the action potential are not significantly altered by killing all neurons except M4, which is necessary for viability of the worm. Therefore, there must be some intrinsic capacity for muscle contraction and some trigger for the initial change in membrane voltage that opens the calcium channels. This mechanism of excitation is as of yet unknown.

Although the nervous system is not necessary for contraction, there are neurons that have direct effects on pharyngeal pumping. *C. elegans* hermaphrodites actually contain two main nervous systems: the extrapharyngeal, consisting of 282 neurons, and the pharyngeal, consisting of 20 [1]. Of these 20 pharyngeal neurons, only three are necessary for nearly normal feeding [43, 44]. M3, which is an inhibitory motor neuron that synapses onto the metacorpus and possibly the isthmus [37], regulates proper timing of the relaxation phase of the action potential. Glutamate is most likely the neurotransmitter utilized by this motor neuron, as exogenous application mimics the effects of M3 and *avr-15* mutants, which have a pharynx insenstive to glutumate and no M3 neurotransmission [45, 46].

The second motor neuron necessary for proper pumping is the excitatory neuron MC, which controls the rate of pumping [39, 44]. Firing of MC usually produces muscle action potentials in the pharynx and Raizen et al. [44] propose that this is the pacemaker for pharyngeal pumping. Rate of pumping is affected by environmental cues and neurotransmitters such as serotonin, and most of this regulation requires MC [17] [5, 39, 44]. The neurotransmitter utilized by MC is somewhat unclear, and while pharmacological evidence suggests acetylcholine ([5, 44], antibody staining to CHA-1 or UNC-17, which are choline acetyltransferase and a vesicular acetylcholine transporter, respectively, fail to show localization in the MC motor [38]. However, Raizen et al. (1995) identified two genes necessary for MC function, *eat-2* and *eat-18*, which most likely encode subunits of a

nAChR, further supporting the hypothesis that acetylcholine is the excitatory neurotransmitter for pharyngeal excitation.

Lastly, the action of isthmus paristalsis is controlled by motor neuron M4, which synapses onto the posterior isthmus [37, 47]. Without M4, worms are unable to swallow food and are severely malnourished and fail to grow. Very little is known about the communication and signaling mechanisms between M4 and the pharyngeal muscle. Antibodies to UNC-17 stain M4, so acetylcholine could be the neurotransmitter used, although reduction of endogenous acetylcholine does not result in major isthmus peristalsis [38].

Genetic studies identified signaling proteins functioning in the pharyngeal muscle and neurons to regulate pumping. *goa-1*, encoding $G_o\alpha$, is expressed in both the pharyngeal muscles and neurons (although specific neurons have not been identified) and mutants have reduced pumping rates [18]. It is possible that $G_o\alpha$ could be functioning downstream of serotonin, which is known to stimulate pumping in the worm [17] [5]. However, *goa-1* mutants still respond to exogenous serotonin, suggesting they function either in the neurons or muscles to regulate pumping in response to another neurotransmitter [18].

Another heterotrimeric G protein, $G_q\alpha$, was shown to be essential for pharyngeal function as well. Brundage et al. (1996) illustrated how severe mutations in *egl-30*, which encodes $G_q\alpha$, confer essentially lethal phenotypes and that less severe alleles result in weak pumping and slow growth. Evidence indicates a function for *egl-30* in the pharyngeal muscles, although recent expression studies have shown *egl-30* is localized to both the pharyngeal muscle and several neurons in the nerve ring [48]. *egl-30* mutations allow *eat-11* mutants to survive in the presence of arecoline, a muscarinic agonist which causes pharyngeal muscle contraction and severely abnormal pumping in the *eat-11* mutants, but has little effect on wild type animals [32]. In addition, this phenotypic rescue is independent of the pharyngeal nervous system, arguing that the site of $G_q\alpha$

action is in the pharyngeal muscles [32]. Because arecoline is a muscarinic agonist, it is possible that the *egl-30* product $G_q\alpha$ might function by mediating acetylcholine signaling in the pharyngeal muscles.

As described, mutations in the two G proteins studied, $G_o\alpha$ and $G_q\alpha$, have similar effects on feeding behavior. Future studies might uncover how these pathways are regulated in the pharynx and how they interact to produce behavior. However, the sequence similarities between these G proteins and those found in mammals suggest that the molecular interactions might be similar as well [32]. This illustrates the importance of understanding, at the level of invertebrates, how signal transduction mechanisms interact to regulate behavior.

DEFECATION

Neurons and muscles of the defecation motor program

The defecation motor program, which is conserved in other nematodes, consists of 4 main steps. As described in Avery and Thomas (1997), the first step involves contraction of the posterior body muscles (pBoc), when fluid in the gut is compressed anteriorly. Next, the animal relaxes and most of the fluid is concentrated near the anus, which is referred to as the relaxation step. The animal then contracts the anterior portion of body wall muscle (aBoc), and simultaneously contracts the head such that the pharynx is pressed into the gut. Lastly, while the anterior muscles are still contracted, the enteric muscles contract, which is referred to as an expulsion muscle contraction or EMC, and the anus opens and the gut contents are expelled (Exp step). Although complex, this defecation motor program (DMP) requires each of the motor steps, and their combined control over defecation probably has adaptive value [38]. In addition, defecation is unique compared to

the behaviors discussed thus far, in that there is a distinct periodicity regulating the frequency of contractions [49]. The standard period between contractions is 45 seconds when assayed at temperatures ranging from 19°C to 30°C [50, 51].

Only two steps, aBoc and Exp, have been shown to be mediated directly by the nervous system. The first line of evidence comes from looking at *unc-25* mutants, which have little or no GABA, and are not able to efficiently activate the Exp step [29, 52]. Interestingly, GABA appears to function in an excitatory capacity in this case, whereas in other mammals, and other systems in *C. elegans*, it functions as an inhibitory neurotransmitter [29, 53]. This invites the possibility that in defecation, GABA might act on a novel receptor and signal through some second-messenger system. The gene *exp-1* has been identified as a potential candidate for this receptor [52] [54]. In addition, the G-protein G_o is present in the enteric muscles, and could possibly be the target of GABA [30] [18]. While there is no direct evidence for this, Segalat et al. (1995) did show that there were no major defecation defects in animals mutant for G_o. In addition, serotonin, which inhibits enteric muscle contractions when applied exogenously, has less effect on *goa-1* mutants, and thus, $G_o\alpha$ appears to mediate the effects of serotonin on defecation. This leaves open the question as to how GABA signals to positively regulate defecation.

Immunostaining for GABA identified the neurons AVL and DVB, which make synapses with the enteric muscles and without which, the enteric muscles can not contract [14] [1] [54]. Interestingly, these neurons seem to be, at least in part, redundant: EMCs are normal when either neuron is ablated alone, but when both are killed, EMCs are abolished [14]. In addition, in a GABA (-) backround, residual EMCs require DVB, but not AVL [54], suggesting the DVB neurons contain a transmitter other than GABA. FLRFamide has been localized to the DVB neurons and is a candidate for such a transmitter [24] [55].

Regulation of aBoc was also shown to involve AVL, as ablation of AVL resulted in a strong aBoc deficiency, which is independent of GABA [14] [52]. This indicates that

AVL is functioning as an interneuron, using one transmitter to regulate anterior body muscle contraction, aBoc, and another, GABA, to regulate the enteric muscles during the Exp step of defecation. Interestingly, this describes a role for multifunctional neurons in regulation of defecation, and possibly other behaviors, in the worm. While much is still unknown, analysis of just one step of the defecation motor program illustrates the complexity in regulation of this behavioral pattern.

Genetic analysis of defecation periodicity: involvement of IP_3 signaling

As one of the more interesting aspects of the defecation cycle in *C. elegans*, the periodicity has many properties characteristic of a biological clock [50]. The cycle is unaffected by temperature changes, can be revived after termination due to environmental cues, such as lack of food, and can also be reset by perturbations such as light touch [50] [52]. This suggests that the clock runs separately from the motor program, and the identification of cellular and molecular pathways that underlie it are a present challenge. However, some recent work has uncovered pieces to this puzzle. Many genes that affect the timing of the cycle period have been identified and characterized, although most are not yet cloned and so full analysis of the proteins and their function is not possible [52] [51]. For example, mutations in *clk-1* confer a longer cycle period, but the molecule encoded by this gene has not been identified [56].

However, other genes have been more fully characterized in their role in regulating cycle periods. Dal Santo et al. (1999) identified *itr-1*, which encodes an inositol trisphosphate (IP_3) receptor that affects cycle periodicity. Animals with severe mutations in *itr-1* (*dec-4* in Iwasaki et al., 1994) are extremely sick and fail to initiate the defecation program. Less severe alleles result in a slow but regular cycle. The localization pattern of the *itr-1*::GFP fusion protein shows expression in the intestinal and rectal epithelial cells, and furthermore, mosaic analysis revealed that IP_3 expression in the intestinal cells is

necessary for normal defecation behavior [132]. Thus, it appears that IP_3 receptor signaling, which regulates intracellular calcium levels, functions in the intestinal cells to determine defecation cycle frequency.

Coordination of this multicellular behavior, defecation, is obviously a complex process, and involves both neuronal activity and signaling molecules such as $G_o\alpha$. Interestingly, the time keeper for this motor program involves not the muscles or neurons of the circuit, but the intestinal cells themselves. Further studies might uncover the interactions between the pacemaker in the intestinal cells and muscles and neurons that regulate this behavior, and this novel mechanism might shed light on how biological rhythmic behaviors are regulated in other organisms.

EGG-LAYING BEHAVIOR

The importance of understanding muscle excitability and its regulation by signal transduction mechanisms in *C. elegans* is clear from the investigation of behaviors such as locomotion, feeding and defecation. Signaling cascades involving multiple heterotrimeric G proteins were shown to converge in the nervous system to regulate movement, shedding light on how this cross-regulation might occur in vertebrates. Analysis of defecation revealed that the mechanisms that regulate cycle periodicity involve neither the muscles nor neurons of the defecation circuit, and thus, represents a novel process by which rhythmic behaviors might be regulated in other systems. I hope to gain similar insights into the molecular regulation of behavior through investigation of egg-laying in *C. elegans*.

In this section, I will describe what information was known about egg-laying in *C. elegans*. The anatomy of the egg-laying circuit and pharmacology affecting this behavior

had been studied; however, the temporal pattern of egg-laying events and the roles of specific neurons and signaling molecules in regulating that pattern, was elusive.

The egg-laying circuit had been determined from serial cross-sectioning by White et al. (1986). The process of oviposition occurs through contraction of 8 vulval and 8 uterine muscles, all of which are linked by gap junctions [26]. The vulval muscles are innervated by at least 11 neurons, and the most extensive connections are between the neurons HSNL/ HSNR and VC4/VC5 and the vulval muscles, vm2 [1]. Ablation experiments had identified the vm2 vulval muscles and the HSNs as the only necessary components of the egg-laying circuit, and the absence of either results in gross egg-laying defects, wherein eggs accumulate in the uterus [57] [15].

Genetic and pharmacological studies have identified neurotransmitters that function to regulate this behavior. The HSNs were shown to contain multiple neurotransmitters, including serotonin, acetylcholine and FMRFamide like peptides [15] [24] [58]. In addition, animals lay eggs in response to serotonin, even in the absence of the HSNs [57]. This led to a simple model for regulation of egg-laying, where the HSNs release serotonin to stimulate egg-laying.

However, the regulation of this behavior appeared to involve a more complex interaction of neurotransmitters. *cat-4* and *bas-1*, mutants lacking any detectable serotonin, were shown not to be overtly egg-laying defective, which would be the prediction if serotonin were the sole neurotransmitter involved [59]. In addition, nicotinic and muscarinic acetylcholine agonists, levamisole and oxotremorine, respectively, strongly stimulate egg-laying [57] and aceylcholine is expressed in the egg-laying circuit [58]. Interestingly, animals lacking acetylcholine are not severely egg-laying defective [10] [60] and the response to cholinergic agonists is dependent on the HSNs [59]. The HSN dependence of the response to cholinergic agonists is not due to lack of serotonin, as *cat-4* mutants still resond to the agonists [59]. This led Weinshenker et

al. (1995) to propose that serotonin and acetylcholine were functioning in parallel, and that the response to levamisole was dependent on a transmitter other than serotonin found in the HSNs. This proposal illustrates the complex nature of regulation of egg-laying behavior and how poorly understood the mechanisms behind its regulation were at the time.

Interestingly, egg-laying in other systems appears to involve regulation via similar molecules as *C. elegans*. In the marine mollusks, *Aplysia californica* and *Lymnaea stagnalis*, egg-laying is initiated and coordinated by release of a neuropeptide, or egg-laying hormone (ELH), from specific cells [133, 134]. In *A. californica*, release of this neurohormone also appears to involve PKC-sensitive calcium channels in the bag cell neurons that release ELH [136]. In the leech *Theromyzon tessulatum*, egg-laying is similarly regulated by a peptide with significant sequence similarity to the mollusk neurohormone [135]. Lastly, peptides that stimulate egg-laying, Acessory gland proteins (Acp), have also been identified in *Drosophila melanogaster* [137, 138]. Although these proteins have been identified in many invertebrates, the complexity of systems such as *Aplysia* and *Drosophila* makes it difficult to investigate the molecular mechanisms by which these neuropeptides function to stimulate egg-laying.

The initial aim of this dissertation was to identify the temporal pattern of egg-laying events in *C. elegans* and use this pattern as a tool to uncover the genetic and cellular mechanisms behind behavioral regulation. Further studies have allowed me to use egg-laying behavior as a model for understanding the mechanisms that underlie long term adaptation of behavior.

CHAPTER II ANALYSIS OF THE TEMPORAL EGG-LAYING PATTERN AND CONTROL OF ALTERNATIVE EGG-LAYING STATES BY SEROTONIN IN *CAENORHABDITIS ELEGANS*

ABSTRACT

Serotonin has been implicated in the regulation of a wide range of brain functions involving alternative behavioral states, including the control of mood, aggression, sex and sleep. Here we report that in the nematode *Caenorhabditis elegans*, serotonin controls a switch between two distinct, on/off states of egg-laying behavior. Through quantitative analysis of the temporal pattern of egg-laying events, we determined that egg-laying can be modeled as a novel random process, in which animals fluctuate between discrete behavioral states: an active state, during which eggs are laid in clusters, and an inactive state, during which eggs are retained. Single-cell ablation experiments indicate that two pairs of motorneurons, HSNR/HSNL and VC4/VC5, can induce the active phase by releasing serotonin. These neurons also release acetylcholine, which appears to trigger individual egg-laying events within the active phase. Genetic experiments suggest that determination of the behavioral states observed for *C. elegans* egg-laying may be mediated through PKC-dependent modulation of voltage-gated calcium channels.

INTRODUCTION

Behavior depends on the ability of the nervous system to establish temporal patterns of muscle contraction, and to modulate these patterns in response to sensory information and experience. Ultimately, behavioral control depends on the functional properties of individual neurons acting within the context of the neuronal circuitry. Although the extreme organizational complexity of vertebrate brains makes it impractical

to gain a reductionist understanding of vertebrate behavior, for animals with less complex nervous systems, such as the nematode *Caenorhabditis elegans*, understanding behavior at the molecular and cellular level is a realizable goal. The *C. elegans* nervous system is extremely simple and well characterized at the anatomical level: an adult hermaphrodite contains only 302 neurons, each with a precisely determined and invariant position and cell lineage [1, 2, 26]. This anatomically simple nervous system is capable of perceiving and responding to a wide range of environmental conditions, including heavy and light touch [61], temperature, volatile odorants, osmotic and ionic strength, food, and other nematodes [62]. Each of these sensory modalities in turn regulates many aspects of the animal's behavior, including the rate and direction of movement, the rates of feeding, egg-laying, and defecation, and the process of mating [54]. *C. elegans* is amenable to classical, molecular, and developmental genetic studies; thus, isolation, phenotypic characterization, and molecular analysis of behavioral mutants provides a promising avenue toward identifying the molecular events that underlie the animal's behavior [63].

We have begun an investigation into the molecular and neural mechanisms responsible for establishing and regulating temporal patterns of egg-laying in *C. elegans*. Oviposition in *C. elegans* occurs through the contraction of 16 vulval and uterine muscles, which are extensively linked by gap junctions. At least 11 neurons make anatomical synapses with the egg-laying muscles, the most extensive of which are between two pairs of neurons, HSNL/HSNR and VC4/VC5, and the vm2 vulval muscles [1]. Cell ablation experiments have demonstrated that the HSNs are required for efficient egg-laying [15, 57]. These neurons contain multiple neurotransmitters [15, 24, 58], and pharmacological and genetic studies strongly have indicated that these transmitters function in a complex but poorly understood fashion to control egg-laying behavior [64]. Genetic screens have identified a number of genes whose products are important for the regulation of egg-laying

behavior [15, 18, 30, 57, 65, 66], at least some of which appear to function in the egg-laying muscles to mediate their response to neurotransmitter signaling.

Although these earlier studies have provided important information about the process of egg-laying in *C. elegans*, they provide little information about the temporal pattern of egg-laying in individual animals and how this pattern is controlled by the nervous system. In addition, the specific roles of particular neurons and neurotransmitters in the control of egg-laying behavior are still not well understood. In this study, we have begun to address these questions by recording the long-term behavior of individual animals, and applying statistical modeling techniques to quantitatively analyze the egg-laying pattern. From these analyses, we have formulated a novel mathematical model that describes the pattern of egg-laying behavior in *C. elegans*, and allows us to quantitatively analyze differences between egg-laying patterns. By analyzing the effects of neuronal ablations on egg-laying in the context of this model, we have been able to identify neurons that modulate specific features of the egg-laying pattern and obtain clues to their molecular mechanism of action. In particular, we have found that serotonin increases the rate of egg-laying by switching the animal from a quiescent to an active egg-laying phase, a switch that requires and may be mediated through protein kinase C-dependent (PKC-dependent) signaling.

RESULTS

A three-state stochastic model for egg-laying behavior

Because egg-laying events are relatively infrequent (4-10 eggs per hour), obtaining accurate data on the timing of egg-laying by real-time observation is both tedious and impractical. Thus, to investigate the pattern of egg-laying behavior, it was necessary to use an automated tracking system capable of recording the behavior of an individual animal on videotape over long time periods. By analyzing the recordings generated in this fashion, we could determine when each egg was laid, and thus investigate the timing of egg-laying events. We first investigated the pattern of egg-laying in wild-type animals under favorable conditions: isotonic nematode growth medium (NGM) seeded with abundant bacteria. We observed that egg-laying events were often clustered, with successive events separated by an average of 20 seconds. These clustered events were interrupted by inactive periods, averaging 20 minutes in duration, during which egg-laying did not occur. Quantitative analysis of these data showed that both the duration of the inactive phases (i.e., the long time intervals) and the intervals between clustered egg-laying events (i.e., the short time intervals) could be modeled as exponential random variables with different rate constants. Neither the onset of egg-laying clusters nor the laying of individual eggs within a cluster appeared to be periodic (Figure 1-1a); rather, both resembled stochastic Poisson processes such as in radioactive decay, in which events occur at random with a fixed rate constant.

These results led us to formulate a mathematical model to describe the temporal pattern of egg-laying [67]. Our objectives were two-fold: to devise a model that accurately described the behavior we had observed, and to use this model to derive algorithms that would allow us to quantitatively analyze the effects of mutations and neuronal ablations on the egg-laying pattern. In the model we developed (Figure 1-1b), animals fluctuate between three states: an inactive state, an active state, and an egg-

laying state, during which egg-laying occurs. The overall egg-laying pattern is dictated by three parameters (Figure 1-1c): the exponential rate constant for the duration of the inactive phase (λ_2), the exponential rate constant for egg-laying within the active phase (λ_1), and the probability of remaining in the active phase after an egg-laying event (p). Simulated egg-laying data generated using this model were very similar to real data (Figure 1-1d, 1-1e); thus our formulation appeared to provide a simple yet accurate description of the egg-laying pattern.

Although this mathematical model was devised without prior mechanistic assumptions about egg-laying behavior, certain features of the model had interesting biological implications. In particular, the three formal states in the model suggested the possibility that there might be discrete behavioral states for egg-laying, which could result from distinct functional states of the neurons or muscles involved in egg-laying. We hoped to gain insight into the molecular and cellular mechanisms that might determine these states by identifying neurons and genes that affected specific parameters of egg-laying behavior. Using the model probability density function, we devised an algorithm that could be used to obtain maximum likelihood (ML) estimates of the model parameters from real experimental data [67]. This made it possible to quantitate differences in particular egg-laying parameters for different mutant and lesioned animals, and thus determine how specific nervous system defects affected specific features of the egg-laying pattern.

Serotonin modulates the transition into the active state

The roles of individual neurons in controlling the timing of egg-laying events can be determined with high precision by eliminating specific neurons by laser ablation and assaying the effect of the ablation on behavior. We therefore eliminated the neurons with prominent synaptic input to the egg-laying muscles to determine how their absence

affected the timing of egg-laying events. We first investigated the involvement of the HSNs, a pair of serotonergic motorneurons that are required for efficient egg-laying. By tracking the behavior of animals lacking both HSNs, we found that elimination of the HSNs did not qualitatively alter the pattern of egg-laying: eggs were still laid in clusters, and the intervals between clusters and between egg-laying events within a cluster were still exponentially distributed. However, HSN ablation did cause a substantial lengthening of the inactive phase , which led to a slower overall rate of egg-laying (Figure 1-2a). Since loss of the HSNs decreased the frequency of egg-laying clusters (i.e., λ_2 was decreased, Table 1-1), but did not slow the egg-laying rate within these clusters (λ_1 was actually increased), these results suggest that the HSNs stimulate egg-laying by inducing the active state.

The HSNs contain at least three neurotransmitters: serotonin, acetylcholine, and a FMRFamide-related neuropeptide [15, 24, 58]. Serotonin has been shown to stimulate egg-laying in nematodes [17, 68]; hence, one possibility is that the HSNs might induce the active phase of egg-laying through the release of serotonin. Consistent with this possibility, we observed that serotonin-deficient mutants, like HSN-ablated animals, exhibited an egg-laying pattern in which egg-laying clusters were separated by abnormally long inactive phases (Figure 1-2b). Moreover, continuous exposure of HSN-defective animals to exogenous serotonin (Figure 1-2c) resulted in an egg-laying pattern that resembled a simple Poisson process, with a rate constant close to the rate constant for wild-type animals in the active state (i.e., λ_1). This pattern suggested that in the presence of exogenous serotonin, the animals were continuously in the active phase. Taken together, these experiments argued that serotonin release from the HSNs stimulates egg-laying not by directly exciting the muscles, but by modulating their activity such that they enter an activated state in which they are more likely to undergo contraction. According

to this hypothesis, the frequency of egg-laying clusters (λ_2) is determined by the rate at which serotonin is released to induce this active state.

Genes required to establish the active state

These analyses suggested that serotonin released from the HSNs controls the switch between alternative behavioral states for egg-laying. Since the HSNs synapse directly with the egg-laying muscles, one possibility is that these behavioral states might correspond to different physiological states of the muscles. To identify molecular pathways that might be involved in determining these states, we analyzed the behavioral patterns of previously identified egg-laying-defective (Egl) mutants, which were identified on the basis of their abnormally slow egg-laying rate. Among the Egl mutants we surveyed, two general patterns were observed (Figure 1-3a). Some of these mutants exhibited a pattern similar to that of HSN-ablated animals; they laid eggs in clusters but had abnormally long inactive phases. These mutants defined genes that might affect the induction of the active state but have little effect on egg-laying within the active phase. Interestingly, among the mutants in this category were strains carrying loss-of-function alleles of the gene *tpa-1* [69], which encodes the *C. elegans* homologue of protein kinase C (PKC). Although *tpa-1* mutations were not previously known to affect egg-laying, we found that loss-of-function PKC mutants laid eggs at significantly lower rates than wild-type, an effect mediated primarily by a lengthening of the inter-cluster time constant (Figure 1-3b, Table 1-1). This result suggested that PKC function is required for efficient induction of the active state by serotonin, but not for egg-laying within an established active state. Consistent with this hypothesis, we found that *tpa-1* mutants were highly resistant to stimulation of egg-laying by serotonin (Figure 1-3c). Thus, protein kinase C is a candidate for a molecule that might function downstream of the serotonin receptor to induce the active phase.

Several Egl mutants showed a different egg-laying pattern: they laid eggs singly rather than in clusters (Figure 1-3a, 1-4). This pattern suggested that these mutants were unable to lay eggs efficiently within the active phase, or that the active phase was unstable or short-lived. As expected, most of these mutants laid eggs at a higher rate when treated with serotonin, suggesting they were not defective in induction of the active state. A striking exception was *egl-19*. Although *egl-19* hypomorphic mutants laid eggs at a reduced but significant rate, they were completely non-responsive to serotonin (Figure 1-4 legend; see also [57]), suggesting that the *egl-19* gene is required to mediate the induction of the active state by serotonin. However, stronger *egl-19* hypomorphs showed an unclustered egg-laying pattern, and weaker alleles caused a significant reduction in both the number of eggs laid in each cluster (i.e., p was reduced) and the rate of egg-laying within the cluster (λ_1 was increased; Figure 4). Taken together, these data suggested that *egl-19*, unlike *tpa-1*, is required both for induction of the active state and for efficient egg-laying within the active state.

Acetylcholine may trigger egg-laying events within the active phase

The observation that the HSNs are not required for efficient egg-laying within the active phase raised the question of what triggers these individual egg-laying events. To address this question, we analyzed the effect of a second pair of neurons, VC4 and VC5, on egg-laying behavior. These cells receive synaptic input from the HSNs and make extensive synapses with the egg-laying muscles [1]. Yet ablation of the VCs causes no gross egg-laying defect, and the role, if any, of these cells in egg-laying behavior has up to now been unclear [70]. However, when we analyzed the egg-laying patterns of VC4/VC5-ablated animals quantitatively, we observed that the VC neurons had several important effects. First, VC-ablation caused a small but significant increase (level of confidence < .02) in the inter-cluster time constant (Table 1-1). Since VC4 and VC5 appear to be

weakly serotonergic [58], they may function along with the HSNs to induce the active phase by releasing serotonin. In addition, ablation of the VCs, like ablation of the HSNs, caused an increased rate of egg-laying within the active phase (Table 1-1).

A more striking effect of the VCs on egg-laying was observed when both the HSNs and the VCs were eliminated. Animals defective in both VC4/5 and HSNL/R were much more severely defective in egg-laying than animals defective in only the HSNs. Furthermore, unlike animals defective in either the HSNs alone or the VCs alone, HSN/VC-defective animals failed to lay eggs efficiently even in the presence of high levels of exogenous serotonin (Figure 1-5a, 1-5b). These results suggest that a second neurotransmitter, which can be provided by either the HSNs or VCs, is required in addition to serotonin for efficient egg-laying. One candidate for such a molecule is acetylcholine, which is found in both the HSNs and VCs [58]. Acetylcholine receptor agonists stimulate egg-laying in an HSN-dependent manner [57, 59] and enhance the stimulation of egg-laying by serotonin (D. Weinshenker and J. Thomas, personal communication). In support of this hypothesis, we observed that HSN/VC-deficient animals laid eggs efficiently when provided with both serotonin and the nicotinic receptor agonist levamisole (Figure 1-5b). In contrast, levamisole alone did not stimulate egg-laying in either HSN-ablated or HSN/VC-doubly ablated animals. Thus, the HSNs and VCs appear to release two neurotransmitters, serotonin and acetylcholine, both of which are required for egg-laying. Since the role of serotonin is to induce the active phase, a logical conjecture is that acetylcholine's role is to trigger individual egg-laying events within those active phases. Consistent with this hypothesis, we observed that depletion of endogenous acetylcholine using conditional alleles of the acetylcholine biosynthetic gene *cha-1* caused a significant decrease in the intra-cluster egg-laying rate, (i.e., λ_1 was decreased, Figure 1-5c), but had little or no effect on the inter-cluster rate ($p\lambda_2$ was not changed).

DISCUSSION

Serotonin controls a switch between active and inactive egg-laying states

In summary, we have found that the complex temporal pattern of egg-laying in *C. elegans* can be effectively modeled as a random process involving discrete behavioral states: an active, "on" state and an inactive, "off" state. By analyzing the effects of neuronal ablations and genetic mutations in the context of this model, we have been able to identify neurons that selectively change specific parameters of the egg-laying pattern, and we have obtained information about the molecular mechanism of action of these cells. These studies indicate that the HSNs are not required to directly contract the egg-laying muscles, but rather to switch the egg-laying muscles from a quiescent state to an active state in which they are capable of readily undergoing contraction. At least one mechanism through which the HSNs facilitate the active state is likely to involve serotonin release. Treatment of HSN-deficient animals with exogenous serotonin induces egg-laying in a pattern resembling a continuous active phase. Moreover, serotonin-deficient mutants, like HSN-ablated animals, have significantly longer inactive periods between egg-laying clusters. Mutations in *bas-1* which cause a defect in the decarboxylase step of serotonin biosynthesis [16] cause a specific increase in the inter-cluster time constant, but have little or no effect on the intra-cluster constant. In the *cat-4* mutant, a large increase in the inter-cluster time constant was also observed, along with a smaller increase in the intra-cluster time constant. Since the *cat-4* mutation appears to be somewhat pleiotropic (for example, *cat-4* mutants apparently have a defective cuticle [71]), it is unclear whether the effect on the intra-cluster rate is related to serotonin. We identified several egg-laying-defective mutants whose phenotype resembled that of HSN-ablated animals: active phases occurred with decreased frequency, but egg-laying within the active phase was largely unaffected. Of these mutants, five (*egl-4*, *egl-11*, *egl-21*, *egl-*

24, and *egl-30*) have been shown to have reduced serotonin response [57]; thus, these mutants define candidate genes for mediating induction of the active state by serotonin

Although these studies indicate that serotonin is sufficient to induce the active state, recent work from other investigators indicates that serotonin is not required for active state induction. For example, *C. elegans* mutants that have no detectable serotonin are egg-laying-competent [20]. Moreover, *C. elegans* mutants have been identified that are serotonin-insensitive but can lay eggs in response to other pharmacological agents [64]. In this study, we observed that egg-laying events are still clustered in serotonin-deficient mutants (Figure 1-2b), suggesting that a second molecule, probably released by the HSNs and/or the VCs, may function redundantly with serotonin to induce the active phase of egg-laying. Immunocytochemical experiments have demonstrated that the HSNs and VCs contain one or more FMRFamide-related neuropeptides (FaRPs) [24]; thus, an FaRP is a logical candidate for a second molecule that could induce the active state.

A voltage-gated calcium channel may determine the active state

Our experiments also indicate that the HSNs and VCs release another molecule, probably acetylcholine, which induces individual egg-laying events within the active period. Acetylcholine agonists have long been known to increase egg-laying in nematodes [57], and more recent work has suggested that acetylcholine might function in parallel with serotonin to stimulate egg-laying [64]. In support of this hypothesis, we observed that animals lacking both the HSNs and VCs were largely resistant to stimulation of egg-laying by serotonin, whereas treatment of these animals with both serotonin and an agonist of nicotinic acetylcholine receptors (nAChRs) induced efficient egg-laying. Furthermore, analysis of acetylcholine-deficient mutants indicated that acetylcholine depletion decreases the rate of egg-laying within clusters. Thus, whereas serotonin specifically affected the duration of intervals between clusters, acetylcholine affected the

duration of intervals between egg-laying events within a cluster. Although at present we do not know the precise mechanism by which acetylcholine stimulates egg-laying, the simplest hypothesis is that it depolarizes the vulval muscle by opening nicotinic receptors, which leads to muscle contraction. According to this model, the rate of egg-laying events within the active phase (λ_1) is a function of the rate of acetylcholine release.

We identified several mutants with reduced egg-laying in the active phase, a pattern which suggests a defect in acetylcholine release or response. As expected, most of these mutants were serotonin-sensitive [57]. The exception was *egl-19*, which was completely serotonin resistant, and thus defective in both induction of the active phase and egg-laying within the active phase. *egl-19* encodes an L-type voltage-gated calcium channel homologue which is thought, based on genetic evidence, to function in the vulval muscles [72]. Thus, an appealing hypothesis is that serotonin might modulate the activity of EGL-19 calcium channels in the vulval muscles and thereby make them more amenable to contraction. In vertebrate cells, G-protein-coupled receptors have been shown to facilitate PKC-dependent phosphorylation of L-type calcium channels (see below), which enhances the coupling between excitation and channel opening [73]. In *C. elegans*, serotonin could induce phosphorylation of EGL-19 calcium channels in the vulval muscles, making them more likely to open in response to voltage changes (Figure 1-6). Consistent with this hypothesis, *tpa-1*, *cat-4* and *bas-1* mutations all interacted genetically with *egl-19* hypomorphic alleles, suggesting that they may function in a common pathway (Table 1-2; see also Methods). Moreover, the egg-laying defects of *tpa-1* and *cat-4* were suppressed by a gain-of-function allele of *egl-19* (see Methods). It is possible that modulation of other ion channels could contribute to the active state as well; for example, inhibition of potassium channels could increase the probability of EGL-19 opening by decreasing the polarization of the membrane.

Mechanisms for modulation of excitable cell states by serotonin

As described above, serotonin appears to control a molecular switch between two egg-laying states, which may correspond to functional states of the egg-laying muscles. By investigating the genetics of egg-laying behavior, we have begun to obtain information about the signaling pathways that help determine these functional states and thus regulate the behavioral pattern. Interestingly, serotonin appears to act through a similar mechanism to modulate the activity of excitable cells in other organisms. For example, recent studies of vertebrate smooth muscle have found that serotonin can potentiate I_{Ca} through modulation of L-type calcium channels, an effect that appears to be mediated by G_q activation of the PKC signaling pathway [74-76]. In molluscs, serotonin-dependent activation of calcium channel activity has been directly correlated with modulation of excitation-contraction coupling in smooth muscle cells [77]. Thus, the molecular mechanisms through which serotonin stimulates muscle activity may be largely or completely conserved between the nematodes and vertebrates. This suggests the possibility that further genetic analysis of egg-laying behavior in the worm will provide an avenue to identify and characterize additional components of this signaling pathway (including the candidate genes identified in our study), and to investigate how these molecules function in vivo to determine the functional muscle states.

Biogenic amines, like other neuromodulators that act through G-protein-coupled serpentine receptors, have been widely implicated in the control of alternative behavioral and mental states in the brains of complex animals. In particular, serotonin appears to be involved in the regulation of brain states involved in mood, sleep, addiction, and sexual behavior [78-81]. In addition, a number of studies from organisms as diverse as crustaceans and humans have implicated serotonin in the control of aggression [82-85]. For example, in lobsters, agonistic behavior involves a series of stereotyped, increasingly violent behavioral patterns, between which animals switch during the course of fights [86].

Serotonin does not appear to be involved in producing these patterns per se; rather, it appears to modulate the probability of switching from a more passive to a more aggressive behavioral state [87]. The formal parallels between the roles of serotonin in crustacean aggression and nematode egg-laying suggest the possibility that the molecular mechanisms that underlie these processes may be similar. Thus, the pathway through which serotonin modulates behavioral states in the worm may provide general insights into the modulatory role of serotonin in more complex behaviors in larger nervous systems.

Generation of complex response patterns by polyfunctional neurons

A surprising conclusion of this study is that the complex pattern of *C. elegans* egg-laying behavior can be attributed largely to the functional versatility of the HSN and VC motorneurons, and to the multiplicity of signaling pathways present in the muscle cells onto which they synapse. Both the HSNs and the VCs contain at least three neurotransmitters: a fast acting classical transmitter (acetylcholine), an amine neuromodulator (serotonin), and a peptide (or perhaps several related peptides). Although each of these molecules is released from the same presynaptic cells, they activate distinct signaling pathways in the post-synaptic cells that affect different parameters of the egg-laying pattern. This not only allows a particular class of motorneurons to induce a complex output pattern in the cells onto which they synapse, but it also opens the possibility that the different response pathways in the post-synaptic cell could be differentially modified by experience, extrinsic modulation, or pathway cross-talk.

Neurons that release multiple neurotransmitters are not unique to nematodes; in fact neurons containing a fast-acting transmitter and a peptide, or a fast-acting transmitter and an amine, are relatively common in all nervous systems [88]. Thus, the complex temporal response patterns induced by cotransmitting neurons may be important for computation and behavioral control in more complex nervous systems [89]. Coupled

point process models, such as the one we have used here to describe egg-laying behavior, should be widely applicable for analyzing the output patterns of excitable cells in which response to a fast-acting transmitter is potentiated by a neuromodulator.

METHODS

Assay conditions and growth media

Unless otherwise noted, nematodes were grown and assayed at room temperature on standard nematode growth medium (NGM) seeded with *E. coli* strain OP50 as a food source. For drug experiments, 5-hydroxytryptamine (creatinine sulfate complex, Sigma) was added to NGM agar at 7.5 mM, and levamisole (hydrochloride, Sigma) was added at 6.25 μM. Egg-laying rates for *egl-19* mutants were measured by counting the number of eggs laid after 1 hr three or more trials of 10 animals each on NGM in the presence or absence of 7.5 mM serotonin.

Recording and analysis of *C. elegans* egg-laying behavior

We used a custom tracking system from Mikron Instruments (San Diego), which tracks an animal for unlimited periods of time at up to 60X magnification (the magnification necessary to adequately observe eggs and distinguish them from debris or clumps of food), automatically moving the microscope stage so that the animal remains centered in the visual field as it moves around the plate. Single animals were observed as they crawled across a standard 8 cm agar plate using a stereoscopic dissecting microscope (Zeiss Stemi 2000c) equipped with a Prior motorized stage. Video frames were captured at periodic intervals, and the stage was controlled based on the position of the animal using the ImagePro software package (Media Cybernetics). A video camera (Cohu) recorded the animal's behavior at standard video frame rate; from these videotapes the times that egg-laying events occurred were determined.

ML estimates of model parameters from real data

For the three-state model described above, we can prove that the random variable governing the egg-laying interval times has the following probability density function (p. d. f.) [67]:

$$f_X(x) = k_1\ \lambda_1\ e^{-\lambda_1 x} + k_2\ (p\lambda_2)\ e^{-(p\lambda_2)x}, \qquad x \geq 0,$$

$$k_1 = \frac{p(\lambda_1 - \lambda_2)}{\lambda_1 - p\lambda_2}, \qquad k_2 = \frac{\lambda_1(1-p)}{\lambda_1 - p\lambda_2}.$$

Given N observations of the intervals x = [x_1,x_2,...,x_N], the likelihood function is given by

$$f(\mathbf{x}|\boldsymbol{\theta}) = \prod_{i=1}^{N} \left[k_1\ \lambda_1\ e^{-\lambda_1 x_i} + k_2\ (p\lambda_2)\ e^{-(p\lambda_2)x_i}\right]$$

which is a function of the parameter vector θ = [p, λ_1, λ_2]. The maximum likelihood 'estimate is defined as the θ that maximizes f(x|θ) over all possible θ 's. The bi-modal peak information provided by the histogram of the log intervals (see Figure 1-1d for example) was used to obtain a rough estimate of θ and initialize the non-linear ML algorithm, which adjusted the parameters to maximize the likelihood function.

To demonstrate the effectiveness of the ML procedure, we simulated egg-laying data using the model p.d.f. Close agreement between the true and estimated value was observed. The true parameters and the ML parameter estimates obtained from 100 independent realizations, each of 216 intervals, (mean ± standard deviation) were: p: true = 0.5891, estimated = 0.5967 ± .0356; λ_1: true = 0.0501, estimated = 0.0497 ± 0.0059; λ_2: true = 0.0014, estimated = 0.0014 ± 0.0002. Standard deviations in Table 1-1 were estimated in a similar fashion.

For the special case of animals on serotonin (where a pattern resembling a homogeneous Poisson process is observed), the time constant was estimated by performing a weighted least-squares linear regression to the log tail distribution (see below).

Log tail distribution

It can be shown that the log tail probability (i.e., the log of the probability that a given interval will be longer than time x) for intervals generated by the three-state model is given by

$$\ln \Pr(X \geq x) = \ln(k_1\ e^{-\lambda_1 x} + k_2\ e^{-p\lambda_2 x})$$

For long intervals ($x > 5/\lambda_1$), ln Pr(X≥x) becomes approximately linear with respect to x, with slope $-p\lambda_2$. Thus, the slope of this plot is inversely proportional to the average duration of the inactive phase.

Histogram of log intervals

Since the intervals between eggs laid are clustered at short intervals and sparse at long intervals, it is meaningful for reasons of better dynamic range to study the distribution of the log intervals (denoted by Y = ln X). Both real and simulated data exhibit a bimodal pattern (as in Figure 1-1c) which can be shown to be represented by the following p. d. f. of Y:

$$f_Y(y) = \left[k_1\lambda_1\, e^{-\lambda_1 e^y} + k_2(p\lambda_2)\, e^{-(p\lambda_2)e^y}\right] e^y,$$

$$k_1 = \frac{p(\lambda_1 - \lambda_2)}{\lambda_1 - p\lambda_2}, \qquad k_2 = \frac{\lambda_1(1-p)}{\lambda_1 - p\lambda_2}.$$

We can show that when λ_1 and $p\lambda_2$ are sufficiently different, $f_Y(y)$ peaks at $y \approx \ln(1/\lambda_1)$ and $\ln(1/p\lambda_2)$ with corresponding peak heights k_1/e and k_2/e. When the values of λ_1 and $p\lambda_2$ are close, only a single peak is observed.

Ablation of egg-laying neurons

The two HSNs were eliminated either by introducing a mutation in the gene *egl-1*, which causes the HSNs to undergo cell death in the hermaphrodite [15] or by ablating the HSN nuclei in the first larval stage. The VC4 and VC5 neurons were ablated in early 4th stage larvae of the strain CF453 (genotype: *dpy-20(e1282)IV; muIs16[dpy-20(+) mab-5:GFP]*; kindly provided by Craig Hunter and Cynthia Kenyon), a strain carrying an integrated *mab-5::GFP* fusion. Expression of GFP in the developing VC4 and VC5 cells was used to identify them prior to the ablation process; cell killing was verified the next day by scoring for the absence of VC nuclei and GFP-expressing neurons and neuronal processes in the vulval region. For HSN/VC- animals, VC4 and VC5 nuclei were ablated as described in the strain AQ112, which carries the *egl-1(n986)* mutation and the *mab-5::GFP* fusion; unablated AQ112 animals were used as the HSN- control.

Construction and characterization of double mutants

Double mutants were generated using standard methods. Mutant homozygotes were identified in the F2 generation on the basis of the following phenotypes: *tpa-1*--resistance to tetradecanoyl phorbol acetate [90], *egl-19* recessive alleles--long body length and sluggish movement, *egl-19(n2368)*--short body length [72], *cat-4*--hypochlorite hypersensitivity [71], *bas-1*--failure to slow in the presence of bacteria [91]. The pattern of egg-laying in the *tpa-1; egl-19* double mutants resembled that of *egl-19*single mutants. The *egl-19(n2368)* single mutant as well as the *tpa-1(k501); egl-19(n2368)* and *cat-4(e1141); egl-19(n2368)* double mutants all laid eggs extremely hyperactively, such that no mature (>8 cells) embryos accumulated in the uterus, and embryo production rather than vulval muscle contraction was limiting for egg-laying. For the doubles with recessive

egl-19 alleles, ML parameter estimates for p, λ_1 and λ_2 were [.158, .020, .0036] for *k501; n582* (6 animals, 38 hr, 13 intervals) and [.255, .016, .0030] for *k501; ad1015* (4 animals, 20 hr, 55 intervals) respectively. Egg-laying rates in Table 1-2 were determined after 1 hour incubation on seeded NGM plates (3 trials of 10 animals each). The percentage of animals forming "bags of worms" (animals in which unlaid embryos hatch within the uterus due to an extreme egg-laying defect) was determined at 2 days following the L4/adult molt.

This chapter, in full, is a reprint of the material as it appears in L.E. Waggoner et al. (1998) "Control of Alternative Behavioral States by Serotonin in *Caenorhabditis elegans*". The dissertation author was the primary investigator and author of this paper. The co-authors directed and supervised the research that forms the basis for this chapter.

Table 1-1. ML estimates of egg-laying parameters for wild-type, mutant, and ablated animals.

Animal type (#, hrs, intervals)	Intra-cluster time constant ($1/\lambda_1$; s)	Inter-cluster time constant ($1/p\lambda_2$; s)	p	λ_1 (s^{-1})	λ_2 ($s^{-1} \times 10^{-3}$)
N2[a]	18	1200	.572	.056	1.46
(7, 40, 216)	±2	±170	±.037	±.008	±0.23
N2, HSN-	8	3400	.534	.125	0.55
(9,44, 66)	±2	±900	±.069	±.049	±0.16
egl-1(n986)	9	3630	.685	.106	0.40
(9,46, 94)	±2	±1020	±.056	±.019	±0.10
bas-1(pa4)	17	3480	.619	.060	0.46
(6,38, 52)	±4	±1080	±.072	±.014	±0.15
bas-1(ad446)	22	1870	.446	.046	1.22
(12,52, 63)	±6	±450	±.072	±.016	±0.42
cat-4(e1114)	43	4550	.609	.023	0.36
(12,52, 63)	±9	±1240	±.073	±.007	±0.13
tpa-1(k501)	33	3060	.448	.030	0.73
(7,35, 66)	±8	±630	±.059	±.016	±0.19
tpa-1(k530)	18	1970	.508	.055	1.04
(5,24, 49)	±6	±540	±.083	±.021	±0.42
CF453[b]	26	1770	.539	.038	1.05
(7, 24, 96)	±6	±360	±.062	±.009	±0.28
CF453, VC4/5-	11	2300	.555	.089	0.75
(8,53, 133)	±2	±430	±.047	±.017	±0.16

[a] N2 (a.k.a. *C. elegans* Bristol) is a standard wild-type strain; all mutants are in this genetic background.

[b] CF453 is a strain carrying a *mab-5:GFP* fusion, which is expressed in VC4 and VC5 and allows identification of these cells for laser ablation.

Table 1-2. Genetic interactions between *egl-19* and putative signaling genes.

Genotype	% bag-of worms (n=40; ± sem)	egg-laying rate (eggs/worm/hr)
wild-type	0 ± 0	3.5 ± 0.5
egl-19(n582)	20 ± 6	5.0 ± 0.6
egl-19(ad1015)	3 ± 2	3.9 ± 0.3
tpa-1(k501)	5 ± 3	3.0 ± 0.6
cat-4(e1141)	5 ± 3	2.4 ± 0.8
bas-1(pa4)	13 ± 5	2.8 ± 0.1
tpa-1(k501); egl-19(n582)	62 ± 8	0.4 ± 0.3
tpa-1(k501); egl-19(ad1015)	8 ± 4	1.5 ± 0.4
cat-4(e1141); egl-19(n582)	90 ± 5	0.5 ± 0.3
bas-1(pa4); egl-19(ad1015)	8 ± 4	1.5 ± 0.6

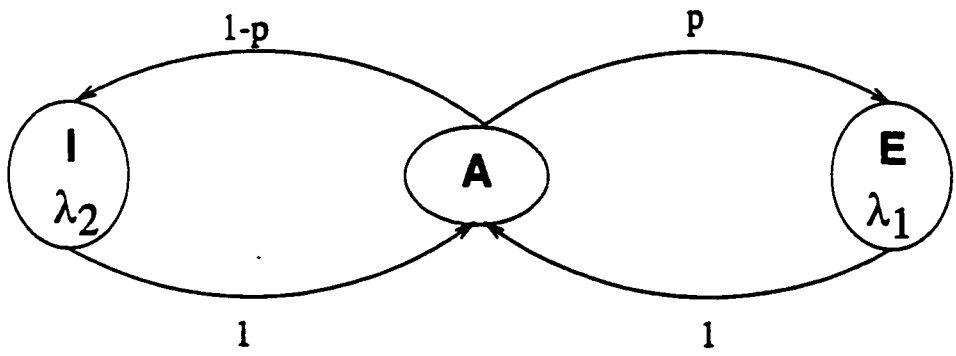


Figure 1-1a. Aperiodicity of the egg-laying pattern. Seven wild-type animals were tracked for a total of 40 hours, and the intervals between egg-laying events (contractions of the egg-laying muscles that led to the expulsion of one or more eggs) were determined. The timing of egg-laying events was analyzed by computing the Fourier transform of the interval data. If the eggs had been laid in a periodic or almost periodic fashion, then the Fourier transform would have exhibited a peak at a non-zero frequency.

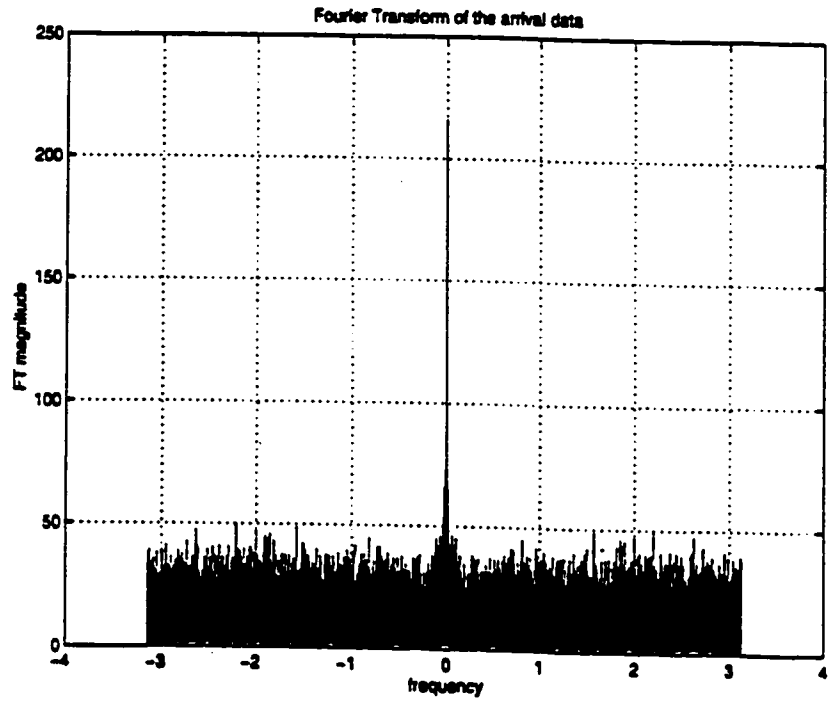


Figure 1-1b. Formal model for egg-laying behavior. According to the model, the animal can exist in one of three states: an inactive state (I), an active state (A), and an egg-laying state (E). Eggs are laid upon entry into the E state; probabilities of state transitions are indicated on the branches of the state diagram. λ_1 is the rate constant for the egg-laying state, and λ_2 is the rate constant for the inactive state.

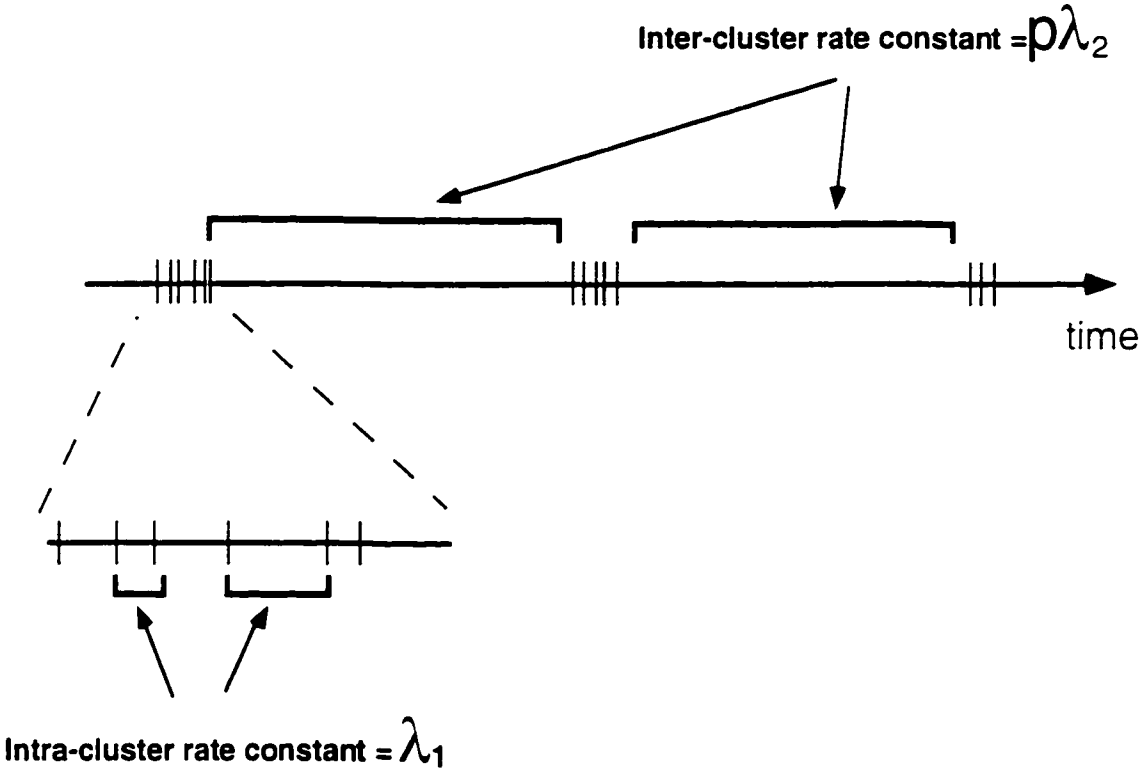
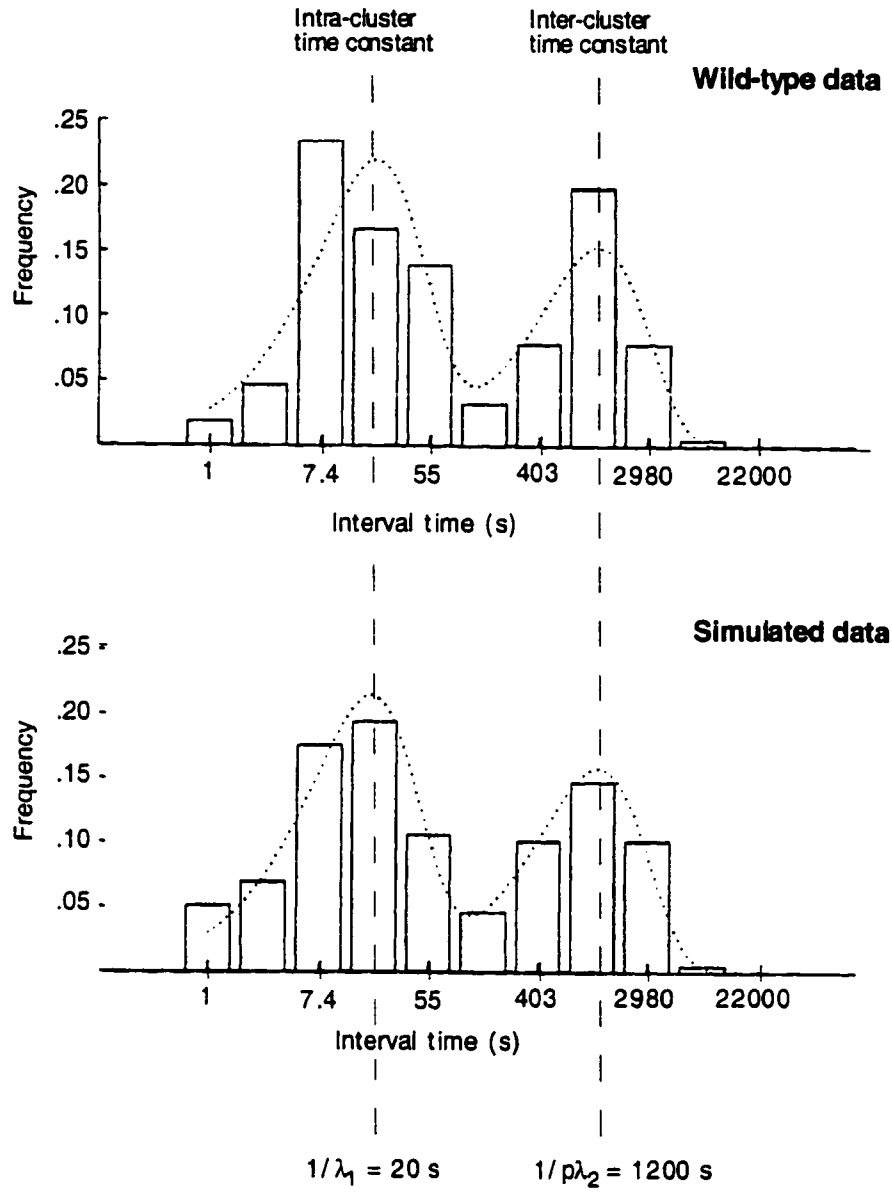


Figure 1-1c. Parameters of egg-laying behavior. Shown is a representative time line, with egg-laying events indicated by hash marks. According to the model, p is the probability that after a given egg-laying event, another egg will be laid before the animal enters the inactive phase; these short intervals resulting from a single visit to the A state are governed by the rate constant λ_1 . Long intervals result from one or more visits to the I (inactive) state (i.e, from E to A to I to A to E, or from E to A to I to A to I to A to E, etc.); the rate constant for these intervals is equal to $p\lambda_2$ (for proof, see [67]

Figure 1-1d. Histograms of observed and simulated log intervals between egg-laying events.

Shown is a histogram of the natural log of the interval times, with the relative frequency on the y-axis. The graph shows a bimodal distribution, with one peak corresponding to the intervals between events in a cluster and the other peak to the intervals between clusters. According to the model, if λ_1 and $p\lambda_2$ are sufficiently different, the left and right peak locations correspond to the time constants for the short and long intervals, respectively (For an exponential random variable, the time constant equals the reciprocal of the rate constant and is the expected value of the mean interval time; for the data shown, these are 20s and 1210s). Shown are a histogram of 216 samples of temporal egg-laying data by wild-type *C. elegans* and one of simulated data based on the model (parameters $p=0.5891$, $\lambda_1=0.0501$ and $\lambda_2=0.0014$) in the bottom figure. The dotted lines indicate the ideal distribution based on ML analysis of the real data


Intra-cluster time constant
Inter-cluster time constant
Wild-type data
Frequency
.25
.20
.15
.10
.05
1
7.4
55
403
2980
22000
Interval time (s)
Simulated data
Frequency
.25
.20
.15
.10
.05
1
7.4
55
403
2980
22000
Interval time (s)
1/λ1 = 20 s
1/pλ2 = 1200 s

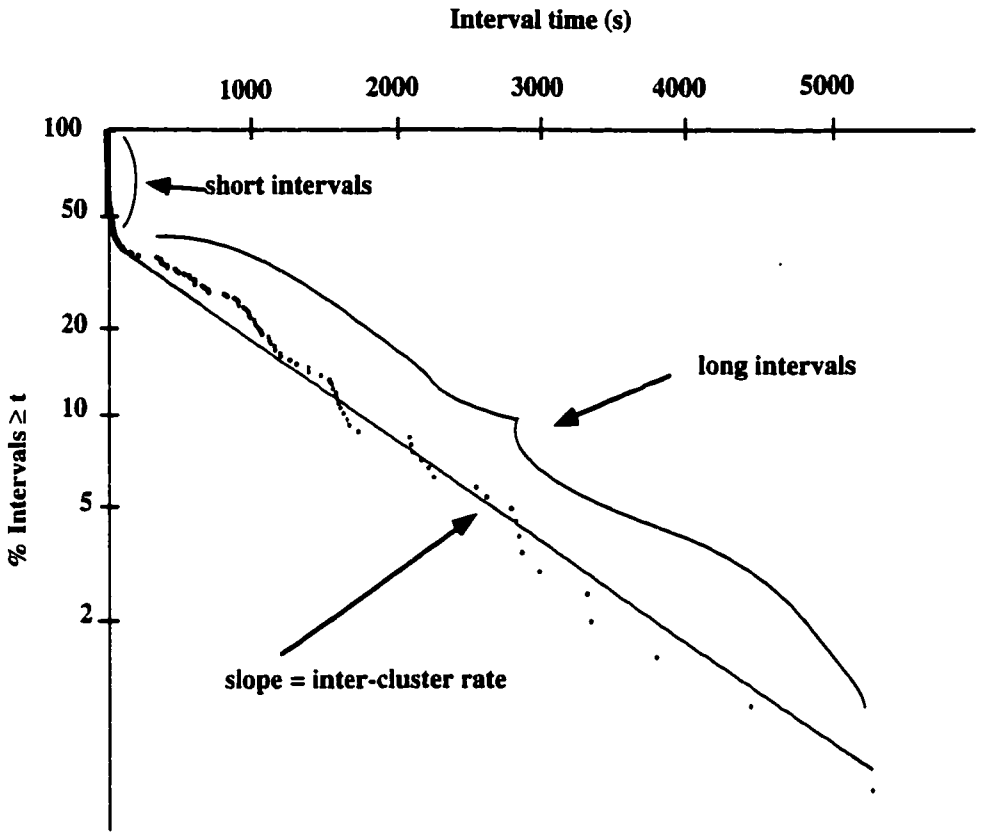


Figure 1-1e. Log tail distribution of intervals between egg-laying events, showing close agreement between theoretical probability and real data. Seven wild-type animals were tracked for a total of 40 hours, and the intervals between egg-laying events (contractions of the egg-laying muscles that led to the expulsion of one or more eggs) were determined.. The model predicts that this distribution should be biphasic, with the steep part of the curve corresponding to the short intervals (i.e., intervals within a cluster) and the more gradual part to the long intervals (i.e., intervals between clusters). The slope of the long intervals (i.e., the right part of the curve) is equal to $-p\lambda_2$ (as noted, $p\lambda_2$ is the inter-cluster rate constant). The solid line shows the theoretical log tail probability ($p=0.5891$, $\lambda_1=0.0501$ and $\lambda_2=0.0014$); the points correspond to the measured wild-type data from part d.

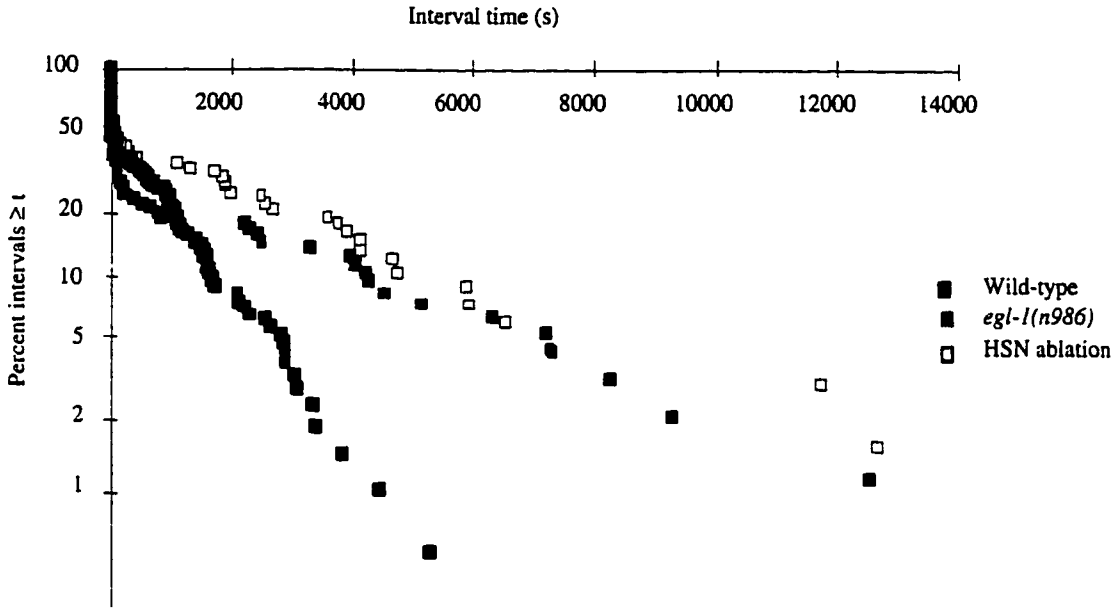


Figure 1-2a. Effect of HSN ablation on egg-laying pattern. Shown are the log tail distributions of the interarrival times for HSN-ablated wild-type (N2) animals or *egl-1(n986)* mutant animals (in which the HSNs undergo cell death) [15] compared to non-ablated N2 animals on NGM. The more gradual slope (see Figure 1e legend) of the *egl-1* and HSN-ablated curves show that the inter-cluster intervals are significantly longer in the HSN-deficient animals. The duration of the long intervals (i.e., >300s) for HSN-ablated and *egl-1* animals were statistically different from unablated wild-type (level of confidence < .001) according to the Mann-Whitney rank sum test.

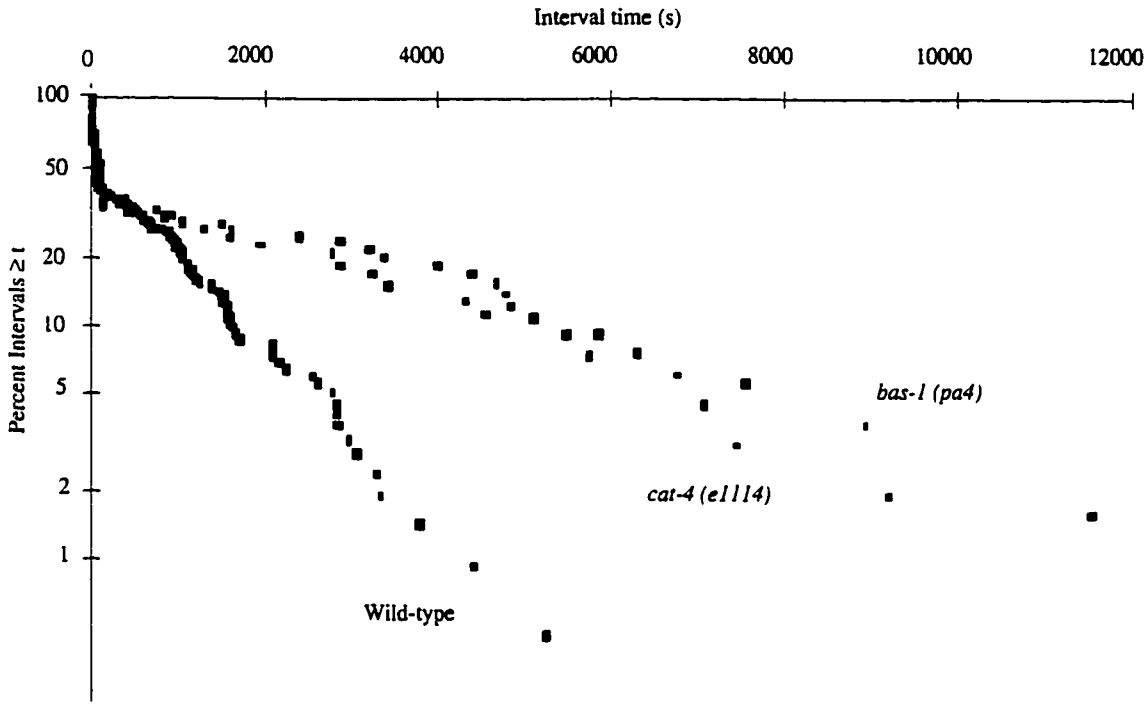


Figure 1-2b. Effect of serotonin deficiency on egg-laying pattern. Shown are log tail distributions for egg-laying intervals of *bas-1* and *cat-4* mutants, which are defective in serotonin biosynthesis [16], compared to the wild-type control. The more gradual slope of the *bas-1* and *cat-4* tails indicates a longer inter-cluster time constant. The long intervals in the mutant animals (including *bas-1(ad446)*; see Table 1-1) were statistically longer than in wild-type (level of confidence < .005 for *pa4* and *e1141*; <.02 for *ad446*) according to the Mann-Whitney rank sum test.

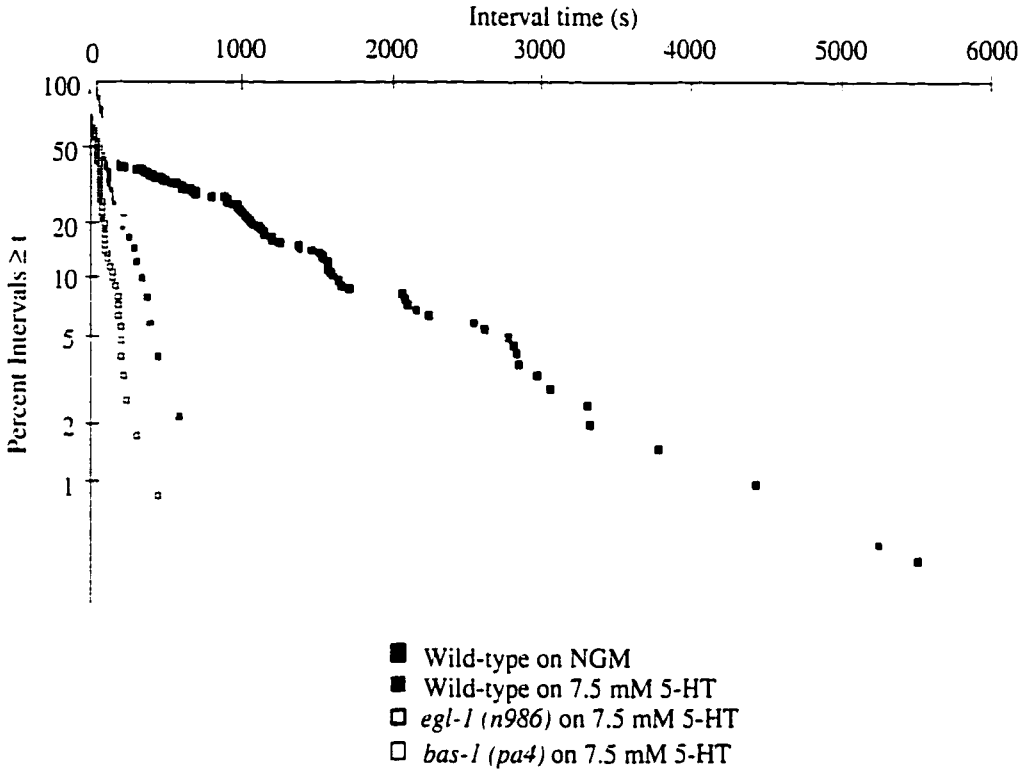
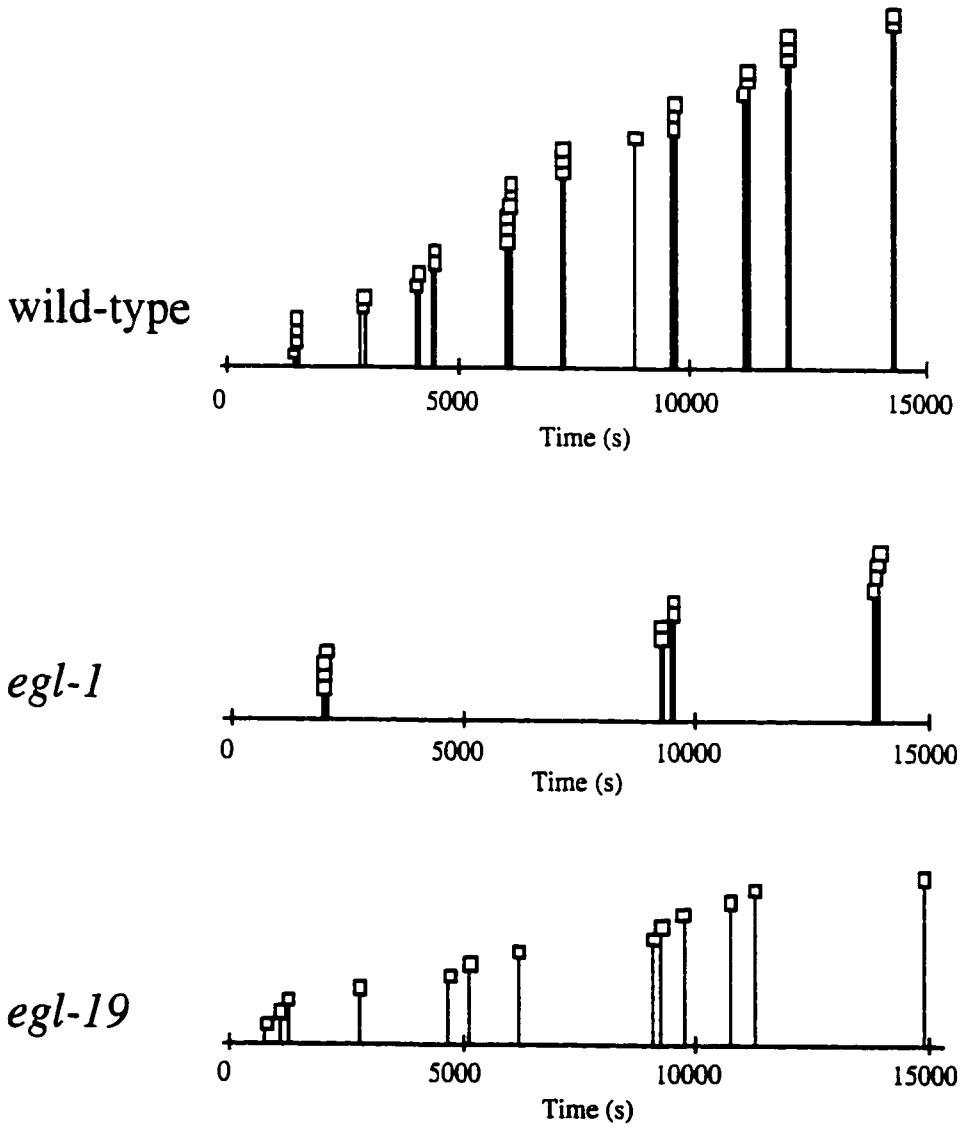


Figure 1-2c. Reversal of the effects of HSN ablation by serotonin. Shown is the log tail distribution of interval times for *egl-1(n986)*, *bas-1(pa4)*, and wild-type hermaphrodites on 7.5 mM serotonin (5-HT). The uniform linear distributions, whose slopes indicate a time constant ($1/\lambda_1$ = 53 s for *egl-1*, for 125 s for *bas-1*, 127 s for N2) close to that for the clustered intervals in wild-type animals, indicates that the animals are almost exclusively in the active state. Animals were tracked as described, and egg-laying patterns were observed until the uterus usually essentially empty (10 minutes for *egl-1*, 20 minutes for N2 and *bas-1*). The number of animals and total intervals analyzed were: N2--9 animals, 49 intervals; *egl-1*--8 animals, 117 intervals; *bas-1*--9 animals, 49 intervals.

Figure 1-3a. Egg-laying patterns for wild-type and mutant animals.

Shown are representative traces of egg-laying behavior for wild-type *C. elegans*, as well as examples of two typical mutant egg-laying patterns. Each open box indicates an egg-laying event. The following mutants showed clustered egg-laying with long inactive phases (i.e., the middle pattern): *egl-1(n986)* (shown), *egl-3(n150)*, *egl-4(n478)*, *egl-11(n587)*, *egl-21(n611)*, *egl-24(n572)*, *egl-30(n686)*, *daf-7(e1372)*, and *tpa-1(k501)*. The following mutants typically laid eggs singly rather than in clusters (the bottom pattern): *egl-7(n575)*, *egl-10(n692)*, *egl-12(n602)*,and *egl-19(n582)* (shown). Of the second group (in which egg-laying within the active phase appears defective), only *egl-19* is serotonin non-responsive [57].


wild-type
0
5000
10000
15000
Time (s)
egl-1
0
5000
10000
15000
Time (s)
egl-19
0
5000
10000
15000
Time (s)

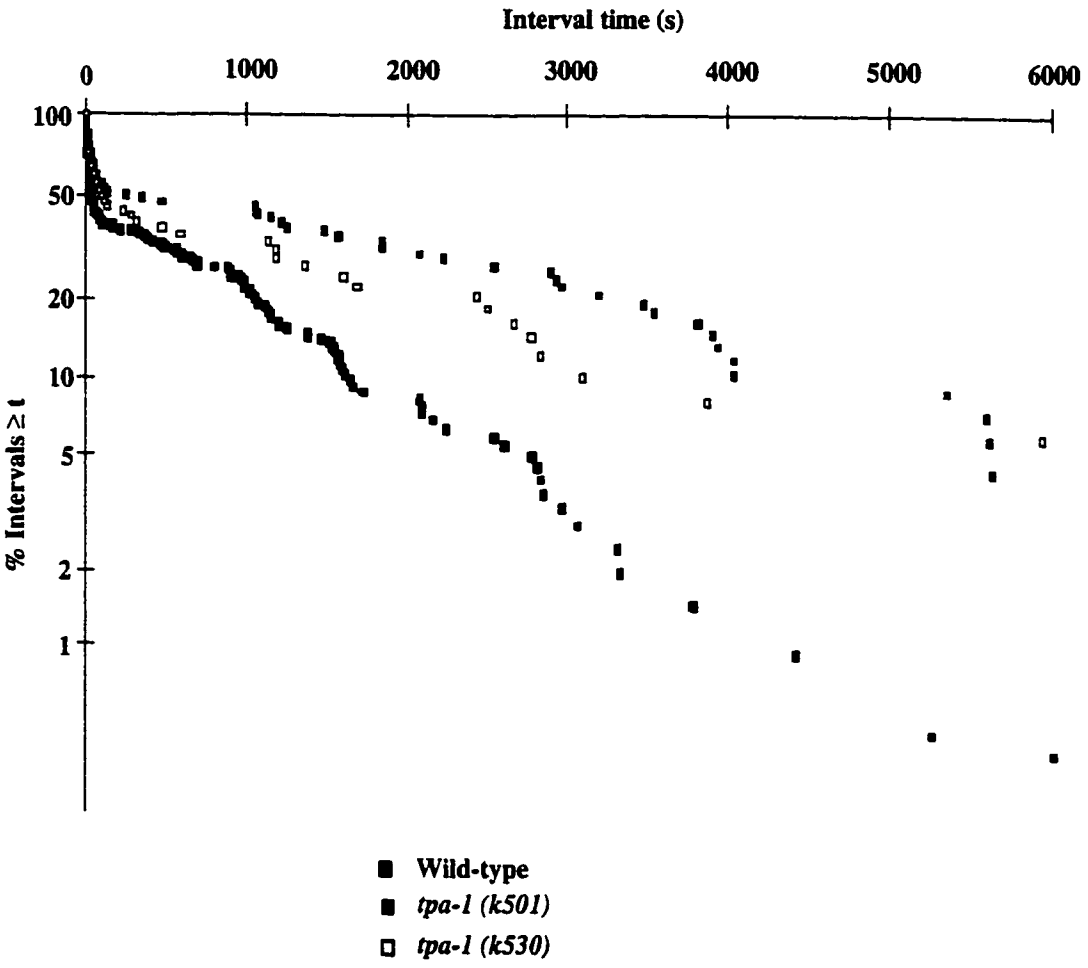


Figure 1-3b. Effect of *tpa-1* on egg-laying behavior. Shown are log tail distributions of egg-laying intervals for two *tpa-1* mutants compared to wild-type animals. The more gradual slope of distributions for the *tpa-1* mutants (Table 1-1) indicates a longer inter-cluster time constant . The long intervals (> 300s) in the mutant animals were statistically longer than in wild-type (level of confidence < .001 for *k501*; ,< .02 for *k530*) according to the Mann-Whitney rank sum test.

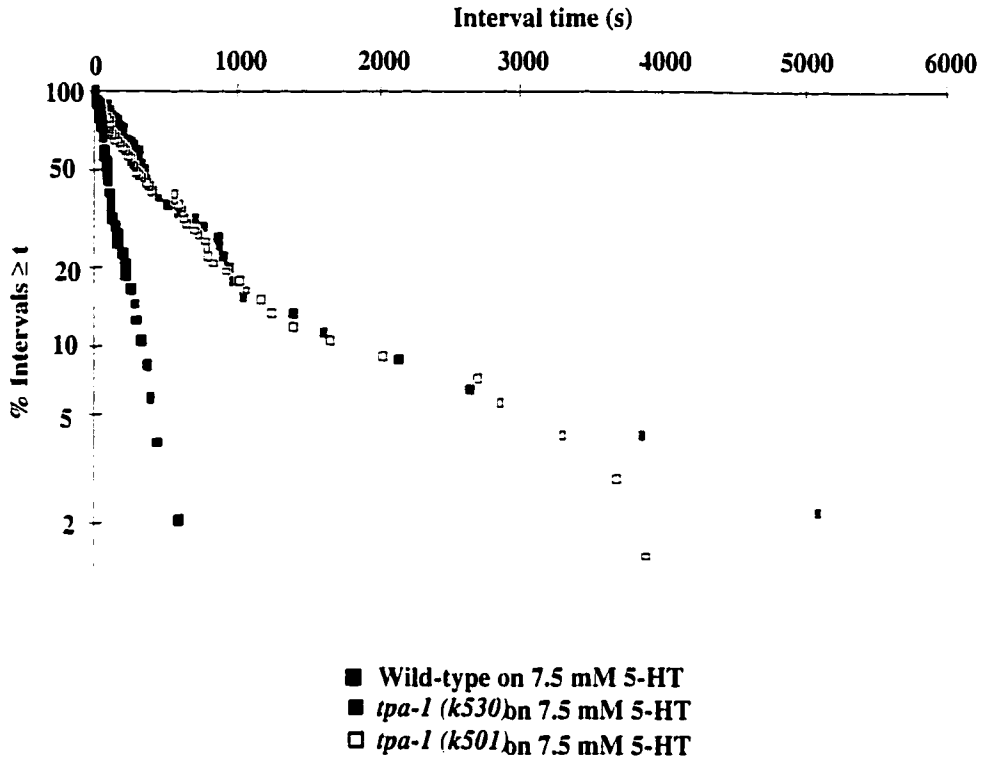
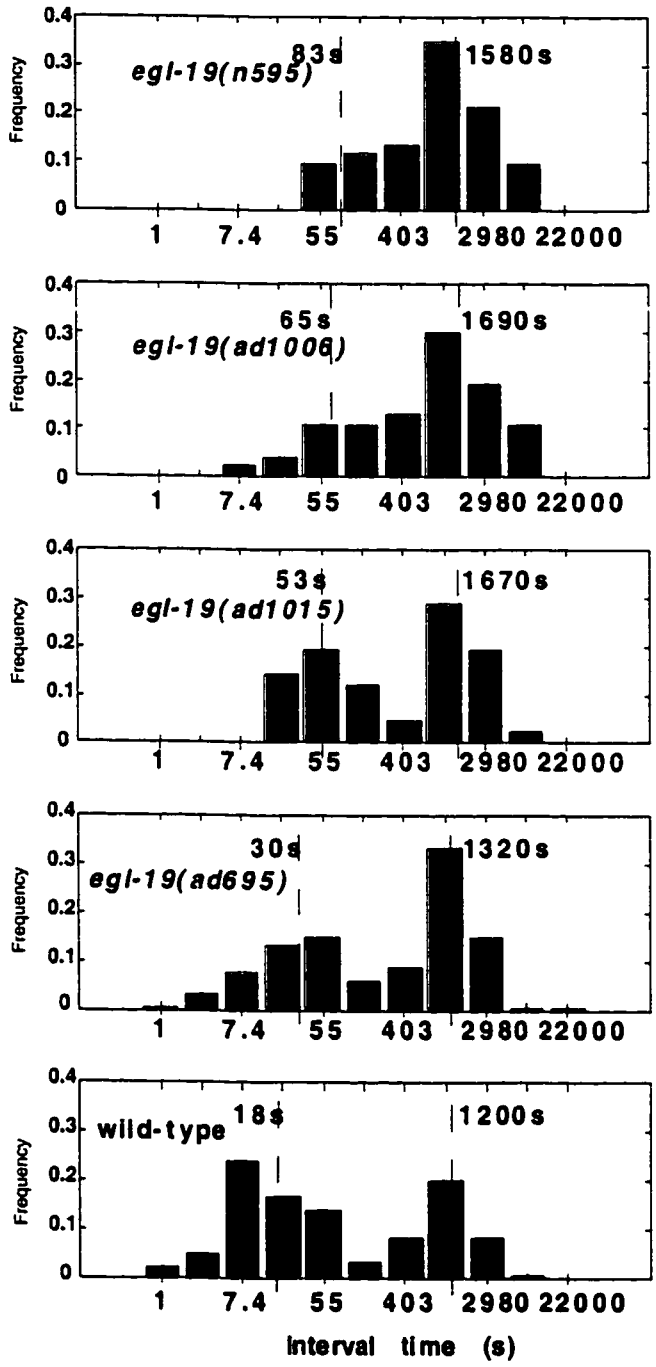


Figure 1-3c. Effect of *tpa-1* on serotonin response. Shown are log tail distributions of egg-laying intervals for *tpa-1* (two alleles) and wild-type animals in the presence of 7.5 mM serotonin (5-HT). The more gradual slope of the *tpa-1* lines (time constant = 429 s for *k501*; 400 s for *k530*, 129 s for wild-type) indicates a slower rate of egg-laying. The intervals in the mutant animals were statistically longer than in wild-type (level of confidence < .001) according to the Mann-Whitney rank sum test. For *tpa-1* mutants, 45 intervals from a total of 7 animals (*k530*) or 67 intervals from a total of 6 animals (for *k501*) were analyzed.

Figure 1-4. Effect of *egl-19* on the egg-laying pattern.

egl-19 animals were tracked on NGM as described. Shown is the histogram of log egg-laying intervals. Loss-of function alleles increased the time constant for egg-laying in the active phase as well as decreasing the number of clustered events. The degree to which these parameters were affected correlated with the severity of the allele. *ad695*, a semidominant, myotonic allele that affects inactivation kinetics [72], also decreased the intra-cluster egg-laying rate. The durations of the short intervals (i.e., < 300s) in the mutants were significantly longer than wild-type in all mutants according to the Mann-Whitney rank sum test (significance <.001 for all recessive alleles; <.02 for *ad695*). ML parameter estimates [p, λ_1 , λ_2] for each allele were: *n582*, [.171, .012, .0037]; *ad1006*, [.246, .016, .0024]; *ad1015*, [.400, .019, .0015]; *ad695*, [.334, .033, .0023]. The number of animals, hours tracked, and total intervals analyzed were: *n582*--3 animals, 32 hr, 53 intervals; *ad1006*--5 animals, 34 hr, 47 intervals; *ad1015*--3 animals, 20 hr, 42 intervals; *ad695*--3 animals, 24 hr, 69 intervals. All recessive *egl-19* mutants were completely serotonin-resistant. Egg-laying rates (eggs/hr; mean ± sem) in the presence and absence of 7.5 mM serotonin were: *ad1006*--2.7 ± 0.3 (no serotonin), 0.7 ± 0.2 (with serotonin); *ad1015*--3.9 ± 0.3 (no serotonin), 1.1 ± 0.3 (with serotonin); *n582*--5.0 ± 0.6 (no serotonin), 1.3 ± 0.4 (with serotonin).


egl-19(n595)
83s
1580s
egl-19(ad1006)
65s
1690s
egl-19(ad1015)
53s
1670s
egl-19(ad695)
30s
1320s
wild-type
18s
1200s
Frequency
0.4
0.3
0.2
0.1
0
1
7.4
55
403
2980
22000
interval time (s)

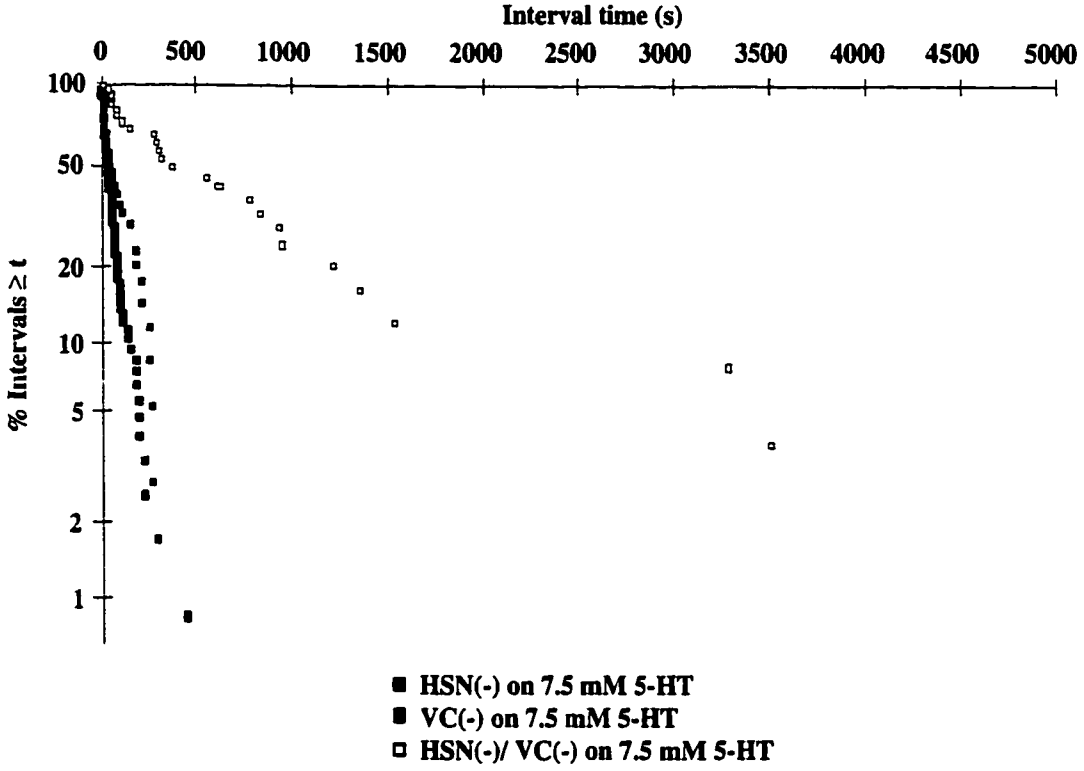


Figure 1-5a. Effect of HSN and VC neurons on serotonin-induced egg-laying. Shown are log tail distributions of egg-laying intervals of HSN-, VC-, and HSN/VC-deficient animals on serotonin. In all cases, a monophasic, linear distribution indicating a homogenous Poisson process was observed; the more gradual slope of the HSN/VC-ablated curve (time constant $1/\lambda_1$ = 405 s for HSN/VC-, 83 s for HSN-, 99 s for VC-) indicated that the rate of egg-laying in response to serotonin was greatly reduced (level of significance < .001). Curves were based on analysis of 35 intervals from 6 animals (VC-), 70 intervals from 6 animals (HSN-), and 25 intervals from 12 animals (HSN-VC-). HSN- and VC- animals were analyzed for 20 minutes, since they became depeleted of embryos after this time. To compare egg-laying in the absence of serotonin, animals were picked as larvae to individual plates and allowed to lay eggs. Both HSN- and HSN-/VC- animals (but not VC- animals) laid eggs slowly, such that the animals "bagged", i.e., were consumed by hatched embryos that had been retained in the uterus. However, in HSN- animals, the mean number of eggs released before bagging was 66.4 (SEM 8.6), while in HSN-/VC- animals, the mean number of eggs released was 24.5 (SEM 4.7).

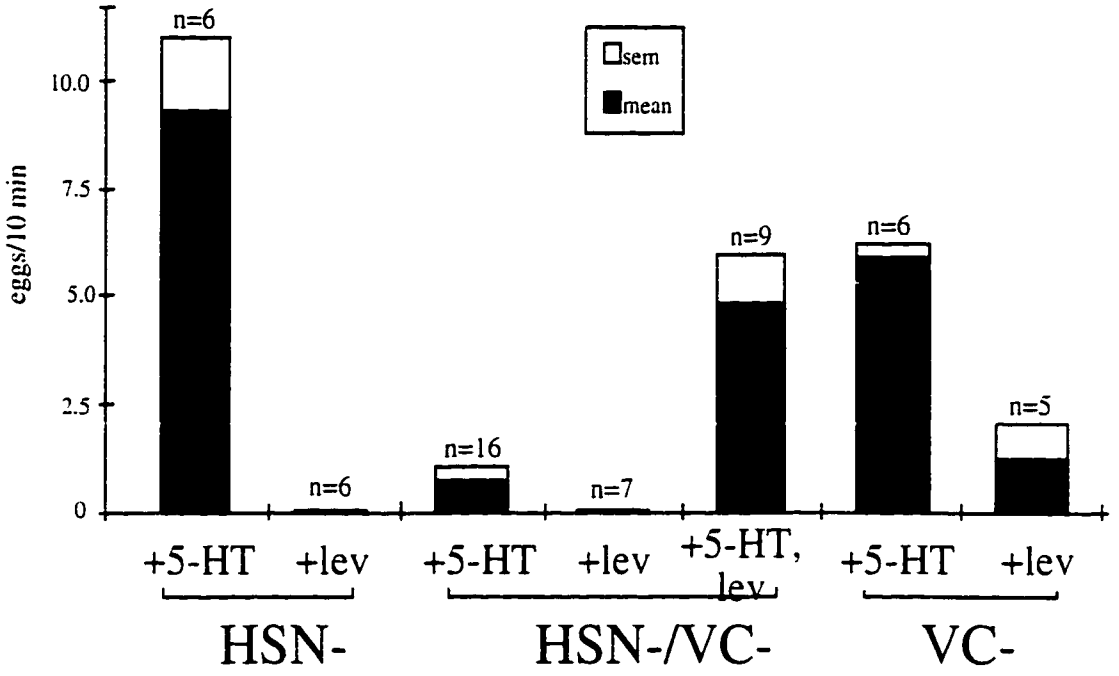
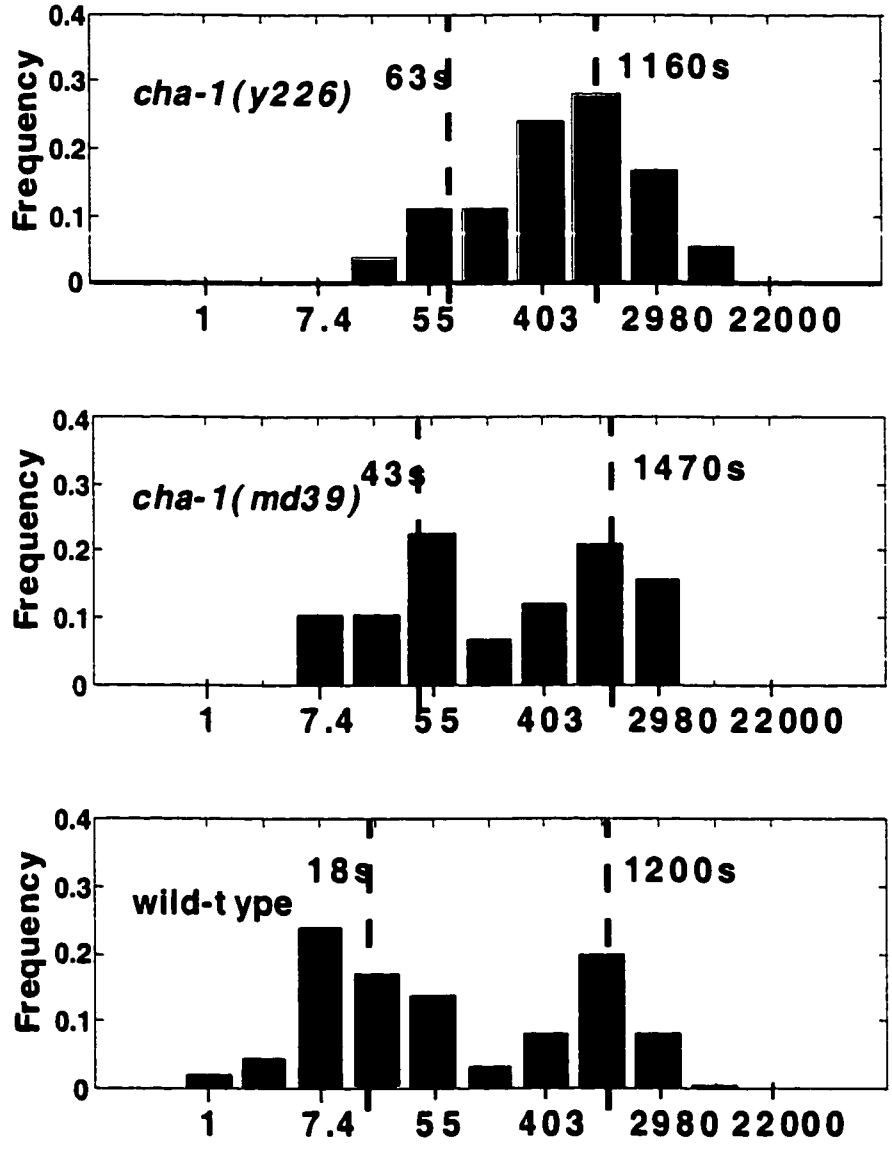


Figure 1-5b. Egg-laying in HSN-/VC- animals requires both serotonin and acetylcholine. HSN/VC-deficient animals laid eggs efficiently only when provided with both serotonin and the cholinergic agonist levamisole. The histogram indicates the egg-laying rate under the indicated condition. The indicated number of animals were tracked for 10 minutes as described in Methods; solid and open boxes indicate mean and standard error of the mean (SEM) respectively. HSN/VC- animals on serotonin and levamisole laid eggs at a higher rate than on serotonin alone (level of confidence < .005) or levamisole alone (level of significance < .001) according to the Mann-Whitney rank sum test.

Figure 1-5c. Effect of acetylcholine depletion on egg-laying.

Depletion of endogenous acetylcholine using a conditional choline acetyltransferase mutant allele caused a decreased rate of egg-laying in the active phase. Shown are histograms of egg-laying intervals for animals carrying a conditional allele of *cha-1* (either *y226* or *md39*) grown to adulthood at 15° and shifted to 22° before tracking. Estimated time constants for long and short intervals are indicated. The short intervals (i.e., <300s) in the mutants were statistically different from wild-type (level of confidence < .001) according to the Mann-Whitney rank sum test. ML parameter estimates for p, λ_1 and λ_2 were [.205, .016, .0042] and [.463, .023, .0015], for *y226* and *md39* respectively.


cha-1(y226)
63s
1160s
cha-1(md39)
43s
1470s
wild-type
18s
1200s
Frequency
0.4
0.3
0.2
0.1
0
1
7.4
55
403
2980
22000

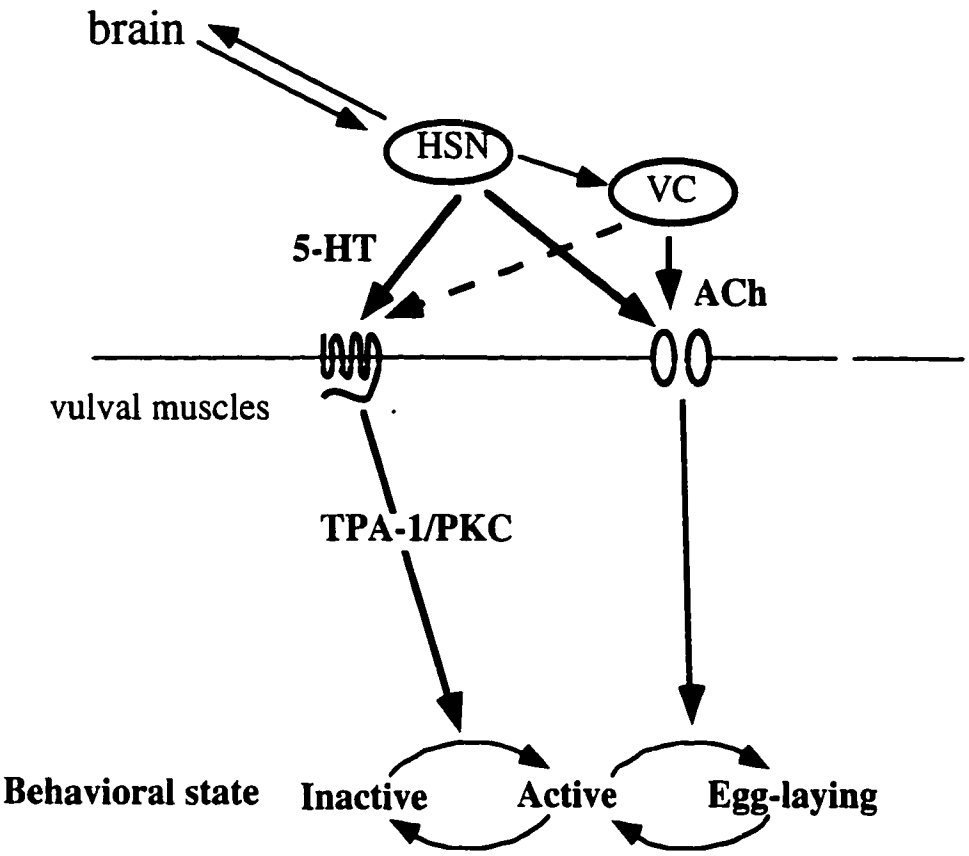


Figure 1-6. Molecular model for the control of egg-laying. Serotonin, released primarily from the HSNs and to a lesser extent from VC4 and VC5, facilitates the onset of the active phase. This could occur through activation of EGL-19 calcium channels in the vulval muscles through PKC-dependent phosphorylation. The HSNs and VCs can both trigger individual muscle contractions within the active phase by releasing acetylcholine; by binding to nicotinic receptors and thus depolarizing the muscle cell membrane, this would lead to opening of activated EGL-19 channels and stimulation of muscle contraction.

CHAPTER III THE EFFECT OF A NEUROPEPTIDE GENE, *FLP-1*, ON EGG-LAYING IN *C. ELEGANS*

ABSTRACT

Egg-laying behavior in the nematode *Caenorhabditis elegans* involves fluctuation between alternative behavioral states: an inactive state, during which eggs are retained in the uterus, and an active state, during which eggs are laid in bursts. We have found that the *flp-1* gene, which encodes a group of structurally related neuropeptides, functions specifically to promote the switch from the inactive to the active egg-laying state. Recessive mutations in *flp-1* caused a significant increase in the duration of the inactive phase, yet egg-laying within the active phase was normal. This pattern resembled that previously observed in mutants defective in the biosynthesis of serotonin, a neuromodulator implicated in induction of the active phase. Although *flp-1* mutants were sensitive to stimulation of egg-laying by serotonin, the magnitude of their serotonin response was abnormally low. Thus, the *flp-1*-encoded peptides and serotonin function most likely in concert to facilitate the onset of the active egg-laying phase. Interestingly, we observed that *flp-1* is necessary for animals to down-regulate their rate of egg-laying in the absence of food. Since *flp-1* is known to be expressed in interneurons that are post-synaptic to a variety of chemosensory cells, the FLP-1 peptides may function to regulate the activity of the egg-laying circuitry in response to sensory cues.

INTRODUCTION

Many aspects of behavior, including mood, aggression, sleep, and sexual arousal, involve discrete, alternative behavioral states. At the most basic level, these different behavioral states result from differences in the functional properties of the neurons and muscle cells in the circuits that produce the behavior. The regulation of switching between these functional states occurs largely through the action of molecules known as neuromodulators. In general, neuromodulators function by activating signaling pathways that regulate the activity of receptors and ion channels in excitable cells. A wide variety of molecules are known to function as neuromodulators, including biogenic amines (e.g. dopamine, serotonin, norepinephrine, and histamine), adenosine, glutamate, acetylcholine (through their action at muscarinic receptors), and a diverse array of neuropeptides. Identification of the mechanisms by which neuromodulators influence behavior at the molecular, cellular, and circuit levels is essential for understanding the function of both simple and complex nervous systems.

We have employed a genetic approach to a simple animal, the nematode *Caenorhabditis elegans,* to investigate the molecular mechanisms by which neuromodulators control behavioral states. *C. elegans* is particularly well-suited to molecular studies of nervous system function. It has a simple nervous system consisting of 302 neurons, and the position, cell lineage, and synaptic connectivity of each of these neurons is precisely known [1, 2, 26]. Because a particular neuron can be positively identified based on its position, it is possible to evaluate the function of an individual neuron or group of neurons through single cell laser ablation [92]. Moreover, because of their short generation time, small genome size, and accessibility to germline transformation, these animals are highly amenable to molecular and classical genetics [93]. Thus, in *C. elegans*, it is relatively easy to identify genes involved in specific

behaviors, and to characterize the functions of their products using molecular, behavioral, and immunocytochemical analyses. Although its nervous system contains relatively few neurons, *C. elegans* makes use of a surprisingly broad array of neuromodulators, including a large number of putative neuropeptides and the biogenic amines serotonin, dopamine, and octopamine [58]. In addition, it exhibits a number of easily assayed and quantifiable behaviors that are affected by a wide range of neuroactive substances [94]. For these reasons, it is an excellent model organism for studying the molecular and cellular basis of neuromodulator action.

The largest class of neuromodulators in *C. elegans* are the FMRFamide-related peptides, or FaRPs. These peptides are characterized by a common carboxy-terminal motif of Arg-Phe-NH_2, and generally range in size from 4-20 amino acids. FaRPs are the predominant family of neuropeptides in invertebrates, where they have been shown to play a role in cardioregulation, learning, and the modulation of muscle contraction [95]. FaRPs have also been identified in vertebrates, where they have been implicated in the regulation of pain responses [96]. In *C. elegans* , immunocytochemical experiments have shown that at least 30 neurons and gland cells contain peptides which have the C-terminal RFamide epitope characteristic of the FaRP family [24]. The *C. elegans* genomic sequence contains at least 20 genes (designated *flp*- genes, for FMRFamide-l ike peptide deficient) which encode predicted polypeptide precursors of more than 50 distinct FaRPs [97]. Most of these *flp* genes have subsequently been shown to be expressed in larval and/or adult *C. elegans,* and in some cases the predicted peptide products have actually been purified from *C. elegans* extracts. At present, only one *flp* gene, *flp-1*, has been extensively characterized at the molecular and genetic level [23, 98]. *flp-1* is expressed in a specific subset of head neurons, and encodes two alternatively spliced products that can be processed to give rise to seven closely related FaRPs. *flp-1* loss-of-function mutants

exhibit a number of behavioral abnormalities, including hyperactive locomotion, nose touch insensitivity, and defective osmotic avoidance [98]. Although many questions remain concerning the molecular and cellular mechanisms through which the *flp-1* encoded peptides influence these behaviors, it is clear that the peptides encoded by *flp-1* have many specific effects on behavior which are at least partially distinct from the other *C. elegans* FaRPs.

In this study, we have investigated the role of *flp-1* in another behavior --egg-laying. *C. elegans* hermaphrodites are self-fertile, and continuously produce embryos and retain them in their uterus for at least two days following the adult molt. Egg-laying in *C. elegans* occurs when embryos are expelled from the uterus through the contraction of 16 vulval and uterine muscles [1]. In the presence of abundant food, wild-type animals lay eggs in a specific temporal pattern: egg-laying events (i.e., contractions of the egg-laying muscles leading to the expulsion of one or more eggs) tend to be clustered in short bursts, or active phases, which are separated by longer inactive phases during which eggs are retained. Both the onset of the active phase and egg-laying within the active phase are aperiodic, and model closely as Poisson processes with distinct rate constants (Figure 1a-c). This egg-laying pattern can be accurately modeled as a three-state probabilistic process, in which animals fluctuate between discrete inactive, active, and egg-laying states [99]. This process has three parameters: the rate constant for the duration of the inactive phase (λ_2), the rate constant for egg-laying within the active phase (λ_1), and the probability of remaining in the active phase after an egg-laying event (p). Using a maximum-likelihood algorithm, it is possible to estimate these egg-laying parameters from real behavioral data, and thereby compare the egg-laying patterns of wild-type and mutant strains [67].

Genetic, pharmacological, and cell ablation studies have provided important insight into the roles of particular neurons and neurotransmitters in the control of egg-laying [17, 57, 64]. Two classes of motorneurons make extensive synapses with the vulval muscles: the 2 HSNs and 6 VCs, each of which expresses multiple neurotransmitters and neuromodulators. For example, the HSNs express serotonin, acetylcholine, and one or more FaRPs, while the VCs express acetylcholine, FaRPs, and possibly a biogenic amine [15, 24, 58]. Both serotonin and acetylcholine have been shown pharmacologically to increase the overall rate of egg-laying [57, 64]. By characterizing the egg-laying patterns of mutant and ablated animals, it has been possible to distinguish neurons and genes that modulate the switching between behavioral states from those that promote egg-laying within the active phase. For example, in both HSN-ablated animals and serotonin-deficient mutants, the inactive egg-laying phase is abnormally long, whereas egg-laying within the active phase is unimpaired [99]. Thus, serotonin released from the HSNs apparently stimulates egg-laying by facilitating the switch from the inactive to the active egg-laying state. Similar experiments have implicated acetylcholine, released from both the HSNs and VCs, in the induction of egg-laying events within the active phase [99].

In this study, we show that the peptides produced by the *flp-1* gene function in the regulation of egg-laying behavior. Specifically, the *flp-1* encoded peptides appear to promote the onset of the active phase of egg-laying, an activity that is at least partially independent of the HSN motorneurons. In addition, we provide evidence that these peptides may participate in the regulation of egg-laying by sensory cues.

RESULTS

flp-1 affects the transition between behavioral states involved in egg-laying

To test the possible involvement of FLP-1-encoded peptides in the modulation of egg-laying behavior, we analyzed the egg-laying patterns of *flp-1* mutants. We first analyzed the egg-laying patterns of mutants carrying recessive loss-of-function mutations in the *flp-1* gene. These animals were not grossly defective in the ability to lay eggs, and their egg-laying patterns were qualitatively similar to wild-type animals: egg-laying events were still clustered in active phases, and both the switch into the active phase and the laying of eggs within the active phase still modeled as Poisson processes. However, the duration of the inactive phase was substantially longer in the *flp-1* mutants (λ_2 for *flp-1(yn2)* and *flp-1(yn4)* were 4.7 and 7.0 x 10^{-4} s^{-1}, respectively) than in wild-type animals (λ_2 was 1.4 x 10^{-3} s^{-1}; Table 2-1) (Figure 2-2a, b). In contrast, egg-laying within the active phase was unimpaired in *flp-1* mutants; in fact, the intra-cluster time constants were actually faster in the *flp-1(yn2)* and *flp-1(yn4)* deletion mutants than in wild-type (Table 2-1, Figure 2-2a). Thus, loss of *flp-1* function appeared to specifically decrease the probability of switching from the inactive to the active phase of egg-laying, suggesting that the function of the wild-type *flp-1* gene products is to promote the onset of the active egg-laying state.

flp-1 and serotonin function in concert to promote the active egg-laying phase

The egg-laying defect exhibited by *flp-1* mutants--longer-than normal inactive phase but rapid egg-laying within the active phase--was quantitatively and qualitatively similar to the defect seen in serotonin-deficient mutants. In principle, these two modulators could function in a common biological pathway, or they could affect distinct parallel pathways. To examine these possibilities, we constructed a double mutant carrying loss-of-function mutations in both *flp-1* and *cat-4*, a gene required for serotonin

biosynthesis [16]. Simple measurements of egg-laying rates indicated that the severity of the egg-laying defect in the double mutant was comparable to that of *flp-1* or *cat-4* single mutants (Figure 2-3a). Moreover, both the inactive phase rate constant λ_2 and the inter-cluster time constant of the double mutant were essentially identical to those of either single mutant (Table 2-1). These results supported the hypothesis that serotonin and *flp-1* most likely function in the same pathway to induce the active egg-laying state.

To further explore the relationship between the effects of these two modulators on the regulation of egg-laying, we assayed the responses of *flp-1* mutants to exogenous serotonin. We first measured the ability of serotonin to stimulate egg-laying under conditions which are normally inhibitory for egg-laying (i.e., in the hypertonic salt solution M9). In this assay, we observed that *flp-1* loss-of function mutants were still responsive to serotonin (Figure 2-3b). Moreover, the serotonin sensitivity of *flp-1* mutants, as measured by the concentration of serotonin that gave half-maximal stimulation, was comparable to that of wild-type animals. However, the magnitude of their response (i.e., the number of eggs laid when stimulated by serotonin) was reduced relative to wild-type. Thus, while a functional *flp-1* gene did not appear to be essential for serotonin to stimulate egg-laying, neither was exogenous serotonin able to completely bypass the effect of *flp-1* on egg-laying. To further investigate the effect of *flp-1* mutations on the egg-laying response to serotonin, we analyzed the egg-laying patterns of wild-type and *flp-1* mutants in the presence of exogenous serotonin. In wild-type animals, treatment with serotonin not only increased rate of egg-laying, it also caused eggs to be laid in a monophasic pattern resembling a simple Poisson process. This pattern implied that in the presence of exogenous serotonin, wild-type animals were mostly in the active egg-laying phase. In *flp-1* mutants, we observed that serotonin treatment affected egg-laying behavior in a similar manner; eggs were laid at a higher rate and in a more

monophasic pattern in the presence of serotonin than on drug-free medium, though the rate of egg-laying in the presence of serotonin was slower in one of the *flp-1* mutants than that in wild-type (Figure 2-3c). Taken together, these experiments led us to conclude that *flp-1* is not necessary for the stimulation of egg-laying by serotonin, though a functional *flp-1* product is required for a maximal serotonin response.

HSN-independence of the *flp-1* mutant phenotype

The serotonergic neurons most strongly implicated in the control of egg-laying are the HSN motorneurons. Although the HSNs themselves do not express *flp-1*, a number of *flp-1* expressing cells in the head lie in close proximity to, and in some cases actually make synapses with, the dendrite of the HSN in the nerve ring. This raised the possibility that the effects of *flp-1*-encoded peptides might be mediated through modulation of the HSNs. Alternatively, given the small size of *C. elegans*, it was also possible that *flp-1* could regulate egg-laying through a neuroendocrine mechanism that bypassed the HSNs. To distinguish between these models, we tested the effect of a cell-specific ablation of the HSNs on the egg-laying behavior of *flp-1* mutants. We observed that HSN-ablated *flp-1* animals were no more severely egg-laying defective than HSN-ablated wild-type animals: both their overall egg-laying rates and their egg-laying patterns were essentially identical (Figure 2-4a). This result was consistent with our earlier observations indicating that serotonin, a neuromodulator known to be released from the HSNs, affects the same aspect of egg-laying behavior as *flp-1*. However, when we analyzed the behavior of HSN-ablated *flp-1* animals in the presence of exogenous serotonin, we found that they laid eggs significantly more slowly than HSN-ablated wild-type animals under the same condition (Figure 2-4b). Thus, the ability of *flp-1* to potentiate the stimulation of egg-laying by serotonin did not appear to require the HSNs. Thus, the effects of the FLP-1 peptides on egg-laying, in particular their ability to

facilitate egg-laying in response to serotonin, were at least partially independent of the HSNs.

flp-1 is necessary for regulation of egg-laying by food signals

What functional role might the FLP-1 peptides play in the control of egg-laying behavior? Among the neurons that express *flp-1* are several pairs of interneurons, which are major recipients of synaptic input from sensory cells and have been implicated in processing and relaying integrated sensory information to motor circuits. The expression of *flp-1* within these cells raised the possibility that *flp-1*-encoded peptides might be involved in the regulation of egg-laying behavior by sensory cues. Egg-laying is affected by a number of environmental conditions, including the presence or absence of a bacterial food source. To determine if *flp-1* affects the regulation of egg-laying by food, we tested the effects of *flp-1* loss-of-function mutations on the ability of animals to control their egg-laying rate in response to the presence or absence of a bacterial lawn. We observed that wild-type animals maintained on agar plates seeded with *E. coli* laid eggs at a significantly higher rate than animals maintained on agar plates that lacked food. Strikingly, however, we observed that *flp-1* loss-of-function mutants laid eggs at essentially the same rate in the presence of a bacterial lawn as in the absence of a lawn (Figure 2-5). This defect in food regulation of egg-laying was fairly specific to *flp-1* mutants, and was not merely a consequence of their general egg-laying defect. For example, egg-laying behavior in *egl-1* mutants, which lacked HSN neurons as a result of inappropriate programmed cell death, was strongly regulated by food, even though their egg-laying rate and pattern in the presence of food was similar to that of *flp-1* mutants. Thus, *flp-1* appeared to function specifically in mediating the control of egg-laying behavior in response to the availability of food.

goa-1 functions independently from _flp-1_ and serotonin in the control of egg-laying

What genes might function downstream of _flp-1_ and serotonin in the control of egg-laying? One possible candidate is _goa-1_, which encodes a G_o homologue that has been hypothesized to mediate the effects of serotonin [18] and the FLP-1 peptides [98] on locomotion. _goa-1_ is expressed in the egg-laying neurons as well as the vulval muscles, and mutations in _goa-1_ have been shown to enhance (in the case of recessive alleles) or inhibit (in the case of dominant gain-of-function alleles) egg-laying behavior [18, 30]. Thus, the _goa-1_ gene product was a plausible candidate for a gene that might function downstream of _flp-1_ and/or serotonin as a negative regulator of egg-laying.

To investigate this possibility, we analyzed the egg-laying patterns of _goa-1_ mutants. We observed that the inactive phase was substantially shorter in _goa-1_ recessive mutant animals than in wild-type (Table 2-1; see also Figure 2-6a, b), implicating GOA-1 as a negative regulator of the switch into the active phase. Since this effect was roughly converse to the effect of mutations in _flp-1_ and _cat-4_ , one possible interpretation of this result was that GOA-1 activity might be negatively regulated by serotonin and/or _flp-1_. Alternatively, _goa-1_ could function independently from, and antagonistically to the pathway(s) activated by serotonin and _flp-1_. To distinguish these possibilities, we constructed double mutants carrying recessive mutations in _goa-1_ and either _flp-1_ or _cat-4_ and analyzed their egg-laying behavior. In each case, the double mutant showed a phenotype intermediate between that of the two single mutants (Figure 2-6a, b; Table 2-1). For example, in the case of _flp-1_, both the inactive phase rate constant λ_2 (.0014 s^{-1}) and the inter-cluster time constant (1950 s) for the _goa-1_; _flp-1_ double mutant were intermediate between those of the _goa-1_ single mutant (.0031 s^{-1}; 890 s) and the _flp-1_ single mutant (.0005; 3840 s). Similarly, both λ_2 and the inter-cluster time constant for

the *cat-4*; *goa-1* double mutant were intermediate between the *goa-1* single mutant and the *cat-4* single mutant. Pharmacological experiments also supported the hypothesis that serotonin, *flp-1* and *goa-1* functioned independently. For example, *goa-1* loss-of function mutants responded to serotonin at abnormally low threshold concentrations, an effect that was suppressed by mutations in *flp-1* (Figure 2-6c). Together, these results suggested that *goa-1* defined a new pathway, independent of the ones activated by *flp-1* and serotonin, regulating the switch into the active egg-laying phase.

Pharmacological experiments have suggested that GOA-1 functions in both neurons and muscle cells to inhibit egg-laying [30]. In principle, GOA-1 might control the onset of the active egg-laying phase by negatively regulating the activity of the HSNs; alternatively, it might negatively regulate the response of the vulval muscles to modulatory inputs from neurons. To distinguish between these models, we analyzed the egg-laying behavior of HSN-ablated *goa-1* mutant animals. Surprisingly, we observed that the inactive phase in HSN-ablated *goa-1* mutants was no shorter than in HSN-ablated wild-type animals (Figure 2-6d; Table 2-1). This indicated that the shortening of the inactive phase by *goa-1* mutations was dependent on the HSNs, and suggested that GOA-1 controls the switch into the active phase by directly or indirectly modulating HSN function. HSN-ablation was not completely epistatic to mutations in *goa-1*; the number of eggs laid within a given active phase (a function of the clustering parameter p) was higher in HSN-ablated *goa-1* mutants than in HSN-ablated wild-type (Table 2-1). Since *goa-1* recessive mutants appeared to have longer active phases, this implied that the function of GOA-1 in the vulval muscles may be to promote the switch from the active egg-laying phase back to the inactive phase.

DISCUSSION

Modulation of egg-laying behavioral states by FaRPs

Egg-laying behavior involves switching between two alternative behavioral states: an active state, during which eggs are laid in bursts, and an inactive phase, during which eggs are retained in the uterus. We observed that loss-of-function mutants defective in the gene *flp-1*, which encodes a set of FMRFamide-related peptides, displayed a specific abnormality in their temporal pattern of egg-laying: the inactive phase was abnormally long, whereas egg-laying within the active phase was normal. Thus, the *flp-1* gene products appeared to function specifically to facilitate the switch from the inactive to the active egg-laying phase. Previous work had shown that the serotonergic HSN motorneurons also were specifically required to promote the onset of the active phase [99]. Nonetheless, at least some of the effects of *flp-1* on egg-laying appeared to be HSN-independent: *flp-1* mutant animals whose HSNs had been eliminated through laser ablation had more severe egg-laying defects, and responded less strongly to serotonin, than HSN-ablated wild-type animals. These results were perhaps surprising, since all the chains of synaptic connections between the *flp-1*-expressing neurons in the head and the vulval muscles involve the HSNs [1]. In fact, *flp-1* mutations only slightly enhanced the egg-laying defect of HSN-ablated animals, suggesting that some of the effects of the FLP-1 peptides are likely to be HSN-dependent. Thus, the FLP-1 peptides may regulate the egg-laying muscles both through modulation of the HSNs as well as through an HSN-independent humoral mechanism (Figure 2-7).

Many questions remain about the cellular mechanism through which FLP-1 peptides regulate egg-laying. *flp-1* expression has been detected in a number of neurons in the head, including AIA, AIY, AVA, AVK, AVE, RIG, and RMG [98]. Based on the results presented here, the simplest hypothesis is that humoral release of FLP-1 peptides from

one or more of these neuronal classes modulates the egg-laying muscles directly. Alternatively, it is possible that some or all of the effects of the FLP-1 peptides on egg-laying could be indirect. For example, FLP-1 peptides could modulate the activity of other neurosecretory cells in the head, affecting the release of a hypothetical neurohormone that modulates the egg-laying muscles. Some of the effects of *flp-1* on egg-laying might also involve the VC neurons, although the fact that *flp-1* mutations lengthen the inactive phase much more than ablations of the VCs do [99] (L. Waggoner, unpublished) argues that the VCs are not the primary target of the FLP-1 peptides. Laser ablations of various combinations of *flp-1*-expressing neurons, as well as neurons postsynaptic to these cells, may provide more detailed information about the cellular basis for the effect of *flp-1* on egg-laying behavior.

Interactions between *flp-1* and serotonin in the control of egg-laying

The effects of *flp-1* on egg-laying are quite similar to the effects of another neuromodulator, serotonin. We observed here that loss of *flp-1* function did not confer resistance to the effects of serotonin on egg-laying, though it did significantly reduce the magnitude of the serotonin response. Therefore, the *flp-1*-encoded peptides appear to stimulate egg-laying at least in part by enhancing the response of the egg-laying muscles to serotonin. This hypothesis is consistent with previously published work, which demonstrated that synthetic peptides identical to the shared carboxy-terminus of the 7 *flp-1* peptides (FLRF-NH_2) increased the average number of eggs laid in response to serotonin [24]. Serotonin and FLP-1 appear to function synergistically not only in the stimulation of egg-laying, but in other *C. elegans* behaviors as well. For example, both serotonin and FLP-1 inhibit locomotion, and the ability of serotonin to inhibit movement has been shown to require a functional *flp-1* gene [98]. Thus, for locomotive as well FLP-1 peptides appear to be necessary to potentiate the effects of serotonin on

locomotion. Although the molecular pathways through which serotonin and *flp-1* control egg-laying are likely to differ in some respects from those involved in locomotion, it is tempting to speculate that the parallel actions of these two modulators on these two different behaviors might depend on a conserved molecular mechanism. Since comodulation of neuromuscular activity by biogenic amines and FaRPs is observed in many organisms [100, 101], the molecular interactions between the *flp-1* and serotonin-activated signaling pathways in the egg-laying cells may provide a useful model for similar processes in other animals.

Insights into the regulation of egg-laying by sensory information

The analysis of the *flp-1* mutants also revealed a role for the FLP-1 peptides in the control of egg-laying behavior by sensory cues. We observed that whereas wild-type worms laid eggs at a much slower rate in the absence of a bacterial food source, *flp-1* loss-of-function mutants laid eggs at the same rate in either presence or absence of food. This insensitivity to the presence of bacteria was not merely a consequence of the *flp-1* animals' generally slower egg-laying rate, as other egg-laying defective animals (e.g., *egl-1* mutants which lacked the HSN motorneurons) still showed significant regulation of egg-laying by food availability. Therefore, the *flp-1*-encoded peptides may be specifically dedicated to relaying signals of food abundance to the egg-laying circuit. In the absence of bacteria, levels of FLP-1 release could be low, leading to long inactive phases and slow egg-laying, whereas abundant food would lead to increased FLP-1 release and more active egg-laying. Other aspects of the *flp-1* mutant phenotype are consistent with FLP-1 functioning as an indicator of food availability. For example, when nematodes, maintained in the absence of food, encounter a lawn of bacteria, they slow their rate of movement [91]. Both the hyperactive locomotion and the "wandering" behavior previously noted in *flp-1* recessive mutants [98] could plausibly stem from a defect in this

response to food. Thus, *flp-1* may function quite generally to facilitate a variety of behavioral patterns that are appropriate for conditions of food abundance.

The expression pattern of *flp-1* in the *C. elegans* nervous system is well suited for a gene that encodes a food signal. The presence of bacteria in the environment is thought to be detected primarily through olfactory or chemosensory cues [62]. The primary route through which nematodes gather chemosensory information is by using a pair of polymodal sense organs known as amphids. Synaptic output from the amphid sensory neurons is relayed to four pairs of amphid interneurons: AIA, AIB, AIY and AIZ [1]. In thermotaxis behavior, the amphid interneurons have been shown to be an important site for integrating and processing sensory information that is used to modulate behavioral outputs [102]. Both AIA and AIY express *flp-1*; thus a simple model for how egg-laying behavior could be controlled by food signals is that under conditions favorable to egg-laying (i.e., abundant food), the AIA and AIY neurons release FLP-1 peptides, switching the animal into the active egg-laying state. This release of FLP-1 peptides from the amphid interneurons could likewise switch the animal into a more inactive state with respect to locomotion.

Evidence that *goa-1* modulates neural states involved in egg-laying behavior

In addition to the pathways activated by FLP-1 and serotonin, a third pathway, defined genetically by the *goa-1* gene, also appears to regulate the onset of the active phase of egg-laying, in a manner antagonistic to and apparently independent of both *flp-1* and serotonin. A recessive mutation in *goa-1*, which encodes the *C. elegans* homologue of the G_o alpha subunit [18, 30], increased the rate of egg-laying by shortening the inactive phase. Genetic analysis indicated that *goa-1* probably functions to regulate egg-laying in a pathway distinct from the ones activated by *flp-1* and serotonin. Interestingly,

the effect of *goa-1* on the onset of the active phase appeared to be completely dependent on the HSNs. Thus, *goa-1* may function by negatively regulating release from the HSNs of a neuromodulator that facilitates the switch from the inactive to the active egg-laying state.

An interesting implication of this hypothesis is that the behavioral states involved in egg-laying may correspond not only to functional states of the egg-laying muscles themselves, but also to distinct functional states of neurons (such as the HSNs) dedicated to egg-laying. Our earlier studies led to the hypothesis that the active egg-laying state depends on a functional activation of the vulval muscles, which allows the excitatory transmitter acetylcholine to readily induce muscle contraction. Our analysis of *goa-1* mutants suggests that the active and inactive egg-laying states may also correspond to functional states of the HSNs--an inactive state in which the HSNs release neurotransmitter with low probability, and an active state in which the probability of neurotransmitter release is high. According to this model, activated GOA-1 may inhibit the switch of the HSNs into this active state; thus, when GOA-1 is inactive or absent, the switch into the active state becomes more frequent. The likely involvement of GOA-1 in controlling HSN activity implies that additional neuromodulators, possibly released from neurons in the head, may regulate egg-laying behavior by controlling GOA-1 activity.

Regulation of egg-laying muscle activity by multiple neuromodulators

Although the effect of *goa-1* on the switch into the active phase was dependent on the HSNs, it was not completely dependent on serotonin. These observations imply that the HSNs contain another neuromodulator, whose release may be regulated by GOA-1, that facilitates the switch into the active phase. Although at present we can only speculate as to the identity of such a molecule, there appear to be a number of candidates.

For example, the HSNs almost certainly contain one or more non-*flp-1* encoded FaRPs, since they contain FaRP immunoreactivity which is not eliminated by deletions of the *flp-1* gene [24, 98]. In addition, pharmacological experiments indicate that muscarinic acetylcholine agonists stimulate egg-laying [64]. Since the HSNs are cholinergic, acetylcholine released from the HSNs might modulate egg-laying through muscarinic receptors. In principle, GOA-1 regulated release of any of these molecules could facilitate the onset of the active egg-laying state. The diversity of neurotransmitter usage in the HSNs is also a hallmark of other neurons and gland cells that participate in the control of egg-laying. For example, non-*flp-1*-encoded FaRPs are present in both the VC motorneurons and the uv1 uterine gland cells [24]. In addition, the VCs contain acetylcholine and, since they express the vesicular monoamine transporter, possibly an unidentified biogenic amine as well [103]. Thus, egg-laying behavior is likely to be regulated by a surprisingly diverse array of neurotransmitters and neuromodulators, which are likely to activate complex, interacting signaling pathways in the vulval muscle cells. The elucidation of these signaling mechanisms represents an important challenge for future studies, and may be a useful model for the functional interaction of neuromodulatory pathways in other organisms.

Modulation of egg-laying behavioral states by FaRPs

Egg-laying behavior involves switching between two alternative behavioral states: an active state, during which eggs are laid in bursts, and an inactive phase, during which eggs are retained in the uterus. We observed that loss-of-function mutants defective in the gene *flp-1*, which encodes a set of FMRFamide-related peptides, displayed a specific abnormality in their temporal pattern of egg-laying: the inactive phase was abnormally long, whereas egg-laying within the active phase was normal. Thus, the *flp-1* gene products appeared to function specifically to facilitate the switch from the inactive to the

active egg-laying phase. Previous work had shown that the serotonergic HSN motorneurons also were specifically required to promote the onset of the active phase [99]. Nonetheless, at least some of the effects of *flp-1* on egg-laying appeared to be HSN-independent: *flp-1* mutant animals whose HSNs had been eliminated through laser ablation had more severe egg-laying defects, and responded less strongly to serotonin, than HSN-ablated wild-type animals. These results were perhaps surprising, since all the chains of synaptic connections between the *flp-1*-expressing neurons in the head and the vulval muscles involve the HSNs [1]. In fact, *flp-1* mutations only slightly enhanced the egg-laying defect of HSN-ablated animals, suggesting that some of the effects of the FLP-1 peptides are likely to be HSN-dependent. Thus, the FLP-1 peptides may regulate the egg-laying muscles both through modulation of the HSNs as well as through an HSN-independent humoral mechanism (Figure 2-7).

Many questions remain about the cellular mechanism through which FLP-1 peptides regulate egg-laying. *flp-1* expression has been detected in a number of neurons in the head, including AIA, AIY, AVA, AVK, AVE, RIG, and RMG [98]. Based on the results presented here, the simplest hypothesis is that humoral release of FLP-1 peptides from one or more of these neuronal classes modulates the egg-laying muscles directly. Alternatively, it is possible that some or all of the effects of the FLP-1 peptides on egg-laying could be indirect. For example, FLP-1 peptides could modulate the activity of other neurosecretory cells in the head, affecting the release of a hypothetical neurohormone that modulates the egg-laying muscles. Some of the effects of *flp-1* on egg-laying might also involve the VC neurons, although the fact that *flp-1* mutations lengthen the inactive phase much more than ablations of the VCs do [99]; L. Waggoner, unpublished) argues that the VCs are not the primary target of the FLP-1 peptides. Laser ablations of various combinations of *flp-1*-expressing neurons, as well as neurons

postsynaptic to these cells, may provide more detailed information about the cellular basis for the effect of *flp-1* on egg-laying behavior.

Interactions between *flp-1* and serotonin in the control of egg-laying

The effects of *flp-1* on egg-laying are quite similar to the effects of another neuromodulator, serotonin. We observed here that loss of *flp-1* function did not confer resistance to the effects of serotonin on egg-laying, though it did significantly reduce the magnitude of the serotonin response. Therefore, the *flp-1*-encoded peptides appear to stimulate egg-laying at least in part by enhancing the response of the egg-laying muscles to serotonin. This hypothesis is consistent with previously published work, which demonstrated that synthetic peptides identical to the shared carboxy-terminus of the 7 *flp-1* peptides (FLRF-NH_2) increased the average number of eggs laid in response to serotonin [24]. Serotonin and FLP-1 appear to function synergistically not only in the stimulation of egg-laying, but in other *C. elegans* behaviors as well. For example, both serotonin and FLP-1 inhibit locomotion, and the ability of serotonin to inhibit movement has been shown to require a functional *flp-1* gene [98]. Thus, for locomotive as well FLP-1 peptides appear to be necessary to potentiate the effects of serotonin on locomotion. Although the molecular pathways through which serotonin and *flp-1* control egg-laying are likely to differ in some respects from those involved in locomotion, it is tempting to speculate that the parallel actions of these two modulators on these two different behaviors might depend on a conserved molecular mechanism. Since comodulation of neuromuscular activity by biogenic amines and FaRPs is observed in many organisms [100, 101], the molecular interactions between the *flp-1* and serotonin-activated signaling pathways in the egg-laying cells may provide a useful model for similar processes in other animals.

Insights into the regulation of egg-laying by sensory information

The analysis of the *flp-1* mutants also revealed a role for the FLP-1 peptides in the control of egg-laying behavior by sensory cues. We observed that whereas wild-type worms laid eggs at a much slower rate in the absence of a bacterial food source, *flp-1* loss-of-function mutants laid eggs at the same rate in either presence or absence of food. This insensitivity to the presence of bacteria was not merely a consequence of the *flp-1* animals' generally slower egg-laying rate, as other egg-laying defective animals (e.g., *egl-1* mutants which lacked the HSN motorneurons) still showed significant regulation of egg-laying by food availability. Therefore, the *flp-1*-encoded peptides may be specifically dedicated to relaying signals of food abundance to the egg-laying circuit. In the absence of bacteria, levels of FLP-1 release could be low, leading to long inactive phases and slow egg-laying, whereas abundant food would lead to increased FLP-1 release and more active egg-laying. Other aspects of the *flp-1* mutant phenotype are consistent with FLP-1 functioning as an indicator of food availability. For example, when nematodes, maintained in the absence of food, encounter a lawn of bacteria, they slow their rate of movement [91]. Both the hyperactive locomotion and the "wandering" behavior previously noted in *flp-1* recessive mutants [98] could plausibly stem from a defect in this response to food. Thus, *flp-1* may function quite generally to facilitate a variety of behavioral patterns that are appropriate for conditions of food abundance.

The expression pattern of *flp-1* in the *C. elegans* nervous system is well suited for a gene that encodes a food signal. The presence of bacteria in the environment is thought to be detected primarily through olfactory or chemosensory cues [62]. The primary route through which nematodes gather chemosensory information is by using a pair of polymodal sense organs known as amphids. Synaptic output from the amphid sensory neurons is relayed to four pairs of amphid interneurons: AIA, AIB, AIY and AIZ [1]. In

thermotaxis behavior, the amphid interneurons have been shown to be an important site for integrating and processing sensory information that is used to modulate behavioral outputs [102]. Both AIA and AIY express *flp-1*; thus a simple model for how egg-laying behavior could be controlled by food signals is that under conditions favorable to egg-laying (i.e., abundant food), the AIA and AIY neurons release FLP-1 peptides, switching the animal into the active egg-laying state. This release of FLP-1 peptides from the amphid interneurons could likewise switch the animal into a more inactive state with respect to locomotion.

Evidence that *goa-1* modulates neural states involved in egg-laying behavior

In addition to the pathways activated by FLP-1 and serotonin, a third pathway, defined genetically by the *goa-1* gene, also appears to regulate the onset of the active phase of egg-laying, in a manner antagonistic to and apparently independent of both *flp-1* and serotonin. A recessive mutation in *goa-1*, which encodes the *C. elegans* homologue of the G_o alpha subunit [18, 30], increased the rate of egg-laying by shortening the inactive phase. Genetic analysis indicated that *goa-1* probably functions to regulate egg-laying in a pathway distinct from the ones activated by *flp-1* and serotonin. Interestingly, the effect of *goa-1* on the onset of the active phase appeared to be completely dependent on the HSNs. Thus, *goa-1* may function by negatively regulating release from the HSNs of a neuromodulator that facilitates the switch from the inactive to the active egg-laying state.

An interesting implication of this hypothesis is that the behavioral states involved in egg-laying may correspond not only to functional states of the egg-laying muscles themselves, but also to distinct functional states of neurons (such as the HSNs) dedicated to egg-laying. Our earlier studies led to the hypothesis that the active egg-laying state

depends on a functional activation of the vulval muscles, which allows the excitatory transmitter acetylcholine to readily induce muscle contraction. Our analysis of *goa-1* mutants suggests that the active and inactive egg-laying states may also correspond to functional states of the HSNs--an inactive state in which the HSNs release neurotransmitter with low probability, and an active state in which the probability of neurotransmitter release is high. According to this model, activated GOA-1 may inhibit the switch of the HSNs into this active state; thus, when GOA-1 is inactive or absent, the switch into the active state becomes more frequent. The likely involvement of GOA-1 in controlling HSN activity implies that additional neuromodulators, possibly released from neurons in the head, may regulate egg-laying behavior by controlling GOA-1 activity.

Regulation of egg-laying muscle activity by multiple neuromodulators

Although the effect of *goa-1* on the switch into the active phase was dependent on the HSNs, it was not completely dependent on serotonin. These observations imply that the HSNs contain another neuromodulator, whose release may be regulated by GOA-1, that facilitates the switch into the active phase. Although at present we can only speculate as to the identity of such a molecule, there appear to be a number of candidates. For example, the HSNs almost certainly contain one or more non-*flp-1* encoded FaRPs, since they contain FaRP immunoreactivity which is not eliminated by deletions of the *flp-1* gene [24, 98]. In addition, pharmacological experiments indicate that muscarinic acetylcholine agonists stimulate egg-laying [64]. Since the HSNs are cholinergic, acetylcholine released from the HSNs might modulate egg-laying through muscarinic receptors. In principle, GOA-1 regulated release of any of these molecules could facilitate the onset of the active egg-laying state. The diversity of neurotransmitter usage in the HSNs is also a hallmark of other neurons and gland cells that participate in the control of egg-laying. For example, non-*flp-1*-encoded FaRPs are present in both the VC

motorneurons and the uv1 uterine gland cells [24]. In addition, the VCs contain acetylcholine and, since they express the vesicular monoamine transporter, possibly an unidentified biogenic amine as well [103]. Thus, egg-laying behavior is likely to be regulated by a surprisingly diverse array of neurotransmitters and neuromodulators, which are likely to activate complex, interacting signaling pathways in the vulval muscle cells. The elucidation of these signaling mechanisms represents an important challenge for future studies, and may be a useful model for the functional interaction of neuromodulatory pathways in other organisms.

METHODS

Strains and Genetic Methods

Routine culturing of *Caenorhabditis elegans* was performed as described [104]. The chromosomal locations of the genes studied in these experiments are as follows: LGI: *goa-1;* LGIV: *flp-1* ; LGV: *cat-4, egl-1.* Unless otherwise indicated, all mutant strains are in the N2 genetic background. Behavioral assays were performed at room temperature (approximately 22^{o} C). Serotonin (creatinine sulfate complex) was obtained from Sigma. The *flp-1(yn2)* allele was chosen for use in double mutant constructions because its phenotype and behavior in genetic crosses suggests that it causes a more severe loss of gene function [98]. *goa-1(n1134)* was used for behavioral analysis and double mutant construction because it encodes a putatively non-functional product, and because the near sterility conferred by the *n363* deficiency allele (brood size is approximately 35 [18]) makes embryo production rather than egg-laying muscle contraction limiting for egg-laying in *n363* mutant animals.

Egg-laying Assays

Unless otherwise stated, nematodes were grown and assayed at room temperature on standard NGM seeded with *E. coli* strain OP50 as a food source. For dose response experiments, individual young, gravid hermaphrodites were placed in microtiter wells containing liquid M9 and the indicated concentration of drug. After a 1 hour incubation at room temperature, the eggs laid by each animal were counted. Experiments measuring egg-laying rate on standard growth medium were performed as described above, and after 1 hour, the eggs laid by each animals were counted. Plates in which the animal had crawled off the agar surface before the end of the assay period were not included in the analysis.

Egg-laying behavior of individual animals on solid media (NGM agar) was recorded for 4-8 hours as described [99] using an automated tracking system. For tracking experiments on serotonin, 5-hydroxytryptamine (creatinine sulfate complex, Sigma) was added to NGM agar at 7.5 mM. Our tracking system was unable to record the behavior of animals on plates lacking a bacterial lawn, since the animals were prone to crawl to the edge of the plate where our system could not follow them.

Analysis of egg-laying patterns

Intervals between egg-laying events were determined from analysis of videotapes obtained using the automated tracking system. Quantitative analysis of the egg-laying pattern using this interval data was performed as described [67]. Briefly, egg-laying events in *C. elegans* are clustered, with periods of active egg-laying, or active phases, separated by long inactive phases during which eggs are retained. Both the duration of the inactive phases ("inter-cluster intervals") and the duration of intervals between egg-laying events in a cluster ("intra-cluster intervals") model as exponential random variables with different time constants [99]. Thus, the probability density function for the intervals

between events is

$$f_X(x) = k_1\ \lambda_1\ e^{-\lambda_1 x} + k_2\ (p\lambda_2)\ e^{-(p\lambda_2)x}, \quad x \geq 0,$$

$$k_1 = \frac{p(\lambda_1 - \lambda_2)}{\lambda_1 - p\lambda_2}, \qquad k_2 = \frac{\lambda_1(1-p)}{\lambda_1 - p\lambda_2}.$$

, where the intra-cluster time constant is $1/\lambda_1$ and the inter-cluster time constant is $1/p\lambda_2$. The parameters were determined using the maximum likelihood estimation technique described previously [67]. The expected variance of estimated parameters and time constants was determined by generating 100 independent sets of simulated egg-laying data using the model probability density function, and computing the standard deviation of the parameters estimated from these simulations. All data in Table 2-1 were obtained and analyzed in this manner.

For analysis of egg-laying patterns on serotonin (Table 2-2), a single exponential time constant was estimated using a weighted least-squares linear regression to the log tail distribution [99]. The expected variance of these time constants was determined by generating 100 independent sets of simulated egg-laying data using a simple exponential probability density function, and by computing the standard deviation of the parameters estimated from these simulations.

Construction of Double Mutant Strains

For *flp-1; cat-4* double mutants, *cat-4* and *flp-1* single mutants were mated, and double mutant progeny were identified in the F2 generation by scoring for bleach sensitivity (*cat-4*[71]) and the presence of a diagnostic PCR product using sequence specific primers (*flp-1* [98]). For the *goa-1; cat-4* and *goa-1; flp-1* double mutants, single mutants were crossed as above, and *goa-1* homozygotes were identified in the F2 generation as hyperactive, egg-laying constitutive animals. These were picked individually and then allowed to self-

fertilize; those F2s that were heterozygous for *cat-4* or *flp-1* segregated double mutant progeny that could be identified using the bleach or PCR assays described above.

This chapter is, in full, a reprint of material in L. E. Waggoner et al. (1999) "The effect of a neuropeptide, *flp-1*, on egg-laying in *C. elegans*" *(in press)*. The dissertation author was the primary investigator and author of this paper.

Table 2-1. Egg-laying behavior of mutant and ablated animals.

Animal type (#, hrs, intervals)	p	λ_1 (s^{-1})	λ_2 ($s^{-1} \times 10^{-3}$)	Intra-cluster time constant ($1/\lambda_1$; s)	Inter-cluster time constant ($1/p\lambda_2$; s)
N2	0.537	0.055	1.4	18	1340
(12, 61, 305)	±0.031	±0.005	±0.18	±2	±140
flp-1(yn2)	0.554	0..079	0.47	13	3820**
(6, 37, 50)	±0.086	±0.022	±0.20	±3	±1170
flp-1(yn4)	0.651	0.090	0.75	11	2050*
(8, 37, 131)	±0.049	±0.016	±0.20	±2	±460
cat-4(e1141)	0.609	0.023	0.36	43	4550**
(12, 52, 63)	±0.073	±0.007	±0.13	±9	±1240
flp-1(yn2); cat-4(e1141)	0.772	0.063	0.30	16	4320
(7, 35, 60)	±0.058	±0.014	±0.15	±3	±1520
goa-1(n1134)	0.357	0.028	3.1	36	890**
(3, 27, 98)	±.055	±.009	±0.81	±16	±160
goa-1(n1134); flp-1(yn2)	0.377	0.174	1.4	6	1950*
(6, 23, 66)	±0.069	±0.138	±0.38	±2	±420
goa-1(n1134); cat-4(e1141)	0.515	0.141	1.4	7	1380*
(6, 32, 101)	±0.056	±0.038	±0.30	±1	±270
N2; HSN-ablated	0.534	0.125	0.55	8	3400**
(9, 44, 66)	±0.069	±0.019	±0.16	±2	±900
flp-1(yn2); HSN-ablated	0.382	0.041	1.1	25	2440
(5, 54, 77)	±0.068	±0.018	±0.29	±8	±440
goa-1(n1134); HSN-ablated	0.720	0.072	0.27	14	5140
(4, 24, 51)	±0.062	±0.020	±0.10	±3	±2060

*/** Long intervals (> 120s) statistically different either from wild-type (for single mutants) or from both constituent single mutants (for double mutants) according to the Mann-Whitney rank test. * indicates significance at .05>p>.01; ** indicates significance at $p < .01$ or less.

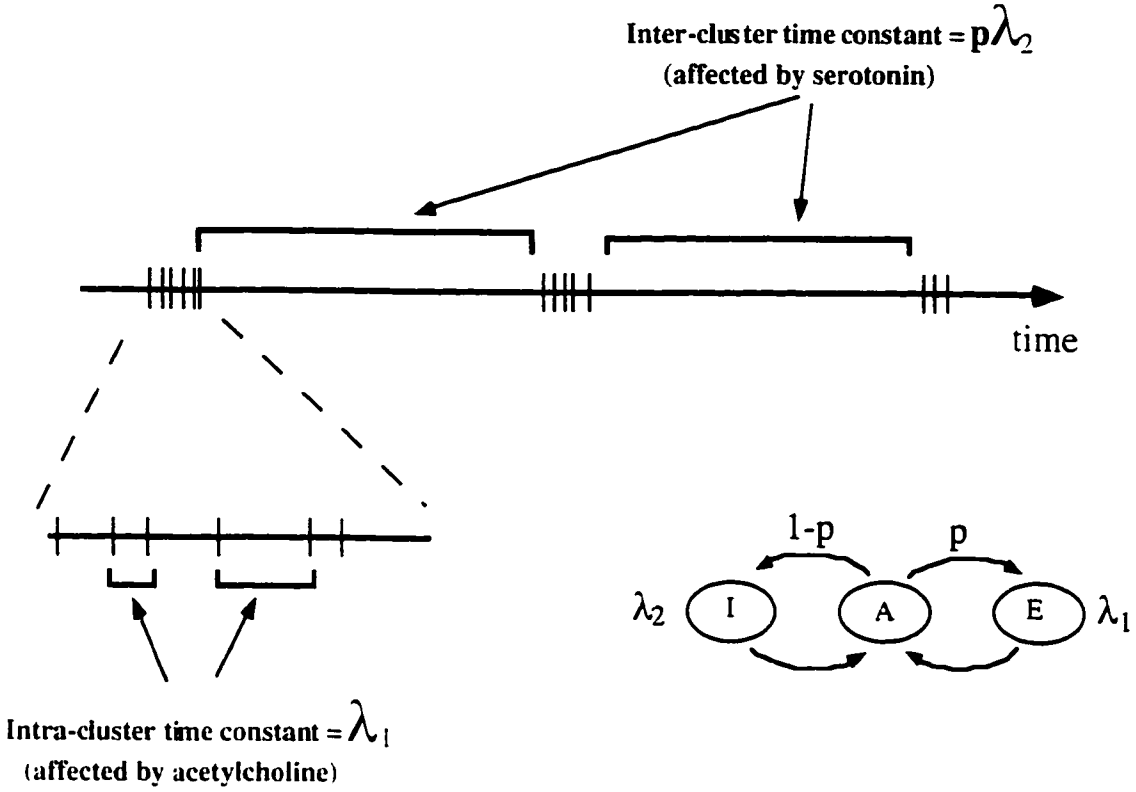


Figure 2-1a. Temporal pattern of egg-laying. A more detailed description of the egg-laying pattern of *C. elegans* and its analysis is described in [99]. Shown is a representative egg-laying pattern, with egg-laying events indicated by hash marks. According to the model, the animal can exist in one of three states: an inactive state (I), an active state (A), and an egg-laying state (E). Eggs are laid upon entry into the E state. λ_1 is the rate constant for the egg-laying state, λ_2 is the rate constant for the inactive state, and p is the probability that after a given egg-laying event, another egg will be laid before the animal enters the inactive phase. Short intervals (resulting from a single visit to the A state) are governed by the time constant $1/\lambda_1$. Long intervals result from one or more visits to the I (inactive) state (i.e., from E to A to I to A to E, or from E to A to I to A to I to A to E, etc.); the time constant for these intervals is equal to $1/p\lambda_2$ (for proof, see [67]).

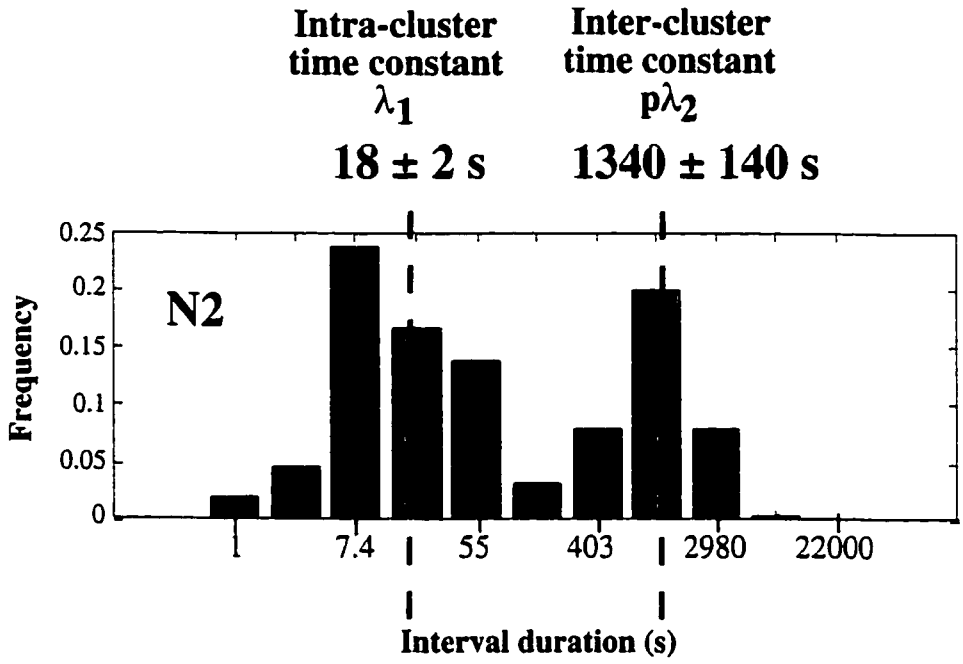


Figure 2-1b. Histogram of log interval times. Shown is a histogram of intervals between egg-laying events recorded from wild-type animals; the bins are on a log scale. According to the coupled-Poisson process model, the location of the two peaks corresponds to the intra-cluster and inter-cluster time constants. Values for these time constants were estimated using maximum likelihood analysis (see Methods).

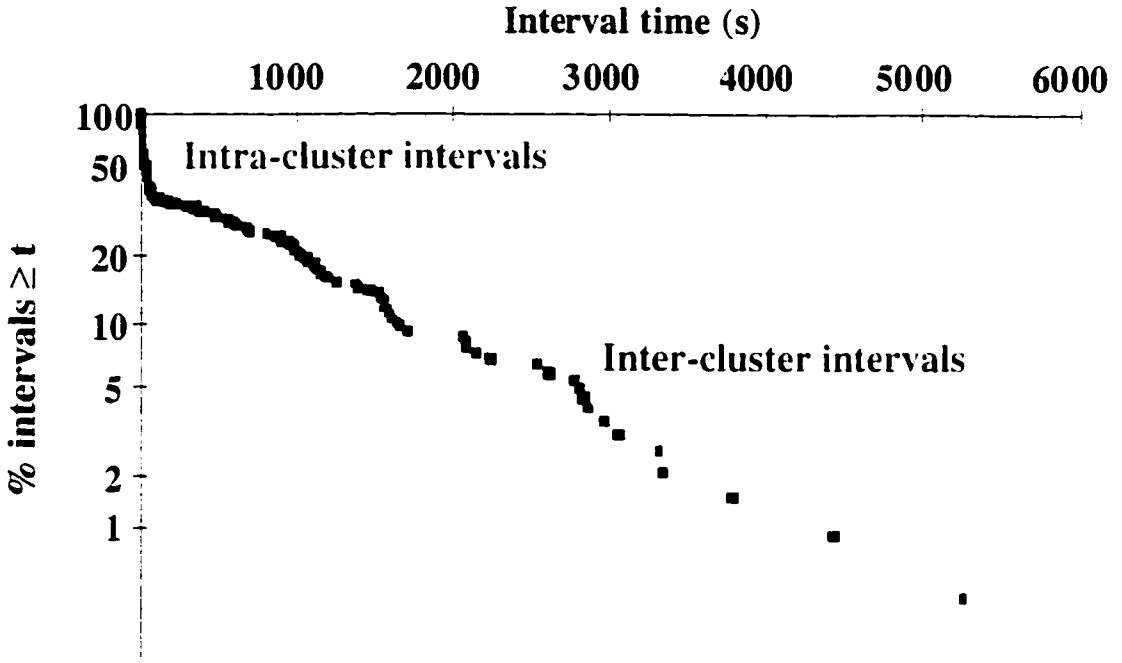
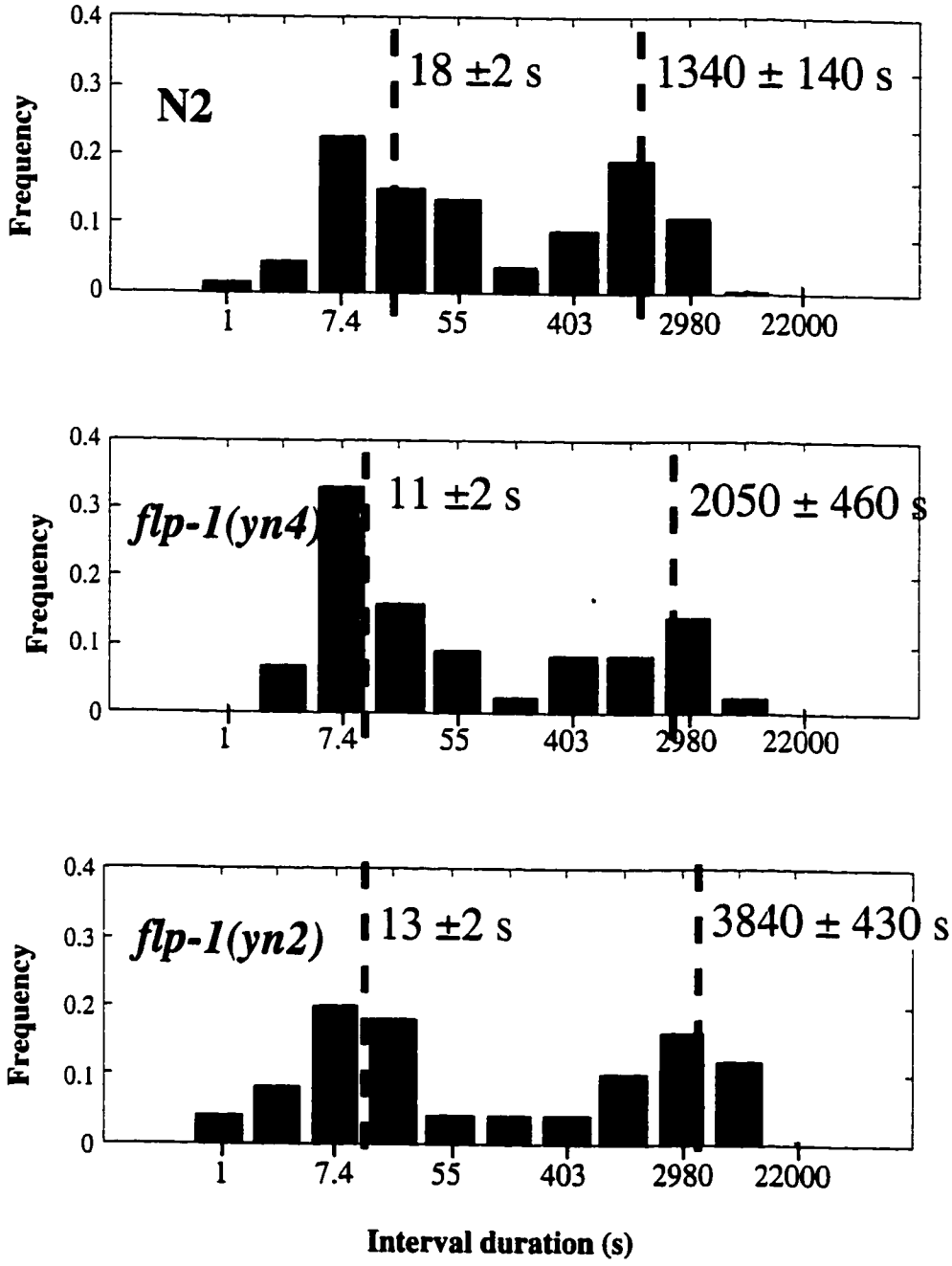


Figure 2-1c. Log-tail distribution of egg-laying intervals. The distribution of egg-laying intervals from wild-type animals is shown. The model predicts that this distribution should be biphasic, with the steep part of the curve corresponding to the intra-cluster intervals and the more gradual part to the inter-cluster intervals. The slope of the long intervals (i.e., the right part of the curve) is equal to the inter-cluster rate constant.

Figure 2-2a. Effect of *flp-1* recessive mutations on the pattern of egg-laying.

flp-1 loss-of-function mutations increase the duration of the inactive phase. For these and other tracking experiments, the numbers of animals tracked, hours observed, and intervals analyzed, along with the estimated model egg-laying parameters, are in Table 2-1. Shown is a histogram of intervals between egg-laying events (log scale); intra-cluster and intra-cluster time constants estimated using maximum likelihood analysis are indicated. Both the *yn2* and *yn4* alleles of *flp-1* specifically lengthen the inter-cluster constant (indicated by the location of the right-most peak). The long intervals (>120 s) were significantly longer in *yn2* ($p < .001$) and *yn4* ($p < .05$) according to the Mann-Whitney rank sum test.


N2
18 ±2 s
1340 ± 140 s
flp-1(yn4)
11 ±2 s
2050 ± 460 s
flp-1(yn2)
13 ±2 s
3840 ± 430 s
Frequency
0.4
0.3
0.2
0.1
0
1
7.4
55
403
2980
22000
Interval duration (s)

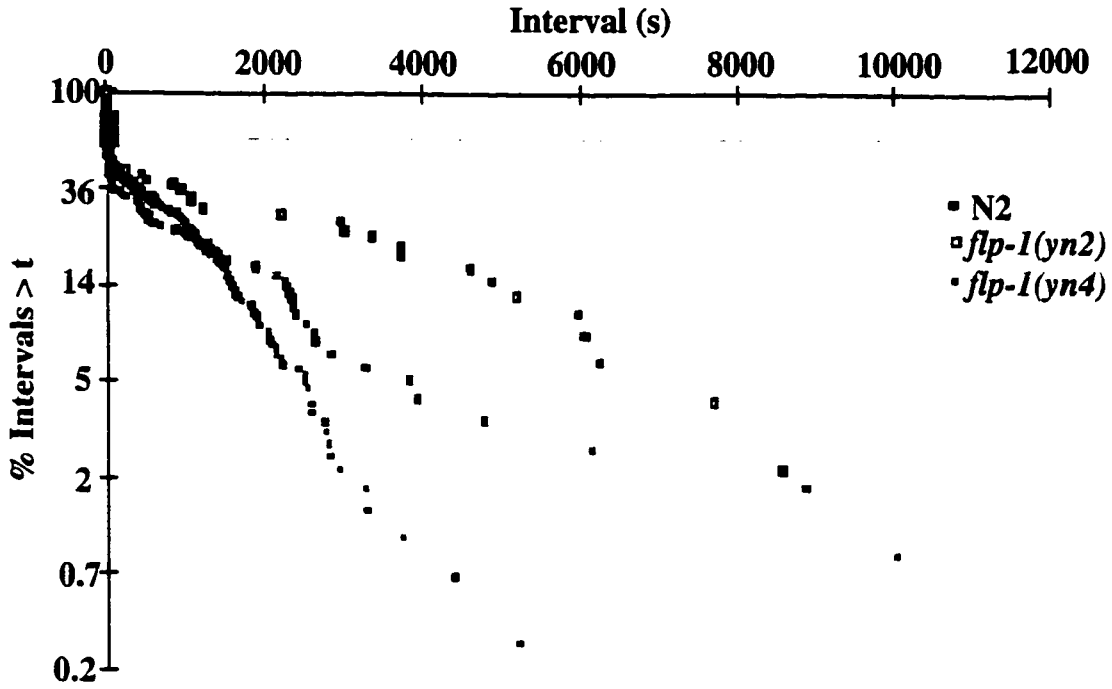


Figure 2-2b. Effect of *flp-1* recessive mutations on inactive phase duration. Shown are the log tail distributions of egg-laying intervals for wild-type and *flp-1* mutant animals. The more gradual slopes of the *yn2* and *yn4* curves indicate longer inter-cluster time constants for these mutants.

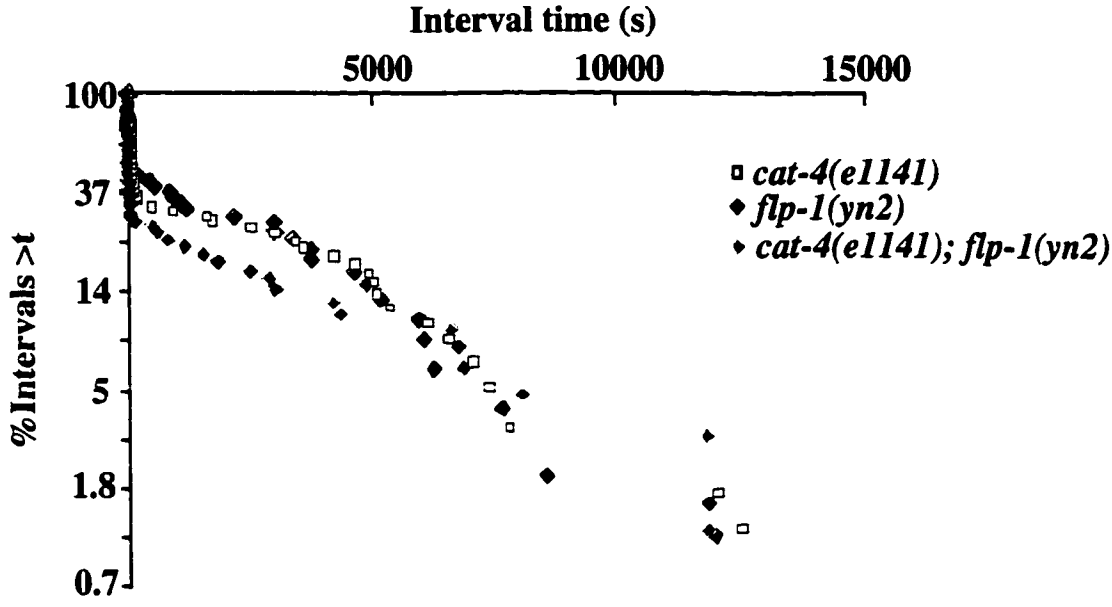


Figure 2-3a. Egg-laying pattern of serotonin-deficient *flp-1* animals. Shown are log tail distributions of egg-laying interval times for *flp-1*, *cat-4* , and *flp-1; cat-4* double mutant animals. The inter-cluster time constants (indicated by the curve tails) were essentially identical (see Table 2-1). Egg-laying rates for each strain were: *flp-1* = 1.94 ± 0.50 (n=6), *cat-4* = 2.35 ± 0.40 (n=12) , *flp-1; cat-4* = 3.03 ± 0.26 (n=7).

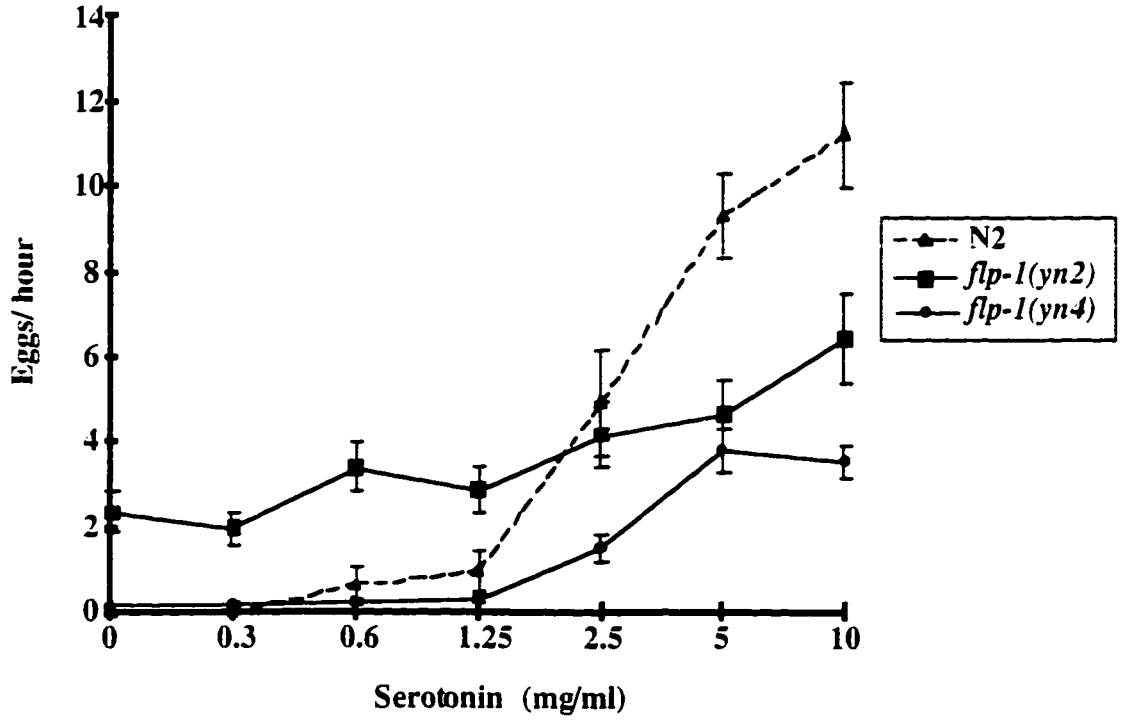
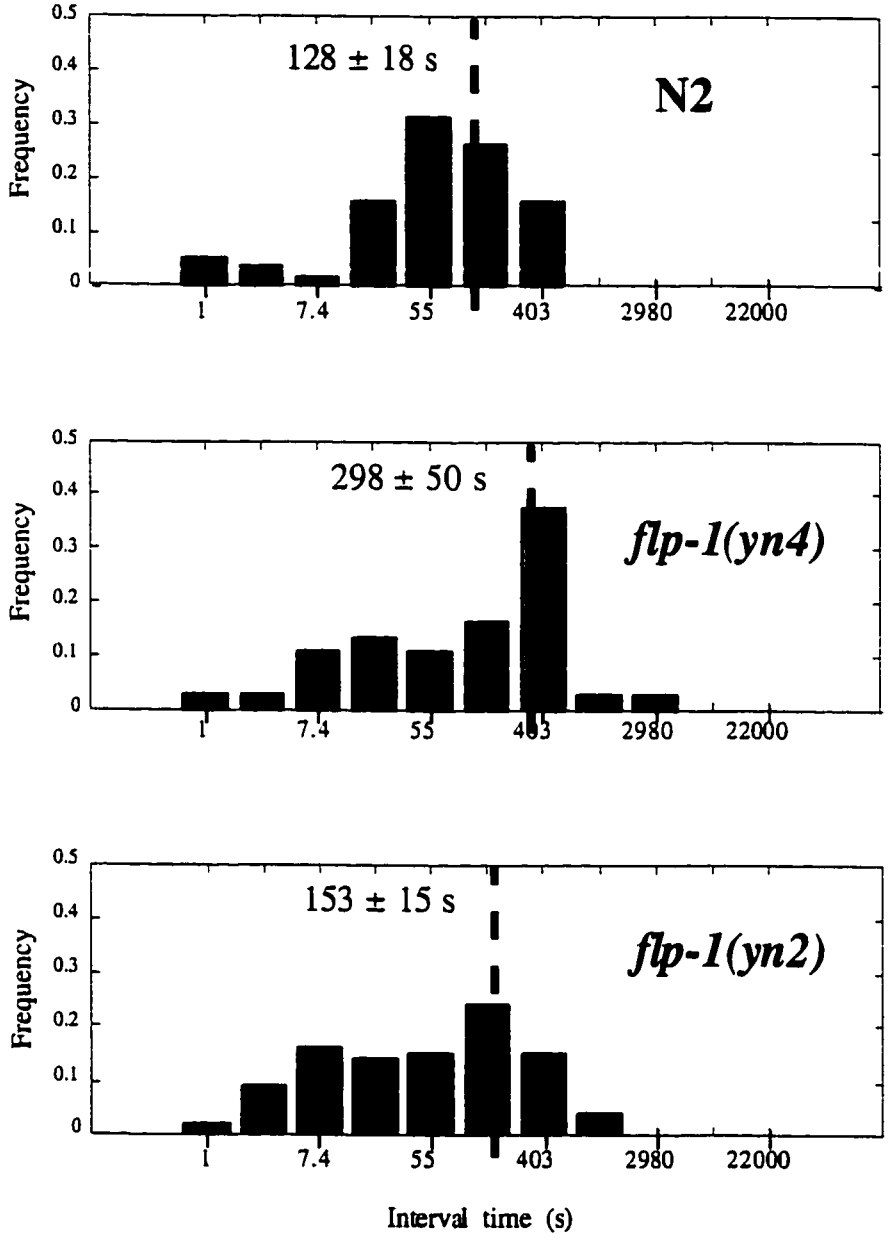


Figure 2-3b. Serotonin response of *flp-1* mutants. Egg-laying responses to serotonin were determined for wild-type and *flp-1* mutants by placing individual animals in liquid M9 at the indicated concentration of serotonin, and counting the number of eggs laid by each animal after 1 hour. Individual points and error bars indicate the mean and SEM of the following numbers of trials: N2= 20, *yn2*= 58, *yn4*= 45. For both *flp-1* mutants, the number of eggs laid at 10 mg/ml was significantly higher than in the no drug control ($p < .001$), and significantly lower than in wild-type at the same concentration ($p < .002$) according to the Mann-Whitney rank sum test.

Figure 2-3c. Egg-laying patterns of *flp-1* mutants on serotonin.

The histograms show the distribution of interval times for wild-type and *flp-1* mutants on 7.5 mM serotonin; bins are on a log scale. A single exponential time constant for each data set (indicated by the dashed line) was estimated as described in Methods. Intervals for *flp-1(yn4)* were significantly ($p < .05$) longer than for wild-type according to the Mann-Whitney rank sum test.


128 ± 18 s
N2
298 ± 50 s
flp-1(yn4)
153 ± 15 s
flp-1(yn2)
Frequency
0.5
0.4
0.3
0.2
0.1
0
1
7.4
55
403
2980
22000
Interval time (s)

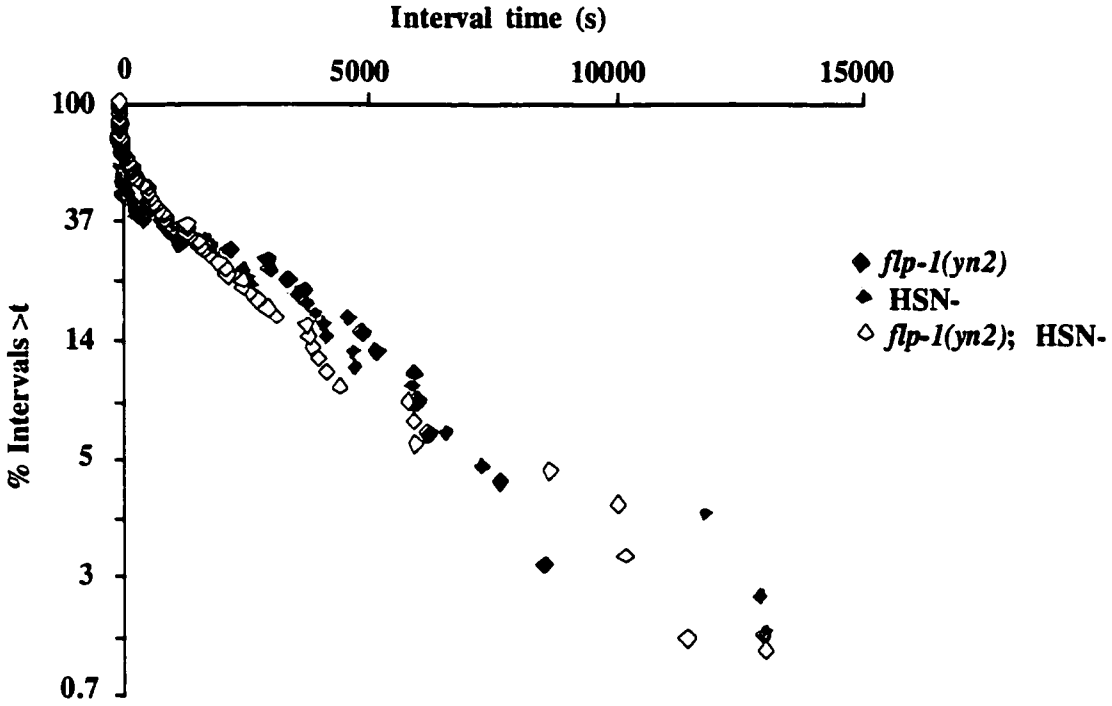


Figure 2-4a. Egg-laying pattern of HSN-ablated *flp-1* animals. Shown are the log tail distributions of egg-laying interval times for HSN-ablated wild-type and *flp-* mutant animals. The inter-cluster time constant (indicated by the slope of the curve tails) was no higher in the ablated mutant animals than in ablated wild-type or unablated mutant animals (see Table 2-1). Egg-laying rates were also measured independently on NGM agar by counting eggs laid by individual animals in an hour; mean and SEM of these experiments were (in eggs/hr): HSN-ablated wild-type= 1.35 ± 0.25 (n=4), unablated *flp-1*= 1.94 ± 0.50 (n=6), HSN-ablated *flp-1*= 1.05± 0.41 (n=11).

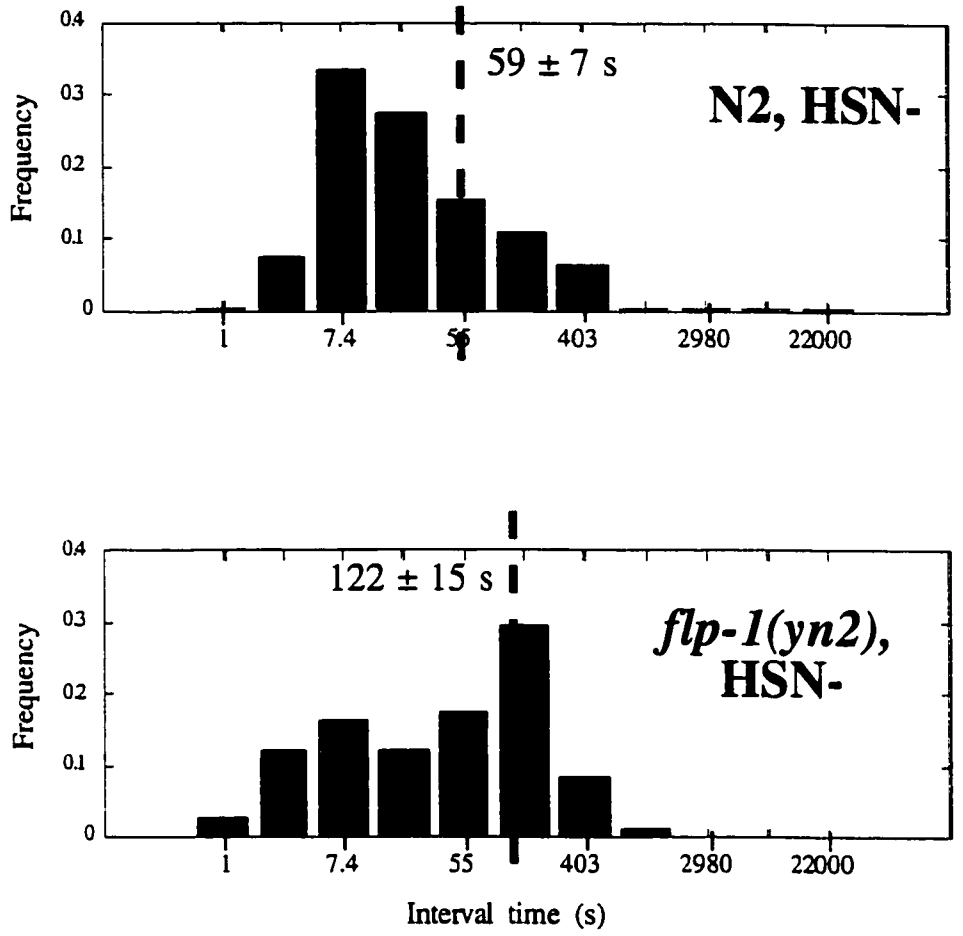


Figure 2-4b. Egg-laying pattern of HSN-ablated *flp-1* mutants on serotonin. The histograms show the distribution of interval times for HSN-ablated wild-type and *flp-1* mutants on 7.5 mM serotonin; bins are on a log scale. A single exponential time constant for each data set was estimated as described in Methods. Intervals for HSN-ablated *flp-1(yn2)* were significantly ($p < .001$) longer than for HSN-ablated wild-type according to the Mann-Whitney rank sum test.

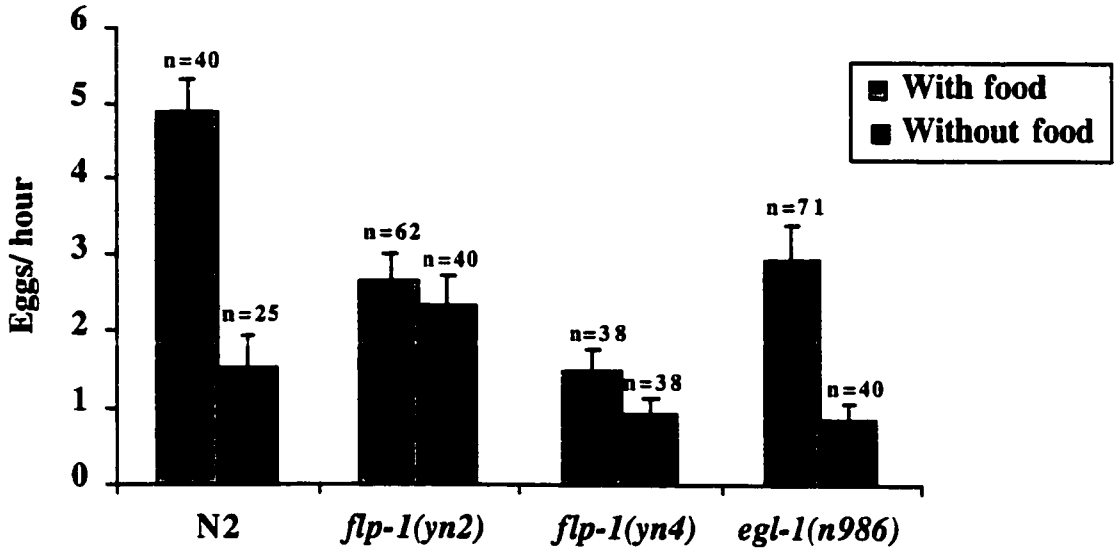


Figure 2-5. Effect of *flp-1* mutations on regulation of egg-laying by food. The histogram shows the mean egg-laying rates of wild-type, *egl-1* and *flp-1* mutant animals in the presence and absence of food. Error bars indicate the SEM; the number of independent trials under each condition are indicated. The wild-type and *egl-1* mutant strains showed a significantly lower ($p < .001$ for wild-type, $p < .02$ for *egl-1*) egg-laying rate in the absence of food according to the Mann-Whitney rank sum test. In contrast, there was no statistically significant difference between the egg-laying rates in the presence or absence of food for either *flp-1* mutant ($p > .5$ for *yn2*, $p > .2$ for *yn4*).

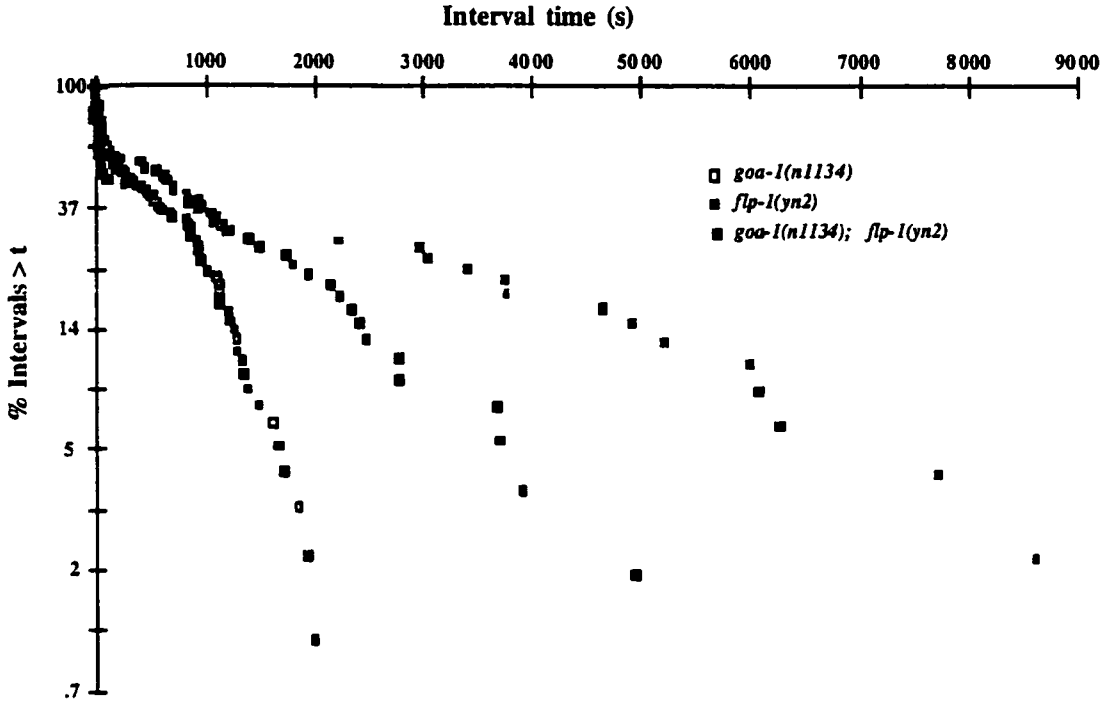


Figure 2-6a. Independence of *flp-1* and *goa-1* egg-laying phenotypes. Shown are the log tail distributions of egg-laying intervals for *flp-1* and *goa-1* mutant animals. The inter-cluster time constant of the *goa-1; flp-1* double mutant curve (indicated by the slope of the curve) is intermediate between that of the *flp-1* and *goa-1* single mutants (see Table 2-1). The long intervals (> 120s) in the double mutant were significantly shorter than in the *flp-1* single mutant ($p < .05$) and significantly longer than in the *goa-1* single mutant ($p < .002$) according to the Mann-Whitney rank sum test.

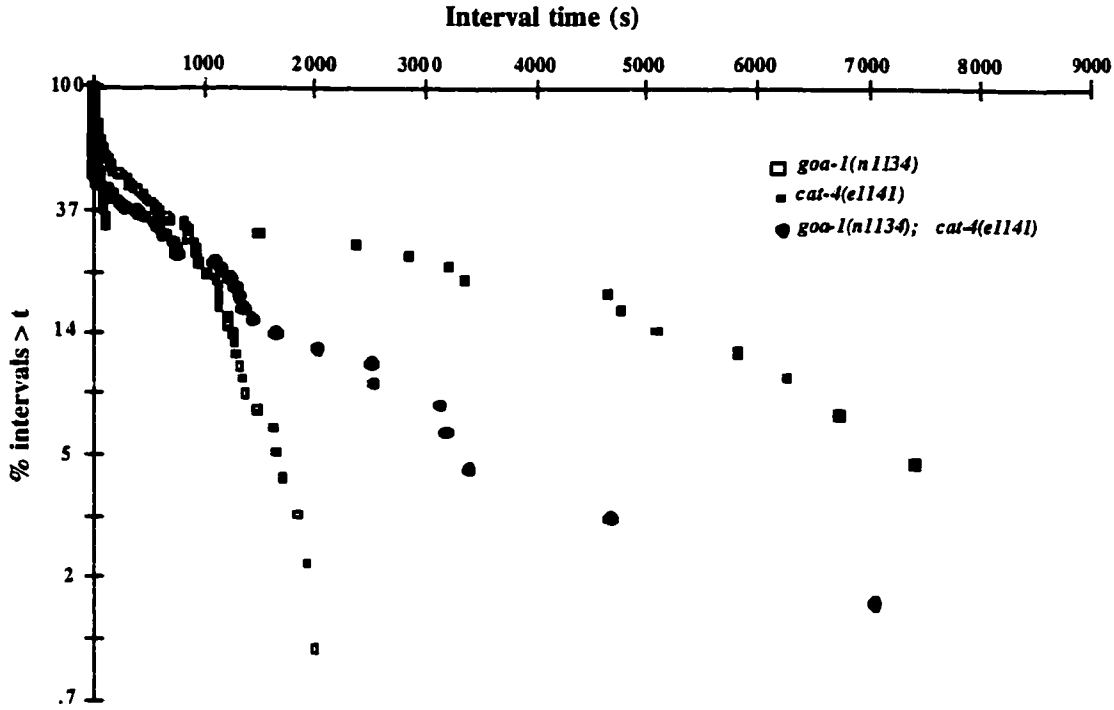


Figure 2-6b. Independence of *goa-1* and *cat-4* egg-laying phenotypes. Shown are the log tail distributions of egg-laying intervals for *cat-4* and *goa-1* mutant animals. The inter-cluster time constant of the *goa-1; cat-4* double mutant curve (indicated by the slope of the curve) is intermediate between that of the *cat-4* and *goa-1* single mutants. The long intervals (> 120s) in the double mutant were significantly shorter than in the *cat-4* single mutant ($p < .001$) and significantly longer than in the *goa-1* single mutant ($p < .02$) according to the Mann-Whitney rank sum test.

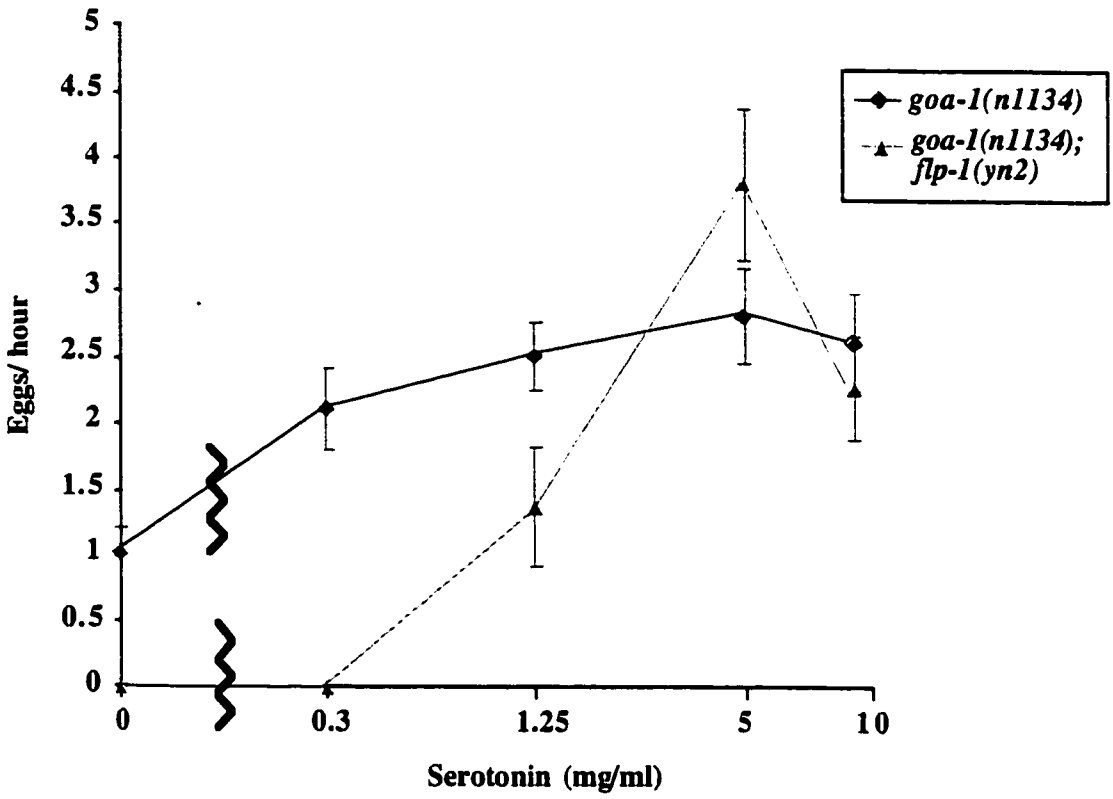


Figure 2-6c. Serotonin responses of *goa-1* mutants. Egg-laying responses to serotonin were determined for *goa-1* and *goa-1; flp-1* mutants as described in Figure 2-3. Individual points and error bars indicate the mean and SEM of the following numbers of trials: *goa-1(n1134)*= 40, *goa-1(n1134); flp-1(yn2)*= 25.

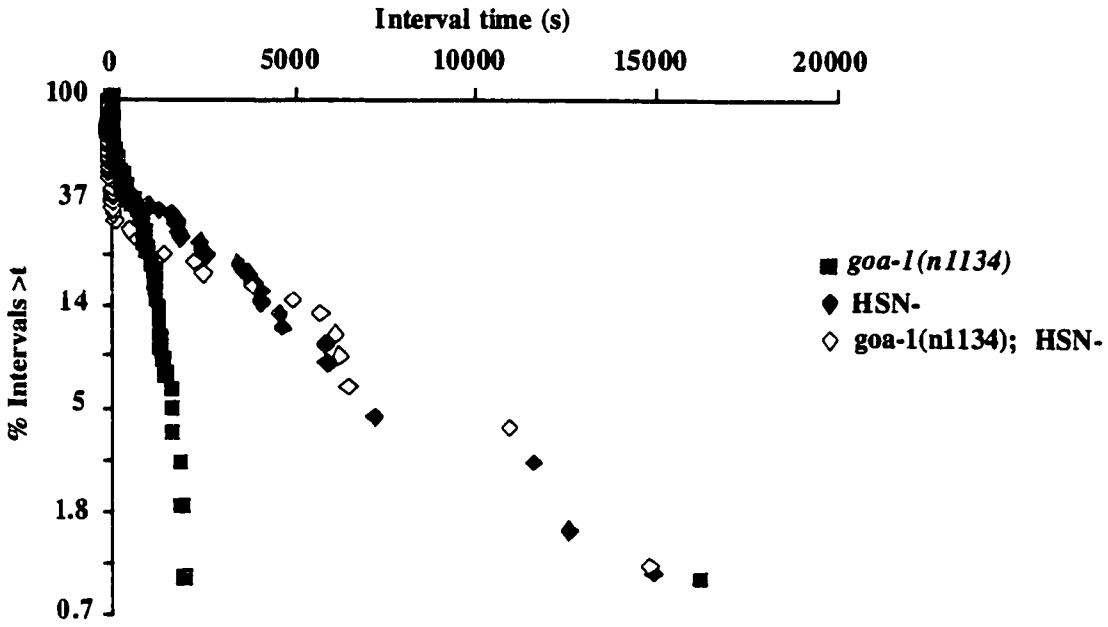


Figure 2-6d. HSN-dependence of the *goa-1* hyperactive egg-laying phenotype. Shown are the log tail distributions of egg-laying intervals for HSN-ablated and *goa-1* mutant animals. The inter-cluster time constant of the HSN-ablated *goa-1* mutant curve (indicated by the slope of the curve) is as long or longer than that of HSN-ablated wild-type.

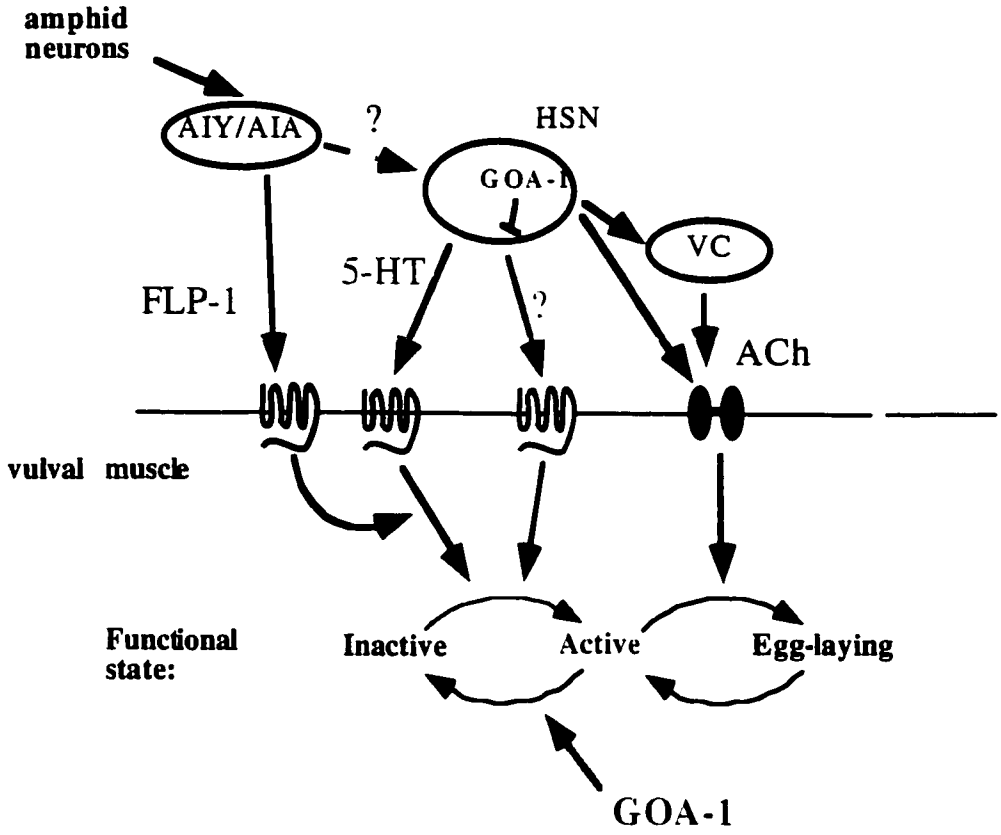


Figure 2-7. Model for neural and molecular regulation of egg-laying. Egg-laying in *C. elegans* is affected by at least two parameters: the rate of switching from the inactive to the active phase, and the rate of egg-laying within the active phase. FLP-1 peptides, released from neurons in the head, specifically regulate the switch into the active phase, a process also regulated by serotonin and possibly another neuromodulator whose release from the HSNs is controlled by GOA-1. The FLP-1 peptides may also modulate the activity of HSNs. The activity of the FLP-1 pathway may be sensitive to chemosensory cues indicating the abundance of food.

CHAPTER IV LONG TERM NICOTINE ADAPTATION OF EGG-LAYING BEHAVIOR IN *C. ELEGANS*: INVOLVEMENT OF NEURONAL NICOTINIC RECEPTORS AND PROTEIN KINASE C

ABSTRACT

Chronic exposure to nicotine leads to the long-term functional inactivation of nicotinic acetylcholine receptors (nAChRs), a process thought to contribute to nicotine addiction. We have found that in *C. elegans*, prolonged nicotine treatment results in long-term adaptation of nicotinic receptors that control egg-laying. In naive animals, acute exposure to cholinergic agonists led to the efficient stimulation of egg-laying, a response mediated by a nicotinic receptor functionally expressed in both neurons and muscle cells. Overnight exposure to nicotine led to a specific and long-lasting change in egg-laying behavior, which rendered the nicotine-adapted animals insensitive to simulation of egg-laying by nicotinic agonist. Mutant animals defective in the gene *tpa-1*, which encodes a homologue of protein kinase C (PKC), failed to undergo adaptation to nicotine, and remained sensitive to cholinergic agonists even after long nicotine exposure. Long treatments with phorbol esters also inhibited the acute response to cholinergic agonists in a *tpa-1* dependent manner. These results suggest that nicotine adaptation may involve long-term inactivation of nicotinic receptors by PKC.

INTRODUCTION

After prolonged exposure to nicotine or other agonists, nicotinic acetylcholine receptors undergo a significant and long-term reduction in activity, a process (or processes) known as functional inactivation. Functional inactivation is qualitatively and mechanistically distinct from classically defined nAChR desensitization; while nAChR desensitization occurs with rapid kinetics and reverses within minutes in the absence of drug, functional inactivation has a much slower time course of induction, and appears to reverse extremely slowly, if at all [105, 106]. Although desensitization is a consequence of the allosteric properties of the receptor itself, functional inactivation is thought to involve the action of additional regulatory molecules. Despite the likely importance of nicotinic receptor inactivation to nicotine tolerance and dependence, the molecular mechanisms underlying this process remain poorly understood.

One way to investigate the mechanisms underlying nicotinic receptor inactivation is to use a genetically tractable animal such as *Caenorhabditis elegans*. *C. elegans* has a simple, well-characterized nervous system and is well-suited for investigating how specific neurotransmitters, receptors, and signaling molecules function within the context of the nervous system to produce behavior. *C. elegans* contains a diverse family of nicotinic receptors [107-109], including both neuromuscular and neuronal receptor subtypes [11-13]. Moreover, nicotinic receptor agonists have specific and easily assayed effects on several aspects of *C. elegans* behavior, including locomotion, feeding, and egg-laying[5, 10, 57]. A number of paradigms for behavioral plasticity have been defined in *C. elegans* [19, 110-113], demonstrating that these animals are capable of at least simple forms of learning. Thus, it seemed reasonable to ask whether chronic exposure to nicotine resulted in long-term adaptation in *C. elegans*, and if so, to attempt to identify molecules required for this process.

Here we report egg-laying behavior in *C. elegans* is indeed modified in a specific and persistent manner by long-term nicotine treatment. In particular, we have found that the acute stimulation of egg-laying by nicotinic agonists, which is mediated by nicotinic receptors that function in both neurons and muscle cells, is stably inactivated by chronic exposure to nicotine. Intriguingly, we have found that this long-term adaptation to nicotine is dependent on the gene *tpa-1*, which encodes a specific form of protein kinase C.

RESULTS

Both nicotine and the more specific nicotinic agonist levamisole have dramatic effects on nematode egg-laying behavior. For example, both nicotine and levamisole stimulate egg-laying in hypertonic liquid medium (M9), a condition that normally inhibits egg-laying [57, 64]. To identify the receptor that mediates this response, we assayed the effect of these drugs on the egg-laying behavior of mutants known to be defective in specific nAChR subunit proteins. Three genes have been identified that appear to encode subunits of the levamisole-sensitive nAChR in body muscle: *unc-29*, *lev-1*, which encode putative non-α subunits, and *unc-38*, which encodes a putative α subunit [11]. We observed that mutants defective in either *unc-29*, *unc-38*, or *lev-1* showed little or no response to levamisole (Figure 3-1a,b,c). *unc-29* mutants also were largely insensitive to stimulation of egg-laying by acute nicotine treatment (Figure 3-1d). In addition, we observed that all *unc-29* mutants tested exhibited an abnormal pattern of egg-laying in the absence of drug that was roughly converse to the pattern of levamisole-treated wild-type animals (Table 3-1; see also Methods). These results indicated that a nicotinic receptor containing the UNC-29, UNC-38, and LEV-1 subunit proteins facilitated egg-laying.

To understand how the UNC-29-containing receptor promotes egg-laying, we investigated the functional expression pattern of the UNC-29 protein. Although *unc-29* mutants were originally identified based on their effect in body muscle [114], recently published studies indicated that UNC-29 might also be expressed in neurons [11]. In principle, therefore, the UNC-29-containing nAChR involved in egg-laying could act within the egg-laying muscles themselves, or it could act in one or more classes of motorneurons that excite the egg-laying muscles. To determine whether the egg-laying neurons or muscle cells contained UNC-29 receptors, we used deconvolution microscopy to investigate the expression pattern of a chimeric UNC-29 protein tagged with GFP at its carboxy-terminus. This UNC-29:GFP chimera was expressed under the control of its own promoter, and could functionally rescue the movement [10] and egg-laying (data not shown) phenotypes of *unc-29* recessive mutants. In multiple independent transgenic lines, we observed expression of UNC-29:GFP protein in many ventral cord neurons, including the VC egg-laying motorneurons, as well as in the vulval muscles (Figure 3-2a).

To determine whether UNC-29 functioned in the egg-laying neurons and/or the vulval muscles, we performed mosaic analysis to identify the cellular focus of the *unc-29* egg-laying phenotype. Genetic mosaics containing clones of mutant cells in an otherwise wild-type organism were generated and assayed for egg-laying behavior under both inhibitory (M9 liquid) and permissive (NGM plates containing bacteria) conditions. Interestingly, these analyses indicated that *unc-29* functioned in neurons as well as muscles to stimulate egg-laying (Figure 3-2b,c,d). Mosaics lacking functional *unc-29* in the AB or AB.p lineages, which give rise to the egg-laying neurons, showed no stimulation of egg-laying by levamisole when assayed on NGM plates, although most of these animals could be stimulated by levamisole in the M9 liquid assay. Similarly, mosaics containing array losses in the MS cell lineage, which gives rise to the egg-laying muscles, were also insensitive to stimulation of egg-laying by levamisole on NGM, but responded to

levamisole in M9 (although this response was reduced relative to the *unc-29(+)* control). Thus, on NGM, *unc-29* function was required in both neuronal and muscle lineages for stimulation of egg-laying, whereas in the more sensitive M9 assay, expression of functional UNC-29 in either neurons or muscle lineages was sufficient to provide at least some levamisole response. In the absence of drug, the egg-laying patterns of both neuronal and muscle mosaics resembled the pattern of the *unc-29(e1072)* mutant that lacked functional receptors in all cells (Table 3-1). Together these results indicated that UNC-29 receptors function in both muscles and neurons to stimulate egg-laying.

Both our expression studies and our mosaic analysis suggested that the VC motorneurons might be an important site of action for UNC-29 in egg-laying. To test this possibility, we eliminated these cells through cell-specific laser ablations and assayed the effect of this ablation on the response to levamisole. We found that when we eliminated all six VCs by ablating their neuroblast precursors, response to levamisole was lost (Figure 3-2e). However, neuroblast ablations that allowed one or more VC neurons to be produced did not abolish levamisole response, nor did direct ablation of only a subset of the VCs (i.e., VC4 and VC5 alone; see Figure 3-2e). Thus, the ablation results supported the hypothesis that levamisole-sensitive nAChRs function in the VCs. Another prediction of this hypothesis is that the egg-laying response to levamisole might be dependent on acetylcholine, a key neurotransmitter released by all the VC neurons. In fact, we observed that when endogenous acetylcholine was depleted through use of a temperature-sensitive choline acetyltransferase mutant, the animals were levamisole-resistant for egg-laying (Figure 3-2e). This result indicated that the stimulation of egg-laying by UNC-29 nicotinic receptors was dependent on the release of endogenous acetylcholine, and that UNC-29 receptors in the VCs might promote acetylcholine release.

Our results thus far indicated that *C. elegans* egg-laying behavior was stimulated by UNC-29-containing nicotinic receptors, raising the possibility that egg-laying could be used as an assay for nicotine adaptation. To investigate the effect of long nicotine treatments on egg-laying behavior, wild-type hermaphrodites were cultured overnight in the presence of 0.5% nicotine, then removed from nicotine and assayed for egg-laying. We observed that overnight nicotine treatment did not result in a gross defect in the ability to lay eggs, and the nicotine-adapted animals retained approximately normal numbers of eggs in the uterus. However, the nicotine-adapted animals were strongly resistant to stimulation of egg-laying by levamisole, although they still laid eggs in response to serotonin (Fig. 3-3a). Thus, long-term nicotine treatment appeared to specifically inactivate or antagonize the egg-laying response to levamisole. This adaptive response to nicotine was induced slowly, as loss of levamisole response occurred only after 1-3 hours of nicotine exposure (Figure 3-3b). Adaptation to nicotine was also surprisingly long-lasting; when nicotine-adapted animals were transferred to drug-free medium, even 24 hours after removal from nicotine most animals remained levamisole-insensitive (Figure 3-3c). Thus, prolonged exposure to nicotine induced long-term plasticity in egg-laying behavior, which attenuated or inactivated the response to levamisole. Since these nicotine-adapted animals failed to respond to levamisole under conditions in which either neuronal or muscle expression of UNC-29 receptors were sufficient for at least partial levamisole response, the activities of both neuronal and neuromuscular UNC-29 receptors appeared likely to be affected by this adaptation.

Long-term nicotine treatment also caused changes in egg-laying behavior in the absence of drug. Egg-laying in *C. elegans* occurs in a specific temporal pattern: egg-laying events are clustered, with periods of active egg-laying, or active phases, separated by long inactive phases during which eggs are retained. Both the duration of the inactive phases ("inter-cluster intervals") and the duration of intervals between egg-laying events

in a cluster ("intra-cluster intervals") model as exponential random variables with different time constants [99]. . Although wild-type hermaphrodites that had been exposed to nicotine overnight could still lay eggs, their pattern of egg-laying behavior was abnormal: the inactive egg-laying phase under drug-free conditions was approximately 2-fold longer than that of naive animals (Figure 3-3d, Table 3-1). This temporal pattern was quite similar to that of *unc-29* mutant animals, suggesting that the abnormalities in egg-laying pattern induced by long-term nicotine exposure resulted at least in part from reduced nAChR activity.

What genes are required for nicotine adaptation in egg-laying behavior? To investigate this question, we assayed *C. elegans* mutants with known defects in signaling molecules that function in the nervous system. In particular, we analyzed mutants defective in the gene *tpa-1*, which encodes a major isoform of protein kinase C . Recessive mutations in *tpa-1* confer resistance to phorbol esters, but do not dramatically impair the health, fertility, or behavior of the worm [90]. To assess the possible role of *tpa-1* in nicotine adaptation, we assayed the effect of *tpa-1* mutations on the induction of egg-laying by levamisole, and on the inactivation of this response by long-term nicotine treatment. We found that naive *tpa-1* mutant animals laid eggs in response to levamisole over the same range of concentrations as wild-type, although the magnitude of this stimulation was somewhat lower in the *tpa-1* mutant (Figure 3-4a). Our earlier work had indicated that TPA-1 participates in the acute response to serotonin [99], a positive modulator of egg-laying; thus, the smaller levamisole response of the *tpa-1* mutant was not surprising. Strikingly, however, we observed that *tpa-1* mutants showed little or no reduction in levamisole-induced egg-laying after overnight exposure to nicotine (Figure 3-4b), indicating that *tpa-1* mutant animals were completely defective in nicotine adaptation. Thus, PKC appeared to play a second, probably distinct role in egg-laying behavior: to promote long-term inactivation of nAChRs in the egg-laying circuitry in

response to chronic nicotine treatment. Since *tpa-1* mutants exhibited an adaptation defect even when the neurons expressing UNC-29 receptors (the VCs) had been eliminated through laser ablation (Figure 3-4c), we inferred that *tpa-1* was necessary for inactivation of nAChR activity in the vulval muscles; this result did not rule out a similar role for TPA-1 in the VC neurons as well.

To determine whether activation of PKC was sufficient as well as necessary for nicotine adaptation, we tested the long-term effect of the PKC activator tetraphorbol acetate (TPA) on the egg-laying response to nicotinic agonist. In the absence of levamisole, these TPA-treated animals laid a significant number of eggs under normally inhibitory conditions, a result consistent with TPA-1's role in activating serotonin signaling (see above). Strikingly, we also found TPA treatment, like nicotine treatment, dramatically reduced the egg-laying response to levamisole (Figure 3-4d). The levamisole response of the *tpa-1* mutant was unaffected by long-term TPA treatment, indicating that the inhibition of nAChR activity by phorbol esters required the TPA-1 isoform of PKC. Together, these results suggested that activation of the TPA-1 protein kinase is not only necessary for nicotine adaptation, but also sufficient for inhibition of nAChR activity in the egg-laying circuit.

DISCUSSION

Nicotinic agonists have both acute and chronic effects on egg-laying behavior in *C. elegans*. We have shown that the acute stimulation of egg-laying by nicotine and the nicotinic agonist levamisole is mediated by nAChRs containing the subunit protein UNC-29. In the egg-laying circuitry, UNC-29 receptors were shown to be functionally expressed in at least two locations: the ventral cord egg-laying neurons (VCs) and the vulval muscles (Figure 3-6). Although normal egg-laying behavior required both neuronal and neuromuscular expression of UNC-29 receptors, expression of UNC-29 in either

neurons or muscle alone was sufficient to stimulate egg-laying under some conditions. In the VC neurons, UNC-29 receptors appears to promote egg-laying by facilitating neurotransmitter release, a function also attributed to neuronal nicotinic receptors in vertebrates [115-118]. The resistance of acetylcholine-deficient mutants to levamisole indicates that neuronal UNC-29 receptors stimulate egg-laying by promoting release of acetylcholine from the VCs. The observation that levamisole treatment shortens, and mutations in *unc-29* lengthens, the inactive egg-laying phase suggests that UNC-29 receptors in the VCs may also promote release of a modulator, perhaps an FaRP, that controls the switch from the active to the inactive phase. In the egg-laying muscles, UNC-29 receptors most likely promote egg-laying by direct muscle excitation. However, since *unc-29* mutants are not strongly egg-laying defective, UNC-29 receptors do not appear to be necessary for egg-laying muscle contraction. The dispensability of UNC-29 for muscle contraction could be due to a second, levamisole-insensitive nAChR in the vulval muscles which displays some functional redundancy with the UNC-29 receptor.

Chronic exposure to nicotinic agonists also had dramatic effects on *C. elegans* egg-laying behavior, which were both long-lasting and specific. Overnight treatment with nicotine induced a strong and persistent resistance to the stimulation of egg-laying by levamisole, and nicotine-adapted animals tracked in the absence of nicotine displayed abnormal egg-laying patterns. Yet nicotine adaptation did not seriously impair the general ability to lay eggs, nor did it affect the stimulation of egg-laying by non-cholinergic agents such as serotonin. Thus, the effects of long-term nicotine treatment on egg-laying can be most simply explained as resulting at least in part from lost or attenuated nicotinic receptor activity. Since nicotine-adapted animals failed to respond to levamisole under conditions in which either neuronal or muscle expression of UNC-29 receptors was sufficient for at least partial levamisole response, the activities of both neuronal and neuromuscular UNC-29 receptors are likely to be inhibited by chronic nicotine treatment.

Adaptation of *C. elegans* egg-laying behavior to nicotine is similar in time-course, persistence, and dependence on neuronal nAChRs to functional inactivation in the vertebrate CNS; thus, genetic analysis of nicotine adaptation in nematodes may depend on processes formally analogous, and perhaps mechanistically similar, to nAChR inactivation in brain neurons.

One gene that we have found to be essential for nicotine adaptation in *C. elegans* is *tpa-1*, which encodes a homologue of protein kinase C. *tpa-1* mutant animals fail to undergo loss of levamisole response after long-term nicotine treatment; thus, the PKC isoform encoded by the *tpa-1* gene is apparently required for, and may participate in, nicotine adaptation. We hypothesize that prolonged activation of UNC-29 receptors by nicotine may lead to PKC-dependent inactivation of UNC-29 receptors in the vulval muscles, and possibly the VCs as well (Figure 3-5). Recent results are consistent with the possibility that PKC may promote nAChR inactivation in organisms other than *C. elegans*. For example, PKC activity is elevated by long nicotine treatments in some vertebrate cell types [119, 120], and at least in some cases, differences between neuronal receptor subtypes in their ability to be phosphorylated correlate with their ability to be inactivated by nicotine [121]. Intriguingly, a recent report indicates that in some nAChR-expressing cells, protein kinase C may positively modulate nAChR activity [122]. Thus, PKC may modulate the activity of different nicotinic receptors in different ways, acting in some contexts as a positive regulator and in others as a negative regulator.

What are the possible mechanisms through which PKC could promote nicotine adaptation? Probably the simplest model is that direct phosphorylation of nAChR subunits could down-regulate the activity of the receptor, either by inactivating the receptor per se, or by targeting the receptor for inactivation. The subunits of the *C. elegans* levamisole receptor (i.e., UNC-29, LEV-1, and UNC-38) contain consensus

sequences for PKC phosphorylation within the M3-M4 cytoplasmic loop [11, 123]; thus, direct phosphorylation of one or more subunits of the levamisole receptor is a reasonable possibility. In the nAChR from the *Torpedo* electric organ, phosphorylation of this loop by PKC or other kinases has been shown to enhance the desensitization rate of the receptor [124-128], an effect also observed with nAChRs from chick muscle [129] and sympathetic ganglia [130]. Thus, it is reasonable to suppose that modulation of receptor desensitization kinetics by PKC may be linked to functional inactivation. Alternatively, some or all of the effects of PKC could be indirect, and the actual substrates of PKC could be unidentified nAChR regulatory molecules. The availability of a simple and robust assay for nicotine adaptation in *C. elegans* should make it possible to identify such additional molecules that participate in long-term responses to nicotine.

METHODS

Analysis of egg-laying patterns

Egg-laying in *C. elegans* occurs in a specific temporal pattern: egg-laying events are clustered, with periods of active egg-laying, or active phases, separated by long inactive phases during which eggs are retained. Both the duration of the inactive phases ("inter-cluster intervals") and the duration of intervals between egg-laying events in a cluster ("intra-cluster intervals") model as exponential random variables with different time constants [99]. We found (Table 3-1) that in wild-type animals treated with levamisole, the duration of the inactive phase was dramatically shortened, and the rate of egg-laying within the active phase was significantly increased. Conversely, all *unc-29* alleles tested exhibited either a longer-than-normal inactive phase (e.g., *x29*), a slower rate of egg-laying within the active phase (e.g., *e1072*), or both (e.g., *e193*). Thus, the UNC-29 receptor appeared to both facilitate the switch into the active phase of egg-laying, as well

as stimulate egg-laying muscle contractions within the active phase. Egg-laying behavior of individual animals on solid media (NGM agar) was recorded for 4-8 hours as described using an automated tracking system [99]. Maximum likelihood estimates of inter-cluster and intra-cluster time constants were determined as described [67]. Briefly, the intra-cluster time constant is the reciprocal of the estimated parameter λ_1, and the inter-cluster time constant is the reciprocal of the product of the parameters p and λ_2. Expected variance of estimated time constants was determined by generating 100 independent sets of simulated egg-laying data using the coupled Poisson probability density function, and computing the standard deviation of the parameters estimated from these simulations.

Egg-laying Assays

Unless otherwise stated, nematodes were grown and assayed at room temperature on standard NGM seeded with *E. coli* strain OP50 as a food source. Egg-laying behavior of individual animals on solid media (NGM agar) was recorded for 4-8 hours as described using an automated tracking system [99]. For drug experiments, 5-hydroxytryptamine (creatinine sulfate complex, Sigma) was added to NGM agar at 7.5 mM, and levamisole (hydrochloride, Sigma) was added at 50 μM. For dose response experiments, individual animals were placed in microtiter wells containing liquid M9 and the indicated concentration of drug. Unless otherwise stated, the number of eggs laid in response to levamisole was assayed at 1 hour. For nicotine adaptation experiments, worms were placed on 0.5% nicotine (Sigma) seeded NGM plates for varying lengths of time, and tested for response to levamisole in individual liquid M9 assays. To maximize the possibility that a levamisole-sensitive animal would lay eggs, eggs were counted after 4 hours in levamisole in these assays.

Mosaic Analysis

We identified potential mosaics using an *unc-29* mutant strain carrying an intact copy of the *unc-29* gene on an unstable free extrachromosomal array (genotype: *unc-29(e1072)I; ncl-1(e1942)III; gaEx25[unc-29+, lin-31+, ncl-1+].* Mitotic loss of the array during development resulted in mosaic animals containing clones of genetically mutant cells in an otherwise wild-type organism [114]. Individual worms with either partial Unc or non-Unc phenotypes were analyzed by Nomarski optics for mosaic expression of the Ncl phenotype. To ascertain the point of loss for the array, the Ncl phenotype was scored in the following cells: HSNR, HSNL, PDEL, PDER, M2L, M2R, M1, M5, I6, NSMR, NSML, MCL, MCR, ISL, ISR, I3, and MI. The egg-laying phenotype of the mosaics were scored in one of two ways. Some mosaics were first tracked for 6 hours on NGM, then transferred to NGM containing 50 μM levamisole and tracked for an additional 2 hours. Other mosaic animals were transferred to microtiter wells containing M9 salts and 25 μM levamisole; after 4 hours incubation at room temperature, the eggs laid by each animal were counted. AQ322 animals that contained the array in all cells, or which had lost the array zygotically and were therefore mutant in all cells, were used as positive and negative controls, respectively.

Cell Ablation Experiments

Ablation of the VC4 and VC5 neurons was performed as described [99]. For ablations of VC1-6, we ablated the neuroblasts P1.a-P9.a, which are the larval precursors of the VCs. Although only P3.a-P8.a normally give rise to the VC's, adjacent Pn.a cells can generate VC's in the absence of P3.a-P8.a unless killed [131]. Animals of strain AQ242 (genotype: *dpy-20(e1282)IV; ljEx1[dpy-20(+), egl-36::GFP]*) were grown at 20° C; approximately 10 hours after hatching, the Pn.a cell nuclei were identified by position in the ventral cord and killed. In this strain, GFP is expressed in all VC neurons; thus cell

killing was verified in late L4 by scoring for the absence of fluorescence in the ventral cord and vulval area. Three other ventral cord neurons which also descend from the ablated neuroblasts (VA7, VB8, and VD7) potentially make single synapses with the egg-laying muscles; thus, it was formally possible these neurons might contribute to this effect. However, ablations of a subset of Pn.a neuroblasts (for example, P3.a-P.8.a) which eliminated VA7, VB8, and VD7 but spared one or more VCs (as indicated by *egl-36::GFP* expressing cells that sent processes to the vulva) did not prevent levamisole response (data not shown).

Microscopy

The strain ZZ2001 (genotype: *unc-29(x29); Ex[rol-6d, unc-29::GFP],* kindly provided by Jim Lewis), was used for localization of UNC-29 receptors. Worms were placed on agar pads and immobilized with 30mM sodium azide. GFP was visualized using standard immunofluorescence techniques at a magnification of 90x. Images were collected using deconvolution microscopy with Delta Vision 2.1 software (Applied Precision Instruments). *unc-29::GFP*-expressing lines generated in our own laboratory using *dpy-20* as a coinjection marker showed fluorescence in essentially the same set of cells as ZZ2001 (data not shown); in all lines, the relative level of fluorescence in particular cells was quite variable.

This chapter, in full, is a reprint of the material in L. E. Waggoner et al. (1999) "Long term nicotine adaptation of egg-laying behavior in *C. elegans*: involvement of neuronal nicotinic recepors and protein kinase C" *(in revision).* The dissertation author was the primary investigator and author of this paper.

Table 3-1. Egg-laying parameters for mutant and drug-treated animals.

Animal type (#, hrs, intervals)	Intra-cluster time constant ($1/\lambda_1$; s)	Inter-cluster time constant ($1/p\lambda_2$; s)	**p**	λ_1 (s^{-1})	λ_2 (s^{-1} x 10^{-3})
N2	18	1240	0.545	0.057	1.5
(8, 46, 237)	±2	±160	±0.035	±0.008	±0.22
N2 + levamisole (50	6	360	0.406	0.172	6.9
μM)	±2	±80	±0.073	±0.076	±0.20
(9, 18, 64)					
unc-29 (x29)	11	1980**	0.673	0.090	0.75
(3, 19, 78)	±2	±480	±0.050	±0.015	±0.38
unc-29 (e1072)	33*	1220	.484	0.031	1.7
(3, 18, 75)	±11	±300	±0.069	±0.013	±0.49
unc-29 (e193)	34*	1800	0.427	0.030	1.3
(3, 18, 78)	±10	±380	±0.067	±0.013	±0.38
N2; nicotine-adapted	11	2040**	0.545	0.057	1.5
(@24h) (8, 46, 105)	±2	±160	±0.051	±0.023	±0.28

*The intra-cluster intervals (<300s in duration) were significantly longer than in wild-type as determined by the Mann-Whitney rank sum test ($p < .002$).
**The inter-cluster intervals (>300s in duration) were significantly longer than in wild-type as determined by the Mann-Whitney rank sum test ($p < .05$).

Table 3-2. Egg-laying parameters for *unc-29* mosaics.

Animal type (#, hrs, intervals)	Intra-cluster time constant ($1/\lambda_1$; s)	Inter-cluster time constant ($1/p\lambda_2$; s)	p	λ_1 (s^{-1})	λ_2 ($s^{-1} \times 10^{-3}$)
AQ322 (*unc-29(+)*)	9	1290	0.343	0.113	2.2
(3, 18, 58)	±6	±280	±0.071	±0.103	±0.74
AQ322; *unc-29* array lost	20*	1300	0.437	0.049	1.8
(3, 16, 65)	±6	±320	±0.076	±0.016	±0.58
AQ322; AB mosaics	29*	1760	0.379	0.034	1.5
(6, 34, 75)	±10	±380	±0.071	±0.016	±0.46
AQ322; MS mosaics	20*	2240	0.503	0.050	0.9
(5, 25, 78)	±5	±440	±0.068	±0.016	±0.23

*The intra-cluster intervals (<300s in duration) were significantly longer than in the *unc-29(+)* control as determined by the Mann-Whitney rank sum test ($p < .002$).

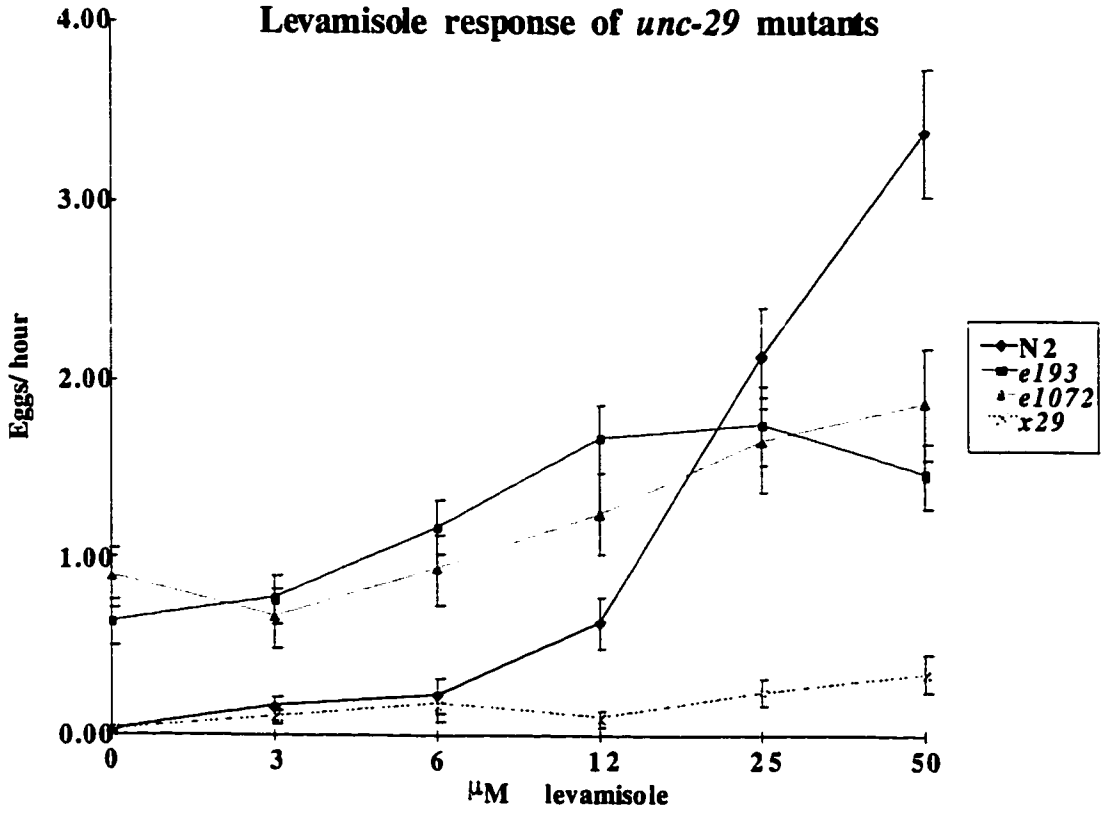


Figure 3-1a. Requirement of UNC-29 receptor proteins for acute response to nicotinic agonists. Egg-laying responses to levamisole or nicotine were determined for the different mutant alleles by placing individual animals in liquid M9 and the indicated concentration of levamisole, and counting the number of eggs laid by each animal after 1 hour of drug exposure. Animals carrying any of three *unc-29* mutant alleles showed a dramatic reduction in levamisole-induced egg-laying. Individual points and error bars indicate the mean and SEM of the following numbers of trials: N2=155, *e193*=144, *e1072*=96, *x29*=96.

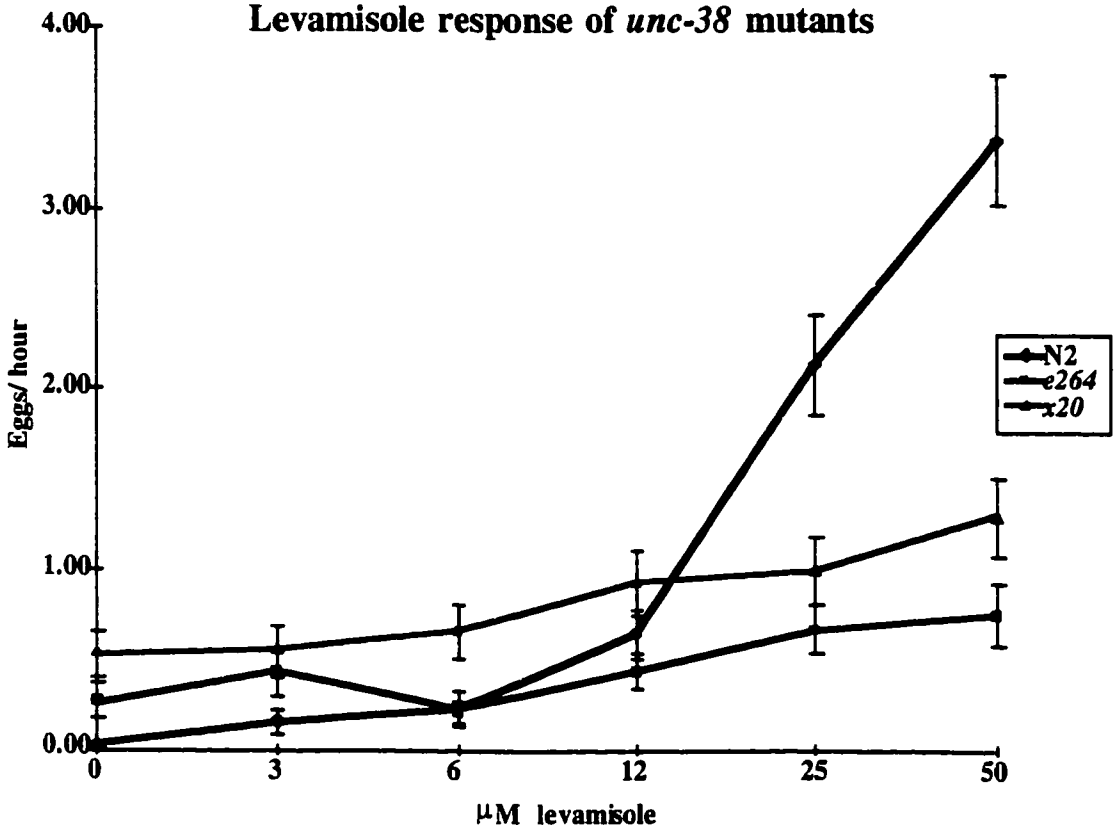


Figure 3-1b. Requirement of UNC-38 receptor proteins for acute response to nicotinic agonists. Egg-laying responses to levamisole or nicotine were determined for the different mutant alleles by placing individual animals in liquid M9 and the indicated concentration of levamisole, and counting the number of eggs laid by each animal after 1 hour of drug exposure. Three different *unc-38* mutants (including a putative null, *x20*) showed dramatically reduced levamisole responses. Points and error bars indicate the mean and SEM of the following number of trials: N2=155, *x20*=96, *e264*=96.

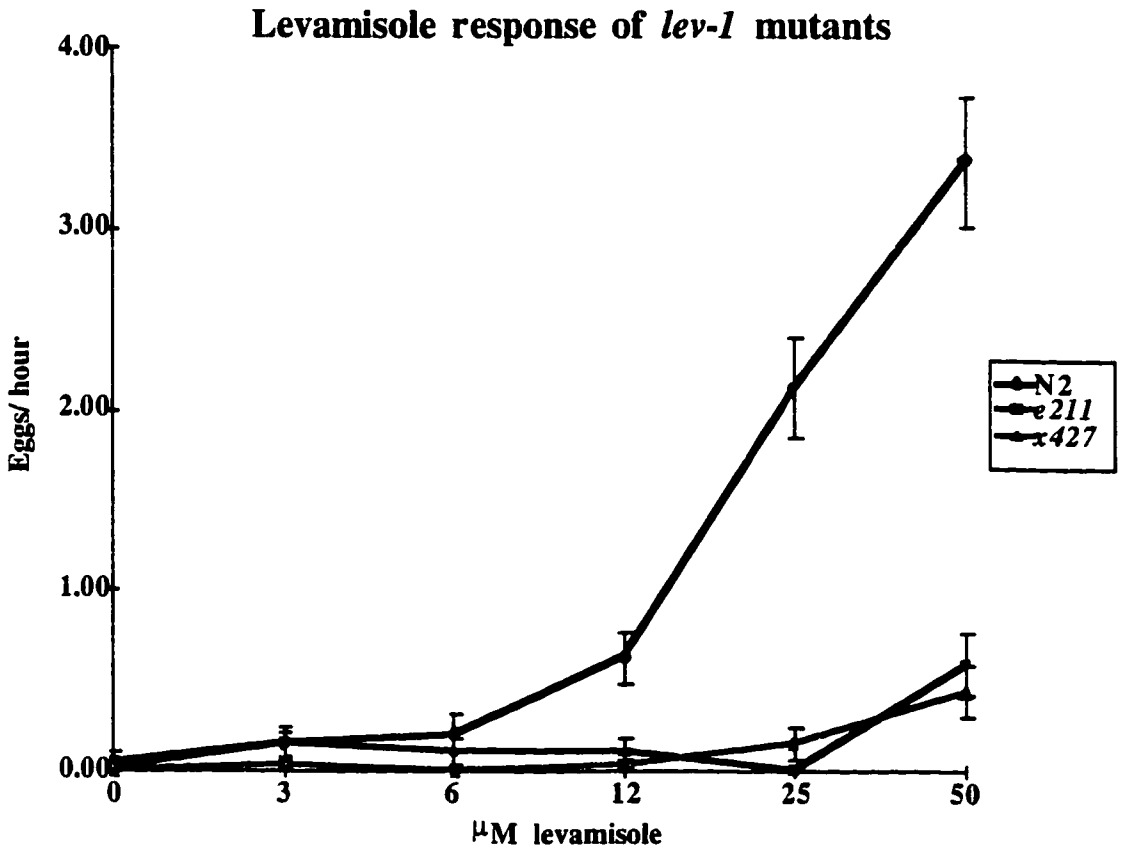


Figure 3-1c. Requirement of LEV-1 receptor proteins for acute response to nicotinic agonists. Egg-laying responses to levamisole or nicotine were determined for the different mutant alleles by placing individual animals in liquid M9 and the indicated concentration of levamisole, and counting the number of eggs laid by each animal after 1 hour of drug exposure. *lev-1* mutants had dramatically reduced levamisole responses. Points and error bars indicate the mean and SEM of the following number of trials: N2=107, *e211*=96, *x427*=72.

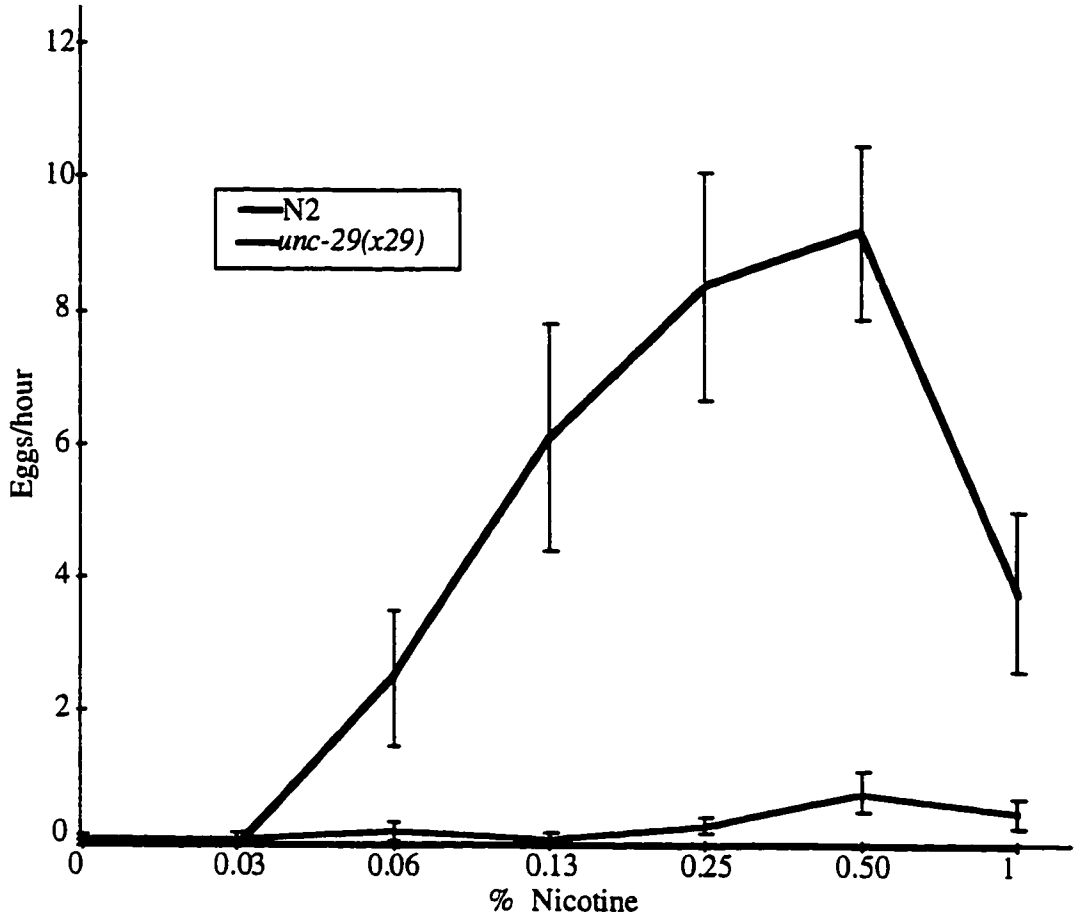


Figure 3-1d. Response of *unc-29* mutants to nicotine. *unc-29* mutants showed a dramatic reduction in nicotine-induced egg-laying. Individual points and error bars indicate the mean and SEM of at least 12 trials.

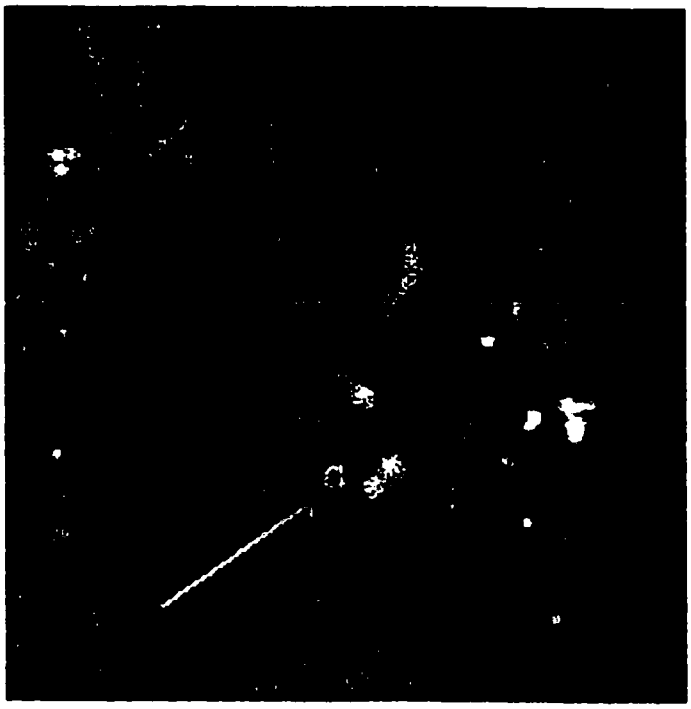


Figure 3-2a. Localization of GFP-tagged UNC-29 protein. Shown is an optical section of a ZZ2001 adult hermaphrodite expressing *unc-29::GFP*. In this ventral/lateral view, the VC5 neuronal cell body (arrow) and one set of vulval muscles are strongly fluorescent. Not shown in this view are other fluorescent cells, including many ventral cord motorneurons and various unidentified head and tail neurons. Punctate fluorescence in body is due to auto-fluorescence of gut granules.

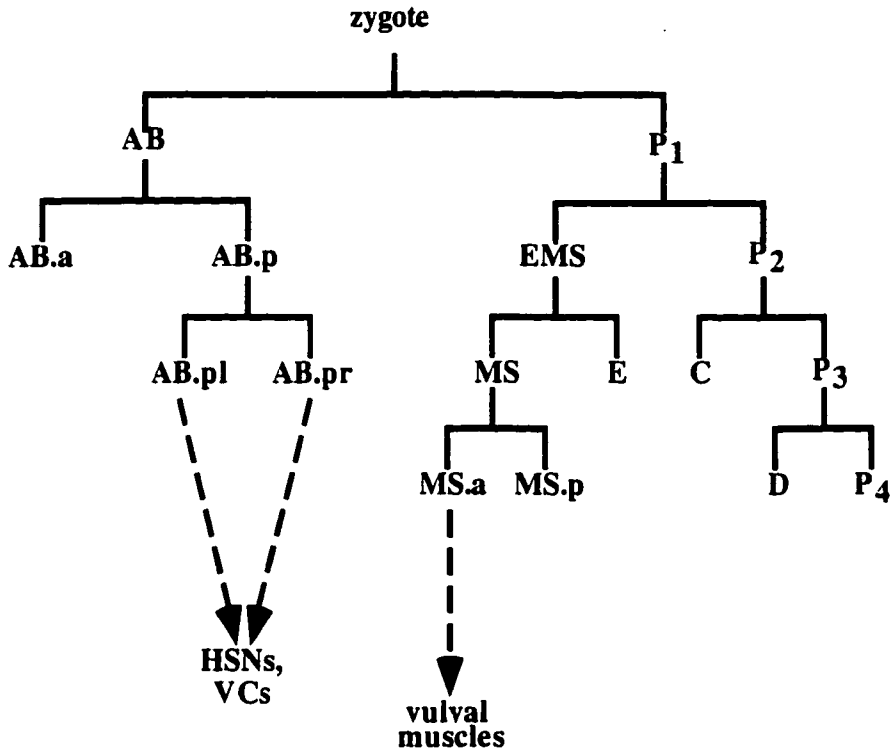


Figure 3-2b. Mosaic analysis of *unc-29* egg-laying phenotype. Shown is the cell lineage for the *C. elegans* hermaphrodite. Indicated are the precursor cells for the egg-laying and body muscles, the motorneurons involved in egg-laying, and the germline. Mosaic animals were generated as described.

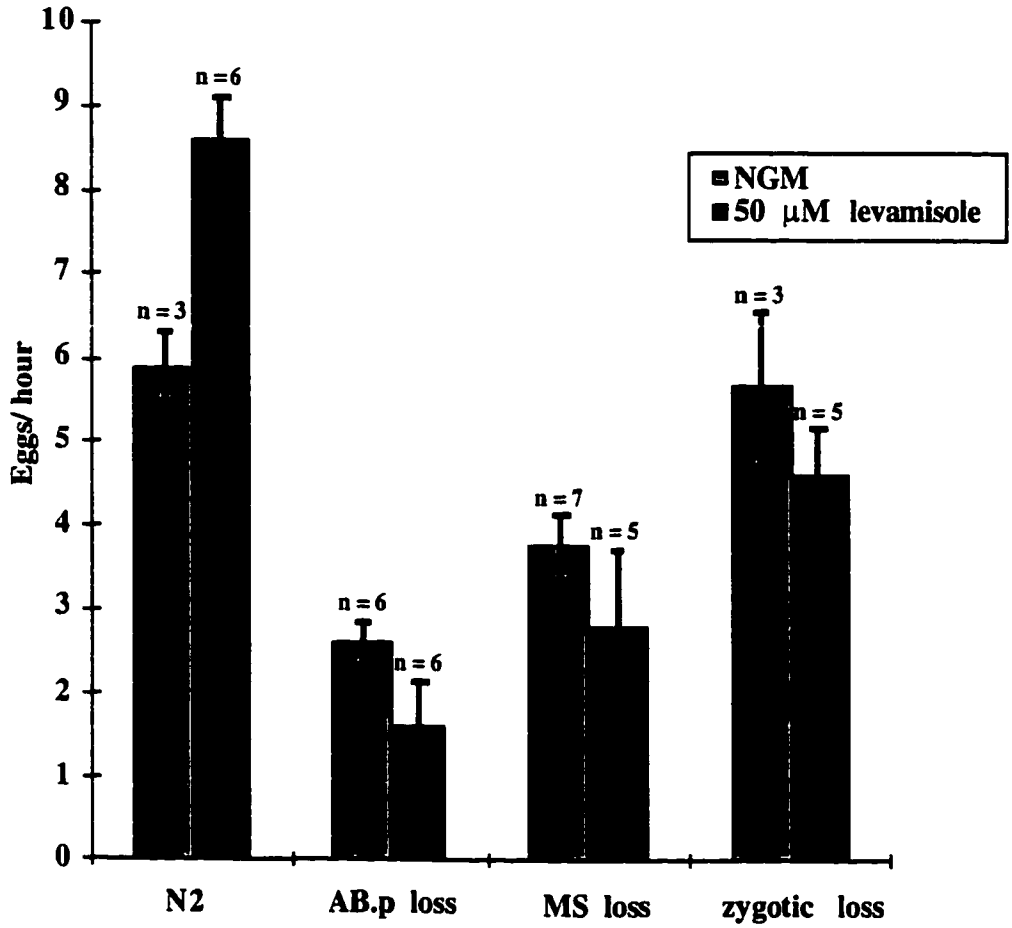


Figure 3-2c. Levamisole responses of mosaic animals (NGM). The histogram represents the egg-laying rates on seeded NGM plates in the presence or absence of 50 µM levamisole. Error bars indicate mean and SEM of the indicated number of trials. Estimated inter-cluster and intra-cluster time constants, along with the number of animals and intervals analyzed, are shown in Table 3-1. "AB.p loss" represents composite data for 5 AB and 1 AB.p mosaic; "MS loss" represents composite data for 5 mosaic animals with EMS or MS losses. "Zygotic loss" refers to animals in which the array was absent in the zygote due to a meiotic loss, making them mutant in all cells.

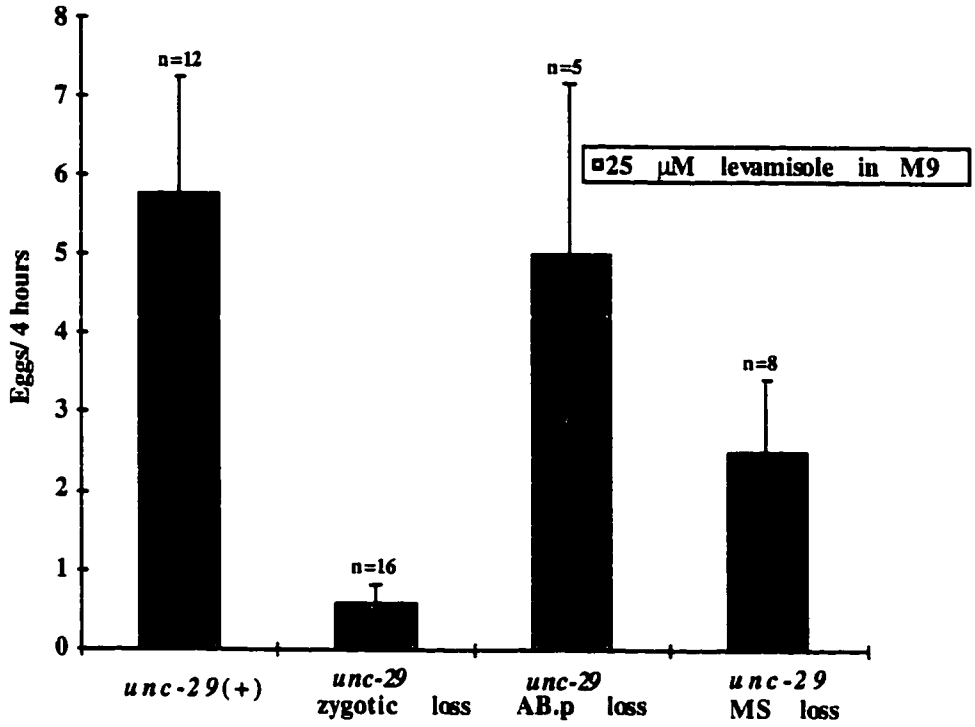


Figure 3-2d. Levamisole responses of mosaic animals (M9). The histogram represents number of eggs laid by animals of the indicated type when assayed individually for egg-laying in M9 salt solution with 25 µM levamisole. Each animal was transferred to M9 salts as a young adult, and eggs were counted after a 4 hour incubation as described in Methods. Histogram boxes and error bars indicate mean and SEM of the indicated number of trials. The levamisole responses of both AB- and MS- *unc-29* mosaics were significantly greater than the *unc-29* mutant strain under this condition according to the Mann Whitney rank sum test ($p < .025$).

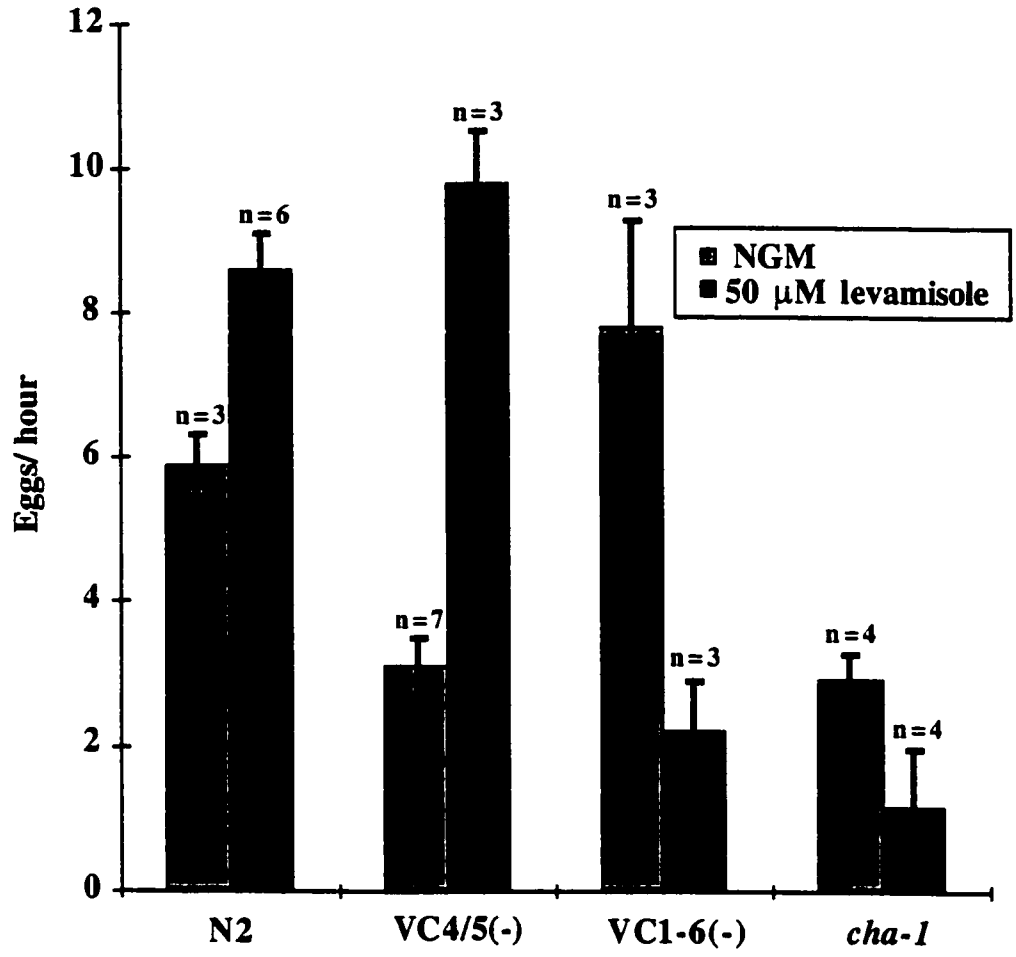


Figure 3-2e. Levamisole responses of VC-ablated and ACh-deficient animals. The histogram represents the egg-laying rates on seeded NGM plates in the presence or absence of 50 μM levamisole. Boxes and error bars indicate mean and SEM of the indicated number of trials. For ablation experiments, VC4/5 and P1.a-P9.a ablations were performed as described in Methods. The *cha-1* experiments were performed using a temperature sensitive *cha-1* (the gene for choline acetyltransferase) mutant, *cha-1(y226)*. Animals were grown at permissive temperature (15° C) until adults, and then tracked in the presence or absence of 50 μM levamisole at the restrictive temperature, 22° C.

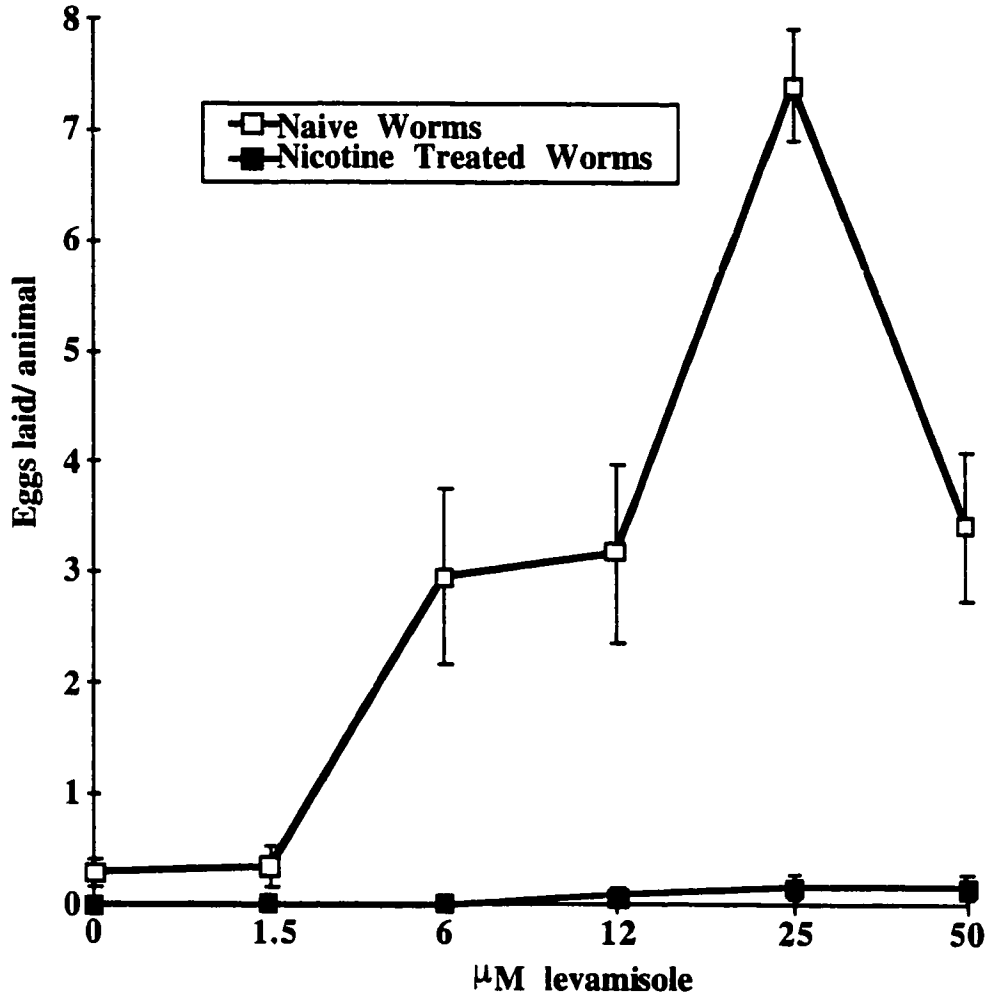


Figure 3-3a. Effect of nicotine treatment on levamisole response. Shown are levamisole dose response curves for wild-type animals grown in the presence of nicotine; the dose response of naive animals under the same conditions is shown as a control. N2 hermaphrodites were cultured overnight on NGM containing 0.5% nicotine and then transferred individually to wells containing liquid M9 with 25 μM levamisole; the number of eggs laid by each animal was counted after 4 hours. Error bars indicate mean and SEM of 18 or more trials. These nicotine-adapted animals responded normally to serotonin; egg-laying rates in M9 salts containing 7.5 mM serotonin were: naive-- 2.7 ± 1.2 eggs/hr; nicotine-adapted-- 2.5 ± 0.6 (n= 12 in both cases).

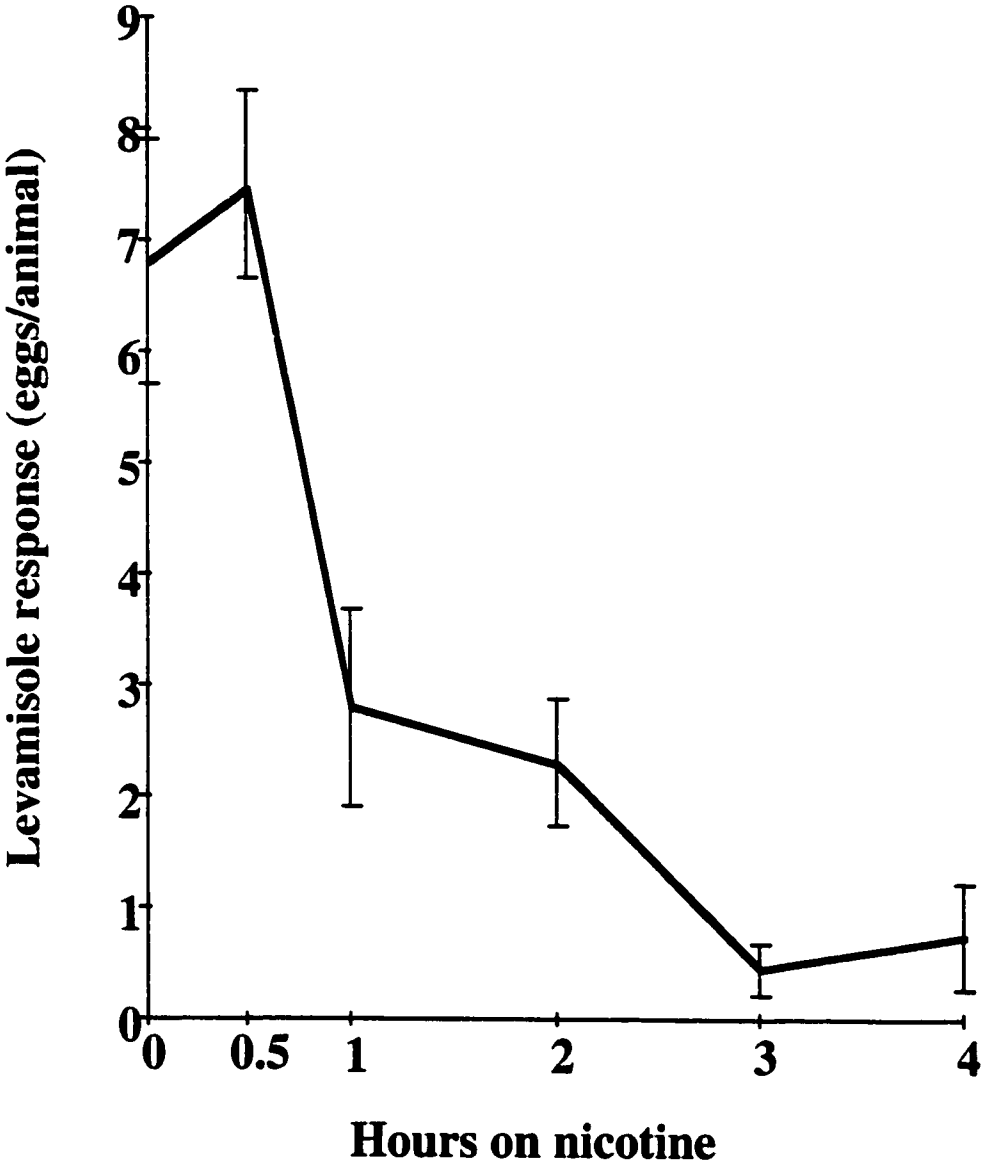


Figure 3-3b. Time course for nicotine adaptation. N2 hermaphrodites were placed on seeded NGM containing 0.5% nicotine for the indicated length of time, then assayed individually for egg-laying in M9 salt solution containing 25 μM levamisole as described Fig. 3-3.a. Points and error bars indicate the mean and SEM of 18 trials.

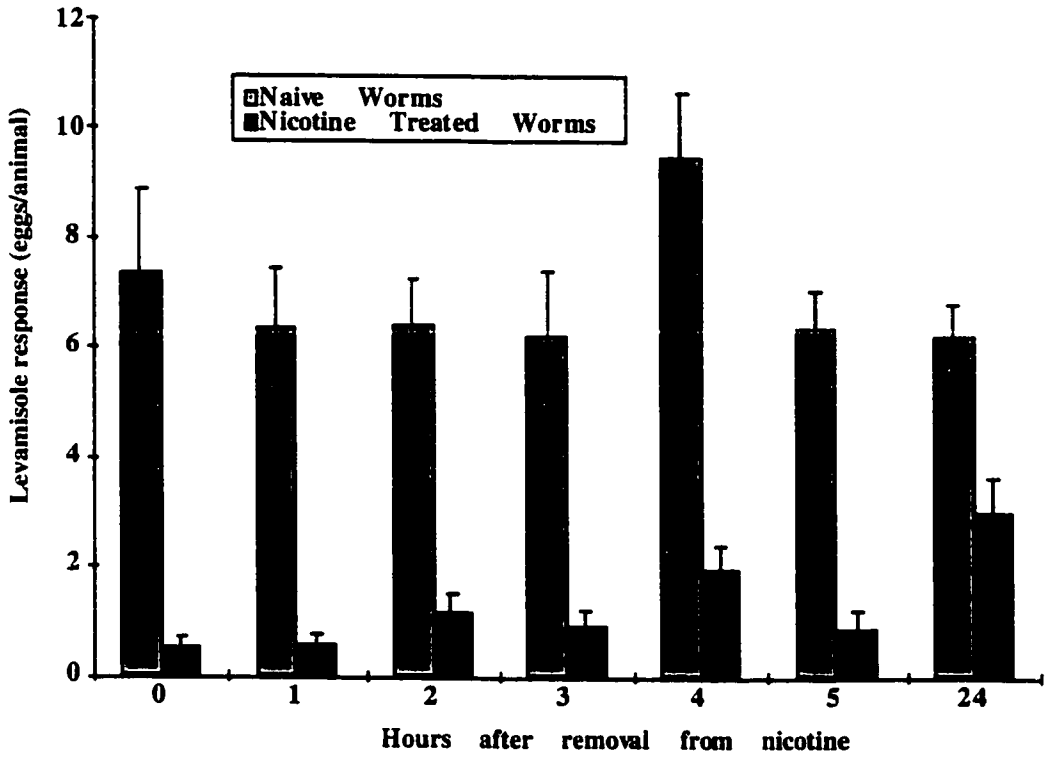


Figure 3-3c. Long-term persistence of nicotine adaptation. The histogram shows the levamisole response of nicotine-adapted animals after incubation for various times on drug-free medium. N2 animals were grown overnight on NGM plates with 0.5% nicotine as described in part a, then transferred to drug-free NGM plates for the indicated times. Egg-laying in response to 25 μM levamisole was tested in individual 4 hour liquid M9 assays as described in Fig. 3-3a. Control experiments with wild-type hermaphrodites mock treated with an overnight incubation on NGM without nicotine were performed in parallel. Error bars indicate mean and SEM of at least 18 trials.

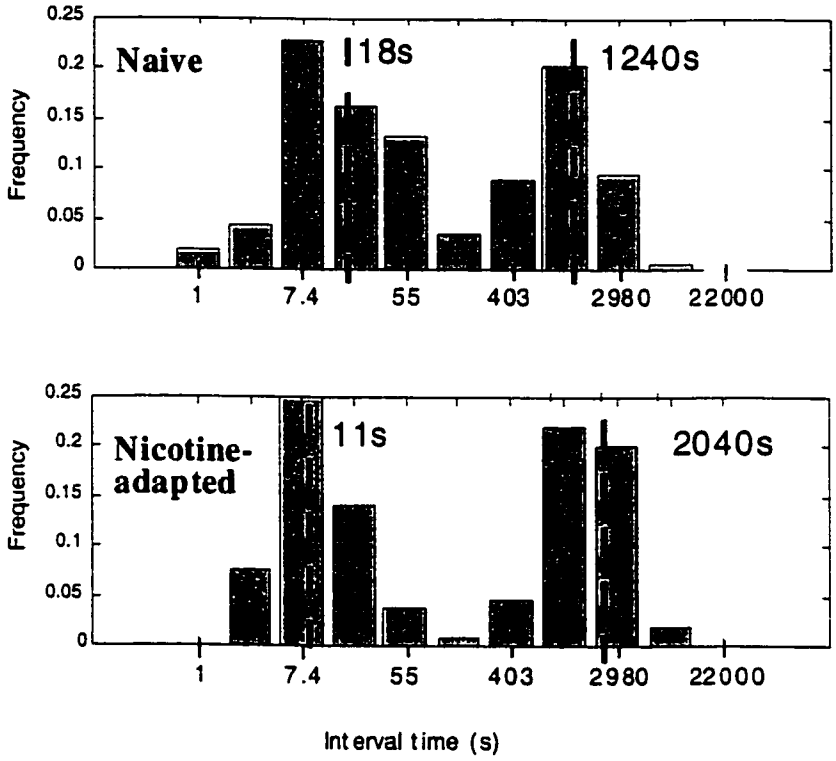


Figure 3-3d. Levamisole dependence of egg-laying in nicotine-adapted animals. Wild-type hermaphrodites were cultured overnight on NGM plates with 0.5% nicotine, then tracked on either drug-free NGM plates or NGM plates with 50 μM levamisole for 6 hours. The histogram shows the mean egg-laying rates for naive and nicotine-adapted animals in the presence and absence of levamisole; error bars indicate the SEM of the indicated number of trials. The egg-laying parameters, number of animals, hours tracked, and total intervals analyzed are indicated in Table 3-1.

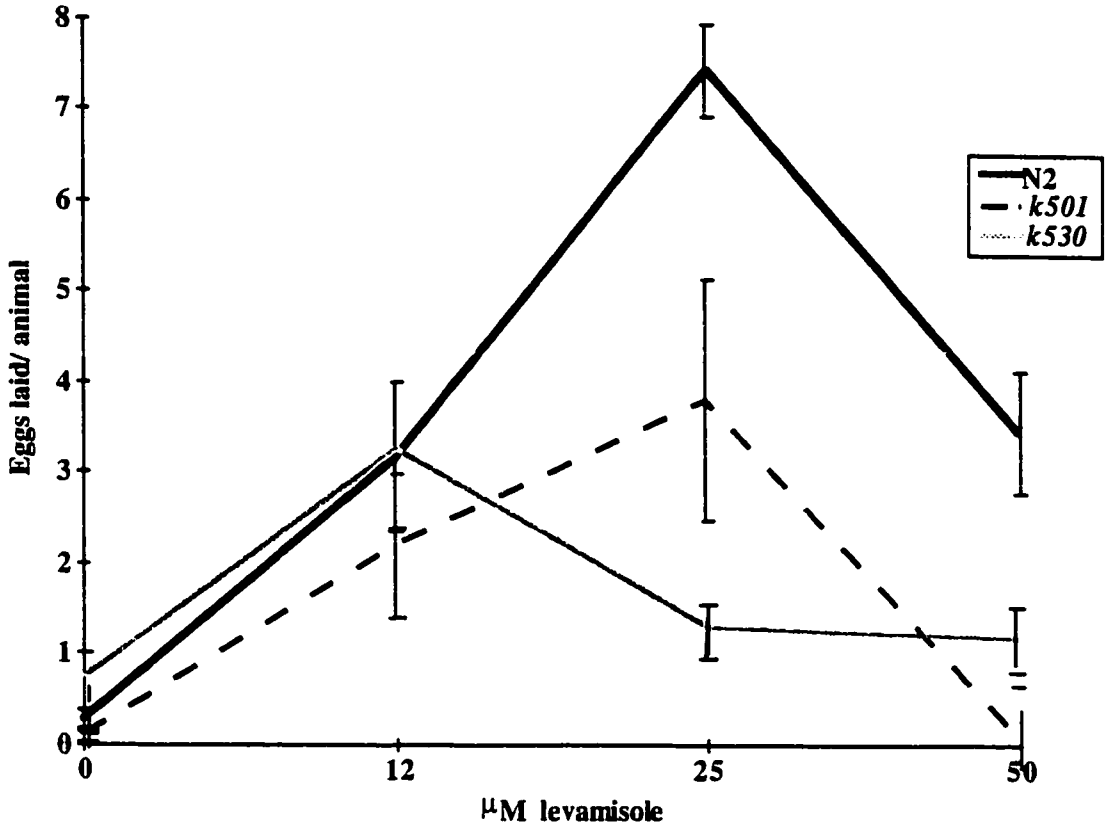


Figure 3-4a. Response of *tpa-1* mutants to levamisole. In each case, animals were tested for egg-laying in individual 4 hour liquid M9 assays at the indicated levamisole concentration as previously described (Figure 3-3). Levamisole dose responses for wild-type and *tpa-1* mutant animals are shown. Points and error bars indicate the mean and SEM of at least 12 trials.

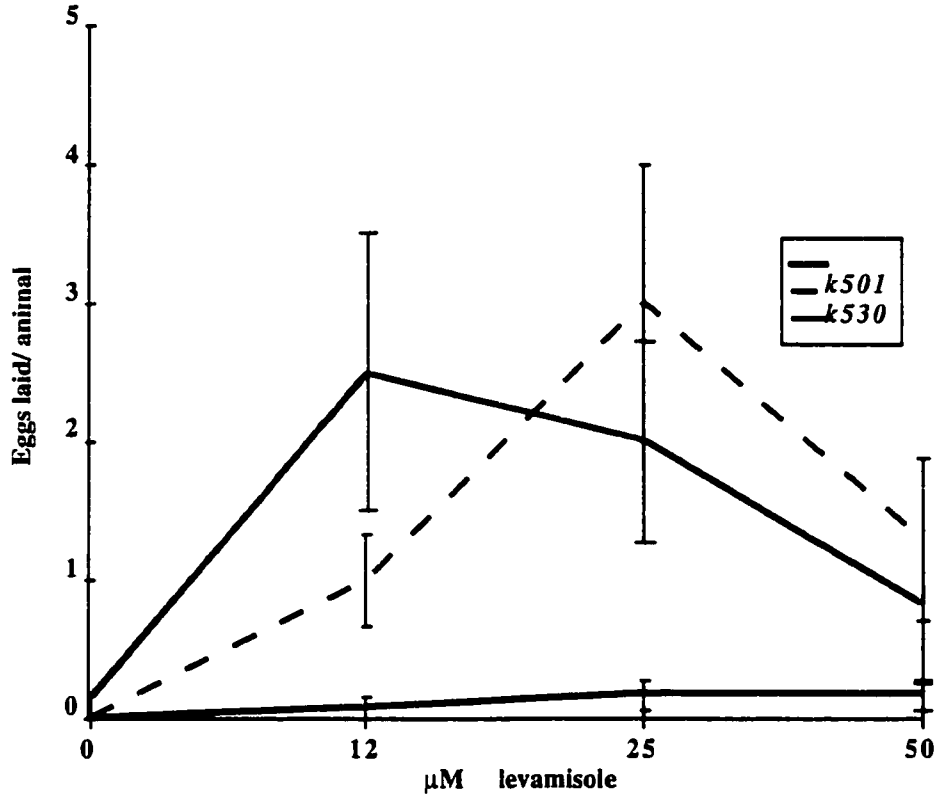


Figure 3-4b. Defect of *tpa-1* mutants in nicotine adaptation. In each case, animals were tested for egg-laying in individual 4 hour liquid M9 assays at the indicated levamisole concentration as previously described (Figure 3-3). Levamisole dose response curves for wild-type and *tpa-1* mutant strains after overnight treatment with 0.5% nicotine are shown. Points and error bars indicate the mean and SEM of at least 12 trials. The responses of both *tpa-1* mutants were significantly greater than wild-type under this condition according to the Mann Whitney rank sum test ($p < .001$). By the same test, the *tpa-1* mutants showed no significant reduction in response to levamisole after overnight nicotine treatment ($p > 0.5$).

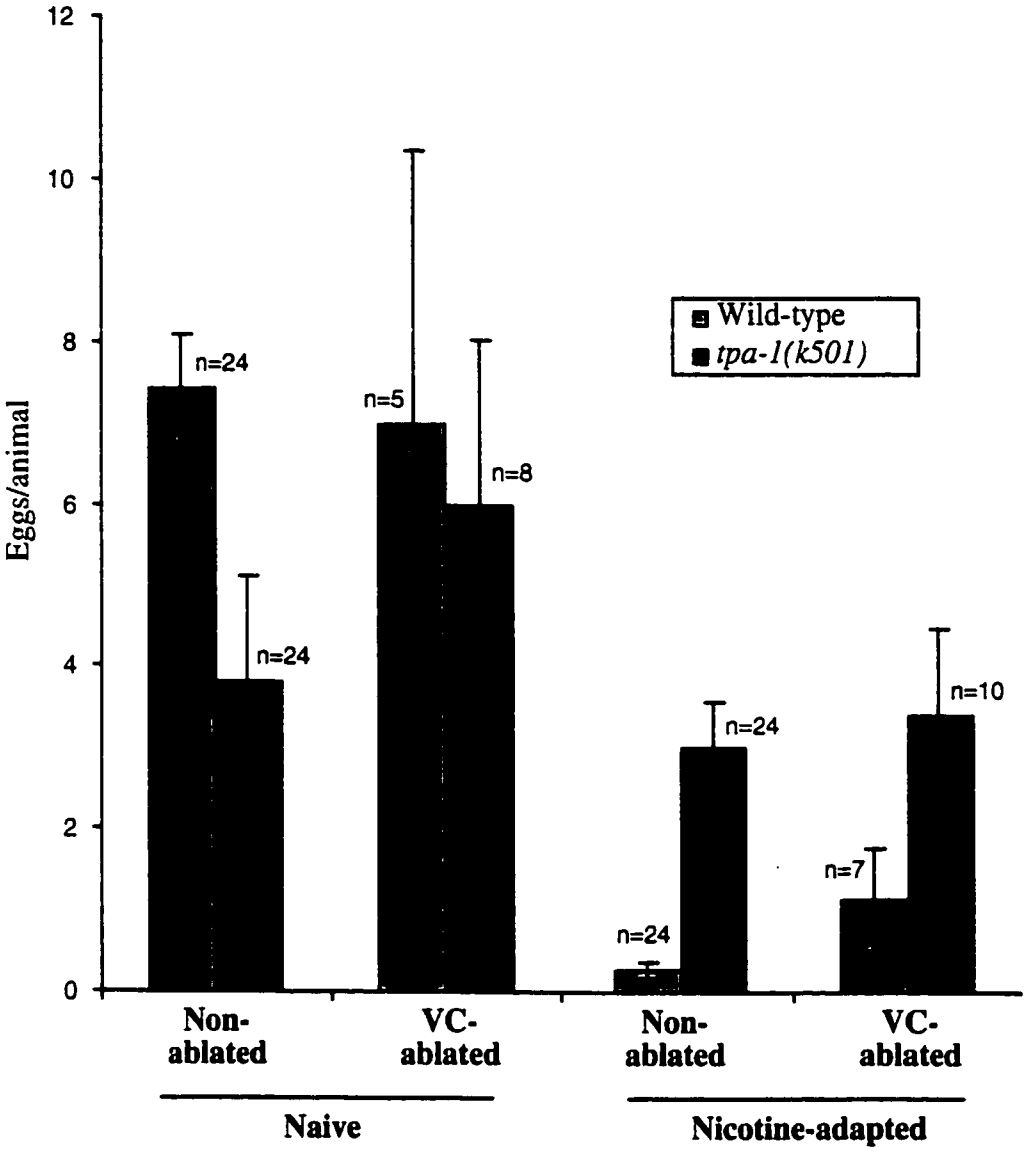


Figure 3-4c. *tpa-1* adaptation defect in VC-ablated animals In each case, animals were tested for egg-laying in individual 4 hour liquid M9 assays at the indicated levamisole concentration as previously described (Figure 3-3). The histogram shows the eggs laid in response to levamisole under the indicated condition for animals in which the neurons expressing UNC-29 receptors (the VCs) had been eliminated. The VC precursors were ablated as described; points and error bars indicate the mean and SEM of the indicated number of trials. The VC-ablated *tpa-1* mutants showed no significant reduction in response to levamisole after overnight nicotine treatment according to the Mann-Whitney rank sum test ($p > 0.5$).

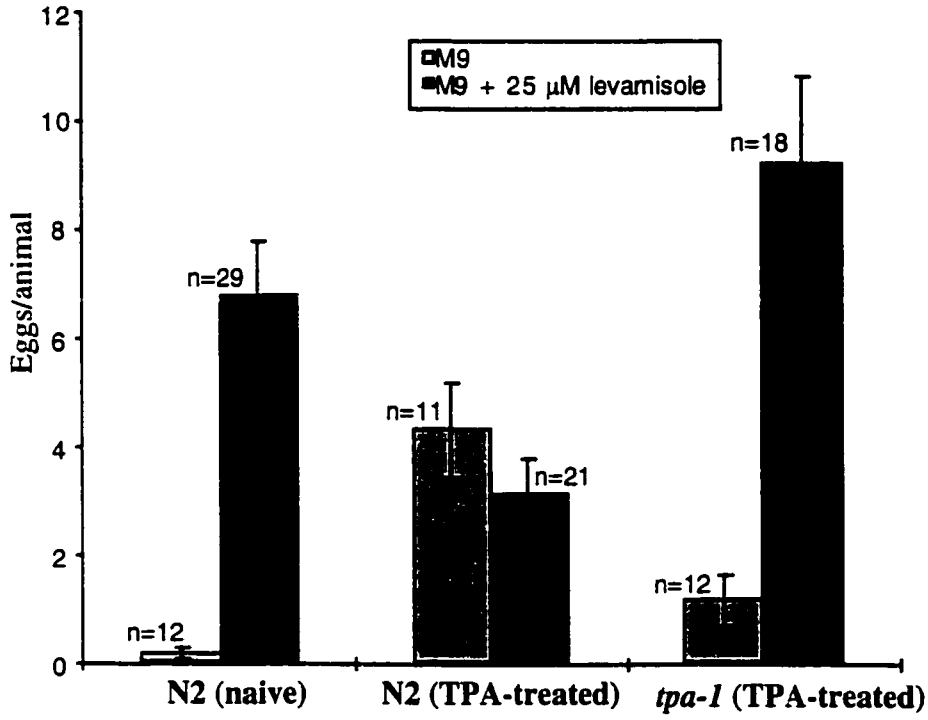


Figure 3-4d. Inactivation of levamisole response by phorbol esters. In each case, animals were tested for egg-laying in individual 4 hour liquid M9 assays at the indicated levamisole concentration as previously described (Figure 3-3). The histogram shows the levamisole responses of naive or TPA-treated (1 µg/ml in NGM) wild-type animals and TPA-treated *tpa-1(k501)* mutants . Points and error bars indicate the mean and SEM of the indicated number of trials. The response of TPA-treated wild-type animals to 25 µM levamisole was significantly less than that of both naive wild-type animals and TPA-treated *tpa-1* mutant animals according to the Mann-Whitney rank sum test ($p<.01$).

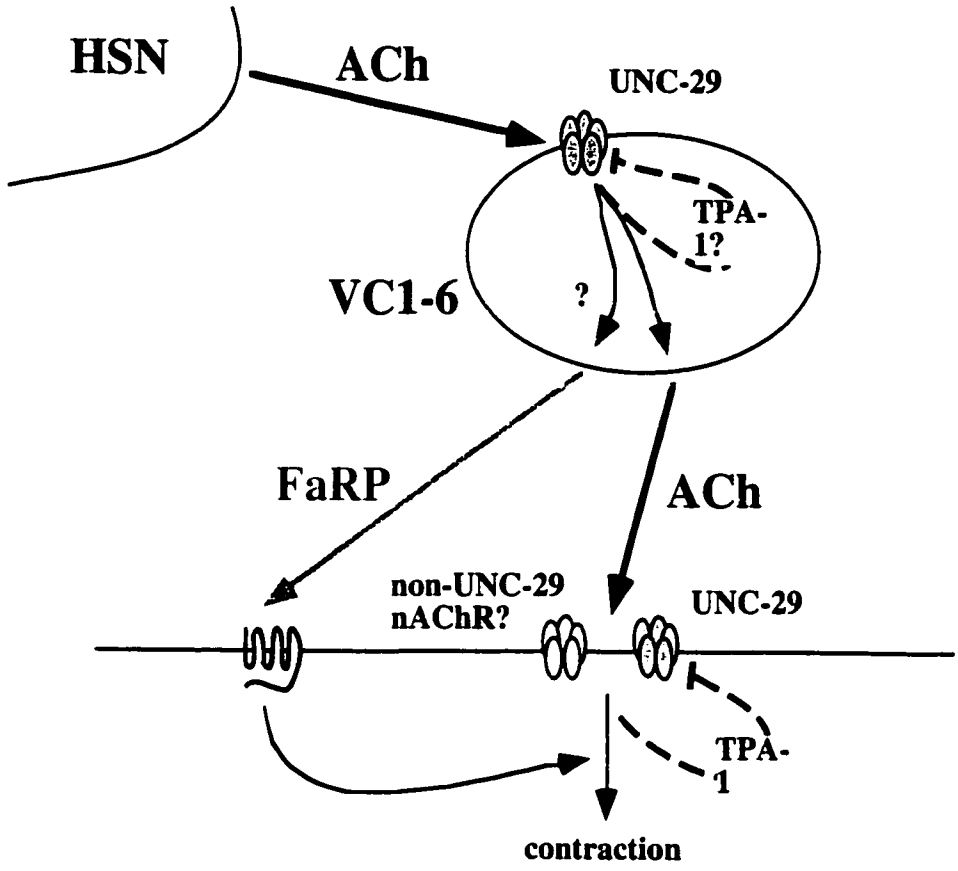


Figure 3-5. Model for nicotinic receptor function and adaptation in the egg-laying circuitry. UNC-29-containing nAChRs are expressed in both the VC egg-laying neurons and in the vulval muscles. Based on genetic and behavioral observations, we propose that activation of neuronal receptors promotes release of acetylcholine, and perhaps a FMRFamide-related peptide (FaRP), from the VCs. We also propose that UNC-29 receptors in the vulval muscles mediate muscle excitation in response to acetylcholine; if the muscles have been activated by an FaRP (or serotonin [99]), this excitation leads to muscle contraction. Prolonged activation of UNC-29 receptors by nicotine leads to PKC-dependent inactivation of UNC-29 receptors in the vulval muscles (and possibly in the VCs as well).

CHAPTER V CONCLUSION

As with most behaviors, egg-laying in *C. elegans* is complex and is regulated by interactions between signaling molecules and pathways found both in neurons and muscles. However, prior to the work presented in this thesis, the temporal pattern of egg-laying events had not been identified, and thus, detailed genetic and molecular analysis was not possible. Identification of the pattern of egg-laying has allowed me to analyze genetic and cellular mutants and gain insight into the molecular mechanisms that regulate this behavior. The pattern of egg-laying events can be modeled as a novel random process, in which the animal fluctuates between two behavioral states: an active egg-laying state and an inactive state. Through analysis of this pattern, I have been able to identify neurotransmitters that regulate distinct aspects of egg-laying, either by inducing entry into the active state or by triggering individual egg-laying events within the active state itself.

Serotonin, which is released primarily from the HSNs, promotes entry in the active egg-laying state. In addition, a FMRF-amide like peptide (FaRP), encoded by *flp-1*, can also induce the active egg-laying state. Pharmacological evidence suggests that serotonin and FaRPs function in concert to promote the active state, as the FLP-1 peptides potentiate the effects of serotonin on egg-laying. In contrast, acetylcholine, released from either the HSNs or VC neurons, triggers individual egg-laying events during the active state. Although these neurotransmitters are often released from the same neuron, they activate distinct signaling pathways in the downstream cells. Thus, neuromodulators, most likely acting through G protein-coupled receptors, can potentiate the effects of fast acting neurotransmitters such as acetylcholine, which allows for multiple levels of behavioral control.

It is possible that the behavioral states observed in *C. elegans* egg-laying correspond to functional states of the vulval muscles themselves. Studies indicate that the functional

states might involve modulation of the voltage gated calcium channel, *elg-19*, through phosphorylation by protein kinase C. This hypothesis is supported by work in vertebrate cells, in which muscle contraction and excitability is coupled to functional states of calcium channels, a process which is mediated by G protein-coupled receptors and protein kinase C (Boland et al., 1991). Furthermore, the switch from an active egg-laying state back to an inactive state, appears to involve $G_o\alpha$, the heterotrimeric G protein encoded by *goa-1*, functioning in the vulval muscles. Interestingly, this provides an example of a behavioral circuit in which the role of neurotransmitters is not to directly induce contraction of the muscles, but to modulate the functional states of the muscles, thus altering ability to contract in response to stimulus.

In addition, evidence indicates that modulation of the neurons themselves might also generate different behavioral states. The *goa-1* pathway, which negatively regulates entry into the active state, involves modulation of HSN activity. In addition, because the FLP-1 peptides are found only in head neurons, some of which synapse directly with the HSNs, these peptides might regulate vulval muscle activity through an HSN dependent mechanism as well. The FLP-1 peptides are implicated in regulation of egg-laying in response to sensory cues, and thus, FLP-1 peptides might signal through *goa-1* to regulate HSN activity in the presence and absence of food. It is then possible that the active and inactive egg-laying states might correspond to functional states of the HSNs: during the active state, the HSNs release neurotransmitter with a high probability and during the inactive state, the release probability is low. Advances in *C. elegans* electrophysiological techniques might soon resolve these questions and aid in our understanding of how multiple neuromodulatory pathways interact in more complex systems.

The study of egg-laying behavior has also provided a means for understanding the molecular mechanisms that underlie long term adaptation of behavior. *C. elegans* is capable of undergoing adaptation to prolonged exposure to nicotine, through long term

inactivation of nicotinic acetylcholine receptors (nAChRs). In egg-laying behavior, acute nicotine treatment results in stimulation of egg-laying, but long term exposure inhibits further stimulation by nicotinic agonists. In analyzing this process, I discovered that this adaptation was dependent on the protein kinase C homolog, *tpa-1*. Although the nAChRs function both in vulval muscles and VC neurons to regulate egg-laying, TPA-1 is localized to the vulval muscles, and thus, inactivation of muscle nAChRs appears to be the basis of nicotinic adaptation. Because this nicotinic adaptation is similar in time-course and persistence to functional inactivation of receptors in vertebrate CNS, the mechanisms might be analogous and future studies identifying signaling molecules and processes could further our understanding of the mechanisms involved in human nicotine addiction.

Lastly, while these studies have advanced our knowledge of *C. elegans* egg-laying and the processes by which adaptation of behavior in nematodes occurs, they also have provided a valuable model for understanding how a complicated behavior is controlled by multiple neurotransmitters released from a limited set of neurons. Hopefully, the analysis of egg-laying regulation in *C. elegans* will further our understanding of how signaling cascades converge to regulate behaviors in more complex organisms such as humans.

REFERENCES

1. White, J., E. Southgate, N. Thomson, and S. Brenner, *The structure of the Caenorhabditis elegans nervous system.* Philos. Trans. R. Soc. Lond. (Biol.), 1986. **314**: p. 1-340.

2. Sulston, J.E., E. Schierenberg, J.G. White, and J.N. Thomson, *The embryonic cell lineage of the nematode Caenorhabditis elegans.* Dev. Biol., 1983. **100**: p. 64-119.

3. Rand, J.B. and M.L. Nonet, *Synaptic transmission*, in *C. elegans II*, D.L. Riddle, *et al.*, Editors. 1997, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

4. Rand, J.B., *Genetic analysis of the cha-1-unc-17 gene complex in Caenorhabditis.* Genetics, 1989. **122**: p. 73-80.

5. Avery, L. and H.R. Horvitz, *Effects of starvation and neuroactive drugs on feeding in Caenorhabditis elegans.* J. Exp. Zool., 1990. **253**: p. 263-70.

6. Alfonso, A., K. Grundahl, J.S. Duerr, H.-P. Han, and J.B. Rand, *Cloning and characterization of the choline acetyltransferase structural gene (cha-1) from the nematode C. elegans.* J. Neurosci., 1994. **3**: p. 359-368.

7. Bejanin, S., R. Cervini, J. Mallet, and S. Berrard, *A unique gene organization for two cholinergic markers, choline acetyltransferase and a putative vesicular transporter of acetylcholine.* J. Biol. Chem., 1994. **269**: p. 21944-21947.

8. Lewis, J.A., C.-H. Wu, H. Berg, and J.H. Levine, *The genetics of levamisole resistance in Caenorhabditis elegans.* Genetics, 1980. **95**: p. 905-928.

9. Culotti, J.G. and W.L. Klein, *Occurrance of muscarinic acetylcholine receptors in wild-type and cholinergic mutants of C. elegans.* J. Neurosci., 1983. **3**: p. 359-368.

10. Lewis, J.A., C.-H. Wu, J.H. Levine, and H. Berg, *Levamisole-resistant mutants of the nematode Caenorhabditis elegans appear to lack pharmacological acetylcholine receptors.* Neuroscience, 1980. **5**: p. 967-89.

11. Fleming, J.T., M.D. Squire, T.M. Barnes, C. Tornoe, K. Matsuda, J. Ahnn, A. Fire, J.E. Sulston, E.A. Barnard, D.B. Sattelle, and J.A. Lewis, *Caenorhabditis elegans levamisole resistance genes lev-1, unc-29, and unc-38 encode functional nicotinic acetylcholine receptor subunits.* J. Neurosci., 1997. **17**: p. 5843-5857.

12. Treinin, M. and M. Chalfie, *A mutated acetylcholine receptor subunit causes neuronal degeneration in C. elegans.* Neuron, 1995. **14**.

13. Squire, M.D., C. Tornoe, H.A. Baylis, J.T. Fleming, E.A. Barnard, and D.B. Sattelle, *Molecular cloning and functional co-expression of a Caenorhabditis elegans nicotinic acetylcholine receptor subunit (acr-2).* Receptors and Channels, 1995. **3**: p. 107-115.

14. McIntire, S.L., E. Jorgensen, J. Kaplan, and H.R. Horvitz, *The GABAergic nervous system of Caenorhabditis elegans.* Nature, 1993. **364**: p. 337-341.

15. Desai, C., G. Garriga, S. McIntire, and H.R. Horvitz, *A genetic pathway for the development of the Caenorhabditis elegans HSN motor neurons.* Nature, 1988. **336**: p. 638-46.

16. Loer, C.M. and C.J. Kenyon, *Serotonin-deficient mutants and male mating behavior in the nematode Caenorhabditis elegans.* J. Neurosci., 1993. **13**: p. 5407-17.

17. Horvitz, H.R., M. Chalfie, C. Trent, and P.D. Evans, *Serotonin and octopamine in the nematode Caenorhabditis elegans.* Science, 1982. **216**: p. 1012-4.

18. Segalat, L., D.A. Elkes, and J.M. Kaplan, *Modulation of serotonin-controlled behaviors by G_o in Caenorhabditis elegans.* Science, 1995. **267**: p. 1648-51.

19. Schafer, W.R. and C.J. Kenyon, *A calcium channel homologue required for adaptation to dopamine and serotonin in Caenorhabditis elegans.* Nature, 1995. **375**: p. 73-8.

20. Sulston, J., M. Dew, and S. Brenner, *Dopaminergic neurons in the nematode Caenorhabditis elegans.* J. Comp. Neur., 1975. **163**: p. 215-226.

21. Hart, A.C., S. Sims, and J.M. Kaplan, *Synaptic code for sensory modalities revealed by C. elegans GLR-1 glutamate receptor.* Nature, 1995. **378**: p. 82-85.

22. Maricq, A.V., E. Peckol, M. Driscoll, and C.I. Bargmann, *Mechanosensory signaling in C. elegans mediated by the GLR-1 glutamate receptor.* Nature, 1995. **378**: p. 78-81.

23. Rosoff, M.L., T.R. Burglin, and C. Li, *Alternatively spliced transcripts of the flp-1 gene encode distinct FMRFamide-like peptides in Caenorhabditis elegans.* J. Neurosci., 1992. **12**: p. 2356-2361.

24. Schinkmann, K. and C. Li, *Localization of FMRFamide-like peptides in Caenorhaditis elegans.* J. Comp. Neurol., 1992. **316**: p. 251-60.

25. Driscoll, M. and J. Kaplan, *Mechanotransduction*, in *C. elegans II*, D.L. Riddle, *et al.*, Editors. 1997, Cold Spring Harbor Press: Cold Spring Harbor, NY.

26. Sulston, J.E. and H.R. Horvitz, *Post-embryonic cell lineages of the nematode Caenorhabditis elegans.* Dev. Biol., 1977. **56**: p. 110-156.

27. Walrond, J.P. and A.O. Stretton, *Reciprocol inhibition in the motor nervous system of the nematode Ascaris: Direct control of ventral inhibitory motorneurons by dorsal excitatory motorneurons.* J. Neurosci., 1985. **5**: p. 9-15.

28. Getting, P.A., *Comparative analysis of invertebrate central pattern generators.*, in *Neural control of rhythmic movements*, A.H.C.e. al., Editor. 1988, John Wiley: New York. p. 101-128.

29. McIntire, S.L., E. Jorgensen, and H.R. Horvitz, *Genes required for GABA function in Caenorhabditis elegans.* Nature, 1993. **364**: p. 334-337.

30. Mendel, J.E., H.C. Korswagen, K.S. Liu, Y.M. Hadju-Cronin, M.I. Simon, R.H. Plasterk, and P.W. Sternberg, *Participation of the protein G_o in multiple aspects of behavior in C. elegans.* Science, 1995. **267**: p. 1652-5.

31. Nurrish, S., L. Segalat, and J.M. Kaplan, *Serotonin inhibition of synaptic transmission: $G\alpha_o$ decreases the abundance of UNC-13 at release sites.* Neuron, 1999. **24**: p. 231-242.

32. Brundage, L., L. Avery, A. Katz, U.-J. Kim, J.E. Mendel, P.W. Sternberg, and M.I. Simon, *Mutations in a C. elegans $G_q\alpha$ gene disrupt movement, egg-laying, and viability.* Neuron, 1996. **16**(5): p. 999-1009.

33. Eglen, R.M., H. Feddy, N. Watson, and R.A.J. Challiss, *Muscarinic acetylcholine receptor subtypes in smooth muscle.* Trends. Pharmacol. Sci., 1994. **15**: p. 114-119.

34. Singer, W.D., H.A. Brown, and P.C. Sternweis, *Regulation of eukaryotic phosphotidalinositol-specific phospholipase C and phospholipase D.* Annual. Rev. Biochem., 1997. **66**: p. 475-509.

35. Miller, K.G., M.D. Emerson, and J.B. Rand, *$G_o\alpha$ and diacylglycerol kinase negatively regulate the $G_q\alpha$ pathway in C. elegans.* Neuron, 1999. **24**: p. 323-333.

36. Hajdu-Cronin, Y.M., W.J. Chen, G. Patikoglou, M.R. Koelle, and P.W. Sternberg, *Antagonism between $G_o\alpha$ and $G_q\alpha$ in C. elegans: the RGS protein EAT-16 is necessary for $G_o\alpha$ signaling and regulates $G_q\alpha$ activity.* Genes Dev., 1999. **13**: p. 1780-1793.

37. Albertson, D.G. and J.N. Thomson, *The pharynx of Caenorhabditis elegans.* Philos. Trans. R. Soc. Lond. B Biol. Sci., 1976. **275**: p. 299-325.

38. Avery, L. and J.H. Thomas, *Feeding and defecation*, in *C. elegans II*, D.L. Riddle, *et al.*, Editors. 1997, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

39. Avery, L. and H.R. Horvitz, *Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of C. elegans.* Neuron, 1989. **3**: p. 473-485.

40. Doncaster, C.C., *Nematode feeding mechanisms. I. Observations on Rhabditis and Pelodera.* Nematologica, 1962. **8**: p. 313-320.

41. Kim, Y.K., H.H. Valdivia, E.B. Maryon, P. Anderson, and R. Coronado, *High molecular weight proteins in the nematode C. elegans bind [3H]ryanodine and form a large conductance channel.* Biophys., 1992. **63**: p. 1379-1384.

42. Raizen, D.M. and L. Avery, *Electrical activity and behavior in the pharynx of Caenorhabditis elegans.* Neuron, 1994. **12**: p. 483-95.

43. Avery, L., *Motor neuron M3 controls pharyngeal muscle relaxation timing in Caenorhabditis elegans.* J. Exp. Biol., 1993. **175**: p. 283-297.

44. Raizen, D.M., Y.N. Lee, and L. Avery, *Interacting genes required for pharyngeal excitation by motorneuron MC in Caenorhabditis elegans.* Genetics, 1995. **141**: p. 1365-1382.

45. Arena, J.P., K.K. Liu, P.S. Paress, J.M. Schaeffer, and D.F. Cully, *Expression of a glutamate-activated chloride current in Xenopus oocytes injected with Caenorhabditis elegans RNA- Evidence for modulation by avermectin.* Mol. Brain. Res., 1992. **15**: p. 339-348.

46. Cully, D.F., D.K. Vassilatis, K.K. Liu, P.S. Paress, L.H.T. Van Der Ploeg, J.M. Schaeffer, and J.P. Arena, *Cloning of an avermectin-sensitive glutamate-gated chloride channel from Caenorhabditis elegans.* Nature, 1994. **371**: p. 707-711.

47. Avery, L. and H.R. Horvitz, *A cell that dies during wild-type C. elegans development can function as a neuron in a ced-3 mutant.* Cell, 1987. **51**: p. 1071-1078.

48. Lackner, M.R., S.J. Nurrish, and J.M. Kaplan, *Facilitation of synaptic transmission by EGL-30 $G_q\alpha$ and EGL-8 PLCβ: DAG binding to UNC-13 is required to stimulate acetylcholine release.* Neuron, 1999. **24**: p. 335-346.

49. Croll, N.A. and J.M. Smith, *Integrated behaviour in the feeding phase of Caenorhabditis elegans (Nematoda).* J. Zool., Lond., 1978. **184**: p. 507-517.

50. Liu, D.W. and J.H. Thomas, *Regulation of a periodic motor program in C. elegans.* J. Neurosci., 1994. **14**: p. 1953-1962.

51. Iwasaki, K., D.W.C. Liu, and J.H. Thomas, *Genes that control a temperature-compensated clock in Caenorhabditis elegans.* Proc. Natl. Acad. SCi., 1995. **92**: p. 10317-10321.

52. Thomas, J.H., *Genetic analysis of defecation in Caenorhabditis elegans.* Genetics, 1990. **124**: p. 855-872.

53. Tobin, A.J., *Molecular biological approaches to the synthesis and action of GABA.* Semin. Neurosci., 1991. **3**: p. 183-190.

54. Avery, T. and J.H. Thomas, *Feeding and Defecation*, in *C. elegans II*, D.L. Riddle, *et al.*, Editors. 1997, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY. p. 679-716.

55. Rand, J.B. and M.L. Nonet, *Synaptic Transmission*, in *C. elegans II*, D.L. Riddle, *et al.*, Editors. 1997, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY. p. 611-644.

56. Wong, A., P. Boutis, and S. Hekimi, *Mutations in the clk-1 gene of Caenorhabditis elegans affect developmental and behavioral timing.* Genetics, 1995. **139**: p. 1247-1259.

57. Trent, C., N. Tsung, and H.R. Horvitz, *Egg-laying defective mutants of the nematode Caenorhabditis elegans.* Genetics, 1983. **104**: p. 619-47.

58. Rand, J.B. and M.L. Nonet, *Neurotransmitter assignments for specific neurons*, in *C. elegans II*, D.L. Riddle, *et al.*, Editors. 1997, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

59. Weinshenker, J.H., G. Garriga, and J.H. Thomas, *Genetic and pharmacological analysis of neurotransmitters controlling egg-laying in C. elegans.* Journal of Neuroscience, 1995. **15**: p. 6975-6985.

60. Rand, J.B. and R.L. Russell, *Choline acetyltransferase-deficient mutants of the nematode Caenorhabditis elegans.* Genetics, 1984. **106**: p. 227-248.

61. Driscoll, M. and J. Kaplan, *Mechanotransduction*, in *C. elegans II*, D.L. Riddle, *et al.*, Editors. 1997, Cold Spring Harbor Press: Cold Spring Harbor, NY. p. 645-678.

62. Bargmann, C.I. and I. Mori, *Chemotaxis and thermotaxis*, in *C. elegans II*, D.L. Riddle, *et al.*, Editors. 1997, Cold Spring Harbor Laboratory Press: Cold Spring Harbor.

63. Bargmann, C.I., *Genetic and cellular analysis of behavior in C. elegans.* Ann. Rev. Neurosci., 1993. **16**: p. 47-71.

64. Weinshenker, D., G. Garriga, and J.H. Thomas, *Genetic and pharmacological analysis of neurotransmitters controlling egg-laying in C. elegans.* J. Neurosci., 1995. **15**: p. 6975-6985.

65. Thomas, J.H., D. Birnby, and J. Vowels, *Evidence for parallel processing of sensory information controlling dauer formation.* Genetics, 1993. **134**: p. 1105-1117.

66. Koelle, M.R. and H.R. Horvitz, *Egl-10 regulates G-protein signaling in the C. elegans nervous system and shares a conserved domain with many mammalian proteins.* Cell, 1996. **84**: p. 115-125.

67. Zhou, G.T., W.R. Schafer, and R.W. Schafer, *A three-state biological point process model and its parameter estimation.* IEEE Trans. on Signal Processing, 1997. **46**: p. 2698-2707.

68. Croll, N.A., *Indolealkylamines in the coordination of nematode behavioral activities.* Can. J. Zool., 1975. **53**: p. 894-903.

69. Sano, T., Y. Tabuse, K. Nishiwaki, and J. Miwa, *The tpa-1 gene of Caenorhabditis elegans encodes two proteins similar to Ca(2+)-independent protein kinase Cs: evidence by complete genomic and complementary DNA sequences of the tpa-1 gene.* J. Mol. Biol, 1995. **251**: p. 477-485.

70. Garriga, G., C. Desai, and H.R. Horvitz, *Cell interactions control the direction of outgrowth, branching and fasciculation of the HSN axons of Caenorhabditis elegans.* Development, 1993. **117**: p. 1071-1087.

71. Loer, C.M., *Characterization of serotonin-deficient mutants in the nematode C. elegans.* Soc. Neurosci. Abstr., 1995. **21**: p. 511.9.

72. Lee, R.Y.N., L. Lobel, M. Hengartner, H.R. Horvitz, and L. Avery, *Mutations in the $\alpha 1$ subunit of an L-type voltage-activated Ca^{2+} channel cause myotonia in Caenorhabditis elegans.* EMBO J., 1997. **16**: p. 6066-6076.

73. Boland, L.M., A.C. Allen, and R. Dingledine, *Inhibition by bradykinin of voltage-activated barium current in a rat dorsal root ganglion cell line: role of protein kinase C.* J. Neurosci., 1991. **11**: p. 1140-1149.

74. Worley, J.F., J.M. Quayle, N.B. Standen, and M.T. Nelson, *Regulation of single calcium channels in cerebral arteries by voltage, serotonin, and dihydropyridines.* Am. J. Physiol., 1991. **261**: p. H1951-H1960.

75. Hirakawa, Y., T. Kuga, S. Kobayashi, H. Kanaide, and A. Takeshita, *Dual regulation of L-type Ca2+ channels by serotonin receptor stimulation in vascular smooth muscle cells.* Am. J. Physiol., 1995. **268**: p. H544-H549.

76. Yang, M.M., Y.-L. Yo, J.-T. Hseih, and R. Ong, *5-hydroxytryptamine receptor-mediated phosphoinositide hydrolysis in canine cultured tracheal smooth muscle cells.* Br. J. Pharmacol., 1994. **111**: p. 777-786.

77. Nelson, I.D. and H. Huddart, *Neuromodulation in molluscan smooth muscle: the action of 5-HT, FMRFamide and purine compounds.* Gen. Pharmacol., 1994. **25**: p. 539-552.

78. Extein, I.L. and M.S. Gold, *Hypothesized neurochemical models for psychiatric syndromes in alchohol and drug dependence.* J Addictive Diseases, 1993. **12**: p. 29-43.

79. Soubrie, P., *Serotonin and behavior, with special regard to animal models of anxiety, depression, and waiting ability.*, in *Neuronal serotonin*, N.N. Osborne and M. Hamon, Editors. 1988, Wiley: New York.

80. Meston, C.M. and B.B. Gorzalka, *Psychoactive drugs and human sexual behavior: the role of serotonergic activity.* J. Psychoactive Drugs, 1992. **24**: p. 1-40.

81. Portas, C.M., M. Thakkar, D. Rainnie, and R.W. McCarley, *Microdialysis perfusion of 8-hydroxy2-(di-n-propylamino)tetralin (8-OH-DPAT) in the dorsal Raphe nucleus decreases serotonin release and increases rapid eye movement sleep in the freely moving cat.* J. Neurosci., 1996. **16**: p. 2820-2828.

82. Olivier, B. and J. Mos, *Rodent models of aggressive behavior and serotonergic drugs.* Prog. Neuro-Psychopharmacol. and Biol. Psychiat., 1992. **16**: p. 847-870.

83. Yeh, S.R., R.A. Fricke, and D.H. Edwards, *The effect of social experience on serotonergic modulation of the escape circuit of crayfish.* Science, 1996. **271**: p. 366-369.

84. Sandou, F., D.A. Amara, A. Dierich, M. LeMeur, S. Ramboz, L. Segu, M.-C. Buhot, and R. Hen, *Enhanced aggressive behavior in mice lacking 5HT1B receptor.* Science, 1994. **265**: p. 1875-1878.

85. Brunner, H.G., M. Nelen, X.O. Breakfield, H.H. Ropers, and B.A. van Oost, *Abnormal behavior associated with a point mutation in the structural gene for monoamine oxidase A.* Science, 1993. **262**: p. 578-580.

86. Huber, R. and E.A. Kravitz, *A quantitative analysis of agonistic behavior in juvenile American lobsters.* Brain Beh. Evol., 1995. **46**: p. 72-83.

87. Huber, R., K. Smith, A. Delago, K. Isaksson, and E.A. Kravitz, *Serotonin and aggressive motivation in crustaceans: Altering the decision to retreat.* Proc. Natl. Acad. Sci. USA, 1997. **94**: p. 5939-5942.

88. Kupfermann, I., *Functional studies of cotransmission.* Physiol. Rev., 1991. **71**: p. 683-732.

89. Marder, E., A.E. Christie, and V.L. Kilman, *Functional organization of cotransmission systems: lessons from small nervous systems.* Invert. Neurosci., 1995. **1**: p. 105-112.

90. Tabuse, Y., K. Nishiwaki, and J. Miwa, *Mutations in a protein kinase C homolog confer phorbol resistance in Caenorhabditis elegans.* Science, 1989. **243**: p. 1713-1716.

91. Sawin, E.R., *Genetic and cellular analysis of modulated behaviors in Caenorhabditis elegans*, in *Biology*. 1996, M. I. T.: Cambridge, MA.

92. Bargmann, C.I. and L. Avery, *Laser killing of cells in Caenorhabditis elegans. Meth. Cell Biol.*, 1995. **48**: p. 225-250.

93. Wood, W.B., ed. *The nematode Caenorhabditis elegans.* . 1988, Cold Spring Harbor Laboratory: Cold Spring Harbor, NY.

94. Rand, J.B. and C.D. Johnson, *Genetic pharmacology: interactions between drugs and gene products in Caenorhabditis elegans.* Meth. Cell Biol., 1995. **48**: p. 187-204.

95. Maule, A.G., J.W. Bowman, D.P. Thompson, N.J. Marks, A.R. Friedman, and T.G. Geary, *FMRFamide-related peptides (FaRPs) in nematodes: occurrence and neuromuscular physiology.* Parasitology, 1996. **113**: p. S119-S135.

96. Yang, H.-Y.T., W. Fratta, E.A. Majane, and E. Costa, *Isolation, sequencing, synthesis and pharmacological characterization of two brain neuropeptides that modulate the action of morphine.* Proc. Natl. Acad. Sci. USA, 1985. **82**: p. 7757-7761.

97. Nelson, L.S., K. Kim, J.E. Memmott, and C. Li, *FMRFamide-related gene family in the nematode Caenorhabditis elegans.* Mol. Brain Res., 1998. **58**: p. 103-111.

98. Nelson, L.S., M.L. Rosoff, and C. Li, *Disruption of a neuropeptide gene, flp-1, causes multiple behavioral defects in Caenorhabditis elegans.* Science, 1998. **281**: p. 1686-1690.

99. Waggoner, L., G.T. Zhou, R.W. Schafer, and W.R. Schafer, *Control of behavioral states by serotonin in Caenorhabditis elegans.* Neuron, 1998. **21**: p. 203-214.

100. Scott, M.L., V. Brezina, and K.R. Weiss, *Ion currents and mechanisms of modulation in the radula opener muscles of Aplysia.* J. Neurophysiol., 1997. **78**: p. 2372-2387.

101. Klein, M., B. Hochner, and E.R. Kandel, *Facilitatory transmitters and cAMP can modulate accomodation as well as transmitter release in Aplysia sensory neurons: evidence for parallel processing in a single cell.* Proc. Natl. Acad. Sci. USA, 1986. **83**: p. 7994-7998.

102. Mori, I. and Y. Ohshima, *Neural regulation of thermotaxis in Caenorhabditis elegans.* Nature, 1995. **376**: p. 344-8.

103. Duerr, J.S., D.L. Frisby, J. Gaskin, A. Duke, K. Asermely, D. Huddleston, L.E. Eiden, and J.B. Rand, *The cat-1 gene of Caenorhabditis elegans encodes a vesicular monoamine transporter required for specific monoamine-dependent behaviors.* J. Neurosci., 1999. **19**: p. 72-84.

104. Brenner, S., *The genetics of Caenorhabditis elegans.* Genetics, 1974. **77**: p. 71-94.

105. Dani, J.A. and S. Heinemann, *Molecular and cellular aspects of nicotine abuse.* Neuron, 1996. **16**: p. 905-908.

106. Simasko, S.M., J.R. Soares, and G.A. Weiland, *Two components of carmamylcholine-induced loss of nicotinic acetylcholine receptor function in the neuronal cell line PC12.* Mol. Pharmacol., 1986. **30**: p. 6-12.

107. Ballivet, M., C. Alliod, S. Bertrand, and D. Bertrand, *Nicotinic acetylcholine receptors in the nematode Caenorhabditis elegans.* J. Mol. Biol., 1996. **258**: p. 261-269.

108. Fleming, J.T., C. Tornoe, H.A. Riina, J. Coadwell, J.A. Lewis, and D.B. Satelle, *Acetylcholine receptor molecules of the nematode Caenorhabditis elegans.* EXS, 1993. **63**: p. 65-80.

109. Bargmann, C.I., *Neurobiology of the Caenorhabditis elegans genome.* Science, 1998. **282**: p. 2028-2033.

110. Rankin, C.H., C. Chiba, and C. Beck, *Caenorhabditis elegans: a new model system for the study of learning and memory.* Behav. Brain Res., 1990. **37**: p. 89-92.

111. Colbert, H.A. and C.I. Bargmann, *Odorant-specific adaptation pathways generate olfactory plasticity in C. elegans.* Neuron, 1995. **14**: p. 803-12.

112. Hedgecock, E.M. and R.L. Russell, *Normal and mutant thermotaxis in the nematode Caenorhabditis elegans.* Proc. Natl. Acad. Sci. USA, 1975. **72**: p. 4061-5.

113. Wen, J.Y., N. Kumar, G. Morrison, G. Rambaldini, S. Runciman, J. Rousseau, and D. van der Kooy, *Mutations that prevent associative learning in C. elegans.* Behav. Neurosci., 1997. **111**: p. 354-368.

114. Miller, L.M., D.A. Waring, and S.K. Kim, *Mosaic analysis using a ncl-1 (+) extrachromosomal array reveals that lin-31 acts in the Pn.p cells during Caenorhabditis elegans vulval development.* Genetics, 1996. **143**: p. 1181-1191.

115. Pontieri, F.E., G. Tanda, F. Orzi, and G. Di Chiara, *Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs.* Nature, 1996. **382**: p. 255-257.

116. Pidoplichko, V.I., M. DeBiasi, J.T. Williams, and J.A. Dani, *Nicotine activates and desensitizes midbrain dopamine neurons.* Nature, 1997. **390**: p. 401-404.

117. McGehee, D.S., M.J. Heath, S. Gelber, P. Devay, and L.W. Role, *Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors.* Science, 1995. **269**: p. 1692-1696.

118. Gray, R., A.S. Rajan, K.A. Radcliffe, M. Yakehiro, and J.A. Dani, *Hippocampal synaptic transmission enhanced by low concentrations of nicotine.* Nature, 1996. **383**(6602): p. 713-716.

119. Tuominen, R.K., M.K. McMillan, H. Ye, M.K. Stachkowiak, P.M. Hudson, and J.S. Hong, *Long-term activation of protein kinase C by nicotine in bovine adrenal chromaffin cells.* J. Neurochem., 1992. **58**: p. 1652-1658.

120. Hsu, Y.-N., S.E. Edwards, and L. Wecker, *Nicotine enhances the cyclic AMP-dependent kinase-mediated phosphorylation of α4 subunits of neuronal nicotinic receptors.* Journal of Neurochemistry, 1997. **69**(6): p. 2427-2431.

121. Hsu, Y.-N., J. Amin, D.S. Weiss, and L. Wecker, *Sustained nicotine exposure differentially affects α3β2 and α4β2 neuronal nicotinic receptor expressed in Xenopus oocytes.* J. Neurochem., 1996. **66**: p. 667-675.

122. Eilers, H., E. Schaeffer, P.E. Bickler, and J.R. Forsayeth, *Functional deactivation of the major neuronal nicotinic receptor cuased by nicotine and a protein kinase C-dependent mechanism.* Molecular Pharmacology, 1997. **52**: p. 1105-1112.

123. Pearson, R.B. and B.E. Kemp, *Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations.* Meth. Enzymol., 1991. **200**: p. 62-81.

124. Huganir, R.L. and P. Greengard, *cAMP-dependent protein kinase phosphorylates the nicotinic acetylcholine receptor.* Proc. Natl. Acad. Sci. USA, 1983. **80**: p. 1130-1134.

125. Safran, A., R. Sagi-Eisenberg, D. Neumann, and S. Fuchs, *Phosphorylation of the acetylcholine receptor by protein kinase C and identification of the phosphorylation site within the receptor δ subunit.* J. Biol. Chem, 1987. **262**: p. 10506-10510.

126. Huganir, R.L., K. Miles, and P. Greengard, *Phosphorylation of the nicotinic acetylcholine receptor by an endogenous tyrosine-specific protein kinase.* Proc. Natl. Acad. Sci. USA, 1984. **81**: p. 6968-6972.

127. Huganir, R.L., A.H. Delcour, P. Greengard, and G.P. Hess, *Phosphorylation of the nicotinic acetylcholine receptor regulates its rate of desensitization.* Nature, 1986. **321**: p. 774-776.

128. Hopfield, J.F., D.W. Tank, P. Greengard, and R.L. Huganir, *Functional modulation of the nicotinic acetylcholine receptor by tyrosine phosphorylation.* Nature, 1988. **336**: p. 677-680.

129. Eusebi, F., M. Molinaro, and B.M. Zani, *Agents that activate protein kinase C reduced acetylcholine sensitivity in cultured myotubes.* Journal of Cell Biology, 1985. **100**: p. 1339-1342.

130. Downing, J.E. and L.W. Role, *Activators of protein kinase C enhance acetylcholine receptor desensitization in sympathetic ganglion neurons.* Proc. Natl. Acad. Sci. USA, 1987. **84**: p. 7739-7743.

131. Li, C. and M. Chalfie, *Organogenesis in C. elegans: positioning of neurons and muscles in the egg-laying system.* Neuron, 1990. **4**(5): p. 681-695.

132. Dal Santo, P., Logan, M.A., Chisholm, A.D., and E.M. Jorgensen, *The inositol trisphosphate receptor regulates a 50-second behavioral rhythm in C. elegans.* Cell, 1999. **98**: 757-767.

133. Chui, A. Y., Hunkapiller, M.W., Heller, E., Stuart, D. K., Hood, L.E., and F. Stumwasser, *Purification and primary structure the neuropeptide egg-laying hormone in Aplysia californica.* PNAS, 1979. **76**: p. 6656-6660.

134. Ebberink, R.H.M., Van Loenhout, H., Geraerts, W.P.M, and J. Joosse, *Purification and amino acid sequence of the ovulation neurohormone of Lymnaea stagnalis.* PNAS, 1985. **82**: p. 7767-7771.

135. Salzet, M., Verger-Bocquet, M., Vandenbulcke, F., and J. Van Minnen, *Leech egg-laying-like hormone: structure, neuronal distribution and phylogeny.* Molecular Brain Research, 1997. **49**: p. 211-221.

136. White, B.H. and L.K. Kaczmarek, *Identification of a vesicular pool of calcium channels in the bag cell neurons of Aplysia californica.* Journal of Neuroscience, 1997. **17**: p. 1582-1595.

137. Monsma, S.A. and M.F. Wolfner, *Structure and expression of a Drosophila male accessory gland gene whose product resembles a petpide prehormone precursor.* Genes and Development, 1988. **2**: p. 1063-1073.

138. Scheller, F.H., Jackson, J.F., McAlister, L.B., Rothman, B.S., Mayeri, E., and R. Axel, *A single gene encodes multiple neuropeptides mediating sterotyped behavior.* Cell, 1983. **32**: 7-22.