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Control of Alternative Behavioral States by Serotonin in *Caenorhabditis elegans*

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Summary

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 motor neurons, HSNL/HSNR 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 protein kinase C–dependent (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 (Sulston and Horvitz, 1977; Sulston et al., 1983; White et al., 1986). This anatomically simple nervous system is capable of perceiving and responding to a wide range of environmental conditions, including heavy and light touch (Driscoll and Kaplan, 1997), temperature, volatile odorants, osmotic and ionic strength, food, and other nematodes (Bargmann and Mori, 1997). 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 (Avery and Thomas, 1997). *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 (Bargmann, 1993).

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 (White et al., 1986). Cell ablation experiments have demonstrated that the HSNs are required for efficient egg laying (Trent et al., 1983; Desai et al., 1988). These neurons contain multiple neurotransmitters (Desai et al., 1988; Schinkmann and Li, 1992; Rand and Nonet, 1997), and pharmacological and genetic studies have strongly indicated that these transmitters function in a complex but poorly understood fashion to control egg-laying behavior (Weinshenker et al., 1995). Genetic screens have identified a number of genes whose products are important for the regulation of egg-laying behavior (Trent et al., 1983; Desai et al., 1988; Thomas et al., 1993; Mendel et al., 1995; Segalat et al., 1995; Koelle and Horvitz, 1996), 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 switch-

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ing 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/hr), 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 s. These clustered events were interrupted by inactive periods, averaging 20 min 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 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 (Zhou et al., 1997). Our objectives were 2-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 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 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 (Figures 1d and 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 (Zhou et al., 1997). 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 motor neurons 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 2a). Since loss of the HSNs decreased the frequency of egg-laying clusters (i.e., λ_2 was decreased; Table 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 (Desai et al., 1988; Schinkmann and Li, 1992; Rand and Nonet, 1997). Serotonin has been shown to stimulate egg laying in nematodes (Croll, 1975; Horvitz et al., 1982); 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 2b). Moreover, continuous exposure of HSN-defective animals to exogenous serotonin (Figure 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

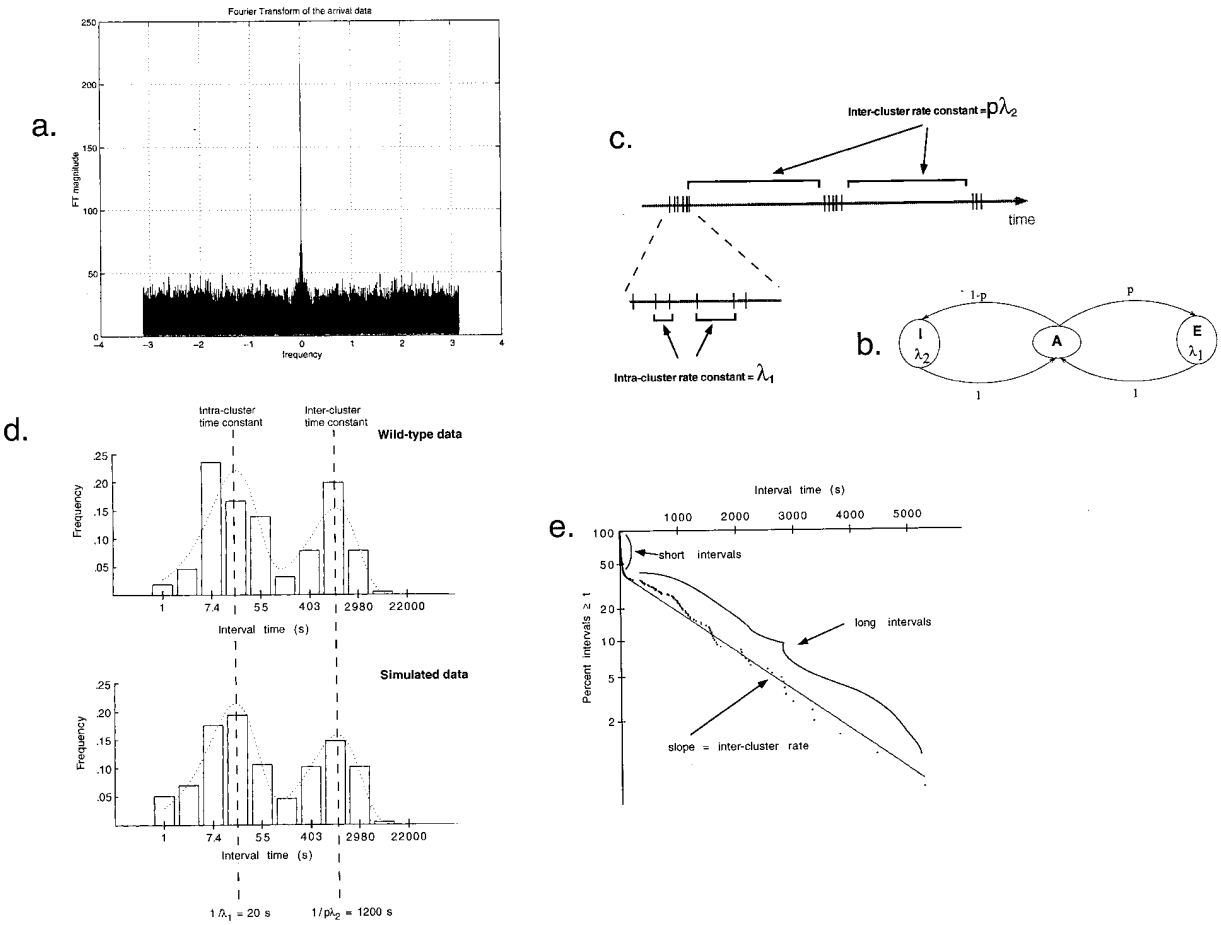

Figure 1. Egg Laying as a Three-State Point Process

Seven wild-type animals were tracked for a total of 40 hr, 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.

(a) Aperiodicity of the egg-laying pattern. 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 nonzero frequency.

(b) 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 (Zhou et al., 1997).

(c) 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 Zhou et al., 1997).

(d) 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 graph. The dotted lines indicate the ideal distribution based on ML analysis of the real data.

(e) Log tail distribution of intervals between egg-laying events, showing close agreement between theoretical probability and real data. 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 intercluster 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 (d).

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

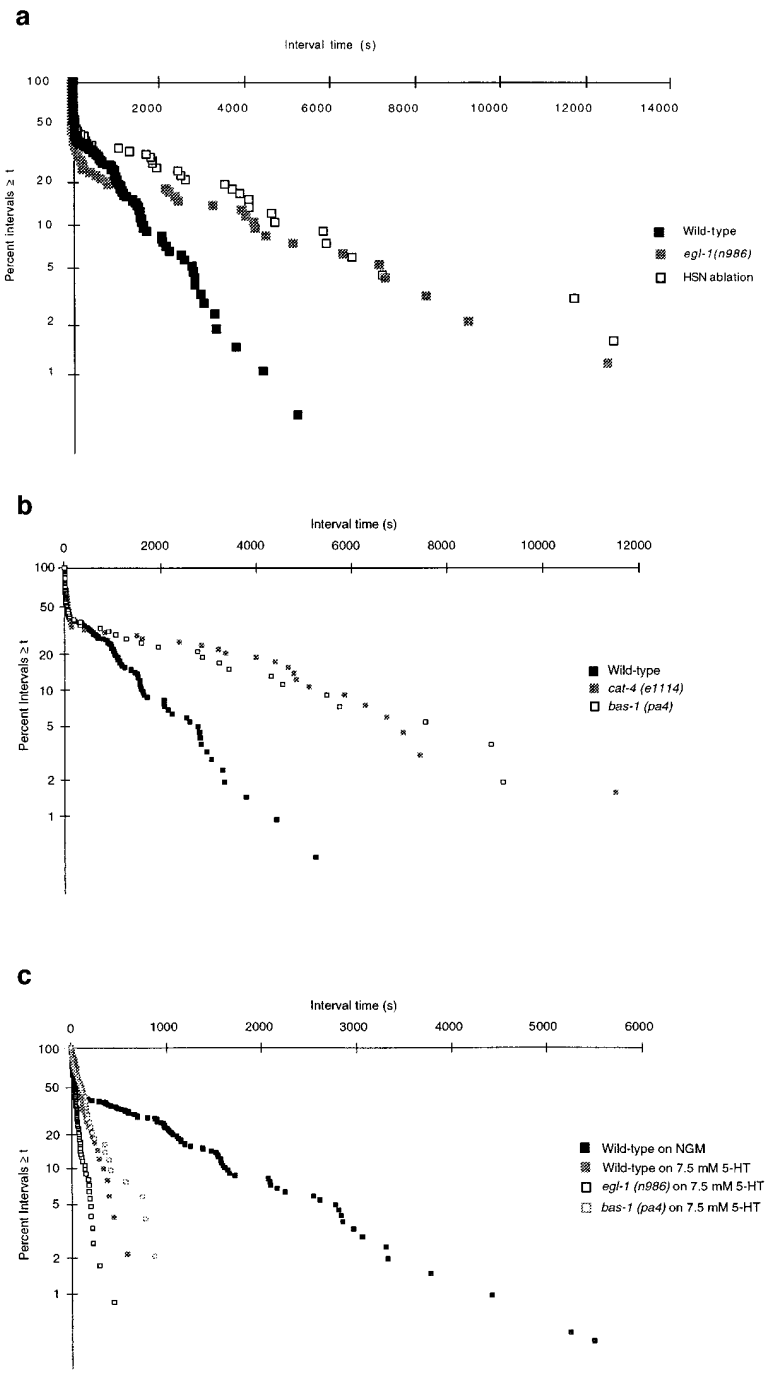

Figure 2. Induction of Active State by Serotonin

(a) 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) (Desai et al., 1988) compared to nonablated N2 animals on NGM. The more gradual slope (see Figure 1e legend) of the *egl-1* and HSN-ablated curves show that the intercluster intervals are significantly longer in the HSN-deficient animals. The duration of the long intervals (i.e., >300 s) for HSN-ablated and *egl-1* animals were statistically different from unablated wild type (level of confidence <0.001) according to the Mann-Whitney rank sum test.

(b) 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 (Loer and Kenyon, 1993), compared to the wild-type control. The more gradual slope of the *bas-1* and *cat-4* tails indicates a longer intercluster time constant. The long intervals in the mutant animals (including *bas-1(ad446)*; see Table 1) were statistically longer than in wild type (level of confidence <0.005 for *pa4* and *e1141*; <0.02 for *ad446*) according to the Mann-Whitney rank sum test.

(c) 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*, 125 s for *bas-1*, and 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 was usually essentially empty (10 min for *egl-1* and 20 min 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; and *bas-1*, 9 animals, 49 intervals.

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 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

Table 1. ML Estimates of Egg-Laying Parameters for Wild-Type, Mutant, and Ablated Animals

Animal Type (number, hours, intervals)	Intracluster Time Constant ($1/\lambda_1$; s)	Intercluster Time Constant ($1/p\lambda_2$; s)	p	λ_1 (s^{-1})	λ_2 ($s^{-1} \times 10^{-3}$)
N2[a]	18	1200	0.572	0.056	1.46
(7, 40, 216)	±2	±170	±0.037	±0.008	±0.23
N2, HSN−	8	3400	0.534	0.125	0.55
(9, 44, 66)	±2	±900	±0.069	±0.049	±0.16
egl-1(n986)	9	3630	0.685	0.106	0.40
(9, 46, 94)	±2	±1020	±0.056	±0.019	±0.10
bas-1(pa4)	17	3480	0.619	0.060	0.46
(6, 38, 52)	±4	±1080	±0.072	±0.014	±0.15
bas-1(ad446)	22	1870	0.446	0.046	1.22
(12, 52, 63)	±6	±450	±0.072	±0.016	±0.42
cat-4(e1114)	43	4550	0.609	0.023	0.36
(12, 52, 63)	±9	±1240	±0.073	±0.007	±0.13
tpa-1(k501)	33	3060	0.448	0.030	0.73
(7, 35, 66)	±8	±630	±0.059	±0.016	±0.19
tpa-1(k530)	18	1970	0.508	0.055	1.04
(5, 24, 49)	±6	±540	±0.083	±0.021	±0.42
CF453[b]	26	1770	0.539	0.038	1.05
(7, 24, 96)	±6	±360	±0.062	±0.009	±0.28
CF453, VC4/5-	11	2300	0.555	0.089	0.75
(8, 53, 133)	±2	±430	±0.047	±0.017	±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.

this category were strains carrying loss-of-function alleles of the gene *tpa-1* (Sano et al., 1995), which encodes the *C. elegans* homolog of 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 intercluster time constant (Figure 3b; Table 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 3c). Thus, PKC 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 (Figures 3a and 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 nonresponsive to serotonin (Figure 4 legend; see also Trent et al., 1983), 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 (White et al., 1986). 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 (Garriga et al., 1993). 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 <0.02) in the intercluster time constant (Table 1). Since VC4 and VC5 appear to be weakly serotonergic (Rand and Nonet, 1997), 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).

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/VC5 and HSNL/HSNR were much more severely defective in egg laying than animals defective in only the HSNs (Figure 5 legend). 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 (Figures 5a and 5b). These results suggest that a second neurotransmitter, which can be provided by either the HSNs or the 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 (Rand and

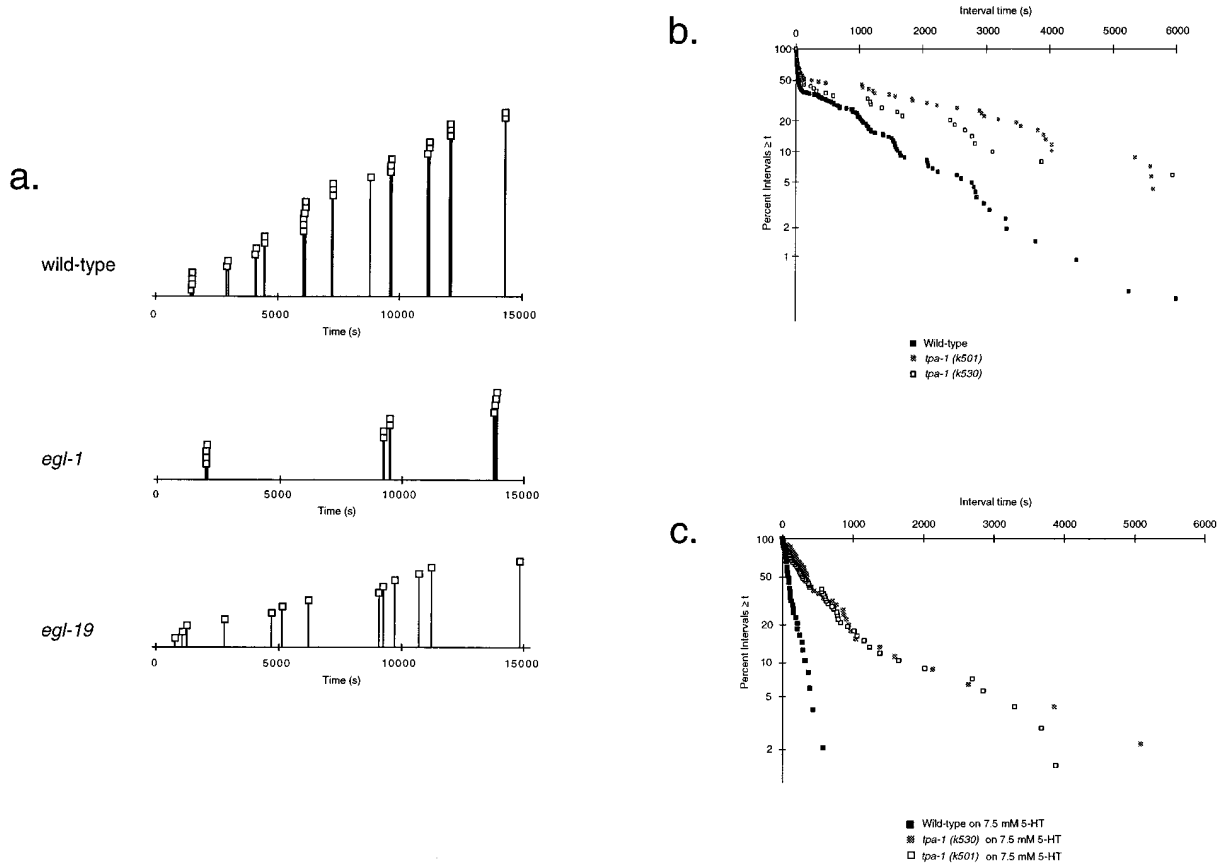

Figure 3. Dependence of Active State Induction on Protein Kinase C

(a) 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 nonresponsive (Trent et al., 1983).

(b) 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) indicates a longer intercluster time constant. The long intervals (>300 s) in the mutant animals were statistically longer than in wild type (level of confidence <0.001 for *k501* and <0.02 for *k530*) according to the Mann-Whitney rank sum test.

(c) 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*, and 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 <0.001) 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.

Nonet, 1997). Acetylcholine receptor agonists stimulate egg laying in an HSN-dependent manner (Trent et al., 1983; Weinshenker et al., 1995) 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 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 intracluster egg-laying rate (i.e., λ_1 was decreased; Figure 5c) but had little or no effect on the intercluster 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

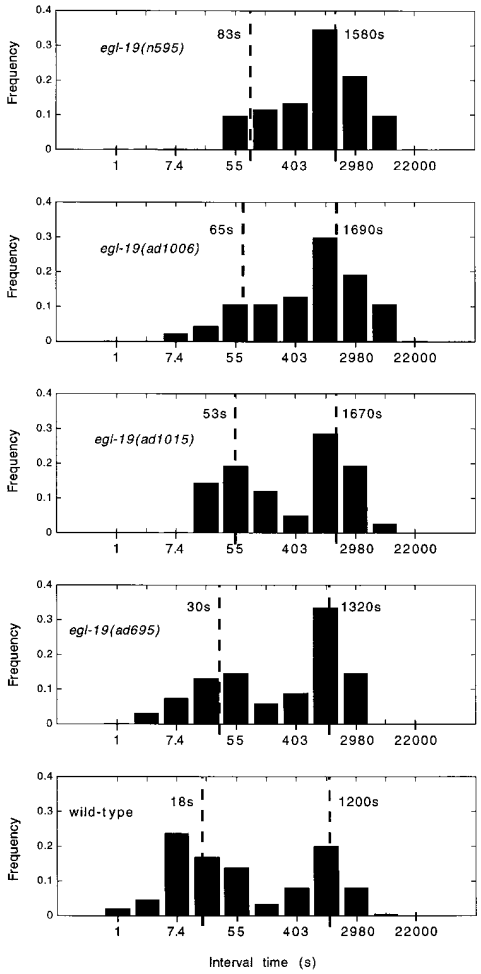

Figure 4. Effect of *egl-19* on the Egg-Laying Pattern

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 (Lee et al., 1997) also decreased the intracluster egg-laying rate. The durations of the short intervals (i.e., $<$300 s) in the mutants were significantly longer than in wild type in all mutants according to the Mann-Whitney rank sum test (significance $<$0.001 for all recessive alleles and $<$0.02 for *ad695*). ML parameter estimates (p, λ_1 , λ_2) for each allele were: 0.171, 0.012, and 0.0037 for *n582*; 0.246, 0.016, and 0.0024 for *ad1006*; 0.400, 0.019, and 0.0015 for *ad1015*; and 0.334, 0.033, and 0.0023 for *ad695*. 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; and *ad695*, 3 animals, 24 hr, 69 intervals. All recessive *egl-19* mutants were completely serotonin resistant. Egg-laying rates (eggs/hr; mean $\pm$ SEM) in the presence and absence of 7.5 mM serotonin were: *ad1006*, 2.7 $\pm$ 0.3 (no serotonin), 0.7 $\pm$ 0.2 (with serotonin); *ad1015*, 3.9 $\pm$ 0.3 (no serotonin), 10.1 $\pm$ 0.3 (with serotonin); and *n582*, 50.0 $\pm$ 0.6 (no serotonin), 1.3 $\pm$ 0.4 (with serotonin).

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 (Loer and Kenyon, 1993), cause a specific increase in the intercluster time constant, but have little or no effect on the intracluster constant. In the *cat-4* mutant, a large increase in the intercluster time constant was also observed, along with a smaller increase in the intracluster time constant. Since the *cat-4* mutation appears to be somewhat pleiotropic (e.g., *cat-4* mutants apparently have a defective cuticle [Loer, 1995, Soc. Neurosci., abstract]), it is unclear whether the effect on the intracluster 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 (Trent et al., 1983); 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 (Sulston et al., 1975). Moreover, *C. elegans* mutants have been identified that are serotonin insensitive but can lay eggs in response to other pharmacological agents (Weinshenker et al., 1995). In this study, we observed that egg-laying events are still clustered in serotonin-deficient mutants (Figure 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) (Schinkmann and Li, 1992); 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 (Trent et al., 1983),

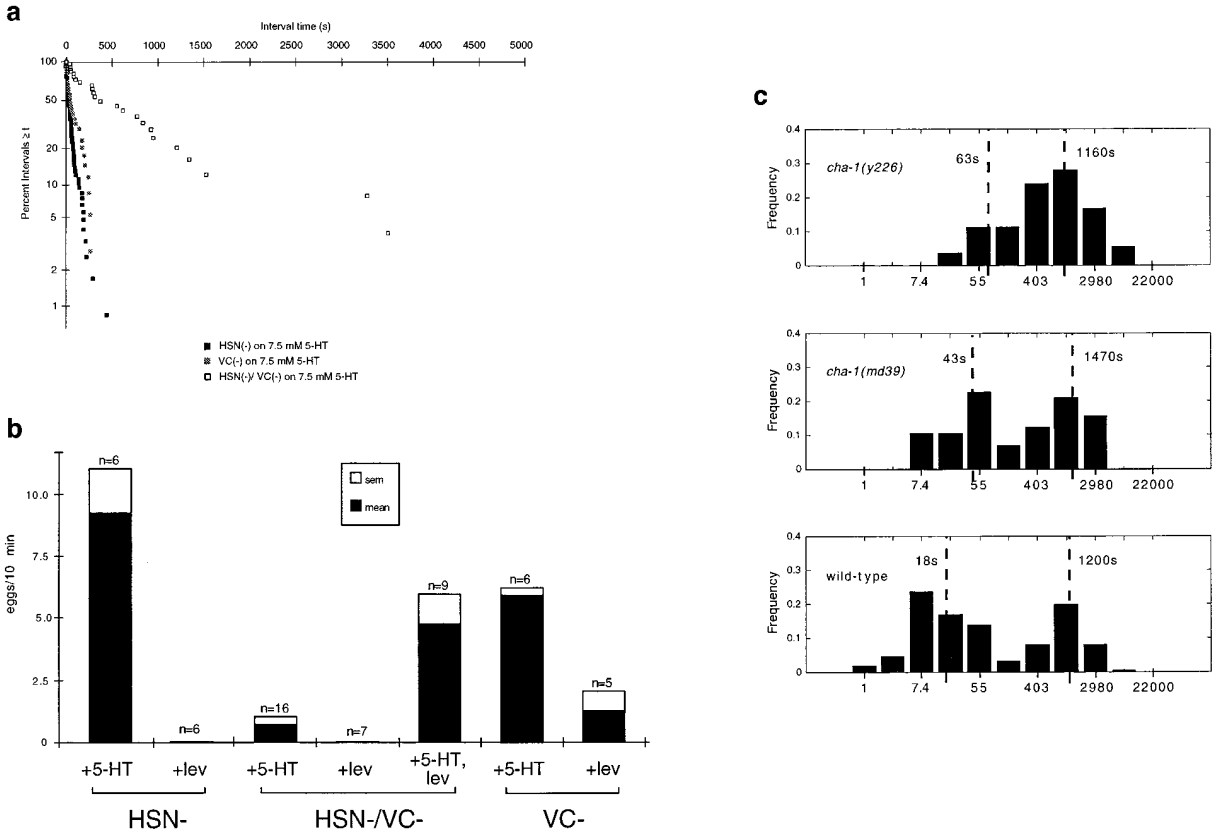

Figure 5. Dependence of Egg Laying on Acetylcholine

(a) 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−, and 99 s for VC−) indicated that the rate of egg laying in response to serotonin was greatly reduced (level of significance <0.001). 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 min, 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).

(b) 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 min as described in Experimental Procedures; 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 <0.005) or levamisole alone (level of significance <0.001) according to the Mann-Whitney rank sum test.

(c) 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 log egg laying intervals for animals carrying a conditional allele of *cha-1* (either *y226* or *md39*) grown to adulthood at 15°C and shifted to 22°C before tracking. Estimated time constants for long and short intervals are indicated. The short intervals (i.e., <300 s) in the mutants were statistically different from wild type (level of confidence <0.001) according to the Mann-Whitney rank sum test. ML parameter estimates for p, λ_1, and λ_2 were 0.205, 0.016, and 0.0042 and 0.463, 0.023, and 0.0015 for *y226* and *md39*, respectively.

and more recent work has suggested that acetylcholine might function in parallel with serotonin to stimulate egg laying (Weinshenker et al., 1995). 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

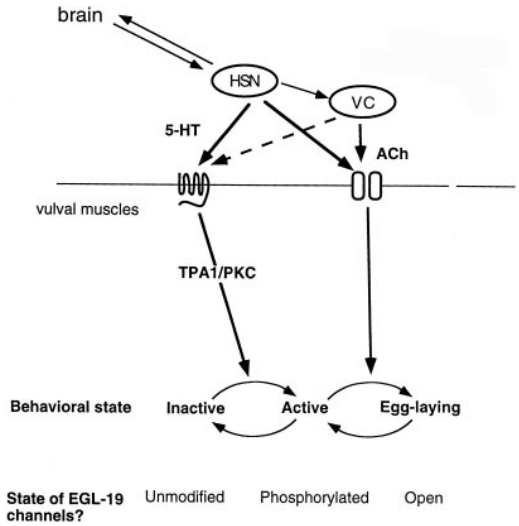

Figure 6. Molecular Model for the Control of Egg Laying

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.

in the active phase, a pattern that suggests a defect in acetylcholine release or response. As expected, most of these mutants were serotonin sensitive (Trent et al., 1983). 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 homolog, which is thought, based on genetic evidence, to function in the vulval muscles (Lee et al., 1997). 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 (Boland et al., 1991). 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 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 2; see also Experimental Procedures). Moreover, the egg-laying defects of *tpa-1* and *cat-4* were suppressed by a gain-of-function allele of *egl-19* (see Experimental Procedures). It is possible that modulation of other ion channels could contribute to the active state as well; for example, inhibition of potassium channels in the egg-laying muscles or neurons could increase the probability of egg-laying events 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 (Worley et al., 1991; Yang et al., 1994; Hirakawa et al., 1995). In mollusks, serotonin-dependent activation of calcium channel activity has been directly correlated with modulation of excitation-contraction coupling in smooth muscle cells (Nelson and Huddart, 1994). 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 (Soubrie, 1988; Meston and Gorzalka, 1992; Extein and Gold, 1993; Portas et al., 1996). In addition, a number of studies from organisms as diverse as crustaceans and humans have implicated serotonin in the control of aggression (Olivier and Mos, 1992; Brunner et al., 1993; Sandou et al., 1994; Yeh et al., 1996). For example, in lobsters, agonistic behavior involves a series of stereotyped, increasingly violent behavioral patterns, between which animals switch during the course of fights (Huber and Kravitz, 1995). 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 (Huber et al., 1997). 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

Table 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

VC motor neurons 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 postsynaptic cells that affect different parameters of the egg-laying pattern. This not only allows a particular class of motor neurons 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 postsynaptic 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 (Kupfermann, 1991). Thus, the complex temporal response patterns induced by cotransmitting neurons may be important for computation and behavioral control in more complex nervous systems (Marder et al., 1995). 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.

Experimental Procedures

Assay Conditions and Growth Media

Unless otherwise noted, nematodes were grown and assayed at room temperature on standard 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 for 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 60× magnification (the magnification necessary to observe eggs adequately and to 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 in Figure 1, we can prove that the random variable governing the egg-laying interval times has the following probability density function (p. d. f.) (Zhou et al., 1997):

$$fx(x) = k_1 \lambda_1 e^{-\lambda_1 x} + k_2 (p\lambda_2) e^{-(p\lambda_2)x},\ x \geq 0,$$
$$k_1 = \frac{p(\lambda_1 - \lambda_2)}{\lambda_1 - p\lambda_2},\ k_2 = \frac{\lambda_1(1 - p)}{\lambda_1 - p\lambda_2}.$$

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

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

which is a function of the parameter vector $\theta = [p, \lambda_1, \lambda_2]$. The maximum likelihood estimate is defined as the θ that maximizes $f(x|\theta)$ over all possible θs. The bimodal peak information provided by the histogram of the log intervals (see Figure 1d for example) was used to obtain a rough estimate of θ and initialize the nonlinear 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 ± 0.0356; λ_1: true = 0.0501, estimated = 0.0497 ± 0.0059; and λ_2: true = 0.0014, estimated = 0.0014 ± 0.0002. Standard deviations in Table 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 ($x > 5/\lambda_1$), $\ln \Pr(X \geq x)$ 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 1c), which can be shown to be represented by the following p. d. f. of Y:

$$f_Y(y) = [k_1\lambda_1\, e^{-\lambda_1 e^y} + k_2(p\lambda_2)\, e^{-(p\lambda_2)e^y}]\, e^y,$$
$$k_1 = \frac{p(\lambda_1 - \lambda_2)}{\lambda_1 - p\lambda_2},\ k_2 = \frac{\lambda_1(1-p)}{\lambda_1 - p\lambda_2}.$$

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 (Desai et al., 1988), or by ablating the HSN nuclei in the first larval stage. The VC4 and VC5 neurons were ablated in early fourth-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 (Tabuse et al., 1989); *egl-19* recessive alleles, long body length and sluggish movement; *egl-19(n2368)*, short body length (Lee et al., 1997); *cat-4*, hypochlorite hypersensitivity (Loer, 1995, Soc. Neurosci., abstract); and *bas-1*, failure to slow in the presence of bacteria (Sawin, 1996). 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 0.158, 0.020, and 0.0036 for *k501; n582* (6 animals, 38 hr, 13 intervals) and 0.255, 0.016, and 0.0030 for *k501; ad1015* (4 animals, 20 hr, 55 intervals), respectively. Egg-laying rates in Table 2 were determined after 1 hr 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.

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