Effect of a Neuropeptide Gene on Behavioral States in *Caenorhabditis elegans* Egg-Laying

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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 function 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. Because *flp-1* is known to be expressed in interneurons that are postsynaptic 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.

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 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 (Sulston and Horvitz 1977; Sulston *et al.* 1983; White *et al.* 1986). 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 (Bargmann and Avery 1995). 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 (Wood 1988). 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 (Rand and Nonet 1997). In addition, it exhibits a number of easily assayed and quantifiable behaviors that are affected by a wide range of neuroactive substances (Rand and Johnson 1995). 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 (FaRPs). These peptides are characterized by a common carboxy-terminal

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motif of Arg-Phe-NH_2 and generally range in size from 4 to 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 (Maule *et al.* 1996). FaRPs have also been identified in vertebrates, where they have been implicated in the regulation of pain responses (Yang *et al.* 1985). In *C. elegans*, immunocytochemical experiments have shown that at least 30 neurons and gland cells contain peptides that have the C-terminal RFamide epitope characteristic of the FaRP family (Schinkmann and Li 1992). The *C. elegans* genomic sequence contains at least 20 genes (designated *flp* genes, for *F*MRFamide-*l*ike *p*eptide deficient), which encode predicted polypeptide precursors of >50 distinct FaRPs (Nelson *et al.* 1998a). 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 (Rosoff *et al.* 1992; Nelson *et al.* 1998b). *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 (Nelson *et al.* 1998b). 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 that are at least partially distinct from the other *C. elegans* FaRPs.

In this study, we 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 2 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 (White *et al.* 1986). 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 1, a–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 (Waggoner *et al.* 1998). 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 (Zhou *et al.* 1997).

Genetic, pharmacological, and cell ablation studies have provided important insights into the roles of particular neurons and neurotransmitters in the control of egg-laying (Horvitz *et al.* 1982; Trent *et al.* 1983; Weinshenker *et al.* 1995). Two classes of motorneurons make extensive synapses with the vulval muscles: the two HSNs and six 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 (Desai *et al.* 1988; Schinkmann and Li 1992; Rand and Nonet 1997). Both serotonin and acetylcholine have been shown pharmacologically to increase the overall rate of egg-laying (Trent *et al.* 1983; Weinshenker *et al.* 1995). 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 (Waggoner *et al.* 1998). 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 (Waggoner *et al.* 1998).

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.

MATERIALS AND METHODS

Strains and genetic methods: Routine culturing of *C. elegans* was performed as described (Brenner 1974). The chromosomal locations of the genes studied in these experiments are as follows: LGI, *goa-1*; LGIV, *flp-1*; LGV, *cat-4*, *egl-1*. The *ynIs9* integrated array contains the wild-type *flp-1* allele and the coinjection *marker lin-11(+)*. Unless otherwise indicated, all mutant strains are in the N2 genetic background. Behavioral assays were performed at room temperature (~22°). Serotonin (creatinine sulfate complex) was obtained from Sigma (St. Louis). 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 (Nelson *et al.* 1998b). *goa-1(n1134)* was used for behavioral analysis and double mutant construction because it encodes a putatively nonfunctional product and because the near sterility conferred by the *n363* deficiency allele (brood size is ~35; Segalat *et al.* 1995) 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 nematode growth medium (NGM) seeded with *Escherichia 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-hr incubation at room temperature, the eggs laid by each animal were counted.

Experiments comparing egg-laying rates in the presence or absence of food were performed by transferring well-fed individual animals to NGM plates that were either seeded with a lawn of *E. coli* OP50 or were left unseeded. After 1 hr at room temperature, the eggs laid by each animal 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. We were unable to record the behavior of individual animals on plates completely lacking a bacterial lawn, because the animals were prone to crawl to the edge of the plate where our tracking system could not follow them. However, we were able to track wild-type (but not *flp-1*) animals on plates containing only a very small (1–2 mm diameter) bacterial spot; the egg-laying parameters of these animals are indicated in Table 1.

Egg-laying behavior of individual animals on solid media (NGM agar) was recorded at room temperature (22°) for 4–8 hr as described (Waggoner *et al.* 1998) using an automated tracking system. The *flp-1(yn2); ynIs9[hs:flp-1(+)]* strain was grown at 15° until early adulthood, then shifted to room temperature for tracking. For tracking experiments on serotonin, 5-hydroxytryptamine (creatinine sulfate complex; Sigma) was added to NGM agar at 7.5 mm.

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 (Zhou *et al.* 1997). 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 ("intercluster intervals") and the duration of intervals between

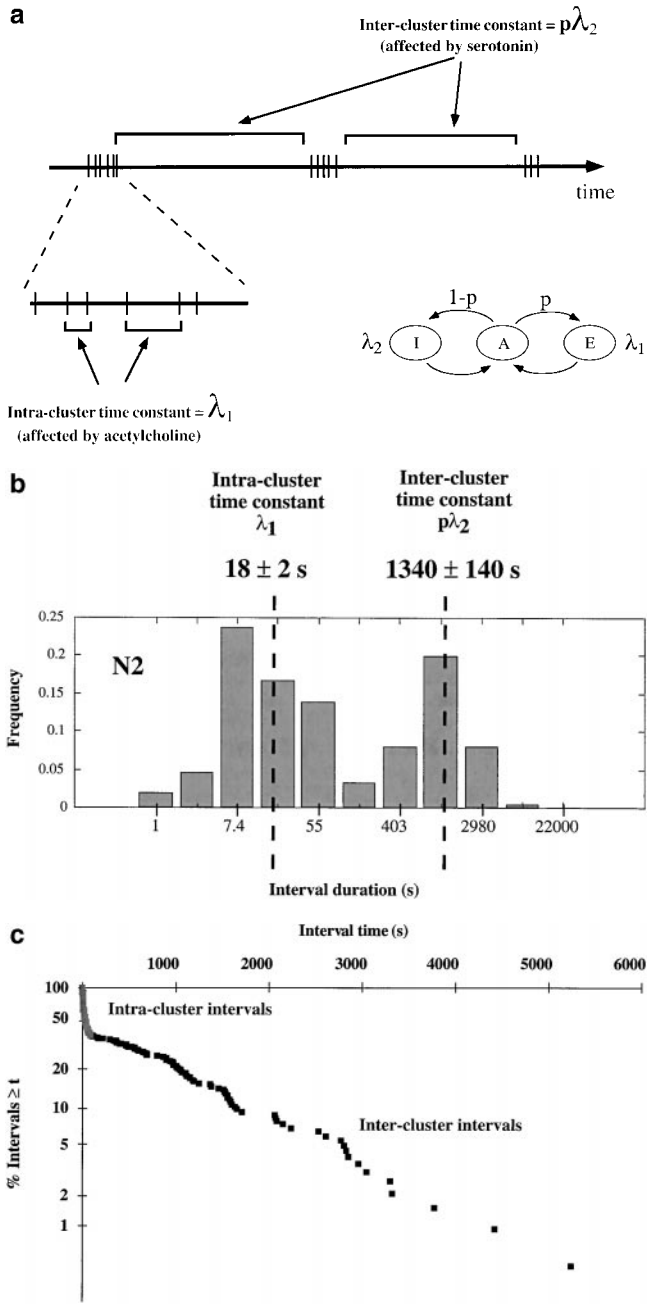


Figure 1.—Behavioral states in *C. elegans* egg-laying behavior. A more detailed description of the egg-laying pattern of *C. elegans* and its analysis is described in Waggoner *et al.* (1998). (a) Temporal pattern of egg-laying. Shown is a representative egg-laying pattern, with egg-laying events indicated 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 Zhou *et al.* 1997). (b) 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 intracluster and intercluster time constants. Values for these time constants were estimated using maximum-likelihood analysis (see materials and methods). (c) Log-tail distribution of egg-laying intervals. The log-tail distribution of egg-laying intervals from wild-type animals is shown. The duration of each interval (on the *x*-axis) was plotted against the percentage of intervals greater than or equal to that interval (on the *y*-axis; logarithimic scale). The model predicts that this distribution should be biphasic, with the steep part of the curve corresponding to the intracluster intervals and the more gradual part to the intercluster intervals. The slope of the long intervals (*i.e.*, the right part of the curve) is equal to the intercluster rate constant.

TABLE 1

Egg-laying behavior of mutant and ablated animals

Animal type (no., hr, intervals)	p	λ_1 (sec^{-1})	λ_2 ($sec^{-1} \times 10^{-3}$)	Intracluster time constant ($1/\lambda_1$; sec)	Intercluster time constant ($1/p\lambda_2$; sec)
N2 (12, 61, 305)	0.537 ± 0.031	0.055 ± 0.005	1.4 ± 0.18	18 ± 2	1340 ± 140
flp-1(yn2) (6, 37, 50)	0.554 ± 0.086	0.079 ± 0.022	0.47 ± 0.20	13 ± 3	3820** ± 1170
flp-1(yn4) (8, 37, 131)	0.651 ± 0.049	0.090 ± 0.016	0.75 ± 0.20	11 ± 2	2050* ± 460
flp-1(yn2); ynIs9[hs:flp-1(+)] (6, 25, 50)	0.468 ± 0.074	0.059 ± 0.019	1.25 ± 0.41	17 ± 5	1800 ± 430
cat-4(e1141) (12, 52, 63)	0.609 ± 0.073	0.023 ± 0.007	0.36 ± 0.13	43 ± 9	4550** ± 1240
flp-1(yn2); cat-4(e1141) (7, 35, 60)	0.772 ± 0.058	0.063 ± 0.014	0.30 ± 0.15	16 ± 3	4320 ± 1520
goa-1(n1134) (3, 27, 98)	0.357 ± 0.055	0.028 ± 0.009	3.1 ± 0.81	36 ± 16	890** ± 160
goa-1(n1134); flp-1(yn2) (6, 23, 66)	0.377 ± 0.069	0.174 ± 0.138	1.4 ± 0.38	6 ± 2	1950* ± 420
goa-1(n1134); cat-4(e1141) (6, 32, 101)	0.515 ± 0.056	0.141 ± 0.038	1.4 ± 0.30	7 ± 1	1380* ± 270
N2; HSN-ablated (9, 44, 66)	0.534 ± 0.069	0.125 ± 0.019	0.55 ± 0.16	8 ± 2	3400** ± 900
flp-1(yn2); HSN-ablated (5, 54, 77)	0.382 ± 0.068	0.041 ± 0.018	1.1 ± 0.29	25 ± 8	2440 ± 440
goa-1(n1134); HSN-ablated (4, 24, 51)	0.720 ± 0.062	0.072 ± 0.020	0.27 ± 0.10	14 ± 3	5140 ± 2060
N2 (food-deprived) (4, 18, 59)	0.816 ± 0.055	0.112 ± 0.025	0.3 ± 0.4	9 ± 2	4540** ± 2260

* and **, long intervals (>120 sec) 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. *, significance at $0.05 > P > 0.01$; **, significance at $P < 0.01$ or less.

egg-laying events in a cluster ("intra-cluster intervals") model as exponential random variables with different time constants (Waggoner *et al.* 1998). Thus, the probability density function for the intervals between events is

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

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

where the intracluster time constant is $1/\lambda_1$ and the intercluster time constant is $1/p\lambda_2$. The parameters were determined using the maximum-likelihood estimation technique described previously (Zhou *et al.* 1997). 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 1 were obtained and analyzed in this manner.

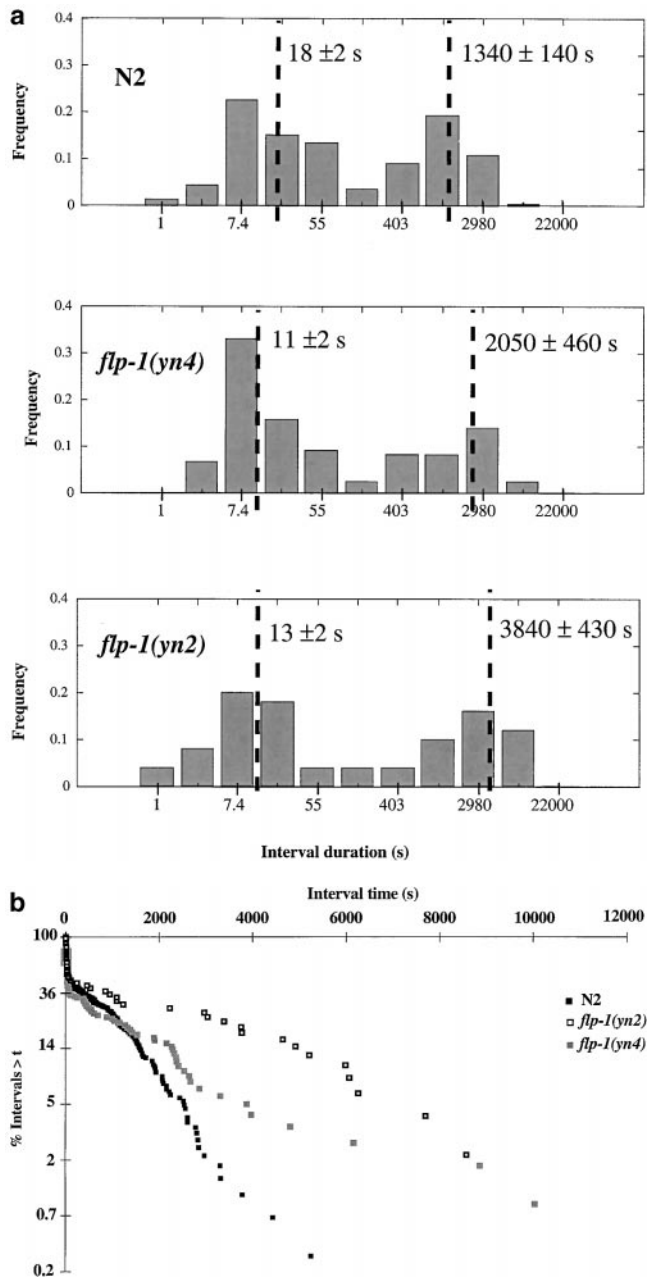
For analysis of egg-laying patterns on serotonin (Table 1), a single exponential time constant was estimated using a weighted least-squares linear regression to the log-tail distribution (Waggoner *et al.* 1998). The expected variance of these time constants was determined by generating 100 independent 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 F_2 generation by scoring for bleach sensitivity (*cat-4*; Loer 1995) and the presence of a diagnostic PCR product using sequence-specific primers (*flp-1*; Nelson *et al.* 1998b). 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 F_2 generation as hyperactive, egg-laying constitutive animals. These were picked individually and then allowed to self-fertilize; those F_2's 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.

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-

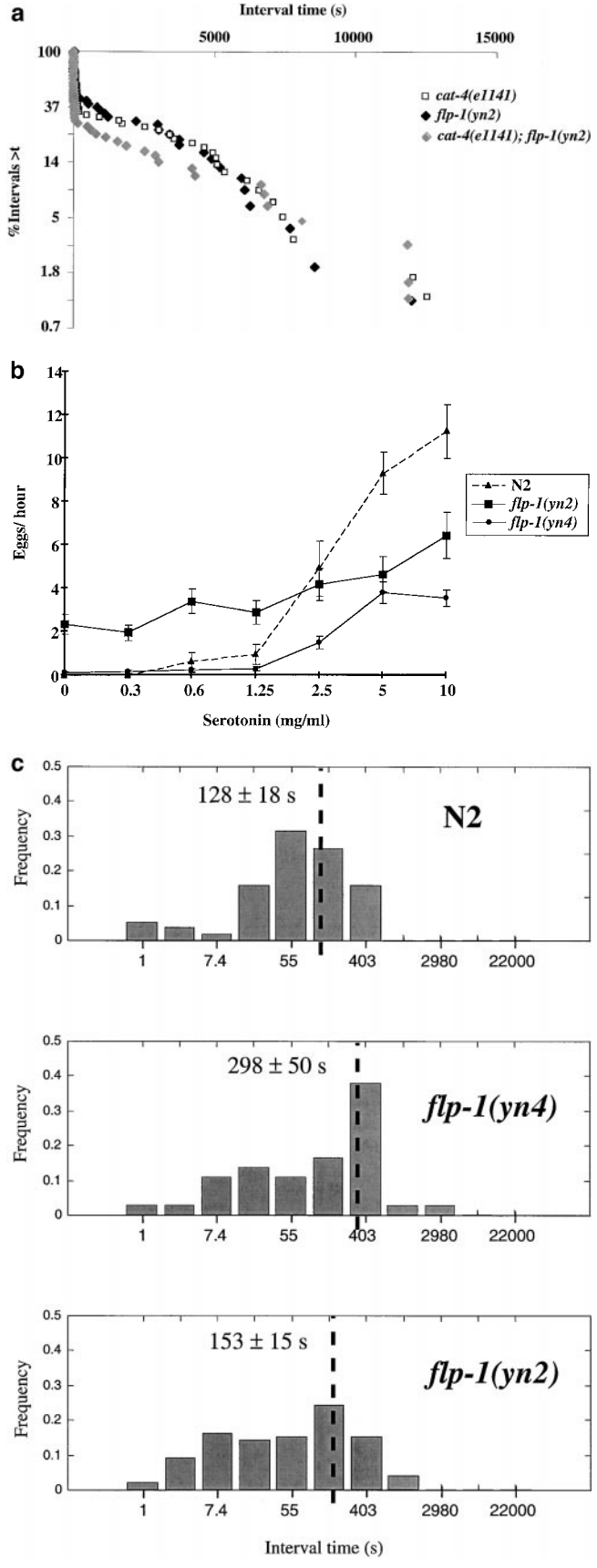
laying events were still clustered in active phases, and both the switch into the active phase and the laying of eggs within the active phase still modeled as Poisson processes. However, the duration of the inactive phase was substantially longer in the *flp-1* mutants (λ_2 for *flp-1(yn2)* and *flp-1(yn4)* were 4.7 and 7.0 $\times$ 10^{-4} sec^{-1}, respectively) than in wild-type animals (λ_2 was 1.4 $\times$ 10^{-3} sec^{-1}; Table 1; Figure 2, a and b). In contrast, egg-laying within the active phase was unimpaired in *flp-1* mutants, and the intracluster time constants were no slower in the *flp-1(yn2)* and *flp-1(yn4)* deletion mutants than in wild type (Table 1, Figure 2a). Expression of a wild-type *flp-1* transgene in a *flp-1(yn2)* background rescued its egg-laying defect (Figure 2a legend, Table 1). 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. For example, a mutation in *cat-4*, a gene required for efficient biosynthesis of serotonin and dopamine (Loer and Kenyon 1993), slows the rate of egg-laying and lengthens the inactive phase. In principle, serotonin and FLP-1 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*. 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 3a). Moreover, both the inactive phase rate constant λ_2 and the intercluster time constant of the double mutant were essentially identical to those of either single mutant (Table 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 that are normally

Figure 2.—Effect of *flp-1* mutations on the pattern of egg-laying. *flp-1* loss-of-function mutations increase the duration of the inactive phase. Two independent, extensively outcrossed alleles (*yn2* and *yn4*) were analyzed. 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 1. (a) Effect of *flp-1* recessive mutations on the pattern of egg-laying. Shown are histograms of intervals between egg-laying events (log scale); intracluster and intercluster time constants estimated using maximum-likelihood analysis are indicated. Both the alleles of *flp-1* specifically lengthened the intercluster constant (indicated by the location of the right-most peak). The long intervals (>120 sec) were significantly longer in *yn2* ($P < 0.001$) and *yn4* ($P < 0.05$) than in wild type according to the Mann-Whitney rank sum test. The long intervals were also significantly longer ($P < 0.001$) in the *flp-1(yn2)* strain than in the strain expressing the wild-type transgene (genotype: *flp-1(yn2); ynIs9[hs:flp-1(+)]*). The short intervals (<120 sec) in the *flp-1* mutant strains were not significantly different from wild type ($P > 0.2$ for *yn2*, $P > 0.05$ for *yn4*). (b) 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 intercluster time constants for these mutants.



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 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

Figure 3.—Relationship between the effects of *flp-1* and serotonin on egg-laying. (a) Egg-laying pattern of serotonin-deficient *flp-1* animals. *cat-4* mutants are defective in serotonin and dopamine biosynthesis; because dopamine inhibits rather than stimulates egg-laying, the slower egg-laying rate of *cat-4* mutant animals is thought to be a consequence of serotonin deficiency. Shown are log-tail distributions of egg-laying interval times for *flp-1*, *cat-4*, and *flp-1; cat-4* double mutant animals. The intercluster time constants (indicated by the curve tails) were essentially identical (see Table 1). The numbers of animals tracked, hours observed, and intervals analyzed, along with the estimated model egg-laying parameters, are in Table 1. Egg-laying rates (eggs per hour) for each strain were as follows: *flp-1*, 1.94 ± 0.50 ($n = 6$); *cat-4*, 2.35 ± 0.40 ($n = 12$); *flp-1; cat-4*, 3.03 ± 0.26 ($n = 7$). The slower intracluster egg-laying rate characteristic of the *cat-4* mutant (Waggoner *et al.* 1998) was not observed in *flp-1; cat-4* animals (Table 1). (b) 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 hr. 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 ($P < 0.001$) and significantly lower than in wild type at the same concentration ($P < 0.002$) according to the Mann-Whitney rank sum test. The constitutive egg-laying observed in *yn2* animals may result from a defect in osmosensation (Nelson *et al.* 1998b), which could reduce the egg-laying inhibition produced by M9 salts. (c) 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 materials and methods. Intervals for *flp-1(yn4)* were significantly ($P < 0.05$) longer than for wild type according to the Mann-Whitney rank sum test. The intervals for the *flp-1(yn2)* allele were not statistically different from wild type.

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 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 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 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 environ-

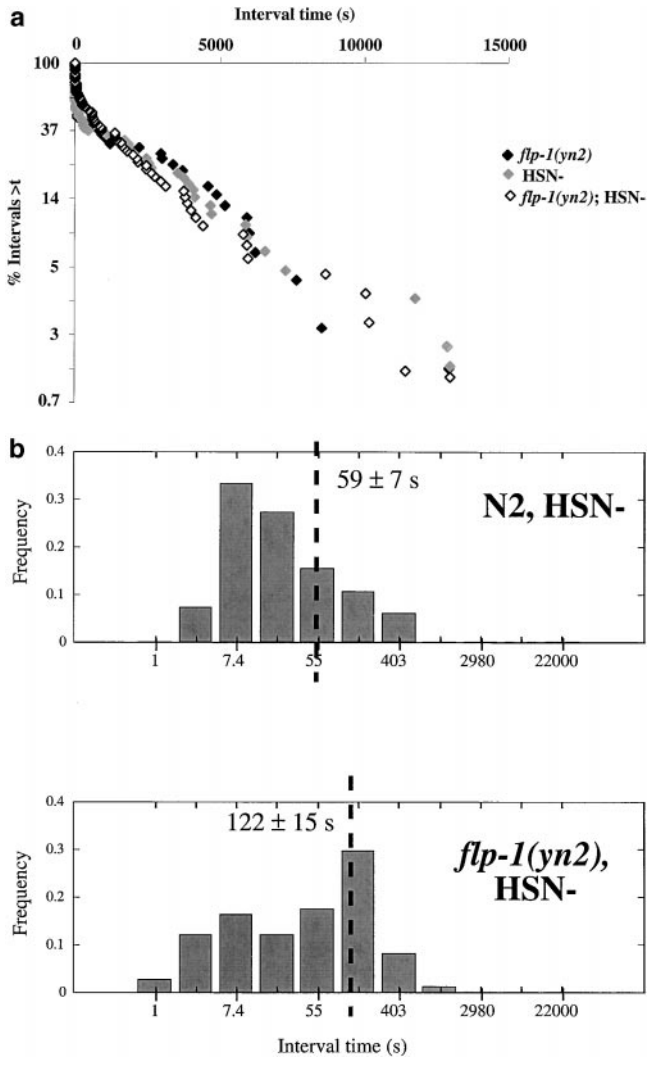


Figure 4.—Relationship between the effects of *flp-1* and the HSN neurons on egg-laying. (a) Egg-laying pattern of HSN-ablated *flp-1* animals. Shown are the log-tail distributions of egg-laying interval times for HSN-ablated wild-type and *flp-1* mutant animals. The intercluster time constant (indicated 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 1). The numbers of animals tracked, hours observed, and intervals analyzed, along with the estimated model egg-laying parameters, are in Table 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 as follows (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). (b) 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 materials and methods. Intervals for HSN-ablated *flp-1(yn2)* were significantly ($P < 0.001$) longer than for HSN-ablated wild type according to the Mann-Whitney rank sum test.

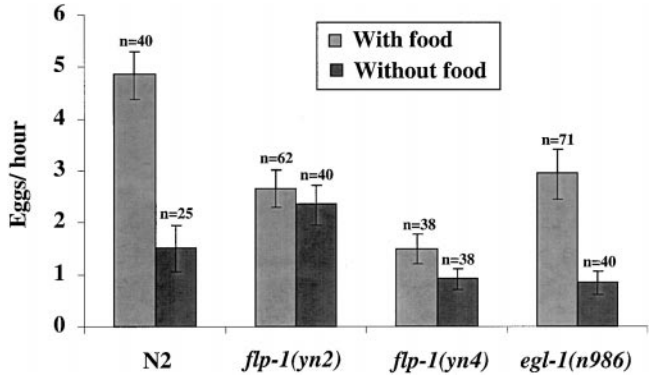


Figure 5.—Effect of *flp-1* mutations on regulation of egg-laying by food. The histogram shows the mean egg-laying rates of wild type, *egl-1*, and *flp-1* mutant animals in the presence and absence of food. Error bars indicate the SEM; the numbers of independent trials under each condition are indicated. The wild type and *egl-1* mutant strains showed a significantly lower ($P < 0.001$ for wild type, $P < 0.02$ 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 ($P > 0.5$ for *yn2*, $P > 0.2$ for *yn4*).

mental 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 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 (Segalat *et al.* 1995) and the FLP-1 peptides (Nelson *et al.* 1998b) 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 (Mendel *et al.* 1995; Segalat *et al.* 1995). 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 1; see also Figure 6, a and b), implicating GOA-1 as a negative regulator of the switch into the active phase. Because 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 6, a and b; Table 1). For example, in the case of *flp-1*, both the inactive phase rate constant λ_2 (0.0014 sec^{-1}) and the intercluster time constant (1950 sec) for the *goa-1*; *flp-1* double mutant were intermediate between those of the *goa-1* single mutant (0.0031 sec^{-1}; 890 sec) and the *flp-1* single mutant (0.0005 sec^{-1}; 3840 sec). Similarly, both λ_2 and the intercluster 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 still responded to serotonin, though weakly and at abnormally low threshold concentrations. In contrast, the *goa-1*; *flp-1* double mutant's serotonin dose response was similar though not identical to that of the *flp-1* single mutant (Figure 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 (Mendel *et al.* 1995; Segalat *et al.* 1995). 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 6d; Table 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 1). Because *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

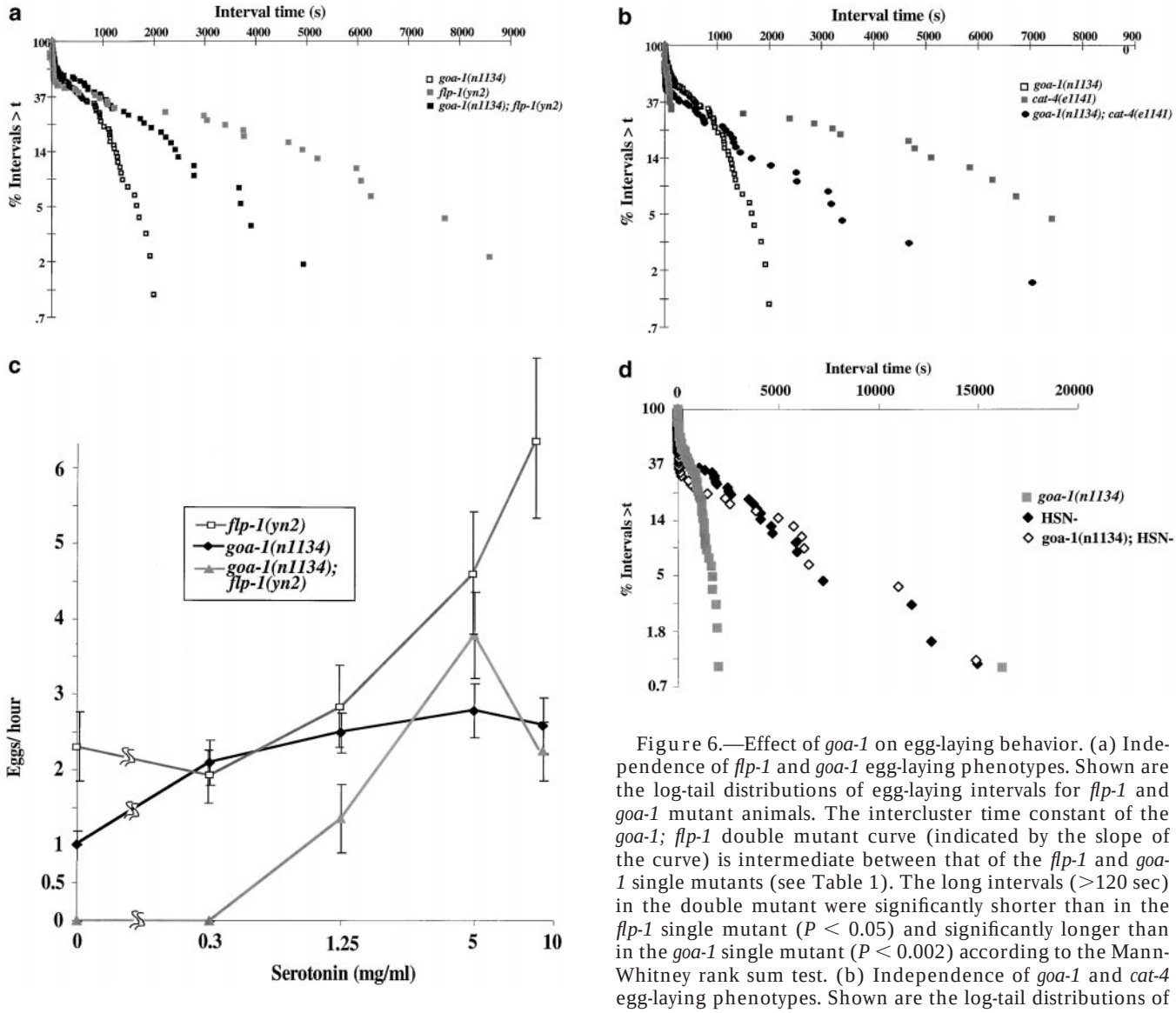


Figure 6.—Effect of *goa-1* on egg-laying behavior. (a) Independence of *flp-1* and *goa-1* egg-laying phenotypes. Shown are the log-tail distributions of egg-laying intervals for *flp-1* and *goa-1* mutant animals. The intercluster time constant of the *goa-1; flp-1* double mutant curve (indicated by the slope of the curve) is intermediate between that of the *flp-1* and *goa-1* single mutants (see Table 1). The long intervals (>120 sec) in the double mutant were significantly shorter than in the *flp-1* single mutant ($P < 0.05$) and significantly longer than in the *goa-1* single mutant ($P < 0.002$) according to the Mann-Whitney rank sum test. (b) 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 intercluster 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 (>120 sec) in the double mutant were significantly shorter than in the *cat-4* single mutant ($P < 0.001$) and significantly longer than in the *goa-1* single mutant ($P < 0.02$) according to the Mann-Whitney rank sum test. (c) 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 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. (d) 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 intercluster 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.

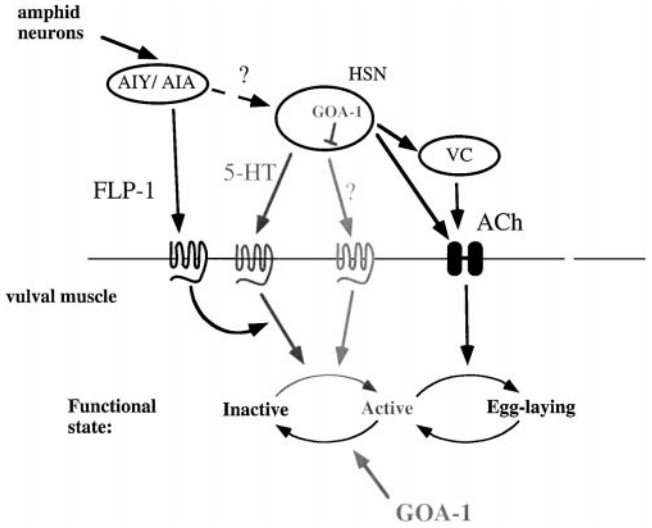


Figure 7.—Model for neural and molecular regulation of egg-laying. Egg-laying in *C. elegans* is affected by at least two parameters: the rate of switching from the inactive to the active phase and the rate of egg-laying within the active phase. FLP-1 peptides, released from neurons in the head, specifically 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.

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 were also specifically required to promote the onset of the active phase (Waggoner *et al.* 1998). Nonetheless, at least some of the effects of *flp-1* on egg-laying appeared to be HSN independent because *flp-1* mutant animals whose HSNs had been eliminated through laser ablation responded less strongly to serotonin than HSN-ablated wild-type animals. These results were perhaps surprising because all the chains of synaptic connections between the *flp-1*-expressing neurons in the head and the vulval muscles involve the HSNs (White *et al.* 1986). 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 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 (Nelson *et al.* 1998b). 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 (Waggoner *et al.* 1998; L. Waggoner, unpublished results) 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 neuromusculature to serotonin. This hypothesis is consistent with previously published work, which demonstrated that synthetic peptides identical to the shared carboxy terminus of the seven *flp-1* peptides (FLRF-NH_2) increased the average number of eggs laid in response to serotonin (Schinkmann and Li 1992). Serotonin and FLP-1 appear to function in concert 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 (Nelson *et al.* 1998b). Thus, for locomotive behavior, FLP-1 peptides appear to be necessary to potentiate the effects of serotonin. 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. Because comodulation of neuromuscular activity by biogenic amines and FaRPs is observed in many organisms (Klein *et al.* 1986; Scott *et al.* 1997), 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 re-

vealed 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 that 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. Consistent with this model, we found that the egg-laying patterns of wild-type animals in the presence of low levels of food resembled that of *flp-1* animals (Table 1). 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 (Sawin 1996). Both the hyperactive locomotion and the "wandering" behavior previously noted in *flp-1* recessive mutants (Nelson *et al.* 1998b) 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 (Bargmann and Mori 1997). 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 (White *et al.* 1986). 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 (Mori and Ohshima 1995). 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 (Mendel *et al.* 1995; Segalat *et al.* 1995), 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 the wild-type *cat-4* gene. The *cat-4* mutation is not a precise serotonin knockout: *cat-4* mutants are defective in the synthesis of other amines, and it is possible that they may contain very low levels of serotonin. However, despite these caveats, the simplest explanation for the difference between the egg-laying patterns of HSN-ablated *goa-1* and *cat-4; goa-1* animals is that GOA-1 has HSN-dependent effects on egg-laying that are serotonin independent. This implies that the HSNs may contain another neuromodulator in addition to serotonin that facilitates the switch into the active phase, whose release may be regulated by GOA-1.

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, because they contain FaRP immunoreactivity, which is not eliminated by deletions of the *flp-1* gene (Schinkmann and Li 1992; Nelson *et al.* 1998b). In addition, pharmacological experiments indicate that muscarinic acetylcholine agonists stimulate egg-laying (Weinshenker *et al.* 1995). Because 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 (Schinkmann and Li 1992). In addition, the VCs contain acetylcholine and, because they express the vesicular monoamine transporter, possibly an unidentified biogenic amine as well (Duerr *et al.* 1999). 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.

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