

## Genes Affecting the Activity of Nicotinic Receptors Involved in *Caenorhabditis elegans* Egg-Laying Behavior

Jinah Kim, Daniel S. Poole, Laura E. Waggoner, Anthony Kempf, David S. Ramirez, P. Alexandra Treschow and William R. Schafer

Division of Biology and Group in Neuroscience, University of California, San Diego, California 92093-0349

Manuscript received October 19, 2000

Accepted for publication December 12, 2000

### ABSTRACT

Egg-laying behavior in *Caenorhabditis elegans* is regulated by multiple neurotransmitters, including acetylcholine and serotonin. Agonists of nicotinic acetylcholine receptors such as nicotine and levamisole stimulate egg laying; however, the genetic and molecular basis for cholinergic neurotransmission in the egg-laying circuitry is not well understood. Here we describe the egg-laying phenotypes of eight levamisole resistance genes, which affect the activity of levamisole-sensitive nicotinic receptors in nematodes. Seven of these genes, including the nicotinic receptor subunit genes *unc-29*, *unc-38*, and *lev-1*, were essential for the stimulation of egg laying by levamisole, though they had only subtle effects on egg-laying behavior in the absence of drug. Thus, these genes appear to encode components of a nicotinic receptor that can promote egg laying but is not necessary for egg-laying muscle contraction. Since the levamisole-receptor mutants responded to other cholinergic drugs, other acetylcholine receptors are likely to function in parallel with the levamisole-sensitive receptors to mediate cholinergic neurotransmission in the egg-laying circuitry. In addition, since expression of functional *unc-29* in muscle cells restored levamisole sensitivity under some but not all conditions, both neuronal and muscle cell UNC-29 receptors are likely to contribute to the regulation of egg-laying behavior. Mutations in one levamisole receptor gene, *unc-38*, also conferred both hypersensitivity and reduced peak response to serotonin; thus nicotinic receptors may play a role in regulating serotonin response pathways in the egg-laying neuromusculature.

NICOTINIC acetylcholine receptors (nAChRs) are heteropentameric ligand-gated ion channels that induce fast depolarization of excitable cells in response to acetylcholine binding (GALZI *et al.* 1991). In muscle cells, nicotinic receptors present at the neuromuscular junction mediate rapid excitation that leads to muscle contraction. Neurons also contain nicotinic receptors, which are widely expressed in the brain and other neural tissues, and function in the modulation of neurotransmission (SARGENT 1995). The activities of nicotinic receptors are known to be subject to both short-term and long-term regulation. For example, long-term exposure to nicotine leads to long-lasting changes in both the abundance and functional activity of nicotinic receptors in brain neurons, processes thought to be critical for nicotine addiction (DANI and HEINEMANN 1996). Yet the molecular mechanisms responsible for regulating nicotinic receptor activity are not well understood in any organism.

One way to approach the question of nAChR function and regulation *in vivo* is to use a genetically tractable animal such as the nematode *Caenorhabditis elegans*. *C. elegans*, with its simple well-characterized nervous system

and its amenability to classical and molecular genetic studies, is well suited for investigating how specific neurotransmitters, receptors, and signaling molecules function within the context of the nervous system to produce behavior. In *C. elegans*, a number of genes encoding homologues of nAChR subunits have been identified (FLEMING *et al.* 1993, 1997; TREININ and CHALFIE 1995; BALLIVET *et al.* 1996; BAYLIS *et al.* 1997; MONGAN *et al.* 1998). Several of these genes have been shown to encode functional receptor subunits when expressed ectopically in oocytes (SQUIRE *et al.* 1995; FLEMING *et al.* 1997); however, the roles most of them play in nervous system function and behavior are not known.

The cells in which nicotinic receptor function has been best characterized in *C. elegans* are the body muscles, which mediate locomotion. Electrical recordings from these muscles indicate that two distinct nicotinic receptor subtypes mediate excitation of the body muscles (RICHMOND and JORGENSEN 1999). The first of these is activated by the antihelminthic drug levamisole and is therefore known as the levamisole receptor. Activation of this receptor by levamisole causes body muscle hypercontraction and, at high doses, spastic paralysis (LEWIS *et al.* 1980b). Screens for levamisole-resistant mutants have led to the identification of multiple genes affecting the function of this receptor (LEWIS *et al.* 1980a). Three of these levamisole-resistance genes, *unc-38*, *unc-29*, and *lev-1*, encode receptor subunits (FLEMING *et al.* 1997):

Corresponding author: William Schafer, Division of Biology, 9500 Gilman Dr., University of California, San Diego, CA 92093-0349.  
E-mail: wschafer@ucsd.edu

*unc-29* and *lev-1* encode candidate non- $\alpha$ -subunits of the levamisole receptor, whereas *unc-38* encodes a candidate  $\alpha$ -subunit. A second levamisole-insensitive nicotinic receptor has also been identified in the body muscle through electrophysiological methods (RICHMOND and JØRGENSEN 1999); the genetics and molecular biology of this receptor have not been characterized.

Nicotinic receptors also function in the pharynx, a specialized muscular organ responsible for feeding. Although the pharynx has intrinsic myogenic contractile activity, cholinergic neurotransmission from the pharyngeal motoneuron MC is necessary for rapid pharyngeal pumping (RAIZEN *et al.* 1995). Since nicotine but not levamisole induces pharyngeal muscle contraction in the absence of the pharyngeal nervous system, a nicotinic receptor distinct from the levamisole receptor appears to be at least partially responsible for mediating cholinergic transmission in the pharynx (AVERY and HORVITZ 1990). *eat-2* and *eat-18* are candidates for encoding subunits of this pharyngeal nAChR, since loss-of-function mutations in these genes cause a phenotype similar to ablation of the MC neurons and in some cases alter pharmacological responses to nicotine (RAIZEN *et al.* 1995). Another gene, *deg-3*, encodes a nicotinic receptor subunit that is expressed in the pharynx; however, *deg-3* loss-of-function mutants do not appear to exhibit abnormal feeding behavior (TREININ and CHALFIE 1995).

Another *C. elegans* behavior that involves the activity of nicotinic receptors is egg laying. Egg laying requires the activity of a set of eight specialized vulval muscles, which are extensively innervated by cholinergic motoneurons (WHITE *et al.* 1986; RAND and NONET 1997a). Nicotinic agonists including levamisole have been shown to stimulate egg laying, suggesting that cholinergic neurotransmission involving nicotinic receptors promotes egg-laying muscle contraction (TRENT *et al.* 1983; WEINSHENKER *et al.* 1995). When applied in combination with serotonin, the nicotinic agonist levamisole is capable of stimulating egg-laying muscle contraction in animals carrying ablations of the egg-laying motoneurons (WAGGONER *et al.* 1998). This result indicates that stimulation of egg laying by cholinergic agonists is mediated at least in part by nicotinic receptors in the vulval muscles. *unc-29* recessive mutants are resistant to stimulation of egg laying by levamisole, and expression of an *unc-29* wild-type transgene in the vulval muscles is sufficient to restore levamisole response (WAGGONER *et al.* 2000a). Thus, UNC-29-containing nicotinic receptors in the vulval muscles appear to be at least partially responsible for the acute effects of nicotinic agonists on egg laying.

In this study, we describe a more detailed analysis of the function and regulation of the nicotinic receptors involved in egg-laying behavior. In particular, we demonstrate that the genes encoding subunits of the well-characterized body muscle levamisole receptor also

function in the control of egg laying, specifically by mediating the stimulation of egg laying by levamisole, controlling the timing of egg-laying events, and regulating the response of the egg-laying neuromusculature to serotonin. We also present evidence that the *unc-29* receptor functions in neurons as well as muscle cells.

## MATERIALS AND METHODS

**Strains and genetic methods:** The chromosomal locations of the genes studied in these experiments are as follows: LGI, *unc-74*, *unc-38*, *unc-63*, *unc-29*, *lev-10*; LGII, *tph-1*; LGIV, *dpy-20*, *lev-1*; LGV, *egl-1*; LGX, *lev-9*, *lev-8*. Routine culturing of *C. elegans* was performed as described (BRENNER 1974). All mutant strains but one [*lev-9(x66)*] had been backcrossed once to wild type when we received them; for the tracking experiments (Table 1), the *unc-29* and *lev-1* mutant strains were each backcrossed an additional four times to wild type.

**Behavioral assays:** Drug response assays in M9 salts were performed essentially as described (WAGGONER *et al.* 2000a). Unless otherwise stated, nematodes were grown 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. For studies of egg-laying behavior on NGM solid media, single animals were transferred to agar plates seeded with *E. coli* OP50 as a food source. The egg-laying behavior of each animal was recorded for 4–12 hr as described using an automated tracking system (WAGGONER *et al.* 1998). Because levamisole treatment emptied the uterus of wild-type animals, wild-type worms were tracked on levamisole for only 2 hr.

**Analysis of egg-laying patterns:** Intervals between egg-laying events were determined from analysis of videotapes obtained using the tracking system. Quantitative analysis of the egg-laying pattern using this interval data was performed as described (ZHOU *et al.* 1998). Briefly, egg-laying events in *C. elegans* are clustered, with periods of active egg laying, or active phases, separated by long inactive phases during which eggs are retained. Both the duration of the inactive phases (“inter-cluster intervals”) and the duration of intervals between egg-laying events in a cluster (“intracluster 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 maximum-likelihood estimation method used to derive timing parameters was essentially the one described previously (ZHOU *et al.* 1998), with one improvement: multiple histograms of log interval time were used to initialize the maximum-likelihood (ML) algorithm, thereby avoiding the possibility of the program fixing on a local rather than the global maximum.

The experimental variance and statistical significance of the timing data were evaluated in two ways. The theoretical expected variance of estimated parameters and time constants based on the two-state model was determined by using the model probability density function to generate 100 independent sets of simulated egg-laying data (containing the same

TABLE 1  
Egg-laying patterns of levamisole-receptor mutants

Strain (no., hours, intervals)	Intracluster time constant ( $1/\lambda_1$ ; sec)	Intercluster time constant ( $1/\rho\lambda_2$ ; sec)	Clustering parameter ( $P$ )
N2 (10, 58, 357)	$16 \pm 2$	$1386 \pm 201$	$0.555 \pm 0.031$
<i>unc-29(x29)</i> (8, 38, 196)	$14 \pm 2$	$2236^* \pm 494$	$0.700 \pm 0.057$
<i>unc-29(e193)</i> (8, 44, 155)	$16 \pm 4$	$2042^* \pm 267$	$0.327 \pm 0.051$
<i>unc-29(e1072)</i> (8, 44, 162)	$31^* \pm 6$	$1733^* \pm 236$	$0.444 \pm 0.045$
<i>unc-38(x20)</i> (6, 25, 127)	$18 \pm 3$	$1738 \pm 389$	$0.669 \pm 0.043$
<i>unc-38(e264)</i> (15, 62, 207)	$18 \pm 4$	$1555 \pm 166$	$0.357 \pm 0.035$
<i>lev-1(e211)</i> (10, 45, 161)	$32^* \pm 5$	$2171^* \pm 296$	$0.497 \pm 0.040$
<i>lev-1(x427)</i> (11, 52, 149)	$28^* \pm 4$	$2896^* \pm 477$	$0.508 \pm 0.048$
<i>unc-29(x29); lev-1(e211)</i> (7, 31, 143)	$30^* \pm 5$	$2067^* \pm 417$	$0.576 \pm 0.047$
<i>unc-38(x20); lev-1(e211)</i> (12, 40, 138)	$27^* \pm 4$	$2007^* \pm 305$	$0.453 \pm 0.048$
<i>unc-38(sy576) unc-29(e1072)</i> (7, 27, 113)	$29^* \pm 5$	$1740^* \pm 324$	$0.479 \pm 0.053$
<i>unc-38(sy576) unc-29(e1072); lev-1(e211)</i> (8, 30, 118)	$32^* \pm 7$	$1850^* \pm 330$	$0.492 \pm 0.050$

\*Intervals statistically different (level of confidence  $P < 0.05$ ) from wild type according to the Mann-Whitney rank sum test. A cutoff point of 120 sec, or 300 sec in the case of strains with intracluster time constants  $>20$  sec, was used to separate short and long intervals.

number of intervals as the real data and using the same parameters) and computing the standard deviation of the parameters estimated from these simulations. Analysis of real data using this method indicates that real variation between individual animals of the same strain is comparable to the theoretical expectation. For example, when eight recordings of different wild-type worms made on different days were individually analyzed, the average intra- and intercluster time constants estimated from each were 14 sec and 1374 sec, with standard deviations of 7 sec and 422 sec, respectively. Simulations using the same parameters and data sets of comparable size ( $\sim 40$  intervals) had similar variation to what was observed experimentally (standard deviation: 4 sec and 504 sec). To test for the statistical significance of differences in egg-laying interval times, a nonparametric test was used (the Mann-Whitney rank test). The ability of such tests to determine statistical significance is independent of the nature and degree of variation in the data (ZAR 1996). To increase our confidence that differences between mutant and wild-type strains were due to mutations in the levamisole receptor genes, all *unc-29* and *lev-1* mutant strains were backcrossed four times to wild type (in addition to the one backcross performed prior to our receiving the strains) before being analyzed in the tracking assay.

**Sequencing of *unc-29* mutations:** Genomic DNA (SULSTON and HODGKIN 1988) or single worms (WILLIAMS 1995) were used as a template to obtain PCR products for sequencing. PCR products were purified using the QIAquick gel extraction kit (QIAGEN, Chatsworth, CA). DNA sequencing was performed by the Molecular Pathology Shared Resource, UCSD Cancer Center, which is funded in part by National Cancer Institute Cancer Center support grant no. 5P0CA23100-16. The sequence alterations were confirmed by both sequencing DNA from additional independent PCR reactions and by restriction digest of multiple PCR products from single worms.

**Construction of double and triple mutant strains:** Double mutants carrying mutations in *tph-1* or *egl-1* and in one of the levamisole receptor genes were constructed by crossing the single mutants and screening the second generation self-progeny for nicotine-resistant animals whose progeny were all egg-laying defective. The *unc-38(sy576) unc-29(e1072)* double mutant was derived by crossing a *unc-38(sy576) unc-29(e1072); him-5(e1490)* strain (provided by Rene Garcia and Paul Stern-

berg) to wild type, and isolating an Unc non-Him F<sub>2</sub> segregant. *lev-1(e211)* was introduced into this double mutant or into *unc-38(x20)* or *unc-29(x29)* in the following manner. *lev-1(e211)* males were mated to the Unc strain and the nicotine-sensitive hermaphrodite progeny were then mated to *lev-1(e211)* males. Non-Unc nicotine-resistant (*i.e.*, *lev-1/lev-1*) hermaphrodite progeny from this backcross were picked individually and allowed to self-fertilize. Some of these segregated Unc animals, which were picked individually and allowed to self-fertilize. The presence of *unc-38*, *unc-29*, and *lev-1* was confirmed by test cross with males heterozygous for *unc-38(x20)* or *unc-29(x29)* or males homozygous for *lev-1(e211)*.

**Cell ablation experiments:** For ablations of VC1–6, we ablated the neuroblasts P1.a–P9.a, which are the larval precursors of the VCs. Although only P3.a–P8.a normally give rise to the VCs, adjacent Pn.a cells can generate VCs in the absence of P3.a–P8.a unless killed (LI and CHALFIE 1990). Wild-type animals were grown at 20°C;  $\sim 10$  hr after hatching, the Pn.a cell nuclei were identified by position in the ventral cord and killed; cell killing was verified by scoring for the absence of adult ventral cord motorneuron nuclei in the midbody region. We also confirmed the ability of this ablation to eliminate the VCs by performing the same procedure on strain AQ242 (genotype *dpy-20(e1282)IV; ljEx1[dpy-20(+), egl-36::GFP]*), which expressed green fluorescent protein (GFP) in all VC neurons; cell killing was verified in late L4 by scoring for the absence of fluorescence in the ventral cord and vulval area. Three other ventral cord neurons that also descend from the ablated neuroblasts (VA7, VB8, and VD7) potentially make single synapses with the egg-laying muscles; thus, it was formally possible these neurons might contribute to this effect. However, ablations of a subset of Pn.a neuroblasts (for example, P3.a–P8.a) that eliminated VA7, VB8, and VD7 but spared one or more VCs (as indicated by *egl-36::GFP*-expressing cells that sent processes to the vulva) did not prevent levamisole response (data not shown).

## RESULTS

**Genes required for egg laying in response to nicotinic agonists:** To identify genes required for nAChR function

in egg-laying cells, we assayed egg-laying behavior in levamisole-resistant mutants, which were originally identified on the basis of their resistance to the effects of levamisole on body muscle. Mutations conferring resistance to high concentrations of levamisole (*i.e.*, 1 mM) have been identified in several genes, including *unc-29*, *unc-38*, *unc-50*, *unc-63*, *unc-74*, and *lev-1* (LEWIS *et al.* 1980a). All of these “strong” levamisole resistance genes affect the assembly of functional levamisole-binding receptors as assayed *in vitro* (LEWIS *et al.* 1987), and three of them, *unc-38*, *unc-29*, and *lev-1*, are known to encode nicotinic receptor subunits (FLEMING *et al.* 1997). Mutations in three additional genes (*lev-8*, *lev-9*, and *lev-10*) confer only partial resistance to levamisole (*i.e.*, to concentrations  $\leq 100 \mu\text{M}$ ) and have no detectable effect on the biochemical properties of the receptor as assayed *in vitro*. These “weak” levamisole resistance genes have been hypothesized to regulate the activity of the levamisole receptor indirectly (LEWIS *et al.* 1980a, 1987).

To assess the possible involvement of these levamisole resistance genes on egg-laying behavior, we assayed mutant animals for egg laying in response to acute levamisole treatment. Levamisole treatment results in a dose-dependent stimulation of egg laying in hypertonic liquid medium (M9 salts), a condition that normally inhibits egg laying (TRENT *et al.* 1983). We had previously found that mutants defective in the nicotinic receptor subunit gene *unc-29* were insensitive to stimulation of egg laying by levamisole (WAGGONER *et al.* 2000a). When we assayed the effects of other levamisole resistance genes, we found that many of them were also levamisole resistant with respect to egg laying (Figure 1). For example, recessive alleles of the *unc-38* and *lev-1* genes, which encode  $\alpha$ - and non- $\alpha$ -nicotinic receptor subunits, respectively, conferred partial or complete resistance to levamisole in the M9 assay. Likewise, mutants defective in the two other strong levamisole resistance genes, *unc-63* and *unc-74*, also showed no significant stimulation of egg laying by levamisole. Finally, *lev-8* and *lev-9* mutants, which were only partially levamisole resistant in the body muscle, were highly resistant to the stimulatory effects of levamisole on egg laying. In contrast, *lev-10* mutant animals exhibited a robust stimulation of egg laying by levamisole. *unc-50* mutants were not tested because their extremely low brood size made egg-laying behavior difficult to evaluate. Together, these results indicated that many of the same genes required for levamisole response in body muscle, including all the known receptor subunit genes, were also required for the acute effects of levamisole on egg laying. Thus, a nicotinic acetylcholine receptor with a similar subunit composition to the levamisole receptor in body muscle also appeared to promote egg laying.

To investigate the possible involvement of other acetylcholine receptors in egg-laying behavior, we assayed the egg-laying responses of the levamisole receptor mutants on responses to the general nicotinic agonist nico-

tine. Like levamisole, nicotine caused robust dose-dependent stimulation of egg laying by wild-type animals in M9. Mutations in all three known levamisole receptor genes (*i.e.*, *unc-29*, *unc-38*, and *lev-1*) led to a reduced response to nicotine in this assay, although significant stimulation was observed in all three mutants, especially at the later time point (Figure 2, a and b). Interestingly, the nicotine response observed in the levamisole receptor mutants displayed different dose-response kinetics from the wild-type response; the half-maximal point for the mutant response was at  $\sim 0.2$  mM, compared to 0.8 mM for the wild-type response. Thus, these results indicate that although the levamisole receptor genes mediate some of the stimulatory effect of nicotine on egg laying, a significant component of this stimulation is levamisole receptor independent and may be mediated through a different nicotinic receptor subtype.

**Effects of the levamisole receptor on the temporal pattern of egg laying:** To investigate in more detail the role of nicotinic receptors in egg-laying behavior, we analyzed the effect of the agonist levamisole on the timing of egg-laying events. Under conditions favorable to egg laying (*i.e.*, NGM seeded with abundant food), wild-type worms fluctuate between two discrete behavioral states: an inactive egg-laying state during which eggs are retained in the uterus and an active state during which eggs are laid in clusters. By recording and analyzing an animal's behavior over long time periods, it is possible to determine exponential time constants for the onset of the active phase and for egg laying within the active phase (WAGGONER *et al.* 1998). When we analyzed the egg-laying pattern of wild-type animals during acute levamisole treatment, we observed that the time constant for the onset of the active phase as well as the time constant for egg laying within the active phase were significantly reduced: on levamisole, the intracluster interval displayed a twofold reduction, and the intercluster interval displayed a more than threefold reduction (Figure 3). Thus, levamisole treatment appeared to both facilitate onset of the active phase of egg laying and stimulate egg-laying muscle contractions during the active phase. These effects of levamisole on the timing of egg-laying events were dependent on the levamisole receptor genes (*unc-29*, *unc-38*, and *lev-1*). By themselves, these genes had only subtle effects on the temporal pattern of egg laying (Table 1). Yet all mutants retained the characteristic biphasic pattern of egg laying, and the mutant time constants differed significantly from those of wild type by no more than a factor of two. Yet in contrast to its effect on wild-type animals, levamisole treatment had little or no effect on the egg-laying patterns of the levamisole receptor mutants (Figure 3; *unc-38* and *lev-1* data not shown). Thus, although the levamisole receptor genes were not critical for egg laying in the absence of drug, they were apparently

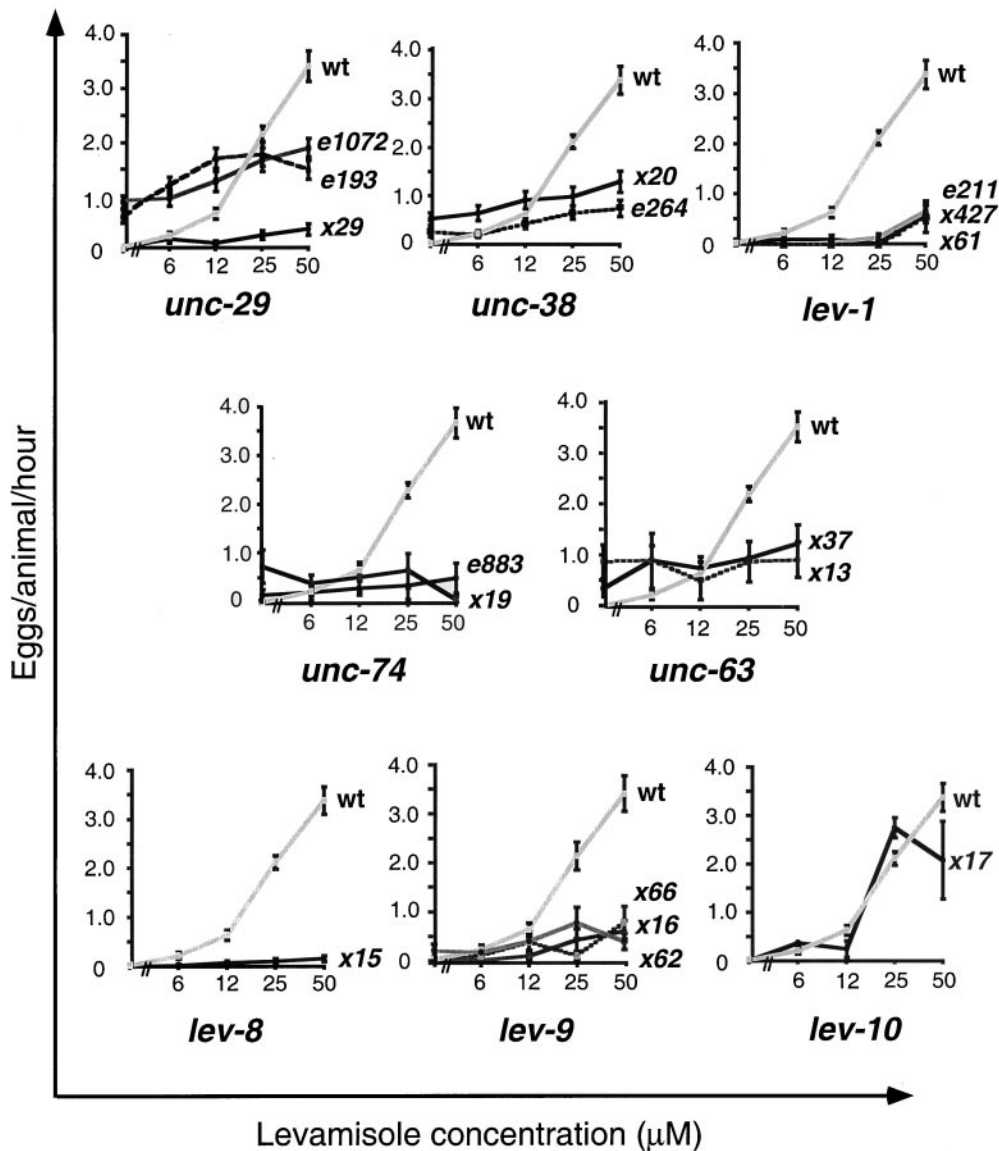


FIGURE 1.—Effects of levamisole resistance genes on egg laying in response to levamisole. Egg-laying responses to levamisole were determined for the different mutant alleles by placing individual animals in liquid M9 and the indicated concentration of levamisole and counting the number of eggs laid by each animal after 1 hr of drug exposure. *unc-29* data (adapted from WAGGONER *et al.* 2000a) are shown for comparison. Animals carrying mutations in *unc-38*, *unc-63*, *unc-74*, *lev-1*, *lev-8*, and *lev-9* showed a statistically significant reduction in levamisole-induced egg laying according to the Mann-Whitney rank sum test (level of confidence  $P < 0.001$ ). Individual points and error bars indicate the mean and SEM of the following numbers of trials: N2, 197; *unc-29*(*e193*), 144; *unc-29*(*e1072*), 96; *unc-29*(*x29*), 96; *unc-38*(*x20*), 96; *unc-38*(*e264*), 96; *lev-1*(*e211*), 96; *lev-1*(*x427*), 96; *lev-1*(*e61*), 48; *unc-74*(*e883*), 38; *unc-74*(*x19*), 26; *unc-63*(*x37*), 40; *unc-63*(*x13*), 28; *lev-8*(*x15*), 72; *lev-9*(*x16*), 72; *lev-9*(*x62*), 30; *lev-9*(*x66*), 30; *lev-10*(*x17*), 96.

necessary for the acute effects of levamisole on egg laying on NGM as well as in M9.

How likely is it that these mutations in the nicotinic receptor genes completely eliminate the functional activity of the encoded receptor protein? The previously reported sequences of the *unc-38*(*x20*) and *lev-1*(*x427*) alleles suggest that they cause severe if not complete loss of receptor function (FLEMING *et al.* 1997). By sequencing the complete coding regions of the *unc-29* mutant alleles, we determined that the *unc-29*(*x29*) mutation introduced a nonsense mutation (tat → taa) within the fourth transmembrane  $\alpha$ -helix. *unc-29*(*e193*) was found to be a missense mutation (cca → tca) that changes a universally conserved proline residue at position 258 to a serine; the sequence alteration in the *unc-29*(*e1072*) allele could not be identified. Thus, *unc-29*(*x29*) probably causes a severe loss of nicotinic receptor function and might represent a molecular null allele. To further address the egg-laying phenotype of a com-

plete levamisole receptor knockout, we analyzed the egg-laying behavior of *unc-29/unc-38/lev-1* double and triple mutant strains, which were defective in two (or in the latter case three) of the identified levamisole receptor subunits. In each case, the pattern of egg laying was grossly normal, and although abnormalities in the timing of egg-laying events were observed, they were quantitatively not much more severe than in the single mutant strains (Figure 3; Table 1). Together, these results suggested that while a nicotinic receptor containing UNC-29, UNC-38, and LEV-1 subunits is necessary for stimulation of egg laying by levamisole, it is unnecessary for egg-laying muscle contraction *per se*.

**The cellular basis for *unc-29* egg-laying phenotypes:** UNC-29 is expressed in both neurons and muscle cells (FLEMING *et al.* 1997). Specifically, UNC-29 has been shown to be expressed in the vulval muscles as well as the VC egg-laying motorneurons (WAGGONER *et al.* 2000a; see Figure 4a). Thus, it was of interest to deter-

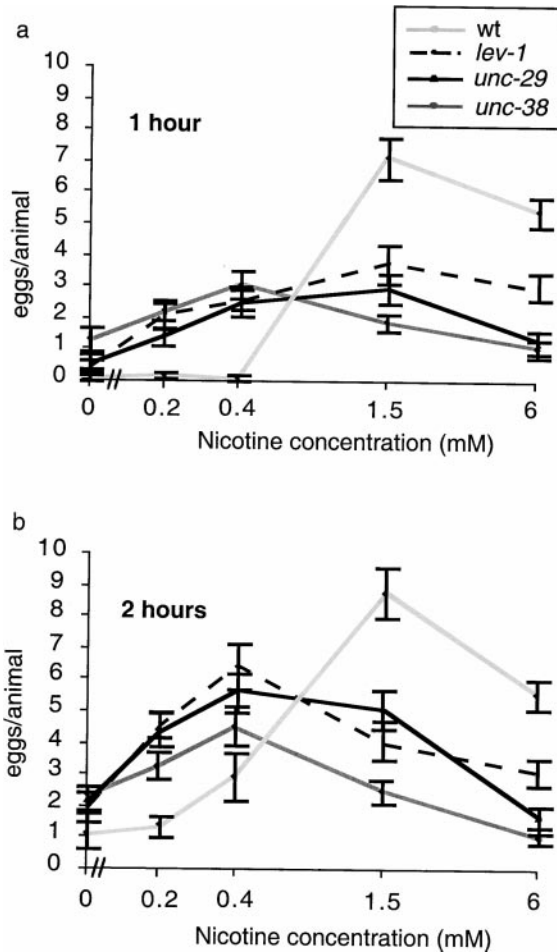


FIGURE 2.—Effects of levamisole receptor genes on egg laying in response to the general nicotinic agonist nicotine. Egg-laying responses to nicotine were determined using the M9 assay described in Figure 1. Data collected after 1 and 2 hr are shown in a and b, respectively. The *unc-29(x29)*, *lev-1(e211)*, and *unc-38(x20)* alleles were used for these experiments. Points and error bars indicate the mean and standard error of 36 trials at each concentration.

mine the cellular foci for the egg-laying phenotypes of *unc-29*. In principle, the egg-laying abnormalities in *unc-29* loss-of-function mutants could be the result of a lack of UNC-29 receptor function in the vulval muscles, the VCs, or perhaps some other cell type. To address these possibilities, we generated transgenic animals with the *unc-29* mutant background that expressed the wild-type allele of *unc-29* only in muscle cells (using the *myo-3* promoter; OKKEMA *et al.* 1993) or only in the vulval muscles (using the *ndE-box* promoter; HARFE and FIRE 1998). When we tested these lines for the ability to lay eggs in M9 in response to levamisole, we observed that both the *myo-3::unc-29* line and the *ndE-box::unc-29* line showed robust stimulation (Figure 4b). Thus, functional UNC-29 receptors in the vulval muscles apparently were sufficient to rescue the levamisole-insensitive phenotype as measured in the M9 assay.

We also assayed the responses of our muscle-specific transgenic lines to levamisole on NGM agar plates. Surprisingly, the egg-laying patterns of both the *myo-3::unc-29* line (Figure 4c) and the *ndE-box::unc-29* line (not shown) were largely unaffected by the presence of levamisole. Thus, whereas vulval muscle-specific expression of wild-type *unc-29* rescued the levamisole-resistant phenotype of *unc-29(x29)* in the M9 egg-laying assay, muscle-specific expression failed to rescue the levamisole-resistant phenotype on NGM plates. These results implied that vulval muscle UNC-29 receptors were sufficient to provide levamisole response in M9, whereas levamisole response on NGM might require the activity of neuronal UNC-29 receptors. To test this possibility, we performed ablations that eliminated the egg-laying motorneurons that express UNC-29 (*i.e.*, the VCs) and determined the effect of this ablation on egg-laying responses to levamisole. We observed that, like the *myo-3::unc-29* animals, animals lacking the VCs were largely levamisole insensitive on NGM (Figure 4d). These results were consistent with the hypothesis that levamisole receptors in the VC neurons participate in the control of egg laying.

**Effect of levamisole receptor genes on serotonin responses:** Previous studies indicated that acetylcholine and serotonin act in parallel to activate egg laying in *C. elegans*. To gain further information about the relationship between serotonin and acetylcholine in the control of egg laying, we analyzed the behavior of double mutants defective in both nicotinic acetylcholine receptor function and serotonergic neurotransmission. We first analyzed the phenotypes of double mutants carrying a loss-of-function mutation in *tph-1*, which encodes the serotonin biosynthetic enzyme tryptophan hydroxylase (SZE *et al.* 2000). We observed that, by itself, a *tph-1* loss-of-function mutation resulted in a small but significant increase in the duration of the inactive egg-laying phase. When a *unc-29* or *lev-1* mutation was crossed into a *tph-1* mutant background, no significant enhancement of the egg-laying phenotype was observed (Figure 5, a–c). In contrast, both *unc-29* and *lev-1* dramatically enhanced the egg-laying defect caused by an *egl-1* mutation, which causes inappropriate cell death of the serotonergic HSN neurons (DESAI *et al.* 1988). In an *egl-1* mutant background, *unc-29* and *lev-1* mutations decreased the overall rate of egg laying and significantly decreased the rate of egg laying within the active phase (Figure 5, d–f). Thus, the activity of UNC-29/LEV-1 containing nicotinic receptors appeared to be particularly important for egg laying in the absence of the HSNs.

We also assayed the effect of mutations in the levamisole receptor genes on the egg-laying response to exogenous serotonin (Figure 6). We observed that *unc-38* mutants exhibited a greatly overall diminished response to serotonin; at peak concentrations, *unc-38* mutants showed at least a threefold reduction in the number of eggs laid (Figure 6c). In addition, *unc-38* mutants also

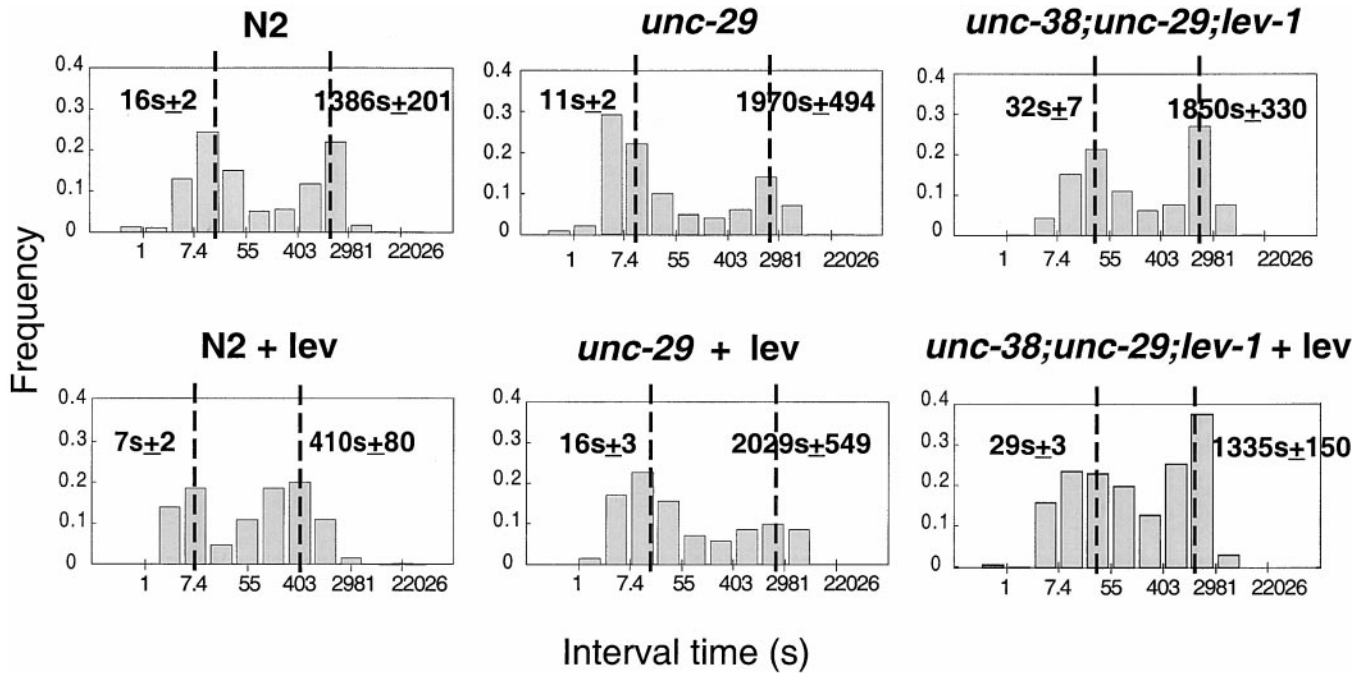


FIGURE 3.—Effect of the levamisole receptor on the timing of egg-laying events. Shown are histograms of interval times between egg-laying events for wild-type or mutant animals in the presence or absence of levamisole. For each histogram, the left peak contains the intracluster intervals (*i.e.*, the intervals between events within a cluster), and the right peak contains the intercluster intervals (*i.e.*, the intervals between clusters). Dashed lines indicate the estimated intracluster and intercluster time constants. Animals were tracked as described on nematode growth medium (NGM) or on NGM containing 50  $\mu\text{M}$  levamisole. This concentration effectively stimulates egg laying in wild-type animals, but does not cause body muscle paralysis. For animals tracked on NGM, the numbers of animals tracked, hours observed, and intervals analyzed, along with the estimated model egg-laying parameters, are in Table 1. For animals tracked on levamisole, the numbers of animals, hours tracked, and total intervals analyzed were as follows: N2, nine animals, 19 hr, 64 intervals; *unc-29*(*x29*), five animals, 21 hr, 100 intervals; *lev-1*(*e211*), six animals, 28 hr, 101 intervals; *unc-38*(*x20*), seven animals, 35 hr, 104 intervals; *unc-38*(*sy576*) *unc-29*(*e1072*); *lev-1*(*e211*), nine animals, 47 hr, 275 intervals. Both the long ( $\geq 120$  sec) and short ( $< 120$  sec) intervals were significantly shortened by levamisole in wild type (confidence level  $P < 0.001$ ). In the *unc-29* single mutant neither parameter was significantly shortened, and in the *unc-38;unc-29;lev-1* triple mutant the long interval was slightly shortened (confidence level  $P < 0.05$ ) according to the Mann-Whitney rank sum test. In testing the significance for the mutants, a cutoff point of 300 sec was used in separating long and short intervals to adapt to the shifts in the curves representing intracluster intervals.

showed hypersensitivity to serotonin in that they consistently responded more than wild type to abnormally low concentrations of drug. Since *unc-38* mutations also conferred serotonin hypersensitivity in an *egl-1* mutant background (data not shown), this effect was apparently independent of the HSN neurons. Thus, *unc-38* appeared to both potentiate and negatively regulate serotonin response in the egg-laying circuitry. In contrast, the dose responses of *unc-29* and *lev-1* mutants were more similar to wild type: although both mutants did experience a reduction in peak response, it was not nearly as pronounced as that seen in *unc-38* mutants, and statistically significant serotonin hypersensitivity to low doses was seen in only one allele of *unc-29* (*e193*; Figure 6, a and b).

## DISCUSSION

**Genetic requirements for cholinergic neurotransmission in egg-laying behavior:** Cholinergic agonists, including the nicotinic agonists nicotine and levamisole, have

long been known to stimulate egg laying. We recently found that the stimulation of egg laying by levamisole requires a nicotinic receptor containing the subunit protein UNC-29, which is also an essential component of the levamisole receptor in body muscle (WAGGONER *et al.* 2000a). In this study we observed that several other genes that affect the activity of the body muscle levamisole receptor are also required for the acute effects of levamisole on egg laying. These include the genes *unc-38* and *lev-1*, which encode  $\alpha$ - and non- $\alpha$ -subunits, respectively, of the body muscle levamisole receptor (FLEMING *et al.* 1997). Thus, the levamisole-sensitive nicotinic receptor that promotes egg-laying muscle contraction appears to have a similar if not identical subunit composition to the levamisole receptor in the body muscle. Interestingly, *lev-1* loss-of-function mutants, which are only partially resistant to levamisole with respect to body muscle contraction, were completely levamisole resistant with respect to egg laying. Therefore, the LEV-1 protein may be a nonessential subunit of the body muscle levamisole receptor, but an essential subunit of the

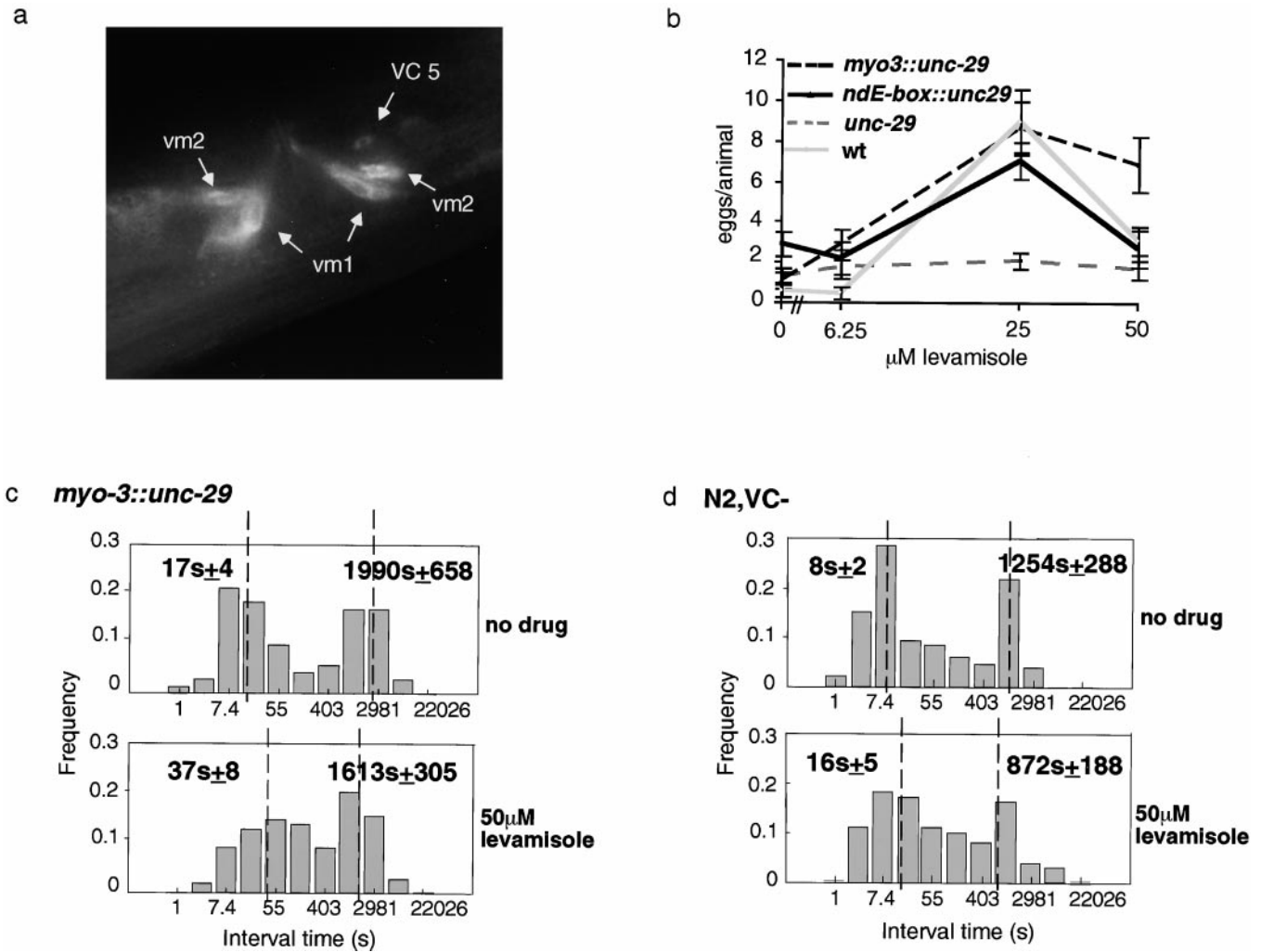


FIGURE 4.—Partial rescue of *unc-29* egg-laying phenotypes by muscle-specific gene expression. (a) Expression pattern of UNC-29 in the egg-laying neuromusculature. An UNC-29::GFP chimeric protein shows pattern of expression in the vulval muscles (vm1 and vm2) and VC motoneurons (WAGGONER *et al.* 2000a). (b) Dose response curves showing egg laying in response to levamisole by wild-type animals, *unc-29* mutants, and transgenic animals expressing functional *unc-29* only in the vulval muscles (*ndE-box::unc-29(+)*; strain AQ497) or in vulval and body muscles (*myo-3::unc-29(+)*; strain AQ548) in an *unc-29* mutant background. Individual points and error bars indicate the mean and SEM of at least the following numbers of trials: N2, 16; *unc-29(x29)*, 16; *ndE-box::unc-29(+)*, 20; *myo-3::unc-29(+)*, 30. (c and d) Effect of levamisole on the egg-laying patterns of animals with muscle-specific *unc-29(+)* expression (c) or with ablation of the Pn.a VC neuronal precursors (d). Dashed lines indicate the estimated intracluster and intercluster time constants. The number of animals, hours tracked, and total intervals analyzed were as follows: AQ548/*myo-3::unc-29* (no drug), five animals, 26 hr, 71 intervals; AQ548/*myo-3::unc-29* (levamisole), five animals, 30 hr, 109 intervals; N2, VC- (no drug), three animals, 20 hr, 42 intervals; N2, VC- (levamisole), three animals, 24 hr, 69 intervals.

levamisole receptor involved in egg laying. Several other levamisole resistance genes that have not been cloned, including *unc-74*, *lev-8*, and *lev-9*, also appeared to affect egg laying in response to nicotinic agonists (though only a single allele of *lev-8* was available, making effects of genetic background impossible to rule out for this mutant). Thus, the regulatory pathways controlling the activity of levamisole receptors involved in egg laying may be also similar to those affecting the body muscle receptors.

Despite their apparent role in mediating cholinergic neurotransmission in the egg-laying circuit, none of the levamisole resistance genes were critical for egg laying.

Recessive mutants with defects in the levamisole receptor subunit genes not only failed to exhibit a gross egg-laying-defective phenotype, but their temporal patterns of egg laying also showed little deviation from those of wild-type animals. Even double and triple mutants defective in multiple levamisole receptor subunits exhibited only a subtle alteration in egg-laying pattern: the intracluster and intercluster time constants were slower by a factor of no more than two. *unc-29*, *unc-38*, and *lev-1* mutants all exhibited a reduced but measurable response to the general nicotinic agonist nicotine; thus, the vulval muscles, like the body muscles, most likely contain a second nicotinic receptor whose func-



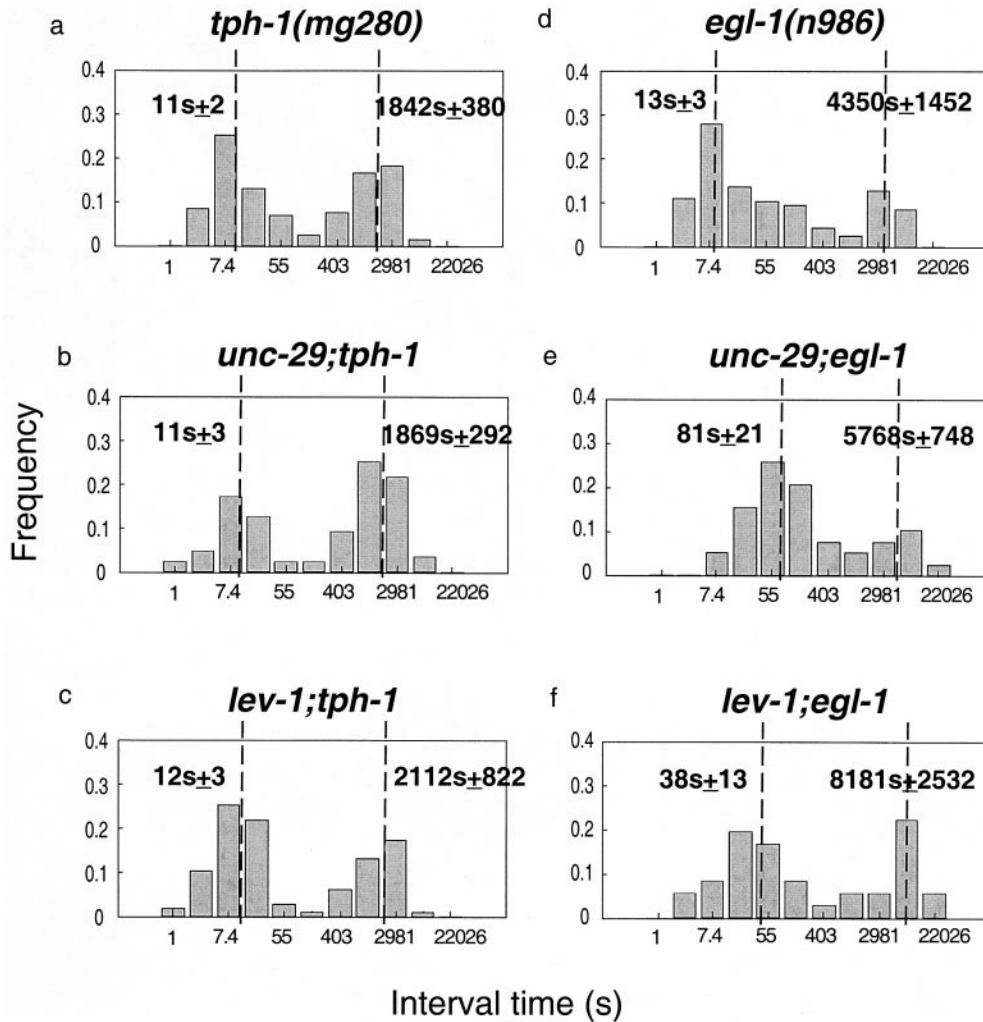


FIGURE 5.—Interactions between the egg-laying phenotypes of *egl-1*, *tph-1*, and the levamisole receptor genes. Shown are histograms of interval times between egg-laying events. Dashed lines indicate the estimated intracluster and intercluster time constants. The number of animals, hours tracked, and total intervals analyzed were as follows: *tph-1(mg280)*, 6 animals, 40 hr, 131 intervals; *tph-1(mg280); lev-1(e211)*, 6 animals, 33 hr, 115 intervals; *tph-1(mg280);unc-29(x29)*, 6 animals, 33 hr, 88 intervals; *egl-1(n986)*, 13 animals, 70 hr, 118 intervals; *egl-1(n986); lev-1(e211)*, 4 animals, 48 hr, 35 intervals; *egl-1(n986);unc-29(x29)*, 6 animals, 72 hr, 40 intervals. The short intervals (<300 sec) in *egl-1*; *lev-1* and *egl-1*; *unc-29* double mutants were statistically different from the *egl-1* single mutant according to the Mann-Whitney rank sum test (level of confidence  $P < 0.05$ ).

tion overlaps that of the levamisole receptor (RICHMOND and JORGENSEN 1999). It is also possible that the vulval muscles, like the pharyngeal muscles, may possess intrinsic myogenic activity, making cholinergic neurotransmission important but not essential for muscle contraction (RAIZEN *et al.* 1995). The egg-laying phenotypes of choline acetyltransferase (*i.e.*, *cha-1*) mutants support this possibility; *cha-1* conditional mutants have a significantly longer intracluster time constant than even the levamisole receptor triple mutant (WAGGONER *et al.* 1998), yet not even the strongest *cha-1* mutants are completely defective for egg laying (RAND 1989). In the future, analyses of vulval muscle physiology may make it possible to address this issue more definitively.

**Evidence that *unc-29* functions in both egg-laying muscles and neurons:** Although the function of UNC-29-containing nicotinic receptors has previously been studied only in muscle cells, GFP reporter studies have indicated that UNC-29 receptors are also expressed in neurons (FLEMING *et al.* 1997). The evidence presented here indicates that neuronal as well as vulval muscle UNC-29 receptors participate in the control of egg-lay-

ing behavior. Expression of a functional *unc-29* transgene under the control of the vulval muscle-specific *ndE-box* promoter restored levamisole sensitivity in the M9 egg-laying assay; thus, it is clear that UNC-29 receptors in the vulval muscles mediate at least some of the effects of nicotinic agonists on egg laying, probably through direct muscle excitation. However, when analyzed on NGM plates, lines expressing *unc-29(+)* under the control of either the *ndE-box* or the muscle-specific *myo-3* promoter appeared levamisole resistant, since their egg-laying pattern was largely unaffected by levamisole treatment. These results illustrate that the genetic requirements for pharmacological stimulation of egg laying can differ when assayed under conditions that are normally permissive for egg laying (*i.e.*, seeded NGM plates) instead of conditions that are normally inhibitory (*i.e.*, M9 liquid medium). Such a discrepancy is not unique to these transgenic lines; we previously observed that mutants defective in the neuropeptide gene *flp-1* showed almost no stimulation of egg laying by serotonin in M9, but displayed an essentially wild-type response on NGM (WAGGONER *et al.* 2000b). Since the *myo-3*

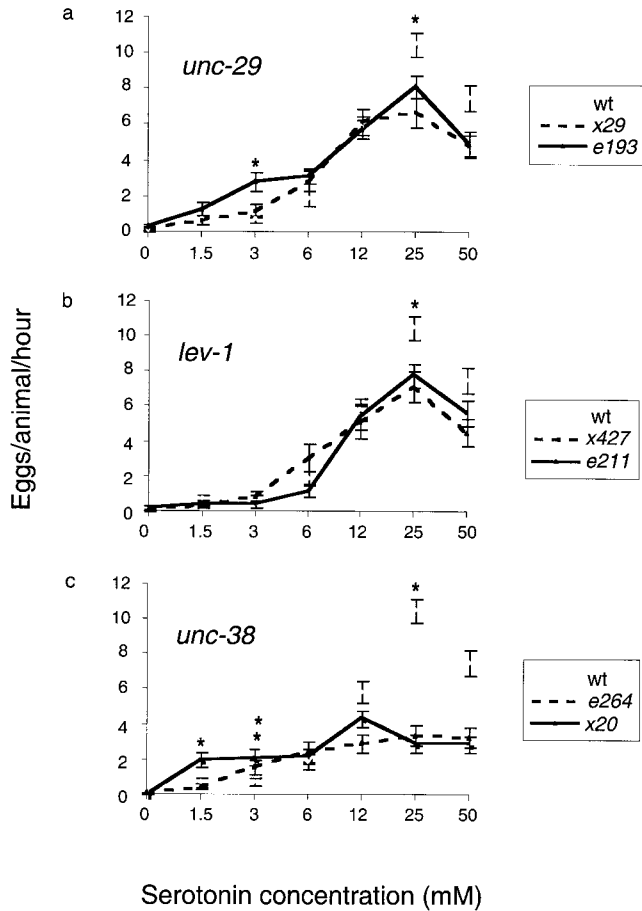


FIGURE 6.—Effect of levamisole receptor genes on egg laying in response to serotonin. Egg-laying responses to serotonin were determined for the different mutant alleles by placing individual animals in liquid M9 and the indicated concentration of serotonin and counting the number of eggs laid by each animal after 1 hr of drug exposure. Both alleles of *unc-38(x20)* and *unc-38(e264)* and one allele of *unc-29(e193)* showed a statistically significant increase in egg laying at low (3.25 mM) serotonin concentrations according to the Mann-Whitney rank sum test (confidence level  $P < 0.05$ ). By the same test, all shown levamisole receptor mutants *unc-29(x29)*, *unc-29(e193)*, *unc-38(x20)*, *unc-38(e264)*, *lev-1(e211)*, and *lev-1(x427)* displayed a statistically significant decrease in egg laying at high (25 mM) serotonin concentrations (confidence level  $P < 0.05$ ). Individual points and error bars indicate the mean and SEM of at least the following numbers of trials: N2, 70; *unc-29(x29)*, 38; *unc-29(e193)*, 36; *unc-38(x20)*, 36; *unc-38(e264)*, 32; *lev-1(e211)*, 33; *lev-1(x427)*, 34.

promoter directs gene expression in all *unc-29*-expressing muscle cells at levels at least as high as those provided by the *unc-29* promoter (see WAGGONER *et al.* 2000a), our results suggest that neuronal UNC-29 receptors are important for the levamisole response on NGM. Consistent with this possibility, ablations that eliminated the VC motoneurons (the only neurons in the egg-laying circuit with detectable UNC-29 expression) also conferred resistance to levamisole in the NGM assay. Since an ectopic promoter that replicated the neuronal UNC-

29 expression pattern was not available, we could not determine whether neuronal UNC-29 receptors were sufficient for levamisole response on NGM. Nonetheless, our results indicate that the alterations in egg-laying pattern caused by levamisole treatment are at least partially dependent on neuronal UNC-29 receptors, possibly functioning in the VCs.

How might neuronal UNC-29 receptors participate in the regulation of egg laying? In vertebrate systems, neuronal nicotinic receptors have been shown to facilitate neurotransmitter release from synaptic terminals. Thus, it is reasonable to suppose that UNC-29 receptors in the VCs function in a similar fashion to promote release of neurotransmitters and/or neuromodulators that function at the VC-vulval muscle neuromuscular junctions. The VCs contain multiple neurotransmitters, including acetylcholine (RAND and NONET 1997a), one or more FMRFamide-related neuropeptides (SCHINKMANN and LI 1992), and an unidentified biogenic amine (DUERR *et al.* 1999). Since levamisole shortens, and levamisole receptor mutations often lengthen, both the intercluster and intracluster time constants, levamisole receptors in the VCs may regulate the release of transmitters that induce egg-laying contractions within the active phase (*i.e.*, acetylcholine) as well as modulators that control the onset of the active phase (perhaps a peptide or amine).

**Levamisole receptors and HSN-independent egg-laying neurotransmission:** Although mutations in the levamisole receptor genes had relatively subtle effects on the pattern of egg laying, their effect was considerably greater in animals lacking the serotonergic HSN motoneurons. In an *egl-1* mutant background, both *unc-29* and *lev-1* mutations lengthened the intracluster time constant more than threefold. These results imply that the levamisole receptor is specifically important for cholinergic neurotransmission between the VC motoneurons and the vulval muscles, but less important for HSN/vulval muscle neurotransmission. Neuronal UNC-29 receptors indeed appear to be expressed in the VCs but not the HSNs; thus, importance of *unc-29* for HSN-independent egg laying may reflect this asymmetry in the distribution of neuronal UNC-29 receptors in the egg-laying motor synapses.

Interestingly, no phenotypic synergy was observed between the levamisole receptor genes and *tph-1*, a gene required for the synthesis of the major HSN neurotransmitter serotonin. This is perhaps surprising since serotonin is sufficient to rescue HSN function (TRENT *et al.* 1983) and has been shown to potentiate the induction of egg laying by nicotinic agonists (WAGGONER *et al.* 1998). However, a number of studies have demonstrated that the loss of HSN function can have effects on egg laying that are more severe than those caused by a defect in serotonergic neurotransmission. For example, HSN-deficient animals were found to be resistant to stimulation of egg laying by levamisole, whereas serotonin-

deficient animals were not (WEINSHENKER *et al.* 1995). Likewise, the ability of  $G_{\alpha}/goa-1$  mutations to shorten the inactive egg-laying phase is dependent on the presence of the HSNs, but not on the ability to synthesize serotonin (WAGGONER *et al.* 2000b). The simplest interpretation of these results is that the HSNs contain a second neuromodulator that functions redundantly with serotonin to potentiate the ability of nicotinic receptors to induce vulval muscle contraction. The identity of such a hypothetical stimulatory modulator of egg laying is not known, although since the HSNs contain RFamide immunoreactivity, one possible candidate is a FMRFamide-related neuropeptide.

**Connections between nicotinic receptor activity and serotonin response:** An interesting and unexpected result was the observation that mutants defective in the receptor subunit gene *unc-38* dramatically altered the egg-laying response to serotonin. *unc-38* animals laid eggs in response to serotonin concentrations that were approximately fivefold lower than those required to stimulate egg laying in wild type; however, the magnitude of the serotonin response (*i.e.*, the maximum number of eggs laid in response to serotonin) was much lower in *unc-38* mutants than in wild-type animals. Both the hypersensitivity and reduced response to serotonin appeared to be somewhat specific to *unc-38*, as both these effects were minimal in *unc-29* and *lev-1*; overall, the serotonin dose responses of these two mutants were more similar to wild type. Thus, a nicotinic receptor containing UNC-38 but not UNC-29 or LEV-1 subunits appears to both promote and negatively regulate serotonin responses in the egg-laying circuitry.

What interactions between cholinergic and serotonergic response pathways might explain the effects of *unc-38* mutations on serotonin response? Interestingly, mutations in a number of genes involved in promoting neurotransmitter release from ventral cord neurons are also serotonin hypersensitive and/or show reduced serotonin responses (SCHAFER *et al.* 1996). Preliminary analysis of *unc-38::GFP* promoter fusions (A. GOTTSCHALK and W. SCHAFER, unpublished data) as well as genetic evidence (discussed in RAND and NONET 1997b) suggests that *unc-38* functions in neurons as well as muscles. Perhaps neuronal UNC-38 receptors might control the release of modulators that downregulate or potentiate serotonin response in the egg-laying neuromusculature. Alternatively, the vulval muscles might express an UNC-38-containing nicotinic receptor subtype whose chronic activity regulates the activity of signal transduction pathways downstream of serotonin. In either case, the long-term activity of UNC-38 receptors could directly or indirectly modulate the activity of serotonin-responsive signaling pathways in the vulval muscles. In the future, the identification and characterization of additional genes required for the egg-laying responses to these transmitters are likely to provide insight into

these interactions between cholinergic and serotonergic neurotransmission.

The authors thank Jim Lewis for generously providing strains, reagents, and advice. We also thank Rene Garcia, Josh Kaplan, Curtis Loer, and Caenorhabditis Genetics Center for strains; members of our lab for discussions; and Stanley Shyn, Rex Kerr, and Alexander Gottschalk for comments on the manuscript. This work was supported by grants from the National Institutes of Health (DA11556 and DA12891), the Joseph and Esther Klingenstein Foundation, the Alfred P. Sloan Foundation, and the Arnold and Mabel Beckman Foundation (to W.R.S.); a postdoctoral training grant from the National Institutes of Health (to L.E.W.); and an undergraduate fellowship from the Howard Hughes Medical Institute (D.S.R.).

#### LITERATURE CITED

- EVERY, L., and H. R. HORVITZ, 1990 Effects of starvation and neuroactive drugs on feeding in *Caenorhabditis elegans*. *J. Exp. Zool.* **253**: 263–270.
- BALLIVET, M., C. ALLIOD, S. BERTRAND and D. BERTRAND, 1996 Nicotinic acetylcholine receptors in the nematode *Caenorhabditis elegans*. *J. Mol. Biol.* **258**: 261–269.
- BAYLIS, H. A., K. MATSUDA, M. D. SQUIRE, J. T. FLEMING, R. J. HARVEY *et al.*, 1997 ACR-3, a *Caenorhabditis elegans* nicotinic acetylcholine receptor subunit. *Receptors Channels* **5**: 149–158.
- BRENNER, S., 1974 The genetics of *Caenorhabditis elegans*. *Genetics* **77**: 71–94.
- DANI, J. A., and S. HEINEMANN, 1996 Molecular and cellular aspects of nicotine abuse. *Neuron* **16**: 905–908.
- DESAI, C., G. GARRIGA, S. MCINTIRE and H. R. HORVITZ, 1988 A genetic pathway for the development of the *Caenorhabditis elegans* HSN motor neurons. *Nature* **336**: 638–646.
- DUERR, J. S., D. L. FRISBY, J. GASKIN, A. DUKE, K. ASERMELY *et al.*, 1999 The *cat-1* gene of *Caenorhabditis elegans* encodes a vesicular monoamine transporter required for specific monoamine-dependent behaviors. *J. Neurosci.* **19**: 72–84.
- FLEMING, J. T., C. TORNOE, H. A. RIINA, J. COADWELL, J. A. LEWIS *et al.*, 1993 Acetylcholine receptor molecules of the nematode *Caenorhabditis elegans*. *EXS* **63**: 65–80.
- FLEMING, J. T., M. D. SQUIRE, T. M. BARNES, C. TORNOE, K. MATSUDA *et al.*, 1997 *Caenorhabditis elegans* levamisole resistance genes *lev-1*, *unc-29*, and *unc-38* encode functional nicotinic acetylcholine receptor subunits. *J. Neurosci.* **17**: 5843–5857.
- GALZI, J.-L., F. REVAH, A. BESSIS and J.-P. CHANGEAX, 1991 Functional architecture of the nicotinic acetylcholine receptor: from electric organ to brain. *Annu. Rev. Pharmacol.* **31**: 37–72.
- HARFE, B. D., and A. FIRE, 1998 Muscle and nerve-specific regulation of a novel NK-2 class homeodomain factor in *Caenorhabditis elegans*. *Development* **125**: 421–429.
- LEWIS, J. A., C.-H. WU, H. BERG and J. H. LEVINE, 1980a The genetics of levamisole resistance in *Caenorhabditis elegans*. *Genetics* **95**: 905–928.
- LEWIS, J. A., C.-H. WU, J. H. LEVINE and H. BERG, 1980b Levamisole-resistant mutants of the nematode *Caenorhabditis elegans* appear to lack pharmacological acetylcholine receptors. *Neuroscience* **5**: 967–989.
- LEWIS, J. A., J. S. ELMER, J. SKIMMING, S. MCLAFFERTY, J. FLEMING *et al.*, 1987 Cholinergic receptor mutants of the nematode *Caenorhabditis elegans*. *J. Neurosci.* **7**: 3059–3071.
- LI, C., and M. CHALFIE, 1990 Organogenesis in *C. elegans*: positioning of neurons and muscles in the egg-laying system. *Neuron* **4**: 681–695.
- MONGAN, N. P., H. A. BAYLIS, C. ADCOCK, G. R. SMITH, M. S. P. SANSOM *et al.*, 1998 An extensive and diverse gene family of nicotinic acetylcholine receptor  $\alpha$  subunits in *Caenorhabditis elegans*. *Receptors Channels* **6**: 213–228.
- OKKEMA, P. G., S. W. HARRISON, V. PLUNGER, A. ARYANA and A. FIRE, 1993 Sequence requirements for myosin gene-expression and regulation in *Caenorhabditis elegans*. *Genetics* **135**: 385–404.
- RAIZEN, D. M., Y. N. LEE and L. AVERY, 1995 Interacting genes required for pharyngeal excitation by motoneuron MC in *Caenorhabditis elegans*. *Genetics* **141**: 1365–1382.

- RAND, J. B., 1989 Genetic analysis of the *cha-1-unc-17* gene complex in *Caenorhabditis*. *Genetics* **122**: 73–80.
- RAND, J. B., and M. L. NONET, 1997a Neurotransmitter assignments for specific neurons, pp. 1049–1052 in *C. elegans II*, edited by D. L. RIDDLE, T. BLUMENTHAL, B. J. MEYER and J. R. PRIESS. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- RAND, J. B., and M. L. NONET, 1997b Synaptic transmission, pp. 611–643 in *C. elegans II*, edited by D. L. RIDDLE, T. BLUMENTHAL, B. J. MEYER and J. R. PRIESS. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- RICHMOND, J. E., and E. M. JØRGENSEN, 1999 One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction. *Nat. Neurosci.* **2**: 1–7.
- SARGENT, P. B., 1995 The diversity of neuronal nicotinic acetylcholine receptors. *Annu. Rev. Neurosci.* **16**: 403–443.
- SCHAFFER, W. R., B. M. SANCHEZ and C. K. KENYON, 1996 Genes affecting sensitivity to serotonin in *Caenorhabditis elegans*. *Genetics* **143**: 1219–1230.
- SCHINKMANN, K., and C. LI, 1992 Localization of FMRFamide-like peptides in *Caenorhabditis elegans*. *J. Comp. Neurol.* **316**: 251–260.
- SQUIRE, M. D., C. TORNOE, H. A. BAYLIS, J. T. FLEMING, E. A. BARNARD *et al.*, 1995 Molecular cloning and functional co-expression of a *Caenorhabditis elegans* nicotinic acetylcholine receptor subunit (*acr-2*). *Receptors Channels* **3**: 107–115.
- SULSTON, J., and J. HODGKIN, 1988 Methods, pp. 587–606 in *The Nematode Caenorhabditis elegans*, edited by W. B. WOOD. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- SZE, J. Y., M. VICTOR, C. LOER, Y. SHI and G. RUVKUN, 2000 Food and metabolic signaling defects in a *Caenorhabditis elegans* serotonin-synthesis mutant. *Nature* **403**: 560–564.
- TREININ, M., and M. CHALFIE, 1995 A mutated acetylcholine receptor subunit causes neuronal degeneration in *C. elegans*. *Neuron* **14**: 871–877.
- TRENT, C., N. TSUNG and H. R. HORVITZ, 1983 Egg-laying defective mutants of the nematode *Caenorhabditis elegans*. *Genetics* **104**: 619–647.
- WAGGONER, L., G. T. ZHOU, R. W. SCHAFFER and W. R. SCHAFFER, 1998 Control of behavioral states by serotonin in *Caenorhabditis elegans*. *Neuron* **21**: 203–214.
- WAGGONER, L. E., K. A. DICKINSON, D. S. POOLE, Y. TABUSE, J. MIWA *et al.*, 2000a Long-term nicotine adaptation in *Caenorhabditis elegans* involves PKC-dependent changes in nicotinic receptor abundance. *J. Neurosci.* **20**: 8802–8811.
- WAGGONER, L. E., L. A. HARDAKER, S. GOLIK and W. R. SCHAFFER, 2000b Effect of a neuropeptide gene on behavioral states in *Caenorhabditis elegans* egg-laying. *Genetics* **154**: 1181–1192.
- WEINSHENKER, D., G. GARRIGA and J. H. THOMAS, 1995 Genetic and pharmacological analysis of neurotransmitters controlling egg-laying in *C. elegans*. *J. Neurosci.* **15**: 6975–6985.
- WHITE, J., E. SOUTHGATE, N. THOMSON and S. BRENNER, 1986 The structure of the *Caenorhabditis elegans* nervous system. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **314**: 1–340.
- WILLIAMS, B. D., 1995 Genetic mapping with polymorphic sequence-tagged sites. *Methods Cell Biol.* **48**: 81–96.
- ZAR, J. H., 1996 *Biostatistical Analysis*. Prentice-Hall, Upper Saddle River, NJ.
- ZHOU, G. T., W. R. SCHAFFER and R. W. SCHAFFER, 1998 A three-state biological point process model and its parameter estimation. *IEEE Trans. Signal Process* **46**: 2698–2707.

Communicating editor: R. K. HERMAN