Genes Affecting Sensitivity to Serotonin in *Caenorhabditis elegans*

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ABSTRACT

Regulating the response of a postsynaptic cell to neurotransmitter is an important mechanism for controlling synaptic strength, a process critical to learning. We have begun to define and characterize genes that may control sensitivity to the neurotransmitter serotonin in the nematode *Caenorhabditis elegans* by identifying serotonin-hypersensitive mutants. We reported previously that mutations in the gene *unc-2*, which encodes a putative calcium channel subunit, result in hypersensitivity to serotonin. Here we report that mutants defective in the *unc-36* gene, which encodes a homologue of a calcium channel auxiliary subunit, are also serotonin-hypersensitive. Moreover, the *unc-36* gene appears to be required in the same cells as *unc-2* for control of the same behaviors. Mutations in several other genes, including *unc-8, unc-10, unc-20, unc-35, unc-75, unc-77,* and *snt-1* also result in hypersensitivity to serotonin. Several of these mutations have previously been shown to confer resistance to acetylcholinesterase inhibitors, suggesting that they may affect acetylcholine release. Moreover, we found that mutations that decrease acetylcholine synthesis cause defective egg-laying and serotonin hypersensitivity. Thus, acetylcholine appears to negatively regulate the response to serotonin and may participate in the process of serotonin desensitization.

THE ability of an animal to learn depends on molecular events within neurons that alter the relative strengths of specific synapses. One important mechanism for the modification of synaptic strength is regulation of the postsynaptic cell's sensitivity to neurotransmitter (NESTLER *et al.* 1993). A simple example of this type of regulation is the process of adaptation or desensitization. A neurotransmitter acts by binding to receptors on the postsynaptic cell surface, which then activate intracellular signaling pathways that evoke a response in the postsynaptic cell. Desensitization occurs when prolonged exposure to neurotransmitter activates adaptive pathways that inhibit or attenuate neurotransmitter response. Neurotransmitter desensitization has many important behavioral consequences; for example, adaptive responses to elevated dopamine levels in the brain have been implicated in addiction to drugs such as cocaine (NESTLER *et al.* 1993; COLE *et al.* 1995).

Studies of receptor-activated signaling pathways in nonneuronal cells indicate that desensitization can be a complex process that occurs at multiple steps in a single signaling pathway. For example, the *Saccharomyces cerevisiae* mating-factor response pathway, which triggers the mating process through a G-protein-coupled signaling mechanism, can undergo desensitization after long-term exposure to mating pheromone. Yeast mutants have been identified that are hypersensitive to pheromone (CHAN and OTTE 1982; COURCHESNE *et al.* 1989; STEDEN *et al.* 1989); these mutants define a number of genes whose products appear to be involved in desensitizing the pheromone response pathway. Surprisingly, these gene products do not appear to be components of a single adaptive pathway, but rather act independently at several different steps in the response pathway to down-regulate the response to pheromone. Pheromone-hypersensitive mutants define genes involved in pheromone degradation (MACKAY *et al.* 1988; STEDEN and DUNTZE 1990; MARCUS *et al.* 1991), receptor turnover (KONOPKA *et al.* 1988; RENEKE *et al.* 1988; DAVIS *et al.* 1993), G-protein inactivation (COLE and REED 1991), and inhibition of downstream effector molecules (MIYAJIMA *et al.* 1989; CYERT and THORNER 1992). Thus, in yeast cells, desensitization apparently occurs through many distinct adaptive mechanisms that act on a variety of different signaling components.

To understand the molecular events involved in the control of neurotransmitter response within the nervous system, we have undertaken a similar genetic approach to identify genes that affect sensitivity to the neurotransmitter serotonin in the roundworm *Caenorhabditis elegans*. Serotonin treatment has striking and specific effects on *C. elegans* behavior: serotonin stimulates egg-laying and feeding, activates a step in the male mating program, and inhibits movement (CROLL 1975; HORVITZ *et al.* 1982; AVERY and HORVITZ 1990; LOER and KENYON 1993). Prolonged exposure to serotonin results in desensitization (CROLL 1975; SCHAFER and KENYON 1995). We previously showed that mutations in the gene *unc-2* resulted in hypersensitivity and failure

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to adapt to serotonin, suggesting that the *unc-2* gene product might be an important regulator of serotonin sensitivity. By cloning *unc-2,* we determined that it encodes a homologue of a voltage-gated calcium channel subunit, which may act to trigger calcium-activated signaling pathways that control the serotonin response (SCHAFER and KENYON 1995).

In this study, we report the identification of several additional mutations that cause hypersensitivity to serotonin and therefore define genes that may regulate the serotonin response of the vulval muscles. One of these genes, *unc-36,* encodes a protein that is a strong candidate for an auxiliary subunit of the UNC-2 calcium channel (L. LOBEL and H. R. HORVITZ, personal communication). Other serotonin hypersensitivity genes appear to be required for acetylcholine release. We present genetic evidence that acetylcholine inhibits the responsiveness of the vulval muscles to serotonin and might therefore participate in the process of serotonin desensitization.

MATERIALS AND METHODS

Materials: Serotonin (creatinine sulfate complex) and dopamine (hydrochloride) were obtained from Sigma. BiTek agar and Agar Noble were from Difco.

Strains and genetic methods: The chromosomal locations of the genes studied in these experiments are as follows: LGI: *unc-35, unc-75;* LGII: *snt-1;* LGIII: *pal-1, dpy-17, ncl-1, unc-36, unc-25;* LGIV: *cha-1, unc-77, unc-8, egl-19, unc-43, him-8;* LGV: *him-5, egl-1;* LGX: *unc-2, unc-20, unc-10, unc-9.* Routine culturing of *C. elegans* was performed as described (BRENNER 1974). Behavioral assays were performed at room temperature (~20–22°).

Construction of double mutant strains: The double mutants carrying mutations in both *egl-1* and one of the genes with an Egl-c (egg-laying constitutive) Unc (uncoordinated) mutant phenotype were constructed by mating *egl-1* males (generated by heat-shock) to Unc hermaphrodites. Unc F_2s (homozygous for the *unc-* mutation) were selected, picked to single plates, and allowed to self-fertilize. In all cases, some of these Unc F_2s were found to be Egl-d (egg-laying defective). Since the *egl-1(n986)* mutation is dominant, *egl-1* homozygotes were identified as animals that were Egl-d and whose self-progeny were all Egl-d.

The *egl-19; unc-2* and *unc-36; egl-19* double mutants were generated as follows: wild-type males were mated to *egl-19* hermaphrodites, yielding male cross progeny that were heterozygous for the *egl-19* mutation. These animals were then mated to *unc-2* or *unc-36* hermaphrodites. F_1 animals with the genotype *egl-19/+; unc-2/+* or *unc-36/+; egl-19/+* were identified as wild-type hermaphrodites that gave rise to both Unc and Egl-d self-progeny. Among the self-progeny of these animals, there was also a class of animals with a novel phenotype: these animals were nearly paralyzed and strongly Egl-d. The self-progeny of these animals showed the identical phenotype. We demonstrated that these animals were in fact the double mutants by mating them with wild-type males and observing the presence in the F2 generation of animals with the Egl-19 and the Unc-2 or Unc-36 single-mutant phenotypes.

The *unc-36; unc-2* double mutants were constructed in the following manner: *dpy-17(e164)* males were mated to *unc-2* hermaphrodites, giving rise to *dpy-17/+; unc-2* males. These animals were then mated to *unc-36* hermaphrodites. F_1 cross progeny of the genotype *dpy-17 +/+ unc-36; unc-2/+* were identified as wild-type hermaphrodites that gave rise to both Dpy (dumpy) and Unc self-progeny. Single Unc non-Dpy animals from these plates were picked individually to new plates and allowed to generate self-progeny. These F_2 animals were homozygous for either *unc-2* or *unc-36* (or possibly both). Since *dpy-17* and *unc-36* are tightly linked on chromosome *3, + unc-36/dpy-17 +; unc-2 /unc-2* animals were identifiable as Unc non-Dpy animals that gave rise to Unc Dpy F_3 progeny. We then picked individual Unc non-Dpy siblings of these F_3s and allowed them to generate self-progeny. *unc-2 unc-36* double homozygotes would be expected to give rise to only non-Dpy self-progeny, whereas + *unc-36/dpy-17* + heterozygotes would generate both Dpy and non-Dpy animals. Of 20 F_3s picked (derived from five different F_2s and two different F_1s), 10 gave rise only to non-Dpys. The genotype of these putative double mutants was confirmed by mating them to *him-5(e1490)* males. All the male F_1 cross progeny were Unc; thus, the animals were homozygous for *unc-2.* In addition, four independent F_1 hermaphrodite cross-progeny gave rise to *unc-36* homozygotes in their self-progeny (these animals were *unc-36* rather than *unc-2* because they gave rise to wild-type male cross-progeny when crossed with wild-type males).

Behavioral assays: Sensitivity to serotonin and constitutive egg-laying rates were measured in M9 liquid medium as described previously (TRENT *et al.* 1983; SCHAFER and KENYON 1995). In Figures 1A and 4A, each bar represents the overall mean computed from five or more such trials; error bars indicate the sample standard deviation of the individual means. In Figures 1B, 3 and 5, each point represents the average of three to five trials of 10 animals each; the points in the *egl-1* curve represent the mean of eight such trials. Error bars indicate the sample standard deviation. Serotonin desensitization (Figures 1C and 4C) was assayed by determining the percentage of animals that accumulated late-stage embryos after overnight treatment with 8.7 mM serotonin (SCHAFER and KENYON 1995). Early egg-laying (Figure 4B) was measured by determining the developmental stage of the most mature embryo present in the uterus of at least 25 gravid hermaphrodites grown on NG plates at 20° in the presence of food. Egl-c animals contained no embryos later than the eight-cell stage of development. Assays for sensitivity and adaptation to dopamine were performed as described (SCHAFER and KENYON 1995). Animals were scored as paralyzed if they made no spontaneous body movements during a 10-sec observation. The assay plates were prepared using either Bitek agar or Agar Noble (Difco). These agar preparations contained very low calcium content relative to other grades of agar; calcium salts have been observed to inhibit the ability of dopamine to paralyze *C. elegans* (W. SCHAFER and C. KENYON, unpublished results).

Mosaic analysis: The strain used to perform mosaic analysis was constructed in the following manner: male animals of the strain CF383 (genotype: *pal-1(ct224) dpy-17(e164) ncl-1(e1865) unc-36(e251); him-8(e1489); sDp3*) were mated with hermaphrodites of the strain CF91 (genotype: *dpy-17(e164) ncl-1(e1865) unc-36(e251); him-5(e1490)*). Since *sDp3* complements the mutations in *pal-1, dpy-17, ncl-1* and *unc-36,* cross progeny carrying the duplication were identified as non-Unc non-Dpy hermaphrodites. These animals were picked individually and allowed to generate self-progeny; non-Unc non-Dpy hermaphrodites were then picked individually from this F_2 population. Since the *pal-1(ct224)* mutation causes lethality, animals that are homozygous for *pal-1* and complemented by the free duplication will only give rise to non-Dpy non-Unc progeny. Thus, *pal-1(+)* animals could be identified as animals that gave rise to Dpy Unc progeny and no dead eggs, and whose

non-Dpy non-Unc progeny all also generated Dpy Unc progeny. Subsequently, animals carrying the wild-type alleles of *him-5* and *him-8* were selected by identifying animals that did not give rise to male self-progeny in the F_1 or the F_2 generations.

Mosaic analysis was performed as described in the text. The Ncl (abnormal nucleolus) phenotype was scored in the following cells: HSNL, HSNR, PDEL, PDER, SDQR, AVM, ALML, ALMR, M2L, M2R, M1, M5, I6, NSML, NSMR, MCL, MCR, I2L, and I2R. In some animals, the cells I4, BDUL, BDUR, and the ventral cord motor neurons were also scored.

RESULTS

Genetic characterization of *unc-36*: To identify gene products that might participate in controlling the response to serotonin, we began to search for mutations that cause serotonin hypersensitivity. In these studies we focused on one well-characterized serotonin-regulated behavior, egg-laying. Adult *C. elegans* hermaphrodites contain a pair of serotonergic neurons, called HSNs (hermaphrodite-specific neuron), that form synapses with the vulval muscles that expel the egg from the uterus (WHITE *et al.* 1986). Animals that lack functional HSNs are unable to lay eggs efficiently unless treated with exogenous serotonin. This has been demonstrated by killing the HSN nuclei by laser microsurgery (H. R. HORVITZ and J. SULSTON, unpublished results, cited in CHALFIE and WHITE 1988) and also by introducing mutations in the *egl-1* gene, which cause the HSNs to undergo inappropriate programmed cell death in hermaphrodites (TRENT *et al.* 1983; DESAI *et al.* 1988). These results argue that serotonin released from the HSNs acts to stimulate contraction of the vulval muscles and thus increase the rate of egg-laying.

In previous work, we demonstrated that mutant strains carrying recessive mutations in the gene *unc-2*, which encodes a homologue of a calcium channel subunit, are hypersensitive to serotonin. *unc-2* mutants have an egg-laying constitutive (Egl-c) phenotype: they lay eggs under conditions that inhibit egg-laying in the wild type and release a fraction of their embryos at an abnormally early stage of development. To determine whether this Egl-c phenotype might result from serotonin hypersensitivity, we constructed an *egl-1; unc-2* double mutant strain that lacked the HSNs and assayed its response to exogenous serotonin. In this way, it was possible to separate and distinguish effects of *unc-2* on serotonin response by the postsynaptic cell (the vulval muscle) from possible effects on serotonin release from the presynaptic cell (HSN); moreover, since the *egl-1; unc-2* strain, like the *egl-1* single mutant, was egg-laying defective (Egl-d), we could compare the ability of serotonin to stimulate egg-laying in experimental (*egl-1; unc-2*) and control (*egl-1*) animals that contained approximately equal numbers of embryos of comparable developmental age. Since *egl-1; unc-2* double mutants were approximately eightfold more sensitive to exogenous serotonin than wild-type or *egl-1* animals, we concluded that a loss of *unc-2* function caused an increased sensitivity of the vulval muscles to serotonin (SCHAFER and KENYON 1995).

TABLE 1

Genetic interactions between *unc-2* and *unc-36*

Genotype	Movement	Egg-laying
N2	+++ (active)	Normal
unc-2(mu74)	+ (sluggish)	Egl-c
unc-2(e55)	+ (sluggish)	Egl-c
unc-36(e251)	+ (sluggish)	Weak Egl-c
unc-36(e873)	+ (sluggish)	Weak Egl-c
egl-19(n582)	++ (slow)	Weak Egl-d
unc-36(e251); unc-2(mu74)	+ (sluggish)	Weak Egl-c
unc-36(e873); unc-2(e55)	+ (sluggish)	Weak Egl-c
egl-19; (n582); unc-2(mu74)	− (paralyzed)	Strong Egl-d
egl-19; (n582); unc-2(e55)	− (paralyzed)	Strong Egl-d
unc-36(e251); egl-19(n582)	− (paralyzed)	Strong Egl-d
unc-36(e873); egl-19(n582)	− (paralyzed)	Strong Egl-d

Since the *unc-2* gene encodes a putative subunit of a voltage-gated calcium channel, mutations in the genes that encode the other subunits of that channel might also be expected to cause hypersensitivity to serotonin. All known voltage-sensitive calcium channels contain at least three accessory subunits: α-2, β, and δ. The *C. elegans* genome project recently identified a coding region with high sequence similarity to the α-2/δ accessory subunits of vertebrate calcium channels (WILSON *et al.* 1994); this coding region has been shown to correspond to the gene *unc-36* (L. LOBEL and H. R. HORVITZ, personal communication). Therefore, we investigated whether *unc-36* mutations might also lead to constitutive egg-laying and serotonin hypersensitivity.

unc-36 mutants exhibit many phenotypic similarities with *unc-2* mutants (Table 1, Figure 1). Both *unc-2* and *unc-36* animals are sluggish, move in an uncoordinated "kinking" manner, and are slightly longer in body length than wild-type animals (BRENNER 1974; WOOD 1988). We observed that like *unc-2* mutants, *unc-36* mutants were egg-laying constitutive (Egl-c), since they laid eggs under conditions that inhibit egg-laying by wild-type animals (Figure 1A). To determine whether this Egl-c phenotype might result from serotonin hypersensitivity, we constructed an *unc-36; egl-1* double mutant strain and measured its sensitivity to exogenous serotonin. We found that *unc-36; egl-1* double mutants were approximately fivefold more sensitive to serotonin than an *egl-1* single mutant, indicating that *unc-36* mutations caused serotonin hypersensitivity (Figure 1B). *unc-36* animals were also defective (although less severely than *unc-2* mutants) in an assay for serotonin adaptation (Figure 1C). Finally, *unc-2* animals were shown previously to be defective in adaptation to another neurotransmitter, dopamine; however, their initial dopamine sensitivity was close to that of wild-type animals. We observed that *unc-36* mutants likewise failed to adapt to

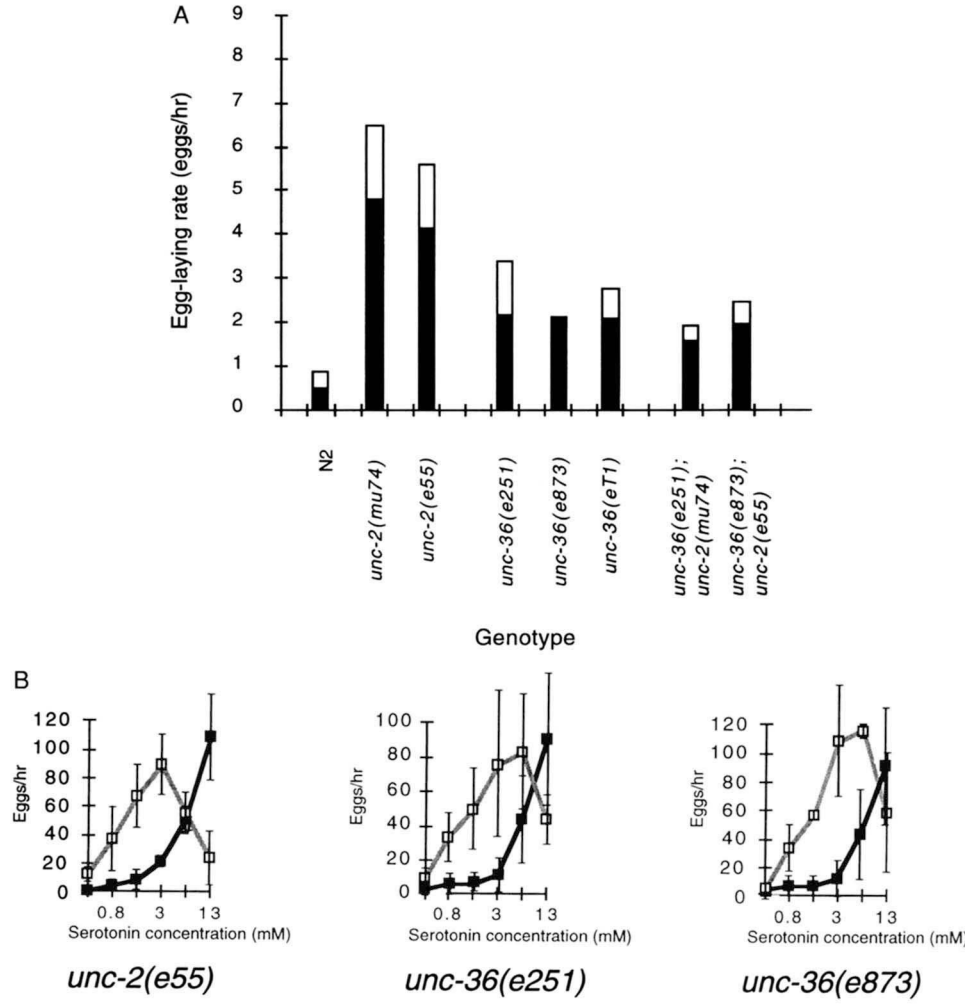

FIGURE 1.—Characterization of the *unc-36* mutant phenotype. (A) Constitutive egg-laying. Egg-laying rates were measured after a 1-hr incubation at room temperature (20°) in hypertonic aqueous solution (M9) as described in MATERIALS AND METHODS. ■, the average rate of egg-laying per animal (eggs/hr); □, the sample standard deviation of the means of at least four independent trials of 10 animals each. All mutant strains gave results that were statistically different from wild type according to the Mann-Whitney rank sum test ($P < 0.01$). (B) Serotonin sensitivity. Dose response curves for *egl-1(n986)* (■) and the indicated *unc; egl-1* double mutant (□) were performed as described (SCHAFER and KENYON 1995). The *y* axis is on a linear scale and indicates the mean rate of egg-laying (eggs/10 worms/hr); the *x* axis is on a logarithmic scale and indicates the serotonin concentration (mM). Each point represents the mean of at least four trials of 10 animals each; the *egl-1* single mutant control represents the mean of eight trials. Error bars denote the sample standard deviation of the individual trials. The *unc-2* curves were computed from data presented previously (SCHAFER and KENYON 1995); the *egl-1* allele was erroneously reported as *n987* in that paper. (C) Serotonin and dopamine desensitization. Adult hermaphrodites were incubated overnight (15 hr) at 20° on 1.5% low-calcium agar plates containing 8.7 mM serotonin or 16 mM dopamine. Desensitization to serotonin was assayed by counting the number of Egl animals that had accumulated unlaid late-stage embryos under these conditions. Dopamine desensitization was assayed by counting the percentage of animals that failed to move independently within a 10-sec interval. At least 40 animals were tested for each strain under each condition. All mutants gave results that were statistically different from wild type according to the *z* test ($P < 0.01$). (D) Dopamine sensitivity. Animals were incubated for 45 min on 1.5% agar plates containing the indicated concentration of dopamine, and paralysis was measured as described above. Error bars, SE. Each point represents the following number of animals: N2, 160; *unc-2(mu74)*, 40; *unc-36(e251)*, 40; *egl-19(n582)*, 60.

long-term dopamine exposure, although their initial dopamine sensitivity was similar to wild type (Figure 1, C and D).

The phenotypes of *unc-36; unc-2* double mutants were essentially identical to the phenotypes of *unc-2* and *unc-36* single mutants: sluggish kinky movement, constitutive egg-laying, and long body length (Table 1, Figure 1A). No significant enhancement of any of these phenotypes was observed, nor were any obvious new synthetic phenotypes such as slow growth or reduced fertility seen in the double mutant. *unc-2* and *unc-36* mutations also showed similar genetic interactions with the gene *egl-19*, mutations in which cause a weak egg-laying defect, slow movement, and dopamine hypersensitivity. Two *egl-19; unc-2* strains and two *unc-36 egl-19* strains showed the same synthetic phenotype: they were nearly paralyzed and completely defective in egg-laying (Table 1). Taken together, these genetic data support the hypoth-

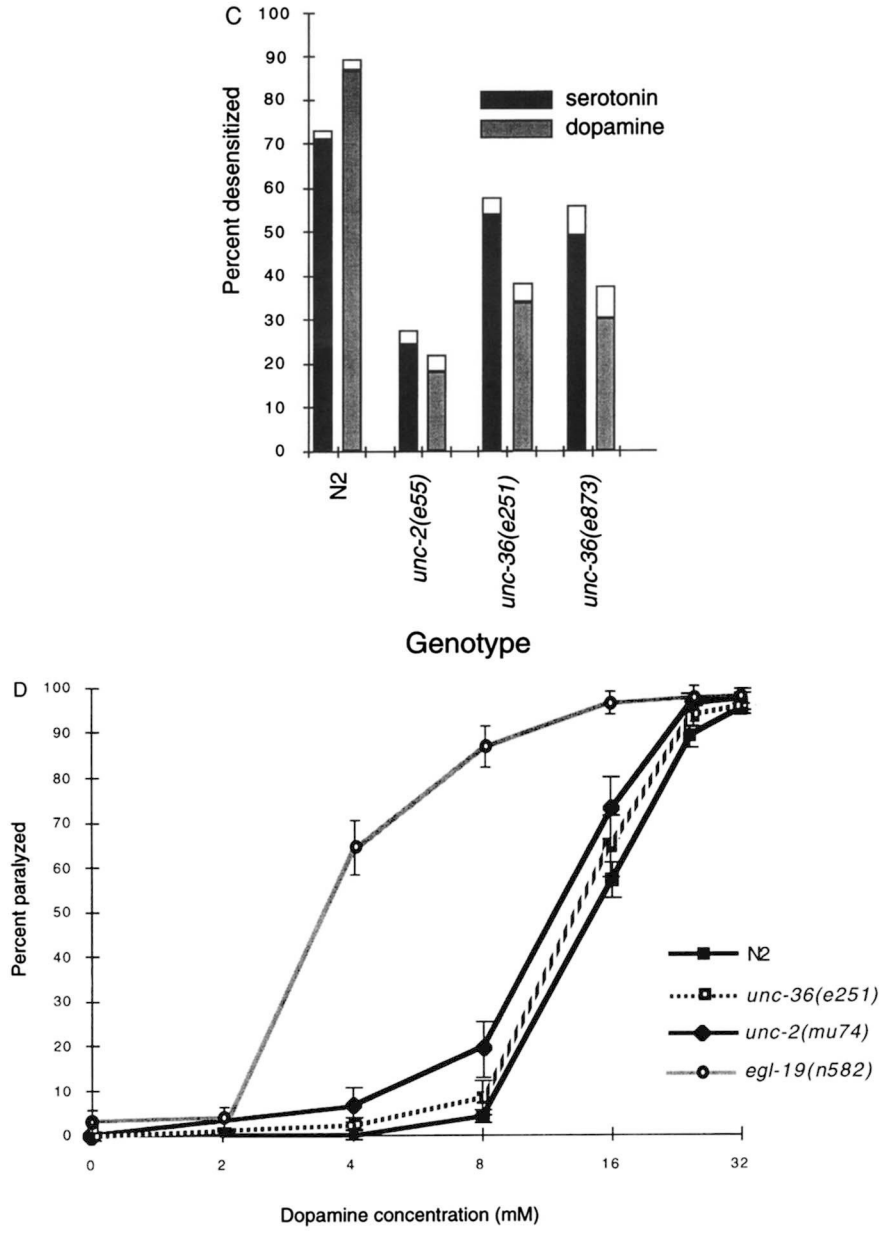

FIGURE 1.—Continued

esis that *unc-2* and *unc-36* are involved in a common molecular function.

To determine whether *unc-2* and *unc-36* might function within the same cells, we performed mosaic analysis on the *unc-36* behavioral phenotypes. Our earlier work indicated that the uncoordinated phenotype of *unc-36* had a focus in the descendants of the AB.p blastomere (KENYON 1986). However, analysis of *unc-2* mosaics indicated that the kinking and sluggish phenotypes of *unc-2* were separable and had distinct cellular foci (SCHAFER and KENYON 1995). Therefore, we performed a new mosaic analysis with the aim of determining the specific foci of the sluggish and kinking phenotypes of *unc-36* mosaics. (Figure 2, Table 2). We observed that the kinking phenotype had a focus in the descendants of the cell AB.p. More specifically, the kinking phenotype appeared to arise from loss of functional *unc-36* in the lineages that give rise to the adult and juvenile motor neurons. In contrast, the sluggish phenotype appeared to have foci in both neuronal and muscle lineages. Partially sluggish mosaics arose from loss of functional *unc-36* in the descendants of P1, which gives rise to the body muscle, and also from loss in the descendants of AB and AB.p, which give rise to nearly all nonpharyngeal neurons. However, none of these mosaics were as sluggish as the *unc-36* mutant itself. This result suggests that *unc-36* may act in both neurons and muscle cells to enhance motor activity. Because the Egl-c phenotype of *unc-36* is relatively weak, it is difficult to score in single animals; thus, we were unable to ascertain the focus of this phenotype. However, the cellular foci of both the kinking and the sluggish phenotypes mapped

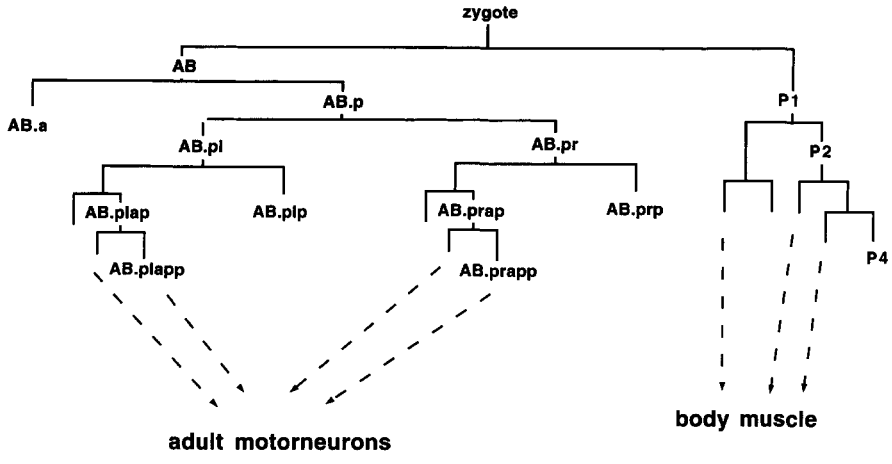

FIGURE 2.—Mosaic analysis of *unc-36*. Mosaic animals were isolated as described, and the point of duplication loss was determined by scoring the *ncl-1* phenotype. The phenotypes of these mosaics are summarized in Table 2. Phenotypes of AB and AB.p mosaics are consistent with previous results (KENYON 1986). One P2-P4 mosaic had an unusual uncoordinated phenotype that was neither sluggish nor kinking(*).

to the same lineages in *unc-36* mosaics as had previously been observed in *unc-2* mosaics. Thus, *unc-2* and *unc-36* may function in some of the same processes in the same cells.

Other serotonin hypersensitive mutants: Our results suggested that the constitutive egg-laying by *unc-2* and *unc-36* mutants might be caused by their hypersensitivity to serotonin. We reasoned that other *C. elegans* behavioral mutants that exhibited constitutive or hyperactive egg-laying (Egl-c) phenotypes might also be hypersensitive to serotonin. A number of "uncoordinated" (Unc) mutants, isolated on the basis of their abnormal locomotion (BRENNER 1974), had been observed to lay early-stage embryos (W. SCHAFER, unpublished observation; B. WIGHTMAN and G. GARRIGA, personal communication). To determine whether some of these Unc Egl-c mutants might be serotonin-hypersensitive, we focused on nine that were not reported to cause gross nervous system or muscle disorganization: *unc-8(e15), unc-9(e101), unc-10(e102), unc-20(e112), unc-25(e156), unc-35(e259), unc-43(e755), unc-75(e950)* and *unc-77(e625)*. Strains were constructed that carried mutations in both *egl-1* and one of the Egl-c Unc genes, and their egg-laying behavior was assayed. In the absence of exogenous serotonin, all these double mutants were egg-laying defective (Egl-d); thus, none of the Egl-c Unc mutations caused constitutive egg-laying by bypassing the requirement for HSN for efficient egg-laying (as in MENDEL *et al.* 1995; SEGALAT *et al.* 1995). Next, each double mutant was assayed for sensitivity to exogenous serotonin (Figure 3). Five of the mutants (*unc-8, unc-10, unc-20, unc-75* and *unc-77*) were strongly hypersensitive to serotonin: half-maximal stimulation of egg-laying was observed at serotonin concentrations five- to 10-fold lower than for the wild-type strain. Another mutant (*unc-35*) was twofold more sensitive to serotonin than wild type. Thus, a total of six additional mutants were identified as serotonin-hypersensitive, defining genes that may regulate response to serotonin by the egg-laying muscles. The remaining three strains either responded to serotonin at approximately the same concentrations as wild type (*unc-25* and *unc-43*) or responded poorly to serotonin (*unc-9*).

To better characterize the egg-laying behavior of these mutants, we assayed constitutive egg-laying under three conditions. First we measured the ability of these strains to lay eggs in a hypertonic salt solution (M9), a condition that inhibits egg-laying in wild-type animals (TRENT *et al.* 1983). The *unc-8, unc-10,* and *unc-20* mutants laid eggs efficiently in M9, whereas the *unc-35, unc-75,* and *unc-77* mutants did not (Figure 4A). Next we determined whether the serotonin-hypersensitive mutants laid eggs abnormally early in development under normal growth conditions. Wild-type animals typically lay embryos no earlier than the 32-cell stage of development (TRENT *et al.* 1983). Although all of the mutants laid early embryos (eight-cell or younger) at least occasionally, *unc-8, unc-20,* and *unc-75* animals were especially defective in their ability to retain early embryos (Figure 4B). Finally, because long-term exposure to serotonin causes serotonin desensitization, overnight treatment with serotonin causes accumulation of unlaid embryos in wild-type animals (SCHAFER and KENYON 1995). All the serotonin-hypersensitive mutants ex-

TABLE 2

Classes of *unc-36* mosaics

Duplication loss[a]	Kinking	Activity
AB (4)	4 kinker	4 sluggish
AB.p (4)	4 kinker	4 +/− sluggish
AB.pl-AB.plap (4)	4 kinker	4 non-sluggish
AB.pr-AB.prap (3)	2 kinker, 1 +/− kinker	2 non-sluggish, 1 +/− sluggish
AB.plapp-HSNL (1)	1 +/− kinker	1 non-sluggish
AB.prapp-HSNR (2)	1 +/− kinker, 1 non-kinker	2 non-sluggish
AB.prp- - (1)	1 +/− kinker	1 non-sluggish
P1 (1)	1 non-kinker	1 +/− sluggish
P2-P4 (2)	2 non-kinker	1 +/− sluggish, 1 Unc*

See Figure 2.
[a]Number of animals in parentheses.

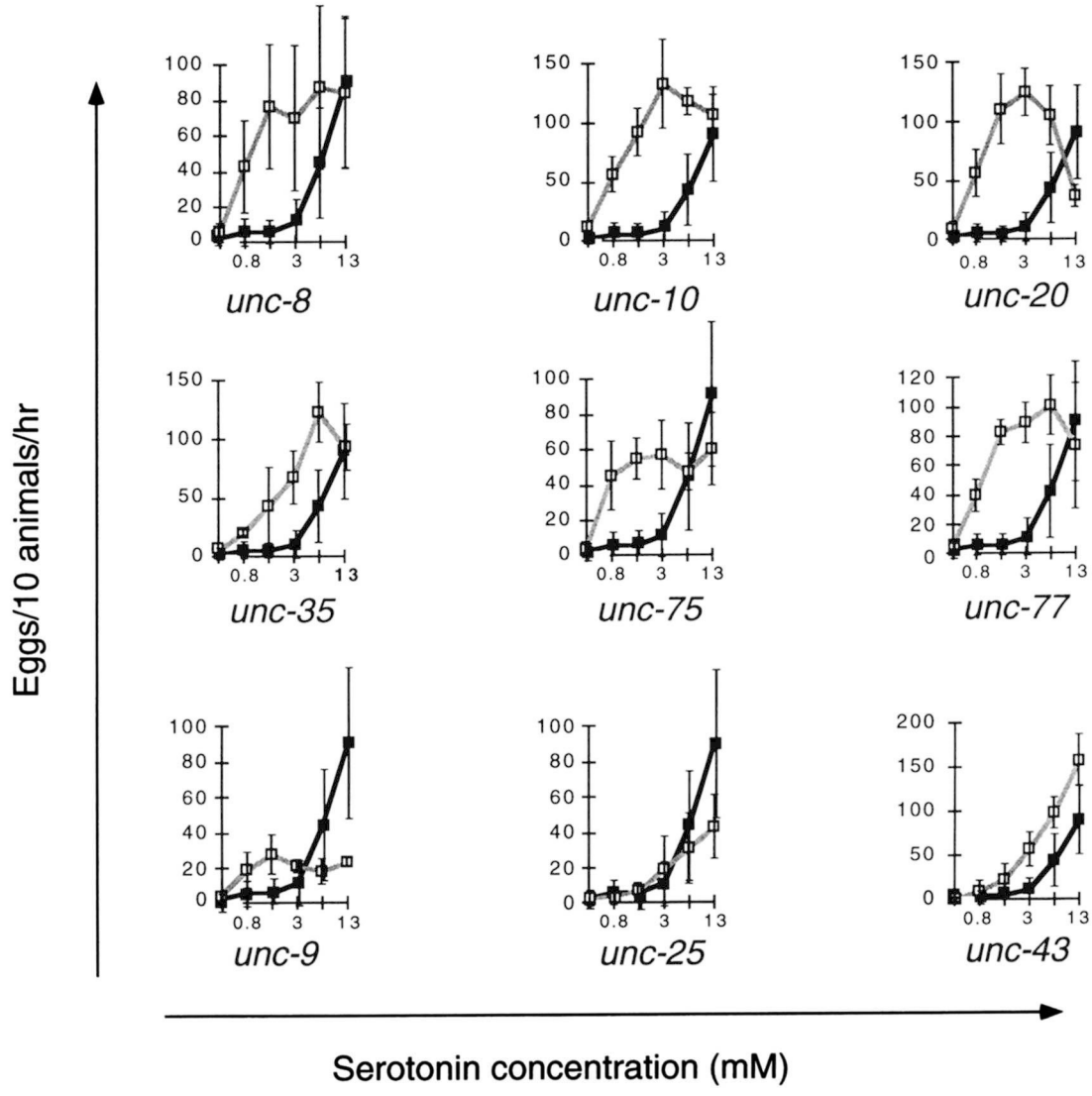

FIGURE 3.—Serotonin sensitivity of Egl-c Unc mutants. Dose response curves for *egl-1(n986)* (■) and the indicated *unc; egl-1* double mutant (□) were determined as described in Figure 1B. Each point represents the mean of at least four trials of 10 animals each; the *egl-1* control represents the mean of eight trials. Error bars indicate the sample standard deviation of the individual trials. The dose response curves obtained in these experiments were used to estimate the serotonin concentration (EC_{50}) that resulted in half-maximal stimulation of egg-laying (RAND and JOHNSON 1995); serotonin hypersensitive mutants were defined operationally as strains whose EC_{50} differed from the *egl-1* single mutant by a factor of two or more. The following mutant alleles were used in this experiment: *unc-8(e15), unc-10(e102), unc-20(e112), unc-35(e259), unc-75(e950), unc-77(e625), unc-9(e101), unc-25(e156),* and *unc-43(e755).*

cept *unc-10* were significantly less inhibited for egg-laying by long-term serotonin treatment than the wild-type, indicating that they were defective in serotonin desensitization (Figure 4C). Thus, all the serotonin-hypersensitive mutations caused an altered response to exogenous serotonin and/or an increased rate of egg-laying in animals with an intact HSN.

Acetylcholine and the regulation of serotonin response: A clue to the possible function of some of these gene products was provided by the observation that several serotonin-hypersensitive strains, including *unc-10, unc-75, unc-36,* and *unc-2* mutants, are resistant to acetylcholinesterase inhibitors such as aldicarb and trichlorfon (NGUYEN *et al.* 1995; K. MILLER and J. RAND, personal communication). The lethality caused by aldicarb results from excessive cholinergic transmission, and the aldicarb-resistance genes that have been cloned so far affect either general neurotransmitter release (HALL and HEDGECOCK 1991; OTSUKA *et al.* 1991; GENGYO-ANDO *et al.* 1992; NONET *et al.* 1993), release of acetylcholine specifically (ALFONSO *et al.* 1993, 1994) or acetylcholine response (LEWIS *et al.* 1980a,b). Since in other organisms voltage-gated calcium channels have been shown to activate vesicle fusion at presynaptic nerve terminals, it is reasonable to suppose that *unc-2, unc-36,* and perhaps other serotonin-hypersensitivity genes may be required for efficient release of acetylcholine or other neurotransmitters. This might suggest a

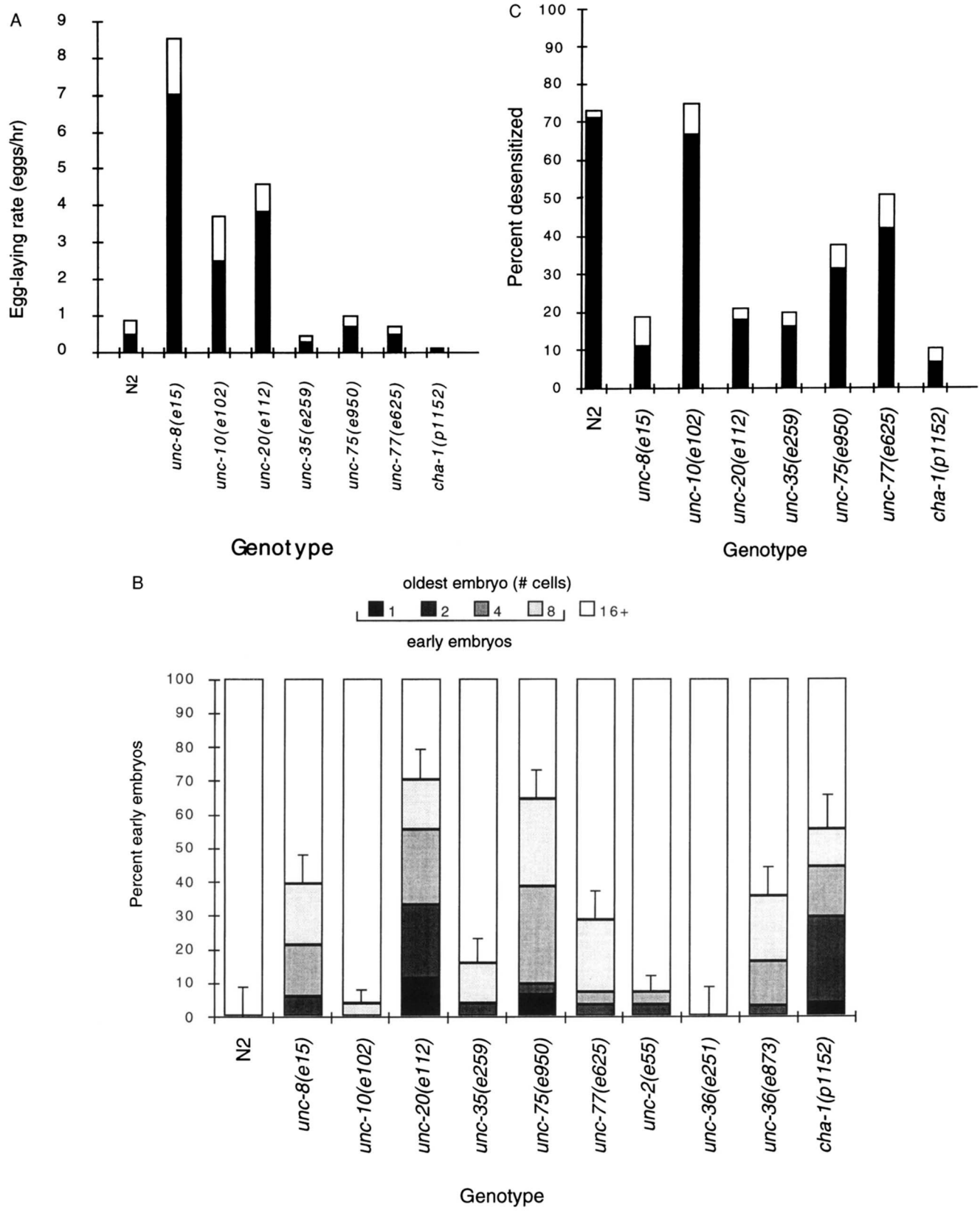

FIGURE 4.—Egg-laying behavior of serotonin-hypersensitive mutants. (A) Constitutive egg-laying. Egg-laying rates were measured as described in Figure 1A. ■, average rate of egg-laying per animal (eggs/hr); □, sample standard deviation of the means of at least four independent trials of 10 animals each. The following mutants gave results that were statistically different from wild type ($P < 0.01$) according to the Mann-Whitney rank sum test: *unc-8(e15)*, *unc-20(e112)*, and *unc-10(e102)*. (B) Early egg-laying. The developmental age of the oldest embryo retained in the uterus of a gravid hermaphrodite of a given genotype was determined by observation under a high-power microscope using Nomarski optics. Animals were grown at 20° on nematode

role for acetylcholine in desensitizing the egg-laying muscles to serotonin. Alternatively, these genes could act more generally at presynaptic terminals and facilitate the release of a different neurotransmitter that inhibits serotonin response by the muscles and thus mediates serotonin desensitization.

To determine whether either of these models might be correct, we tested the serotonin sensitivity of two aldicarb-resistant mutants with known molecular defects. The gene *cha-1* encodes the single *C. elegans* isozyme of choline acetyltransferase, which catalyzes the synthesis of acetylcholine (ALFONSO *et al.* 1994). Mutations in *cha-1* result in aldicarb resistance and uncoordinated movement (RAND and RUSSELL 1984). Likewise, the *snt-1* gene encodes the only *C. elegans* isoform of synaptotagmin, a protein that is important for the fusion of neurotransmitter-containing vesicles at presynaptic terminals; *snt-1* mutants are also aldicarb resistant and Unc (NONET *et al.* 1993). If acetylcholine inhibits the responsiveness of the vulval muscles to serotonin, both *cha-1* and *snt-1* mutants would be predicted to be serotonin-hypersensitive. In contrast, if serotonin sensitivity is inhibited by release of a neurotransmitter other than acetylcholine, mutations in *snt-1,* but not *cha-1,* would confer serotonin hypersensitivity. If neither model is correct, neither mutation would be expected to affect serotonin sensitivity. In fact, mutations in both *cha-1* and *snt-1* resulted in increased sensitivity to exogenous serotonin (Figure 5). Moreover, strong alleles of *cha-1* caused animals to lay early embryos, suggesting that failure to release acetylcholine caused hypersensitivity to endogenous as well as exogenous serotonin (Figure 4B). *cha-1* mutants were also defective in serotonin desensitization (Figure 4C). Taken together, these results argue that acetylcholine negatively regulates egg-laying behavior by inhibiting the serotonin sensitivity of the vulval muscles.

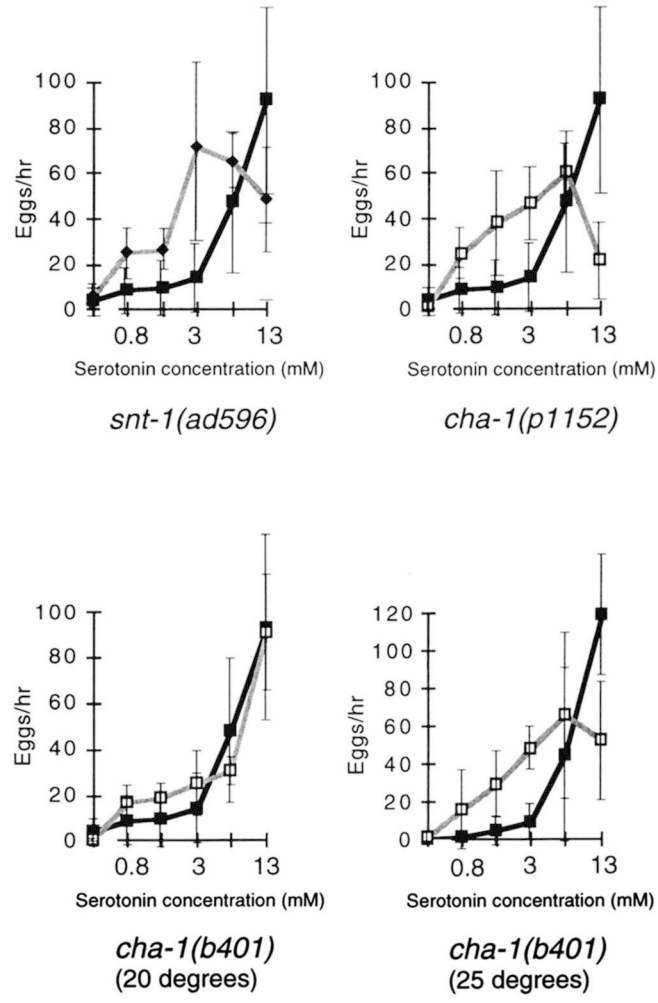

FIGURE 5.—Increased serotonin sensitivity of mutants defective in acetylcholine release. Dose response curves for *egl-1(n986)* (■) and the indicated double mutant (□) were determined as described for Figure 1B. Except as indicated, strains were grown and assayed at 20°. For the experiment using the *cha-1(b401)* temperature-sensitive mutant, mutant and control animals were also grown to adulthood at 15°, then incubated for 15 hr at 25°; animals grown in this manner were assayed at 25°. Each point represents the mean of at least three trials of 10 animals each; error bars indicate the sample standard deviation of the individual trials.

DISCUSSION

In previous work, we determined that mutations in a gene encoding a voltage-gated calcium channel subunit, *unc-2,* caused hypersensitivity to serotonin and constitutive egg-laying behavior. In this study, we have determined that mutations in at least nine additional genes confer an egg-laying-constitutive, serotonin-hypersensitive phenotype. These genes define additional candidate molecules that may regulate the sensitivity of the vulval muscles to serotonin and thereby regulate the rate of egg-laying. Based on previous molecular and genetic analysis of some of these genes, it is now possible to propose more detailed molecular hypotheses for the regulation of egg-laying behavior in *C. elegans.*

***unc-36* may encode an auxiliary subunit of the UNC-2 calcium channel:** The gene whose mutant phenotype most resembles that of *unc-2* is *unc-36.* Both mutant strains are Egl-c, long, and move in a sluggish uncoordinated manner. Mutations in the two genes have qualitatively and quantitatively similar effects on serotonin sen-

growth medium (NGM). At least 25 animals of a particular genotype were tested; the error bars indicate the standard error for the percentage of Egl-c animals retaining no 16-cell or older embryos. The following strains were statistically different from wild type according to the *z* test ($P < 0.01$): *cha-1(p1152), unc-20(e112), unc-8(e15), unc-35(e259), unc-36(e873),* and *unc-75(e950). unc-77(e625)* was also statistically different from wild type ($P < 0.05$). (C) Serotonin desensitization. Desensitization to serotonin was assayed as described in Figure 1C. At least 30 animals were assayed for each strain. With the exception of *unc-10(e102),* all strains gave results that were statistically different from wild type according to the *z* test ($P < 0.01$).

sitivity and dopamine adaptation, and both show identical genetic interactions with mutations in the *egl-19* gene. In addition, mosaic analysis suggests that the two genes act in the same cells to control the same behaviors, and the phenotypes of *unc-36; unc-2* double mutants resemble the *unc-2* and *unc-36* single mutant phenotypes. *unc-36* has recently been shown to encode a homologue of the α-2/δ large auxiliary subunit of voltage-gated calcium channels (L. LOBEL and H. R. HORVITZ, personal communication). Thus, it is reasonable to hypothesize that *unc-36* and *unc-2* encode subunits of the same calcium channel that controls desensitization to serotonin.

Although *unc-2* and *unc-36* loss-of-function mutants are nearly identical in appearance, there are some subtle but significant differences in the two mutant phenotypes. The most striking difference is that *unc-36* males are completely unable to mate, whereas males with strong recessive alleles of *unc-2* mate very well, despite the fact that they are uncoordinated and slow-moving (HODGKIN 1983). This suggests that a calcium channel containing UNC-36 but not UNC-2 protein may function in neurons and/or muscles that are involved in male mating. Conversely, *unc-2* mutants, but not *unc-36* mutants, exhibit a subtle but reproducible defect in the migrations of the descendants of the QR neuroblast (M. SYM and C. KENYON, unpublished results). This suggests that a calcium channel consisting of UNC-2 but not UNC-36 protein may be required for proper execution of these migrations. Although one must be cautious when interpreting the phenotypes of mutations not known to be nulls, these results suggest the possibility that the *C. elegans* nervous system not only contains multiple calcium channel proteins, but that different calcium channel subunits may assort differently in different cells to create additional functional diversity.

Serotonin desensitization and acetylcholine: The analysis of serotonin hypersensitive mutants resulted in the identification of another molecule that is likely to play a role in the regulation of serotonin response by the vulval muscles: the neurotransmitter acetylcholine. Mutations in six of the genes that we have determined to cause serotonin hypersensitivity had previously been shown to confer resistance to acetylcholinesterase inhibitors, including *unc-2, unc-10, unc-36, unc-75, snt-1,* and *cha-1. cha-1* mutants are defective in the enzyme choline acetyltransferase, which is required specifically for the synthesis of acetylcholine. This suggests strongly that release of acetylcholine from certain neurons acts to inhibit the sensitivity of the egg-laying muscles to serotonin. Perhaps the best candidates for the neurons that might mediate this regulation are the VC cells of the ventral nerve cord, which contain choline acetyltransferase (J. DUERR and J. RAND, personal communication), receive synaptic input from the HSNs, and make many synapses with the vulval muscles (WHITE *et al.* 1986). Since UNC-2 is expressed in these cells

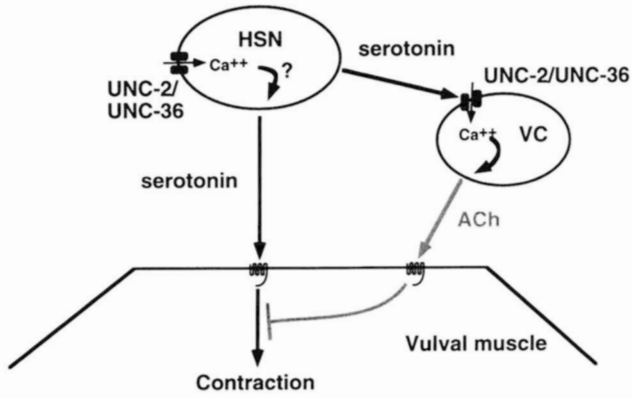

FIGURE 6.—Model for serotonin desensitization. Acetylcholine released from the VCs may inhibit the response of the vulval muscles to serotonin. Release of acetylcholine from the VCs could be triggered by a calcium influx though the putative UNC-2/UNC-36 voltage-gated calcium channel, which might be stimulated by serotonin released from the HSNs. The effect of *unc-2* and other serotonin-hypersensitive, aldicarb-resistant mutations on neurotransmitter release from the HSNs is unclear.

(SCHAFER and KENYON 1995), a parsimonious model for serotonin desensitization is that the UNC-2 calcium channel facilitates release of acetylcholine from the VCs to inhibit the serotonin response of the vulval muscles. Serotonin released from the HSNs might increase the rate of acetylcholine release and thus promote desensitization (Figure 6). Of course, although this model explains the available data in a simple and consistent manner, it represents only one of several possibilities for how acetylcholine might modulate the serotonin response of the egg-laying muscles.

These results implicating acetylcholine as a negative regulator of egg-laying were somewhat unexpected, since previous pharmacological studies demonstrated that cholinergic agonists stimulate, rather than inhibit, egg-laying (TRENT *et al.* 1983). How can we reconcile these observations? Although *cha-1* mutants showed increased sensitivity to serotonin, the magnitude of their response to serotonin appeared to be diminished, especially at high serotonin concentrations. Thus, acetylcholine may act as both an activator and an inhibitor of egg-laying. Perhaps neuronal and neuromuscular acetylcholine receptors have opposing effects on egg-laying behavior. Another possibility, not inconsistent with the first, is that different classes of acetylcholine receptor affect the activity of the egg-laying muscle in opposite ways. Further genetic studies of acetylcholine receptor mutants and *cha-1* mosaics, as well as pharmacological studies using different cholinergic agonists and antagonists, should provide insight into the potentially complex role of acetylcholine in regulating egg-laying behavior.

Other genes that affect serotonin sensitivity: We identified three additional mutant strains that were five- to 10-fold more sensitive to serotonin than wild type,

containing mutations in the genes *unc-8, unc-20,* and *unc-77.* Mutations in these three genes have not been identified in large-scale screens for aldicarb-resistant mutants (J. RAND, personal communication). *unc-8, unc-20,* and *unc-77* may therefore encode novel regulators of serotonin response by the egg-laying muscles (although since only single alleles of these genes were tested, it is possible that serotonin hypersensitivity was in fact caused by a second linked mutation). Some genetic evidence suggests that *unc-8* may be functionally similar to the degenerin family of neuronal ion channels (SHREFFLER *et al.* 1995); little is known about the possible molecular functions of *unc-20* and *unc-77.* The *unc-20* mutant was qualitatively most similar to *unc-2* and *unc-36* mutants in its response to serotonin: like *unc-2* and *unc-36* mutants, *unc-20* animals laid significantly fewer eggs at concentrations of serotonin greater than the optimal dose. *unc-20* animals express what appear to be a subset of the *unc-2/unc-36* mutant phenotypes: they are serotonin-hypersensitive, Egl-c, and uncoordinated kinkers, but they are not dopamine-adaptation-defective, sluggish, or long. One explanation for this phenotype is that the UNC-20 gene product affects the same signaling pathway as the UNC-2/UNC-36 calcium channel in some, but not all, of the cells that express the channel.

The phenotypes of Egl-c mutants that are not serotonin-hypersensitive also provide insight into the control of egg-laying behavior in *C. elegans.* For example, the *unc-43* mutation had very little effect on the threshold for serotonin response by the egg-laying muscles; however, it appeared to have a substantial effect on the magnitude of the response. Rather than specifically controlling serotonin sensitivity, the *unc-43* gene may instead regulate the activity of the egg-laying muscles in a more general way. Consistent with this possibility, *unc-43* has recently been shown to affect the activity of a variety of muscle types, including enteric muscles not known to be regulated by serotonin (REINER *et al.* 1995).

Other insights on the regulation of egg-laying behavior: Our results also provide clues to the nature of a long-standing mystery about egg-laying behavior: although mutations in *egl-1* and other genes that eliminate the HSNs cause severe egg-laying defects, mutations in genes that greatly reduce serotonin levels in the HSNs, such as *cat-4,* have little effect on egg-laying behavior (SULSTON *et al.* 1975; DESAI *et al.* 1988). However, the available evidence argues strongly that serotonin released from the HSNs is an important activator of egg-laying behavior. For example, exogenous serotonin is sufficient to rescue the egg-laying defect of *egl-1* animals (TRENT *et al.* 1983), and serotonin-deficient mutants have been demonstrated to exhibit a synthetic Egl-d phenotype in certain genetic backgrounds (AVERY *et al.* 1993). Moreover, mutations in a putative effector of serotonin signaling, *goa-1,* cause either an Egl-c phenotype that is epistatic to *egl-1* or a serotonin-resistant Egl-d phenotype (MENDEL *et al.* 1995; SEGALAT *et al.* 1995). Analysis of certain egg-laying-defective mutations has led to the hypothesis (WEINSHENKER *et al.* 1995) that the HSNs contain a second neurotransmitter that can stimulate contraction of the egg-laying muscles. The phenotype of *unc-9* is consistent with this hypothesis: *unc-9* animals are dependent on the HSN for efficient egg-laying, yet they respond poorly to exogenous serotonin. These results could be explained if *unc-9* mutants are defective in serotonin response yet remain sensitive to a hypothetical second activator of egg-laying expressed in the HSNs.

In summary, we have identified and begun characterization of several genes that can mutate to confer hypersensitivity to serotonin. One of these, *unc-36,* is a strong candidate for the gene that encodes the α-2 and δ subunits of the voltage-gated calcium channel that is required for inhibiting serotonin sensitivity. Among the other serotonin-hypersensitive mutants, several appear to affect acetylcholine release (or in one case, acetylcholine synthesis). Acetylcholine may therefore function as an inhibitory neuromodulator to desensitize the egg-laying muscles to serotonin, and thus mediate serotonin adaptation, in *C. elegans.* Further molecular and genetic characterization of serotonin-hypersensitive mutants should provide additional insights into the molecular mechanisms that underlie desensitization in the *C. elegans* nervous system.

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