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Regulation of the Nicotinic Acetylcholine Receptor in *Caenorhabditis elegans*: Insights into the Molecular Basis of Behavior.

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

Neurosciences

by

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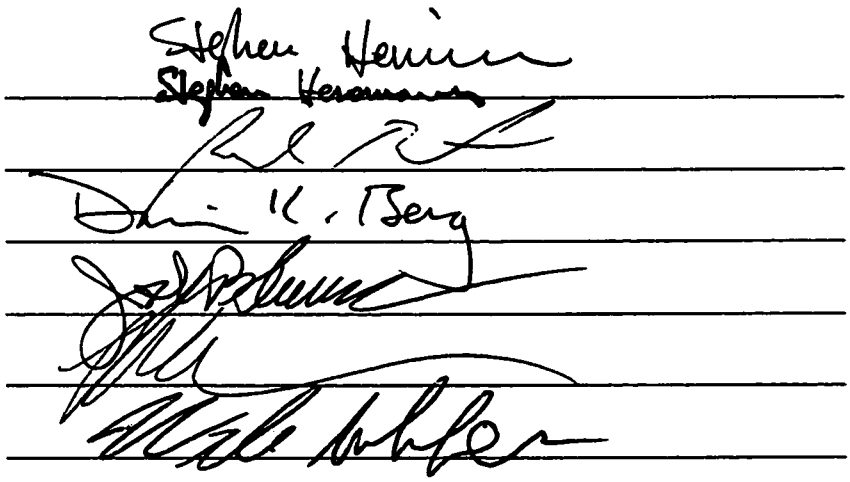

Chair

University of California, San Diego

2001

DEDICATION

This work is dedicated to my family. To my parents, whose hard work and sacrifice allowed me to attain my goals: my mother, for her abounding love, faithful prayers, and for teaching me to have joy for life; and my father, for his tremendous wisdom and guidance, integrity, and selflessness. To my sisters, my role models, whom I respect and love dearly, for their advice, support, and encouragement. Finally, to my husband, who has been my wonderful source of strength and joy through his continuous support and encouragement, understanding, steadfastness, humor, and love.

I would also like to thank my advisor, William Schafer, for his guidance and mentorship; and the Schafer Lab members, for their help, advice, and friendship.

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Kim J, Moore L, Kindt R, Haslam S, van Halbeek H, Gottschalk A, Richmond J, Dickinson KA and Schafer WR (2002) "The NIC-1 glycosyltransferase modifies the functional activity of nicotinic receptors in *Caenorhabditis elegans*." (manuscript in preparation for submission)

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ABSTRACT OF THE DISSERTATION

Regulation of the Nicotinic Acetylcholine Receptor in *Caenorhabditis elegans*: Insights into the Molecular Basis of Behavior.

by

Jinah Kim

Doctor of Philosophy in Neurosciences

University of California, San Diego, 2002

Professor William R. Schafer, Chair

I have studied *Caenorhabditis elegans* behavior to investigate the genetic and molecular basis for regulation of the nicotinic acetylcholine receptor (nAChR). First, I describe genes involved in regulating the activity of the levamisole receptor in the *C. elegans* egg-laying circuitry. Seven of these genes, including nAChR subunit genes *unc-29*, *unc-38*, and *lev-1*, were essential for levamisole-induced egg-laying stimulation, but were not essential for egg-laying muscle contraction. The levamisole-receptor mutants responded to nicotine, so other acetylcholine receptors are also likely to mediate cholinergic neurotransmission in egg laying. Additionally, expression of *unc-29*(+) in muscle cells restored levamisole sensitivity under some, but not all, conditions,

suggesting that both neuronal and muscle UNC-29 receptors contribute to regulating egg-laying behavior.

Next, I describe the gene, *nic-1*, discovered in a screen for nicotine hypersensitive mutants. *nic-1* encodes a homolog of the yeast α-1,3/α-1,6 mannosyltransferase Alg2, which catalyzes two early steps in assembling the dolichol-linked core glycan $Glc_2Man_9GlcNAc_2$-PP-Dol in asparagine(N)-linked glycosylation. NIC-1 is a 483aa protein with 25% amino acid sequence similarity to Alg2 and contains a dolichol-binding site. FAB-mass spectra of PNGase-F released *N*-glycans in wild type and *nic-1* extracts revealed drastically decreased signals of the high mannose glycans $Man_6GlcNAc_2$-$Man_9GlcNAc_2$ in *nic-1*, while signals for $Man_5GlcNAc_2$, truncated, and complex glycans were similar to wild type. This suggests NIC-1 may function as an α-1,2 mannosyltransferase in high-mannose *N*-glycan assembly, or in the mechanism wherein $Man_5GlcNAc_2$ "flips" from the cytosolic to the lumenal side of the ER. Interestingly, NIC-1 appeared to negatively regulate nAChR activity. *nic-1* mutants were hypersensitive for cholinergic agonist-induced body muscle hypercontraction, egg laying and spicule protraction. Mutants also displayed increased egg laying within the active egg-laying phase, a pattern characteristic of animals experiencing increased cholinergic neurotransmission. Furthermore, *nic-1* caused hypersensitivity for nicotine-induced paralysis in animals with neuron- and

muscle-specific expression of UNC-38, suggesting that NIC-1 downregulated the activity of UNC-38 receptors in neurons and possibly in muscle. In summary, in this dissertation I identify key genes required for proper activity of the nAChR in *C. elegans* egg-laying behavior; furthermore, I demonstrate that glycosylation is essential for proper downregulation of nAChR activity in *C. elegans*.

CHAPTER I. INTRODUCTION

In modern biology, we know that the blueprint for any living organism exists in every cell of that organism in the form of DNA. Defined segments of DNA, or genes, encode for proteins that make up components of cells. Cells make up an organism that produces behavior in response to endogenous and external stimuli. One of the basic questions in biology is: what produces behavior? Understanding the nervous system is key to deciphering how a stimulus is sensed, then processed to produce a behavior. In doing so, it is important to examine the roles of genes, proteins, and signaling pathways involved within cells to fully understand what is happening at the molecular level to produce such behavior. In the quest for this knowledge it is essential to ask simple questions that give clear-cut, definite answers. However, the nervous systems of higher organisms are much too complicated to obtain clear answers: humans have upwards of 10^{11} neurons in their central nervous system (Kandel 1991), with many more times that number in synaptic connections between neurons. This makes it difficult for scientists to obtain clear, unambiguous results to human brain studies. One way around this problem is to study the systems of simple organisms such as *Caenorhabditis elegans*. This small (1.5-mm-long adult), free-living, soil nematode has a life cycle of about three days, is easy to cultivate in the laboratory, and is genetically tractable and easily manipulated for classical genetic, molecular and behavioral studies. Research of simple organisms such as this one will allow scientists to search for homologous genes, molecules and their functions in higher organisms, and gain insight into the nervous system of humans.

The focus of my study was to investigate the genetic and molecular mechanisms underlying nicotinic acetylcholine receptor (nAChR) activity and

regulation in the nematode *Caenorhabditis elegans*. Tobacco use has been cited as one of the most significant risk factors for cancer, heart disease, and emphysema in the modern world, and nicotine addiction is widely acknowledged as the cause of the high rates of tobacco use. In the brain, nicotine exerts its effects through the activity of the nicotinic acetylcholine receptor, and long term changes in expression and sensitivity of neuronal nicotinic receptors have been implicated as the underlying cause of nicotine addiction, tolerance, and withdrawal. However, the molecular mechanisms underlying these events are largely unknown. Therefore, I wished to use the power of genetics and molecular biology in *C. elegans*, a simple, well-characterized organism that is amenable to genetic and molecular studies, to identify components of the pathway that regulates the activity of the nicotinic acetylcholine receptor.

AN OVERVIEW OF THE *CAENORHABDITIS ELEGANS* NERVOUS SYSTEM

The invertebrate *C. elegans* has a well-defined nervous system of 302 neurons in the hermaphrodite (381 in the male). The lineage, position (White et al. 1986) and synaptic connectivity (Sulston et al. 1988) have been determined through cell division/migration studies and serial-section electron microscopy for every neuron in the hermaphrodite nervous system. Cell number and position are constant from animal to animal and most neurons are relatively simple in structure: each neuron contains one or at most a few processes. Most of the neuronal processes form a ring around the basement membrane surrounding the pharynx (the nerve ring), or along the dorsal and ventral nerve cords, and the cell bodies of most neurons are concentrated around the pharynx, along the ventral midline and in the tail. The *C. elegans* nervous system contains approximately 2000 neuromuscular

junctions, 5000 chemical synapses between neurons, and 700 gap junctions. Presynaptic terminals are often associated with a thick, electron-dense vesicle release site juxtaposed against a less obvious post synaptic density (PSD). However, postsynaptic junctional folds such as those observed in mammalian neuromuscular junctions are absent, and morphologies of neuron-neuron junctions and neuromuscular junctions are not as dissimilar as they are in vertebrate synapses (Rand and Nonet 1997).

Despite its simple anatomy, *C. elegans* boasts an impressive array of classical neurotransmitters. Among them, acetylcholine (ACh) appears to act as the primary excitatory neurotransmitter for motor neurons, and has been the only neurotransmitter shown so far to be required for *C. elegans* viability: animals mutant for *cha-1*, the gene for the enzyme choline acetyltransferase (ChAT) necessary for acetylcholine synthesis, are able to hatch but can barely move or feed and end up dying as L1 larvae (Rand 1989; Avery and Horvitz 1990; Alfonso et al. 1994a). Animals lacking the synaptic vesicle acetylcholine transporter, *unc-17*, display phenotypes similar to those of *cha-1* mutants (Brenner 1974; Rand and Russell 1984, Alfonso et al. 1993), further demonstrating that ACh is necessary for proper neuron function and viability. Cholinergic cells identified through anti-ChAT and anti-UNC-17 antibody staining show that almost all appear to be motor neurons, including six of the eight ventral cord motor neurons, pharyngeal motor neurons, and sublateral motor neurons (Rand and Nonet 1997). In *C. elegans*, acute nicotine treatment leads to hypercontraction of body muscle and rapid pharyngeal pumping: ACh neurotransmission at the neuromuscular junction (NMJ) is most likely mediated by ligand-gated ion channels of the nicotinic acetylcholine receptor family (nAChRs) (Lewis et al. 1980b; Avery and Horvitz 1990). A number of genes encoding homologues of nAChR subunits have been identified in the *C. elegans*

genome (Ballivet *et al.* 1996; Baylis *et al.* 1997; Fleming *et al.* 1997; Fleming *et al.* 1993; Mongan *et al.* 1998; Treinin and Chalfie 1995). Several have been shown to encode functional receptor subunits when expressed ectopically in oocytes (Fleming *et al.* 1997; Squire *et al.* 1995). Though the vertebrate and *C. elegans* nAChRs at the NMJ have similarities, they are also different. For example, the potent vertebrate nAChR-binding toxin α-bungarotoxin does not effectively bind to the *C. elegans* receptor. Rather, the anti-helminthic drug levamisole is a potent agonist of *C. elegans* NMJ nAChRs, and this has helped significantly in characterizing the levamisole receptor in *C. elegans* (Lewis et al. 1980a).

GABA has also been found to act in *C. elegans*, in both an inhibitory and excitatory manner. Twenty-six neurons, mostly motor neurons, have been found to contain GABA immunoreactivity to anti-GABA antibodies. Additionally, mutants defective for proper GABAergic neurotransmission have been identified, including *unc-25* (mutants lack glutamic acid decarboxylase activity), *unc-47* (mutants accumulate elevated levels of GABA due to a defective GABA vesicular transporter), and *unc-49* (mutants are resistant to the GABA receptor agonist muscimol due to a defective $GABA_A$ receptor) (McIntire et al. 1993a).

Serotonin (5-hydroxytryptamine, or 5-HT) also functions to mediate a variety of behaviors in *C. elegans*. This includes stimulation of egg laying and pharyngeal pumping, inhibition of locomotion and defecation (Horvitz et al. 1982; Segalat et al. 1995), and proper male mating behavior (Loer and Kenyon 1993). Serotonin has been detected by formaldehyde-induced fluorescence (FIF; Sulston et al. 1975) (Horvitz et al. 1982) and by anti-serotonin immunostaining (Desai et al. 1988; McIntire et al. 1992). At least ten cells contain serotonin, including the pharyngeal NSM cells, the male CP neurons involved in male mating behavior, and the hermaphrodite HSN cells, which are required for egg laying. Several mutants

have been identified that lack serotonin: *bas-1* (biogenic amine synthesis-defective) (Loer and Kenyon 1993), *cat-4* (originally found because of its dopamine deficiency), and *tph-1* (tryptophan hydroxylase-deficient). A related aminergic neurotransmitter, dopamine (3,4-dihydroxyphenylethylamine) has been detected in *C. elegans* sensory cells as well as in several ray neurons, also using FIF (Sulston et al. 1975; Sulston and Horvitz 1977). Five *cat* (catecholamine-deficient) mutants have been identified: these animals display reduced or absent levels of dopamine (*cat-2* and *cat-4)* or abnormal morphology of the neurons and subcellular localization of the FIF (*cat-1, cat-3, cat-5).* Dopamine appears to be required for the function of several sensory neurons: behaviors mediated by these sensory neurons, such as foraging and bacterial lawn sensation, are defective in *cat-2, cat-4,* and *bas-1.*

Glutamate functions both as an excitatory and an inhibitory neurotransmitter in *C. elegans. glr-1* encodes a homolog of a subunit of the vertebrate AMPA glutamate receptor. Analysis of *glr-1* mutants suggests that certain classes of sensory neurons utilize glutamate as an excitatory neurotransmitter; and several other genes have also been identified through sequence analysis to encode homologs of vertebrate excitatory glutamate receptors (Hart et al. 1995; Maricq et al. 1995). However, in the pharynx, the M3 motorneurons appear to be glutamatergic and inhibitory: they appear to act through the opening of a chloride channel (Dent et al.)

Octopamine (*p*-hydroxyphenylethanolamine) is another neurotransmitter found in *C. elegans* and appears to antagonize the action of serotonin: it stimulates movement and inhibits egg laying in *C. elegans*, and has been detected in *C. elegans* extracts (Horvitz et al. 1982), though it is not known which cells contain octopamine.

CHOLINERGIC NEUROTRANSMISSION IN *C. ELEGANS* BEHAVIOR

LOCOMOTION

Nicotinic acetylcholine receptors are present in body muscle and mediate locomotion. Locomotion in *C. elegans* involves the simultaneous contraction and relaxation of the opposing dorsal and ventral body wall muscles to generate a pattern of sinusoidal body movement (Driscoll and Kaplan 1997). Body wall muscles of *C. elegans* are organized into two dorsal rows and two ventral rows (Sulston and Horvitz 1977). The ventral body muscles are innervated by the VA, VB, VC, and VD motor neurons, while the dorsal body muscles are innervated by the AS, DA, DB, and DD motor neurons (White et al. 1986). During sinusoidal movement, the contraction and relaxation of the opposing body muscles are out of phase: this involves the action of motor neurons causing excitation in one set of muscles while simultaneously causing inhibition in the other set of muscles. Thus, this process involves the cooperative action of excitatory and inhibitory neurons. The motor neurons of classes A and B and the AS neuron are thought to be excitatory and have been shown to contain ACh, while the class D motor neurons are thought to be inhibitory and contain GABA (McIntire et al. 1993b). At the NMJ, the A and B neuronal termini each synapse onto two distinct postsynaptic elements: a body wall muscle and a D neuron dendrite (White et al. 1986). As seen through physiological evidence in *Ascaris* (Walrond and Strettoo 1985), genetic and cell ablation studies in *C. elegans*, and the pattern of synaptic connectivity, it is likely that the inhibitory VD and DD neurons function as cross-inhibitors. For example, during a dorsal bend, the dorsal body muscle (which contains nAChRs) would receive excitatory (ACh) input from the DA and DB, causing contraction, at the same time that VD receives

input from the same neurons. In response, VD sends an inhibitory (GABA) signal to the ventral muscle causing relaxation.

The cells in which the nicotinic receptor has been most well studied in *C. elegans* are the body muscle. Levamisole, a nAChR agonist, causes muscle hypercontraction and at high doses, spastic paralysis. Screens for levamisole-resistant mutants have led to the identification of multiple genes affecting the function of the levamisole receptor (Lewis *et al.* 1980a). Several of these levamisole-resistance genes, including *unc-38*, *unc-29*, and *lev-1*, function in muscle and encode receptor subunits (Fleming *et al.* 1997): *unc-29* and *lev-1* encode candidate non-α subunits of the levamisole receptor, whereas *unc-38* encodes a candidate α subunit. A second, levamisole-insensitive nicotinic receptor has also been identified in the body muscle through electrophysiological methods (Richmond and Jorgensen 1999); the genetics and molecular biology of this receptor have not been characterized.

FEEDING

Feeding in *C. elegans* involves the synchronized contraction and relaxation of the various pharyngeal muscles. There are eight muscle types in the pharynx, separated into three distinct groups: the corpus (comprised of the procorpus and the metacarpus), the isthmus, and the terminal bulb. The act of feeding involves the near-simultaneous contraction of all three groups of pharyngeal muscles (pumping), followed by near-simultaneous relaxation of the muscles (peristalsis)(Albertson and Thomson 1976; Avery and Horvitz 1989).

While the pharynx possesses some intrinsic myogenic activity (Avery et al. 1989), pumping of the pharynx is regulated by three main neurons of the pharyngeal nervous system: MC, M3 and M4. The excitatory MC neuron controls

the rate of pumping and is proposed to be the pacemaker of pharyngeal pumping (Raizen et. al 1995). The rate of pumping is affected by environmental cues and drugs such as serotonin and nicotine (Horvitz et al. 1982; Avery et al. 1990). The neurotransmitter used by MC is still unclear, but there is pharmacological evidence for the use of acetylcholine. *unc-29* mutants have normal pharyngeal pumping (Avery 1990), thus it seems unlikely that pharyngeal pumping is mediated by the levamisole receptor. However, two genes, *eat-2* and *eat-18*, which encode potential subunits of a nicotinic acetylcholine receptor, have been shown to be necessary for proper MC function (Desai et al. 1988; Raizen et al. 1995; Avery and Thomas 1997). Another candidate neuronal nAChR subunit expressed in the pharynx is *deg-3*, though *deg-3* loss-of-function mutants do not appear to exhibit abnormal feeding behavior (Treinin and Chalfie 1995).

EGG LAYING

Egg laying in *C. elegans* involves the action of 8 uterine and 8 specialized vulval muscles receiving input from two main types of egg-laying motor neurons, VC4/VC5 and HSNL/HSNR (White et al. 1986). Cell ablation experiments have identified the HSNs and the vulval muscles vm2 as the necessary components of egg laying, as killing of these cells leads to gross egg-laying defects (Trent et al. 1983; Desai et al. 1988). The HSNs contain various neurotransmitters, including serotonin, acetylcholine, and FMRFamide like peptides (Rand and Nonet 1997; Schinkmann and Li 1992; Desai et al. 1988). Serotonin was thought to be the main neurotransmitter responsible for egg laying, as exogenous serotonin leads to egg-laying stimulation in HSN-ablated animals (Trent et al. 1983). However, mutants lacking serotonin, *cat-4* and *bas-1*, were shown not to be grossly egg-laying defective. Moreover, cholinergic agonists, including levamisole (nicotinic agonist)

and oxotremorine (muscarinic agonist), stimulate egg laying, and this was shown to be dependent on the HSNs. This response was not dependent on serotonin, as *cat-4* mutants respond to levamisole(Weinshenker et al. 1995). Mutants lacking cholinergic neurotransmission, including *cha-1* and *unc-29*, are not grossly egg-laying defective as well (Lewis et al. 1980; Rand and Russell 1984). These results led to the suggestion that serotonin and acetylcholine, along with another as yet unidentified neurotransmitter, function in parallel in the HSNs to stimulate egg laying. Furthermore, through analysis of the temporal pattern of egg laying, it was discovered that wild type *C. elegans* fluctuate between discrete egg-laying behavioral states: an active state, during which eggs are laid in clusters, and an inactive state, during which eggs are retained in the uterus. Serotonin was shown to affect the switch between the active and inactive states, while acetylcholine was shown to affect the rate of egg laying within the active phase (Waggoner et al. 1998).

MATING

The *C. elegans* male nervous system contains 381 neurons, and at least 79 of them function in facilitating mating behavior (Sulston et al. 1980; Loer and Kenyon 1993; Liu and Sternberg 1995). Mating behavior is initiated when the male tail contacts the hermaphrodite and searches for the vulva. When the vulva is contacted, the male inserts his copulatory spicules and transfers sperm in a series of characterized steps (Chalfie and White 1988; Liu and Sternberg 1995). Garcia et. al. found that protraction of the spicules during penetration of the vulva involves cholinergic neurotransmission. The acetylcholine agonists levamisole, nicotine and arecoline induced spicule protraction, and the male tail neurons PCB, PCC, and SPC were found to be involved in aldicarb-induced protraction. At least two different types of nicotinic acetylcholine receptors appear to function in mediating protraction,

as *unc-38* and *unc-29*, mutants of subunits of the levamisole receptor, were resistant to levamisole-induced protraction but displayed wild type behavior in nicotine and arecoline (Garcia et al. 2001).

AN OVERVIEW OF *N*-GLYCOSYLATION

Most secreted and membrane proteins in eukaryotes undergo glycosylation, a process during which a protein is modified by the addition of carbohydrate moieties to specific amino acid residues. There are two main types of protein glycosylation: *N*- linked glycosylation, wherein a glycan is attached to an asparagine (Asn) residue of the tripeptide consensus sequence Asn-x-Ser/Thr of a protein (where x refers to any amino acid except Pro), and *O*-linked glycosylation, where a glycan is attached to a Ser or a Thr residue of a protein (Kornfeld and Kornfeld 1985; Herscovics and Orlean 1993). Early stages of *N*-linked glycosylation that take place in the ER are surprisingly well conserved in eukaryotic organisms (Varki et al. 1999). In *N*-linked glycosylation, a lipid (dolichol)-linked core oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, is synthesized in the endoplasmic reticulum (ER) during a step-wise assembling process involving an array of step-specific glycosyltransferases and monosaccharide donors. UDP-GlcNAc and GDP-mannose are utilized as donors for the assembly of the two core GlcNAc monosaccharides and the first five mannose residues, respectively. These first seven linkage reactions occur on the cytosolic side of the ER membrane. Then, by a mechanism not fully understood, the $Man_5GlcNAc_2$-Dol flips across the membrane before four additional mannoses are added, this time in the lumen of the ER, using Dol-P-Mannose as the donor. Finally, three GlcNAc residues are added before the oligosaccharide precursor is added to an Asn of a nascent protein. Further modification by ER and Golgi glycosidases and glycosyltransferases results in glycans of varying sugar composition: high mannose- or

oligomannose-type, $Man_{5-9}GlcNAc_2$; truncated-type (present largely in invertebrates), $Man_{1-4}GlcNAc_2$; and complex-type, which involves addition of sugars to the distal side of the core oligosaccharide and is the most abundant class of mammalian glycans (Varki et al. 1999).

In *Saccharomyces cerevisiae*, where the process of *N*-linked glycosylation has been well characterized, we know the genes that encode specific glycosyltransferases involved in catalyzing specific steps of the core oligosaccharide assembly in the ER. These have been termed *alg*- genes, for 'asparagine-linked glycosylation'. The yeast *alg2* gene encodes an α-1,3 and α-1,6 mannosyltransferase that transfers a mannose from GDP-Man to $Man_1GlcNAc_2$-PP-Dol and $Man_2GlcNAc_2$-PP-Dol to form $Man_3GlcNAc_2$-PP-Dol. *alg2* mutants abnormally accumulate $Man_1GlcNAc_2$-PP-Dol and $Man_2GlcNAc_2$-PP-Dol and exhibit a temperature-sensitive lethal phenotype due to cell cycle arrest (Huffaker and Robbinson 1983; Jackson et al. 1993). The *alg2* homolog in the zygomycete fungus *Rhizomucor pusillus* has been found to encode a 455-aa protein with remarkable amino acid sequence similarity to yeast Alg2 and contain a dolichol-binding site (Val/Ile-x-Phe-x-x-Ile; x is any amino acid) near its C-terminus (Yamazaki et al. 1999).

NICOTINIC ACETYLCHOLINE RECEPTORS AND GLYCOSYLATION

Nicotinic acetylcholine receptors are ligand gated ion channels that cluster at the postsynaptic membrane and mediate rapid excitatory synaptic transmission. For the vertebrate muscle nAChR, individual subunits form an array around the pore (channel), and undergo a conformational change upon binding of the ligand

acetylcholine. This change opens the channel, allowing ions to flow through and subsequently changing the membrane potential (Karlin 1993; Unwin 1993). The nAChR is a pentamer with different subunits making up the assembled receptor. Each subunit type is encoded by its own gene, transcribed then translated into a protein, then undergoes post-translational processing in the endoplasmic reticulum (ER), including glycosylation (Anderson and Blobel 1981) and protein folding. The folded subunits assemble in a defined pathway, then the pentameric AChR is transported to the Golgi where, among other things, it undergoes sugar chain modification (Anderson and Blobel 1981; Smith et al. 1987; Gu et al. 1989; Yu and Hall 1991; Gu et al 1991; Verall and Hall 1992; Chavez et al. 1992; Gu and Hall 1988; Nomoto et al. 1986; Olson et al. 1984).

Each of the known vertebrate subunits of the AChR contains consensus sequences for glycosylation; however, little is known about the functional role of the oligosaccharide chains. Research on other glycoproteins has shown that glycosylation can affect anything from the structural conformation and stability of a protein to its solubility and its ability to associate with other proteins (Dwek 1995). Studies on vertebrate nAChRs have also shown some interesting results. Addition of a simple high mannose glycan to the single glycosylation site of the α subunit metabolically stabilizes the polypeptide, as it undergoes rapid degradation when glycosylation is blocked (Merlie et al. 1982; Blount and Merlie 1990). The β subunit has a single glycosylation site, the δ subunit has three potential sites, and the ε and γ subunits both have two potential sites. All sites are located in the N-terminal extracellular domain. Mutating a highly conserved glycosylation site in each of the subunits α, γ, and δ, eliminates cell surface expression of assembled nAChRs. It appears glycosylation of those three subunits is important for proper folding and assembly of the receptor (Gehle et al. 1997; Ramanathan and Hall 1999).

C. elegans nAChRs appear to be glycosylated as well. The sequences for the UNC-29, UNC-38, and LEV-1 subunits of the levamisole-sensitive nAChR predict two, three, and five asparagine-linked glycosylation sites (Asn-x-Ser/Thr), respectively (Fleming et al. 1997). However, it is unknown what role(s) glycosylation plays in the assembly, stability and function of the *C. elegans* nAChR.

This chapter, in full, is a reprint of the material as it appears in *Genetics* 2001, Kim, Jinah; Poole, Daniel; Waggoner, Laura E.; Kempf, Anthony; Ramirez, David S.; Treschow, P. Alexandra; Schafer, William R. The dissertation author was the primary investigator and author of this paper.

CHAPTER II. GENES AFFECTING THE ACTIVITY OF NICOTINIC RECEPTORS INVOLVED IN *CAENORHABDITIS ELEGANS* EGG-LAYING BEHAVIOR

ABSTRACT

Egg-laying behavior in *C. 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.

INTRODUCTION

NICOTINIC acetylcholine receptors (nAChRs) are heteropentameric ligand-gated ion channels which 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 using 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 (Ballivet *et al.* 1996; Baylis *et al.* 1997; Fleming *et al.* 1997; Fleming *et al.* 1993; Mongan *et al.* 1998; Treinin and Chalfie 1995). Several of these genes have been shown to encode functional receptor subunits when expressed ectopically in oocytes (Fleming *et al.* 1997; Squire *et al.* 1995); 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): *unc-29* and *lev-1* encode candidate non-α subunits of the levamisole receptor, whereas *unc-38* encodes a candidate α subunit. A second, levamisole-insensitive nicotinic receptor has also been identified in the body muscle through electrophysiological methods (Richmond and Jorgensen 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 motorneuron 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 motorneurons (Rand and Nonet 1997a; White *et al.* 1986). 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 motorneurons (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.

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 μM) 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.* 1987; Lewis *et al.* 1980a).

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 α and non-α 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 nicotine. 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 2a, 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 approximately 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

In order 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, nematode growth medium 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 intra-cluster interval displayed a 2-fold reduction, and the inter-cluster interval displayed a more than 3-fold 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 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 complete 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 determine 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 5a-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 5d-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 three-fold reduction in the number of eggs laid (Figure 6c). In addition, *unc-38* mutants also 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 6a, 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 α and non- α 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 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 intra-cluster and inter-cluster time constants were slower by a factor of no more than 2. *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 function 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 intra-cluster 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-laying 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* 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 motorneurons (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 inter-cluster and intra-cluster 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 motorneurons. In an *egl-1* mutant background, both *unc-29* and *lev-1* mutations lengthened the intra-cluster time constant more than three-fold. These results imply that the levamisole receptor is specifically important for cholinergic neurotransmission between the VC motorneurons 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_o/*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, though 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 five-fold 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) 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.

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 *E. coli* strain OP50 as a food source. For dose response experiments, individual young, gravid hermaphrodites were placed in microtiter wells containing liquid M9 and the indicated concentration of drug. After a 1 hour incubation at room temperature, the eggs laid by each animal were counted. 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 hours 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 hours.

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

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

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

, where the intra-cluster time constant is $1/\lambda_1$ and the inter-cluster 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 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 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 8 recordings of different wild-type worms made on different days were individually analyzed, the average intra- and inter-cluster time constants estimated from each were 14 s and 1374 s, with standard deviations of 7 s and 422 s, respectively. Simulations using the same parameters and data sets of comparable size (approximately 40 intervals) had similar variation to what was observed experimentally (standard deviation: 4s and 504s). To test for the statistical significance of differences in egg-laying interval times, a non-parametric

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 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. DNA sequencing was performed by the Molecular Pathology Shared Resource, UCSD Cancer Center, which is funded in part by NCI Cancer Center Support Grant #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 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 Sternberg) to wild-type, and isolating an Unc non-Him F2 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 VC's, adjacent Pn.a cells can generate VC's in the absence of P3.a-P8.a unless killed (Li and Chalfie 1990). Wild-type animals were grown at 20° C; approximately 10 hours after hatching, the Pn.a cell nuclei were identified by position in the ventral cord and killed; cell killing was verified by scoring for the absence of adult ventral cord motorneuronal nuclei in the mid-body 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 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 which also descend from the ablated neuroblasts (VA7, VB8, and VD7) potentially make single synapses with the egg-laying muscles; thus, it was formally possible these neurons might contribute to this effect. However, ablations of a subset of Pn.a neuroblasts (for example, P3.a-P.8.a) which eliminated VA7, VB8, and VD7 but spared one or more VCs (as indicated by *egl-36::GFP* expressing cells that sent processes to the vulva) did not prevent levamisole response (data not shown).

ACKNOWLEDGMENTS

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Table 1-1: Egg-laying patterns of levamisole-receptor mutants

Strain (#, hrs, intervals)	Intra-cluster time constant ($1/\lambda_1$; s)	Inter-cluster time constant ($1/p\lambda_2$; s)	Clustering parameter (p)
N2 (10, 58, 357)	16 ±2	1386 ±201	0.555 ±0.031
unc-29(x29) (8, 38, 196)	14 ±2	2236* ±494	0.700 ±0.057
unc-29 (e193) (8, 44, 155)	16 ±4	2042* ±267	0.327 ±0.051
unc-29 (e1072) (8, 44, 162)	31* ±6	1733* ±236	0.444 ±0.045
unc-38 (x20) (6, 25, 127)	18 ±3	1738 ±389	0.669 ±0.043
unc-38 (e264) (15, 62, 207)	18 ±4	1555 ±166	0.357 ±0.035
lev-1 (e211) (10, 45, 161)	32* ±5	2171* ±296	0.497 ±0.040
lev-1 (x427) (11, 52, 149)	28* ±4	2896* ±477	0.508 ±0.048
unc-29(x29); lev-1(e211) (7, 31, 143)	30 ±5	2067 ±417	0.576 ±0.047
unc-38(x20); lev-1(e211) (12, 40, 138)	27* ±4	2007* ±305	0.453 ±0.048
unc-38(sy576) unc-29(e1072) (7, 27, 113)	29* ±5	1740* ±324	0.479 ±0.053
unc-38(sy576) unc-29(e1072); lev-1(e211) (8, 30, 118)	32* ±7	1850* ±330	0.492 ±0.050

* short(<120s) or long (>120s) intervals statistically different (level of confidence $p < .05$) from wild type according to the Mann-Whitney rank sum test.

Figure 1-1: 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 hour 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 $p < .001$). 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.

Figure 1-1

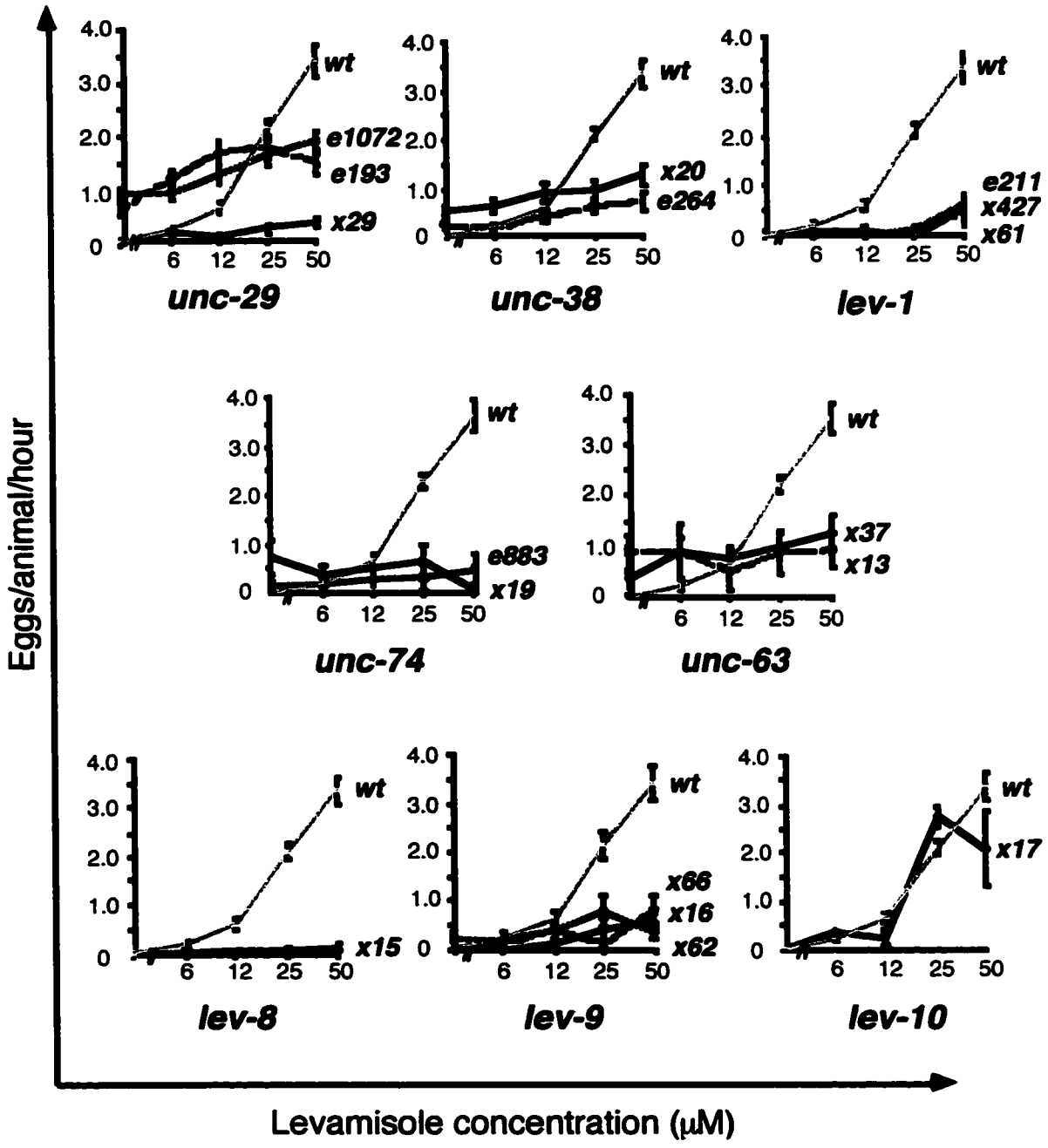

Eggs/animal/hour
4.0
3.0
2.0
1.0
0
6 12 25 50
wt
e1072
e193
x29
unc-29
wt
x20
e264
unc-38
wt
e211
x427
x61
lev-1
wt
e883
x19
unc-74
wt
x37
x13
unc-63
wt
x15
lev-8
wt
x66
x16
x62
lev-9
wt
x17
lev-10
Levamisole concentration (μM)

Figure 1-2a: 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 hour are shown in part a. 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.

Figure 1-2a

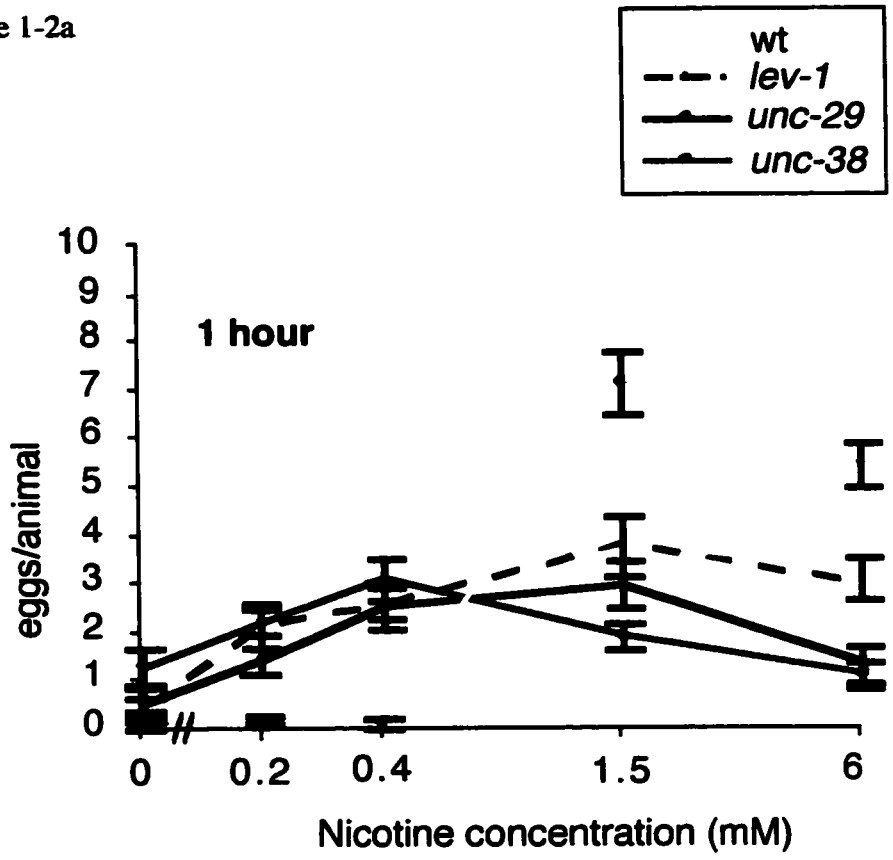

wt
lev-1
unc-29
unc-38
1 hour
eggs/animal
10
9
8
7
6
5
4
3
2
1
0
0
0.2
0.4
1.5
6
Nicotine concentration (mM)

Figure 1-2b: 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 2 hour are shown in part b. 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.

Figure 1-2b

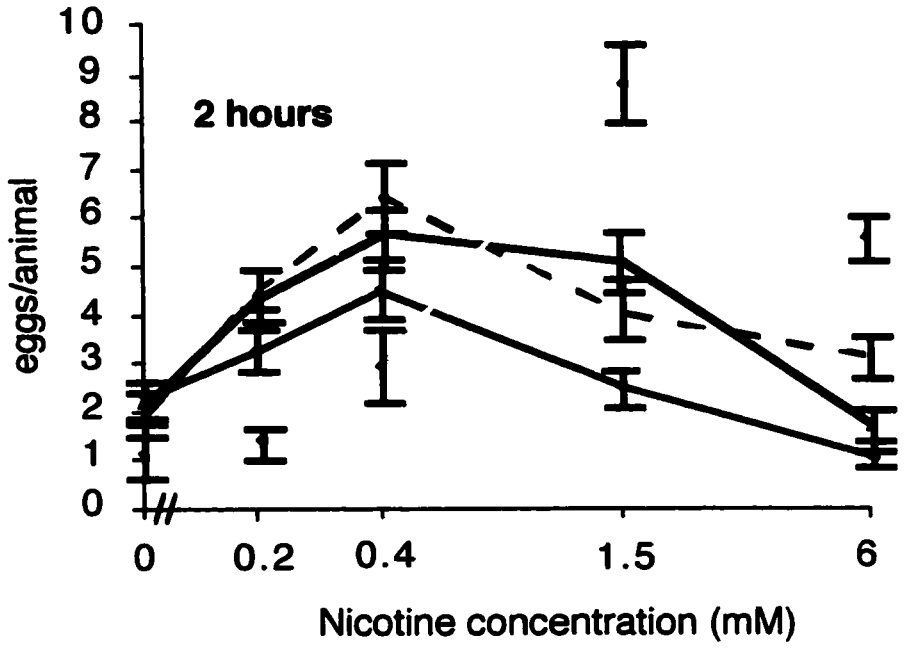

10
9
8
7
6
5
4
3
2
1
0
2 hours
eggs/animal
0
0.2
0.4
1.5
6
Nicotine concentration (mM)

Figure 1-3: 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 intra-cluster intervals (i.e., the intervals between events within a cluster), and the right peak contains the inter-cluster intervals (i.e., the intervals between clusters). Dashed lines indicate the estimated intra-cluster and inter-cluster time constants. Animals were tracked as described on nematode growth medium (NGM) or on NGM containing 50 μM 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: N2--9 animals, 19 hrs, 64 intervals; *unc-29(x29)*--5 animals, 21 hrs, 100 intervals; *lev-1(e211)*--6 animals, 28 hrs, 101 intervals; *unc-38(x20)*--7 animals, 35 hrs, 104 intervals; *unc-38(sy576) unc-29(e1072); lev-1(e211)*--9 animals, 47 hrs, 275 intervals. Both the long (>120s) and short (<120s) intervals were significantly shortened by levamisole in wild-type (level of confidence $p<.001$). 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 (level of confidence $p<.05$) according to the Mann-Whitney rank sum test. In testing the significance for the mutants, a cut-off point of 300s was used in separating long and short intervals to adapt to the shifts in the curves representing intra-cluster intervals.

Figure 1-3

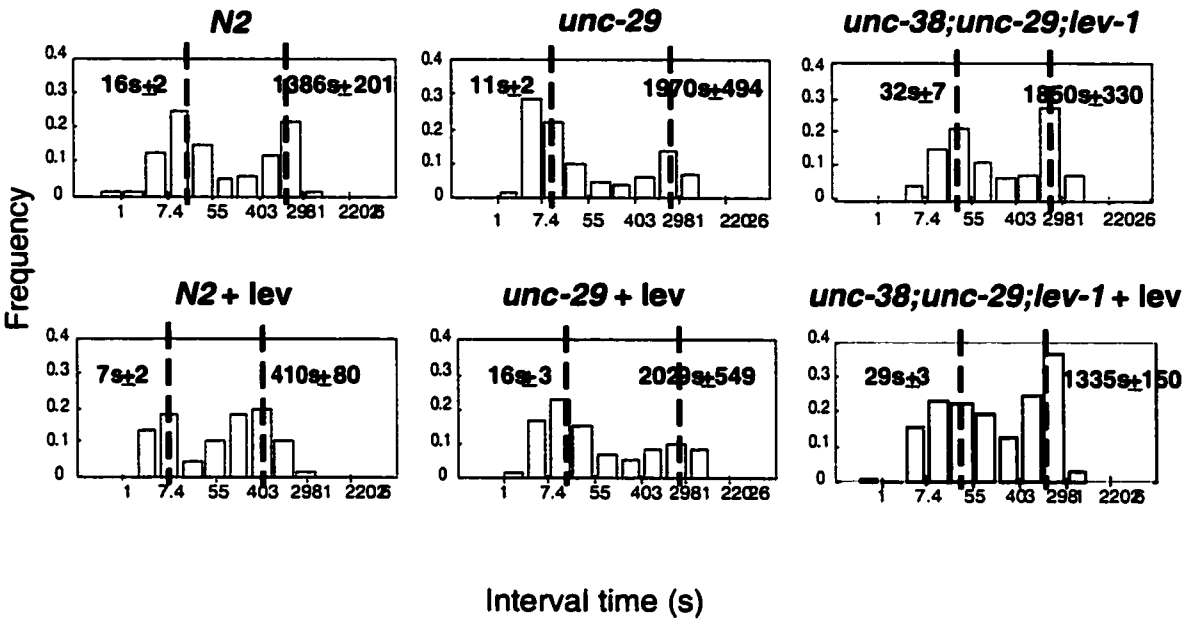

N2
16s±2
1386s±201
unc-29
11s±2
1970s±494
unc-38;unc-29;lev-1
32s±7
1850s±330
N2 + lev
7s±2
410s±80
unc-29 + lev
16s±3
202s±549
unc-38;unc-29;lev-1 + lev
29s±3
1335s±150
Frequency
0.4
0.3
0.2
0.1
0
1
7.4
55
403
2981
22026
Interval time (s)

Figure 1-4a: 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 motorneurons (Waggoner *et al.* 2000a).

Figure 1-4a

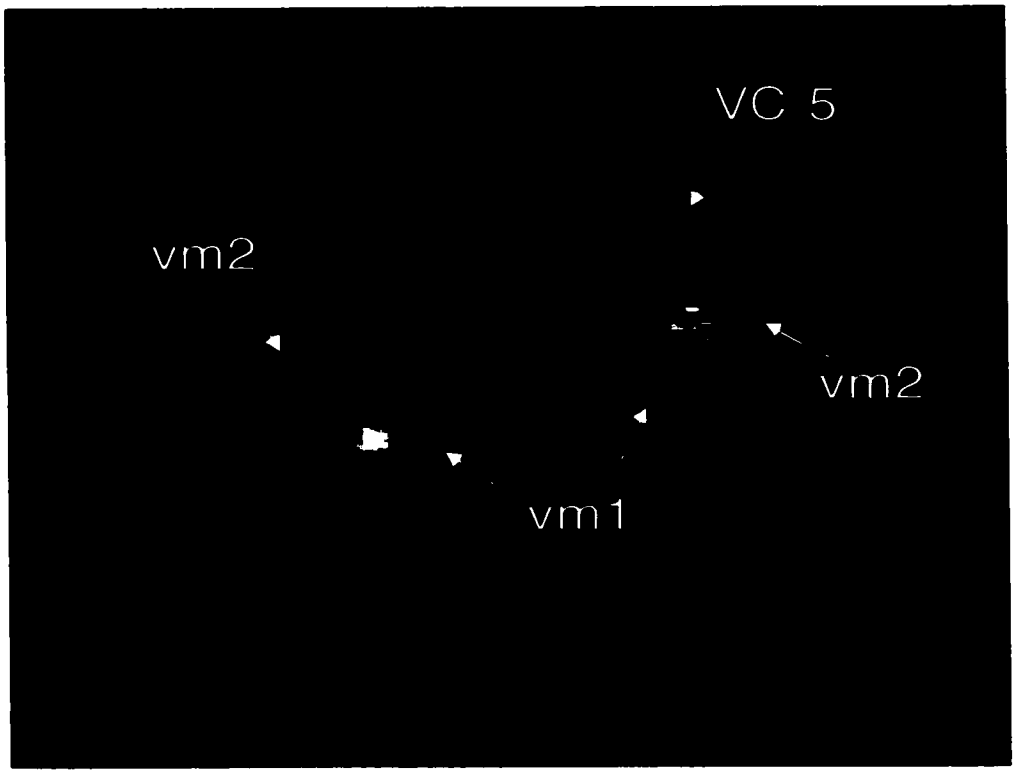


Figure 1-4b: Partial rescue of *unc-29* egg-laying phenotypes by muscle-specific gene expression. b. Dose response curves showing egg-laying in response to levamisole by wild-type animals, *unc-29* mutants, and transgenic animals expressing functional *unc-29* only in the vulval muscles (*ndE-box::unc-29(+)*; 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.

Figure 1-4b

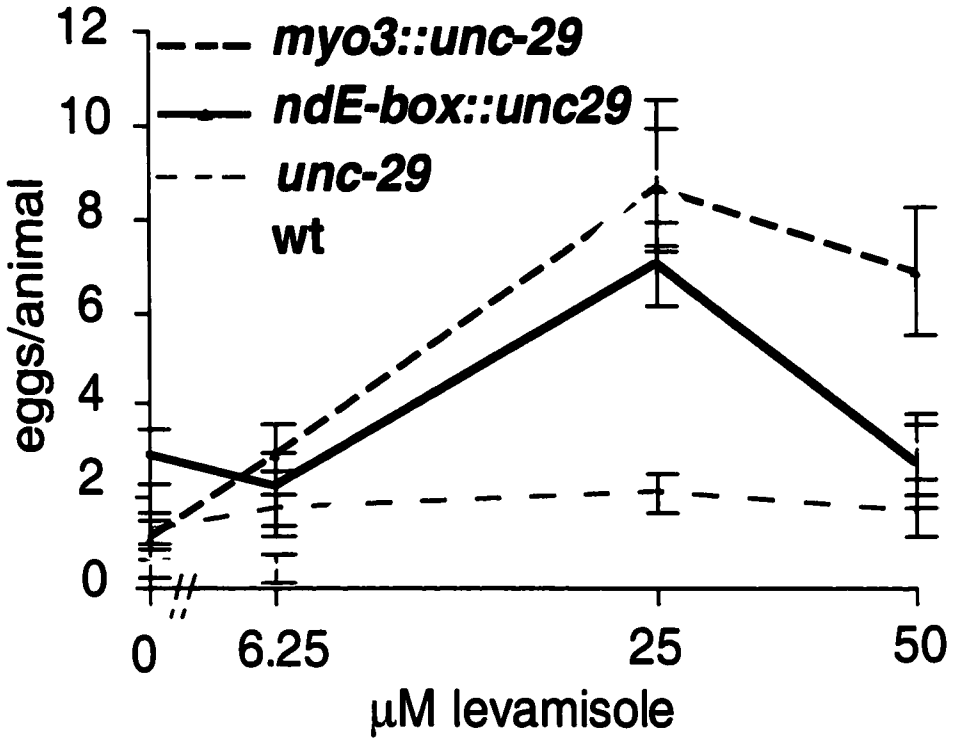

12
10
8
6
4
2
0
eggs/animal
myo3::unc-29
ndE-box::unc29
unc-29
wt
0
6.25
25
50
μM levamisole

Figure 1-4c: Partial rescue of *unc-29* egg-laying phenotypes by muscle-specific gene expression. c. Effect of levamisole on the egg-laying patterns of animals with muscle specific *unc-29(+)* expression. Dashed lines indicate the estimated intra-cluster and inter-cluster time constants. The number of animals, hours tracked, and total intervals analyzed were: AQ548/*myo-3::unc-29* (no drug): 5 animals, 26 hr, 71 intervals; AQ548/*myo-3::unc-29* (levamisole): 5 animals, 30 hr, 109 intervals.

Figure 1-4c

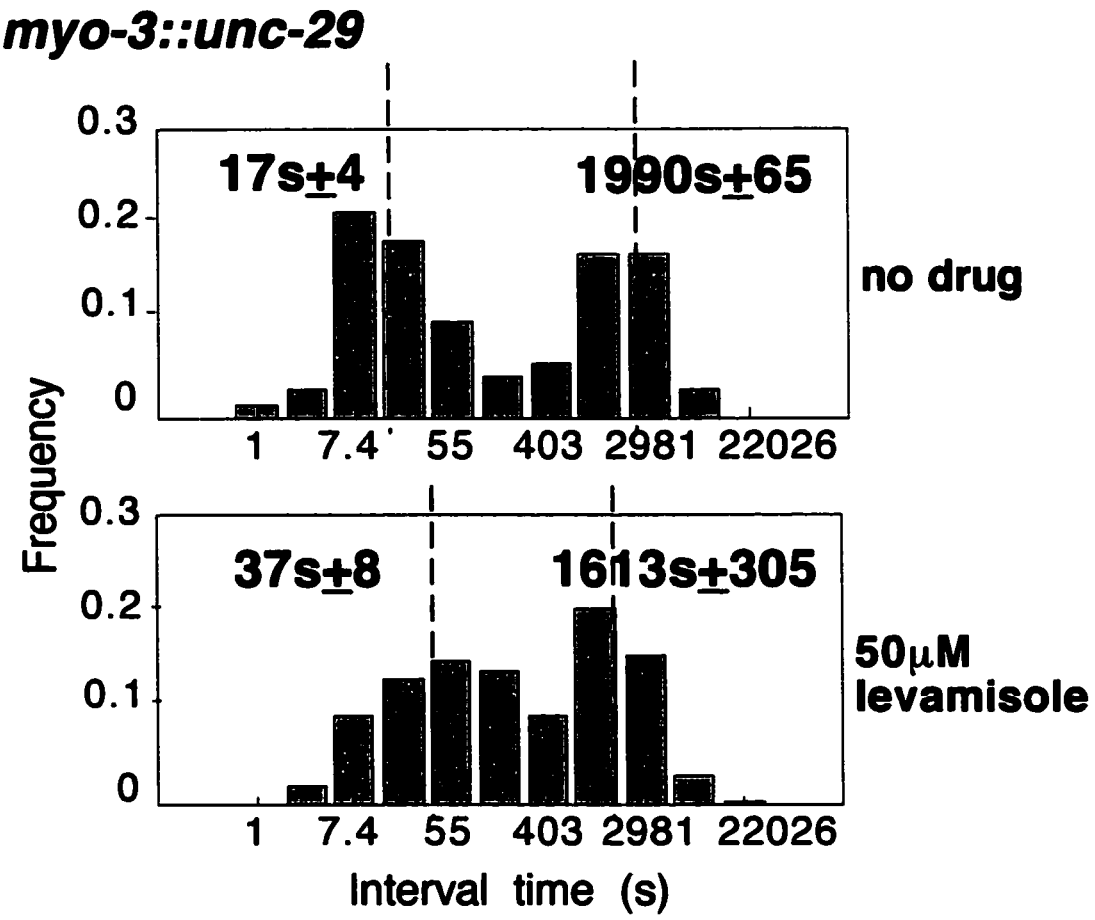

myo-3::unc-29
0.3
0.2
0.1
0
17s±4
1990s±65
no drug
Frequency
0.3
0.2
0.1
0
37s±8
1613s±305
50μM
levamisole
1 7.4 55 403 2981 22026
1 7.4 55 403 2981 22026
Interval time (s)

Figure 1-4d: Partial rescue of *unc-29* egg-laying phenotypes by muscle-specific gene expression. d. Effect of levamisole on the egg-laying patterns of animals with ablation of the Pn.a VC neuronal precursors. Dashed lines indicate the estimated intra-cluster and inter-cluster time constants. The number of animals, hours tracked, and total intervals analyzed were: N2, VC- (no drug): 3 animals, 20 hr, 42 intervals; N2, VC- (levamisole): 3 animals, 24 hr, 69 intervals.

Figure 1-4d

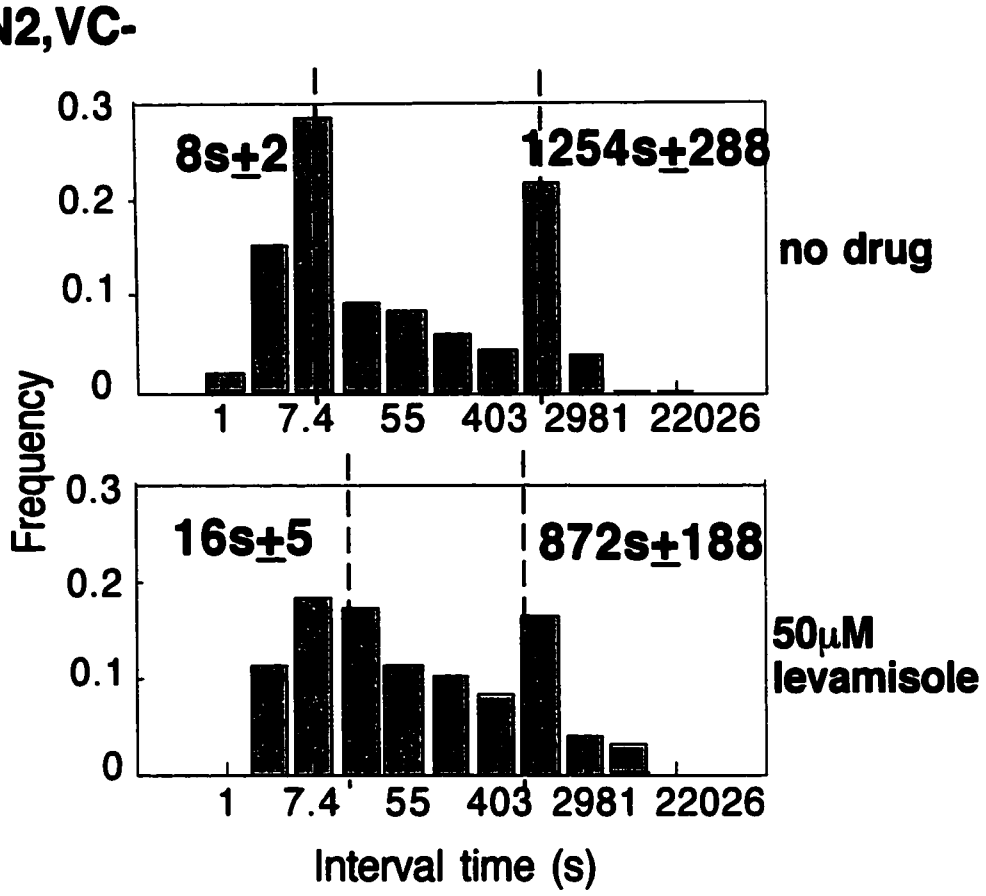

N2,VC-
0.3
0.2
0.1
0
8s±2
1254s±288
no drug
Frequency
1 7.4 55 403 2981 22026
16s±5
872s±188
50μM
levamisole
Interval time (s)

Figure 1-5: Interactions between the egg-laying phenotypes of *egl-1*, *tph-1* and the levamisole receptor genes *lev-1* and *unc-29*. Shown are histograms of intervals times between egg-laying events. Dashed lines indicate the estimated intra-cluster and inter-cluster time constants. The number of animals, hours tracked, and total intervals analyzed were: *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 (< 300s) 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 $p < .05$).

Figure 1-5

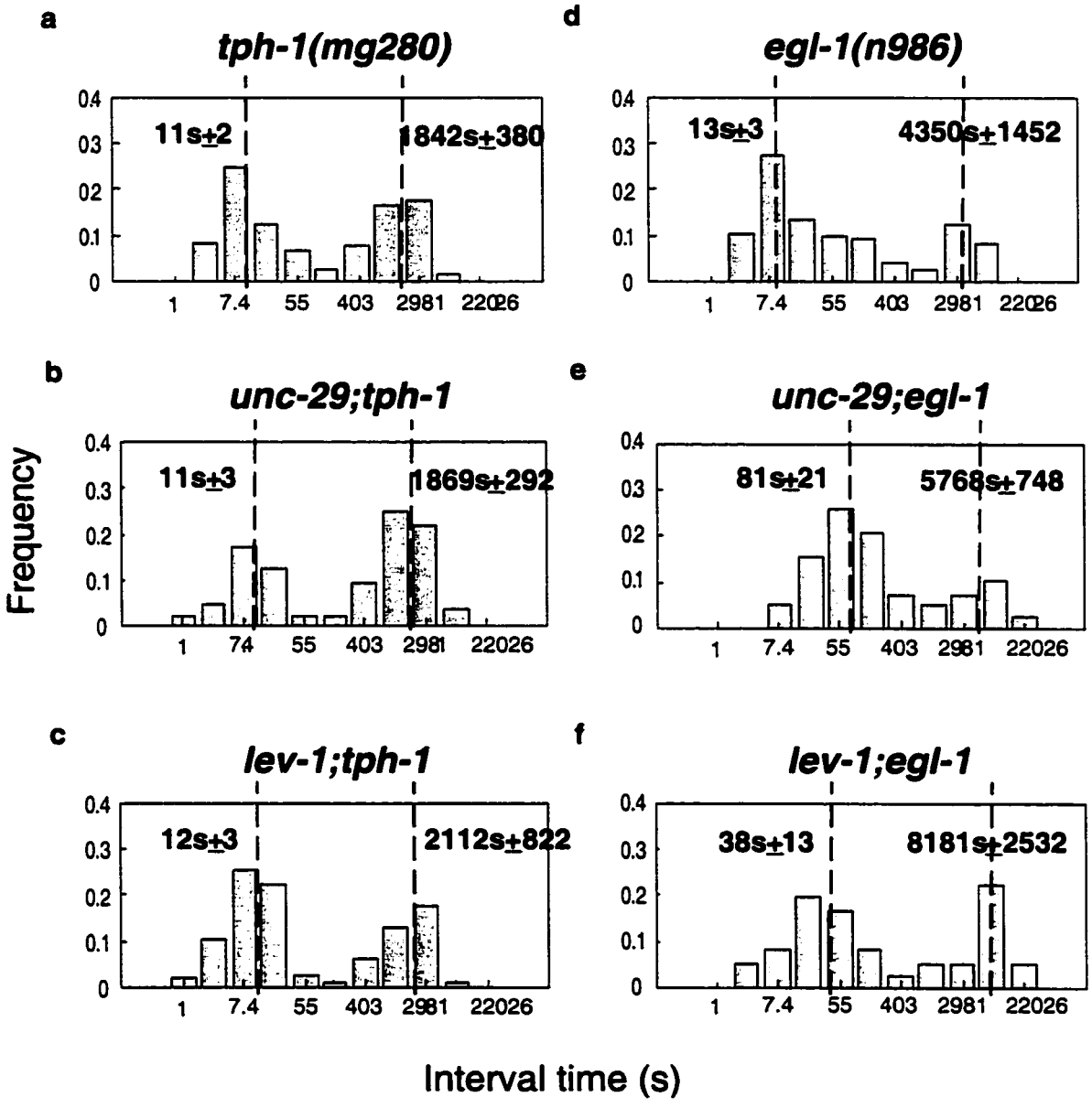
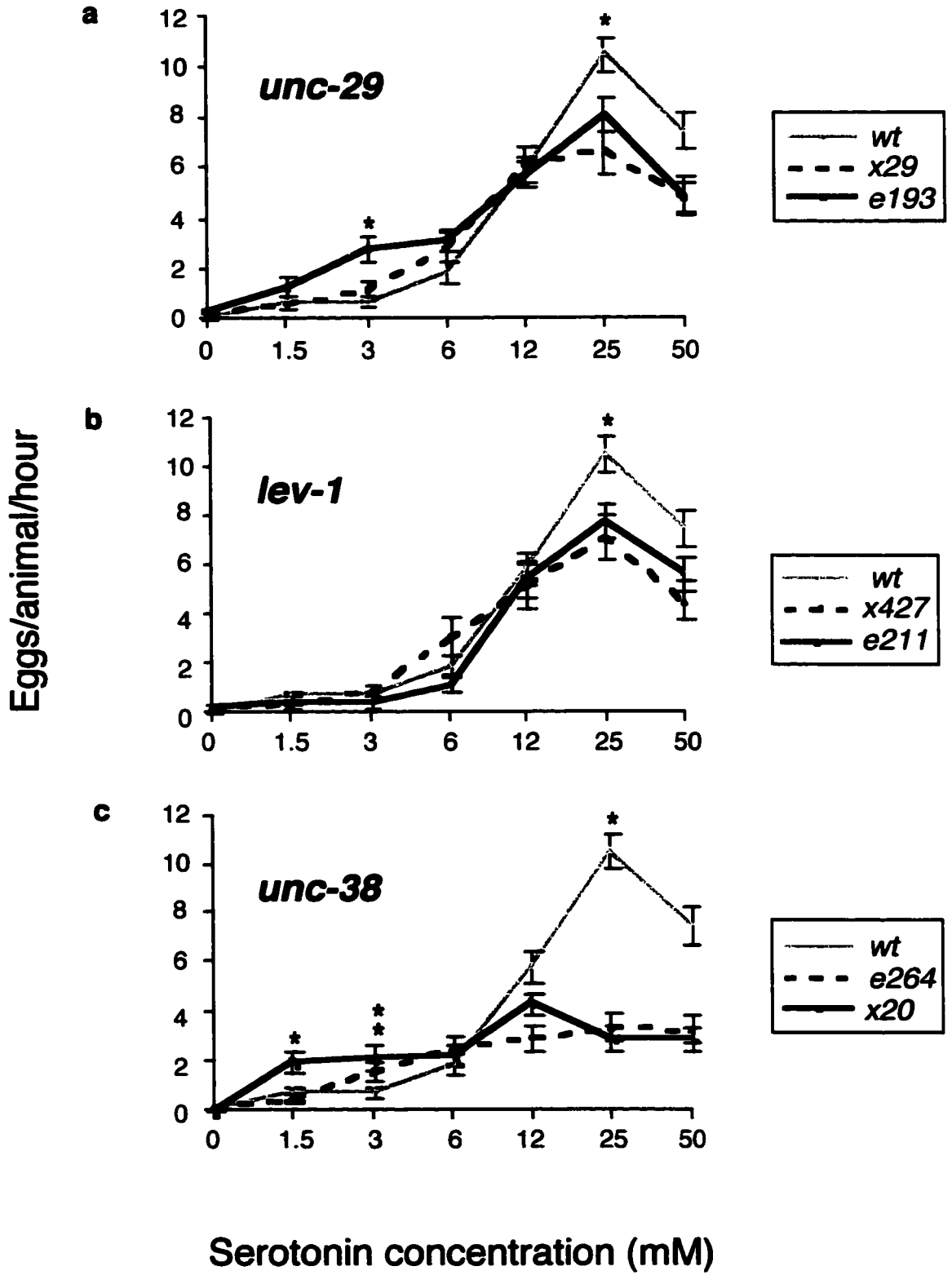

a
tph-1(mg280)
11s±2
1842s±380
d
egl-1(n986)
13s±3
4350s±1452
b
unc-29;tph-1
11s±3
1869s±292
e
unc-29;egl-1
81s±21
5768s±748
c
lev-1;tph-1
12s±3
2112s±822
f
lev-1;egl-1
38s±13
8181s±2532
Frequency
0
0.1
0.2
0.3
0.4
1
7.4
55
403
2981
22026
Interval time (s)

Figure 1-6: 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 hour 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 (level of confidence $p < .05$). 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 (level of confidence $p < .05$). 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.

Figure 1-6


a
unc-29
wt
x29
e193
b
lev-1
wt
x427
e211
c
unc-38
wt
e264
x20
Eggs/animal/hour
Serotonin concentration (mM)
0
1.5
3
6
12
25
50

CHAPTER III. THE NIC-1 GLYCOSYLTRANSFERASE MODIFIES THE FUNCTIONAL ACTIVITY OF NICOTINIC RECEPTORS *IN C. ELEGANS*

ABSTRACT

The *Caenorhabditis elegans* gene *nic-1* encodes a homolog of the yeast α-1,3 and α-1,6 mannosyltransferase Alg2, which catalyzes two early steps in the assembly in the ER of a lipid-linked core oligosaccharide $Glc_2Man_9GlcNAc_2$-PP-Dol in *N*-linked glycosylation. NIC-1 is a 483 aa protein with 25% amino acid sequence similarity to Alg2 and contains a dolichol-binding consensus sequence (Val/Ile-x-Phe-x-x-Ile). FAB-mass spectra of PNGase-F released perdeuteromethylated *N*-glycans in wild type and *nic-1* extracts revealed drastically decreased signals of the high mannose-type glycans $Man_6GlcNAc_2$-$Man_9GlcNAc_2$ (m/z 1863-2502) in *nic-1* mutants compared to wild type, while signals for the high mannose ($Man_5GlcNAc_2$; m/z 1649), truncated ($Man_{3-4}ManNAc_2$; m/z 1222-1793), and complex ($FucMan_3GlcNAc_2$; m/z 1403)–type glycans were similar to those seen for wild type. This suggested that NIC-1 may function either as an α-1,2 mannosyltransferase in high-mannose type *N*-glycan assembly, or as an affector in the mechanism wherein $Man_5GlcNAc_2$ "flips" from the cytosolic side to the lumenal side of the ER. Interestingly, NIC-1 appeared to negatively regulate the activity of nicotinic acetylcholine receptors. *nic-1* mutants were hypersensitive for the nicotine and/or levamisole-induced behaviors, body muscle hypercontraction, egg laying and spicule protraction; and mutants also displayed increased egg laying within the active egg-laying phase, a pattern characteristic of animals experiencing increased cholinergic

neurotransmission. Furthermore, *nic-1* caused hypersensitivity for nicotine-induced paralysis in animals with neuron- and muscle-specific expression of UNC-38, suggesting that NIC-1 down regulated the activity of UNC-38 receptors in neurons and possibly in muscle.

INTRODUCTION

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

One way to investigate the mechanisms underlying nicotinic receptor inactivation is to use a genetically tractable animal such as *Caenorhabditis elegans. C. elegans* has a simple, well-characterized nervous system and is well suited for investigating how specific neurotransmitters, receptors, and signaling molecules function within the context of the nervous system to produce behavior. *C. elegans* contains a diverse family of nicotinic receptors, including both neuromuscular and neuronal receptor subtypes. Moreover, nicotinic receptor agonists have specific and easily assayed effects on several aspects of *C. elegans* behavior, including locomotion, feeding, and egg-laying. A number of paradigms for behavioral plasticity have been defined in *C. elegans*, demonstrating that these animals are capable of at least simple forms of learning.

Acute nicotine treatment has several effects on *C. elegans*, including hypercontraction of the body muscles, protraction of male spicules and stimulation of egg laying. These effects are mediated at least in part in part by acute stimulation of the *unc-*

29/unc-38/lev-1-dependent levamisole receptor, which is localized, among other places, on the body wall, vulval, and male sex muscles. Long-term exposure to nicotine has been shown to result in what we termed adaptation: an attenuation of nicotine response on the body muscle levamisole receptor, and an inhibition of the response of these muscle receptors to other cholinergic agonists.

Most secreted and membrane proteins in eukaryotes undergo glycosylation, a process during which a protein is modified by the addition of carbohydrate moieties to specific amino acid residues. There are two main types of protein glycosylation: *N*- linked glycosylation, wherein a glycan is attached to an asparagine (Asn) residue of the tripeptide consensus sequence Asn-x-Ser/Thr of a protein (where x refers to any amino acid except Pro), and *O*-linked glycosylation, where a glycan is attached to a Ser or a Thr residue of a protein (Kornfeld and Kornfeld 1985; Herscovics and Orlean 1993). Early stages of *N*-linked glycosylation that take place in the ER are surprisingly well conserved in eukaryotic organisms (Varki et al. 1999). In *N*-linked glycosylation, a lipid (dolichol)-linked core oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, is synthesized in the endoplasmic reticulum (ER) during a step-wise assembling process involving an array of step-specific glycosyltransferases and monosaccharide donors. UDP-GlcNAc and GDP-mannose are utilized as donors for the assembly of the two core GlcNAc monosaccharides and the first five mannose residues, respectively. These first seven linkage reactions occur on the cytosolic side of the ER membrane. Then, by a mechanism not fully understood, the $Man_5GlcNAc_2$-Dol flips across the membrane before four additional mannoses are added, this time in the lumen of the ER, using Dol-P-Mannose as the donor. Finally, three GlcNAc residues are added before the oligosaccharide precursor is added to an Asn of a nascent protein. Further modification by ER and Golgi glycosidases and glycosyltransferases results in glycans of varying sugar composition: high mannose- or

oligomannose-type, $Man_{5-9}GlcNAc_2$; truncated-type (present largely in invertebrates), $Man_{1-4}GlcNAc_2$; and complex-type, which involves addition of sugars to the distal side of the core oligosaccharide and is the most abundant class of mammalian glycans (Varki et al. 1999).

In *Saccharomyces cerevisiae*, where the process of *N*-linked glycosylation has been well characterized, we know the genes that encode specific glycosyltransferases involved in catalyzing certain steps of the core oligosaccharide assembly in the ER. These have been termed *alg-* genes, for 'asparagine-linked glycosylation'. The yeast *alg2* gene encodes an α-1,3 and α-1,6 mannosyltransferase that transfers a mannose from GDP-Man to $Man_1GlcNAc_2$-PP-Dol and $Man_2GlcNAc_2$-PP-Dol to form $Man_3GlcNAc_2$-PP-Dol. *alg2* mutants abnormally accumulate $Man_1GlcNAc_2$-PP-Dol and $Man_2GlcNAc_2$-PP-Dol and exhibit a temperature-sensitive lethal phenotype due to cell cycle arrest (Huffaker and Robbinson 1983; Jackson et al. 1993). The *alg2* homolog in the zygomycete fungus *Rhizomucor pusillus* has been found to encode a 455-aa protein with remarkable amino acid sequence similarity to yeast Alg2 and contain a dolichol-binding site (Val/Ile-x-Phe-x-x-Ile; x is any amino acid) near its C-terminus (Yamazaki et al. 1999).

RESULTS

Screen for nicotine hypersensitive mutants

To identify candidate negative regulators of nicotinic receptor activity, we performed a genetic screen for nicotine hypersensitive mutants (Figure 1). To increase the sensitivity of our screen and decrease false positives (e.g., a mutation causing a cuticle defect that renders the animal hypersensitive to virtually all drug treatments and has nothing to do with nAChR function), we conducted our screen in a sensitized genetic background. The starting

strain contained a mutation in the levamisole receptor non-α subunit gene *lev-1* that weakly cripples the levamisole receptor (Lewis et. al. 1980, Fleming et. al. 1997). These *lev-1* mutants are weakly resistant to nicotine for body muscle paralysis and recover more rapidly and more reproducibly from nicotine treatment than wild-type animals (Dickinson and Schafer, unpublished data), and are resistant for egg-laying stimulation by levamisole in M9 (Kim et. al. 2001). The F_2 self-progeny of EMS treated *lev-1* hermaphrodites were transferred to plates containing 1% nicotine, a concentration that leads to spastic paralysis for wild type, but that which leads to initial paralysis, then recovery and resistance to nicotine within one hour for *lev-1* mutants. Mutants that remained paralyzed after one hour on 1.0% nicotine plates were isolated and allowed to self fertilize. The progeny were then retested for nicotine hypersensitivity and also tested for recovery of levamisole sensitivity for egg-laying behavior, and animals that bred true for both phenotypes were identified as nicotine hypersensitive mutants. Mutations of this type identified genes encoding potential negative regulators of nAChR function. We isolated eight such candidates, of which one we termed *nic-1*(lj22) showed especially strong hypersensitivity to nicotine and exhibited uncoordinated movement and decreased ability of males to mate, both behaviors that suggest a defect in motorneuronal signaling.

nic-1 mutants are hypersensitive to nicotine

To characterize the effect of the *nic-1* on nicotinic receptor activity, we assayed *nic-1* mutants for behaviors known to be caused by acute nicotine treatment: body muscle paralysis, egg-laying stimulation, and male spicule protraction (Figure 2). First, we analyzed the effect of the *nic-1* mutation on the activity of body muscle nAChRs by measuring dosage response to nicotine-induced paralysis. As characterized by the nature of the screen, *lev-1;nic-1* mutants were sensitive to nicotine for body muscle paralysis and were unable to recover within one hour, unlike the *lev-1* single mutant. To examine the

effect of the *nic-1* mutation on its own, without the *lev-1* mutation, we crossed *nic-1* out of the *lev-1* background by mating *lev-1;nic-1* hermaphrodites to wild type males, then Likewise, the *nic-1* single mutant, crossed out of the *lev-1* background and backcrossed 2x to wild type, was also hypersensitive to nicotine for paralysis compared to wild type. A nicotine dose-response test on normal nematode growth medium (NGM) plates containing nicotine revealed that the half-maximal concentration for paralysis in *nic-1* mutants was around 0.3mM, compared to 4mM for wild type, a more than ten-fold difference. Next, we investigated *nic-1*'s effect on the activity of nAChRs in the vulval muscles of the egg-laying neuromusculature. Wild type animals treated with levamisole or nicotine in M9, a hypertonic salt solution inhibitory for egg laying, produces robust, dose dependent stimulation of egg laying (Waggoner *et.al.* 2000; Kim *et.al.* 2000). Analysis of egg-laying behavior revealed that *nic-1* mutants were hypersensitive for egg laying. *lev1;nic-1* animals were hypersensitive to levamisole compared to *lev-1* animals (their half-maximal responses at 25μM levamisole were 2 eggs/animal versus 0.75 eggs/animal, respectively). Additionally, preliminary results showed *nic-1* animals hypersensitive to nicotine compared to wild type at low doses of 0.2-0.4mM (1.5 eggs/animal for *nic-1* and 0.1 eggs/animal for wild type at 0.2mM). Finally, we investigated *nic-1*'s effect on the activity of nAChRs in the protractor muscles of the male tail by measuring male spicule protraction on nicotine. Extensive genetic studies done on male mating behavior have shown that the sequence of events characterizing male mating behavior in *C. elegans* involves a complex network of neurons and muscles in the male tail that requires cholinergic neurotransmission. In particular, the protractor muscles which contract to extend the spicules, male-specific structures that facilitate in sperm transfer during mating, receive input from neurons expressing *unc-17::GFP*, an acetylcholine vesicular transporter promoter driving expression of green fluorescent protein. Furthermore, treatment with nicotine causes inappropriate spicule protraction during periods of non-mating (Garcia and Sternberg

2000). Investigation of nicotine-induced spicule protraction in *nic-1* males showed they were hypersensitive also for this behavior (half-maximal response at <0.1mM nicotine) compared to wild type (half-maximal response at 2mM nicotine). Examination of the different nicotine-induced behaviors in *nic-1* animals revealed unequivocally that this mutant was hypersensitive to nicotinic agonists. To test whether these behaviors were the result of a cuticle defect in the mutant leading to a non-specific hypersensitivity to all drugs, we assayed *nic-1*'s sensitivity to agonists of other known non-cholinergic targets. Assays for paralysis in response to serotonin, agonist to a seven transmembrane G-protein coupled receptor, and to ivermectin, , were performed. We saw that the *nic-1*response to these drugs was not significantly greater than that of wild type, thus failing to support the possibility of a non-specific cuticle defect. These results were consistent with the wild type role of the NIC-1 gene product functioning specifically as a downregulator of nAChR activity, and a mutation in *nic-1* thereby resulting in increased activity through nAChRs in the different muscle groups assayed.

nic-1 affects behaviors involving cholinergic neurotransmission

To further investigate the possible role of NIC-1 in regulating nAChR activity, we examined *nic-1*'s effect on intrinsic behaviors due to acetylcholine (ACh)-induced cholinergic neurotransmission, such as the timing of egg-laying events under normal conditions. Egg-laying in *C. elegans* occurs in a distinct pattern, in which wild type worms fluctuate between two discrete egg-laying behavioral states: an active egg-laying state during which eggs are laid in clusters, and an inactive state during which eggs are retained in the uterus. By observing and analyzing an animal's egg-laying behavior over long periods, one can estimate exponential time constants for both the onset of the active phase and for egg laying within the active phase (Waggoner *et.al.* 1998). Analysis of egg-laying patterns of various nervous system mutants has shown that multiple neurotransmitters contribute to the timing

of egg-laying events. Mutants defective for serotonin signaling, such as *cat-4* (defective catecholamine synthesis) and *egl-1* (inappropriate cell death of the HSNs, major serotonergic egg-laying motorneurons) show increased inter-cluster time constants and lower overall rate of egg laying. Meanwhile, mutants with lowered cholinergic neurotransmission, such as *unc-29 unc-38;lev-1 (*defective levamisole receptor) and *cha-1* conditional mutants (lacking choline acetyltransferase), show increased intra-cluster time constants, indicating a slower rate of egg laying within the active state. Serotonin appears to promote the onset of egg-laying clusters, while ACh largely stimulates egg-laying events within a cluster (Waggoner *et.al.* 1998). When we analyzed the egg-laying pattern for *nic-1* animals, we found that the mutants experienced a four-fold shorter intra-cluster time constant compared to wild type (4.1s vs. 16s, respectively), while their inter-cluster time constant was not significantly different from that of wild type (1632s vs.1386s, respectively). As expected for a mutant with higher than normal nAChR activity, *nic-1* displayed an increased rate of egg laying within the active state. This was precisely the converse of the effect shown by *unc-29 unc-38;lev-1* mutants, animals that have a defective levamisole receptor, and consequently, lower than normal nAChR activity (Figure 3). This result clearly showed that the *nic-1* mutation specifically increased egg laying within the active state, and suggested that the wild type NIC-1 gene product directly or indirectly downregulates cholinergic neurotransmission in the egg-laying circuitry.

In addition, we observed other subtle phenotypes in *nic-1* that were consistent with the mutant having defects in cholinergic neurotransmission. First, they displayed a unique, uncoordinated (Unc) movement phenotype, which was evident in their movement tracks made in a lawn of bacteria. This movement defect was unlike other Unc phenotypes where the animals are lethargic and have a decreased ability to move. Rather, *nic-1* animals constantly made efforts to move yet appeared unable to coordinate their muscles in the manner of the characteristically wild type sinusoidal bends, thus making it difficult for a *nic-*

1 animal to mobilize from one location to another. Second, *nic-1* males displayed decreased ability to mate. Although the Unc phenotype of these mutants might be thought to play a role in decreasing the efficiency of mating, it seemed unlikely to be the sole cause of the mating defect. *nic-1* hermaphrodites, just as Unc as their male counterparts, appeared to be able to mate as efficiently as wild type: *nic-1* hermaphrodites placed on mating plates with wild type males gave rise to the normal ratio of male to hermaphrodite progeny, about 1:1. *nic-1* animals were also shorter in length than wild type and a bit Dumpy (Dpy), which appeared possibly to be the result of their body muscles being in a baseline hypercontracted state. Body muscle contraction, movement, and male mating behavior all require cholinergic motor input during normal function. These characteristics of *nic-1*, though not concrete enough to form an argument on their own as the basis for the role of NIC-1, are certainly consistent with phenotypes one might see for mutants experiencing increased cholinergic neurotransmission. These observations and results taken together support the role of NIC-1 as a negative regulator of nAChR activity in various muscle groups.

nic-1 sensitivity to other drugs

To examine whether *nic-1* specifically affects the activity of nicotinic acetylcholine receptors or causes a more general non-specific effect, we assayed *nic-1* animals for sensitivity to other drugs that act on other neurotransmitter targets (Figure 4). We tested the following drugs: serotonin, dantrolene, and muscimol. The neurotransmitter serotonin (5-hydroxytryptophan, or 5-HT) is agonist of the metabotropic G-protein coupled seven-transmembrane 5-HT receptor. Serotonin is present in *C. elegans* motor neurons, and causes paralysis in wild type at 10 mg/ml. A dose response assay for paralysis caused by serotonin showed that *nic-1* mutants displayed a similar level of sensitivity to serotonin for paralysis as that shown by wild type; *nic-1* did not display hypersensitivity to the drug

serotonin. Dantrolene is an antagonist of the ryanodine receptor, another metabotropic receptor located on the sarcoplasmic reticulum of muscle cells that, when activated, signals the release of intracellular stores of calcium into the cytosol and further promotes muscle contraction. In wild type, dantrolene prevents the release of intracellular calcium, inhibiting the effective calcium activation of muscle fibers, thereby causing flaccid paralysis. The results of a dose response assay to dantrolene also showed that *nic-1* animals were not hypersensitive to dantrolene compared to wild type; both *nic-1* and wild type revealed a half-maximal dose response at about 75 mM. Muscimol is an agonist of the ionotropic GABA-A receptor, a neurotransmitter-gated chloride channel which, upon binding its agonist, opens to allow Cl^- ions to exit the cell, causing hyperpolarization of the membrane and an overall inhibitory action in neurons. A dose response assay on muscimol revealed that *nic-1* mutants were hypersensitive to this drug compared to wild type between the concentrations 0.025 mM and 5 mM. These results suggested that *nic-1* mutants were not merely hypersensitive to all drugs, as seen by the evidence that *nic-1* animals did not display increased sensitivity to the drugs serotonin and dantrolene compared to wild type. However, since we did observe that *nic-1* animals displayed hypersensitivity to muscimol, a non-nicotinic receptor agonist, we concluded that the effects of NIC-1 were not confined to only nicotinic acetylcholine receptors. One possible explanation for this result is that since nicotinic receptors and GABA-A receptors are both types of heteromeric, ligand-gated ion channels, while 5-HT receptors and ryanodine receptors are both metabotropic, G-protein coupled receptors, NIC-1's role may be limited to acting on ligand-gated ion channels and not on G-protein coupled receptors.

NIC-1 affects the activity of UNC-38 receptors

We have previously seen that the different known levamisole receptor subunits show slightly different expression patterns. *unc-29::*GFP and *lev-1::*GFP are expressed mostly

in body muscle and variably in vulval muscles, as well as in some neurons in the ventral cord and in the head. *unc-38* shows more abundant expression in the head neurons as well as in body muscle. To investigate whether *nic-1* affected the activity of neuronal nAChRs or muscle nAChRs or both, we generated *nic-1* mutants that carried cell-specific *unc-38* rescue arrays (generously given to us by Rene Garcia) in an *unc-38* mutant background: a *myo-3* promoter driving muscle-specific rescue (*unc-38(x20)I; nic-1(lj22)X; Ex[myo-3p::unc-38, myo-2p::*GFP]), and a *unc-119* promoter driving neuron-specific rescue (*unc-38(x20)I; nic-1(lj22)X; Ex[unc-119p::unc-38, myo-2p::*GFP]) of wild type UNC-38 protein. We performed nicotine-induced body muscle paralysis assays for these cell-specific UNC-38 rescue mutants to determine if the *nic-1* mutation would cause nicotine hypersensitivity (Figure 5). *unc-38* mutants are resistant to concentrations as high as 1mM levamisole for body muscle paralysis, while *nic-1* mutants showed hypersensitivity at 0.5 mM. *unc-38;nic-1* double mutants displayed slight sensitivity to 0.5 mM levamisole but were largely resistant, as body muscle levamisole receptors were not functional in this mutant. Likewise, *unc-38* mutants with cell-specific rescue in wild type *nic-1* background were largely resistant to acute treatment with levamisole, though both strains showed some sensitivity, with the muscle-specific rescued animals displaying more sensitivity than the neuron-specific rescued animals. In the *nic-1* mutant background, acute treatment with 0.5 mM levamisole showed that both the neuron-specific and muscle-specific *unc-38* rescued animals displayed hypersensitivity almost as high as that shown by *nic-1* single mutants. However, neither strain fully rescued the *nic-1* hypersensitivity to levamisole. One way to interpret this result would be to suggest that the *nic-1* mutation increased the activity of UNC-38 containing receptors in both neurons and muscle, and that UNC-38 receptors in both neurons and body muscle were necessary to fully restore *nic-1*'s effect. Therefore, in this theory, the wild type NIC-1 protein appears to function in both neurons and muscle to downregulate the activity of both neuronal and muscle nAChRs. A different way to

interpret this result would be to suggest that NIC-1 functions only in neurons to downregulate the activity of neuronal nicotinic receptors. For this possibility, we would say that in the case of neuron-specific UNC-38 rescue, the *nic-1* mutation was causing body muscle hypercontraction by increasing the neuronal UNC-38 receptor activity, and allowing for increased cholinergic neurotransmission onto the neuromuscular junction (NMJ). In the case of the muscle-specific UNC-38 rescue, *nic-1* may have been causing increased activity of other neuronal nicotinic receptors, or decreased activity of GABA receptors, which would also have the effect of increased excitatory neurotransmission onto overexpressed muscle UNC-38 receptors. This would also lead to body muscle hypercontraction.

nic-1 and nAChR abundance

We hypothesized that the increase in cholinergic neurotransmission in *nic-1* mutants might be due to higher abundance of nAChRs. To investigate this possibility, we generated a *unc-29(x29);nic-1(lj22)* transgenic strain that expressed wild type *unc-29(+)* under its own promoter tagged by a GFP reporter construct to measure the relative intensity of UNC-29::GFP fluorescence in the vulval muscles of wild type (*unc-29(x29);Ex[punc-29::unc-29(+)::GFP, rol-6d]*) and *nic-1* (*unc-29(x29);nic-1(lj22);Ex[punc-29::unc-29(+)::GFP, rol-6d]*). Comparison of UNC-29::GFP expression in the wild type and mutant showed no apparent differences in intensity; however, the signal expressed through this transgene was overall very weak. Therefore, we examined UNC-29::GFP expression more closely by using a different construct, *myo-3::unc-29::GFP*, which drove expression through a muscle-specific, *myo-3* promoter that gave a stronger signal. Comparison of relative GFP intensity of wild type and *nic-1* again showed no significant difference, and clearly no increase in fluorescence in the vulval muscles of *nic-1*. Furthermore, analysis of LEV-1::GFP expression in *nic-1* mutants also showed no apparent increase in receptor

abundance in body muscle compared to wild type. We further investigated *nic-1*'s role in nAChR abundance by examining the amount of α-bungarotoxin binding in *nic-1* compared to wild type

However, examination of an *unc-38::unc-38-3xMYC* expression in *nic-1* mutants showed an overabundance of UNC-38 receptor expression in the head neurons compared to that seen in wild type. This result supports a role for NIC-1 either acting primarily in neurons to downregulate the abundance of UNC-38 subunit containing nAChRs and regulating the release of acetylcholine (ACh) onto the neuromuscular junction (NMJ), or of NIC-1 having a role in both neurons and muscle but acting in a different mechanism for each type of cell.

nic-1 encodes a glycosyltransferase

In order to understand the role of NIC-1 in nAChR regulation more clearly, we cloned the *nic-1* gene. With the sequencing of the *C. elegans* genome complete and readily accessible, cloning of a *C. elegans* gene is a relatively straightforward procedure. First, *nic-1* was mapped to the X chromosome (LG X) by crossing hermaphrodites to wild type males and scoring the F_1 male cross progeny for the Unc and nicotine hypersensitive phenotypes. If *nic-1*(lj22), a recessive mutation, were located on one of the autosomes, all of the F_1 cross progeny would display the wild type phenotypes: non-Unc, non-nicotine hypersensitive. However, because the gene was located on LG X, and since *C. elegans* males inherit only one copy of the X chromosome, from their mother, all of the F_1 male cross progeny displayed the *nic-1* phenotypes. In contrast, the F_1 hermaphrodite cross progeny, having two copies of the X chromosome, one from their *nic-1* mother and the other from their wild type father, were all phenotypically wild type. The mutation was then mapped to a small 2-cosmid interval (10 ORFs, ~30kb) through PCR-mapping with sequence-tagged polymorphisms (stPs) and single nucleotide polymorphisms (SNP) as genetic markers.

Finally, *nic-1* was narrowed down to a single open reading frame (ORF) by double-stranded RNA interference (dsRNAi). Wild type animals treated with dsRNA spanning the sequence for T23F2.1 phenocopied the Unc and nicotine hypersensitive (for paralysis) characteristics of *nic-1* mutants. As a result, we found that *nic-1* encodes a 435 aa protein identified through sequence analysis as a putative glycosyltransferase with 25% homology to the yeast gene *alg2*. The yeast *alg2* gene encodes an α-1,3 and α-1,6 mannosyltransferase that transfers mannose from GDP-Mannose to $Man_1GlcNAc_2$-PP-Dol and $Man_2GlcNAc_2$-PP-Dol, forming $Man_3GlcNAc_2$-pp-Dol in the lumen of the endoplasmic reticulum (ER). This Alg2 protein catalyzes 2 steps of a step-wise assembly of a lipid (dolichol)-linked oligosaccharide that eventually gets transferred to a nascent protein in the ER on its way to becoming a glycoprotein. We found that the *nic-1* sequence contained a dolichol recognition domain (Val/Ile-x-Phe-x-x-Ile; x is any amino acid).

Other glycosyltransferases affect nicotinic receptor activity

We wished to obtain further evidence to substantiate the identity of NIC-1 as a mannosyltransferase that affects nAChR activity. One approach we took was to knock out the function of *C. elegans* homologs of other glycosyltransferases in the yeast *Alg2* pathway, then to see if the Unc and nicotine hypersensitive characteristics of *nic-1* mutants were phenocopied in these "knockout" mutants. Double stranded RNA interference (dsRNAi) was used as the method for removing the functions of *C. elegans* homologs for yeast glycosyltransferases *Alg2* through *Alg11*. Table 1 shows the results of a post-RNAi drug assay in which sensitivity to nicotine for body muscle paralysis was tested. Wild type animals were grown on bacteria that were transformed with a feeding vector (pL4440) containing *C. elegans* ORF sequences that would be transcribed into dsRNA, which then could be taken up by the animals through feeding. A control group was mock treated by being fed the same bacteria that was transformed with an empty feeding vector devoid of

any ORF sequence to be transcribed into RNA. This control group displayed no Unc movement and little sensitivity to 0.25% nicotine treatment (Table 1), while all of the experimental groups treated with dsRNA of *C. elegans* ORF sequences homologous to yeast glycosyltransferase genes (F09E5.2—*Alg2/11*; K09E4.2—*Alg3*; C08H9.3—*Alg6/8*; C08B11.8—*Alg6/8*; T27F7.3—*Alg9*; K12H6.3; T14B4.9; D2085.6) displayed nicotine hypersensitive behavior. In particular, the animals that underwent RNAi treatment for F09E5.2 (Alg2/11) and T27F7.3 (Alg9) displayed both Unc movement and apparent hypersensitivity comparable to that seen by the *nic-1* positive control group animals. They were also slow-growing, a phenotype also seen in *nic-1* mutants. From this experiment, we saw that knocking out each of the potential glycosyltransferase homologs tested resulted in Unc movement and nicotine hypersensitive behavior for the animals. These results gave supporting evidence that *nic-1* encodes a glycosyltransferase that acts in a similar pathway as that of the yeast Alg2p, and that this dolichol-linked oligosaccharide chain-building pathway is somehow involved in negatively regulating nAChR activity.

Mass spectrometry reveals differences in *nic-1* and wild type *N*-glycans

In order to determine if NIC-1 truly functions as a mannosyltransferase in the *N*-linked glycosylation pathway, we explored whether or not there was a fundamental difference in *N*-linked glycan content between wild type and *nic-1* animals. Using the method of Fast Atom Bombardment (FAB) Mass Spectrometry, we analyzed the differences in molecular weight distribution of *N*-glycans from wild type and *nic-1* extracts. Wild type and *nic-1* animals grown on HGM plates were collected and extracts of each sample were obtained following homogenization in CTAB. The extract was then treated with Peptide-N-glycosidase F (PNGase F) enzyme to release asparagine-linked (*N*-linked) sugar chains from glycoproteins and perdeuteromethylated to increase volatility and to label the non-reducing end of the sugars (the point of attachment to glycopeptide). The results

from these samples showed distinct differences between the spectra obtained from N2 and from *nic-1* (Figure 8). The N2 spectra, as expected, was dominated by small truncated structures $Hex_3HexNAc_2$-$Hex_4HexNAc_2$ (m/z 1222-1793) and structures with compositions consistent with high mannose type structures $Hex_5HexNAc_2$-$Hex_9HexNAc_2$ (m/z 1649-2502). Interestingly, we also saw a signal for Hex10HexNAc2 (m/z 2715). In comparison, the *nic-1* spectra showed that the levels of the truncated structures $Hex_3HexNAc_2$-$Hex_4HexNAc_2$ (m/z 1222-1793) appeared to be similar, as did the signal for the smallest high mannose type structure $Hex_5HexNAc_2$ (m/z 1649). However, the signals for the other high mannose structures, $Hex_6HexNAc_2$-$Hex_9HexNAc_2$ (m/z 1863-2502), were greatly reduced but still observable. Both structures also contained minor amounts of complex structures with levels that were similar in both spectra. Therefore, we observed from this data that *nic-1* worms had reduced levels of the high mannose structures $Hex_6HexNAc_2$-$Hex_9HexNAc_2$ compared to wild type, but interestingly had similar levels of truncated, complex and the high mannose glycan $Hex_5HexNAc_2$ as wild type. In parallel, a portion of each of the extract samples were also put through PNGase A digest, which releases *O*-linked glycans and PNGase F resistant *N*-linked glycans. FAB-MS of these samples revealed no significant differences between the N2 and *nic-1* spectras. These results together suggest that NIC-1 functions in the *N*-linked glycan pathway, more specifically in catalyzing one or more steps in the assembly of high-mannose type structures but not truncated or complex type structures.

DISCUSSION

The role of NIC-1 as a high-mannose specific glycosyltransferase

The most convincing evidence to support NIC-1's role as a glycosyltransferase was shown from the mass spectra (MS) of PNGase F-released *N*-glycans from wild type (N2) and *nic-1*. The molecular weight profiles from the spectra showed that while wild type and *nic-1* both contained similar amounts of the oligosaccharides $Man_3GlcNAc_2$-$Man_5GlcNAc_2$, *nic-1* displayed drastically decreased profiles of the high-mannose type *N*-glycans ($Man_6GlcNAc_2$-$Man_9GlcNAc_2$). This leads us to speculate that NIC-1 functions in the *N*-glycosylation pathway downstream of $Man_5GlcNAc_2$. Thus, NIC-1 is most likely an α-1,2 mannosyltransferase, as each subsequent mannose residue added onto the Man_5 through Man_9 high-mannose type *N*-glycan structures have been shown to be attached by α-1,2 linkages (Kornfeld and Kornfeld 1985; Varki et al.1999). Alternatively, NIC-1 may play a role in the "flipping" mechanism by which $Man_5GlcNAc_2$ switches orientation along the ER membrane, from the cytosolic side to the lumenal side of the ER. As $Man_6GlcNAc_2$-$Man_9GlcNAc_2$ are normally assembled on the lumenal side of the ER, an inability of the Man_5 oligosaccharide to reach its proper site may prevent the successful assembly of the high-mannose glycan.

NIC-1 and nicotinic acetylcholine receptors

The sequences of *C. elegans* nAChR subunit genes revealed potential sites for glycosylation: in each of the sequences for the genes *unc-29, unc-38, lev-1* and *unc-63*, there were sequences with the motif Asn-x-Ser/Thr , the consensus amino acid sequence for *N*-linked glycosylation sites (Fleming et al. 1997). nAChRs are well known to be glycosylated membrane proteins and glycosylation of these proteins has been shown to be important in certain cases for proper receptor function, degradation and turnover. Therefore, it is reasonable to initially suspect that NIC-1 might be directly affecting the functional activity of the nicotinic acetylcholine receptor by promoting glycosylation of the receptor proteins. If this were the case, however, and if glycosylation of the receptor proteins were important for proper functional activity, it does not seem likely that an enzyme that promotes glycosylation of the receptor would down regulate the activity of the receptor. On the other hand, if glycosylation were promoting degradation of the nAChR, thereby increasing the rate of receptor degradation and leading to decreased cholinergic neurotransmission due to lower number of receptors at the synapse, a mutation in *nic-1* would be unable to degrade receptors efficiently, thus allowing for an increased number of receptors at the synapse, and increased cholinergic response leading to nicotine hypersensitive behavior. This also does not seem likely, however, since *nic-1* did not appear to increase the levels of UNC-29::GFP and LEV-1::GFP fluorescence in vulval and body muscles. Furthermore, α-Bgt injected into wild type and *nic-1* showed no visible difference in the amount of toxin binding to nAChRs (Gottschalk and Schafer, unpublished). A more likely role for NIC-1 in nAChR regulation is that of an indirect affector. The results from the mass spectrometry as well as the dsRNAi of *C. elegans* homologs for other glycosyltransferases (*alg* genes) in the *N*-glycosylation pathway support the idea that NIC-1 functions as a mannosyltransferase in the assembly of the core glycan for *N*-glycosylation in the ER. The mass spectra readings suggest that NIC-1 is important for high-mannose-type glycan synthesis, specifically that of

$Man_6GlcNAc_2$-$Man_9GlcNAc_2$ and higher. It is possible that NIC-1 catalyzes the addition of mannose to of $Man_5GlcNAc_2$, thereby making it difficult to produce the higher mannose chains. Alternatively, NIC-1 may play a crucial role in the flipping of the of $Man_5GlcNAc_2$ oligosaccharide from the cytosolic to the lumenal side of the ER. In either case, *N*-glycosylation of proteins would be blocked or at least drastically reduced in the *nic-1* mutant, and any affector proteins that may interact with the nAChR or other ligand-gated ion channel receptors to down regulate their function would not be able to carry out their wild type functions.

METHODS

Assay conditions and growth media. Unless otherwise noted, nematodes were grown and assayed at room temperature on standard nematode growth medium (NGM) seeded with *E. coli* strain OP50 as a food source. Drug response assays in M9 salts were performed as described (Waggoner et. al. 2000). Egg-laying dose-response experiments were performed as described (Kim et. al. 2001). The egg-laying behavior of each animal was recorded for 4-12 hours as described using an automatic tracking system (Waggoner et. al. 1998). For other drug experiments, 5-hydroxytryptamine (creatinine sulfate complex, Sigma) was added to NGM agar at 7.5 mM, and levamisole (hydrochloride, Sigma) was added at 6.25 μM.

Mutant screen. L4 to young adult *lev-1(e211)IV* hermaphrodites were mutagenized by treating with methane sulfonic acid ethyl ester (EMS) (Sigma), which commonly causes point mutations (G/C-A/T transitions), washed, transferred to seeded NGM plates and allowed to propagate. F2 self progeny were transferred to 1% nicotine plates and scored for paralysis after 1 hour. Potential *nic* mutants were then isolated onto individual plates. Self-progeny were retested for nicotine induced paralysis and assayed for egg laying in levamisole as described above. *nic-1* mutants were crossed out of the *lev-1* background by mating hermaphrodites to N2 males, picking non-Unc F1 hermaphrodites to individual plates, and selecting Unc, nicotine hypersensitive F2. Those that bred true for both phenotypes were selected as *nic-1* and backcrossed an additional 1x to wild type.

Cloning and molecular biology. *nic-1* was first mapped to LG X by crossing *nic-1* hermaphrodites and scoring the F1 males for the Unc movement phenotype. The region containing the gene was further narrowed down using a PCR based sequence-tagged polymorphisms (stP) mapping strategy (Williams et. al. 1992). Single nucleotide polymorphisms (SNPs) were then used to narrow the region even further, then finally to a single open reading frame (ORF). dsRNAi was performed to confirm the result as

described. Soaking: T7-flanking sequences of forward and reverse primers were designed to cover all but one exon of T23F2.1. PCR was performed from genomic template using the Expand Kit (Roche). After amplification, in vitro transcription was performed using RiboMAX Large Scale RNA Production System-T7 (Promega). ssRNA was precipitated and resuspended in IM Buffer, then made into dsRNA by incubating at 68°C for 20 min then at 37°C for 30 minutes to anneal.

Mass Spectrometry. 1-2 grams of wild type (N2) and *nic-1* animals were grown at room temperature and collected from 30 large (15 cm) High Growth Medium (HGM) plates, rinsed 2x with 0.1 M NaCl. Worms were homogenized in CTAB (Sigma)/SDS extraction buffer in eppendorf tubes, then placed in 4°C on a rocker for an additional 24 hours. Sold debris was spun down at 13,000rpm for 10 minutes, and the supernatant was collected and placed under dialysis to remove detergent, using a 7000 MW membrane and several changes of 50 mM ammonium bicarbonate buffer, for 24 hours at 4°C. Dialyzed extract was then collected and vacuum dried using a vacuum centrifuge. Extract was resolubilized in buffer, treated with PNGase F to release asparagine (*N*)-linked glycans, perdeuteromethylated (1) to confer volatility of the sugars to obtain mass spectra readings and (2) to label the non-reducing end (the original site of attachment to the polypeptide/protein), and placed through the mass spectrometer. Half of each of the extract samples was saved and treated with PNGase A rather than PNGase F to release other non-PNGase-F released *N*-glycans and *O*-glycans, and put through the same process as described above.

Table 2-1: RNAi of *C. elegans* homologs of yeast glycosyltransferases in *N*-glycosylation.

Genotype of animals tested	n	dsRNAi of C. elegans ORF	Yeast glycosyl-transferase homolog of C. elegans ORF	Fraction of animals affected	Notable RNAi phenotypes
nic-1	74	pL4440 (empty vector)	--	1.00	Unc, Slow-growing
N2	30	pL4440	--	0.10	
		unc-22	--	0.48	Twitcher
		T23F2.1	Alg2	1.00	Unc, Slow-growing
	30	F09E5.2	Alg2/Alg11	0.93	Unc, Slow-growing
	37	K09E4.2	Alg3	0.83	
	37	C08H9.3	Alg6/Alg8	0.63	
	33	C08B11.8	Alg6/Alg8	0.75	
	42	T27F7.3	Alg9	1.00	Unc, Slow-growing
	36	K12H6.3		1.00	
	25	T14B4.9		0.63	
	21	D2085.6		0.81	

Figure 2-1 Screen for nicotine hypersensitive mutants

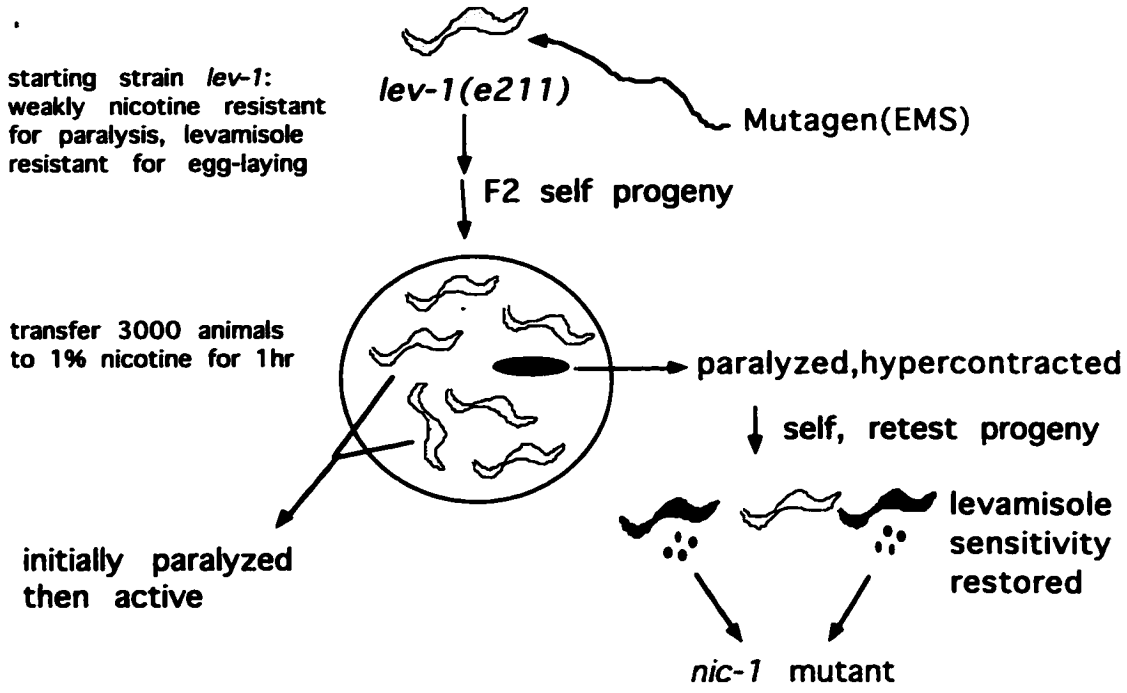


Figure 2-2 ***nic-1* hypersensitivity to nicotine and levamisole**

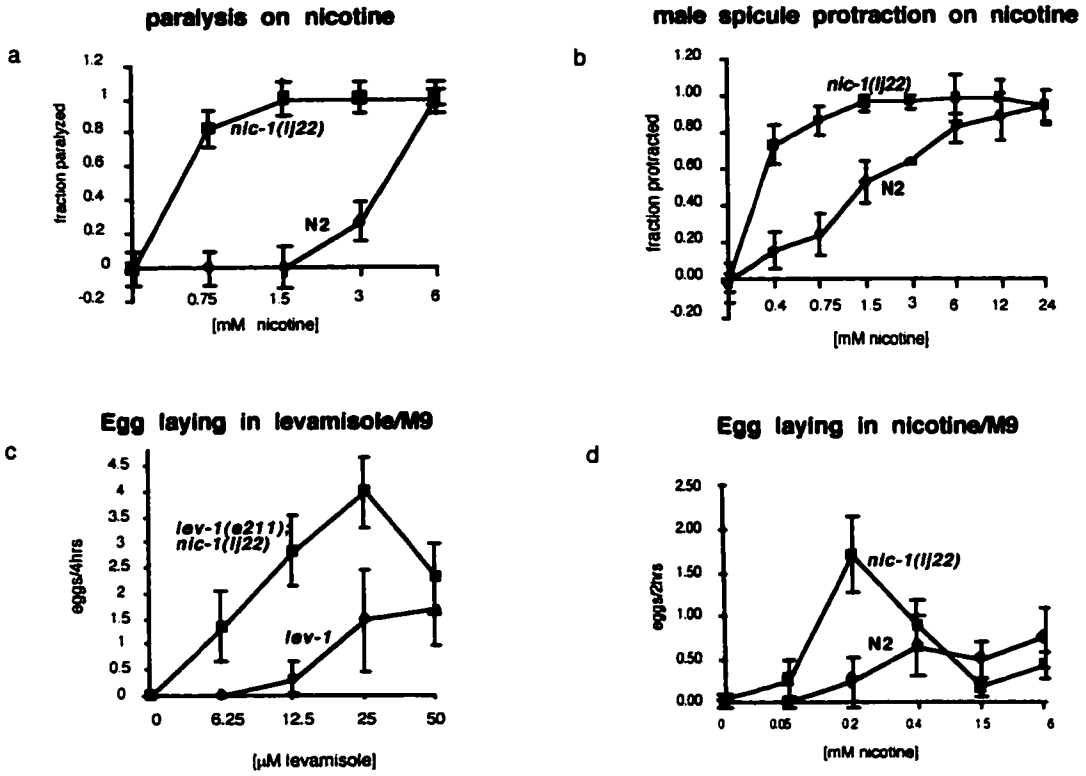


Figure 2-3

nic-1 displays increased egg-laying within the active phase

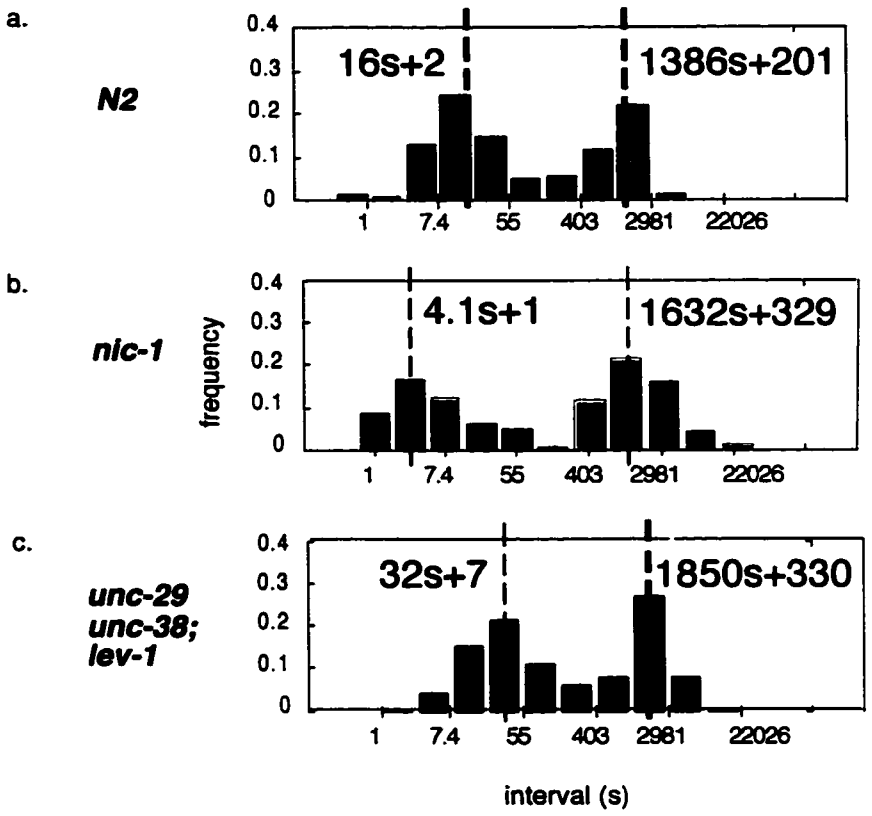


Figure 2-4

nic-1 sensitivity to other drugs

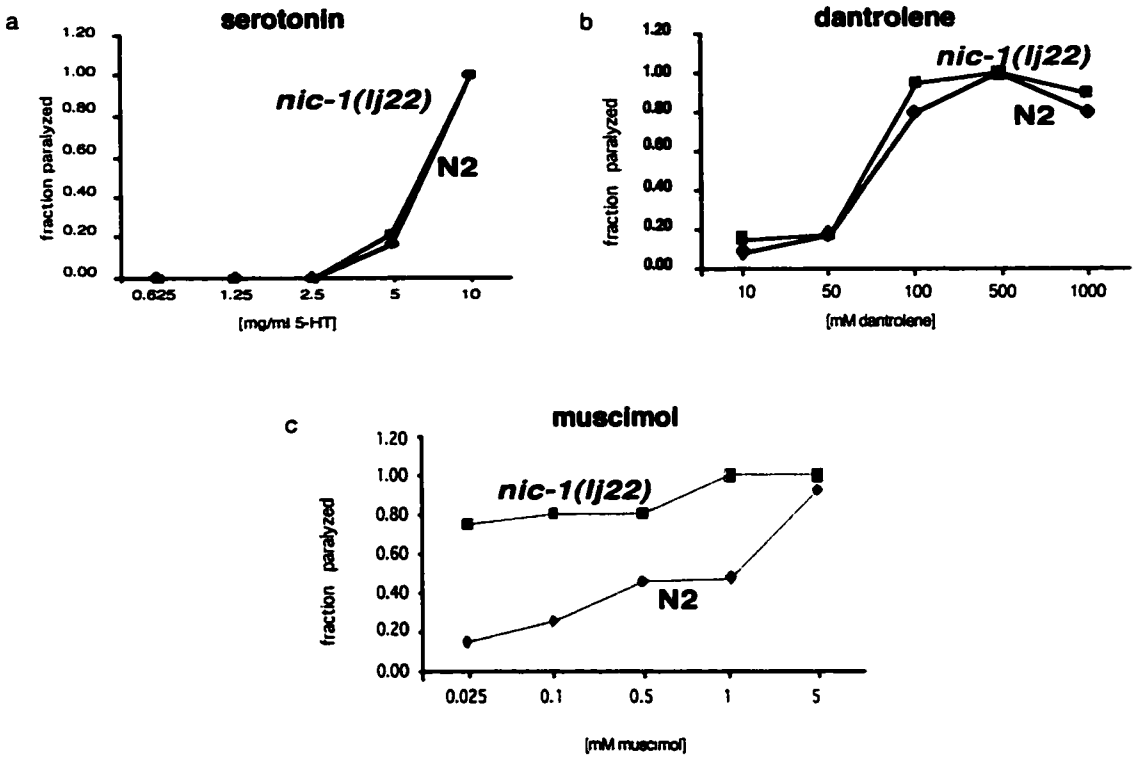


Figure 2-5

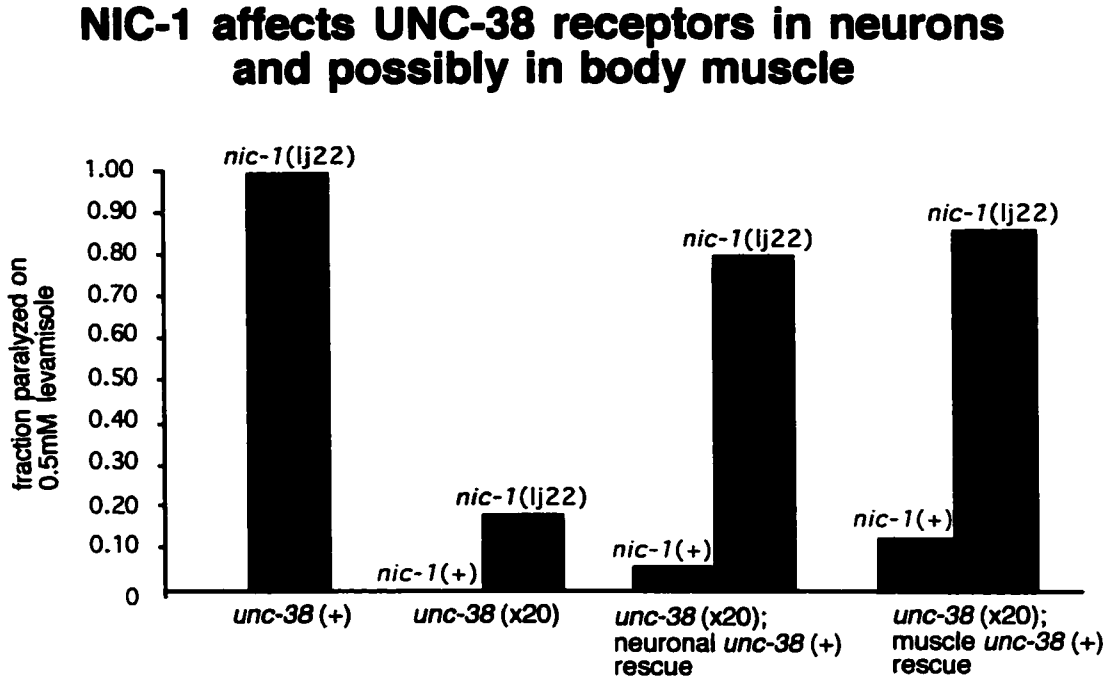
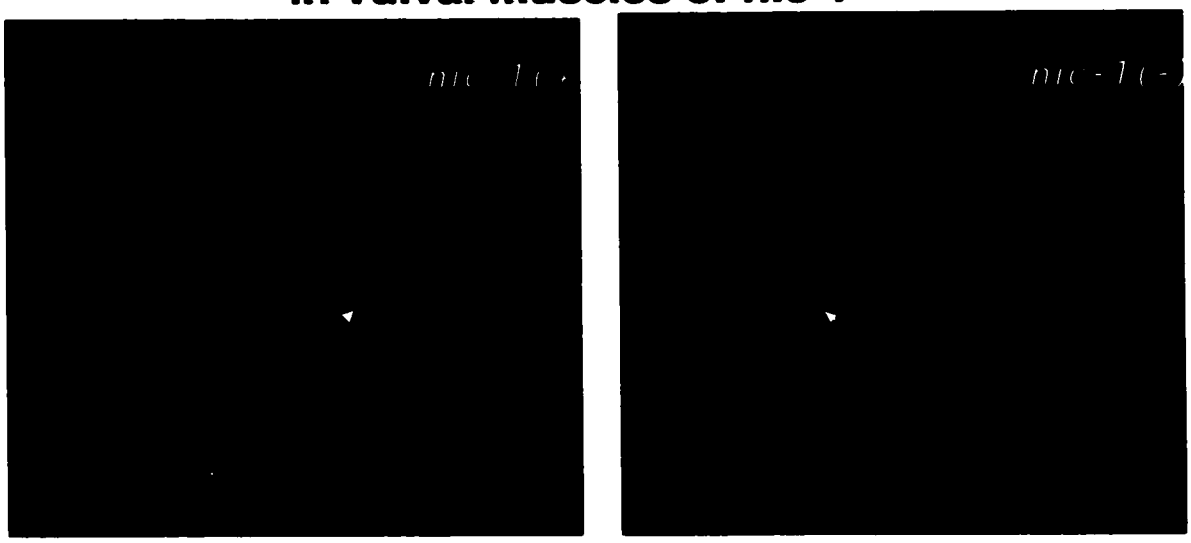

NIC-1 affects UNC-38 receptors in neurons and possibly in body muscle
fraction paralyzed on 0.5mM levamisole
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
nic-1(lj22)
nic-1(+)
nic-1(lj22)
nic-1(+)
nic-1(lj22)
nic-1(+)
nic-1(lj22)
unc-38 (+)
unc-38 (x20)
unc-38 (x20); neuronal unc-38 (+) rescue
unc-38 (x20); muscle unc-38 (+) rescue

Figure 2-6

No increase in UNC-29 receptor protein abundance in vulval muscles of *nic-1*



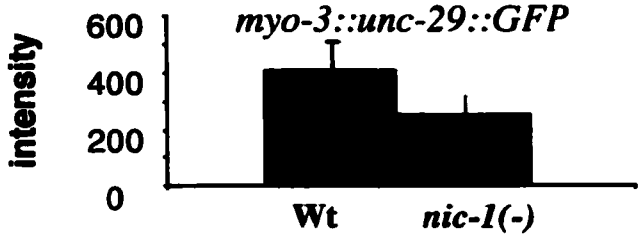
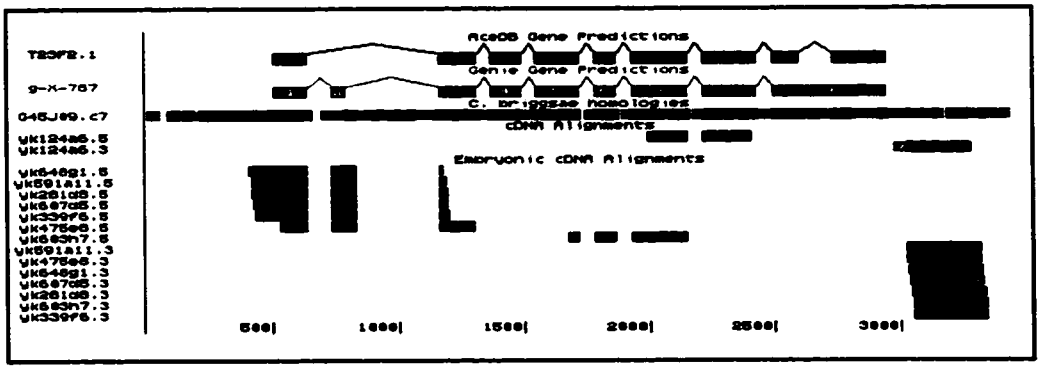


Figure 2-7

nic-1 encodes a glycosyltransferase



437aa protein:

25% homology to Yeast Alg2p

```
T23F2.1          MHVVIVEPEQWHGGSDRCTVALIRHPVSQGHRVTWLFTMIDEYWKHTPDGVEIR   55
YEAST ALG2P   MTSKSLNVAFIHPDLGIGGAERLVVDAAVGIQKHGHQVIFYTSHHDP---HHCPE--ETR   55

T23F2.1          EVGLKLHP-GDWWSQHVALGNHHVFSHLNP----------------DVAIIDHSASCVPMI   99
YEAST ALG2P   DGTLKVQVRGDWLPRTIPGRFYILCAILRQFVLVASLILWERHSYDIFFVDQLSACVPLL  115

T23F2.1          KWRFPQCKILFYCHFPQQLVTPSRFFLYRHYAKLIGIVKKKLFGHIDQIFVNSNFTATQF  159
YEAST ALG2P   KW-FTTAKILFYCHFPDKLLTQRNSTIKKLIRAPVDKMKKKLTTGMSDLIAVNSGFTAGMF  174

T23F2.1          CKVMPNIEKNKVRVVYPPCDIDWIVSASERPVSR-----AQRAKNETYTFLSMNRFWPRKR  215
YEAST ALG2P   KKSFPSVHQTP-QILIPPINFD-----AYDRPVDRNDPTVKILETDKRVLLSINRFERKKN  229

T23F2.1          LDIIIEAASILKQKGY--------HFHVQLAGSVMPHIPESRIYYEVLQRM-TKKLNVTDN  267
YEAST ALG2P   VKLALRAFAALKIKNNVPKDVFANYRLVLAGGYDKRVRENVEYLKELDQLATEKFGLQTP  289

T23F2.1          ----------------VTFIPSPTDKVKPQLYQQCDTALITPPNEHFGIVPIEALDQRRP  311
YEAST ALG2P   TIHPSSAAADVPADAQVVFLCSFNDAQRTFLLDQAKLLLYTPSNEHFGITPVEGMYASVP  349

T23F2.1          VIVCDSGGPAETVLEDITGTKIAKPCGKLLAEAMLHHNNKRDMPELDTDEGYAKQRHRLE  371
YEAST ALG2P   VIAVNTGGPVETVKNKETGLLLPSD-PDVWAEGIRDFIIEKYNGKQNGQHG-----RQHVQ  404

T23F2.1          TEPSTRGPCGNIDRAIAEMGTLEISSSEPTTTPLVDTI--VHQPTATEVYSKPQQYNKA  429
YEAST ALG2P   SKFSLPAFADRLE-----AMGIELETSTPDQSSSGAVYLLGAIGVLFACIIYCIKQ       455

T23F2.1          AHSRRAQA
```

Figure 2-8

Role of Alg2p in N-linked glycosylation

$$\left[\text{Man}_{1/2}\text{GlcNAc}_2\text{-PP-Dol} \xrightarrow{\text{Alg2p}} \text{Man}_3\text{GlcNAc}_2\text{-PP-Dol}\right]$$

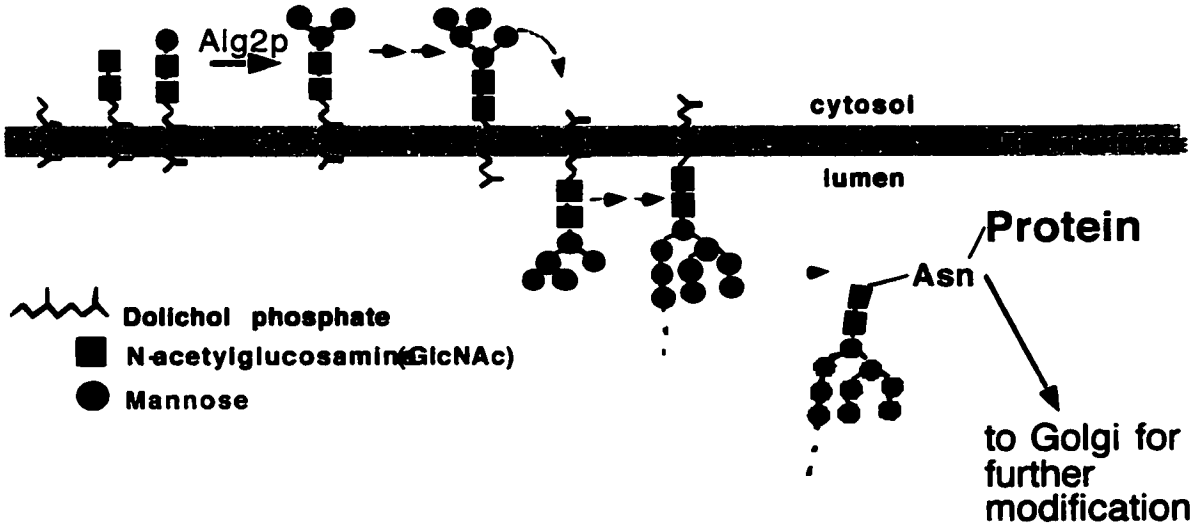


Fig 2-9a

Partial FAB-MS of perdeuteromethylated PNGase-F released *N*-glycans from *C. elegans* extracts

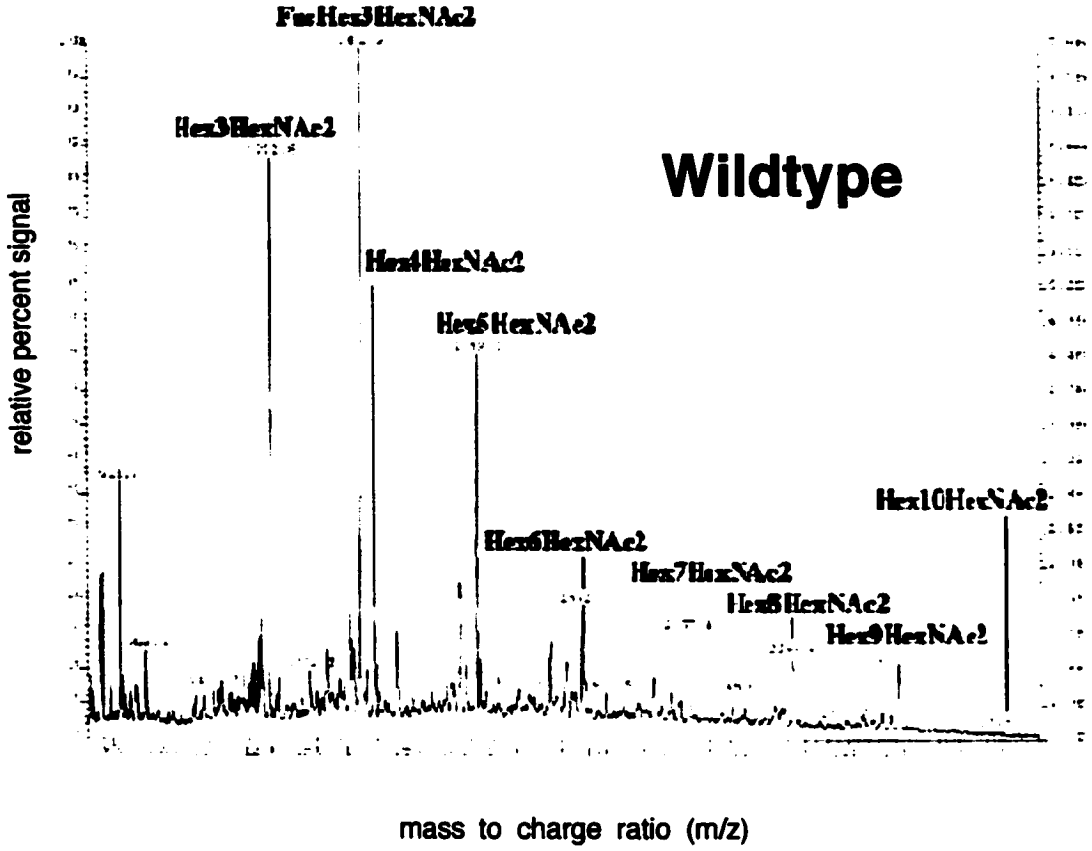


Figure 2-9b

Partial FAB-MS of perdeuteromethylated PNGase-F released *N*-glycans from *C. elegans* extracts

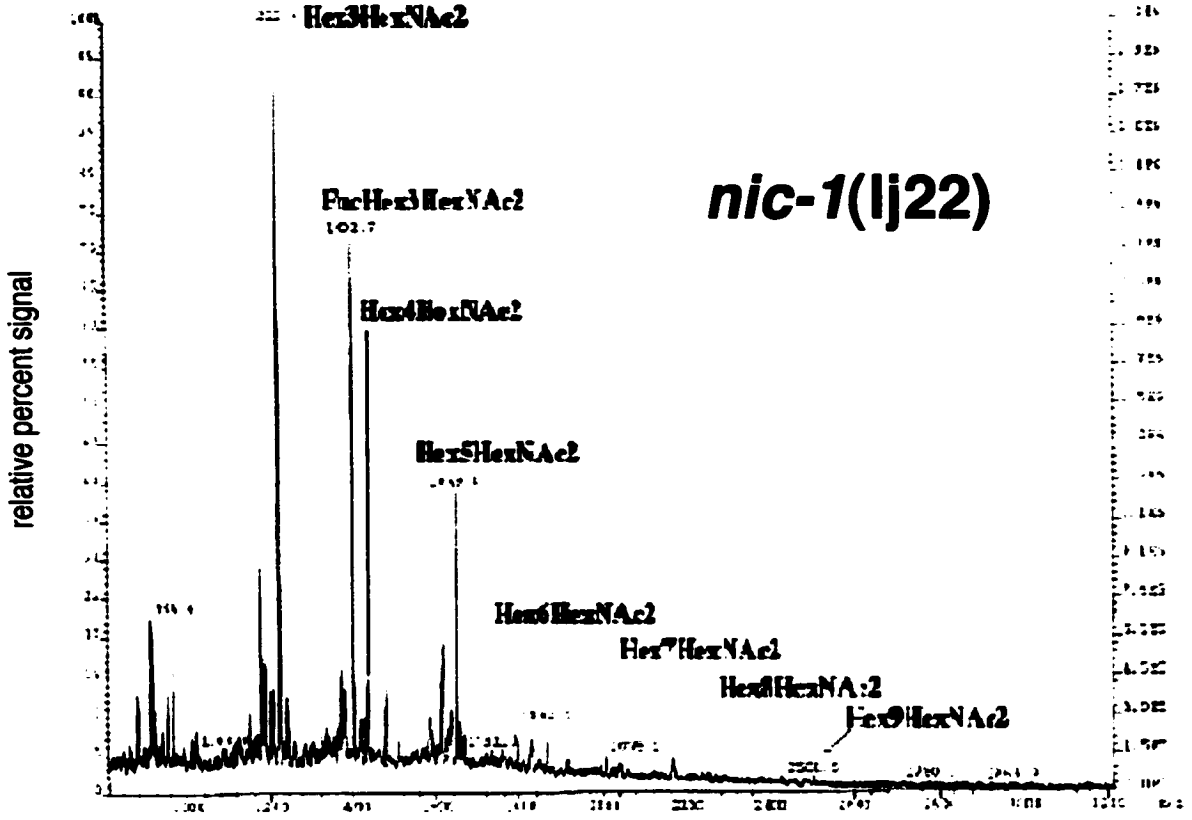


Figure 2-10

Homologs of other Alg2-pathway glycosyltransferases are also involved in the NIC-1 pathway

dsRNA sample		fraction paralyzed on nicotine	phenotypes
nic-1 control (*nic-1*, pL4440)		1.00	Unc, slow-growing
Negative control (N2, pL4440)		0.10	
Positive control (N2, *unc-22*)		0.48	Twitcher
F09E5.2	ALG2/11	0.93	Unc, slow-growing
K09E4.2	ALG3	0.83	
C08B11.8	ALG6/8	0.75	
C08H9.3	ALG6/8	0.63	
T27F7.3	ALG9	1.00	Unc, slow-growing
D2085.6		1.00	
T14B4.9		0.63	
K12H6.3		1.00	

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