

Caenorhabditis elegans Levamisole Resistance Genes *lev-1*, *unc-29*, and *unc-38* Encode Functional Nicotinic Acetylcholine Receptor Subunits

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We show that three of the eleven genes of the nematode *Caenorhabditis elegans* that mediate resistance to the nematocide levamisole and to other cholinergic agonists encode nicotinic acetylcholine receptor (nAChR) subunits. *unc-38* encodes an α subunit while *lev-1* and *unc-29* encode non- α subunits. The nematode nAChR subunits show conservation of many mammalian nAChR sequence features, implying an ancient evolutionary origin of nAChR proteins. Expression in *Xenopus* oocytes of combinations of these subunits that include the *unc-38* α subunit results in levamisole-induced currents that are suppressed by the nAChR antagonists mecamylamine, neosurrogatoxin, and *d*-tubocurarine but not α -bungarotoxin. The mutant phenotypes reveal that *unc-38* and *unc-29* subunits are necessary for nAChR function, whereas the *lev-1* subunit is

not. An UNC-29–GFP fusion shows that UNC-29 is expressed in body and head muscles. Two dominant mutations of *lev-1* result in a single amino acid substitution or addition in or near transmembrane domain 2, a region important to ion channel conductance and desensitization. The identification of viable nAChR mutants in *C. elegans* provides an advantageous system in which receptor expression and synaptic targeting can be manipulated and studied *in vivo*.

Key words: acetylcholine receptor; levamisole resistance genes; receptor mutations; *Caenorhabditis elegans*; evolution; nematode, *unc-29*; *unc-38*; *lev-1*; transmembrane domain mutation; *Xenopus* oocyte expression; GFP; confocal microscopy; receptor localization

Nicotinic acetylcholine receptors (nAChRs) are the most thoroughly characterized receptors in the family of ligand-gated ion

channels (Karlin, 1993; Unwin, 1993a,b, 1995). The vertebrate muscle nAChR is a pentamer of four subunit types in the stoichiometry $\alpha_2, \beta, \gamma,$ and δ . Each subunit contains four hydrophobic, putative transmembrane regions (TM1–TM4), with TM2 in each subunit contributing to the lining of the channel (Devillers-Thiery et al., 1993). A main part of the binding sites for ACh has been localized to each of the two α subunits, close to a pair of vicinal cysteines (positions 192 and 193 in the *Torpedo* α subunit) that define α subunits in all nAChRs (Kao and Karlin, 1986). Residues of the γ and δ subunits also contribute to ACh binding (Karlin, 1993). The subunits of vertebrate neuronal nAChRs are encoded by a separate set of genes and fall into two major classes, either α or non- α , each with several isoforms (α_2 – α_9 and β_2 – β_5) (Sargent, 1993; Ortells and Lunt, 1995).

Beyond cloning the subunits of nAChRs, considerable effort has been expended both *in vitro* and *in vivo* toward understanding the precise contribution of each subunit and the identities and functions of proteins that interact with the nAChR (Gautam et al., 1995; Karlin and Akabas, 1995; Gautam et al., 1996). To aid in this endeavor, *Caenorhabditis elegans* offers a genetic facility for defining the molecules associated with nAChR function that would be difficult to achieve in more complex animals.

Nematodes possess nAChRs (Johnson and Stretton, 1980; Fleming et al., 1993; Ajuh and Egwang, 1994; Squire et al., 1995; Treinin and Chalfie, 1995; Ballivet et al., 1996; Wiley et al., 1996). Although >20 nAChR sequences have been uncovered in the *C. elegans* genome sequencing project, our attention has been fo-

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The GenBank accession numbers for the *lev-1*, *unc-29*, and *unc-38* sequences reported in this paper are X98601, U81144, and X98599, respectively.

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Table 1. Major levamisole resistance loci of *Caenorhabditis elegans*

Levamisole resistance phenotype	Gene	Phenotypes ^a	Number of alleles	[³ H]MAL binding ^b	Gene product
Extremely resistant <i>unc</i>	<i>lev-1</i>	s + w	2 + 13	Variable	Non- α -nAChR subunit ^c
	<i>unc-29</i>	s + w	74 + 2	–	Non- α -nAChR subunit ^c
	<i>unc-38</i>	s + w	44 + 3	+/-	α -nAChR subunit ^c
	<i>unc-63</i>	s + w	58 + 1	+/-	Unknown
	<i>unc-74</i>	s + w	27 + 1	–	Unknown
	<i>unc-50</i>	s	5	–	Not an nAChR subunit ^d
Partially resistant pseudo-wild type	<i>lev-8</i>	w	1	+	Unknown
	<i>lev-9</i>	w	3	+	Unknown
	<i>lev-10</i>	w	1	+	Unknown
Twitcher	<i>unc-22</i>	w	16	+	Muscle-specific ^e
	<i>lev-11</i>	w	1	+	Muscle-specific ^f

^aPhenotypes: s, strong; w, weak. Only a limited effort was made to isolate and characterize weak, partially levamisole-resistant alleles. The number of weak alleles isolated thus mainly reflects their relative ease of isolation. This table summarizes discussion of previously reported work described in the text (Lewis et al., 1980a, 1987b; work cited in other footnotes).

^bThe [³H]MAL binding characteristic of the most levamisole-resistant alleles of each gene is indicated. +, Substantial specific [³H]MAL binding; –, little or no specific [³H]MAL binding.

^cThis work.

^dM. O. Hengartner, N. Tsung, J. A. Lewis, and H. R. Horvitz, unpublished data.

^eMoerman et al. (1988).

^fWilliams and Waterston (1994).

cused on the levamisole receptor. Levamisole is a more potent agonist than acetylcholine at nematode muscle nAChRs (Lewis et al., 1980b; Harrow and Gratton, 1985; Martin et al., 1991; Robertson and Martin, 1993). Mutants resistant to levamisole define 11 genes (Brenner, 1974; Lewis et al., 1980a). These mutants fall into three classes: *uncs*, *pseudo-wild types*, and *twitchers* (Table 1). Mutants in six genes with the *unc* phenotype exhibit extreme levamisole resistance, uncoordinated motor behavior, and resistance to other cholinergic agonists. Weakly resistant mutants (*pseudo-wild types*) move normally but have severalfold higher resistance to levamisole and other cholinergic agonists. All genes mutable to extreme resistance (except *unc-50*) also produce weaker mutant alleles having a partially resistant phenotype (Table 1). *lev-1* is the only locus for which the predominant mutant phenotype is that of partial resistance but for which two rare *unc* extreme resistance alleles, *x21* and *x61*, have also been found. These two alleles are the only extreme levamisole resistance mutations that show any dominance.

Using [³H]meta-aminolevamisole (MAL), nAChR binding has been detected in nematode extracts (Lewis et al., 1987a,b). Mutants of the six loci that give rise to strong resistance have deficient or altered receptor binding. Such receptor mutants are only moderately impaired in motor behavior as adults but are severely incapacitated at early larval stages.

Here we show that three genes associated with levamisole resistance encode nAChR subunits, and that α and non- α subunit combinations of these genes generate functional nAChRs when co-expressed in *Xenopus* oocytes. The eight other levamisole resistance genes are candidates for components of nAChR-mediated synaptic signaling. The ability to exploit *C. elegans* genetics offers the prospect of identifying additional novel molecular components of nicotinic cholinergic synapses.

MATERIALS AND METHODS

Nematode strains. The wild-type *C. elegans* used was Bristol strain N2 (Brenner, 1974). The Bergerac BO transposon mutator strain (Moerman and Waterston, 1984) was obtained from the *Caenorhabditis* Genetics Center, and the TR679 mutator strain was kindly provided by P. Anderson (University of Wisconsin) (Collins et al., 1987)

Mutant isolation. Mutants containing restriction fragment polymorphisms were obtained in the following ways. For the isolation of spontaneous transposon-induced mutations, 30 (BO strain) or 40 (TR679 strain) adult hermaphrodites were placed on a 100 mm diameter Petri dish spread with bacteria. After 4–5 d at 20°C, progeny worms were washed off the plate and placed to one side of a separate plate containing 1 mM levamisole, as described previously (Lewis et al., 1980a). Drug plates were then screened at daily intervals for extreme resistance to levamisole. To isolate γ -ray-induced mutants, worms were first irradiated for 6 min with 1500 rads from a ⁶⁰Co source, and 20 mutagenized adults were transferred to each Petri plate. Mutants were tested for genetic identity as described previously (Lewis et al., 1980a).

Identification of restriction fragment length polymorphisms in mutants. The mutator strains used contain high copy numbers of the transposon Tc1. Genomic probing with Tc1 DNA was used to identify a novel candidate Tc1 element generating a levamisole resistance mutation. Extraneous background Tc1 elements were eliminated by balancing the mutation against a Bristol chromosome containing left- and right-flanking markers and then back-crossing the mutation 12 times into a strain homozygous for the same genetic markers. The candidate mutation was then recombined with the left- and right-flanking markers, and, if it proved necessary to achieve adequate viability, the mutation was separated by further recombination from one or both flanking markers before isolating a strain homozygous for the back-crossed mutation. Control constructs homozygous for approximately the same high-copy Tc1 chromosomal region, but otherwise having a Bristol low-copy Tc1 genetic background, were generated by back-crossing the wild-type allele present in the parent mutator strain into a Bristol strain having an ethylmethane sulfonate (EMS)-induced mutation in the gene of interest, usually flanked by the same left and right genetic markers used in constructing the mutants. For producing *unc-38* mutant constructs, *unc-57(e406)* and *dpy-5(e61)* marker mutations (0.5 map units to either side of *unc-38*) were used. For control constructs, *unc-11(e47)* was used instead of *unc-57*. In the case of *unc-29* constructs, *unc-13(e450)* and *lin-11(n389)*, 1.1 and 1.6 map units to either side of *unc-29*, were used. For *lev-1*, *unc-30(e191)* and *dpy-4(e1166)*, 0.3 and 4.5 map units to either side of *lev-1*, were used.

lev-1 mapping. Several mistakes in the chromosomal placement of *lev-1* were discovered by T.M.B. The needed corrections in its map position were the placement of *lev-1* close to *unc-30*, the inversion of the *unc-26*, *lev-1* gene order, and the finding that the deficiency *sDf23* complements *lev-1(x38)* and therefore does not delete the gene. The correct position of *lev-1* on the genetic map was determined as follows. First, the two-factor distance between *lev-1* and *dpy-4* was measured. From a strain that was *lev-1(x21)tra-3(e1767)dpy-4(e1166)/+++*, 74 Lev non-Dpy and 61 Dpy non-Lev recombinants were found among the 3014 progeny constituting 10 complete broods, giving a distance of 4.5 map units. For deficiency

mapping, the recessive allele *x38* was used. *x38/+* males were mated into *Df/nT1* strains, and outcross males were scored. The deficiencies *sDf21* and *nDf27* failed to complement *x38*, whereas *sDf22*, *sDf23*, and *sDf60* did complement *x38*. For three-factor mapping, Unc non-Dpy and Dpy non-Unc recombinants were picked from a strain that was *dpy-20(e1282) + unc-26(e205)/+ lev-1(x21) +*. Twenty of 22 Dpy recombinants and 2 of 21 Unc recombinants contained *x21*. In a further three-factor mapping experiment, Unc non-Dpy and Dpy non-Unc recombinants were selected from a strain that was *unc-30(e191) + dpy-4(e1166)/+ lev-1 +*, using mutator- or γ -ray-generated *lev-1* alleles (*x505*, *x508*, *x548*, and *x566*) isolated in this study (see above). The cumulative data were that 4 of 64 Unc and 24 of 27 Dpy recombinants contained the *lev-1* marker. In summary these data give a gene order of *unc-30-lev-1-unc-26*. *unc-26* is genetically inseparable from the Bergerac polymorphism *nP33* both from the left (Yuan et al., 1993) and to the right (T. M. Barnes and J. Hodgkin, unpublished data). Thus *lev-1* was inferred to lie physically between the *unc-30*-rescuing cosmid (Jin et al., 1994) and the *nP33*-detecting cosmid, a distance of about 120 kb.

Recombinant DNA techniques. Standard recombinant DNA techniques were used (Sambrook et al., 1989). The Tc1 transposable element used as a genomic probe was an *EcoRV* fragment prepared by D. Bird (North Carolina State University) from an isolate supplied by S. Emmons (Albert Einstein College of Medicine). The flanking genomic DNA associated with a novel Tc1-induced mutation was separated from the Tc1 DNA in any genomic subclone by excision with *EcoRV* and purification by gel electrophoresis. Most hybridizations and washes performed in the course of screening *C. elegans* genomic libraries were performed under high stringency conditions (washing at 65°C with 0.2 \times SSC).

Reduced stringency conditions were used for screening with an 800 bp *ard* probe (from position 500 to position 1350) (Hermans-Borgmeyer et al., 1986). The probe was labeled to a specific radioactivity of about 1 \times 10⁹ cpm/ μ g by random priming. Suitable hybridization conditions were chosen by probing a Southern blot of *C. elegans* genomic DNA with the *ard* cDNA. Hybridization was performed at 30°C in 43% (w/v) formamide and 5 \times SSC for 48 hr. Filters were washed for 30 min each, twice at room temperature, twice at 37°C in 2 \times SSC and 0.1% SDS, and then for at least 45 min at 45°C in 1 \times SSC and 0.1% SDS before autoradiography with intensification at -70°C. Fifty thousand phage from a *C. elegans* genomic library in λ 2001 (approximately eight genome equivalents) were screened.

Phage clones were fingerprinted by A. Coulson [Medical Research Council (MRC), Cambridge, UK] (Coulson et al., 1986). DNA sequencing followed the method of McCombie et al. (1991). Cosmids spanning the *unc-38* and *unc-29* loci were provided by A. Coulson. Cosmid DNA was microinjected after removal of RNA by LiCl precipitation and purification by repeated ethanol precipitation. For reverse transcription (RT)-PCR, except as noted below, poly(A⁺) RNA (Jacobson, 1987) was prepared from total nematode RNA or was obtained as a gift from D. Zarkower (MRC); 5'- or 3'-anchored PCR was performed using standard procedures (Frohman et al., 1988).

lev-1 cDNA. A full-length cDNA of *lev-1* was obtained as follows. An oligonucleotide complementary to bp 2208–2237 of the genomic sequence was used to isolate a partial cDNA clone from a mixed stage *C. elegans* library (provided by I. Maruyama, MRC). The cDNA contained 232 bp of sequence upstream of the predicted start of translation but lacked the predicted 3' end. The 3'-anchored PCR (Frohman et al., 1988) using an internal primer (position 3181–3200) upstream of the *EcoRI* site at 3306 was used to generate a fragment containing the 3' end of the message. The available partial cDNA clone contained what appeared to be an unspliced 48 bp intron, but because there was no termination codon in this intron and the reading frame was maintained, it was not clear whether this sequence was present in the mature mRNA. Therefore we analyzed the splicing pattern across this region using RT-PCR. The data (not shown) were consistent with the quantitative removal of this intron. Therefore, a partial cDNA lacking the 48 bp intron was cleaved with *EcoRI* (which cleaves in the 3' primer as well), and the resulting fragment was cloned into the *EcoRI* site of the original cDNA, thus regenerating a complete cDNA.

Cloning unc-29. The mutator-induced *unc-29* mutation *x513* was backcrossed into the wild-type strain and recombined with the closely linked left and right flanking markers *unc-13(e450)* and *lin-11(n389)* to reduce the background of unrelated Tc1 elements. Hybridization of a Tc1 probe to *EcoRI*-digested DNA from the back-crossed strain showed a novel 3.7 kb *EcoRI* fragment not present in the wild-type strain. The Tc1 element was not separable from the *x513* mutation in 12 recombination events with either the *unc-13* or the *lin-11* flanking markers. The *x513* Tc1 element was cloned, and the genomic DNA flanking the insert was used

as a probe to identify nine wild-type genomic clones in an EMBL4 phage library (supplied by C. Link and W. Wood, University of Colorado). The physical map position of these phage was the same as that of the putative nAChR homolog JF#WA33 isolated by cross-hybridization with *ard* and was consistent with the genetic map location of *unc-29* on chromosome I (Brenner, 1974; Lewis et al., 1980a) (Fig. 1B). Southern blots of a γ -ray-induced mutant and five additional mutator-induced mutants all revealed chromosomal rearrangements when DNA from one of the EMBL4 phage (ZZ#1) was used as a probe (data not shown). Of the putative transposon-induced *unc-29* mutants, four had insertions in either a 1.4 or 5.0 kb *EcoRI* fragment. These *EcoRI* fragments lie immediately downstream from the 2.0 kb *EcoRI* fragment in which both *x513* and another insertion occurred (Fig. 1B). The insertions in two of these six mutants were lost in spontaneously occurring revertants to the wild-type phenotype, further indicating association of the restriction fragment length polymorphisms (RFLPs) with the *unc-29* gene.

A 6.0 kb *HindIII* fragment contained within the 8.7 kb of DNA spanned by the three neighboring *EcoRI* fragments associated with the *unc-29* mutation was sequenced (GenBank accession number U81144). The subcloned 1.4 kb *EcoRI* fragment (Fig. 1B) was used as a probe to isolate a full-length cDNA. The cDNA had an SL1 *trans*-spliced leader sequence at its 5' end, as commonly found on many nematode mRNAs (Krause and Hirsh, 1987; Zorio et al., 1994), eight nucleotides upstream of the ATG start.

Cloning unc-38. A Southern blot containing *HindIII*-digested genomic DNA from three back-crossed *unc-38* mutator-induced mutants, five nonmutant back-crossed control strains, and the Bristol wild-type strain was probed with Tc1. All three *unc-38* mutants contained a 4.8 kb *HindIII* band not present in the wild-type strain and all nonmutant controls. The 4.8 kb fragment from *unc-38(x525)* was subcloned, and the genomic DNA flanking the Tc1 site was used as a probe on Southern blots of five mutator-induced and five γ -ray-induced *unc-38* mutants. Four of the mutator-induced (including the Tc1 mutants probed above) and two of the γ -ray mutants showed a size distinct from that seen in the wild-type strain (3.2 kb). The subcloned DNA flanking the *x525* Tc1 element was used as a hybridization probe to identify corresponding λ phage from a wild-type EMBL4 genomic library. The physical map position of the phage was consistent with the *unc-38* genetic map position on chromosome I (Brenner, 1974; Lewis et al., 1980a) (Fig. 1C). The sequence of the 3' end of the *unc-38* gene, which was missing from the 3.2 kb *HindIII* fragment, was obtained directly from cosmid B0241 (Fig. 1C) by linear amplification sequencing (Craxton, 1991) using oligonucleotide primers derived from the cDNA sequence that was obtained.

The *HindIII* fragment was used to screen two *C. elegans* cDNA libraries (provided by S. Kim, Stanford University; and R. Barstead, Oklahoma Medical Research Foundation). Three cDNAs were isolated from 240,000 clones screened. One cDNA was sequenced and appeared to have the complete 3' end, because it contained a polyA tail 65 bases downstream of the predicted stop codon. Based on the genomic sequence, the 5' coding region was not present on any of the cDNA clones. To recover the missing 5' end, a forward primer was made corresponding to the 5' untranslated leader predicted from the genomic sequence (nucleotides 273–309, but containing an *XbaI* site: ATTCTCTCTA-GAACACTTCTTTCAAGGCTTTTCATA). This forward primer and a reverse primer (nucleotides 854–882) corresponding to the known partial cDNA sequence were used to amplify total first strand cDNA from a mixed stage population of *C. elegans*. The resulting PCR product was cleaved with *XbaI* and *BamHI* (unique *BamHI* site at position 837) and ligated to the partial, but mature, cDNA that itself was cleaved with *BamHI*. The *XbaI* site was ligated to the vector polylinker cleaved with *XbaI*. The resulting hybrid PCR cDNA sequence corresponded to the predicted mature full-length cDNA sequence.

To search for null mutations of *unc-38* and *unc-29*, cDNA clones were generated by RT-PCR using total RNA prepared according to the method of Chomczynski and Sacchi (1987) with Trizol (Life Technologies, Gaithersburg, MD) phenol-guanidine isothiocyanate reagent. The RNA (20 μ g) was treated with 10 U of RQ1 RNase-free DNase and 56 U of RNasin (both from Promega, Madison, WI) in 85 μ l of buffer for 20 min at 37°C. After phenol/CHCl₃ extraction and ethanol precipitation, 1 μ g of the resuspended RNA was used in RT-PCR according to the protocols of the Perkin-Elmer (Norwalk, CT) GeneAmp RNA PCR kit in a Perkin-Elmer Thermocycler 480, using thick-walled GeneAmp tubes. RT-PCR reactions, after a hot start, were conducted for 45 cycles of 1 min denaturation at 94°, 1 min annealing at 59°, and 2 min extension at 72°C with a final extension of 5 min. To produce cDNA products from

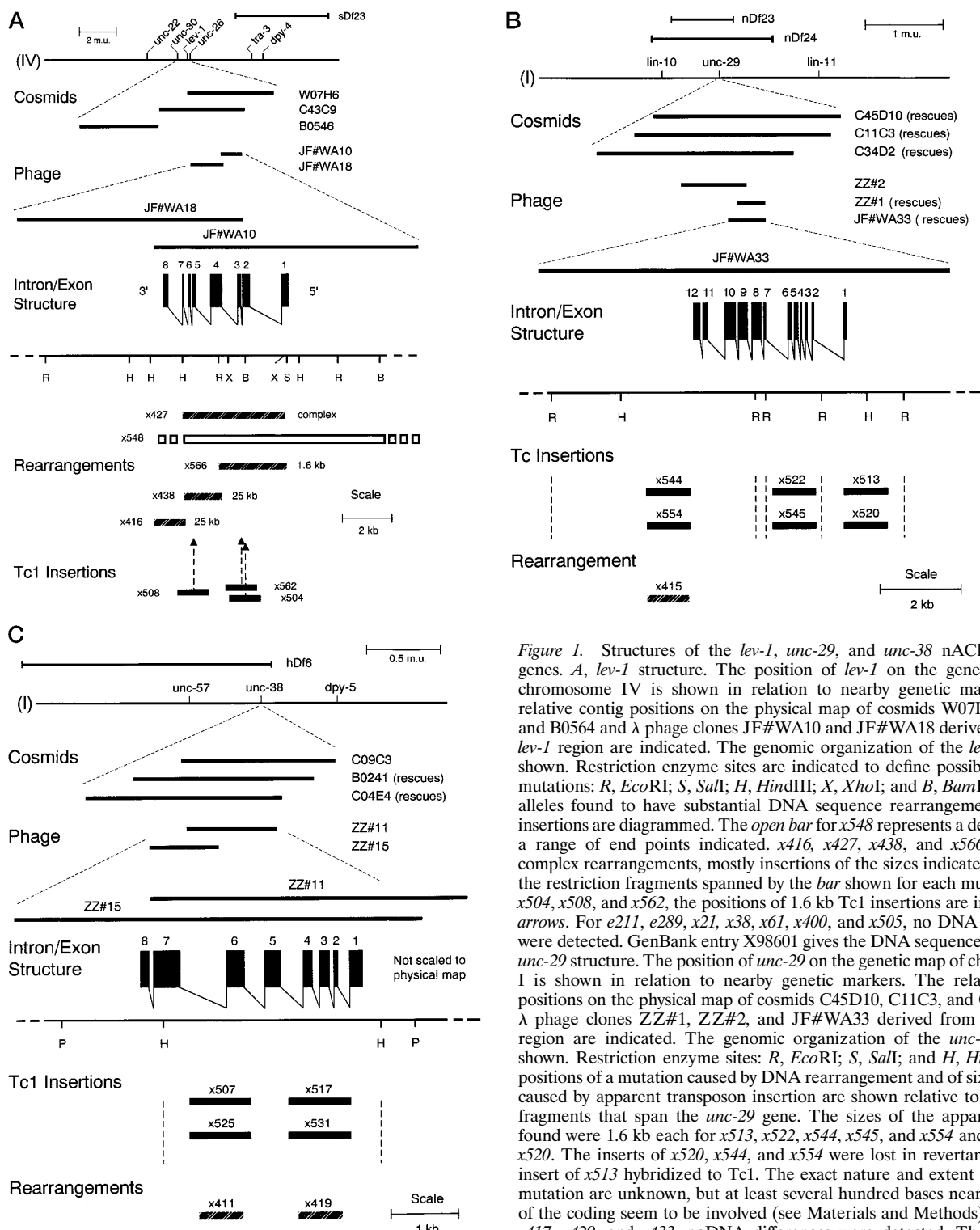


Figure 1. Structures of the *lev-1*, *unc-29*, and *unc-38* nAChR subunit genes. *A*, *lev-1* structure. The position of *lev-1* on the genetic map of chromosome IV is shown in relation to nearby genetic markers. The relative contig positions on the physical map of cosmids W07H6, C43C9, and B0564 and λ phage clones JF#WA10 and JF#WA18 derived from the *lev-1* region are indicated. The genomic organization of the *lev-1* gene is shown. Restriction enzyme sites are indicated to define possible limits of mutations: *R*, *EcoRI*; *S*, *SalI*; *H*, *HindIII*; *X*, *XhoI*; and *B*, *BamHI*. Mutant alleles found to have substantial DNA sequence rearrangements or Tc1 insertions are diagrammed. The open bar for *x548* represents a deletion with a range of end points indicated. *x416*, *x427*, *x438*, and *x566* represent complex rearrangements, mostly insertions of the sizes indicated, affecting the restriction fragments spanned by the bar shown for each mutation. For *x504*, *x508*, and *x562*, the positions of 1.6 kb Tc1 insertions are indicated by arrows. For *e211*, *e289*, *x21*, *x38*, *x61*, *x400*, and *x505*, no DNA differences were detected. GenBank entry X98601 gives the DNA sequence of *lev-1*. *B*, *unc-29* structure. The position of *unc-29* on the genetic map of chromosome I is shown in relation to nearby genetic markers. The relative contig positions on the physical map of cosmids C45D10, C11C3, and C34D2 and λ phage clones ZZ#1, ZZ#2, and JF#WA33 derived from the *unc-29* region are indicated. The genomic organization of the *unc-29* gene is shown. Restriction enzyme sites: *R*, *EcoRI*; *S*, *SalI*; and *H*, *HindIII*. The positions of a mutation caused by DNA rearrangement and of six mutations caused by apparent transposon insertion are shown relative to the *EcoRI* fragments that span the *unc-29* gene. The sizes of the apparent inserts found were 1.6 kb each for *x513*, *x522*, *x544*, *x545*, and *x554* and 2.5 kb for *x520*. The inserts of *x520*, *x544*, and *x554* were lost in revertants, and the insert of *x513* hybridized to Tc1. The exact nature and extent of the *x415* mutation are unknown, but at least several hundred bases near the 3' end of the coding seem to be involved (see Materials and Methods). For *x401*, *x417*, *x429*, and *x433*, noDNA differences were detected. The DNA sequence of *unc-29* is given by GenBank entry U81144. *C*, Structure of the *unc-38* nAChR α subunit gene. The position of *unc-38* on the genetic map of chromosome I is shown in relation to nearby genetic markers. The relative contig positions on the physical map of cosmids C09C3, B0241, and C04E4 and λ phage clones ZZ#11 and ZZ#15 are indicated. The genomic organization of the *unc-38* gene is shown. Restriction enzyme sites: *P*, *PstI*; and *H*, *HindIII*. Mutations associated with Tc1 insertion or DNA rearrangements within the *HindIII* fragment spanning the *unc-38* gene are indicated. For *x402*, *x404*, *x414*, and *x511*, no DNA differences were found in this fragment. Other than causing a size alteration of the 3.2 kb *HindIII* fragment, the exact nature and extent of the *x411* and *x419* mutations are unknown. The DNA sequence of *unc-38* is given by GenBank entry X98599.

the 5' ends of *unc-38* transcripts, a forward primer derived from positions 339-364 in the genomic sequence was used with a reverse primer from positions 2184-2209 antisense. To obtain an overlapping cDNA from the 3'-end of *unc-38* transcripts, forward and reverse primers from positions

1669-1691 and 3517-3541 antisense were used, respectively. The 5' and 3' RT-PCR products obtained for *unc-38(x411)* and *unc-38(x419)* were the same sizes as for wild type, 915 and 847 bp, respectively, as was the 3' PCR product of *unc-38(x20)*. The 5' RT-PCR product from *x20* RNA

was only about 760 bp in length in two independent amplifications done from the same RNA preparation. After purification with the QIAquick PCR Purification System (Qiagen, Chatsworth, CA), the 5' x20 products were ligated to pT7Blue (Novagen, Madison, WI) and electroporated into DH12S. Sequencing of the two independent x20 5' clones showed that the base sequence of the third exon was precisely missing in both clones, accounting for the smaller size of the PCR products. Two independent genomic clones were generated using the Boehringer-Mannheim (Indianapolis, IN) EXPAND PCR kit with forward and reverse primers derived from positions 638–663 and 1120–1143 antisense, respectively. Each PCR amplification was done using the genomic DNA from two adult worms digested with protease K (Williams et al., 1992) using the shortened cycle times recommended (10 cycles without autoextension followed by 20 cycles with an additional 20 sec each). Unlike the cDNA products, the genomic products seemed to be of wild-type length (506 bp). In both clones the G found in the universal 3' splice acceptor AG consensus sequence was found mutated to an A, accounting for the loss of the third exon in the RNA transcript (genomic position 764). The finding of apparently normal sizes found for 5' and 3' RT-PCR products prepared from *unc-38(x411)* and *unc-38(x419)* was surprising, because these mutants showed significant RFLP differences from the wild type on Southern blots (Fig. 1C). To obtain 5' and 3' RT-PCR products of *unc-29* transcripts, sets of forward and reverse primers were used from positions 494 to 521 and 2996 to 2972 antisense and from positions 2632 to 2655 and 4147 to 4170 antisense, respectively. The 5' and 3' RT-PCR products produced from *unc-29(x29)* and *unc-29(e1072)* total RNA were wild type in size (835 and 915 bp, respectively), as was the 5' product of *unc-29(x415)*. No 3' product was obtained for *x415* RNA even when a primer 214 bases downstream from the initial reverse primer position was used (positions 4361–4381). Southern blots showed that the 5.0 kb *EcoRI* fragment containing the 3' coding region of *unc-29* was about 200 bases shorter than the wild-type fragment, consistent with the PCR results and indicating that *x415* contains a rearrangement involving the 3' end of the *unc-29* coding region (Fig. 1B).

Mutant rescue. Germ line transformation of *unc-29* and *unc-38* was accomplished using the methods developed by Fire (1986) and Mello et al. (1991).

Construction of UNC-29::GFP fusions. Two UNC-29::GFP fusions were made for this work, and each was found to rescue the *unc-29* mutant phenotypes (both uncoordinated movement and levamisole insensitivity). Rescue indicates that transgene expression is physiologically relevant. Details of transgene construction are as follows. For LJH5, a genomic clone carrying the *unc-29* coding region and 1.5 kb upstream was fused after the C terminus to the coding region for *Aequora victoria gfp* (Chalfie et al., 1994). For these constructs, the Ser65→Thr variant of GFP (Heim et al., 1994) was used for its improved fluorescent properties. The *gfp* coding region in these constructs also incorporates several consensus nematode introns to facilitate nuclear export of RNA products (A. Fire, G. Seydoux, J. Ahnn, and S. Xu, unpublished observations). The UNC-29-GFP fusion junction is just at the C terminus of UNC-29, so that no UNC-29 amino acids are removed from the construct. The 3' nontranslated region is from *unc-54*. Construct LJH9 is similar to LJH5, except that the *unc-29* upstream regions have been replaced by the body muscle-specific *myo-3* promoter (Okkema et al., 1993) to generate a *myo-3::unc-29::gfp* fusion. The entire coding sequence from *unc-29* is in this construct. There are six introns from *unc-29* (these are introns 6–11 from the 3' end of the endogenous primary transcript), and one upstream intron is provided by the expression vector, whereas three artificial introns are present in *gfp*. The 3' untranslated region is from *unc-54*. The *unc-29* introns are not necessary for rescue by the *myo-3::unc-29* constructs; a simple fusion of the *myo-3* promoter to *unc-29* cDNA was constructed and found to rescue the *unc-29* movement defect. Injection of these constructs, derivation, and maintenance of transgenic lines were by standard protocols (Mello and Fire, 1995), using the selectable marker *rol-6* (Mello et al., 1991). Strain PD9253 carries the *gfp*-tagged *unc-29* clone LJH5, whereas strain PD9254 carries the *myo-3::unc-29::gfp* fusion construct LJH9, each as an unstable, extrachromosomal array. A control transgenic PD9258 strain carries just the *rol-6* marker. Each of these transgenics was constructed by injection into a *unc-29(e193)* genetic background. To examine the *unc-29::gfp* fusions in other genetic backgrounds, hermaphrodites of strains PD9253 and PD9254 were first crossed with N2 males to obtain the arrays in a wild-type background. The arrays were then crossed into the background of other *unc-29* mutants and were found to rescue all other *unc-29* mutants tested (mutant alleles *x29*, *x513*, *x520*, *x522*, *x545*, and *e1072*).

Confocal microscopy on strains carrying *gfp* fusions. Worms were grown at 20°C on NGM plates. Young adult worms that rolled strongly were picked to a 5% agarose pad containing 1× M9 and 10 mM sodium azide (Bargmann and Avery, 1995) and covered with a number 1½ coverslip. Confocal microscopy was done with a Bio-Rad (Richmond, CA) MRC-1024 microscope equipped with a 60×, 1.4 numerical aperture oil immersion objective. Specimens were viewed with 488 nm excitation from a krypton–argon laser at 10% transmission. Emitted light passing through a 522DF32 filter was collected at a normal scan speed with an iris setting of 3.4, gain of 1113, and a black level of –2, using Lasersharp software. Each optical section shown represents the accumulation over a 3 μm vertical distance of images scanned every 0.5 μm.

Transient heterologous expression in *Xenopus* oocytes. *In vitro* RNA transcription was performed using a Riboprobe kit obtained from Promega. Oocytes were obtained from female *Xenopus laevis* from Blades Biological (Kent, UK). The oocytes were kept in standard oocyte saline (SOS) medium [containing (in mM) 100 NaCl, 5 HEPES, 1.8 CaCl₂, 1 MgCl₂, and 2 KCl, pH 7.6]. Under the dissecting microscope, individual oocytes were defolliculated manually (i.e., the outer thecal layer and the follicle cell layers were removed with forceps), leaving the innermost vitelline membrane intact. The oocytes were then transferred to 1 mg/ml collagenase (type 1A; Sigma, St. Louis, MO) in SOS and incubated for 20 min to ensure that any remains of the follicle layer were digested. After a short recovery period in SOS (10–30 min), the oocytes were ready for injection. Test oocytes were injected with 50 nl of RNA solution (1 ng/nl for each subunit transcript) in the vegetal hemisphere. Control oocytes were either left uninjected or were injected with sterile distilled water.

Recordings were performed using a standard two-electrode voltage clamp (Dascal et al., 1984). A single oocyte was placed on the Sylgard base in a 1 ml experimental chamber. The oocyte was impaled with two glass microelectrodes (Clarke Electromedical glass GC 150 GF-150), fabricated using an electrode puller (Scientific and Research Instruments Ltd; catalog number 2001), and filled with 1 M KCl. Electrodes with a resistance of 5 MΩ were used for the voltage electrode, and current electrodes of 1–2 MΩ were used. Oocytes were voltage-clamped using a GeneClamp 500 amplifier. Current responses were monitored on an oscilloscope (Nicolet 3091) and recorded on a Gould BS-272 pen recorder. In some experiments, responses were recorded and stored using pClamp5 software installed on an IBM Personal Computer. Medium (SOS) and drugs were applied by a perfusion pump (Pharmacia LKB pump P-1) at a rate of 4 ml/min. Levamisole, mecamlamine, *d*-tubocurarine, neosurugatoxin, and α-bungarotoxin were each dissolved in the standard SOS medium. Levamisole was bath-applied. In experiments with nAChR antagonists, oocytes were preexposed to either mecamlamine (at the concentration indicated), *d*-tubocurarine (1 × 10⁻⁵ M), or neosurugatoxin (5 × 10⁻⁷ M) for 5 min, and then mecamlamine, *d*-tubocurarine, or neosurugatoxin was co-applied with levamisole for 30 sec. For α-bungarotoxin (M_r ~7800), preexposure was done with a 5.0 × 10⁻⁶ M concentration for 30 min.

Construction of an evolutionary tree. An alignment of 23 receptor sequences was generated using the computer program CLUSTAL V (Higgins et al., 1992) and edited using Genetic Data Environment. The resulting data set was analyzed under maximum parsimony conditions using the program Phylogenetic Analysis Using Parsimony (PAUP) (Swofford, 1991). Briefly, a 1000 replicate heuristic search was performed using independent random number seeds and tree bisection reconnection. The resulting tree of length 2384 changes was evaluated using a 1000 replicate bootstrap analysis in PAUP (Swofford and Olsen, 1990; Felsenstein, 1992). The resulting tree was rooted using the rat GABA_A α1 receptor subunit. The data set contains phylogenetic data as determined using the gl statistic bracket (gl = –0.869766; *p* < 0.01) (Huelsenbeck and Hillis, 1993). An essentially identical tree was generated from the same starting alignment by nearest neighbor-joining analysis using the program CLUSTAL W (Thompson et al., 1994), which uses the neighbor-joining algorithm of Saitou and Nei (1987). The alignment and the PAUP data file are available from J.T.F., D.B.S., or J.A.L.

The following protein sequences, available from the SWISS-PROT protein sequence database, were used in constructing Figures 2 and 4. All except GAA1_RAT are nAChR subunits. Rat α subunits: ACHA_RAT, ACH2_RAT, ACH3_RAT, ACH4_RAT, ACH5_RAT, ACH6_RAT, and ACH7_RAT (muscle α and neuronal α2–7). Rat neuronal β subunits: ACHN_RAT, ACHO_RAT, and ACHP_RAT (β2–β4). Rat muscle non-α: ACHB_RAT, ACHD_RAT, ACHE_RAT, ACHG_RAT (β, δ, ε, and γ). *Drosophila* subunits: ACH1_DRÖME, ACH2_DRÖME, ACH3_DRÖME, ACH4_DRÖME (ALS, SAD, ARD, and SBD). Lo-

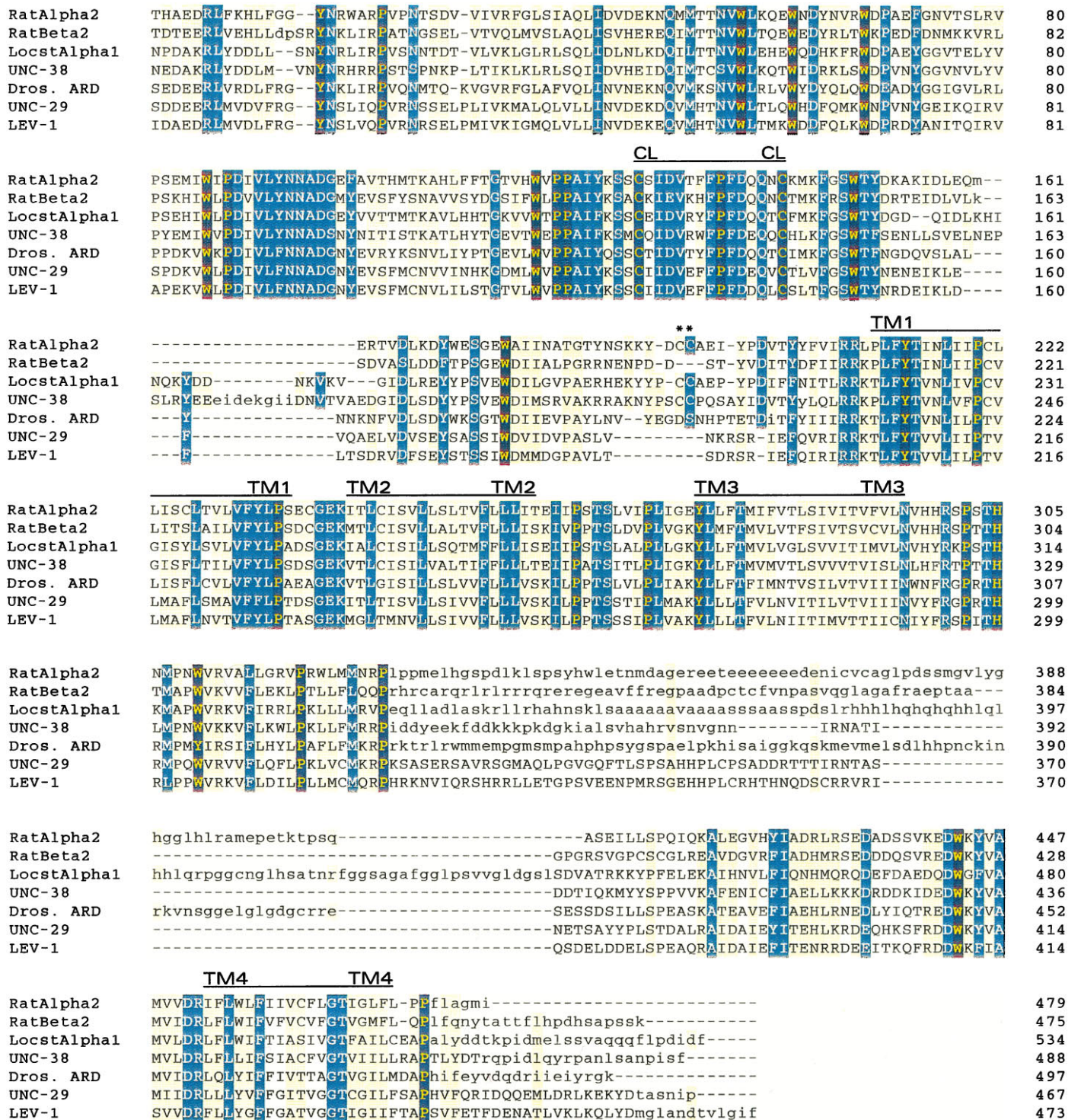


Figure 2. Amino acid sequence alignment of the *Caenorhabditis elegans* presumptive mature nAChR subunit sequences UNC-38, UNC-29, and LEV-1 with locust (*Schistocerca gregaria*) α L1, *Drosophila* (*Dros.*) non- α ARD, and rat α 2 and β 2 nAChR subunit sequences. The alignment was constructed using MACAW, version 2.0.5 (Schuler et al., 1991). Regions of sequence identity or high similarity within blocks of homology are indicated by dark coloring. Regions of moderate similarity or regions at boundaries of homology blocks are indicated by light coloring. Sequences in regions with no significant similarities between subunits are given in lower case letters, and no effort was made to align the amino acids in these regions. The positions of the four transmembrane domains (TM1-TM4) and the extracellular dicysteine loop (CL) characteristic of nAChR subunits are indicated. Asterisks indicate the positions of the vicinal cysteines characteristic of α -acetylcholine-binding subunits. The percent identity and similarity of UNC-38 to the locust and rat α sequences are 48 and 58% and 42 and 55%, respectively, as determined by individual pairwise comparisons. The percent identity and similarity of UNC-29 to the LEV-1, ARD, and rat β sequences are 66 and 77%, 50 and 65%, and 39 and 56%, respectively, determined by pairwise comparisons.

cust *Schistocerca gregaria* α L1: ACH1_SCHGR. Rat GABA α subunit: GAA1_RAT (GABA_A α 1).

RESULTS

Deletion and transposon insertion alleles of the levamisole resistance genes

The isolation and characterization of nAChR subunit genes is an essential beginning to understanding the effects of levamisole resistance mutations on synaptic signaling. To clone the subunit genes, two different experimental approaches were adopted to maximize the chances of success. One approach was to search for *C. elegans* nAChR subunit homologs by cross-hybridization and placement of the clones on the physical map of the nematode genome (Coulson et al., 1986). The other approach was to generate levamisole-resistant mutants by transposon insertion followed by cloning of the insertion site (Greenwald, 1985; Moerman et al., 1986), allowing genes to be cloned regardless of their actual functions or homology to known nAChR subunit genes.

Putative transposon insertion mutants were obtained by screening the progeny of either the Bergerac BO or the TR679 mutator strains (Moerman and Waterston, 1984; Collins et al., 1987) for spontaneous resistance to 1 mM levamisole. Isolates showing strong levamisole resistance were tested by complementation against the six known extreme levamisole resistance loci (Table 1). To confirm the identity of any clone found by transposon tagging or by cross-hybridization, mutants that might represent DNA rearrangements in the same resistance genes were also isolated by a similar selection scheme using the progeny of γ -irradiated worms. New alleles of *unc-29*, *unc-38*, *unc-63*, *unc-74*, *unc-50*, and *lev-1* were isolated. All showed extreme levamisole resistance, except for the *lev-1* isolates, which produced only partial resistance, consistent with partial resistance being the null phenotype for this gene (Lewis et al., 1980a).

Isolation and identification of λ phage containing nAChR subunit gene homologs

To isolate λ phage carrying nAChR subunit genes, a library of *C. elegans* genomic DNA was screened as described in Materials and Methods with an *ard* cDNA probe, which encodes a *Drosophila* non- α subunit (Hermans-Borgmeyer et al., 1986). Fifty-eight positive hybridizing phage were isolated. DNA from the 14 strongest positives was isolated and placed on the physical map by fingerprinting (Coulson et al., 1986). Several of these nAChR homologs mapped to positions on the physical map that were very close to the known genetic locations of levamisole resistance genes or corresponded to clones simultaneously identified by transposon tagging (*unc-38* and *unc-29*). After revision of the genetic map position of *lev-1* by T.M.B., the map position of this gene on chromosome IV was also consistent with the physical map position of two phage clones (JF#WA10 and JF#WA18; Fig. 1A and Materials and Methods).

Characterization of the *lev-1* locus

The identity of *lev-1* as a gene encoding a non- α -nAChR was established as follows. First, sequence analysis of the phage occupying the expected physical map position of *lev-1* identified a 4.8 kb *Hind*III fragment that contained an almost intact nAChR subunit gene, organized into eight exons (GenBank accession number X98601). Second, a complete cDNA sequence recovered from this region was found to encode an open reading frame for a non- α -nAChR subunit of 507 amino acids clearly homologous to vertebrate and insect nAChR subunits (Fig. 2). Third, when the genomic phage clones were used to probe Southern blots of genomic DNA from 11 mutator- and γ -ray-induced *lev-1* mutants and five EMS-

induced mutants, allele-specific rearrangements were found by T.M.B. that defined the *lev-1* locus (Fig. 1A). Three of four γ -ray-induced alleles (*x416*, *x427*, and *x438*) and two of seven mutator-induced alleles (*x548* and *x566*) were found to have rearrangements that included at least part of the 4.8 kb *Hind*III fragment identified by *ard* hybridization or the adjacent 1.4 kb *Hind*III fragment (*x416*). For three other mutator-induced alleles, the 4.8 kb *Hind*III fragment was replaced by a 6.4 kb fragment, consistent with the insertion of a typical 1.6 kb Tc1 transposon element. By using two PCR primers, one an oligonucleotide corresponding to the ends of the Tc1 element together and the other an oligonucleotide corresponding to the sequence of either strand at various positions within the 4.8 kb *Hind*III fragment (Barnes, 1990), it was possible to locate precisely the transposon within the 4.8 kb *Hind*III fragment (Fig. 1A). These results show that the *lev-1* gene and a nAChR homolog co-localize to the same 4.8 kb *Hind*III fragment. The null phenotype of *lev-1* was defined by the finding that most, if not all, of the coding region of the gene is deleted by the *x548* mutation and the homozygous *x548* null mutants are viable, partially levamisole-resistant, pseudo-wild-type mutants.

Characterization of the *unc-29* locus

unc-29 was identified as a non- α -nAChR subunit as follows. Genomic phage fingerprinting to the region of *unc-29* on chromosome I were isolated based on the identification of a novel Tc1 insertion associated with the *unc-29(x513)* mutation. These phage overlapped the phage JF#WA33 identified by cross-hybridization with the *Drosophila ard* probe (Fig. 1B). Hybridization of the phage to Southern blots of DNA from mutator- and γ -ray-induced *unc-29* mutant identified RFLPs through a region spanned by a 6.0 kb *Hind*III fragment (Fig. 1B). The sequence the 6.0 kb *Hind*III fragment contained the entire coding region, divided into 12 exons, of a non- α -nAChR subunit highly homologous to *lev-1* (GenBank accession number X98601). A full-length 1.7 kb cDNA that contained an open reading frame of 493 amino acids (Fig. 2) was obtained from a *C. elegans* mixed stage library (provided by R. Barstead).

Mutant rescue experiments confirmed the cloning of *unc-29*. Three cosmids that encompass the λ clone JF#WA33, and thus include all the regions affected by the *unc-29* transpositions described above (Fig. 1B), could rescue the *unc-29* mutant phenotype in transgenic animals. The cosmids were able to rescue completely the *unc* phenotype and to restore levamisole sensitivity, as were the smaller λ phage clones ZZ#1 and JF#WA33. Even the 6.0 kb *Hind*III fragment that spans the *unc-29* coding region and contains only 490 bp of DNA upstream from the translational start site (Fig. 1B) is capable of rescuing *unc-29* (S. Kim, personal communication) (this work).

Because the mutant phenotype can be rescued by a transgene sequence, it can be inferred that the transgene expression pattern includes those tissues in which the endogenous gene is required. To monitor transgene expression, we constructed a set of plasmids in which the coding region for GFP (*A. victoria* green fluorescent protein) (Chalfie et al., 1994) was fused to the C-terminal coding region for UNC-29. An activated form of GFP (S65T) (Heim et al., 1994) was used to maximize fluorescent signal. The resulting constructs were capable of rescuing the mutant phenotypes of *unc-29* alleles *e193*, *x29*, *x513*, *x520*, *x522*, *x545*, and *e1072*. A major focus of *unc-29* promoter activity was seen in body muscles. To test the hypothesis that UNC-29 expression in body muscles was sufficient for its function, we produced a construct in which *unc-29* upstream sequences ("promoter") were removed and

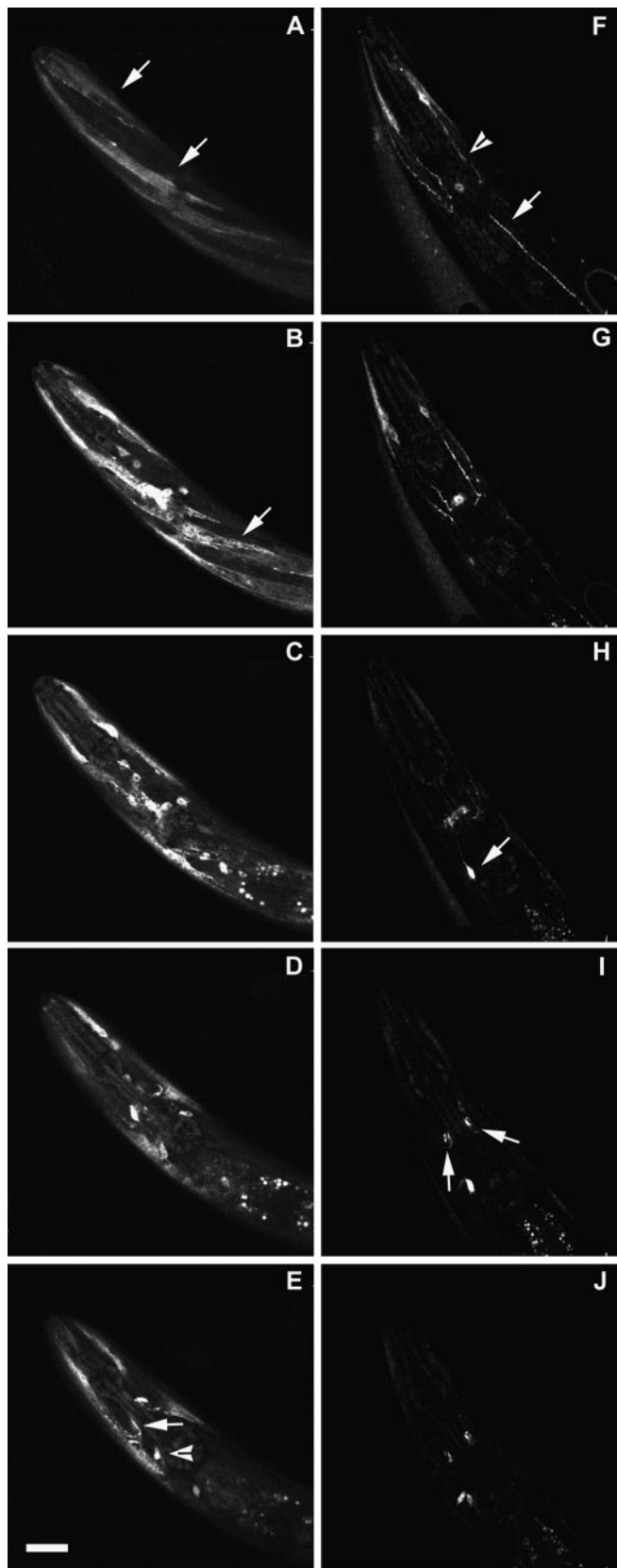


Figure 3. Serial optical sections showing expression of the *unc-29* promoter-driven *unc-29::gfp* fusion L_{JH5} in N2 wild type and in the *unc-29(x29)* mutant strain. Confocal microscopic sections through the head region of a wild-type and an *x29* mutant animal were accumulated as described in Materials and Methods. Each picture represents a successive

UNC-29/GFP expression was driven by the body muscle-specific *myo-3* promoter. The *myo-3::unc-29::gfp* fusion was indeed capable of rescuing the mutant phenotypes of *unc-29* mutant animals. This was consistent with the hypothesis that the primary focus of *unc-29* was in body muscle. The muscle staining is consistent with previous pharmacological data from studying *C. elegans* (Lewis et al., 1980b) and physiological data from *Ascaris* that levamisole-sensitive nAChRs are present on muscle (Harrow and Gratton, 1985). Recent mosaic studies using a *unc-29* clone provided by us also show a major focus of *unc-29* expression to be muscle (Miller et al., 1996b). We observed a low level of neuronal fluorescence, both with the *unc-29::gfp* fusion and with the *myo-3::unc-29::gfp* fusion. It is not clear whether this fluorescence represents bona fide activity in these cells.

The ability of GFP fusions to rescue the mutant phenotype allowed us to examine the intracellular localization of a biologically functional *unc-29* derivative in living cells. Intracellular localization in a wild-type background (i.e., in the presence of a wild-type chromosomal copy of the gene) was somewhat surprising, with activity predominantly internal to muscle cells. Because the presence of the natural UNC-29 product might be expected to affect assembly of the recombinant protein, we examined localization of the protein produced from an L_{JH5}-derived extrachromosomal array in a variety of *unc-29* mutant genetic backgrounds. Several *unc-29* mutant backgrounds behaved similarly to wild type in these assays (Fig. 3*A–E*), with the exogenous GFP fusion present primarily in the cell body (*e193*, *e1072*, *x520*, *x522*, and *x545*). However, when the fusions were crossed into the homozygous mutant background provided by *unc-29(x29)* (and to a lesser extent, *x513*), relative localization was closer to that expected for a neurotransmitter receptor (head region for *x29* homozygote shown in Fig. 3*F–J*). In addition, the overall amount of staining within muscle appeared to decrease. Internal staining of body muscles almost disappeared with punctate staining found along the nerve cords. Because of the background of neuronal staining, it was difficult to say what fraction of the staining in the nerve cords arose from body muscles. Some staining in the *unc-29(x29)* mutant background was still found internally in head muscles, but the relative amount of staining localized to synaptic regions of the ring (the central neuropil) greatly increased. These observations are consistent with the idea that the UNC-29 protein produced from the chromosomal copy of the *unc-29* gene competes in the assembly and localization of nAChR molecules with the UNC-29–GFP fusion protein. Paradoxically, from the decrease in overall staining observed in a *unc-29(x29)* background, the endogenous gene product may also help stabilize the

←

3 μ m thickness of the head. *A–E*, Wild type. Arrows in *A* point to fluorescence in head muscles. Fluorescence is seen more intensely inside the same muscles in *B* and also within a body muscle (arrow). In *E*, a muscle process entering the central neuropil next to the isthmus of the pharynx is stained (arrow). Stain is also accumulated in a neuronal cell body (arrowhead). *F–J*, *unc-29(x29)* mutant. Less staining is seen overall, and the stain is relatively concentrated in head muscle processes and nerve cords with continued neuronal staining. In *F*, process from anterior head muscles stain (arrowhead) and punctate staining is seen in a nerve cord (arrow). In *H*, the arrow points to a brightly staining neuronal cell body with a process running to the central neuropil, appearing as a hazy band in the center. In *I*, arrows point to brightly stained areas in the central neuropil immediately adjacent to the isthmus of the pharynx that appear to be associated with muscle processes running into the neuropil at this point. The same region is stained in the center in *J*, with two neuronal cell bodies below the center.

GFP fusion protein in assembly intermediates, possibly suggesting that more than one UNC-29 subunit can be assembled into an nAChR molecule. The null phenotype of *unc-29* has yet to be defined, but both homozygotes of the *unc-29(x29)* and *unc-29(e1072)* mutations have been shown previously to contain little or no detectable specific high-affinity [³H]MAL binding (Lewis et al., 1987b). Although the focus of *unc-38* and *lev-1* expression has not been defined in this study, the great similarity in mutant phenotypes of *unc-38* mutants and *lev-1* semidominants to *unc-29* mutants makes it likely that muscle is also the major focus for the expression of these genes as well.

Characterization of the *unc-38* locus

The *unc-38* gene was identified by a novel Tc1-containing RFLP found in four of five spontaneously induced *unc-38* mutants (see Fig. 1C and Materials and Methods). Two of five γ -ray-induced *unc-38* mutants also showed a restriction size difference from wild type when the subcloned genomic DNA flanking one of the Tc1 inserts was used as a hybridization probe on genomic Southern blots. DNA extending through and beyond the 3.2 kb *Hind*III fragment that was the site of six *unc-38* mutations was sequenced and found to encode a complete α subunit of an nAChR (GenBank accession X98599). A complete cDNA sequence reconstructed from this region encoded an open reading frame of 511 amino acids with strong homology to known α subunit sequences of vertebrates and insects (Fig. 2). The fingerprint of genomic phage containing this α subunit gene was consistent with the genetic map position of *unc-38* to the left of *dpy-5* on chromosome I (Fig. 1C).

The cloning of *unc-38* was confirmed by mutant rescue. The injection of either cosmid B0241 or C04E4 into the germ line of *unc-38* mutants completely restored normal movement in the L1 stage offspring, and the transgenic worms could now be killed by exposure to 1 mM levamisole. The B0241 and C04E4 cosmids completely encompass the λ phage ZZ#11 and ZZ#15 shown to contain the sites of the *unc-38* mutations (Fig. 1C).

The null phenotype of *unc-38* was defined to be that of an extremely levamisole-resistant *unc*. This finding was originally suggested by sequence analysis that showed that the Tc1 insertion site in *unc-38(x525)* disrupts a reading frame 15 amino acids upstream from the dicysteine loop (amino acids 128–142, *Torpedo* α numbering) that is characteristic of all nAChR subunits. Further mutant analysis showed that the EMS-induced mutation *unc-38(x20)* is an absolute splicing defect in which the third exon is skipped because the universal AG intron consensus sequence at the third exon splice acceptor site is mutated to AA. The mutation causes a transcript to be produced that is 155 bases shorter than the wild type when detected by RT-PCR. Homozygous *unc-38(x20)* mutants grow well and are among the most resistant of all levamisole-resistant mutants to the effects of levamisole and other cholinergic agonists (Lewis et al., 1980b). Because body muscles of *unc-38* mutants are more resistant than head muscles to agonists, it is likely that there is at least one other α -nAChR subunit participating in the formation of other nAChR isotypes, and these isotypes differ in tissue distribution from the receptor formed with the *unc-38* α subunit.

Sequence comparison of LEV-1, UNC-29, and UNC-38

In Figure 2, the deduced amino acid sequences of the LEV-1, UNC-29, and UNC-38 proteins are aligned and compared with vertebrate and insect nAChR subunit sequences. Comparison of the *C. elegans* sequences with database sequences shows that the nematode sequences are most similar to the *Drosophila melano-*

gaster nAChR subunit sequences ARD and ALS (Hermans-Borgmeyer et al., 1986; Bossy et al., 1988) and to a partially sequenced putative nAChR subunit of the parasitic nematode *Onchocerca volvulus* (Ajuh and Egwang, 1994). The nematode non- α subunits UNC-29 and LEV-1 exhibit the highest amino acid sequence identity to ARD (50 and 46%), whereas the α subunit UNC-38 shows 49% identity to the ALS subunit. The predicted mature lengths of the three *C. elegans* nAChR subunits are 488 amino acids for UNC-38, 467 for UNC-29, and 473 for LEV-1. Whereas the α subunit of vertebrate muscle is the smallest muscle nAChR subunit, UNC-38 is the longest of the known nematode nAChR subunits, a relative size difference that holds for other invertebrate and for vertebrate neuronal α subunits compared with their non- α counterparts. The main structural features of the nAChR subunits of higher organisms are strikingly conserved in phylogeny down to the nematode, including the positions of the four transmembrane domains (TM1–4), the high sequence similarity found in each TM region, the variable long loop between TM3 and TM4, and the dicysteine loop, which is a hallmark of this superfamily and invariably found at the equivalent to positions 128–142 of the *Torpedo* α subunit (Barnard, 1992). There seem to be additional regions in all α subunits of invertebrates that have no counterpart in vertebrates. These insertions occur 25 amino acids N-terminal to the vicinal cysteines (equivalent to positions 192 and 193 of the *Torpedo* α subunit) and at the C terminus of the polypeptide. The functional significance of these domains is unknown. The insertion N-terminal to the vicinal cysteines is 13 amino acids longer for the nematode UNC-38 α subunit than for locust and *Drosophila* α subunits and is absent from invertebrate non- α subunits.

UNC-29 and LEV-1 are highly homologous: 66% amino acid identity or, with conservative substitutions, 77% similarity, a resemblance found for few other nAChR subunit pairs from the same species. They are categorized as non- α subunits by the absence of vicinal cysteines (at positions 192 and 193, *Torpedo* α numbering) and when compared with vertebrate nAChRs show the closest homology to neuronal subunits. Both UNC-29 and LEV-1 are about equally similar to neuronal α and non- α of the rat and chick (rat $\alpha 2$ – $\alpha 4$ and $\beta 2$ – $\beta 4$, chick $\alpha 2$ and $\alpha 4$ and $\beta 2$ and $\beta 4$) with UNC-29 showing slightly greater over all sequence similarity, ~55 versus ~50% for LEV-1. Consistent with these results, many aspects of the *in situ* pharmacology of muscle nAChRs of *C. elegans* and *Ascaris* resemble those of vertebrate neuronal nAChRs, including insensitivity to α -bungarotoxin, block by mecamylamine (Lewis et al., 1987a, 1980b; Fleming et al., 1993), and, in the case of *Ascaris suum* muscle nAChRs, block by κ -bungarotoxin (Colquhoun et al., 1993).

Intron–exon structure comparisons and phylogenetic analysis using accepted mutation parsimony trees

Between *unc-29* and the γ , δ , and ϵ subunits of vertebrate muscle (Nef et al., 1984; Buonanno et al., 1989), the positions of 5 of the 11 introns are completely conserved, and an additional 3 intron positions are within four amino acids of the equivalent sites in the vertebrate muscle subunits. The inexact conservation of some intron–exon boundaries may be attributable to splice junction drift or *de novo* creation of introns at “permissible” sites. The locations of four of the seven *lev-1* introns and three of the seven *unc-38* introns are identical in *unc-29*. However, only one intron location, between exons 5 and 6 in *unc-29*, is shared between *unc-38* and *lev-1*, and this site is present in all nAChR subunit genes. The flanking exons seem to form a calcium-binding domain (Godzik

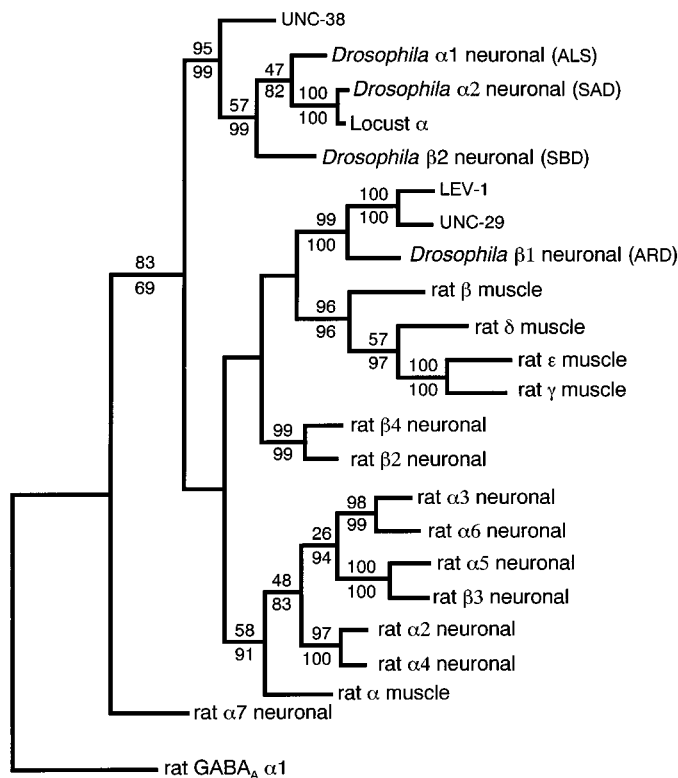


Figure 4. Maximum parsimony phylogenetic tree showing the relationship between the nAChR subunits. The tree is shown rooted using the rat GABA_A receptor $\alpha 1$ sequence. LEV-1 and UNC-29 are shown in a class of polypeptides that include mammalian muscle nAChR non- α subunits. UNC-38 is shown clearly related to other invertebrate α -like AChR subunits. The values over the branches represent the minimum number of times from 1000 random seeds in the bootstrap analysis that a particular branch is expected to appear ($p < 0.01$). Branches without numbers do not have significant probability of appearing at that exact point in the tree. Regions with little homology, such as the intracellular cytoplasmic loop, were not used in the comparison. A tree of the same shape was generated by nearest neighbor-joining analysis, and the bootstrap values for 1000 random seeds are shown for comparison below the branches.

and Sander, 1989), although its significance has not been well defined. A *unc-29–unc-38* common splice site occurring between exons 3 and 4 of *unc-29* is conserved in all nAChR subunit genes examined except *lev-1*. These findings suggest that the conserved splice sites predate the divergence of nematodes, insects, and vertebrates, which occurred about 600 million years ago. Unique to the nematode receptors is an intron that interrupts the coding region between TM4 and the C terminus in each of the three nematode subunits. Although the gene structures of *unc-29* and *lev-1* show considerable similarity, *unc-38* is no more homologous to the other *C. elegans* subunits than any other insect or vertebrate nAChR subunit genes, suggesting that the divergence between α and non- α subunits is very ancient. The intron–exon structure of all three nematode genes is also more typical of the highly interrupted vertebrate muscle nAChR genes than it is of the vertebrate neuronal nAChR genes (Nef et al., 1988), in which there is generally only a single intron between the conserved splice sites in the extracellular domain and at the end of the protein.

A maximum parsimony analysis for nAChR polypeptides is shown in Figure 4. The polypeptide sequences of 22 nAChRs and the rat GABA_A $\alpha 1$ receptor subunit were aligned. Using PAUP, a single tree of length 2384 changes was found (Swofford, 1991).

The significance of this tree was evaluated using bootstrap analysis (Felsenstein, 1992). The resulting tree shows that nAChRs fall into several distinct classes. The *C. elegans* subunits LEV-1 and UNC-29 are very closely related and, of the other sequences analyzed, are most similar to the *Drosophila* neuronal subunit ARD. UNC-38 represents an α subunit type closely related to the *Drosophila* α -like neuronal subunits. Although the distinctiveness of UNC-38 on the tree is not strongly supported by the bootstrap analysis, it is consistent with the unique pharmacological properties of nematode nAChRs as observed for *C. elegans* and *Ascaris*.

Potential glycosylation and phosphorylation signals in UNC-29, UNC-38, and LEV-1

Sequence prediction of glycosylation sites for the vertebrate muscle nAChR subunits, now confirmed biochemically (Claudio et al., 1989), indicated a single glycosylation site for the α and β subunits, two for the γ , and three for the δ muscle subunits. The vertebrate neuronal sequences usually contain two potential glycosylation signals. The sequences of UNC-29, UNC-38, and LEV-1 predict two, three, and five asparagine-linked glycosylation sites, respectively. There is no glycosylation site common to all three subunits, and only one shared between UNC-29 and LEV-1 at asparagine 50 (UNC-29 numbering), a site that is also present in nearly all invertebrate and vertebrate neuronal sequences (but lacking in human $\beta 4$ and goldfish non- $\alpha 3$). UNC-38 is the only invertebrate sequence to lack this site, and the three glycosylation signals UNC-38 contains are not in positions common to any other nAChR. An asparagine 109-linked glycosylation site in LEV-1 is conserved in $\alpha 2$ and $\alpha 4$ neuronal sequences and in the chick muscle δ subunit.

The *Torpedo* nAChR can be phosphorylated by at least three different protein kinases: cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and a tyrosine kinase (TK) (Huganir and Greengard, 1983; Huganir et al., 1984; Huganir, 1988). The region of the *Torpedo* receptor that was shown to be phosphorylated is the intracellular loop between TM3 and TM4. In the equivalent regions, the *C. elegans* UNC-29 subunit has three PKC sites, one PKA site, and one TK site; UNC-38 has single PKC and TK sites; LEV-1 has only a single PKC site. Studies with vertebrate nAChR subunits have shown phosphorylation to be involved in desensitization, receptor turnover, and receptor assembly. The *in vivo* functions of the glycosylation and phosphorylation sites can be investigated in *C. elegans* by mutagenizing a given site and then returning the altered gene to a null mutant strain that cannot otherwise produce the targeted subunit.

Expression of *unc-29*, *unc-38*, and *lev-1* in *Xenopus* oocytes

In earlier studies, RNA isolated from mixed stage wild-type *C. elegans* was injected into *Xenopus* oocytes with the result that a dose-dependent depolarization was detected in response to bath-applied levamisole (Fleming et al., 1991, 1993). The levamisole response was not blocked by α -bungarotoxin (Tornoe et al., 1995), in agreement with [³H]meta-aminolevamisole-binding studies using membrane fragments (Lewis et al., 1987a) and cut worm muscle contraction assays (Lewis et al., 1980b). Cytoplasmic co-injection of cRNAs encoding UNC-29, LEV-1, and UNC-38 resulted in inwardly directed currents (holding potential, -60 mV) in response to levamisole (1×10^{-4} M), whereas oocytes injected separately with message encoding a single subunit or the equivalent volume of distilled water showed no such responses (Fig. 5A–E). Pairwise injection of all possible combinations yielded either no

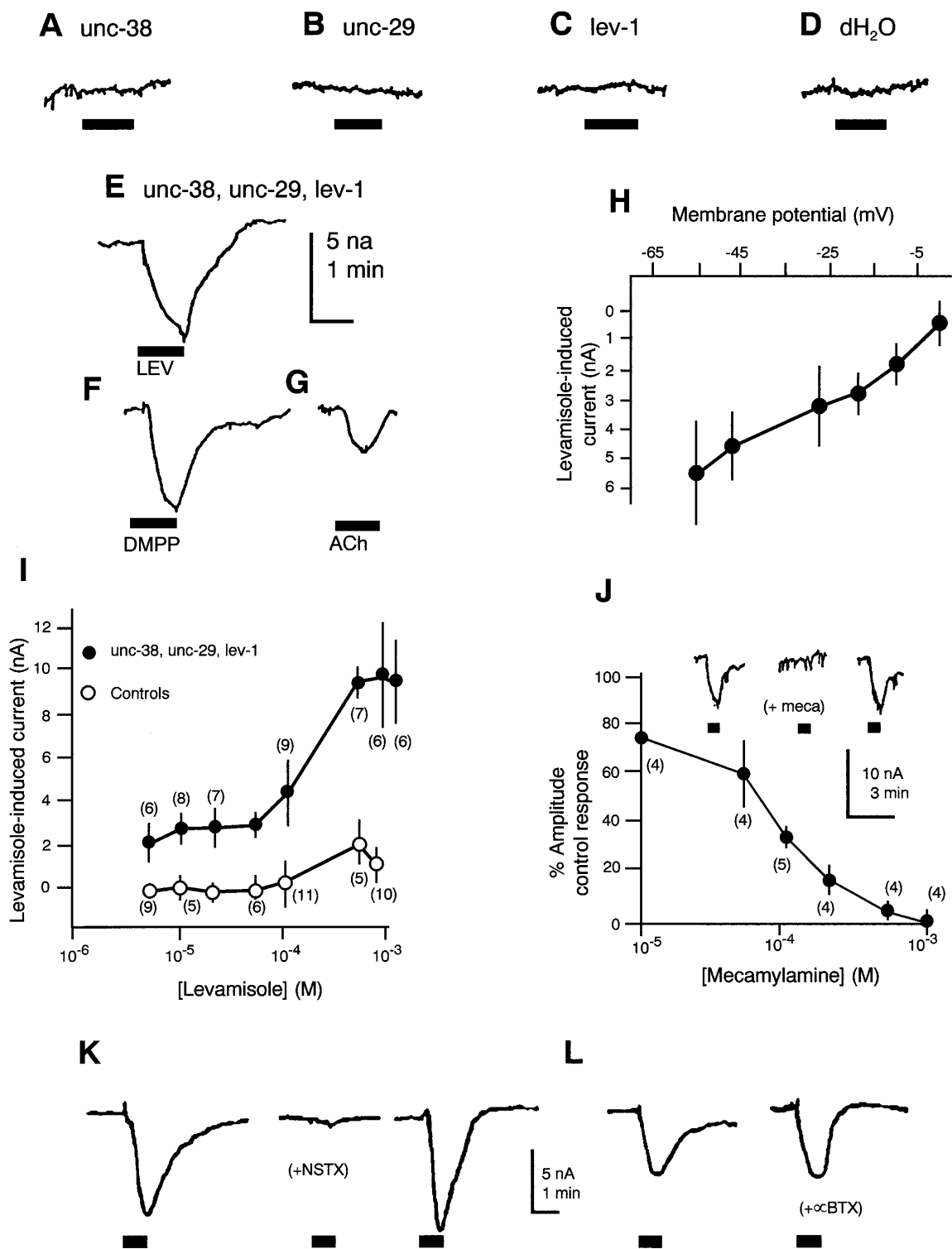


Figure 5. Transient expression of *unc-29*, *unc-38*, and *lev-1* cRNAs in *Xenopus* oocytes. *A–E*, Responses to levamisole (100 μ M) of *Xenopus* oocytes injected cytoplasmically with one (*A–C*) or a combination (*E*) of cRNAs encoding *C. elegans* putative nAChR subunits or the equivalent volume (50 nl) of distilled water (*D*). Whereas, when injected separately (*A–C*) no response was obtained, and all pairwise combinations yielded either no expression or unreliable expression, when all three subunits were co-expressed, small amplitude inward currents were observed in response to levamisole (*E*), DMPP (*F*), and acetylcholine (*G*). Levamisole-induced currents recorded when all three subunits were co-expressed were membrane potential-dependent, and the estimated reversal potential suggested a cationic current (*H*). Such responses to levamisole were dose-dependent (*I*) and were blocked in a dose-dependent manner by the nicotinic antagonist mecamylamine (*J*), which also blocks native muscle nAChRs in *Ascaris suum*. As is also the case for native *Ascaris* muscle nAChRs, on the expressed receptors, neosurugatoxin (0.5 μ M, 10 min) was an effective blocker of levamisole responses (*K*), whereas α -bungarotoxin (5.0 μ M, 30 min) was ineffective (*L*).

	TM1			TM2																				
		237			247																			
<i>lev-1(wt)</i>	Y	L	P	T	A	S	G	E	K	M	G	L	T	M	N	V	L	L	S	I	V	V	F	
<i>lev-1(x21)</i>	Y	L	P	T	A	S	G	K	K	M	G	L	T	M	N	V	L	L	S	I	V	V	F	
<i>lev-1(x61)</i>	Y	L	P	T	A	S	G	E	K	M	G	L	T	M	N	V	L	L	L	S	I	V	V	F
α -7 (wt)	L	L	P	A	D	S	G	E	K	I	S	L	G	I	T	V	L	L	S	L	T	V	F	
α -7 (anionic)	L	L	P	A	D	S	G	P	A	K	I	S	L	G	I	T	V	L	L	S	L	T	F	
α -7(Leu-247)	L	L	P	A	D	S	G	E	K	I	S	L	G	I	T	V	L	G	S	L	T	V	F	

Figure 6. Comparison of TM2 sequences from the two *lev-1* dominant alleles with the chick α 7 nAChR subunit mutations shown to convert cationic to anionic selectivity (Galzi et al., 1992) and a rat α 7 nAChR subunit mutated in leu-247, resulting in altered desensitization (Revah et al., 1991). Mutated amino acids are shown in *bold italic underlined*.

responses or inconsistent responses. In oocytes injected with all three cRNAs, the response to levamisole was mimicked by dimethylphenylpiperazinium (DMPP; 1.0×10^{-4} M; $n = 4$; Fig. 5F) and less efficiently by ACh (1.0×10^{-4} M; $n = 4$; Fig. 5G). The membrane potential of the amplitude of the levamisole-induced currents indicated the gating of a cation ion channel (Fig. 5H). Responses to levamisole were dose-dependent (Fig. 5I) and blocked in a dose-dependent manner by the potent antagonist of *Ascaris* muscle nAChRs mecamylamine (Fig. 5J). Neosurugatoxin (5×10^{-7} M; Fig. 5K), which blocks reversibly *Ascaris* muscle nAChRs, also reversibly blocked the response to levamisole (1.0×10^{-4} M) in oocytes injected with UNC-29, LEV-1, and UNC-38 ($n = 6$), as did *d*-tubocurarine (1.0×10^{-5} M; data not shown; $n = 5$). In contrast, α -bungarotoxin (5×10^{-6} M; Fig. 5L), a weak antagonist of *Ascaris* muscle nAChRs, was ineffective on responses to levamisole even after 30 min exposure of oocytes injected with all three subunits. The observed currents are of small amplitude and do not require a rigorous subunit stoichiometry. Perhaps not all subunits needed for full expression have been identified, or alternatively, some factor useful in assembling the nematode receptor, e.g., one of the other gene functions identified by levamisole resistance mutation, may be lacking in the frog oocyte.

Sequence analysis of the *lev-1* dominant alleles (*x21* and *x61*)

We also determined the sequence alterations in the two unusual *lev-1* semidominant alleles, *x21* and *x61*. The *lev-1* cDNAs from these mutants were amplified by PCR and sequenced. To minimize the possibility of a cloning artifact, cDNAs were amplified and sequenced from two different preparations of each mutant. Both alleles contained mutations in or very near the TM2 membrane-spanning domain of the LEV-1 protein (Fig. 6). For *x21*, a glutamate to lysine (E to K) mutation was found at the hydrophilic site equivalent to the -1 position of *Torpedo* TM2 (numbering from the N-terminal direction), a position known to influence the overall conductance and Ca^{2+} permeability of the ion channel. Mutations at this position in vertebrate nAChR subunits produce the largest changes in channel conductance and ion selectivity (Imoto et al., 1988; Konno et al., 1991; Galzi et al., 1992). For *x61*, a leucine insertion was found at a position equivalent to the +11 position of *Torpedo* TM2, a position in TM2 demonstrated to affect ion conductance by *in vitro* mutagenesis of the rat neuronal α 7 nAChR subunit (Galzi et al., 1992). The sites of the two *lev-1* dominant mutations are thus 11 amino acids apart in the same functional domain of the protein, consistent with their similar mutant phenotypes. The mutations are about three turns of an α helix apart and are presumed to face or be close to the lining of the channel (Unwin, 1989; Revah et al., 1991).

DISCUSSION

We have shown that the *lev-1*, *unc-29*, and *unc-38* genes of *C. elegans* associated with resistance to the cholinergic anthelmintic drug levamisole encode nAChR subunits. Furthermore, subunit combinations that include the *unc-38* α subunit can be expressed in *Xenopus* oocytes to form ion channels activated by levamisole. The viability of *C. elegans* nAChR mutants provides unique opportunities to study both molecular aspects of nAChR function and the interactions of nAChR molecules with other cellular components throughout development.

The non- α subunit UNC-29 is required for agonist binding

Earlier studies implicated UNC-29 as part of the levamisole binding site in nematode nAChRs. *unc-29* mutants had little specific [^3H]MAL binding, whereas *unc-38* mutants retained detectable binding (Lewis et al., 1987b). Levamisole seems to act like nicotine, competing with acetylcholine for binding to the receptor (Lewis et al., 1980b; Harrow and Gratton, 1985; Lewis et al., 1987a). The finding that *unc-38* rather than *unc-29* encodes an nAChR α subunit suggests that the levamisole and acetylcholine binding site may be formed between α and non- α subunits, as occurs for vertebrate muscle nAChRs (Pedersen and Cohen, 1990; Czajkowski et al., 1993). Ten rare mutants of *unc-29* and *unc-38* retain normal motor behavior but are extremely resistant to levamisole, as might result from missense amino acid substitutions that interfere with the binding of levamisole but not of acetylcholine. Sequencing these rare *unc-38* and *unc-29* mutations should help further define the levamisole and acetylcholine binding site.

LEV-1 is highly homologous to UNC-29 but not required for nAChR function

LEV-1 and UNC-29 are much more homologous than almost all other nAChR subunit pairs from the same species. Mutation of *unc-29* is more functionally debilitating than mutation of *lev-1* (Lewis et al., 1980b). *unc-29* mutants have lost high-affinity specific [^3H]MAL binding, are extremely uncoordinated, and are extremely resistant to muscle contraction induced by levamisole. *lev-1* mutants have detectable levamisole binding, wild-type motility, and much weaker levamisole resistance. In vertebrate muscle, the γ and ϵ subunits can functionally replace one another while sharing 52% amino acid identity (65% similarity). UNC-29 with 66% identity and 77% similarity to LEV-1 might substitute for a missing LEV-1 subunit and allow the partial function seen in *lev-1* mutants (including deletion and transposon mutants; Fig. 1A). The inability of LEV-1 to replace the UNC-29 subunit is consistent with UNC-29 but not LEV-1, being required for the function of most levamisole-sensitive nAChR molecules in *C. elegans*.

lev-1 semidominant mutations block nAChR function

Although the LEV-1 subunit is normally not essential, two rare semidominant (*sd*) mutations of *lev-1*, when homozygous, greatly reduce levamisole-sensitive nAChR function. Our results show that these mutations represent either an amino acid substitution (*x21*) or an amino acid addition (*x61*) within or very near the TM2 domain of the LEV-1 subunit. For several hundred other extreme levamisole resistance mutations that have been complemented, including *unc-29* and *unc-38* mutants, the single copy of the wild-type gene in a heterozygote is sufficient for wild-type sensitivity to levamisole, whereas a single copy of the wild-type *lev-1* gene in *lev-1(sd)/+* heterozygote results in partial resistance. Normal assembly of a receptor with a defective LEV-1 subunit that interferes with or alters ion conductance could produce the

lev-1(sd) mutant phenotype, especially if the LEV-1 subunit is normally present in the great majority of levamisole receptor molecules. The hypothesis that most levamisole-sensitive nAChR molecules contain LEV-1 is consistent with the observations that mutation of *lev-1* affects the major portion of high-affinity specific [³H]MAL binding, and that the two *lev-1(sd)* mutants as homozygotes have normal amounts of specific [³H]MAL binding trapped in an unusual high-affinity state (Lewis et al., 1987b). The semi-dominant phenotype could be explained if at least 50% of the assembled molecules in a *lev-1(sd)/+* heterozygote were poisoned by the *lev-1(sd)* subunit, whereas in all other *unc/+* heterozygotes examined, in which the *unc* allele was selected for strong dysfunction in the homozygous state, the limited amount of receptor molecules assembled were preferentially drawn from the wild-type pool of receptor subunits for the gene in question.

The *lev-1(x61)* mutant phenotype is caused by insertion of an additional leucine into TM2 at the equivalent position of leucine 247 in the chick $\alpha 7$ neuronal nAChR. Replacement of the chick $\alpha 7$ Leu-247 with either a threonine or a serine results in a channel with no rectification, reduced desensitization, and increased affinity for ACh (Revah et al., 1991). The change in *x61*, adding an additional hydrophobic leucine residue next to this position, causes the opposite mutant phenotype with respect to agonist sensitivity: reduced sensitivity to agonist and an apparent desensitized, high-affinity binding state insensitive to the addition of the noncompetitive antagonist mecamylamine (Lewis et al., 1987b). Similarly, the charge reversal in *x21* (E to K transition) at a position known to be critical to ion conductance in vertebrate nAChRs is consistent with the insensitivity of the *x21* mutant to agonist and its similarly desensitized binding state (Lewis et al., 1987b). A series of engineered changes in the rat neuronal $\alpha 7$ subunit including an amino acid addition and substitution at the site of the *x21* mutation can even convert the $\alpha 7$ channel to one conducting anions (Galzi et al., 1992). Because $\alpha 7$ forms a homooligomer, the $\alpha 7$ mutant phenotypes are the result of the same mutation being present in all five subunits of a receptor molecule. By contrast, the *lev-1(sd)* mutant phenotype is generated by mutation of a single non- α subunit that only forms a channel when combined with other subunits, as shown both by mutant phenotypes and by oocyte expression studies. The *lev-1* mutations may act by causing desensitization of the entire receptor molecule rather than by simply blocking ionic conductance, consistent with the apparent importance of TM2 to desensitization as well as to ion passage (Unwin, 1995). The lack of similar dominant isolates for other subunit genes such as *unc-29* and *unc-38* may simply be caused by the rarity of dysfunctional subunits that assemble well yet poison the assembled receptor strongly.

The predicted UNC-38 amino acid sequence is consistent with the insensitivity to α -bungarotoxin and lophotoxin observed for nematode nAChRs

The potent neurotoxin lophotoxin is a cyclic diterpene isolated from the gorgonian coral *Lophogorgia chilensis* and has been shown to block all vertebrate muscle, neuronal, and invertebrate neuronal nAChRs tested (Abramson et al., 1988; Bai et al., 1993). This toxin binds covalently to the α subunit of the *Torpedo* receptor, and protease digestion followed by sequence analysis has identified tyrosine 190 as the target amino acid residue. This residue is conserved in all α subunits sensitive to lophotoxin tested to date (Abramson et al., 1989) and is close to the vicinal cysteines that define a main part of the acetylcholine binding site on α subunits (Kao and Karlin, 1986). UNC-38, however, con-

		α -bungarotoxin block	lophotoxin block	‡	192	193	*
Muscle α	S	YES	YES	x Y x	C C	P x x	P Y
Muscle α	R	NO	YES	x Y x	C C	L x x	P/H Y
Neuronal α	YES	YES	YES	x Y x	C C	- x x	P Y
Neuronal α	NO	NO	YES	x Y x	C C	- x x	I Y
UNC-38		NO	NO	Y P x	C C	P x x	A Y

Figure 7. Amino acid sequence comparison of UNC-38 with neuronal α and vertebrate muscle α subunits in the region known to include the α -bungarotoxin and lophotoxin binding sites. In all species in which the sequence and pharmacology are known, a proline is present at position 197 (*Torpedo* α subunit numbering, indicated by asterisk) if the α subunit binds α -bungarotoxin. The muscle α subunits that do not bind α -bungarotoxin used in this alignment (*muscle α R*) are from two snakes (Neumann et al., 1989) and a mongoose (Barchan et al., 1992). The neuronal α subunits that bind bungarotoxin include chick $\alpha 7$ (Couturier et al., 1990), invertebrate locust $\alpha 1$ (Marshall et al., 1990), and the *Drosophila* ALS α -like sequence. The tyrosine present at the equivalent of *Torpedo* α position 190 (‡) is found in subunits binding lophotoxin (see Discussion). Sequence positions important to the comparison are shown in upper case. The letter x is used to indicate positions not considered important to the comparison. R and S, α -Bungarotoxin-resistant and -sensitive, respectively.

tains a proline at the position equivalent to Tyr-190 (Fig. 7), and lophotoxin is unable to block the levamisole-induced responses of *Ascaris suum* muscle (Tornoe et al., 1995). In general, this region of UNC-38, extending through and beyond the vicinal cysteines equivalent to residues 192 and 193 of *Torpedo* α , is more typical of a muscle-type α subunit than a neuronal α subunit (Fig. 7). In addition, UNC-38 has an alanine at position 197 (*Torpedo* α numbering). This position is occupied by a proline in all α subunits that are capable of binding α -bungarotoxin. This is consistent with our finding that the *C. elegans* nAChR is not blocked by α -bungarotoxin in living worms and cut worms, in binding assays, or on expressed receptors in *Xenopus* oocytes (Lewis et al., 1980b; Lewis et al., 1987a; Tornoe et al., 1995).

Conclusions and future prospects

We have shown here that 3 of the 11 *C. elegans* levamisole resistance loci encode nAChR subunits (see Table 1). The complete loss of levamisole sensitivity in mutants of either *unc-29* or *unc-38* suggests that these subunits co-exist in the same receptor molecules. The dominant effect of rare *lev-1* mutations argues that the LEV-1 subunit is also an integral although normally dispensable part of most receptor molecules formed from UNC-29 and UNC-38. Two other loci (*unc-22* and *lev-11*) encode muscle proteins (Moerman et al., 1988; Williams and Waterston, 1994). Of the remaining six levamisole resistance loci, some may encode proteins important to receptor function other than additional nAChR subunits. Two of these genes, *unc-50* and *unc-74*, are essential for nAChR response and [³H]MAL binding (Lewis et al., 1987b). The gene for *unc-50* has recently been cloned (M. O. Hengartner, N. Tsung, J. A. Lewis, and H. R. Horvitz, unpublished data), and it is not an nAChR subunit. The genes *lev-8*, *lev-9*, and *lev-10* are probably not major structural subunits of the levamisole nAChR, because mutants of these genes have relatively normal levamisole binding (Lewis et al., 1987b). *unc-63* is not predicted to encode an nAChR structural subunit because of the isolation of a rare allele (*b404*) that is markedly uncoordinated although still very sensitive to levamisole (Lewis et al., 1980b). The genetic loci identified so far have been discovered on the basis of uncoordination or levamisole resistance. Other mutant types that might identify additional genes important to receptor function, such as revertants or suppressors of the levamisole-resistant mutant phenotype, have yet to be sought extensively.

In conclusion, a number of genes affecting cholinergic neurotransmission have now been identified in *C. elegans* (Nonet et al., 1993; Alfonso et al., 1994; Arpagaus et al., 1994; Squire et al., 1995; Treinin and Chalfie, 1995; Miller et al., 1996a; Baylis et al., 1997) in addition to the novel nAChR subunit genes described in this work. The ease and rapidity of working with *C. elegans* make it well suited for the manipulation and expression *in vivo* of genes that are important to cholinergic synapse formation and function. Receptors from other organisms that are difficult to study, such as parasitic nematodes (e.g., *Onchocerca volvulus*), might be rendered more amenable to analysis by transgenic expression in *C. elegans* in place of the native nicotinic acetylcholine receptor.

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