acr-16 Encodes an Essential Subunit of the Levamisole-resistant Nicotinic Receptor at the Caenorhabditis elegans Neuromuscular Junction*

The Caenorhabditis elegans neuromuscular junction (NMJ) contains three pharmacologically distinct ionotropic receptors: γ-aminobutyric acid receptors, levamisole-sensitive nicotinic receptors, and levamisole-insensitive nicotinic receptors. The subunit compositions of the γ-aminobutyric acid- and levamisole-sensitive receptors have been elucidated, but the levamisole-insensitive acetylcholine receptor is uncharacterized. To determine which of the ~40 putative nicotinic receptor subunit genes in the C. elegans genome encodes the levamisole-resistant receptor, we utilized MAPCeL, a microarray profiling strategy. Of seven nicotinic receptor subunit transcripts found to be enriched in muscle, five encode the levamisole receptor subunits, leaving two candidates for the levamisole-insensitive receptor: acr-8 and acr-16. Electrophysiological analysis of the acr-16 deletion mutant showed that the levamisole-insensitive muscle acetylcholine current was eliminated, whereas deletion of acr-8 had no effect. These data suggest that ACR-16, like its closest vertebrate homolog, the nicotinic receptor α7-subunit, may form homeric receptors in vivo. Genetic ablation of both the levamisole-sensitive receptor and acr-16 abolished all cholinergic synaptic currents at the NMJ and severely impaired C. elegans locomotion. Therefore, ACR-16-containing receptors account for all non-levamisole-sensitive nicotinic synaptic signaling at the C. elegans NMJ. The determination of subunit composition for all three C. elegans body wall muscle ionotropic receptors provides a critical foundation for future research at this tractable model synapse.

Nicotinic acetylcholine receptors are a family of highly conserved pentameric channels used extensively in both vertebrate and invertebrate neurotransmission. These receptors have been implicated in memory formation, nociception, and nicotine addiction as well as in various neuronal disorders such as Parkinson disease and epilepsy (1). Most organisms express multiple nicotinic receptor subunits that combine to form receptors with diverse biophysical and pharmacological properties (2), based largely on receptor composition. The molecular mechanisms controlling nicotinic receptor subunit composition, assembly, trafficking, and localization remain to be fully elucidated.

One approach to this question is the application of molecular genetics to the simple organism Caenorhabditis elegans (3, 4). The most experimentally tractable and therefore best studied synapses in C. elegans are the body wall neuromuscular junctions (NMJs) (5–7). Individual C. elegans body wall muscles have both cholinergic and GABAergic inputs, which trigger contraction and relaxation, respectively. A single gene (unc-49) encodes the ionotropic GABA receptors present at the C. elegans NMJ (8–10). In contrast, the cholinergic receptors at the NMJ fall into two pharmacologically distinct classes (6). The most extensively studied class is activated by the nematode-specific acetylcholine receptor (AChR) agonist levamisole (4, 11). Through the analysis of levamisole-resistant mutants, the subunit composition of this receptor class has been established. In all, five subunits contribute to the levamisole response, including three essential subunits (UNC-38, UNC-29, and UNC-63) (6, 11, 12) and two nonessential subunits (LEV-1 and LEV-8) (4, 12, 13). Studies of the assembly, trafficking, and localization of C. elegans levamisole-sensitive receptors have provided significant insights into the molecular mechanisms that regulate this receptor (14–18). The second class of nicotinic receptor at the NMJ is levamisole-insensitive, and its subunit composition is unknown. Because this receptor class accounts for a major component of the synaptic response at the C. elegans NMJ, identifying the subunit composition is important for future studies that utilize this accessible model synapse. However, the C. elegans genome contains at least 40 genes that have sequence similarity to nicotinic receptor subunits (19), of which 27 appear to be authentic (20). Therefore, to identify the genes that encode the levamisole-insensitive nicotinic receptor subunits, we adopted a recently developed microarray profiling strategy, MAPCeL (micro-array profiling of C. elegans cells) (21). In summary, the microarray screen identified ACR-16, a nicotinic receptor α7-like subunit, which we confirmed by electrophysiological and genetic approaches to be

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an essential subunit of the non-levamisole receptor at the *C. elegans* NMJ.

**EXPERIMENTAL PROCEDURES**

**Strains**

Nematode strains were maintained at 20–25 °C using standard culture methods (3). The strains used in these studies were the wild-type Bristol isolate N2, DS83/uac-119(ed3) III, myo-3::GFP pPD2451 (ccOS4251) I (22), pacr-8::GFP (NC752/wdEx263), NC753/wdEx264), pacr-16::GFP (NC971/wdEx148), NC972/wdEx149), ZZ37::unc-63(x37) I, CB407::unc-49(e407) III, RB918::acr-16(ok789) V, and pmyo-3::ACR-16 (SY1023 [jaEx1023]). The double mutant SY1024::unc-63(x37);acr-16(ok789), SY1025::unc-49(e407) III;acr-16(ok789) V, and SY1026::unc-63(x37) I and the triple mutant SY1027::unc-63(x37);unc-49(e407);acr-16(ok789) were generated using standard genetic techniques.

**Microarray Analysis of myo-3::GFP-labeled Body Muscle Cells**

Detailed descriptions of MAPCeL, including *C. elegans* cell culture, fluorescence-activated cell sorting (FACS), and the microarray methods used in this work, are provided in Ref. 21. Primary in vitro cultures of *C. elegans* embryonic cells were established as described previously (23), with the exception that cells were plated on poly-L-lysine-coated surfaces to ease removal for FACS. myo-3::GFP-labeled cells were isolated by FACS after 24 h in culture. Sorting experiments were performed in a FACStar Plus flow cytometer (BD Biosciences). Nonviable cells were excluded by labeling with propidium iodide. Sorting gates were empirically established to achieve ~90% enrichment of myo-3::GFP-labeled muscle cells. 100 ng of RNA from myo-3::GFP cells (micro-RNA isolation kit, Stratagene) was amplified and labeled using the Affymetrix GeneChip eucaryotic small sample target labeling protocol with modifications as described previously (21). The Affymetrix *C. elegans* GeneChip array was hybridized with 15 μg of labeled mRNA. myo-3::GFP cells were profiled in triplicate. Reference microarray data were obtained from four independent experiments with 24-h cultures of all embryonic cells (from the wild type, N2) after sorting to exclude nonviable cells. Affymetrix hybridization signals were scaled in comparison with a global average value (24). To detect enriched mRNAs, intensity values were normalized by RNA (robust multi-array) analysis and statistically analyzed using SAM (significance analysis of microarrays) software (Stanford University). Muscle enriched ACHR subunit transcripts are defined as ACHR genes that are ≥1.7-fold elevated versus average expression in all cells at a false discovery rate of ≤1.2%. A comprehensive analysis of the muscle microarray data will be described elsewhere.

**GFP Reporters and Transgenic Animals**

*pacr-8::GFP*—A region 2460 bp upstream of the ATG start site was generated by PCR using with primers *acr-8p1* (5′-AAAGGTTTTG-CAGTCTTCAAGATTAC-3′) and *acr-8p2* (5′-GGAGCCTGAGAAGCTG-GAGTGAGAAG-3′). The *acr-8* PCR fragment and an *unc-119* minigene (from plasmid pd98051) (25) were subcloned into the GFP vector pPD985.75 to produce vector *acr-8::GFP-unc-119*. Four independent *acr-8::GFP* transgenic lines were generated by microparticle bombardment with the *acr-8::GFP-unc-119* plasmid into unc-119(ed3) animals. *pacr-16::GFP*—GFP reporters were generated using overlap PCR (26). The primers used for amplification of the GFP fragment have been described by Hobert (26). A region extending from 3000 bp upstream of the *acr-16* start site to 24 bp into exon 1 was PCR-amplified with primers *acr-16sp1* (5′-CACCCTGGTCCTCAGTAGTGAGTGG-3′) and *acr-16sp2* (5′-AGTTAGCTTGACGAGATGAGGGCGGAG-3′). The *acr-16* PCR fragment and an *unc-119* minigene (from plasmid pd98051) (25) were subcloned into the GFP vector pPD985.75 to produce vector *acr-16::GFP-unc-119*. 15 μl of PCR product was co-injected with 25 μg/ml plasmid pRF4 (rol-6). Five independent lines of *acr-16::GFP* were generated.

**Microscopy**

Images of GFP reporter expression were obtained using a Zeiss LSM 510 META confocal microscope. Cultured embryonic cells were photographed using a Zeiss Axiovert inverted microscope.

### References


### Tissue-specific Rescue

For *Pmyo-3::acr-16* genomic fusion, PCR was used to isolate the genomic version of *acr-16*. The sequence of the 5′-end PCR primer was agtacctctcatgctccggtatctgtgctt, and the sequence of the 3′-end PCR primer was agtacctcattgccgaagtagctggtg. BamHI and KpnI sites were introduced into the 5′- and 3′-end primers, respectively, for subsequent cloning. The PCR product was cloned into pPD96.52 downstream of the *myo-3* promoter. The final product was purified using a QIAprep spin mini prep kit and injected into the gonads of *acr-16(ok789)* mutants at a concentration of 5 μg/ml along with *pmyo-3::GFP* (20 μg/ml) as a co-injection marker. Five independent lines were generated. Rescue of the ACR-16 body wall muscle cholinergic response was assessed electrophysiologically.

### Deletion Mutants

Break points in *acr-8(ok1240)* were identified using the following primer pair: CACCGAGGAAATGGAGTGAAC and ACTCAGGCAACATCGTTTCC. The same primers were used for sequencing. Break points in *acr-16(ok789)* after a 2× outcross, identified by the *C. elegans* Knockout Consortium, were confirmed using the following primer pair: ACTCAGGCAACATCGTTTCC and TGTCTGAGACCCCGTGA. PCR products were cloned and sequenced with the same primer pairs at the University of Illinois Sequencing Facility.

### Behavioral Analysis

Behavioral assays were conducted on N2 and the *acr-16(ok789)*, *unc-63(x13)*, and *unc-63(x13);acr-16(ok789)* mutants. Thrashing behavior (7) for individual worms placed in M9 at 20 °C was measured in the number of head thrashes/min averaged over a 5-min period. Locomotion was scored for individual worms placed on an agar plate without a bacterial lawn and allowed to acclimatize for 1 min. The subsequent body bends were counted for the following minute. Head tap assays were also performed on worms acclimatized for 5 min on agar plates lacking bacterial lawns. An eyelash was used to gently tap the worm on the ventral surface of the head to elicit a head bend. The number of head bends was counted. Worms were filmed to ensure accurate scoring of body bends. A body bend is described as the movement in which the head of the worm completes a full sinusoid.

### Electrophysiology

Body wall muscle recordings were obtained from dissected worms as described previously (6). Briefly, worms were immobilized using a cyanoacrylate (Araldite) glue, and the cuticle was cut open longitudinally, exposing body wall muscles that were treated with collagenase to remove the basement membrane. Anterior ventral medial muscles were whole cell voltage-clamped at a holding potential of ~60 mV using an EPC-10 amplifier (HEKA Instruments Inc.). The extracellular solution consisted of 150 mM NaCl, 5 mM KCl, 1 mM CaCl2, 10 mM glucose, 5 mM Hepes (pH 7.3; ~350 mosm). Ligand applications (acetylcholine (ACh) and levamisole) were applied to voltage-clamped muscles via a pressure-ejection electrode. Evoked synaptic responses were obtained by depolarizing the ventral nerve cord using a stimulating electrode placed anterior to the body wall muscle as described previously (28). Subsequent analysis was carried out using Pulsefit (HEKA Instruments Inc.) and Mini Analysis (Synapsoft, Inc.) and graphed using IGOR Pro (Wavemetrics, Inc.). All statistically derived values obtained using InStat (GraphPad Software) are given as means ± S.E.

### RESULTS

**Microarray Experiments Identify Muscle Nicotinic AChR Subunit Genes**—We used a new microarray technology, MAPCeL (21), to identify nicotine receptors known preferentially expressed in body wall muscles. Muscle cells, labeled with the *myo-3::GFP* reporter gene, were obtained from *in vitro* cultures of *C. elegans* embryonic cells (Fig. 1). We have shown previously that *myo-3::GFP* cells differentiate in culture, display a spindle-shaped morphology resembling the body wall muscle cells in *vivo*, express muscle-specific genes, and exhibit largely normal physiological properties (23). In culture, *myo-3::GFP* cells constitute ~15% of all cells, which is comparable with their frequency in *vivo* (81 body muscle cells/550 total embryonic cells = 15%) (23). We used FACS to enrich *myo-3::GFP*
cells to ~90% (Fig. 2). mRNAs from these cells were amplified, labeled, and hybridized to the C. elegans Affymetrix GeneChip array. To identify the subset of genes specifically enriched in body muscle cells, we compared these results with microarray data obtained from all embryonic cells. A total of 945 genes from cultured myo-3::GFP cells were detected at or above a threshold of 1.7-fold enrichment versus an average expression level in all cells. Here, we focused on the nicotinic AChR subunit genes in the muscle enriched data set. As shown in Table I, we detected transcripts encoding all five of the known components of the levamisole-sensitive AChR, i.e. unc-29, unc-38, unc-63, lev-1, and lev-8. Two additional transcripts, acr-8 and acr-16, were also enriched.

To confirm expression of ACR-8 and ACR-16 in body wall muscles, we generated transgenic lines with acr-8::GFP and acr-16::GFP fusions. In both cases, we detected GFP signals in body wall muscles (Fig. 3). acr-8::GFP was strongly expressed in all body muscle cells as well as in anal and vulval muscles and ventral cord motor neurons (Fig. 3A). acr-16::GFP was detected in all body wall muscle cells (but not vulval or anal muscles) and was also present in a subset of neurons, notably the DB motor neurons in the ventral nerve cord (Fig. 3, B and C). Expression of both acr-8::GFP and acr-16::GFP was visible in the embryo.

The AChR α7-Like Subunit ACR-16 Is Expressed in Body Wall Muscle and Contributes to the Synaptic Response—Based

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**FIG. 1. Muscle profiling strategy.** Intact embryos are released from synchronized populations of adult myo-3::GFP hermaphrodites by treatment with hypochlorite solution. Eggshells are degraded by chitinase treatment, and embryos are dissociated by filtration. The resultant blastomeres are cultured in vitro for 24 h. myo-3::GFP muscle cells are enriched by FACS. RNA is isolated for application to the C. elegans Affymetrix GeneChip array.

**FIG. 2. FACS isolation of GFP-labeled body wall muscle cells.** A, shown is a fluorescence scatter plot of wild-type (non-GFP) cells. Propidium iodide (PI)-labeled cells are shown in red. The sorting gate in the lower right-hand corner excludes autofluorescent gut cells in the GFP channel. B, shown is a fluorescence scatter plot of cultured cells from myo-3::GFP embryos. Note the abundant GFP-labeled cells (green). C, GFP-positive cells were also gated within the circumscribed area (purple) to exclude large cell clumps and small debris. D, embryonic cells are shown after 24 h in culture and prior to FACS. E, myo-3::GFP cells in culture (~15%) were enriched to ~90% after sorting. Scale bars = 5 μm.
on the microarray data and expression patterns, we considered ACR-8 and ACR-16 to be potential subunits of the body wall muscle AChRs. To determine whether either of these subunits contributes to the levamisole-insensitive channel, we obtained deletion mutants of acr-8(ok1240) and acr-16(ok789) from the C. elegans Gene Knockout Consortium. The deletion break points and predicted protein disruption of acr-8(ok1240) and acr-16(ok789) are shown in Fig. 4.

To establish whether ACR-8 or ACR-16 contributes to the ACh response of the body wall muscles, we compared the ACh (5 × 10⁻⁷ M) response amplitudes of voltage-clamped body wall muscles in acr-8 and acr-16 mutants with that in the wild type. acr-8(ok1240) mutants exhibited wild-type responses to ACh (Fig. 5A). In contrast, the ACh response of acr-16(ok789) was reduced by 85% compared with that of the wild type (284 ± 29 pA (n = 9) for acr-16(ok789) versus 1898 ± 206 pA (n = 7) for N2; p < 0.0001) (Fig. 5A). To examine whether the levamisole receptors were affected in acr-16(ok789) mutants, we recorded muscle levamisole responses. Levamisole-induced currents in acr-16(ok789) mutants were, however, indistinguishable from those in the wild type (Fig. 5B). This result suggests that ACR-16 is a required component of the levamisole-insensitive AChR in the ventral body wall muscles. Because acr-8(ok1240) mutants showed no reduction in either ACh or levamisole responses, ACR-8 is unlikely to contribute to either of the two electrophysiologically identified AChR classes in body muscle.

To determine the extent to which ACR-16 contributes to the synaptic response at the NMJ, we recorded evoked responses in acr-16(ok789) mutants. The evoked response was reduced by 83% in acr-16(ok789) mutants compared with wild-type worms (Fig. 6A), indicating that ACR-16 is present at postsynaptic sites and contributes to the synaptic response of the body wall muscles.

To confirm that the defects observed in acr-16(ok789) mutants are specifically due to loss of ACR-16 in body wall muscles, we expressed acr-16 genomic DNA fused to the muscle-specific promoter Phmyo-3 along with a Phryo-3::GFP co-injection marker in acr-16(ok789) mutants. Transgenic lines were generated in which the expression of ACR-16 and the GFP co-injection marker was mosaic. Muscle cells that inherited the acr-16 rescuing construct could then be detected as GFP-positive cells. GFP-fluorescing muscles exhibited robust responses to both pressure-ejected ACh (Fig. 6B, right trace) and evoked stimulation (Fig. 6C, right trace), whereas non-fluorescing cells showed responses typical of acr-16(ok789) mutants (Fig. 6, B and C, left traces). These data indicate that the reduced muscle ACh and evoked responses of acr-16(ok789) mutants are due to the deletion of the acr-16 gene locus in muscles and rule out the possibility that a background mutation in the acr-16(ok789) strain produced the electrophysiological phenotype.

**Table 1. Summary of the seven nicotinic receptor subunits identified by MAPCeL**

<table>
<thead>
<tr>
<th>AChR subunit</th>
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<td>Lev-sensitive</td>
<td>+</td>
<td>Motor neurons</td>
</tr>
<tr>
<td>unc-38</td>
<td>1.8</td>
<td>Lev-sensitive</td>
<td>+</td>
<td>Motor neurons</td>
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<td>Lev-sensitive</td>
<td>+</td>
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<td>Lev-sensitive</td>
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<td>+</td>
<td>Motor neurons</td>
</tr>
<tr>
<td>acr-16</td>
<td>2.5</td>
<td>Lev-insensitive</td>
<td>+</td>
<td>Motor neurons</td>
</tr>
</tbody>
</table>

* From microarray data in this work.

**Summary of the seven nicotinic receptor subunits identified by MAPCeL.**

**Fig. 3.** GFP reporters for acr-8 and acr-16 are expressed in body wall muscle cells. A, head region showing acr-8::GFP expression in body muscles. B, combined differential interference contrast and GFP images of acr-8::GFP expression in body muscle cells. The spiral disposition of the body muscle cells is due to the Rol-6 transgenic marker. C, ventral view of the midbody region. acr-16::GFP was expressed in body muscles and in DB motor neurons (arrowheads) in the ventral nerve cord. All images are confocal projections. Scale bars = 10 μm.

**Fig. 4.** acr-16 and acr-8 encode predicted ionotropic receptor subunits. A and C, genomic organization of acr-16 and acr-8, respectively. Shaded boxes represent predicted coding regions. Open boxes represent deleted regions in acr-16(ok789) and acr-8(ok1240). Predicted protein structures of ACR-16 (B) and ACR-8 (D). TM1–TM4 represent the four transmembrane domains, the open boxes indicate the deleted regions in the acr-16(ok789) mutant (amino acids 165–329) (B) and the acr-8(ok1240) mutant (an early stop codon at amino acid 152) (D).
**FIG. 5.** acr-16(ok789) mutants reduce levamisole-resistant muscle ACh responses. A, the representative current traces of voltage-clamped body wall muscle ACh responses (100-ms pulses of $5 \times 10^{-4}$ M ACh) demonstrate that acr-16(ok789) mutants, but not acr-8(ok1240) mutants, reduced ACh current amplitudes compared with the wild type (WT). Plots of the average ACh current amplitude of acr-16(ok789) mutants demonstrate a reduction of $\sim 85\%$ in current amplitude compared with the wild type ($n = 10$ for acr-16 and $n = 10$ for the wild type ($p < 0.0001$) and $n = 3$ for acr-8(ok1240)). B, shown are the responses to 100-ms pulses of $5 \times 10^{-4}$ M levamisole (Lev). Levamisole current amplitudes were unaffected in acr-8(ok1240) and acr-16(ok789) mutants. ***, $p \leq 0.0002$.

**FIG. 6.** Cholinergic synaptic activity reduced in acr-16(ok789) is rescued by muscle-specific expression of ACR-16. A, the evoked synaptic current elicited in voltage-clamped muscle cells by ventral nerve cord depolarization was reduced in acr-16(ok789) mutants by 83% as shown in representative traces and average evoked amplitudes ($n = 4$ for acr-16 and $n = 10$ for the wild type (WT); $p = 0.0005$). B and C, transgenic lines expressing ACR-16 under the control of the muscle-specific myo-3 promoter rescued the responses to both pressure-ejected ACh ($5 \times 10^{-4}$ M) ($n = 10$ for acr-16 and $n = 5$ for Pmyo-3::acr-16; $p < 0.0001$) and evoked release ($n = 4$ for acr-16 and $n = 4$ for Pmyo-3::acr-16; ***, $p = 0.0001$), respectively.
for levamisole), suggesting that, in acr-16 mutants, only the levamisole-sensitive receptors are still functional. To test this hypothesis, we generated unc-63(ok789);acr-16(ok789) double mutants. unc-63 encodes an essential α-subunit of the levamisole-sensitive receptor; we have shown previously that the levamisole response is abolished in unc-63(x37) mutants (12). The muscle ACh current in unc-63(x37);acr-16(ok789) double mutants was eliminated (Fig. 7A). Therefore, we can conclude that the nicotinic receptors on body wall muscle activated by pressure-ejected ACh contain either UNC-63 (levamisole-sensitive) or ACR-16 (levamisole-insensitive) subunits. Thus, unc-63(x37) and acr-16(ok789) mutants can be used to genetically isolate the two muscle AChR subtypes. We have shown previously that, in the absence of levamisole-sensitive AChRs, desensitization of the remaining muscle ACh response is faster, suggesting that the two receptor subtypes have different kinetic properties (12). We confirmed this observation by examining the rates of desensitization to 1-s ACh pulses in unc-63(x37) and acr-16(ok789) mutant muscles. The levamisole-sensitive receptors present in acr-16(ok789) mutants were shown to desensitize at a much slower rate than the levamisole-insensitive receptors present in unc-63 mutants (Fig. 7B). Together, these data indicate that the two muscle receptor subtypes have different subunit compositions, pharmacology, and kinetics.

Exogenous ACh application allowed us to define two classes of nicotinic receptors on the muscle membrane. This analysis did not address whether these two receptors account for all cholinergic synaptic current at the NMJ. To specifically examine the post-synaptic nicotinic receptors, we needed to first isolate the cholinergic synaptic responses from GABAergic synaptic events at the NMJ. To specifically examine the contribution of ACR-16-dependent receptors to cholinergic synaptic activity, we generated unc-49(e407);acr-16(ok789) double mutants. unc-49(e407);acr-16(ok789) mutants exhibited prominent reductions in both endogenous and evoked cholinergic transmission at the NMJ (Fig. 8) compared with unc-49(e407) single mutants. We also examined the impact of removing the levamisole-sensitive receptors by making unc-63(x37);unc-49(e407) double mutants. The evoked and endogenous responses were not significantly reduced in unc-63(x37);unc-49(e407) double mutants (Fig. 8) compared with unc-49(e407) alone. To examine whether all cholinergic synaptic activity could be eliminated in the absence of both nicotinic receptor types, we generated unc-63(x37);unc-49(e407);acr-16(ok789) triple mutants. The endogenous miniature synaptic currents and evoked responses were completely abolished in unc-63(x37);unc-49(e407);acr-16(ok789) triple mutants (Fig. 8).
Thus, we conclude that, in combination with UNC-63-containing receptors, ACR-16-containing receptors account for all cholinergic ionotropic responses in these muscles.

These data suggest that both levamisole-sensitive and levamisole-insensitive receptors contribute to the excitability of body wall muscles in *C. elegans*. Therefore, we predict that *unc-63(x37);acr-16(ok789)* double mutants would have a more severe locomotory defect than the two single mutants. Several behavioral assays were conducted to test this prediction. In all assays, *unc-63(x37);acr-16(ok789)* double mutants exhibited more severe locomotory defects than *unc-63(x37)* mutants alone (Fig. 9), demonstrating that both receptors contribute to *C. elegans* locomotion.

**DISCUSSION**

Using a newly designed microarray profiling strategy (MAP-Cell) combined with reverse genetics and *in situ* electrophysiological analysis, we have identified and characterized ACR-16 as an essential subunit of the levamisole-insensitive nicotinic receptor at the *C. elegans* NMJ. This study highlights the utility of microarray profiling to identify novel genes of interest in *C. elegans*. In this study, genetic ablation of *acr-16* produced no overt behavioral phenotype (Fig. 9). This observation explains why *acr-16* mutants have not been isolated in previous genetic screens that selected for uncoordinated or pharmacological phenotypes. By selectively profiling body muscle cells, we narrowed our search for components of the levamisole-insensitive receptor from ~40 potential subunit genes to only two candidates, *acr-8* and *acr-16*. The *acr-8* deletion had no effect on either the levamisole-sensitive or levamisole-insensitive components of the muscle ACh response. Therefore, we ruled out ACR-8 as an essential subunit of either nicotinic receptor class in the ventral body wall muscles assayed in this study. Examination of the pore-forming M2 domain of ACR-8 in which a highly conserved glutamic acid is replaced by histidine suggested that this subunit may actually contribute to an anion-selective channel of yet unknown function (20). This finding left only one remaining candidate, ACR-16, which we have now demonstrated is an essential subunit of the body wall muscle levamisole-insensitive AChR. With the identification of *acr-16*, we can genetically ablate any of the three neurotransmitter receptor components that contribute to the synaptic readout at the NMJ. Thus, we can now study the physiology and regulation of each receptor class in isolation.

Our finding that ACR-16 is the only muscle enriched subunit that is required for levamisole-insensitive nicotinic responses leads to the possibility that ACR-16 assembles into a homomeric receptor in *C. elegans* body wall muscles. ACR-16 is an α-subunit with 47% homology to the vertebrate nicotinic receptor α7-subunit (29). The vertebrate α7-subunit is capable of forming functional homomeric nicotinic receptors in several cells, including *Xenopus* oocytes, PC12 cells, and rat brain (30–32). Similarly, ACR-16 (previously termed Ce21) has been
shown to form functional nicotinic receptors when expressed in *Xenopus* oocytes (29). By identifying ACR-16 as an essential subunit of the body wall muscle levamisole-insensitive receptor, we are now able to directly compare previous heterologous expression data with those of the native ACR-16-containing receptor. The pharmacological profile of heterologously expressed ACR-16 receptors closely resembles that of the native receptor in situ. Specifically, both heterologously expressed and native ACR-16-dependent receptors are activated by nicotine and ACh, but not levamisole, and maximal responses to nicotine are smaller than those to ACh (∼70% of ACh [29]). Although we cannot fully exclude the possibility that other subunits contribute to the native ACR-16-containing receptor, the present data suggest that ACR-16 may function as a homomeric nicotinic receptor α7-like subunit in the *C. elegans* body wall muscles.

Vertebrate α7-subunit-containing nicotinic receptors are present in diverse areas of the human brain (33) and are involved in higher cognitive functions, including learning and memory (34–36). Alterations in α7-subunit-containing nicotinic receptor signaling have been linked to several neuropathologies, including Parkinson disease and epilepsy (1) and Alzheimer disease (37). α7-Subunit-containing nicotinic receptors are localized presynaptically and postsynaptically (38, 39) in a variety of synapses, yet the molecular mechanisms responsible for this targeted expression remain to be elucidated. Agonist-dependent trafficking of these receptors appears to be regulated by several pathways both on a short time scale via SNARE (soluble NSF attachment protein receptor) protein-mediated endocytosis (40) and on longer time scales (41, 42). By identifying a homologous receptor in *C. elegans*, it is anticipated that mutations that disrupt the localization and trafficking of ACR-16 will provide insights into the regulation of vertebrate nicotinic receptors, including the α7-subunit-containing receptors.

ACR-16-dependent receptors account for 85% of both the muscle exogenous Ach response and the evoked response elicited by ventral nerve cord stimulation. Surprisingly, acr-16 null mutants show no obvious behavioral phenotype. This is in contrast to mutants of the levamisole-sensitive receptor subunits, unc-29, unc-38, and unc-63, which exhibit defective locomotion and exaggerated body bends. Because the double mutants have a more severe locomotory phenotype than either unc-63 or acr-16 alone, we can conclude that both receptor subtypes contribute to neuromuscular transmission. However, these data establish that the levamisole-sensitive receptors play a greater functional role in locomotion. The predominant role of the levamisole-sensitive receptor in locomotion could reflect the observed differences in desensitization rates of the levamisole-sensitive receptor (slower) compared with the ACR-16-dependent receptor (faster). This difference may enable levamisole-sensitive receptors to be more efficiently coupled to the excitation-contraction machinery downstream of AChR activation. For example, the levamisole-sensitive receptors may be more effective in activating voltage-gated calcium channels or releasing calcium from intracellular stores.

Although cholinergic synaptic activity is eliminated at the NMJs of unc-63(x37);acr-16(ok789) double mutants, these worms exhibit residual movement as seen in thrashing assays...
C. elegans ACR-16 Nicotinic Receptor α7-Like Subunit

and locomotion off food. This suggests that some muscle contraction persists in the absence of nicotinic responses. The source of this excitatory input has yet to be identified and could reflect muscarinic, peptidergic, co-transmitter signaling, or myogenic activity.

In summary, we have relied upon microarray results derived from body muscle cells to demonstrate that ACR-16 is a subunit of the levamisole-insensitive nicotinic receptor that functions at NMJs. We have shown that genetic ablation of this subunit abolishes the non-levamisole cholinergic response in C. elegans NMJs. We have shown that genetic ablation of this subunit abolishes the non-levamisole cholinergic response in body wall muscles. The identification of this receptor subunit provides new avenues of exploration for the differential trafficking and regulation of two distinctly targeted nicotinic receptors within the same muscle cells in a genetically tractable organism.

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acr-16 Encodes an Essential Subunit of the Levamisole-resistant Nicotinic Receptor at the Caenorhabditis elegans Neuromuscular Junction

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