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## Activation of Single Nicotinic Receptor Channels from *Caenorhabditis elegans* Muscle

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## Abstract

Nicotinic acetylcholine receptors (nAChRs) are pentameric neurotransmitter-gated ion channels that mediate synaptic transmission throughout the nervous system in vertebrates and invertebrates. *Caenorhabditis elegans* is a non-mammalian model for the study of the nervous system as well as a model of parasitic nematodes. Nematode muscle nAChRs are of considerable interest as they are targets for anthelmintic drugs. We show single-channel activity of *C. elegans* muscle nAChRs for the first time. Our results reveal that in the L1 larval stage ACh activates mainly a levamisole-sensitive nAChR (L-AChR). A single population of 39 pS channels, which are 5-fold more sensitive to levamisole than ACh, is detected. In contrast to mammalian nAChRs, open durations are longer for levamisole than for ACh. Studies in mutant strains reveal that UNC-38, UNC-63 and UNC-29 subunits are assembled into a single L-AChR in the L1 stage and that these subunits are irreplaceable, suggesting that they are vital for receptor function throughout development. Recordings from a strain mutated in the LEV-1 subunit show a main population of channels with lower conductance (26 pS), prolonged open durations, and reduced sensitivity to levamisole. Thus, although LEV-1 is preferentially incorporated into native L-AChRs, receptors lacking this subunit can still function. No single-channel activity from levamisole-insensitive nAChRs is detected. Thus, during neuromuscular transmission in *C. elegans* the majority of ACh-activated current flows through L-AChRs. This study contributes to the understanding of the molecular mechanisms underlying functional diversity of the nAChR family and offers an excellent strategy to test novel antiparasitic drugs.

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Nicotinic acetylcholine receptors (nAChR) mediate fast synaptic transmission throughout the nervous system. A large number of nAChR subunits have been cloned from both vertebrates and invertebrates (Jones et al., 2003). Intriguingly, the free-living helminth *Caenorhabditis elegans* has one of the largest nAChR gene families known (Jones and Sattelle, 2003).

nAChR subunits are classified as  $\alpha$ , which contain a disulphide bridge involved in the binding of agonists, and non- $\alpha$ , which lack this motif. Receptors are pentameric proteins that can be either heteromeric, composed of  $\alpha$  and non- $\alpha$  subunits, or homomeric, composed of five identical  $\alpha$  subunits. Nematode muscle nAChRs are of considerable interest as they are targets for antiparasitic drugs. These drugs behave as full agonists of nematode nAChRs, thus producing muscle paralysis (Martin, 1997). Based on their sensitivity to levamisole, two different types of muscle nAChRs have been described in adult *C. elegans*: L-AChR, or levamisole-sensitive, and N-AChR, which is levamisole-insensitive and nicotine-sensitive (Richmond and Jorgensen, 1999; Culetto et al., 2004; Touroutine et al., 2005). Although several subunits corresponding to each nAChR subtype have been identified, their stoichiometry and activation kinetics remain unknown. UNC-38, UNC-63 and LEV-8 subunits, which are  $\alpha$  subunits, and LEV-1 and UNC-29, which correspond to non- $\alpha$  subunits, are components of the adult *C. elegans* L-AChR. UNC-38, UNC-63 and UNC-29 have been shown to be essential for activation of L-AChRs in adult worms (Fleming et al., 1997; Richmond and Jorgensen, 1999; Culetto et al., 2004; Towers et al., 2005). How these subunits are assembled into functional receptor(s) is still not known. To date, only one subunit, ACR-16, has been reported as a component of the N-

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AChR (Touroutine et al., 2005). ACR-16 is capable of forming homomeric receptors in *Xenopus* oocytes (Ballivet et al., 1996).

In this study, we explore for the first time at the single-channel level the activation properties of nAChRs from *C. elegans* muscle. We used a primary culture system that allows differentiation of embryonic cells into L1 larva muscle cells *in vitro* (Christensen et al., 2002). Our results reveal that levamisole shows an extremely high efficacy for channel activation. The levamisole-activated receptors are the main detected channels, indicating that the majority of current flows through L-AChRs during neuromuscular transmission. No N-AChR activity can be detected in cell-attached patches. Single-channel recordings from mutant strains reveal that, as in the adult stage, UNC-38, UNC-63 and UNC-29 are required to obtain functional L-AChRs, whereas LEV-1 can be replaced by other subunits.

Single-channel studies allow the elucidation of activation properties, composition, and functional roles of nAChRs. Because *C. elegans* is a model of parasitic nematodes, these studies will contribute to the understanding of how parasites acquire resistance to anthelmintics and to the development of novel therapies. Moreover, due to its available genome sequence *C. elegans* has become an invertebrate model of the human nervous system and therefore studies in this nematode are proving valuable in understanding processes involving nAChRs in mammals.

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## Materials and Methods

### *C. elegans* Strains

All nematode strains were obtained from the *Caenorhabditis* Genetic Center, which is funded by the NIH National Center for Research Resources (NCRR). The following strains were used: N2 wild type (Bristol variety), *myo-3::GFP* PD4251(*ccls4251I*) (Fire et al., 1998), CB904 *unc-38(e264) I*, CB1072 *unc-29(e1072) I*, ZZ37 *unc-63(x37) I*, RB918 *acr-16(ok789) V* and CB211 *lev-1(e211) IV*. Nematodes were maintained at 20-25 °C using standard culture methods (Brenner, 1974). The RB918 strain has not been outcrossed and it therefore carries other mutations. However, previous reports have shown reduced ACh-responses due to the lack of muscle N-AChRs in adult worms, which can be rescued by muscle-specific expression of ACR-16 (Touroutine et al., 2005). Thus, it is possible to ensure that the lack of function of N-AChRs in this strain is due to the deletion of the *acr-16* gene and not to the presence of background mutations (Touroutine et al, 2005; Francis et al, 2005).

### *Isolation and culture of C. elegans muscle cells*

Embryonic cells were isolated and cultured as described by Christensen et al. (2002). Briefly, adult nematodes were exposed to an alkaline hypochlorite solution (0.5 M NaOH and 1 % NaOCl). Eggs released were treated with 1.5 units/ml chitinase (Sigma-Aldrich Co., St. Louis, MO) for 30-40 minutes at room temperature. The embryo cells were isolated by gently pipetting and filtered through a sterile 5- $\mu$ m Durapore syringe filter (Millipore Corp., Bedford, MA) to remove undissociated embryos and newly hatched larvae. Filtered cells were plated on glass coverlips coated with poly-O-Ornithine. Cultures were maintained at 24 °C in a humidified incubator in L-15 medium (Hyclone, Logan, UT)

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containing 10 % fetal bovine serum. Complete differentiation to the various cell types that comprise the newly hatched L1 larva were observed within 24 hs. Electrophysiology experiments were performed 1-5 days after cell isolation.

The percentage of neuron and muscle cells in culture is in great agreement with previous reports, and it is similar to that observed in the newly hatched L1 larva (Christensen et al., 2002).

PD4251 strain produces green fluorescence protein (GFP) in body wall muscle cells, thus allowing their identification under fluorescence optics (Fire et al., 1998). Muscle cells are easily identifiable due to their spindle-shaped morphology that resembles the body wall muscle cells *in vivo* (Christensen et al., 2002; Yuan et al., 2003; Touroutine et al., 2005). Therefore, in other strains muscle cells were recognized by their distinctive morphology, which was similar to that of green cells of the PD4251 strain (Yuan et al., 2003).

#### *Single-channel recordings*

Recordings were obtained in the cell-attached patch configuration (Hamill et al., 1981) at 20°C essentially as described previously (Bouzat et al., 1994, 2000). The bath and pipette solutions contained 142 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl<sub>2</sub>, 1.7 mM MgCl<sub>2</sub>, and 10 mM HEPES (pH 7.4). Acetylcholine or anthelmintic agents were added to the pipette solution. Single-channel currents were recorded using an Axopatch 200 B patch-clamp amplifier (Axon Instruments, Inc., CA), digitized at 5 μs intervals with the PCI-6111E interface (National Instruments, Austin, TX), recorded to the hard disk of a computer using the program Acquire (Bruyton Corporation, Seattle, WA), and detected by the half-amplitude threshold criterion using the program TAC 4.0.10 (Bruyton Corporation, Seattle,

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WA) at a final bandwidth of 10 kHz. Open and closed time histograms were plotted using a logarithmic abscissa and a square root ordinate and fitted to the sum of exponentials by maximum likelihood using the program TACFit (Bruxton Corporation, Seattle, WA).

Experimental data are shown as mean  $\pm$  S.D. Statistical comparisons were done using the Student's *t* test. A level of  $p < 0.05$  was considered significant.

#### *RT-PCR for ACR-16*

Total RNA was isolated from synchronized L1 and adult wild-type nematodes by the acid guanidium-phenol-chloroform method. RNA was converted into cDNA using the Molony murine leukaemia virus reverse transcriptase (MLV-RT; Promega, Madison, WI) and random primers (Promega, Madison, WI). Polymerase chain reaction (PCR) was run for 35 cycles in a Mini Cycler<sup>tm</sup> (MJ Research, Reno, NV). Specific primers for PCR were designed to prime in different exons of the *acr-16* gene to differentiate by length cDNA amplification from genomic DNA amplification. The primers used were: sense primer 5'CGTCACTCGGAATCATTGATCC 3' (Exon 9) and antisense primer 5'GCGACAAGATACGGTGCTGACC 3' (Exon 10). A 375 bp RT-PCR product was expected.



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## Results

### *Single-channel currents from L1 muscle cells activated by ACh*

To explore activity of nAChRs from *C. elegans* muscle cells at the single-channel level we used a cell culture technique that allows embryonic cells to differentiate *in vitro*. Cultured cells correspond to the L1 developmental stage (Christensen et al., 2002). We first studied the PD4251 strain, which contains wild-type nAChRs. Single channels activated by ACh (0.5-1000  $\mu$ M) are readily detected in cell-attached patches from muscle cells (Figs. 1 and 2). In contrast, no opening events are observed in the absence of agonist (n=14). ACh-activated channels exhibit a single conductance of  $38.7 \pm 1.6$  pS at positive membrane potentials (Fig. 1).

The minimum ACh concentration that allows channel detection is 0.5  $\mu$ M. The percentage of active patches increases from 26.6 % at 0.5  $\mu$ M (n=15) to 86.6 % at 10  $\mu$ M ACh (n=15). The frequency of channel openings increases as a function of ACh concentration. The number of opening events per second, measured within the first minute of recording, increases from  $376 \pm 112$  to  $1184 \pm 170$  when ACh concentration increases from 1 to 10  $\mu$ M (Fig. 2).

At 0.5-10  $\mu$ M ACh open time distributions are well described by the sum of two exponential components (Fig. 2; Table 1). The durations of these components but not their fractional areas are independent of ACh concentration within the 0.5-10  $\mu$ M range (Fig. 2). At 1  $\mu$ M ACh the fractional area of the briefest component is significantly higher than that observed at 10  $\mu$ M ( $0.90 \pm 0.10$  and  $0.40 \pm 0.10$  for 1  $\mu$ M and 10  $\mu$ M ACh, respectively;  $p < 0.05$ ). This result indicates that brief openings correspond to receptors with incomplete occupation of the binding sites. At ACh concentrations higher than 10  $\mu$ M, flickering

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block occurs, which is evidenced by the presence of brief closings interrupting channel openings (Fig. 2). At 300  $\mu\text{M}$  ACh, open time histograms can be correctly fitted by a single component whose duration is 4-fold briefer than that of the main open component at 10  $\mu\text{M}$  ( $p < 0.05$ ; Table 1).

Closed time distributions can be well fitted by two or three exponential components (Fig. 2; Table 1). The duration of the slowest closed component systematically decreases with ACh concentration up to 50  $\mu\text{M}$  (18-fold between 1  $\mu\text{M}$  and 50  $\mu\text{M}$  ACh;  $p < 0.01$ ). This observation is due to the increase in channel activity as a function of agonist concentration (Fig. 2). The duration of the briefest closed component and its relative area remain constant between 0.5 and 10  $\mu\text{M}$  ACh ( $p > 0.05$ ) (Table 1). This component may represent brief closures during single activation, as described before for vertebrate and *Ascaris* nAChRs (Colquhoun and Sakmann, 1985; Evans and Martin, 1996). At higher ACh concentrations closed time distributions show an increase in the area of the briefest closed component, due to the concentration-dependent increase of brief closures corresponding to blocked periods ( $0.10 \pm 0.04$  and  $0.45 \pm 0.01$  at 10 and 300  $\mu\text{M}$  ACh, respectively;  $p < 0.05$ ). In the histograms, these brief closures cannot be distinguished from the brief closures corresponding to activation.

The concentration-dependent decrease in the mean open time, the concentration-dependent increase in the fractional area of the brief closed component, and the constant mean duration of the brief blocked intervals across all blocker concentrations (Table 1), are indicative of open-channel block produced by ACh (Neher and Sakmann, 1978; Rayes et al., 2001).

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In contrast to the behavior of mammalian muscle nAChRs (Bouzat et al., 2000), clusters of activation periods cannot be clearly distinguished at any ACh concentration (Fig. 2).

Given that macroscopic current recordings have shown that ACh activates both L- and N-AChR subtypes in adult *C. elegans* muscle (Richmond and Jorgensen, 1999) we evaluated the nAChR subtype(s) involved in the channel activity detected at the L1 stage. To this end, we measured channel activity in the presence of 10  $\mu$ M dihydro- $\beta$ -erythroidine (DH $\beta$ E), which has been reported to selectively antagonize the N-AChR subtype (Richmond and Jorgensen, 1999; Martin et al., 2003). The conductance of ACh-activated channels recorded in the presence of DH $\beta$ E is not significantly different from that obtained in the absence of this drug ( $p > 0.05$ ) (Fig. 1B; Table 1). Moreover, no significant changes in channel frequency (data not shown), mean open and mean closed times are observed in the presence of DH $\beta$ E (Table 1), thus indicating that the detected channels do not correspond to the N-AChR subtype.

No changes in channel activity were observed in cultured muscle cells between 1 and 5 days after plating. In addition, cell morphology remained constant during this interval. Longer times of culture could not be tested because cells detach from the dish after 7 days and cannot be used for single-channel recordings.

#### *Single-channel currents activated by levamisole*

Although whole-cell recordings revealed that levamisole activates the muscle L-AChR subtype in *C. elegans* (Richmond and Jorgensen, 1999; Touroutine et al., 2005), no single-channel data describing this activation was available to date. At positive membrane

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potentials, levamisole activates single-channel currents of  $36.9 \pm 0.8$  pS ( $n=34$ ) (Fig. 1B). This conductance value is similar to that obtained from ACh-activated patches ( $p>0.05$ ), thus suggesting that channels detected with levamisole and ACh correspond to the same nAChR subtype. nAChR activity is observed at levamisole concentrations higher than 100 nM (Fig. 3). This concentration is 5-fold lower than the minimum ACh concentration required for channel detection, indicating that this anthelmintic agent is indeed a more potent agonist than the endogenous neurotransmitter. At 100 nM levamisole open time distributions can be correctly fitted by two components (Fig. 3; Table 1). The duration of the second open component is significantly longer than that of ACh-activated channels.

As described for ACh, the mean open time decreases as a function of levamisole concentration. At levamisole concentrations higher than 1  $\mu$ M open time histograms are fitted by a single open component (Fig. 3; Table 1). Such concentration-dependent reduction is typical of an open channel blocker.

Closed time histograms can be well fitted by several components. The duration of the briefest closed component is similar to that of ACh-activated channels (Table 1). The duration of the main closed component, C2, decreases with the concentration of levamisole, indicating an increase in channel activity as a function of levamisole concentration. Although the decrease in open duration reveals that levamisole is an open-channel blocker, neither flickering nor a new closed component corresponding to blocked periods can be detected. This result can be explained by a slow dissociation of levamisole from the channel, making blocked periods too long to be clearly distinguished (Papke and Oswald, 1989; Rayes et al., 2001).

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In agreement with the behavior of channels activated by ACh, no clusters are observed at a wide range of levamisole concentration (100 nM-1 mM).

*Single-channel currents activated by pyrantel and morantel*

To further characterize muscle nAChRs from *C. elegans*, we evaluated activation by other widely used anthelmintic agents, pyrantel and morantel. Both drugs elicit single-channel activity when added to the pipette solution (Fig. 4). At -100 mV, the amplitudes of the unitary currents are  $3.80 \pm 0.30$  pA (n=18) and  $3.60 \pm 0.20$  pA (n=16) for pyrantel- and morantel-activated channels, respectively. These values are similar to those of ACh- and levamisole-activated nAChRs ( $p > 0.05$ ), suggesting that the channels correspond to the same nAChR subtype.

nAChR activity is detected at 1 nM of either pyrantel or morantel in 80 % of the seals, indicating that both anthelmintic agents are potent agonists. Open time distributions of 10 nM pyrantel-activated nAChRs can be well fitted by a main component of  $200 \pm 10$   $\mu$ s (relative area  $> 0.9$ ) and a minor component of  $810 \pm 20$   $\mu$ s (n=4) (Fig. 4). Increasing 100-fold pyrantel concentration (1  $\mu$ M) leads to an increase in the fractional area of the slowest open component together with a reduction in its duration ( $\tau_2 = 510 \pm 50$   $\mu$ s,  $p < 0.05$ , area =  $0.48 \pm 0.10$ ,  $p < 0.01$ ). Again, the concentration-dependent increase in the relative area of the slowest open component can be explained by the fact that it probably arises from activity of fully occupied nAChRs. On the other hand, the reduction in the mean duration as a function of pyrantel concentration is due to open-channel block.

The behavior of morantel-activated channels is very similar to that of pyrantel-activated channels. At 10 nM morantel, open time histograms can be well fitted by two

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components of  $170 \pm 20 \mu\text{s}$  (relative area  $0.83 \pm 0.10$ ) and  $510 \pm 100 \mu\text{s}$  ( $0.16 \pm 0.09$ ) ( $n=3$ ) (Fig. 4). At concentrations higher than  $1 \mu\text{M}$  open time distributions are displaced to briefer durations ( $100 \pm 10 \mu\text{s}$  at  $50 \mu\text{M}$  morantel,  $n=3$ ;  $p<0.05$ ), revealing open-channel block (Fig.4).

As described for levamisole, no flickering block is observed for either pyrantel or morantel-activated nAChRs, suggesting that the three anthelmintic drugs produce a slow open-channel block of *C. elegans* muscle nAChR channels.

#### *Single-channel currents from unc-38, unc-29, unc-63 and acr-16 null mutants*

In order to clearly identify the nAChR subtype detected in the L1 muscle cells and to determine how the different subunits assemble into functional receptors, we evaluated channel activity from *unc-38*, *unc-63*, *unc-29* null mutants. These mutants exhibit impaired locomotion especially at early larval stages. We also evaluated *acr-16* null mutants, which show no evident movement defects at any developmental stage (Lewis et al., 1980; Richmond and Jorgensen, 1999; Touroutine et al., 2005).

ACh or levamisole ( $0.1$ - $1000 \mu\text{M}$ ) are not capable of activating unitary currents in cell-attached patches of L1 muscle cells from *unc-29* ( $n=18$ ), *unc-38* ( $n=23$ ) and *unc-63* ( $n=19$ ) null mutant embryos (Fig. 5). This result strongly reveals that the active nAChR detected in *C. elegans* muscle is composed of UNC-29, UNC-38 and UNC-63 subunits, and that these subunits are essential for channel activity in the L1 developmental stage. Moreover, we also performed experiments in the *unc-63* null mutant using morantel and pyrantel as agonists. No channel activity was observed at a range of pyrantel ( $10$ - $50 \mu\text{M}$ ,  $n=11$ ) and morantel

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(10-300  $\mu$ M, n= 16) concentrations. These results confirm that all anthelmintic agents activate the same type of nAChR.

With the aim of detecting channel activity from the N-AChR subtype and given that no L-AChR activity is observed in muscle cells from these three null mutants, we performed recordings with nicotine (10-1000  $\mu$ M) in the pipette solution. No single-channel currents could be detected in 26 patches. Given that it has been postulated that the N-AChR subtype desensitizes at a much faster rate than the L-subtype (Richmond and Jorgensen, 1999; Touroutine et al., 2005) we also performed cell-attached patches using pipettes in which the tip was filled with buffer (without agonist) and the shaft was filled with nicotine at different concentrations. With rapid sealing, this would allow detection of channel openings before complete desensitization. However, no channels could be detected under this condition in 10 different seals. In contrast, channel activity from wild-type cells appeared 2-4 minutes after the beginning of the recording, thus confirming that the strategy could allow the detection of channels if fast desensitization occurred.

ACh- and levamisole-activated channels are detected in muscle cells from the *acr-16* null mutant strain, RB918 (Fig. 5). *acr-16* encodes for the N-AChR, which is insensitive to levamisole but sensitive to nicotine (Francis et al., 2005). In this mutant strain, channel amplitude, the duration of open and closed components (Table 1), and channel frequency (data not shown) are similar to those observed for ACh and levamisole in the wild-type strain (Fig. 5 and Table 1). These results further confirm that the main single-channel activity arises from the activity of the L-AChR subtype.

Because we were not able to detect N-AChR activity in any strain and under different conditions, we performed RT-PCR to detect mRNA from the ACR-16 subunit.

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Samples were obtained from wild-type worms in the L1 developmental stage. The expected band of 375 pb was observed in these samples, and its size was identical to that obtained by PCR using ACR-16 cDNA as the template. The presence of ACR-16 mRNA in muscle L1 cells has been reported before (Touroutine et al., 2005). However, direct evidence of functional muscle N-AChRs at this early larval stage has not been reported to date. Thus, it may be possible that this nAChR subtype is not functional at L1 stage or that channel activity from this receptor cannot be detected in cell-attached patches.

#### *Single-channel currents from lev-1 mutant strain*

It has been reported that the non- $\alpha$  LEV-1 subunit is an accessory component of the muscle L-AChR of adult *C. elegans* (Fleming et al., 1997; Culetto et al., 2004). In order to clarify the role of this subunit in the L-AChR, we recorded unitary currents of *in vitro* differentiated muscle cells from the *lev-1(e211) IV* mutant strain. This mutant contains a missense mutation (G461E) in the M4 segment of LEV-1, and shows normal movement in the absence of levamisole but uncoordinated movement in its presence (Culetto et al., 2004). Channel activity is detected at ACh concentrations higher than 0.8  $\mu$ M. About 90 % of the recordings show channels with a conductance of  $26 \pm 2$  pS (Figs. 1B and 6, Table 1). This value differs significantly from that of wild-type nAChRs ( $p < 0.05$ ), revealing the presence of a new low-conductance L-AChR population.

Open time histograms of 10  $\mu$ M ACh-activated channels exhibit a single component of about 0.5 ms (Table 1), which is longer than that of wild-type L-AChRs ( $p < 0.05$ ), indicating that not only the conductance but also the kinetics of this receptor are altered (Fig. 6A). In contrast to recordings from wild-type muscle cells, channel activity decays



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during the course of the recording ( $977 \pm 218$  events/ s and  $166 \pm 98$  events/s for the intervals 0-15 s and 300-315 s after the start of the recording, respectively). In addition, clear clusters are observed at ACh concentrations higher than 50  $\mu$ M (Fig. 6B). The increase of ACh concentration displaces the main closed component, which corresponds to closings within clusters, to briefer durations ( $C_2 = 42 \pm 12$  ms and  $6 \pm 2$  ms for 50 and 300  $\mu$ M ACh, respectively) (Fig. 6B). This behavior is similar to that of mammalian muscle nAChRs (Bouzat et al., 2000).

It is interesting to note that although most of the channels correspond to the low-conductance nAChR, 50 % of the recordings exhibit an additional channel population with similar conductance to that of wild-type L-AChRs. The kinetics of these channels is, however, different from that of wild type L-AChR (Fig. 6C). These channels may correspond to receptors carrying the mutant LEV-1 subunit. It is therefore possible that LEV-1 carrying the M4 mutation is inefficiently incorporated into functional receptors. Another explanation for this observation is that channels lacking LEV-1 show an additional conductance state. Supporting this hypothesis is the fact that the kinetics of the high-conductance channels is similar to that of the low-conductance ones. For instance, at 10  $\mu$ M ACh, the mean open time is not significantly different from that of the 26 pS channels ( $\tau_1 = 0.42 \pm 0.05$  ms;  $p > 0.05$ ). Moreover, channel openings appear in clusters at high concentrations, as described for the low-conductance channels (Fig. 6C).

In this mutant strain, levamisole is capable of eliciting single channel currents with an amplitude of 2.8 pA at -100 mV (Table 1), similar to that of ACh-activated channels. Therefore, we can ensure that the low-conductance nAChR, which may lack LEV-1, is also sensitive to levamisole. However, the minimum concentration of levamisole that allows

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channel detection is 0.5  $\mu\text{M}$ , which is 5-fold higher than that of the native L-AChR subtype, suggesting a reduced sensitivity to the anthelmintic. Interestingly, the sensitivity to ACh decreases only 1.6-fold. Open time distributions at 1  $\mu\text{M}$  levamisole exhibit a single component (Table 1), whose duration is significantly longer than that corresponding to wild-type nAChRs at the same drug concentration ( $p < 0.05$ ) (Fig. 6A). As described for ACh, clusters can be identified at levamisole concentrations higher than 100  $\mu\text{M}$  and an additional channel population with a conductance value that resembles that of the native L-AChR is detected in 42 % of the recordings.

In summary, our results reveal that LEV-1 can be replaced by other subunits in the pentameric receptor, leading to L-AChR channels with lower conductance and lower levamisole sensitivity than the wild-type L-AChR.

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## Discussion

*C. elegans* is a genetically tractable and genomically defined non-mammalian model for the study of the nervous system. In particular, this model offers the prospect of better understanding the molecular and functional diversity of the nAChR family. *C. elegans* nAChRs comprise one of the most extensive nicotinic families. The reason for the high diversity of nAChR subunits in the worm as well as the function and composition of the native receptors are not fully understood. The free-living nematode *C. elegans* is also a model for the study of parasitic nematodes (Jones et al., 2005). These parasites affect human population, livestock and food crops. Nematode nAChRs are of clinical significance because they are targets for anthelmintic chemotherapy. Under this scenario, the characterization of single nAChR channel activity in *C. elegans* becomes highly significant. Given the importance of the larval stages in nematode life cycles and because the subunit expression pattern may change during development, studies at each stage are necessary. Here we described single nAChR channel activity from muscle cells of the L1 larval stage for the first time.

ACh activates mainly levamisole-sensitive nAChRs in L1 muscle cells. It was not known if there is one or more L-AChRs. As judged by the single-component amplitude histogram and the homogeneous kinetics, it is highly probable that *C. elegans* at L1 stage contain a single, or at least one highly predominant, L-AChR subtype. Although no unitary currents have been described before for *C. elegans* nAChRs, few reports have shown AChR channels from nematode parasites (Pennington and Martin, 1990; Robertson and Martin, 1993; Robertson et al., 1999; Levandoski et al., 2005). The comparison of channel properties of L-AChRs between parasites and *C. elegans* reveals that they have similar conductances and mean open times (Levandoski et al., 2005). They also share a similar

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potency for activation by ACh and anthelmintic agents. We show that the relative potency is morantel = pyrantel > levamisole > ACh, in full agreement with the observations in *Ascaris* (Harrow and Gratton, 1985; Martin et al., 1996). Thus, L-AChRs seem to be conserved within different nematode clades. Such conservation enhances the usefulness of *C. elegans* as a model of parasitic nematodes.

Wild-type L-AChRs show high channel activity, which does not decay significantly during the course of the recording. The latter observation supports a slow desensitization rate. In agreement with this, macroscopic current recordings have shown that L-AChRs from *C. elegans* desensitize much slower than N-AChRs (Richmond and Jorgensen, 1999). In contrast to recordings from mammalian nAChRs (Sine and Steinbach, 1987), no clusters are observed and closed intervals do not depend on agonist concentration. Only at low concentrations closed times are more prolonged, but this is due to the activation of only a fraction of the entire population of nAChRs. Slow desensitization together with a relatively slow opening rate may account for the absence of distinguishable concentration-dependent closed components of L-AChRs. Also, the high expression of these channels in muscle cells may unmask the presence of clusters.

Open time histograms of L-AChRs activated by ACh or levamisole show a brief component whose area decreases as a function of agonist. Studies in mammalian nAChRs and  $\alpha_7$ -5HT<sub>3A</sub> have shown that this component may correspond to receptors with fewer occupied binding sites than required for optimal activation (Sine et al., 1990; Rayes et al., 2005). We postulate a similar behavior for *C. elegans* L-AChRs. Given that the non- $\alpha$  UNC-29 and LEV-1 subunits are components of the native L-AChR, optimal activation may arise from two or three molecules of agonist bound. The mean duration of the slowest

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component at non-blocking anthelmintic concentrations is more prolonged than that of ACh-activated channels. Thus, not only the potency but also gating kinetics are slightly different between ACh and nematocide drugs.

Channel blockade by agonists is evidenced by the concentration-dependent decrease of the mean open time. ACh causes L-AChRs to flicker rapidly between open and blocked states. In contrast, neither flickering nor closed components associated to blocked periods are observed in the presence of anthelmintics, suggesting that the drugs dissociate from the channel very slowly. Alternatively, their blocking mechanisms may deviate from the simple open-channel block model, as reported for morantel in *Ascaris* nAChR (Evans and Martin, 1996).

The exact stoichiometry of the L-AChR(s) remains unknown. Our observations reveal that UNC-38, UNC-63 and UNC-29 subunits are assembled into a single L-AChR in the L1 stage and that these subunits are essential and irreplaceable in the native receptor. Because these subunits have been previously shown to be essential for adult L-AChRs (Richmond and Jorgensen, 1999), our results demonstrate that they are vital for receptor function throughout development. However, larvae have been shown to be more affected by mutations in the L-AChR than adults, therefore the expression level or the composition of these receptors may vary during development. Our study also confirms that ACR-16 is not part of the L-AChR (Touroutine et al., 2005) given that the *acr-16* null mutant shows channel activity identical to that of wild-type strains.

Recordings from the strain carrying a missense mutation in M4 of LEV-1 demonstrate that nAChRs lacking this subunit can function. A new and main population of low-conductance channels showing activation in clusters and slightly prolonged durations than wild-type nAChRs is detected. The mutation at G461 in M4 is not expected to

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produce a change in channel conductance as this residue is neither part of the ion pore nor it is located at the vestibule of the channel (Blanton and Cohen 1994; Unwin, 2005). Moreover, M4 mutations in mammalian muscle nAChRs affect kinetics but not channel conductance (Bouzat et al., 2000, 2002; Mitra et al., 2004). Thus, if the mutant LEV-1 subunit were incorporated into the pentameric receptor similarly to wild-type LEV-1, no changes in conductance should occur. The fact that a change in conductance is observed strongly suggests that the subunit composition of the receptor has changed. Because the only AChR subunit affected in this strain is LEV-1, the most plausible explanation for this finding is that the mutant subunit is not present in the receptor, and that this receptor lacking LEV-1 shows lower conductance. Generation of *lev-1* null mutants will help to confirm this result. Because we could not detect this channel population in the wild-type strain, our results also reveal that LEV-1 is preferentially incorporated in native L-AChRs. In the low-conductance AChR, LEV-1 may be replaced by another subunit that conforms the L-AChR in L1. Alternatively, LEV-1 may be substituted by a different subunit not normally expressed in this stage. The latter possibility occurs in congenital myasthenic syndromes, in which the fetal  $\gamma$  subunit reappears and it is incorporated into functional receptors when there are null mutations in the  $\epsilon$  subunit gene (Engel et al., 1998).

The presence of a smaller proportion of channels with similar conductance but different kinetics to those of wild-type nAChRs may be due to the fact that, although less efficiently, the mutant LEV-1 is also incorporated into functional channels. Alternatively, L-AChRs lacking LEV-1 may show a minor wild-type like conductance state with similar gating kinetics to the low-conductance channels.

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The sensitivity to ACh and levamisole is reduced in the low-conductance nAChR, albeit the reduction is significantly more pronounced for the anthelmintic. This lower potency may explain the mutant phenotype, which exhibits normal locomotion in the absence of levamisole but it becomes uncoordinated, though not killed, in its presence (Culetto et al., 2004).

Our RT-PCR essay reveals that ACR-16 may be expressed in the L1 stage, in agreement with previous reports (Touroutine et al., 2005). However, no single-channel currents from N-AChRs could be detected. Several reasons can explain this: i) the conductance of this receptor may be too low for single-channel detection. In this sense, N-AChR from *Ascaris* exhibits lower amplitude than that of L-AChRs (Levandovski et al., 2005); ii) receptors may desensitize too fast to allow detection in cell-attached patches; iii) the efficacy for activation may be extremely low; iv) L-AChRs may predominate during larval stages. Regarding to this, phenotypic analyses of *unc-29* and *unc-38* null mutants suggest that the levamisole-sensitive function is critical for the entire motor behaviour of the L1 stage but in the adult it is essential only for forward motion in the anterior part of the body (Lewis et al, 1980); v) other subunits showing different expression patterns during development may contribute to native N-AChRs. In this respect, non- $\alpha$  ACR-16-like subunits have been identified in *C. elegans* (Jones and Sattelle, 2003).

Individual *C. elegans* body wall muscles have both cholinergic and GABAergic inputs, which trigger contraction and relaxation, respectively. It is intriguing how mutant *C. elegans* that show no ACh channel activity can move, although in an uncoordinated way. Alternative excitatory signals, not yet identified, may be involved.

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Studies at the single-channel level will help to determine which receptors are involved in worm locomotion, to elucidate the molecular composition and functional roles of the different nAChRs and to develop novel antiparasitic drugs.

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### Footnotes

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## Legends for Figures

*Figure 1.* Single-channel currents of wild-type ACh-activated receptors from *C. elegans* muscle cells. Recordings were obtained from cultured muscle cells derived from PD4251 strain. a) Traces of single-channel activity at the indicated membrane potential are shown filtered at 9 kHz with channel openings as upward deflections. Corresponding amplitude histograms are shown. ACh concentration: 50  $\mu$ M. b) Amplitude-voltage relationships. Channels were recorded from wild-type strains in the presence of ACh (o), levamisole (■), and ACh plus 10  $\mu$ M DH $\beta$ E ( $\Delta$ ). ( $\blacktriangledown$ ) Correspond to channels activated by ACh recorded from *lev-1* mutant strain. Each point corresponds to at least 3 different recordings for each condition.

*Figure 2.* Single-channel currents as a function of ACh concentration. Recordings were obtained from cultured muscle cells derived from PD4251 strain. nAChRs were recorded at different ACh concentrations. Membrane potential: -100 mV. Channel traces are shown at two different time scales. Channels appear as upward deflections. Filter: 9 kHz. The corresponding open and closed time histograms for each condition are shown.

*Figure 3.* Single-channel currents as a function of levamisole concentration. Recordings were obtained from muscle cultured cells derived from PD4251 strain. nAChRs were recorded at different levamisole concentrations. Membrane potential: -100 mV. Channel traces are shown at two different time scales. Channels appear as upward deflections. Filter: 9 kHz. The corresponding open and closed time histograms for each condition are shown.



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*Figure 4.* Single-channel currents activated by pyrantel and morantel. Recordings were obtained from muscle cultured cells derived from PD4251 strain. Channels activated by pyrantel or morantel are shown at two different time scales. Membrane potential: -100 mV. Channels appear as upward deflections. Filter: 9 kHz. The corresponding open time, closed time and amplitude histograms for each condition are shown.

*Figure 5.* Single-channel activity from mutant strains. L1 muscle cells were obtained from the corresponding mutant strain. No levamisole- and ACh-activated channels are detected in the *unc-38(e264)*, *unc-63(x37)*, *unc-29(e1072)* mutants at a range of agonist concentration. In contrast, channel activity similar to that of wild-type is observed in the *acr-16(ok789)* strain. Channels activated by levamisole and ACh are shown with the corresponding duration and amplitude histograms. Membrane potential: -100 mV. Filter: 9 kHz.

*Figure 6.* Single-channel activity from *lev-1* mutant strain.

- a) Channel traces from cell-attached patches recorded in the presence of ACh and levamisole are shown at two different time scales. Open, closed and amplitude histograms for each condition are shown.
- b) Clusters of low-conductance channels at different ACh concentrations. The corresponding closed time histograms are shown. The main closed component is displaced to briefer durations as a function of ACh concentration.
- c) Clusters of low- and high-conductance channels recorded from the same cell-attached patch. The corresponding amplitude histogram for the entire recording is shown. Channels are shown as upward deflections. Membrane potential: -100 mV. Filter: 9 kHz.

**Table 1. Channel properties of nAChR channels from *C. elegans* muscle cells in culture**

Agonist	pS	Conc. ( $\mu$ M)	$\tau_1$ ( $\mu$ s)	$\tau_2$ ( $\mu$ s)	C1 (ms)	C2 (ms)
<b>PD4251 (WT)</b>						
ACh	38.7 $\pm$ 1.6	1 (n=6)	100 $\pm$ 20	280 $\pm$ 60	0.06 $\pm$ 0.01	180 $\pm$ 90
		10 (n=13)	96 $\pm$ 23	320 $\pm$ 50	0.04 $\pm$ 0.02	40 $\pm$ 24
		50 (n=8)	140 $\pm$ 30	240 $\pm$ 20	0.05 $\pm$ 0.01	11 $\pm$ 6
		300 (n=7)	80 $\pm$ 10	-	0.04 $\pm$ 0.02	9 $\pm$ 4
ACh + 10 $\mu$ M DH $\beta$ E	36.5 $\pm$ 1.7	50 (n=5)	170 $\pm$ 80	-	0.05 $\pm$ 0.02	12 $\pm$ 03
Lev	36.9 $\pm$ 0.8	0.1 (n=6)	140 $\pm$ 30	600 $\pm$ 70	0.05 $\pm$ 0.01	895 $\pm$ 240
		1 (n=5)	270 $\pm$ 10	-	0.08 $\pm$ 0.01	220 $\pm$ 40
		10 (n=8)	150 $\pm$ 50	-	0.09 $\pm$ 0.01	24 $\pm$ 11
		100 (n=4)	120 $\pm$ 20	-	0.19 $\pm$ 0.07	5 $\pm$ 0.8
<b>RB918 (<i>acr-16</i>)</b>						
ACh	36.8 $\pm$ 1.3	10 (n=5)	120 $\pm$ 40	280 $\pm$ 20	0.06 $\pm$ 0.01	68 $\pm$ 20
Lev	36.2 $\pm$ 2.6	0.1 (n=6)	90 $\pm$ 20	480 $\pm$ 60	0.04 $\pm$ 0.01	560 $\pm$ 120
<b>CB211 (<i>lev-1</i>)</b>						
ACh	26.0 $\pm$ 2.0	10 (n=3)	490 $\pm$ 20	-	0.05 $\pm$ 0.02	170 $\pm$ 40
Lev	28.3 $\pm$ 1.2	1 (n=3)	390 $\pm$ 30	-	0.07 $\pm$ 0.01	280 $\pm$ 20

Single-channel recordings were performed from muscle cells in culture obtained from wild-type and the specified mutant strains. ACh, ACh plus 10  $\mu$ M DH $\beta$ E or levamisole were present in the pipette solution. The conductance, expressed in pS, was taken from the slope of the current-voltage relationship (Fig. 1B) or from the current calculated at -100 mV.  $\tau_1$

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and  $\tau_2$  correspond to the open components of the open time distributions. C1 and C2 are the closed components obtained from closed time histograms. n corresponds to the number of recordings for each condition.

Fig.1

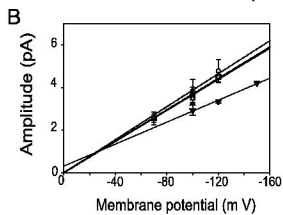
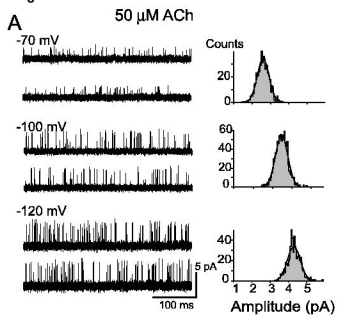


Fig.2

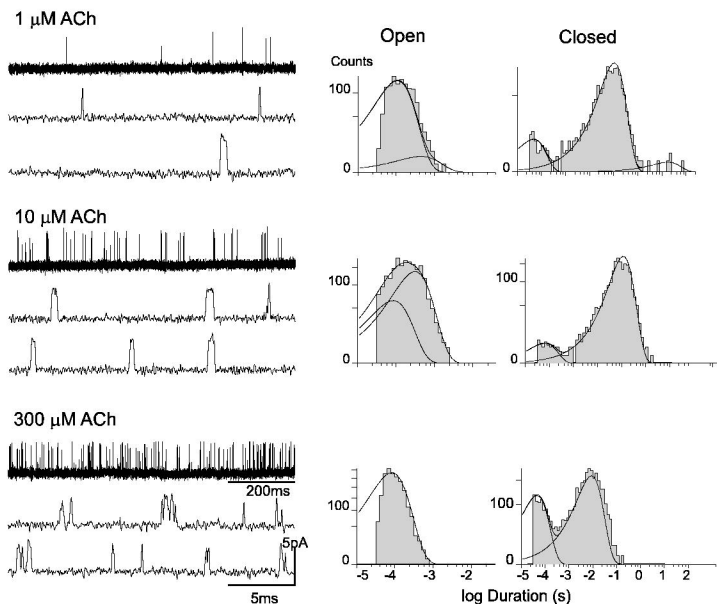


Fig.3

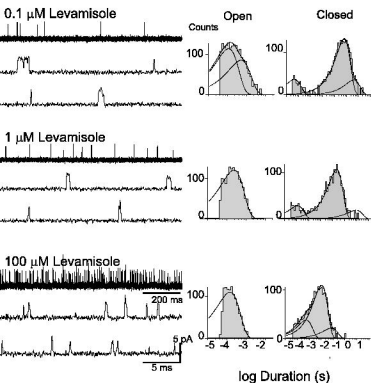


Fig.4

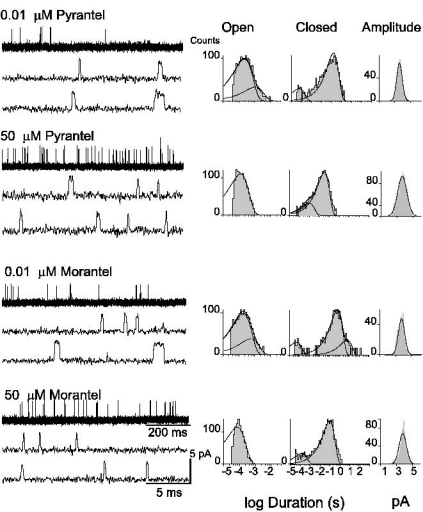


Fig.5

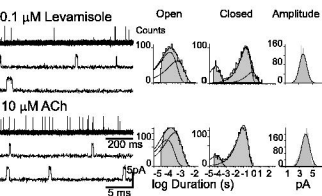
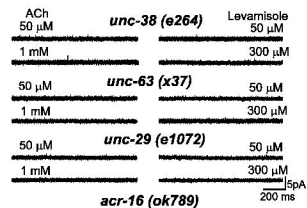




Fig.6

