A Cys-loop Mutation in the Caenorhabditis elegans Nicotinic Receptor Subunit UNC-63 Impairs but Does Not Abolish Channel Function

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The nematode Caenorhabditis elegans is an established model organism for studying neurobiology. UNC-63 is a C. elegans nicotinic acetylcholine receptor (nAChR) α-subunit. It is an essential component of the levamisole-sensitive muscle nAChR (L-nAChR) and therefore plays an important role in cholinergic transmission at the nematode neuromuscular junction. Here, we show that worms with the unc-63(x26) allele, with its αC151Y mutation disrupting the Cys-loop, have deficient muscle function reflected by impaired swimming (thrashing). Single-channel recordings from cultured muscle cells from the mutant strain showed a 100-fold reduced frequency of opening events and shorter channel openings of L-nAChRs compared with those of wild-type worms. Anti-UNC-63 antibody staining in both cultured adult muscle and embryonic cells showed that L-nAChRs were expressed at similar levels in the mutant and wild-type cells, suggesting that the functional changes in the receptor, rather than changes in expression, are the predominant effect of the mutation. The kinetic changes mimic those reported in patients with fast-channel congenital myasthenic syndromes. We show that pyridostigmine bromide and 3,4-diaminopyridine, which are drugs used to treat fast-channel congenital myasthenic syndromes, partially rescued the motility defect seen in unc-63(x26). The C. elegans unc-63(x26) mutant may therefore offer a useful model to assist in the development of therapies for syndromes produced by altered function of human nAChRs.

Nicotinic acetylcholine receptors (nAChRs) are of fundamental importance in synaptic transmission at the neuromuscular junction as well as throughout the nervous system (1, 2).

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**The abbreviations used are:** nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; L-nAChR, levamisole-sensitive nAChR; FCCMS, fast-channel congenital myasthenic syndrome(s); ANOVA, analysis of variance; PB, pyridostigmine bromide; 3,4-DAP, 3,4-diaminopyridine; CMS, congenital myasthenic syndrome(s).
a disrupted Cys-loop motif due to a cysteine-to-tyrosine (UNC-63 C151Y) substitution (13). Here, we show the functional impact of this mutation, reporting for the first time the behavioral and physiological consequences of this mutation at the Cys-loop of the \textit{C. elegans} L-nAChR. Our results show that disruption of the Cys-loop significantly impairs nAChR channel function, thus demonstrating its conserved role throughout the animal kingdom.

Mutations of the human muscle nAChR that produce either loss or gain of function can lead to congenital myasthenic syndromes (16). With much of the machinery required for neuromuscular transmission in mammals conserved in \textit{C. elegans}, the nematode has emerged as a useful model organism for studying neuromuscular diseases and drug testing (17–19). The \textit{unc-63(x26)} worm containing the mutant L-nAChR has uncoordinated locomotion due presumably to deficient cholinergic signaling at the neuromuscular junction. Here, we show that this phenotype is partially rescued by drugs used to treat humans for fast-channel congenital myasthenic syndromes (16). With much of the machinery required for neuromuscular transmission in mammals conserved in \textit{C. elegans}, the nematode has emerged as a useful model organism for studying neuromuscular diseases and drug testing (17–19). The \textit{unc-63(x26)} worm containing the mutant L-nAChR has uncoordinated locomotion due presumably to deficient cholinergic signaling at the neuromuscular junction. Here, we show that this phenotype is partially rescued by drugs used to treat humans for fast-channel congenital myasthenic syndrome (FCCMS) by enhancing neuromuscular transmission. The fact that both molecular and phenotypic changes produced by a mutation at a key site of a muscle \textit{C. elegans} nAChR parallel those observed in humans allows us to propose that the \textit{unc-63(x26)} strain may permit screening for drugs aimed at alleviating the symptoms of diseases arising from mutations of muscle nAChRs.

**EXPERIMENTAL PROCEDURES**

\textit{C. elegans Strains}—Nematodes were raised at 21 °C under standard laboratory conditions on agar plates cultured with \textit{Escherichia coli} (OP50). The following \textit{C. elegans} strains were used: wild-type N2 (Bristol variety), \textit{myo-3::GFP PD4251 (ccls4251I)}, \textit{unc-63(x26)}, and \textit{unc-63(x37)}. N2 and the mutant strains were obtained from the \textit{Caenorhabditis} Genetic Center. All strains were handled according to standard procedures (WormBook site).

**Isolation and Culture of \textit{C. elegans} Muscle Cells**—Embryonic cells were isolated and cultured as described previously (20, 21). Briefly, adult nematodes were exposed to an alkaline hypochlorite solution (0.5 M NaOH and 1% NaOCl), and the eggs released were treated with 1.5 units/ml chitinase (Sigma) and hypochlorite solution (0.5M NaOH and 1% NaOCl), and the eggs released were treated with 1.5 units/ml chitinase (Sigma) for 30–40 min at room temperature. The embryonic cells were isolated by gently pipetting and filtering through a sterile 5-μm Durapore syringe filter (Millipore Corp., Bedford, MA) to remove undissociated embryos and newly hatched larvae. Filtered cells were plated on glass cover-slips coated with poly-O-ornithine. Cultures were maintained at 24 °C in a humidified incubator in L-15 medium (HyClone, Logan, UT) containing 10% fetal bovine serum. Complete differentiation to the various cell types were observed in newly hatched L1 larvae within 24 h. Electrophysiological recordings were performed 1–5 days after cell isolation. The percentages of neurons and muscle cells growing in culture were in good agreement with previous reports (20). As a control, we used the PD4251 strain, which contains the wild-type nAChR and produces GFP in body wall muscle cells, thereby facilitating muscle cell identification under fluorescence optics (22). Muscle cells are easily identifiable due to their spindle-shaped morphology, which resembles that of body wall muscle cells in vivo (10, 20). In the \textit{unc-63(x26)} mutant strain, muscle cell morphology was similar to that of green cells of the PD4251 strain (21). The nAChR channel properties of the PD4251 strain are identical to those of the wild-type N2 Bristol strain (21).

**Single-channel Recording**—Recordings were obtained in the cell-attached patch configuration (23) at 20 °C as described in detail previously (21, 24). The bath and pipette solutions contained 142 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl\textsubscript{2}, 1.7 mM MgCl\textsubscript{2}, and 10 mM HEPES (pH 7.4). Acetylcholine chloride or levamisole was added to the pipette solution. Single-channel currents were recorded using an Axopatch 200 B patch-clamp amplifier (Molecular Devices), digitized at 5-μs intervals with the PCI-6111E interface (National Instruments, Austin, TX), and detected by the half-amplitude threshold criterion using the program TAC 4.0.10 (Bruxton Corp., Seattle, 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 Corp.). Only recordings showing more than ~400 opening events were considered for the generation of duration histograms.

The frequency of openings (number of opening events/s) was calculated by counting bursts within the first minute of the recording to minimize the effects of desensitization and instability of the patch. A burst was considered as a series of channel openings separated by brief closings (<150 μs). Experimental data are shown as the mean ± S.D. Statistical comparisons were done using Student’s \textit{t} test. A probability of \(p < 0.05\) was considered significant.

**Anti-UNC-63 Primary Antibody Production**—A peptide fragment of UNC-63 (LNVPGRHSHKRYPC) that is located in loop C and shows low homology to the equivalent region in other nAChR subunits (13) was used to generate polyclonal antibodies in rabbits at GenScript Corp. The antibodies were purified using the SulfoLink immobilization kit for peptides (Thermo Scientific, Loughborough, United Kingdom).

**Immunocytochemical Staining of Worms and Cultured Cells**—Two-day cultures from isolated embryonic cells were fixed with 2% paraformaldehyde for 30 min, incubated for 2 h with 1% bovine serum albumin to block unspecific sites, and exposed overnight to anti-UNC-63 primary antibodies at 1:5 dilution. After rinsing with PBS, cultures were incubated for 2 h with secondary antibodies (Alexa Fluor 568-labeled goat anti-rabbit; Invitrogen) at 1:1000 dilution. Negative controls were processed in the absence of the primary antibody. Cultured cells derived from N2, \textit{unc-63(x26)}, and \textit{unc-63(x37)} were processed and observed simultaneously using a Nikon Eclipse E600 fluorescence microscope. All images were acquired at the same gain and exposure settings and analyzed with NIH ImageJ software. For each culture, >12 random fields were observed, and 20–30 cells were selected for quantification. The intensity of fluorescence given by the program was normalized to that of the mean value obtained in the wild-type culture of each experiment. Values are expressed as the mean ± S.D. Staining levels significantly different from those found for N2 are indicated by triple asterisks (\(p < 0.0001\)).
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Wild-type, *unc-63(x26)* and *unc-63(x37)* worms were

0.001; one-way analysis of variance (ANOVA) and Dunnett’s

post hoc test).

Wild-type, *unc-63(x26)* and *unc-63(x37)* worms were

freeze-cracked (25) on poly-L-lysine-coated microscope slides

and fixed in methanol for at least 2 min and in acetone for at

least 4 min. After blocking overnight in 5% bovine serum al-

bumin (Sigma), worms were incubated overnight with anti-

UNC-63 primary antibody at 1:5 dilution. After rinsing,

worms were incubated overnight with secondary antibody

(Alexa Fluor 594-labeled goat anti-rabbit; Invitrogen) at

1:1000 dilution. Slides were mounted with ProLong gold anti-

fade reagent (Invitrogen). Antibody staining patterns were

visualized with the use of a Zeiss Axioplan-2 fluorescence mi-

croscope and AxioVision 4.5 software.

**RESULTS**

L-nAChRs of *C. elegans* unc-63(x26) Show Decreased Chan-

nel Activity and Abnormally Brief Openings—The *unc-

63(x26)* strain carries a mutation (C151Y) that removes the

first cysteine residue of the Cys-loop in the UNC-63 subunit

(13). To determine whether the C151Y mutation affects mus-

cle nAChR function, we analyzed the single-channel proper-

ties of L-nAChR channels

of bending at the midbody (see Fig. 5A). All assays were blind

and carried out at 21 °C. Data are plotted as the mean ± S.E.

One-way ANOVA with Dunnett’s multiple comparison was used

for comparing the responses of worms to drugs with control

worms in M9 medium only; drug-induced thrashing rates higher

than those of control worms with *p* < 0.05 were considered sig-

nificant rescue effects.

Automated Analysis of Thrashing—The thrashing (swim-

ning) rates of worms were measured as described previously

(26). Essentially, 30-s movies of thrashing worms were analyzed

by an algorithm that reduces image background using principal

components analysis; calculates the covariance matrix, which

displays intervals between similar worm conformations; and de-

rives the thrashing rate from the covariance matrix.
detected in cell-attached patches from L1 muscle cells (Fig. 1). At −100 mV, L-nAChR channels appeared as 3.8-pA opening events (Fig. 1). At 1–10 μM ACh, open time distributions were well described by the sum of two exponentials (Fig. 1B and Table 1). The relative area of the longest duration component increased with ACh concentration from ~0.2 at 1 μM to ~0.6 at 10 μM ACh and therefore became the main open component at 10 μM ACh (Table 1). At higher ACh concentrations, channel block occurred, which is reflected in the reduction in the mean open time (21). When levamisole was applied, the reduction in the open duration due to open channel block was observed at concentrations lower than those reported for ACh (21). Due to channel block, open time histograms from L-nAChR channels activated by 10–50 μM levamisole showed only one component (Table 1). For both agonists, the frequency of opening events measured within the first minute of recording was not statistically different between 10 and 50 μM, thus revealing that saturation had been achieved at 10 μM (Fig. 2).

We found that, in the unc-63(x37) null mutant, single-channel activity in response to ACh or levamisole was not detected (Fig. 1A) (21). These findings indicate that UNC-63 is an essential subunit of the L-nAChR, in agreement with previous reports (21). They also show that, in cultured embryonic muscle cells, the detected ACh-activated channel openings arise mainly from L-nAChRs.

In contrast to the findings with the UNC-63 null mutant, L-nAChR channel activity was detected in muscle cells of the unc-63(x26) mutant strain (Fig. 1). In the presence of ACh or levamisole, openings of ~3.6 pA were detected in cell-attached patches (Fig. 1 and Table 1). Given that channel openings were not observed in the absence of the cholinergic agonists, we concluded that UNC-63 with the C151Y mutation is able to form functional L-nAChRs. As expected because of the fact that the Cys-loop does not contain determinants of channel conductance, the amplitude of the unitary current is not affected in the unc-63(x26) L-nAChR.

However, striking differences in L-nAChR channel activity in this mutant strain compared with that of the wild type were observed. The frequency of opening events at 10 μM ACh measured within the first minute of recording was ~100-fold lower than that of wild-type channels (Fig. 2). As in the wild-type strain, increasing the ACh or levamisole concentration from 10 to 50 μM did not lead to an increase in the frequency of openings, which suggests that saturation had been achieved at 10 μM (Fig. 2). Due to the low frequency of opening, recordings containing a measurable number of openings were difficult to achieve. For example, at 10 μM ACh, the percentages of cell-attached patches that showed a number of opening events enough to perform appropriate analysis (see “Experimental Procedures”) were ~90% (n = 24) and 20% (n = 21) for wild-type and unc-63(x26) strains, respectively. Closed time distributions of wild-type and unc-63(x26) mutant L-nAChRs could be fitted by two or three components (Fig. 1). The duration of the longest closed component gave an estimation of the frequency of openings in the whole recording (Fig. 1). As expected, in the unc-63(x26) mutant, the duration of this main component was longer than that in the wild type (Fig. 1). To better relate this closed component to the measured frequency (Fig. 2), we constructed closed time histograms with data corresponding only to the first minute of recording. As shown in Table 1, the duration of the main closed component (C) was significantly more prolonged in the mutant than in the wild-type strain, in accordance to the reduced frequency of openings (Fig. 2).

### TABLE 1

| Agonist | Strain        | Amplitude | τ1 (area) | τ2 (area) | C       |
|---------|---------------|-----------|-----------|-----------|---------|
|         |               | pA        | μs        | μs        | ms      |
| ACh     | 1 μM          | Wild-type | 3.7 ± 0.4 | 150 ± 40  | 340 ± 60| 250 ± 70|
|         | unc-63(x26)   | 3.7       | 120 (1)   | 270 ± 30  | 2.1 ± 0.6|
|         | 10 μM         | Wild-type | 3.7 ± 0.2 | 75 ± 40   | 30 ± 150|
|         | unc-63(x26)   | 3.6 ± 0.2 | 95 ± 40   | 2.4 ± 60  | 146 ± 50|
| Levamisole | 10–50 μM | Wild-type | 3.9 ± 0.1 | 210 ± 40  | ND      | 24 ± 10 |
|         | unc-63(x26)   | 3.4 ± 0.2 | 60 ± 20   | ND        | 350 ± 140|

### FIGURE 2. Frequency of L-nAChR channel openings in wild-type and unc-63(x26) C. elegans muscle.

Recordings were obtained from N2 (wild type; gray bars) and unc-63(x26) (black bars) embryonic C. elegans muscle cells. The number of events/s was determined by measuring the total number of bursts during the first minute of recording at saturating ACh or levamisole concentrations (see “Experimental Procedures”). Data are the mean ± S.D. Statistically significant differences from the wild type at the same concentration are indicated (t test): * p < 0.05; ***, p < 0.001.
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Opening events were significantly briefer than wild-type openings. Open time histograms of ACh-activated channels were fitted by one brief component instead of the two observed in wild-type muscle (Fig. 1B). The slowest component, which was the main component at 10 μM ACh in wild-type L-nAChRs, was not observed at any ACh concentration in the mutant channels (Table 1). As shown for ACh, when levamisole was the agonist, the mutant channels were briefer with respect to the wild-type channels (Table 1). Such atypically brief channel openings will lead to abnormally fast decays of the responses to ACh. Thus, the low frequency of openings and the abnormally brief duration of opening events will result in a dramatic reduction in the response to ACh during neuromuscular synaptic transmission.

Surface Expression and Distribution of UNC-63 in the unc-63(x26) Mutant Are Similar to Those in Wild-type Worms—We raised antibodies against the N-terminal ligand-binding region of UNC-63. To better relate the level of surface expression of L-nAChRs to the changes detected at the single-channel level, we performed immunocytochemistry assays on the cultures used for single-channel recordings (Fig. 3). To this end, we used the anti-UNC-63 antibody and a secondary antibody labeled with Alexa Fluor 568. Similar UNC-63 staining was seen in cultured muscle cells derived from N2 and unc-63(x26) strains. Staining was found preferentially in the periphery, and because cells were not permeabilized during the labeling procedure, this indicates that UNC-63 is localized in the plasma membrane. By contrast, only background staining was detected at the muscle surface in the UNC-63 null mutant worms (Fig. 4). Thus, this antibody specifically stained the UNC-63 null mutant strain (Fig. 3A, unc-63(x37) panels) as well as in the absence of the primary anti-UNC-63 antibody (data not shown).

Quantification of the fluorescence intensity of individual cells from two different cultures for each strain showed no significant differences between the control and unc-63(x26) mutant strains. However, a statistically significant reduction in the label was found in the null mutant (unc-63(x37)) compared with the wild-type or unc-63(x26) mutant strains (Fig. 3B).

These assays using cultured cells confirmed that the surface expression of L-nAChRs is not noticeably affected by the UNC-63 C151Y mutation. Given that the frequency of openings was reduced by 100-fold in the unc-63(x26) mutant compared with wild type without the surface expression level being significantly affected, these results strongly suggest that channel activation, which leads to infrequent opening events, is significantly impaired in the mutant receptor.

Immunohistochemistry performed on adult worms showed that UNC-63 was present in the body wall muscle of wild-type worms (Fig. 4B), as has been reported previously using a GFP fusion construct (13). Also, UNC-63 staining occurred as individual puncta in the ventral nerve cord (Fig. 4D), which consists of motor neurons and bundles of neuronal processes that innervate the body musculature (27). Similarly, UNC-63 staining was seen in the body wall muscle (Fig. 4C) and ventral nerve cord (Fig. 4E) of unc-63(x26) worms, suggesting that the mutation in this strain does not affect the distribution of the UNC-63 subunit. In contrast, staining was not detected in the body wall muscle and ventral nerve cord of the unc-63(x37) null mutant strain (data not shown), in agreement with the lack of single-channel activity in muscle cells (Fig. 1) and the lack of staining of cultured muscle cells (Fig. 3A).

Drugs Used to Treat Congenital Myasthenic Syndrome Show Partial Rescue of the unc-63(x26) Mutant Phenotype—In line with deficient function of the neuromuscular junction, the unc-63(x26) mutant strain has a mild but significant uncoordinated phenotype (13). This was confirmed by our findings that unc-63(x26) thrashed at 109 ± 4.9 body bends/min (n = 14 batches of eight worms ± S.E.), which was significantly lower than the 222 ± 3.6 thrashing rate (n = 10 batches of eight worms ± S.E.) of wild-type N2 worms (p < 0.0001, unpaired t test). The unc-63(x26) thrashing rate was significantly higher than that of the unc-63(x37) null mutant (p < 0.0001, unpaired t test), which thrashed at 12 ± 1.3 body bends/min (n = nine batches of eight worms ± S.E.), indicating that the C151Y mutation does not abolish UNC-63 function.

The acetylcholine esterase inhibitor PB and the K+ channel blocker 3,4-DAP are commonly used to treat FCCMS. We measured the effect of these chemicals on unc-63(x26) thrashing rates to determine whether they rescue the mutant uncoordinated phenotype. When exposed to PB at 0.9 or 15.6 mM, the thrashing rates of unc-63(x26) were 137 ± 5.7 and 136 ± 6.8, respectively (Fig. 5B), which were significantly higher than the thrashing rate in M9 medium alone, indicating that PB was able to rescue the mutant uncoordinated phenotype by 24%. A rescue effect was also seen with all the concentrations of 3,4-DAP tested (Fig. 5C). The highest thrashing rate observed was 152 ± 6.5 at 15.6 mM, which corresponds to a 38% rescue. PB or 3,4-DAP did not significantly increase the thrashing rates of N2 worms or the unc-63(x37) null mutant (Fig. 5, B and C) (13). Because PB and 3,4-DAP are commonly co-administered to FCCMS patients, we measured the thrashing of worms exposed to a combination of both chemicals. In 15.6 mM PB combined with different concentrations of 3,4-DAP (Fig. 5D), unc-63(x26) showed a significant increase in thrashing rates. In 1.9 mM 3,4-DAP and 15.6 mM PB, the highest thrashing rate seen was 153 ± 8.5, which corresponds to a 39% rescue, similar to that in 3,4-DAP alone. However, the thrashing rate of the unc-63(x37) null mutant worms significantly increased when exposed to both PB and 3,4-DAP (Fig. 5D), whereas PB or 3,4-DAP alone had no effect (Fig. 5, B and C), indicating that the two drugs have a combined effect, most likely in increasing ACh signaling to the other major nAChR subtype present at the neuromuscular junction, ACR-16 (9, 10). Together, these results show that, of the three worm strains tested, unc-63(x26) is the most sensitive to the rescue effects of drugs clinically used to treat FCCMS.

An algorithm allowing the automated analysis of worm thrashing has been developed (26). To test the potential of using the unc-63(x26) strain in automated drug screens, we used this algorithm to analyze the effects of 3,4-DAP on the thrashing rates of wild-type and unc-63(x26) worms. A rescue effect was detected, as four of the five 3,4-DAP concentrations used significantly increased the thrashing rate of unc-63(x26) mutants, whereas wild-type worms were unaffected (Fig. 5E). The thrashing rates obtained by automated means closely resembled those obtained manually (Fig. 5F), illustrating the
FIGURE 3. Measuring the surface expression levels of UNC-63 in cultured muscle cells from wild-type (N2) and unc-63(x26) worms by immunohistochemistry. Embryonic cells were isolated from the different strains, and UNC-63 expression was analyzed after 2 days in culture. A, anti-UNC-63 antibody staining of fixed cultured cells obtained from wild-type, unc-63(x26) (carrying the UNC-63 C151Y mutant subunit), and unc-63(x37) (null mutant) strains. Representative results visualized under a fluorescence microscope are shown. Scale bars = 10 μm. The insets show the cells (marked with the arrow) at higher resolution (×8). B, fluorescence intensities (relative to the wild type) of selected cells derived from wild-type, unc-63(x26), and unc-63(x37) (null mutant) strains. The intensity values were obtained by visualization of ≥12 random fields in cell cultures derived from each strain. The total number of cells for which the fluorescence was quantified was 45 per strain. The results correspond to data of two independent experiments, and values were normalized to the mean value of the wild type for each experiment. Values are expressed as the mean ± S.D. Staining levels significantly different from those found for N2 are indicated by triple asterisks (p < 0.001, one-way ANOVA and Dunnett’s post hoc test).
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**FIGURE 4. Measuring the distribution of UNC-63 in wild-type (N2) and unc-63(x26) worms by immunohistochemistry.** A, schematic representation of C. elegans somatic body wall muscle, which consists of rhomboid-shaped muscle cells arranged in longitudinal bundles located in quadrants. B, anti-UNC-63 antibody staining of body wall muscle in wild-type worms. C, anti-UNC-63 antibody staining of body wall muscle in unc-63(x26) worms. D, anti-UNC-63 antibody staining of distinct puncta in the ventral nerve cord of wild-type worms. E, anti-UNC-63 antibody staining of distinct puncta in the ventral nerve cord of unc-63(x26) worms. Scale bars = 10 μm.

suitability of this fast automated procedure in assaying the effects of chemicals on unc-63(x26) motility.

**DISCUSSION**

The unc-63(x26) strain is homozygous for a mutation in the nAChR α-subunit unc-63, an essential constituent of L-nAChRs, which play a major role in cholinergic transmission at the neuromuscular junction (8, 13, 21). The C151Y mutation disrupts the Cys-loop, a defining feature of nAChRs and Cys-loop receptors (28) that has been shown to be essential for the correct assembly of nAChR subunits in mammalian cell lines (4) and necessary for the functional coupling between ligand binding and channel opening in mammalian nAChRs (2, 5, 29, 30). The C151Y mutation causes a loss of function as evidenced by a significant decrease in channel open duration and frequency of opening. Increasing agonist concentration does not increase the frequency of openings, which would be expected if saturation had not been achieved due to lower affinity. Moreover, the effects of the mutation on channel properties when activated by levamisole are quantitatively similar to those described for ACh: low frequency of opening and channel openings of brief duration. These results indicate that changes are not dependent on the type of agonist, as would be expected given that the Cys-loop is located at the extracellular-transmembrane interface and not close to the binding site.

We have demonstrated that unc-63(x26) C151Y forms functional receptors (presumably with UNC-38, UNC-29, LEV-1, and LEV-8 (11)) because L-nAChR channel activity was detected, whereas no channel activity was detected in the null mutant (unc-63(x37)) (Fig. 1). In addition, the use of antibodies raised against UNC-63 revealed a punctate staining pattern in the nerve cord of unc-63(x26) worms (Fig. 4E), which is characteristic of nAChRs assembled and clustered at neuromuscular junctions (31), similar to that of wild-type strains but distinct from the null mutant. Moreover, immunocytochemistry assays in cultured cells revealed that the UNC-63 C151Y mutation caused no detectable changes in the level of surface expression of L-nAChRs (Fig. 3). We were unable to estimate the opening rate of L-nAChRs because opening events could not be unambiguously attributed to a single channel, as there may be more than one channel present in the recording (21, 32). Nonetheless, the decrease in the frequency of channel openings observed without a corresponding reduction in surface expression levels suggests that the fewer openings in the unc-63(x26) muscle are due to a lower opening rate.

Mutations in human muscle nAChRs have been associated with congenital myasthenic syndromes (CMS) (33), a group of genetically determined heterogeneous disorders all characterized by muscle weakness (34). The syndromes are grouped into nAChR deficiency and kinetic abnormality, which can cause loss (FCCMS) or gain (slow-channel CMS) of function (16, 33).

FCCMS patients have, in common, greatly reduced nAChR activation in response to nerve impulses. Such a reduction can occur either by nAChRs closing abnormally rapidly, which is observed at the single-channel level as briefer channel openings, or by channels showing impaired opening, which is observed as reduced frequency of openings and prolonged duration of closed periods. It has been shown that most fast-channel mutations inhibit receptor activation by both mechanisms (33). As the C. elegans unc-63(x26) strain mimics the molecular features reported for human FCCMS (reduced open probability and rapid channel closing), this strain may be a candidate in vivo invertebrate model for this type of CMS.

Mutations in the Cys-loop of human AChRs have been found to be associated with CMS. In one case, the mutation was in the position equivalent to C151Y, albeit in the ε-subunit (eC128S) (35). The eC128S mutation is recessive and becomes pathogenic in a patient carrying a mutation in the other allele. Ligand binding studies have shown that the C128S mutant ε-subunit is not incorporated into cell-surface nAChRs, thus behaving as a null mutation (35). Lack of strict functional equivalence between Cys-loops of different nAChR subunits has been reported (36); for example, eC128S is a null mutation, whereas UNC-63 C151Y is not. Moreover, another mutation within the Cys-loop of the human α-subunit, V132L, has been shown to produce FCCMS. This mutation leads to a 7-fold reduction in the duration of the dominant component of the open time histogram and prolongs closings, similar to our observations with the UNC-63 C151Y mutant (36).

The acetylcholine esterase inhibitor PB is used to treat patients with FCCMS, commonly in conjunction with the potassium channel blocker 3,4-DAP. C. elegans possesses homologs of both human acetylcholine esterase (37) and potassium channels (38), and we have shown that the uncoordinated phenotype of unc-63(x26) mutant worms was partially rescued with PB, 3,4-DAP, or both PB and 3,4-DAP (Fig. 5). All concentrations of 3,4-DAP used resulted in partial rescue,
whereas with PB, partial rescue was observed at 0.9 and 15.6 mM. These concentrations of PB also increased the thrashing rate of *unc-63(x26)* worms in a teaching study exploring the classroom use of a *C. elegans* human disease model (39). The statistical power analysis of the thrashing rates of *unc-63(x26)* worms in PB was calculated to be ~30% (Decision Support Systems). Thus, our assay may have missed subtle yet real effects of PB at concentrations between 0.9 and 15.6 mM.

The availability of a fully sequenced genome (40) in which 60% of the genes have vertebrate counterparts (41) has led to the widespread adoption of *C. elegans* as a model organism for investigating neuronal diseases (19) and is increasingly being used in disease-oriented drug screens (42, 43). The finding that drugs used to treat FCCMS also reduce the muscle deficiency of *unc-63(x26)* worms highlights the potential of using this mutant strain in whole-organism high-throughput *in vivo* screens for new therapeutic treatments for human CMS.

In conclusion, we have shown that a mutation at the Cys-loop of the *C. elegans* muscle AChR impairs channel function,

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**FIGURE 5.** PB and 3,4-DAP partially rescue the *unc-63(x26)* uncoordinated phenotype. *A*, thrashing movement of an adult wild-type N2 worm in M9 medium. A single thrash was defined as a change in the direction of bending at the midbody. *B–D*, the thrashing rates of N2 (dashed line), *unc-63(x26)* (solid line), and *unc-63(x37)* (dotted line) worms were detected manually in different concentrations of drugs used to treat FCCMS. Thrashing rates in response to drugs that are significantly higher than the rate in M9 medium alone are indicated by single and double asterisks ($p < 0.05$ and $0.01$, respectively; one-way ANOVA and Dunnett’s post hoc test). Values at 0 concentration indicate worm thrashing in M9 medium only without any drugs. *E and F*, automated measurement of *C. elegans* thrashing (swimming) detects drug rescue of the *unc-63(x26)* uncoordinated phenotype. *E*, thrashing rates of N2 (dashed line) and *unc-63(x26)* (solid line) worms in different concentrations of 3,4-DAP. Values at 0 concentration indicate worm thrashing in M9 medium only without any drugs. Thrashing rates in 3,4-DAP that are significantly higher than those in M9 medium alone are indicated by a single asterisk ($p < 0.05$, one-way ANOVA and Dunnett’s post hoc test). *F*, the thrashing rates of *unc-63(x26)* determined by automated analysis (•; also shown in *E*) were similar to those obtained by manual counting (○; also shown in *C*). The ability to automate the phenotypic rescue by drugs offers the future possibility of screening chemical libraries. Thrashing rates are shown as the mean ± S.E. (three to seven batches of eight worms).
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giving rise to phenotypic changes that resemble, to a certain degree, those found in human diseases. We therefore propose that the C. elegans unc-63(x26) allele may offer a useful model for developing therapies for CMS.

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