Functional genomics in *Brugia malayi* reveal diverse muscle nAChRs and differences between cholinergic anthelmintics

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Many techniques for studying functional genomics of important target sites of anthelmintics have been restricted to *Caenorhabditis elegans* because they have failed when applied to animal parasites. To overcome these limitations, we have focused our research on the human nematode parasite *Brugia malayi,* which causes elephantiasis. Here, we combine single-cell PCR, whole muscle cell patch clamp, motility phenotyping (Worminator), and dsRNA for RNAi for functional genomic studies that have revealed, in vivo, four different muscle nAChRs (*M-, L-, P-,* and *N-*). The cholinergic anthelmintics had different selectivities for these receptors. We show that motility and patch-clamp responses to levamisole and pyrantel, but not morantel or nicotine, require the *unc-38* and/or *unc-29* genes. Derquantel behaved as a competitive antagonist and distinguished *M*-nAChRs activated by morantel (K_b 13.9 nM), *P*-nAChRs activated by pyrantel (K_b 126 nM), and *L*-nAChRs activated by levamisole (K_b 0.96 μM) and bephenium. Derquantel was a noncompetitive antagonist of nicotine, revealing *N*-type nAChRs. The presence of four diverse nAChRs on muscle is perhaps surprising and not predicted from the *C. elegans* model. The diverse nAChRs represent distinguishable drug targets with different functions: Knockdown of *unc-38*+*unc-29* (*L*- and/or *P*-receptors) inhibited motility but knockdown of *acr-16*+*acr-26* (*M*- and/or *N*-receptors) did not.

filaria | *Brugia* | dsRNA | nAChR | patch clamp

The neglected tropical diseases caused by nematode parasites affect some 2 billion people globally, and effective vaccines are not available. Although these parasites by themselves do not produce high mortality, they produce significant morbidity that affects worker productivity and increase susceptibility to AIDS and malaria, and they are strongly associated with poverty in endemic countries (1–4). There are only a limited number of classes of anthelmintic drugs that are used to treat these diseases, and there are concerns about the development of resistance (5). There is a pressing need to advance the study of anthelmintic drugs.

Many useful studies on modes of action of anthelmintic drugs have been conducted on the model nematode, *Caenorhabditis elegans*, but this is not a parasite: It is separated evolutionarily by millions of years from parasitic nematodes and it does not possess "parasitism genes" (6). This separation increases the impetus for anthelmintic studies using real parasites, but until now many techniques developed in *C. elegans* have not have been tractable when applied to animal parasites (7).

The neuromuscular systems of nematodes, which control motility and support feeding, growth, and reproduction, are governed by significant parts of their gene pools (8, 9). Their neuromuscular systems are unlike those of vertebrates because nematode muscles send processes to the motor neurons to form synapses, rather than the other way around. Membrane ion-channels, which regulate nematode neuromuscular systems, are target sites of major classes of anthelmintic drugs, including the cholinergic anthelmintics levamisole, pyrantel, and derquantel (10–17). Here, we have combined a number of techniques for the study of muscle pentameric acetylcholine receptor channels (nAChRs) of the filarial nematode parasite, *Brugia malayi.* Filariasis is one of a group of neglected tropical diseases caused by clade III parasitic nematodes that are transmitted by biting insects. *B. malayi* adults are found in the lymphatic vessels of humans, producing lymphatic filariasis, sometimes associated with swelling of the limbs (elephantiasis). *B. malayi* is also a suitable model parasite because it can be maintained in laboratory rodents, unlike a more common cause of lymphatic filariasis *Wuchereria bancrofti*. Lymphatic filariasis occurs in some 120 million people in developing countries, and regrettably, effective treatments for adult filaria infections are limited. Although a triple combination of ivermectin, diethylcarbamazine, and albendazole seems to be effective (18), development of new treatments to kill adults is still required (19, 20). Cholinergic anthelmintics (levamisole, haloxon) are not regularly used for the treatment of *B. malayi*, but there are reports of their efficacy (21, 22) that have encouraged us to study their nAChRs as anthelmintic targets.

To study the functional genomics of nAChRs of *B. malayi,* we used the following: (*i*) video capture for quantitative phenotyping (23, 24); (*ii*) single-cell PCR to identify nAChR subunit genes expressed in muscle (25); (*iii*) dsRNA for RNAi knockdown of *unc-38*+*unc-29* of nematode levamisole and pyrantel receptors (26, 27), and (*iv*) whole-cell patch clamp to record functional properties of muscle nAChRs (10, 28). Knockdown of *acr-16*+*acr-26* had little effect on motility, but knockdown of *unc-38*+*unc-29* abolished motility, suggesting in *B. malayi* that different physiological functions may be ascribed to different receptors. Our observations revealed that there are four types of nAChR in *B. malayi* muscle.

Significance

Methods for studying functional genomics of ion-channel target sites of antinematodal drugs (anthelmintics) have been limited to the model nematode *Caenorhabditis elegans* because many techniques for studying animal parasitic nematodes have been unsuccessful. Here, we develop preparations of the human parasite *Brugia malayi,* which causes elephantiasis, to allow us to combine RNAi, PCR amplification of RNA from single muscle cells, computer analysis of movement, and patch-clamp electrophysiology to study the action of cholinergic anthelmintics. We find that they are not a homogenous class of drugs and that they act selectively on four distinguishable, *M-, L-, P-,* and *N*-acetylcholine ion-channel receptors. These four receptors are distinct druggable targets with different physiological functions.

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Two types, which require UNC-38 and/or UNC-29 subunits, support motility: They are the *L*-type, which is preferentially activated by levamisole, and the *P*-type, which is preferentially activated by pyrantel. The two other types, which persisted in the absence of UNC-38 and UNC-29 and which did not maintain motility, were the *M*-type, which was preferentially activated by morantel and preferentially antagonized by derquantel, and the *N*-type, which was preferentially activated by nicotine. Separation of the four muscle receptors is significant and contrasts with the model nematode, *C. elegans*, where only *N*- and *L*-receptors are recognized (29). The four receptors could be targeted by future combinations of single receptor selective cholinergic anthelmintics or one that is equally potent on all of the receptors. Either of these approaches are anticipated to overcome or slow the development of resistance due to mutation at a single receptor.

Results

Cholinergic Anthelmintics Inhibit Whole *B. malayi* Motility. Fig. 1*A* shows the concentration-dependent inhibitory effects of cholinergic agonist anthelmintics on motility of *B. malayi* adult females. The effects of each of these agonists were rapid and occurred within 5 min of application. We quantitated the motility of the adult *B. malayi* by using Worminator motility measurements at 5 min to compare the potencies of the anthelmintics. Levamisole was the most potent with an IC_{50} of 99 ± 2 nM; pyrantel was less potent, IC_{50} = 516 ± 30 nM; as was morantel IC_{50} = 3.7 ± 0.7 μM; and nicotine, IC_{50} = 4.8 ± 0.8 μM. The potency series was: levamisole > pyrantel > morantel = nicotine. We also tested the effects of a cholinergic antagonist, derquantel, and found that it was the least potent of the anthelmintics on motility inhibition: Even high concentrations of derquantel (10 μM) only had a small (~30%) inhibitory effect on motility (Fig. 1*B*). Interestingly, despite a poor effect on motility, derquantel antagonized and inhibited the effects of the cholinergic agonists. Fig. 1*B* shows the inhibitory effect of derquantel on the actions of levamisole on whole-worm motility, indicating that its low potency inhibiting motility is not due to the failure to permeate the cuticle of *B. malayi* because it inhibits the effects of levamisole. To examine differences between the actions of these cholinergic anthelmintics, we conducted single-muscle cell RT-PCR to identify nAChR subunits and whole-muscle cell patch clamp to characterize receptors.

Single-Cell PCR Reveals Expression of Multiple nAChR Subunits. We are able to collect cytoplasm from individual somatic muscle cells of *B. malayi* for PCR analysis. Suction on the whole muscle-cell patch pipette following recording allowed us to collect cytoplasm from single muscle cells. After collection of cytoplasm, the 2-mm tip of the patch pipette was broken off and placed in a single-step RT-PCR mix. We designed specific primer pairs (Table S1) to look for and were able to detect amplicons in each of six muscle cells per subunit: *Bma-acr-8, Bma-acr-16*, *Bma-acr*-21, *Bma-acr*-8, *Bma-unc-63*, *Bma-unc-38*, *Bma-unc-29*, and *Bma-acr*-26 (Fig. 2*A*), along with the housekeeping gene, *gapdh* (30). Although we confirmed the expression of *Bma-acr*-27 in whole *B. malayi* worms, expression in single muscle cells was more difficult to detect, and amplicons were visible only in 2 of 8 single muscle cells tested (Fig. S1). Recovery of mRNA for these different subunits encouraged the view that multiple nAChRs are expressed in single *B. malayi* somatic muscles and that the receptor types differ from those seen in *C. elegans* where only levamisole-sensitive, *L*-type, and the nicotine-sensitive, *N*-type, of nAChR are observed (31). Clearly there are additional subunits present indicating the presence of additional nAChR subtypes.

Cholinergic Anthelmintic Agonist Effects on *B. malayi* Muscle nAChR Currents. Fig. 2*B* shows a representative recording of the inward current responses to 100 μM acetylcholine followed by the effects of increasing concentrations of levamisole made on a muscle cell clamped at −40 mV: These current responses illustrate the presence

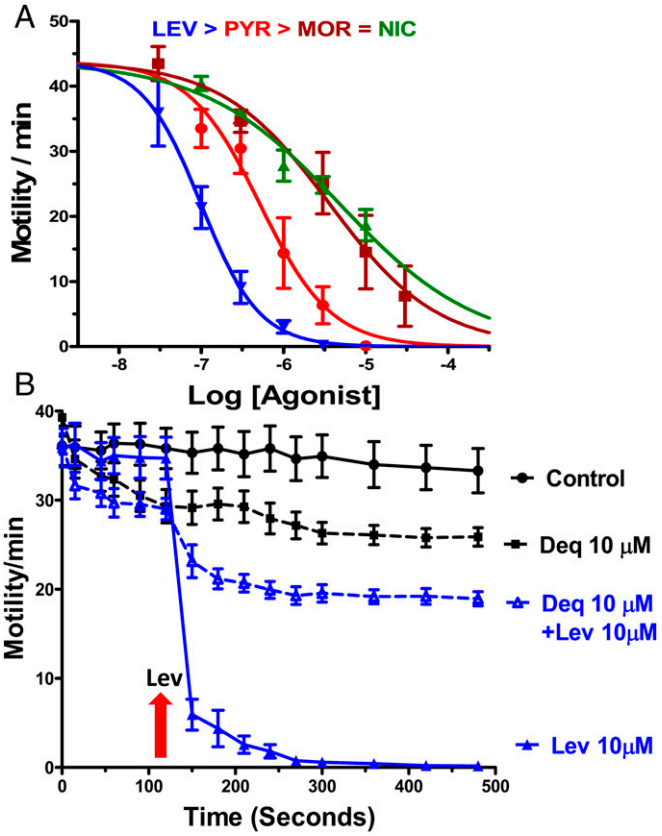

Fig. 1. (*A*) Concentration response plots of the effects of levamisole, pyrantel, morantel, and nicotine on motility of whole adult female worms. Each point is the mean ± SE of four worms, each from a different batches of worms. Curves were fitted with variable slope nonlinear regression model analysis in GraphPad Prism 6. Concentration response curves for different agonists: LEV, levamisole 99.2 ± 2.3 nM; PYR, pyrantel 516 ± 3 nM; MOR, morantel 3.7 ± 0.7 μM; and NIC, nicotine 4.8 ± 0.8 μM (n = 4 for each concentration of agonist, four biological replicates). (*B*) Effects over time on motility of whole *B. malayi* of: 10 μM derquantel (Deq 10 μM); derquantel pretreatment followed by 10 μM levamisole (Deq 10 μM + Lev 10 μM, levamisole at the arrow); 10 μM levamisole (Lev 10 μM, levamisole at the arrow). Note that derquantel only has a modest effect on motility by itself, but clearly, it reduces the inhibitory effect of levamisole. n = 12, four biological replicates, two-way ANOVA and Bonferroni post hoc tests: ***P < 0.001; **P < 0.01.

of functioning nAChRs on *B. malayi* muscle cells. We tested other cholinergic anthelmintics in the same manner, and Fig. 2*C* shows log-concentration plots, normalized to the first application of 100 μM acetylcholine, for levamisole, pyrantel, morantel, nicotine, and bephenium. Acetylcholine had an EC_{50} of 2.4 ± 0.6 μM; pyrantel had an EC_{50} of 63.5 ± 0.2 nM; morantel had an EC_{50} of 101.8 ± 0.4 nM; levamisole had an EC_{50} of 3.4 ± 0.6 μM; bephenium had an EC_{50} of 4.2 ± 0.1 μM with a reduced maximum response and; nicotine had an EC_{50} of 30.1 ± 0.4 μM (n = 7 for all agonists). Thus, the EC_{50} potency series was pyrantel > morantel >> acetylcholine = levamisole = bephenium >> nicotine. This potency series was different from the *B. malayi* whole worm motility assay with pyrantel and morantel being more potent than levamisole. One reason for the difference in the potency series is the cuticle barrier of *B. malayi*, which may be more permeable to levamisole, which is much less polar than pyrantel and morantel at pH 7.2.

Selective Antagonist Effects of Derquantel Distinguish Four Muscle nAChRs. We also tested morantel, pyrantel, levamisole, bephenium, and nicotine as agonists in the presence of the selective anthelmintic antagonist derquantel (n = 5). Fig. S2*A* shows

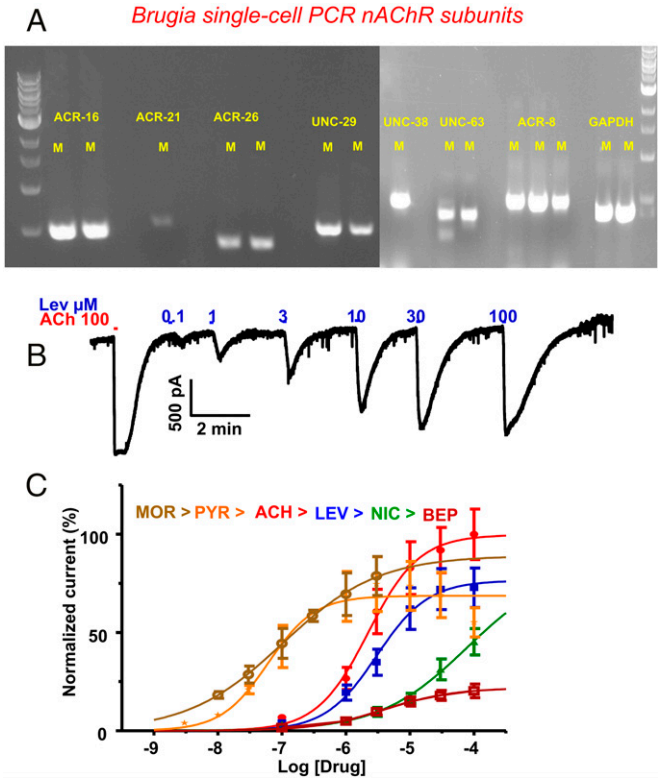

Fig. 2. (*A*) Single muscle cell PCR demonstrating the presence of different nAChR subunits expressed in single muscle cells. Representative gel pictures show the presence of mRNA for *acr-16*, *acr-21*, *unc-29*, *unc-38*, *unc-63*, *acr-8*, and *acr-26*. GAPDH was the internal control for each cell (example of $n = 6$, all positive for each gene). (*B*) Concentration-dependent effect of nicotinic agonists on *B. malayi* muscle cell whole cell patch-clamp recordings. Representative trace illustrating effects of increasing concentrations of levamisole on inward current, response is normalized to ACh 100 μM (first application) for EC_{50} calculation. (*C*) Concentration-response curves for different agonists. ACH, acetylcholine; BEP, bephenium; LEV, levamisole; MOR, morantel; NIC, nicotine; PYR, pyrantel; ($n = 7$ for each agonist).

representative current traces demonstrating that derquantel inhibited all of the agonists. Fig. S2*B* shows bar charts of the mean ± SE currents produced by the different agonists without, and then with, 1 μM derquantel for morantel, or 10 μM derquantel for the other agonists. The inhibition was significant ($P \leq 0.001$, two-way ANOVA and Bonferroni post hoc tests) for all of the agonists and reversible on washing.

The effect of derquantel on concentration–response relationships for these agonists is shown in Fig. 3. We fitted parallel concentration–response plots to quantify and describe the inhibitory action of derquantel by using the competitive antagonist model and determined the concentration ratios (CR) from the ratios. Derquantel had a potent effect on morantel, so we used 1 μM derquantel as the antagonist concentration and found the concentration ratio to be 74.8 ± 5.9 ($n = 6$). We calculated the dissociation constant, K_b, for derquantel from the classic Gaddum–Schild equation:

$$\text{CR-1} = X_b/K_b,$$

where X_b is antagonist concentration, K_b is the antagonist dissociation constant, and CR is the concentration ratio. The K_b for derquantel using morantel as the agonist was 14 nM. We used 10 μM derquantel with the other agonists because we found that it was less potent. The pyrantel concentration-ratio was 81.8 ± 5.2 ($n = 6$) and the K_b was 126 nM; the levamisole CR was 12.6 ± 1.3 ($n = 6$) and the K_b was 956 nM; the bephenium CR was 3.1 ± 0.3 ($n = 6$) and the K_b was 5.4 μM. The antagonism of nicotine was noncompetitive (Fig. 3*E*), demonstrating that nicotine activates a different population of nAChRs to the other agonists. Two-way ANOVA showed that the differences between the agonist CRs were significant ($P < 0.001$), and the Bonferroni multiple comparison tests showed that the CRs for 10 μM derquantel were significantly different for the agonists except for levamisole and bephenium. These observations show that morantel, pyrantel, levamisole, and nicotine activate different populations of receptors, but that levamisole and bephenium activate populations of nAChRs that were not distinguished by derquantel.

The derquantel inhibitory potency sequence is morantel >> pyrantel >> levamisole. We interpret our observations to indicate that there are four separable types of nAChR present on *B. malayi* muscle. The receptors preferentially activated by morantel and inhibited most by derquantel we refer to as the *M*-receptors, the

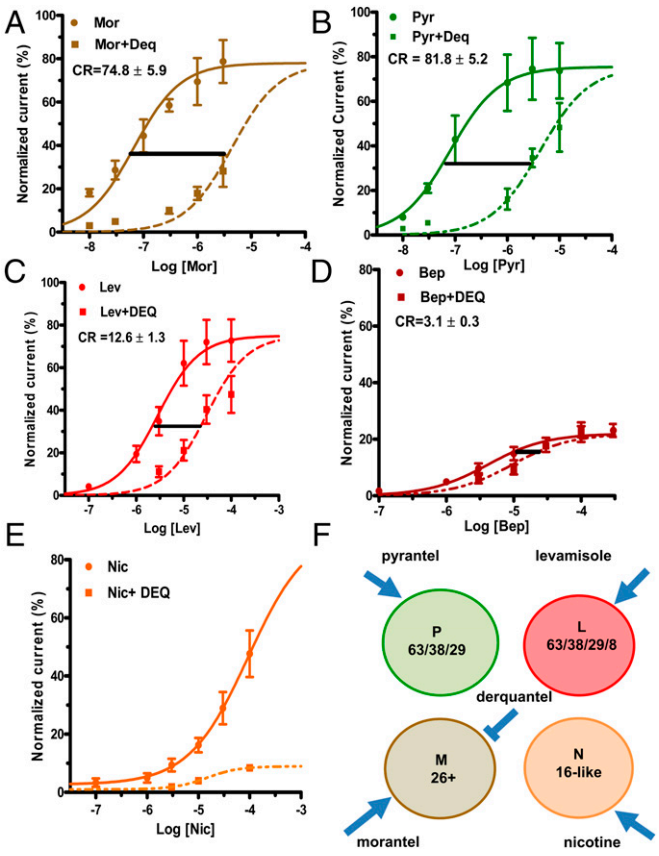

Fig. 3. Selective antagonist effects of derquantel on nAChR agonist concentration response relationships. Control concentration response relationship in the absence (solid line) and then in the presence (broken line) of derquantel. (*A*) Morantel antagonism by 1 μM derquantel. (*B*) Pyrantel was antagonized in the presence of 10 μM derquantel. (*C*) Levamisole concentration–response relationship was antagonized by 10 μM derquantel. (*D*) Bephenium concentration response curve was antagonized in the presence of 10 μM derquantel. (*E*) Derquantel suppressed the nicotine concentration response relationship in noncompetitive manner, $n = 5$ worms for each set of experiments (CR, concentration ratio). (*F*) Diagrammatic representation of the four types of nAChR: *P*, *L*, *M*, and *N*. Also shown is their selective agonists with derquantel being the most potent inhibitor on the *M*-receptor. The *L*-receptor is shown as composed of the subunits ACR-63 (63), UNC-38 (38), UNC-29 (29), and ACR-8 (8); the *P*-receptor is shown as composed of the subunits ACR-63 (63), UNC-38 (38), and UNC-29 (29); the *M*-receptor is shown as composed of the subunits ACR-26 (26) and another (+), possibly ACR-27 subunit; the *N*-receptor is proposed to be composed of ACR-16-like (16-like) subunits and may include ACR-21. Bep, bephenium; CR, concentration ratio; Deq, derquantel; Mor, morantel; Nic, nicotine.

receptors preferentially activated by pyrantel as the *P*-receptors, and the receptors preferentially activated by levamisole as the *L*-receptors. Because derquantel had a noncompetitive effect on nicotine in contrast to the effects on the other agonists, it demonstrates that nicotine acts on a different population of nAChRs. We refer to the preferred population of receptors activated by nicotine as the *N*-receptors. Fig. 3*F* is a schematic representation of the main pharmacological properties of these receptors and their putative subunit compositions.

Knockdown of unc-29 and unc-38mRNA Is High and Selective. RNAi has not been tractable when applied to animal parasitic nematodes, unlike the model nematode, *C. elegans* (32, 33). Success using heterogeneous short interfering RNA (hsiRNA) mixtures has been reported with *B. malayi* (34). We found that we did not need to use hsiRNA and that a high level of mRNA knockdown was achieved when we used 30 μg/mL of double stranded RNA (dsRNA) for 4 d. We designed and screened our dsRNA platform for adult *B. malayi* to knock down two specific nAChR subunit transcripts selected out of a group of some 30 nAChR subunits that might be present in the whole worms (30). We targeted both UNC-29 and UNC-38 nAChR subunits, which contribute to putative levamisole-sensitive, *L*-type, and pyrantel-sensitive, *P*-type nAChRs in parasitic nematodes (14, 35). We used two negative controls: an untreated (no added dsRNA) and *lacZ* from *E. coli* as a nonspecific dsRNA control that is not expressed in part or whole in nematodes. A dual-target dsRNA mixture delivery approach worked well and produced robust results in every experiment. Fig. 4*A* shows the high percentage of mRNA knockdown of *unc-29+unc-38* (specific) dsRNA-treated worms compared with the *lacZ* dsRNA-treated worms: The mRNA levels of the other controls, GABA (*unc-49*) and GAPDH (*gpd-1*), remain little affected for both treatments. The mRNA levels were measured on single worms that were the same worms used for the day 5 electrophysiology recordings described later. We achieved significant (two-way ANOVA and Bonferroni post hoc, $P \leq 0.001$) knockdown of transcript levels for *unc-29* (95 ± 2.8%) and *unc-38* (80 ± 6.9%) in the specific dsRNA-treated worms compared with modest change in the transcript level for *unc-29* (7.3 ± 3.1%) and *unc-38* (7.8 ± 2.9%) in the *lacZ* dsRNA-treated worms. We also tested whole worms for transcript knockdown for the nAChR subunits, ACR-26, ACR-27, and ACR-16. There was no significant knockdown in either of the nonspecific or specific dsRNA-treated worms (Fig. S3).

dsRNA Knockdown of unc-29+unc-38 Produces a Motility-Inhibition Phenotype. We used the Worminator video tracking system to quantify, using pixel changes, the motility of the adult female worms exposed to specific (*unc-29+unc-38*) and *lacZ* (nonspecific) dsRNA over 5 d. Fig. 4*B* shows the recorded motility at daily intervals for control (untreated and *lacZ* dsRNA-treated) worms and test (*unc-38+unc-29* dsRNA-treated) worms. There was a large and significant (ANOVA, $P < 0.001$, $n = 114$) decrease in motility in the worms exposed to *unc-29+unc-38* dsRNA by day 3 but only a modest decrease in motility in the control worms or *lacZ*-treated worms. These observations suggest that UNC-29 and/or UNC-38 are essential for maintaining the motility phenotype and that other nAChR subtypes, which do not contain UNC-29 and/or UNC-38 subunits, do not support motility. To test this hypothesis further, we conducted similar experiments and knocked down *acr-16+unc-26* and found that in contrast to knockdown of *unc-38+unc-29,* there was little effect on motility (Fig. S4).

Only Levamisole and Pyrantel Currents Are Sensitive to unc-29+unc-38 Knockdown. dsRNA directed-target knockdown reduced transcript and motility levels (Fig. 4) and also changed the responses to agonists (Fig. 5). We tested the whole muscle-cell current responses of the two controls (untreated and *lacZ*, $n = 16$ for both) and dsRNA-treated (*unc-29+unc-38*, $n = 16$) worms

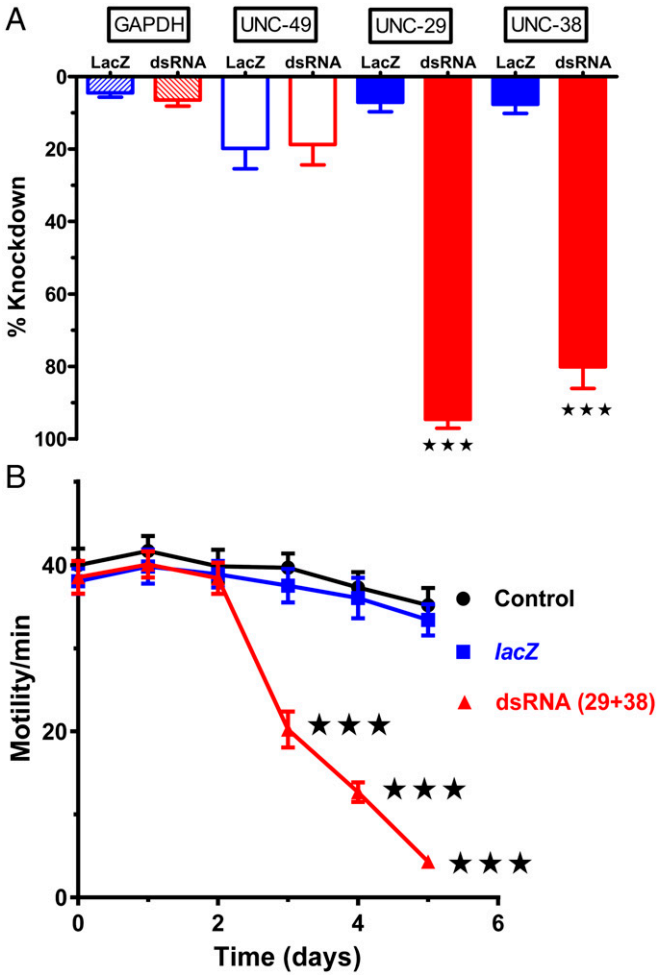

Fig. 4. Effects of dsRNA *unc-38+unc-29* on quantitative PCR and motility analysis demonstrate a selective reduction in *unc-38* and *unc-29* transcript levels and motility in dsRNA mixture soaked *B. malayi*. (*A*) Bar chart demonstrating quantitative PCR results showing a significant reduction in transcript levels of *unc-29* (by 95 ± 2.8%, 29 red bar) and *unc-38* (by 80 ± 6.9%, 38 red bar) from the control transcript levels in *lacZ* dsRNA-treated worms, *unc-29* (by 7.3 ± 3.1%, 29 blue bar) and *unc-38* (by 7.8 ± 2.9%, 38 filled blue bar). Quantitative PCR results are also shown for GAPDH (hatched bars) and for the GABA receptor subunit transcript *unc-49* (open bars). $n = 8$, one-way ANOVA and Bonferroni post hoc tests; ***$P < 0.001$. (*B*) Time series of motility for dsRNA-soaked worms. Black color data points (circles) representing control, blue color data points (squares) *lacZ* (nonspecific dsRNA), and red (triangles) color representing the worms treated with mixture of *unc-29* and *unc-38* dsRNA. $n = 38$ worms for all treatments, two-way ANOVA and Bonferroni post hoc tests; ***$P < 0.001$.

to fixed concentrations of acetylcholine, levamisole, nicotine, morantel, pyrantel, and bephenium on day 5. We included GABA, which is an inhibitory transmitter in nematodes, and which does not activate nAChRs as an additional control. Fig. 5*A* shows representative examples of the inward current responses to GABA, acetylcholine, nicotine, morantel, levamisole, pyrantel, and bephenium recorded at −40 mV in untreated worms and in *unc-29+unc-38* dsRNA-treated worms. We compared effects of the dsRNA on each of the agonist responses. Fig. 5*B* shows that the current responses in *unc-29+unc-38* dsRNA-treated worms were significantly reduced for levamisole, pyrantel, and bephenium compared with untreated worm responses. Similar reductions were seen when we compared *lacZ* controls with the dsRNA-treated worms (Fig. S5). The effects on GABA, acetylcholine, morantel, and nicotine current responses were not statistically significant, suggesting that the UNC-29 and/or UNC-38 subunits

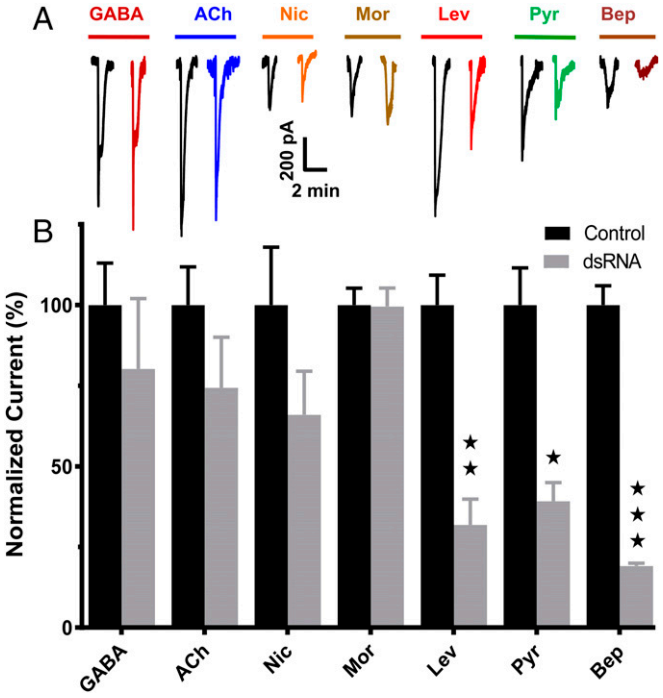

Fig. 5. Levamisole, pyrantel and bephenium currents are sensitive to *unc-29* and *unc-38* knockdown. (*A*) Representative traces demonstrating the inward current responses to acetylcholine, levamisole, nicotine, pyrantel, bephenium and GABA at -40 mV in control untreated and dsRNA-treated worms under whole-cell patch clamp ($n = 8$). (*B*) Bar chart illustrating the agonist current responses of 29+38 dsRNA-treated worm normalized against mean current response of each agonist in control untreated worms. $n = 8$ worms for each agonist. Only levamisole, pyrantel, and bephenium responses were significantly inhibited when 29+38 dsRNA worm's responses were compared with untreated control dsRNA-treated worm currents: one-way ANOVA and Bonferroni post hoc tests: $***P < 0.001$; $**P < 0.01$; $*P < 0.05$. Ach, acetylcholine; Bep, bephenium; Lev, levamisole; Mor, morantel; Nic, nicotine; Pyr, pyrantel.

are an essential part of receptors that respond to levamisole, bephenium, and pyrantel, but these subunits have little or no part to play in the responses to the other agonists. These observations suggest that the *L*-type and *P*-type nAChRs contain UNC-38 and/or UNC-29 subunits but that the *N*-type and the *M*-type do not contain UNC-38 and/or UNC-29 subunits.

No Monepantel-Sensitive Receptors on *B. malayi* Muscle. Monepantel is another cholinergic anthelmintic that acts as a positive allosteric modulator of the DEG-3 family of nAChRs that may be in nematode muscle (36, 37). We tested the effects of 1 mM choline and 10 μM monepantel on *B. malayi* whole-muscle currents. We found that monepantel was without effect when tested in four preparations (Fig. S6). These observations suggest the absence of monepantel receptors [DEG-3 nAChRs (MPLR-1, ACR-23, and ACR-20)] from *B. malayi* muscle.

Discussion

Despite there being a published genome of *B. malayi* (38), functional and genomic studies of the modes of action of anthelmintics in filaria have been difficult, and there have been only a few successful reports of RNAi experiments in *B. malayi* (34, 39–47). Here, we are able to describe a reliable RNAi method, using soaking in dsRNA that we have coupled with movement phenotyping and patch clamp to study the effects of cholinergic anthelmintics on adult *B. malayi* in vitro. Important components were the optimization of the dsRNA concentration and culture duration required for effective and selective RNAi knockdown. We can now conduct functional genomics related to the modes of action of anthelmintics in a significant nematode parasite; there are advantages over the use of the *C. elegans* model, which is evolutionarily distant and does not contain "parasite genes." We are optimistic we can now explore functional properties of their other ion channels, effects of anthelmintics, and examine the role of individual or groups of selected genes in this major parasite. We did not find significant off-target effects with our *lacZ* controls and no significant knockdown of GAPDH, GABA, or other nicotinic channel subunit genes (Fig. 4 and Fig. S3).

Functional Interpretation of the Observations. We have identified four distinct (*M*-, *L*-, *P*-, and *N*-) types of cholinergic receptors on muscle of *B. malayi* (Fig. 3). The dsRNA knockdown dissected out *L*- and *P*-receptors from *M*- and *N*-receptors, and derquantel dissected out *M*-, *P*-, *L*-, and *N*-receptor populations. The names for these receptor types is based on the agonists we used with derquantel as the antagonist. We point out that the agonists are not exclusive to one receptor and will have some overlapping actions, depending on the agonist concentration and receptor type. nAChRs are composed of five subunits with the ion channel in the center as the pore. These ion channels, if they are composed of five identical subunits, are described as homomeric or heteromeric if these subunits are different. In *C. elegans* (31, 35), only two types of muscle nAChR are recognized: the *L*-type, which appears to be composed of UNC-63, UNC-38, UNC-29, LEV-1, and LEV-8 subunits, and the *N*-type, which appears to be composed of ACR-16 subunits. There are also a range of ACR-16–like subunits, including ACR-21 present in other tissues of *C. elegans* (48, 49), which are anticipated to form homomeric receptors in vivo. In animal parasitic nematodes, there is a greater diversity in the nAChRs that are present on somatic muscle. In *Ascaris suum*, there are three separable muscle subtypes [*N*-, *L*-, and *B*- (27)] and four types in *Oesophagostomum dentatum* (11). *Xenopus* expression experiments suggest in *Haemonchus contortus* and *Oesophagostomum dentatum* that: the *L*-type (L-AChR-1) is composed of UNC-63, UNC-38, UNC-29, and ACR-8 subunits; the *P*-type (L-AChR-2) is composed of UNC-63, UNC-38, and UNC-29 subunits (10, 25); and the *N*-type in *Ascaris suum* is composed of ACR-16 subunits (14, 35, 50). A fourth type of muscle nAChR selectively activated by morantel and composed of ACR-26 and ACR-27 subunits has been described in *Haemonchus contortus* (51). We anticipate that our *M*-type could include nAChRs involving ACR-26 subunits and ACR-27 or other subunits.

One of the limitations of these earlier studies has been the difficulty in performing RNAi and in vivo electrophysiology on the same parasite for functional genomic studies. Here, we have been able to overcome those difficulties and were able to knockdown *unc-38* and *unc-29* mRNA to show that the *L*-types, which are sensitive to levamisole, and/or the *P*-types, which are sensitive pyrantel, are required to drive spontaneous motility of *B. malayi.* In contrast, knockdown of *acr-16+acr-26* does not inhibit the motility phenotype, suggesting that the *M*- and *N*-type receptors do not drive motility. We also point out that derquantel was most potent as an inhibitor of the *M*-type of receptors and derquantel only had a weak effect on motility, supporting the view that the *M*-receptors do not drive motility. This lack of effect on motility is interesting because it raises the question of the function of the *M*- and *N*-receptors in the muscle. nAChR subunit genes are not only expressed in body muscle, but they are widely distributed and found on embryos, spermatogonia, and lateral cord of *B. malayi* (52); the distribution suggests that the ion-channels function in development and metabolism and not just in muscle control. nAChRs have functions outside the neuromuscular system in mammals where they are also involved in immune responses, metabolism, and early development (53). Thus, *M*- and *N*-receptors may have paracrine, immune response, and/or developmental functions in parasitic nematodes. Anthelmintics like derquantel targeting *M*- or other anthelmintics targeting *N*-receptors could have effects on

development, responses to host attack, and adaptations to different environments in the host rather than just effects on motility. Future phenotypic anthelmintic screens for drug development should include growth and developmental assays as well as release of excretory substances and exosomes assay (54–56) in addition to motility screens.

Materials and Methods

Details of our methods are provided in *SI Materials and Methods* and are outlined here. Female *B. malayi* were used for studying functional genomics by using: (*i*) Worminator for motility phenotyping; (*ii*) single-cell RT-PCR to identify nAChR subunit genes expression; (*iii*) dsRNA for knockdown of UNC-38+UNC-29 subunits of nematode levamisole and pyrantel receptors and ACR-16+ACR-26 subunits of nicotine and morantel receptors; and (*iv*) whole-cell patch clamp to study functional properties of muscle nAChRs.

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