

Research article

Functional analysis of nematode nicotinic receptors

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Parasitic nematodes pose a significant threat to the health and economic welfare of communities worldwide, both directly through human disease and indirectly through infection of livestock and crop. At present, anthelmintic drugs such as the nicotinic agonists, which target nematode nicotinic acetylcholine receptors (nAChRs), offer a facile and cost-effective method of controlling both human and animal infection. Unfortunately, the continued heavy reliance on such compounds has led to the inevitable emergence of resistance in many different Nematoda species, thus making the subject of nematode nicotinic receptors of great importance. Using various levamisole-resistant, nAChR subunit mutants of the soil-dwelling nematode *Caenorhabditis elegans* (viz. *unc-29*, *unc-38* and *unc-63*) and well-established resistance-monitoring assays, the responses of the different strains to the nicotinic agonists levamisole, morantel, oxantel and pyrantel have begun to be characterized. Wild-type adult *C. elegans* are relatively unaffected by 1 mM oxantel, contrasting with a developmental retardation of larvae. Also, *unc-38* mutants appear to be less refractory to the anthelmintics than the *unc-29* and *unc-63* animals. In preparation for the use of *C. elegans* as an ectopic transformation system for parasitic receptor DNA, preliminary biolistic transformation experiments were performed using microparticle bombardment. It was found that transformation of *unc-38* animals with *myo-3::gfp* construct has no significant effect on their ability to thrash in suspension. The mutant characterization data may possibly reflect differential expression of nAChRs at various stages of development. The transformation data suggest that microparticle bombardment has little or no effect on *C. elegans*, which is essential for future transgenic experiments. In summary, important baseline data on *C. elegans* nicotinic receptors have been generated and a fundamental transgenic control line in *unc-38;myo-3::gfp* has been established. This will allow for exciting rescue experiments using parasitic nematode nAChR DNA.

Key words: parasite resistance, nicotinic acetylcholine receptor (nAChR), *Caenorhabditis elegans*, parasitic nematode, anthelmintic drug, nicotinic agonist.

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Introduction

Parasitic nematode infections are of major global concern as they pose a considerable threat not only to human life, but that of livestock and crops.^{1–3} In excess of 1 billion people are parasitized by intestinal nematodes in the developing world alone.⁴ Consequently, they have an impact on both the health and economic welfare of communities and are now recognized as one of the principal causes of human morbidity.^{1, 5} Fortunately, anthelmintic chemotherapeutic agents offer a simple and inexpensive method of controlling both human and animal infections.⁶

There are three major classes of broad-spectrum anthelmintic in widespread use: the benzimidazoles, the macrocyclic lactones and the nicotinic agonists.⁶ A large quantity of these specifically target and bind to regions of the nematode nervous system, highlighting the importance of its study for medical and veterinary purposes, among others.^{7, 8} The nicotinic agonists, such as levamisole, morantel, oxantel and pyrantel, specifically target nicotinic acetylcholine receptors (nAChRs) found at the nematode neuromuscular junction.^{9, 10} Of particular significance, the identification of many nicotinic agonist targets and potential resistance genes was first achieved using the well-defined, free-living

nematode *Caenorhabditis elegans* rather than parasitic Nematoda.^{11–15}

The nicotinic agonists open ligand-gated receptors found on muscle cell membranes, causing depolarization and hypercontraction.^{9, 10, 12} Levamisole is more potent than acetylcholine at nematode muscle nAChRs.^{16, 17} These anthelmintics are not rapidly degraded, leading to prolonged channel opening and Ca²⁺ entry, causing spastic paralysis and ultimately death. In parasitic nematodes, this paralysis allows expulsion from the host. Anthelmintics can be used to treat infections in animals because they have a selective therapeutic effect on nematode muscular nAChRs, and do not activate host receptors.¹⁸ The molecular basis for this specificity is largely unidentified.¹⁹

Unfortunately, the continued heavy reliance on and widespread application of such compounds have led to the inevitable emergence of resistance to all three major classes of broad-spectrum anthelmintic in many nematode populations.^{20–22} This rise in drug resistance has predictably led to a concomitant decrease in our ability to control infections. Resistance to one anthelmintic usually confers resistance to all other drugs in that chemical class and reversion to an anthelmintic-susceptible state once resistance has been established has never been observed in a population.²³ Consequently, it is imperative that the underlying mechanisms of resistance are better elucidated in order to both prolong the lifetime of currently available anthelmintics and limit the future impact of resistance.

Caenorhabditis elegans has been important to the study of nematode nAChRs; a good proportion of what is known about their function, genetics and pharmacology having been deduced using the small non-parasitic species.¹² More than 29 genes encode nAChR subunits in *C. elegans*, which is the most extensive and diverse collection of any single organism characterized to date.²⁴ *Caenorhabditis elegans* was first used in the study of parasitology over a decade ago²⁵ and its popularity as a model organism to study parasitic nematodes has been on the rise ever since. This is due predominantly to the genetic and morphological similarities it shares with other nematodes and the major difficulties associated with cultivating parasitic species in the lab.²⁶

At least two distinct nAChR subtypes have been identified in *C. elegans* muscle; N-type receptors, which are composed of five ACR-16 subunits and are most sensitive to nicotine,¹⁴ and L-type receptors, which are composed of three α -subunits (LEV-8, UNC-38 and UNC-63) and two non- α -subunits (LEV-1 and UNC-29) and are most sensitive to levamisole.^{11, 13, 15} Disturbance of both receptor subtypes causes near complete debilitation of *C. elegans*, but disruption of either one only causes slight locomotory deficiency, suggestive of partial redundancy.^{14, 27} In addition to the various nAChR subunits, three ancillary proteins (RIC-3, UNC-50 and UNC-74) are essential for heterologous

expression of the nicotine-insensitive L-type receptor in *Xenopus laevis* oocytes.¹⁹ *unc-29*, *unc-38* and *unc-63* null mutants are all highly resistant to levamisole¹² and electrophysiological responses to levamisole, but not nicotine, are completely abolished.²⁸ They also share a similar behaviour of uncoordinated locomotion, especially in early larval stages.²⁹ Surprisingly, the response of *C. elegans* to morantel, oxantel and pyrantel is at present ill-defined. It has often been assumed that the response to levamisole reflects that of the other three anthelmintics. Oxantel is an N-type agonist of *Ascaris suum* receptors, however, and not an L-type,³⁰ suggesting that the effects of oxantel could perhaps be different to levamisole at least.

Transformation techniques such as microparticle bombardment of DNA constructs (aka biolistic transformation) are integral to the use of *C. elegans* as a heterologous transformation system to study parasitic receptors *in vivo*. The generation of transgenic animals is important for the identification of genes involved in anthelmintic resistance and their subsequent functional analysis.²⁵ Microparticle bombardment involves accelerating DNA-coated beads (such as gold particles) to high speeds, allowing the penetration of target organism cells.^{31, 32} Successfully transformed individuals from subsequent generations are identified using a positive marker, such as GFP.³¹ Combining both the ease and speed of using *C. elegans* with the efficiency and low cost of microparticle bombardment, much can be learned about anthelmintic drug action and mechanisms of parasitic nematode resistance.

The main objective of the work presented here was to elucidate nematode nicotinic receptor function by analysing the effects of the nicotinic agonists levamisole, morantel, oxantel and pyrantel on various *C. elegans* strains. Surprisingly, little is currently known about the effects of these anthelmintics, so if *C. elegans* is to be used as a parasite model, there is urgent need to fully characterize the consequences of their use.

Materials and methods

Nematode strains and general methods

Caenorhabditis elegans strains were maintained using standard conditions.³³ The following strains were used: N2 Bristol wild-type, CB904 *unc-38(e264)* I, ZZ13 *unc-63(x13)* I and CB193 *unc-29(e193)* I. All strains were maintained on Nematode Growth Medium (NGM) (3 g/l NaCl, 17 g/l Bactoagar, 2.5 g/l Bactopectone, 1 ml/l 5 mg/ml cholesterol, 1 ml/l 1 M CaCl₂, 1 ml/l 1 M MgSO₄, 25 ml/l potassium phosphate buffer [pH6]) at ~19°C and sustained on freshly seeded OP50 *Escherichia coli*. Where possible, assays were blind and conducted at room temperature (18–22°C). Reagents were obtained from Sigma if not stated otherwise.

Age synchronization

L4 stage animals were identified by the characteristic morphology of the developing vulva, which appears as a translucent, semi-circle region located in the middle of the body.³⁴ Animals aged using this distinctive trait were picked to NGM plates and placed at 18°C for 24 ± 1 h before assaying.

Dose–response assay

Adapted from the larval paralysis test,³⁵ gravid adults were placed in wells of a 96-well microtitre plate containing M9 solution (3 g/l KH_2PO_4 , 6 g/l Na_2HPO_4 , 5 g/l NaCl, 0.25 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) with varying concentrations of anthelmintic or vehicle (99.5% dimethyl sulphoxide [DMSO]). Animals were then left for 10–12 min before being scored for immobilization. As in all experiments, anthelmintics were freshly prepared as 100 mM stocks in 99.5% DMSO. Suitable quantities of the stock were added to the M9 to produce a final concentration of $1 \mu\text{M}$ –1 mM. Equivalent amounts of DMSO were used as a control; the final concentration never exceeding 1%. Animals were categorized as immobilized following 10 s of inactivity.

Anthelmintic drug plates

NGM plates were made as described above with the addition of levamisole, morantel, oxantel, pyrantel or DMSO after autoclaving. A final concentration of 1 mM was used. Plates were foil-sealed and kept at room temperature for no longer than 4 weeks. For drug sensitivity assays, plates were seeded with 150 μl of *E. coli* within 3 h of being poured and then incubated at room temperature for 6 days to allow growth of a robust bacterial lawn. Plates used in body bend assays remained unseeded.

Growth sensitivity assays

Similar to the larval development test,³⁶ *C. elegans* embryos were transferred to plates with an *E. coli* lawn at time 0 h and their development was visually assessed at 48 and 72 h. Animals were classified as being adult, L4, L1–L3 or dead based on body size, vulval development and motility. Animals were presumed dead following 10 s of immobility in combination with a complete lack of response to prodding with a pick.

Thrashing assays

Age-synchronized young adults were placed in individual wells containing M9 with or without drug at 1 mM. Each well contained 55 μl of solution in total; a volume chosen following preliminary experiments. After 5–10 min of acclimatization, thrashes were counted for 30 s and multiplied by 2 to obtain an estimate per minute. A single thrash was defined as a complete change in direction of

bending at the mid-body.³⁷ Animals that moved on to the well sides were discarded from the analysis.

Body bend assays

Age-synchronized young adults were placed on unseeded 1 mM NGM plates and allowed to acclimatize for 5–10 min. This short period is essential because well-fed wild-type *C. elegans* tend to show frequent directional switching immediately post-transferral.^{38, 39} The number of body bends was counted for 30 s and multiplied by 2 to obtain an estimate per minute. A complete body bend was defined as the bending of the head region across the central-line of the animal, in which a full sinusoid was completed.^{14, 40} It has previously been observed that *C. elegans* reduce body bend frequency when encountering bacteria;⁴¹ therefore, animals found within 1 cm of unintentional food deposition during an assay were discarded from the analysis.

Microparticle bombardment

Biolistic transformation was performed using a BioRad Biolistic PDS-1000/He particle delivery system⁴² and was scaled-up using a Hepta adapter gas splitter.⁴³ ApaI-linearized Addgene plasmid pPD118.20 containing a *myo-3::gfp* marker was used in the transformation experiments (courtesy of A. Fire). The following parameters were implemented:³¹ $\frac{1}{4}$ in. gap distance, 9 mm macrocarrier to screen distance, 21 in. of Hg vacuum and 1550 p.s.i. rupture disc. Gold particles (0.3–3 μm) were prepared as described in the PDS-1000/He user manual and stored at 4°C.⁴² Plasmid DNA was coated onto the gold microparticles as outlined in Berezikov *et al.*⁴³

Animals were washed from 4 to 6 crowded 9 cm plates using M9 into a single Falcon tube and allowed to sediment. Following supernatant removal, they were spread onto a single unseeded 9 cm NGM plate and allowed to dry. Animals were cooled in order to reduce movement, improving transformation efficiency and then subjected to bombardment.³¹

Post-bombardment, animals were allowed to recover for 1–2 h at 19°C before being washed with M9 onto seeded NGM plates. These were allowed to dry for ~2 h before being incubated at 19°C for 3–5 days. Early-stage transformants were identified by their positive GFP marker, picked to new plates and allowed to reach the egg-laying stage. Thrashing assays were subsequently performed vs. non-bombarded controls, as described above.

Statistical analysis

Data sets were statistically analysed using either an unpaired two-sample *t*-test or a Mann–Whitney *U*-test according to parameters outlined in Townend.⁴⁴ Normality of the data was assessed using the Kolmogorov–Smirnov test and both Levene's and Bartlett's tests were used to evaluate homogeneity of variance. Minitab 15 Statistical Software

was used for all statistical calculations. Some graphs lack statistical analyses; this is due to the data being presented as percentages.

Results

Mutant characterization

High concentrations of oxantel have no visible effect on wild-type mobility

To assess short-term, visible consequences of anthelmintic exposure, a dose–response assay was performed (Fig. 1). Gravid adults were passaged to wells containing M9 solution with varying drug concentrations and observed after 10–12 min.

All mutants were visibly unaffected by the four drugs; no single animal was paralysed even at the high concentration of 1 mM (data not shown). Some wild-type animals, however, displayed inactivity in levamisole, morantel and pyrantel at $\geq 100 \mu\text{M}$. All three drugs had similar effects; there was a concomitant rise in the percentage of animals immobilized with increasing drug concentration, such that at 1 mM, the majority of animals were paralysed. Using an unpaired two-sample *t*-test, 1 mM levamisole, morantel and pyrantel were all significantly different from the control. Oxantel had no visible effect on wild-type animals; movement was comparable to the control at all concentrations. A 1 mM drug concentration was used in subsequent assays because it had the greatest effect on wild-type with no apparent effect on mutants.

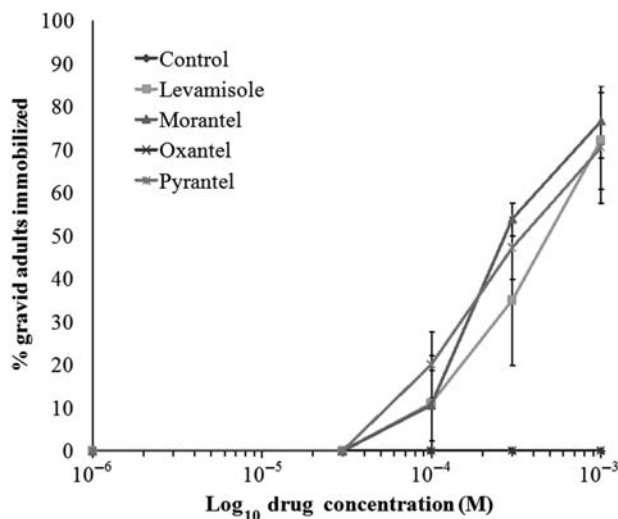


Figure 1. Percentage of gravid wild-type animals immobilized following 10–12 min in M9 solution with varying concentrations of anthelmintics. Values are mean percentages \pm SE derived from three trials performed on separate days. For each treatment, 50 animals of each strain were included in the analysis.

Levamisole has the greatest effect on *C. elegans* development

To investigate the effect of the anthelmintics on development, growth sensitivity assays were performed (Fig. 2). Embryos of the various strains were passaged to 1 mM drug plates and observed 48 and 72 h post-transferral.

At 48 h, there was little difference between the control and drug plates for all four strains; no adult animals were present and very few L1–L3s were unresponsive to prodding with a pick (data not shown). After 72 h, development of wild-type *C. elegans* was retarded when grown on plates containing levamisole (Fig. 2A); the majority of animals were found at the L1–L3 stage and no adults were present. This contrasts with control plates, which harboured a proportion of adults well over 90%. Morantel and pyrantel also altered the timing of animal development; only small percentages reached adulthood, but retardation was less severe than on levamisole. Conversely, a larger percentage of animals on oxantel plates reached maturity ($>60\%$), with many more reaching the L4 stage.

In the previous assay, all four anthelmintics had no obvious effect on the mutants. This remains true for morantel, oxantel and pyrantel, since the proportions of animals at the different stages were comparable to the control for all three strains. *unc-29* and *unc-38* mutants raised on levamisole, however, appeared to have slight developmental retardation; a higher percentage of animals remained at the L1–L3 stage (Fig. 2B and C). *unc-63* development appeared unaffected by all four drugs (Fig. 2D).

Oxantel has no effect on *C. elegans* thrashing rate

To measure the acute impact of the anthelmintics on *C. elegans* locomotion, thrashing assays were performed in M9 solution containing 1 mM drugs (Fig. 3).

Thrashing rates of wild-type and *unc-38* animals were significantly reduced by levamisole, morantel and pyrantel, whereas oxantel had no significant effect (Fig. 3A and C). The reduction was much more marked for wild-type. Only levamisole caused a reduction in *unc-29* thrashing rate (Fig. 3B) and *unc-63* locomotory activity was not significantly affected by any drug (Fig. 3D). The results for wild-type, *unc-29* and *unc-63*, were similar to those of the growth sensitivity assay, in that the same drugs caused the same negative trends (Fig. 2). Conversely, *unc-38* locomotory activity appeared to be more affected than its development by morantel and pyrantel. Plotting thrashing rates as a percentage of control data highlights the insensitivity of the wild-type to oxantel in comparison with the other drugs (Fig. 3E). It should also be noted that control wild-type thrashing rates were about 4-fold faster than those of the mutants (*t*-test, $P < 0.001$), and that control *unc-63* animals thrashed significantly less than the other *unc* mutants.

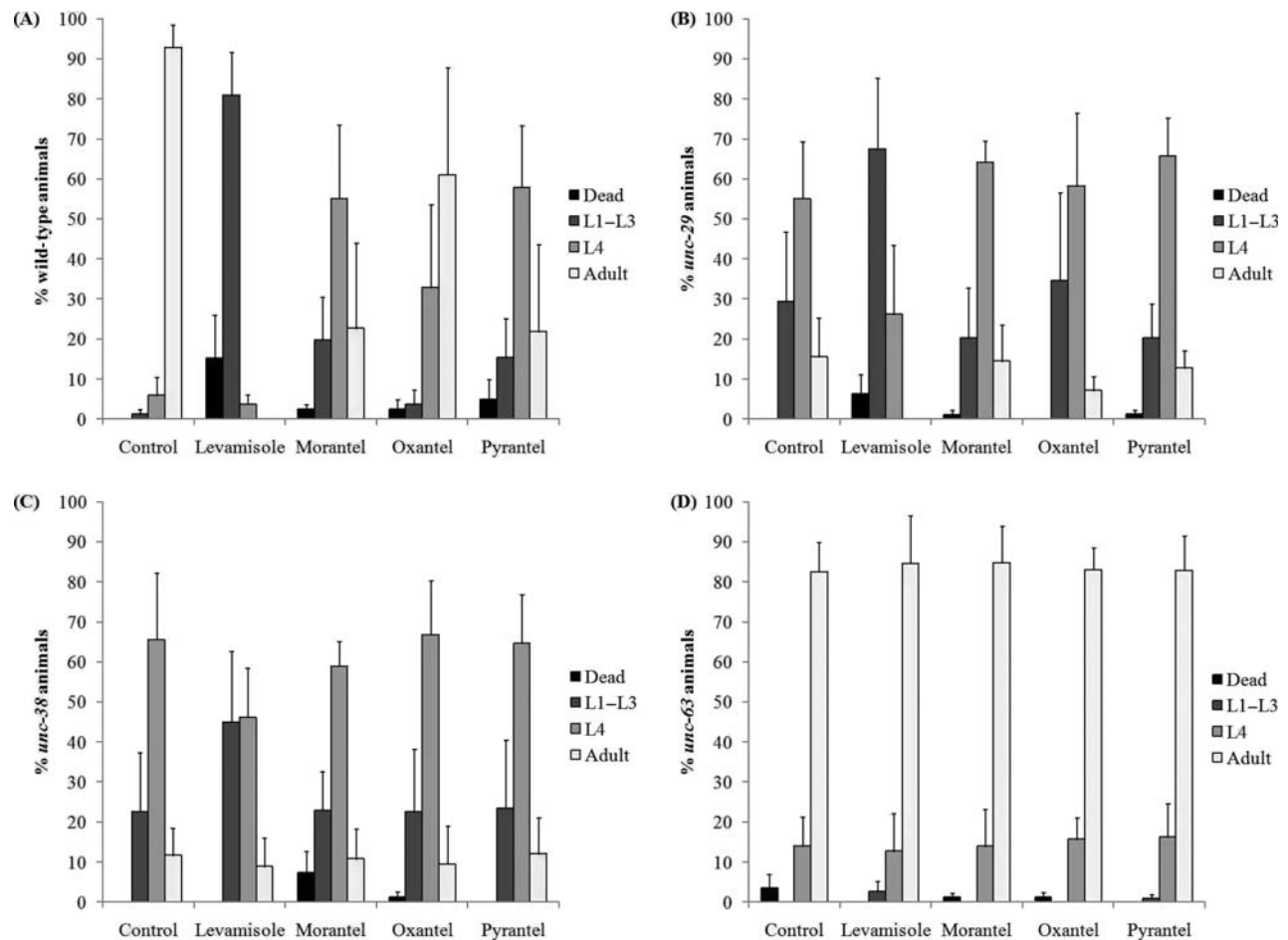


Figure 2. Growth sensitivity data showing the percentage of adult, L4, L1–L3 and dead animals 3 days post-transferral of embryos to different 1 mM anthelmintic plates. (A) Wild-type, (B) *unc-29*, (C) *unc-38* and (D) *unc-63*. Values are mean percentages \pm SE derived from three trials performed on separate days. One hundred animals of each strain were assayed on each drug.

Oxantel has no effect on *C. elegans* body bend rate

Thrashing assays indicated that the anthelmintics could disrupt high-frequency *C. elegans* locomotion (Fig. 3). A body bend assay was subsequently performed to analyse less strenuous, non-maximal locomotion. The number of body bends was counted in a 30 s period, 5–10 min post-transferral and multiplied by 2 to get an estimate per minute (Fig. 4).

Upon passage to plates containing morantel and pyrantel at 1 mM, wild-type animals were immediately incapacitated and did not regain the ability to move within the following hour. When transferred to plates with levamisole at 1 mM, the majority of wild-type animals moved around the plate for ~1–2 min then became rigid and paralysed. A small percentage moved in the characteristic sinusoidal motion for up to 15 min; however, after 20 min, all animals were unable to move. Control and oxantel had no obvious effect up to 1 h post-transferral. Anthelmintics caused the mutants to begin to pirouette (i.e. move around in tight circles) after about

2–3 min, but the body bend frequency was relatively unaffected.

Levamisole, morantel and pyrantel were all found to significantly reduce body bend generation in gravid wild-type adults. Once again, oxantel had no significant effect. No anthelmintic significantly altered the body bend rate of the mutants. The frequency of wild-type body bends on control plates was significantly greater than all three mutants (*t*-test, $P < 0.05$), so rates of body bends were also measured as a percentage of control (Fig. 4E). This emphasizes the insensitivity of the wild-type to oxantel in comparison with the other three drugs. It should be noted that wild-type animals were instantaneously incapacitated on levamisole, morantel and pyrantel, but were able to survive and reach at least the L4 stage in the growth sensitivity assay (Fig. 2A).

Mutant characterization summary

As expected, 1 mM levamisole, morantel and pyrantel significantly altered wild-type behaviour without fail (Table 1). In

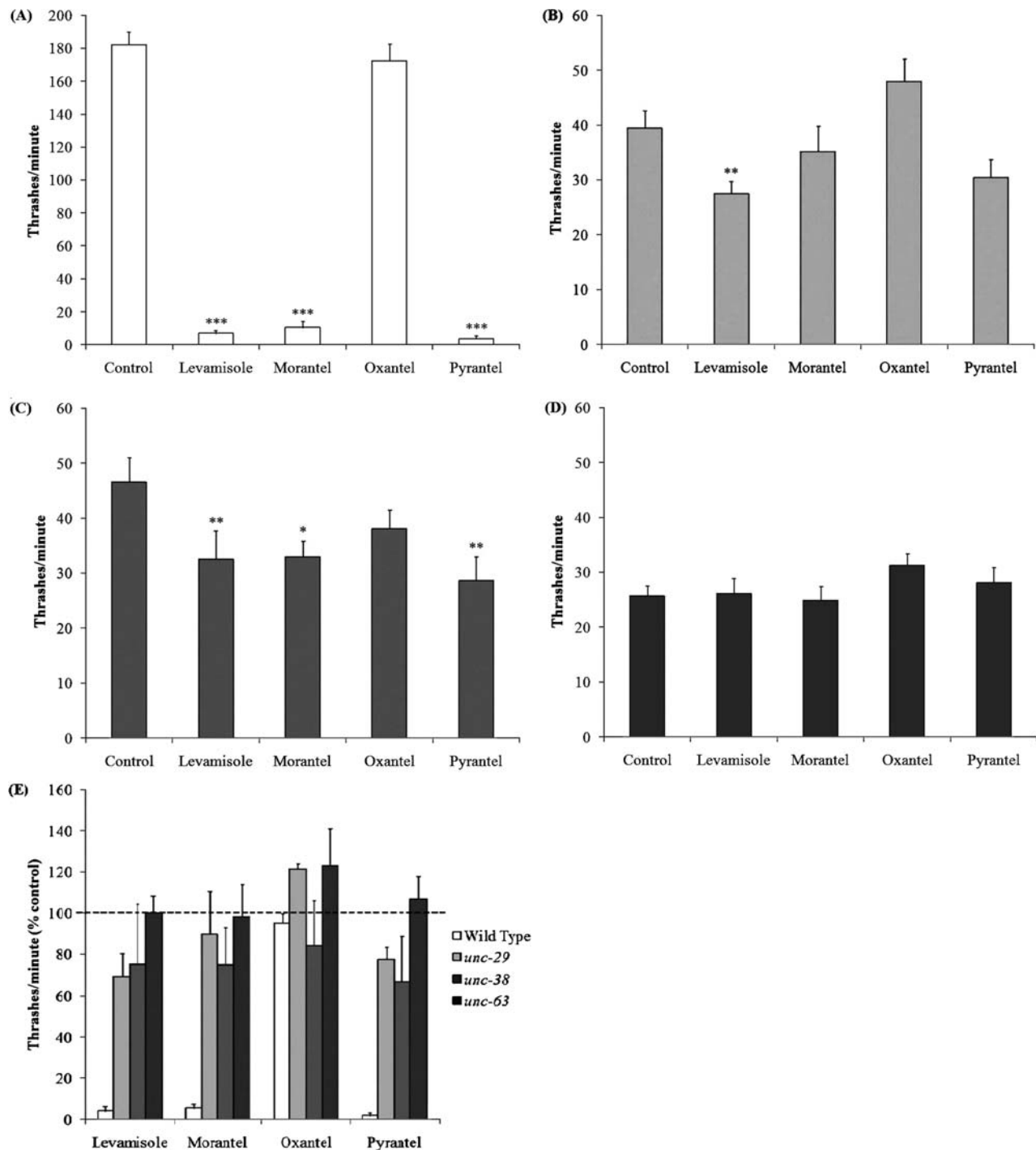


Figure 3. Thrashing rates after 5–10 min in M9 solution with various anthelmintic drugs at 1 mM. (A) Wild-type, (B) *unc-29*, (C) *unc-38* and (D) *unc-63*. Values are composite means \pm SE. Assays were performed on three separate days. For each drug, 18 animals of each strain were assayed. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ Mann–Whitney *U*-test or unpaired two-sample *t*-test. NB: (A) has a larger scale than (B–D). (E) Thrashing rates plotted as a percentage of the control. Values are mean percentages \pm SE derived from three trials performed on separate days.

the dose–response assay (Fig. 1), a concentration of 1 mM caused immobilization of between 70% and 80% of gravid adults. In the growth sensitivity assay, all three caused retardation of development, levamisole being the most potent

(Fig. 2). Finally, in the thrashing (Fig. 3) and body bend (Fig. 4) assays, they all caused a dramatic decrease. In stark contrast to this, oxantel had no significant effect on any of the *C. elegans* behaviours in any strain.

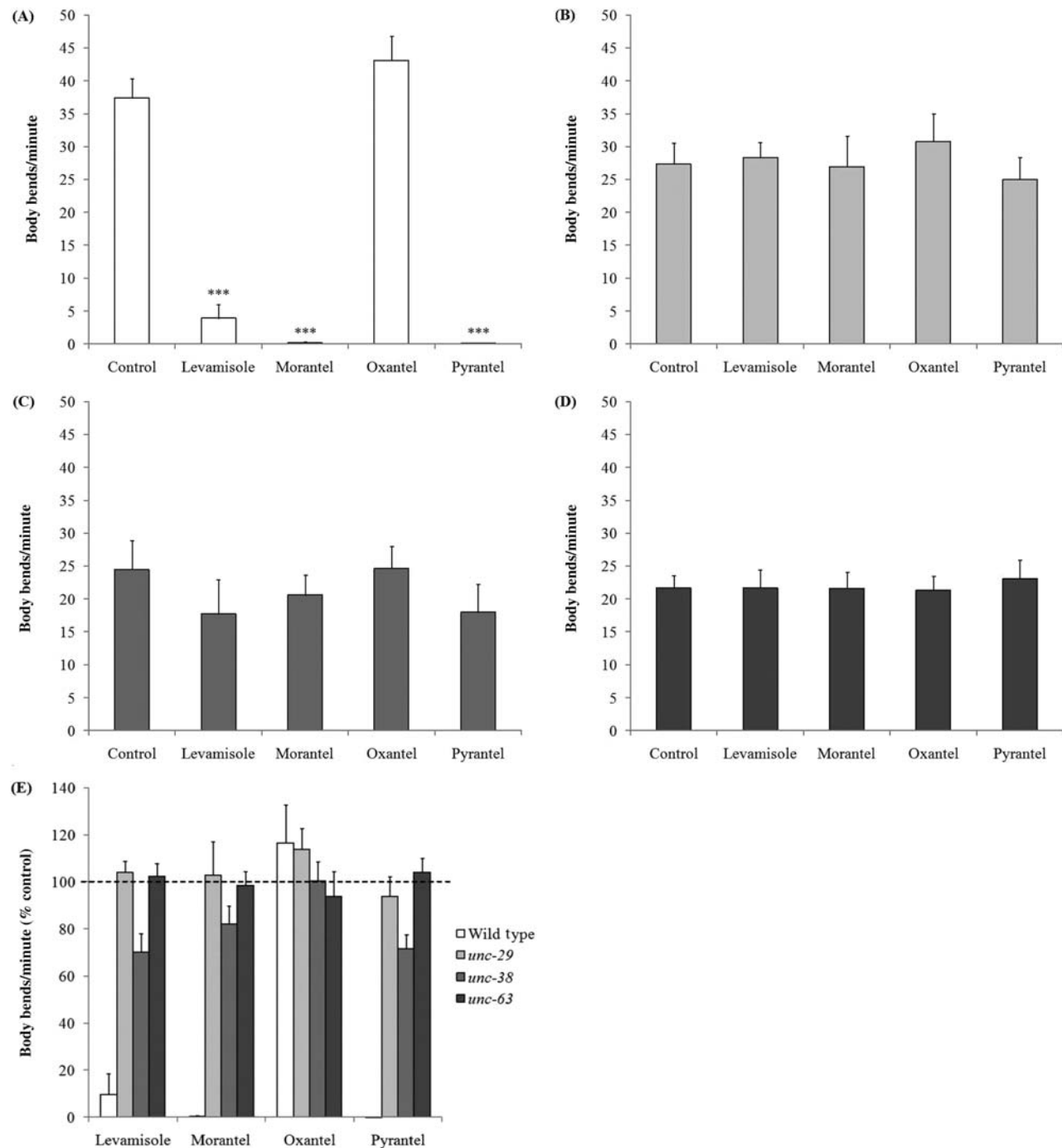


Figure 4. Body bends per minute on NGM plates containing various anthelmintic drugs at 1 mM. (A) Wild-type, (B) *unc-29*, (C) *unc-38* and (D) *unc-63*. Values are composite means \pm SE. Assays were performed on three separate days. For each drug, at least nine animals of each strain were assayed. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ unpaired two-sample t-test. (E) Body bend rates plotted as a percentage of the control. Values are mean percentages \pm SE derived from three trials performed on separate days.

In contrast to wild-type, the mutant animals were relatively unaffected by the anthelmintic drugs. The behavioural phenotypes of *unc-29* animals were completely unaffected except thrashing rate in levamisole (Table 1). *unc-38* animals were the most susceptible mutants assayed;

levamisole, morantel, and pyrantel causing a reduction in thrashing rate (Table 1). *unc-63* animals appeared to be the most refractory to the anthelmintics; not a single behavioural phenotype was significantly altered by the anthelmintics tested (Table 1).

Table 1. Mutant characterization summary

	Wild-type			<i>unc-29</i>		
	1	2	3	1	2	3
Levamisole	**	***	***	NS	**	NS
Morantel	***	***	***	NS	NS	NS
Oxantel	NS	NS	NS	NS	NS	NS
Pyrantel	**	***	***	NS	NS	NS
	<i>unc-38</i>			<i>unc-63</i>		
	1	2	3	1	2	3
Levamisole	NS	**	NS	NS	NS	NS
Morantel	NS	*	NS	NS	NS	NS
Oxantel	NS	NS	NS	NS	NS	NS
Pyrantel	NS	**	NS	NS	NS	NS

1, dose-response assay; 2, thrashing assay; 3, body bend assay; NS, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Microparticle bombardment

To establish a transgenic control strain, biolistic transformation using a BioRad Biolistic particle delivery system was performed. *unc-38* mutants were bombarded with ApaI-linearized plasmid pPD118.20 containing *myo-3* promoter fused to *gfp*. *myo-3* encodes a muscle-type specific myosin heavy-chain isoform and is expressed in all *C. elegans* muscles outside the pharynx.^{45–47} Transformed animals expressing the *myo-3::gfp* construct were identified 3–5 days post-bombardment via green fluorescence observed in body wall and vulval muscles.

To observe whether microparticle bombardment had any significant effect on *C. elegans* locomotion, transformant animals were allowed to reach the egg-laying stage and then assayed for thrashing rate (Fig. 5). Non-bombarded *unc-38* mutants were used as a control. A thrashing assay was used because thrashing of *unc-38* animals appeared to be the most anthelmintic-susceptible behaviour/phenotype (Table 1). There was no significant difference between the thrashing frequencies of *unc-38;myo-3::gfp* and control animals under all five conditions. Biolistic transformation also had no effect on *unc-38* resistance to the anthelmintics, as there was no difference between control thrashing rates and those in the drugs. Similarly, there was no difference between *unc-38* control thrashing rates and rates in the different drugs. Ultimately, an *unc-38;myo-3::gfp* transformant control line was established by the microparticle bombardment technique.

Discussion

Mutant characterization

Oxantel caused retardation of wild-type *C. elegans* development (Fig. 2A); whereas it had no significant effect on

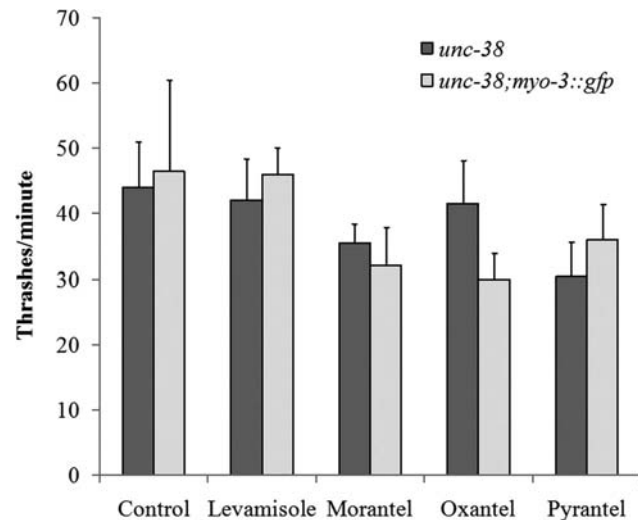


Figure 5. Thrashing rates of transformant and control animals after 5–10 min in M9 solution with various 1 mM anthelmintic drugs. Values are means \pm SE. For each drug, four animals of each strain were assayed. All data were analysed using unpaired two-sample *t*-tests.

phenotypes analysed by the other assays. This could perhaps be due to differing nAChR expression profiles of larval and adult *C. elegans*.⁴⁸ In the growth assay, larvae were exposed to anthelmintics from hatching; whereas adults were assayed in the other experiments. It is possible that larval stage animals have differing proportions and/or distributions of nAChR subunits and receptor subtypes in order to better exploit their surroundings and thus enhance survival.³⁰ This dynamic modulation may be needed in order to rapidly adapt to environmental changes. This is more likely for parasitic nematodes as the external environment experienced by larvae is often very different from that encountered by adults; for instance, the sheep nematode *Haemonchus contortus* has both free-living and parasitic stages.⁴⁹ Sangster and Bjorn⁵⁰ have shown that the level of levamisole resistance of *H. contortus* varies throughout its life-cycle; immature parasites being more susceptible than adults both *in vitro* and *in vivo*. This variation in susceptibility could be explained by differential nAChR expression profiles, for example, in the muscles and neurons. Since *H. contortus* and *C. elegans* both belong to Nematoda Clade V,⁵¹ it is quite possible that this could also manifest in the latter.

Oxantel is a derivative of pyrantel developed to treat children with whipworm infection.^{30, 52} It has since been characterized as an N-type agonist of the Clade III parasite *A. suum*.^{30, 51} Contrastingly, oxantel is an antagonist of *C. elegans* N-type receptors,⁵³ binding to nAChRs composed solely of ACR-16 subunits.¹⁴ The early developmental retardation of wild-type *C. elegans*, in combination with the resistance at adult stages, could possibly be due to differential expression of N-type receptors. If ACR-16 expression is up-regulated in larvae, it is conceivable that larvae would be

more susceptible to oxantel than adults. In contrast, wild-type animals appeared to be relatively more susceptible to levamisole, an L-type agonist,²⁸ at later stages of development. Unlike oxantel, when passaging adult animals to plates containing 1 mM levamisole, the vast majority were completely paralysed within 5 min. In the growth sensitivity assay, despite development being severely retarded compared with the control and more so than oxantel, wild-type *C. elegans* larvae were still alive and moving about the plate. These results are corroborated by the similar data for pyrantel, which is also an L-type agonist.⁵⁴

Altogether, these results are perhaps concordant with greater expression of N-type nAChRs in larvae than adults. They also suggest that L-type receptor expression is possibly up-regulated as *C. elegans* ages. The greater retardation of wild-type development by levamisole over oxantel suggests that L-type receptors are more prevalent throughout the life-cycle. Phosphorylation levels of nAChRs have been shown to have an effect on agonist potency,⁵⁵ which may also explain the differential susceptibilities of the various *C. elegans* stages. Oxantel has no such effect on the development of mutant animals, which all have fully functional N-type receptors. This could be explained by the slower growth rate of *unc-29* and *unc-38* animals, which may mask the developmental retardation seen in the wild-type. *unc-63* mutants seemed to grow faster than wild-type animals (Fig. 2); this enhanced development may also have negated the developmental retardation caused by oxantel.

Contrary to wild-type, *unc-63* mutant behaviours all appeared refractory to the anthelmintics. This was also true for the *unc-29* mutants, excluding the significantly reduced thrashing rate in levamisole (Fig. 3B). In contrast, *unc-38* thrashing frequency was significantly reduced by levamisole, morantel and pyrantel (Fig. 3C). This perhaps implies that both UNC-29 and UNC-63 are more important for binding of the various L-type agonists than UNC-38. When UNC-29 and UNC-63 were present and UNC-38 was absent, mutant thrashing rates were significantly reduced. This reduction in thrashing and not the other phenotypes suggests that restricted movement is the consequence of exposure to anthelmintics. Thrashing is most vulnerable to the anthelmintics and as such is the most sensitive assay to detect weak/mild drug susceptibilities. Eight genes are required for functional reconstruction of the L-type receptor in a *Xenopus* oocyte expression system;¹⁹ however, the significant reduction of thrashing rates of *unc-38* mutants in response to levamisole suggests that some semblance of an L-type receptor is still partially functional *in vivo*.

The conductance dose–response curves of morantel and pyrantel are ‘bell-shaped’, such that past a certain concentration, increasing their molarities will cause a reduction in receptor opening.¹⁶ This phenomenon may serve to explain why morantel and pyrantel did not reduce *unc-29* thrashing rates like levamisole at 1 mM. Additional structural

requirements of morantel and pyrantel may also explain the discrepancy. Pyrantel requires a glutamate at a specific position in loop D of UNC-63 for receptor binding, whereas levamisole does not.⁵⁶ Leading on from this, due to the apparent resistance of *unc-63* to all anthelmintics tested, it is possible that the most important residues for efficient binding of levamisole, morantel and pyrantel reside somewhere within the UNC-63 subunit.

When interpreting these *C. elegans* data, it is important to be wary of the drug molarities used.⁵⁷ The high concentration of 1 mM used in these resistance assays is likely to be toxic to a mammalian host and so cannot be used to therapeutically treat nematode infections. If a concentration of 100 μ M was administered to treat parasitic infection, this would constitute a 10-fold difference when comparing with the *C. elegans* data generated here.

Microparticle bombardment

The GFP expression pattern of *unc-38* animals bombarded with *myo-3::gfp* DNA construct is suggestive of successful transformation because they resemble the endogenous pattern of *myo-3* expression.^{45–47} *myo-3* is usually expressed in body wall and vulval muscles, among others, which can be seen to fluoresce in the *unc-38;myo-3::gfp* animal. Transformants at the egg-laying stage were subsequently shown to thrash at similar rates to non-bombarded *unc-38* controls and were unaffected by the anthelmintics (Fig. 5), suggesting that microparticle bombardment had no significant effect on thrashing rate. Of note, the *unc-38* controls were not significantly affected by the anthelmintics as they were in the previous thrashing assay (Fig. 3). This is most likely due to a lack of age-synchronization and small sample sizes ($n = 4$). Now that a transgenic control line has been established, this can easily be rectified.

Conclusion

It has been shown that the nicotinic agonist oxantel has little effect on adult wild-type *C. elegans* locomotory behaviour, which may reflect differential nAChR subtype expression. It has also been demonstrated that the *C. elegans* nAChR subunits UNC-29 and UNC-63 are potentially more important for anthelmintic binding than UNC-38. The generation of baseline data on *C. elegans* nicotinic receptors in combination with the establishment of an important transgenic control line provides a solid base for future rescue experiments with parasitic nematode DNA. This will undoubtedly aid in the functional analysis of nematode nicotinic receptors and potentially elucidate mechanisms involved in parasitic drug resistance.

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Author biography

James graduated with a First Class MBIol (Hons) degree in Biology from the University of Bath in 2009 and was awarded the Institute of Biology Prize for the best Biology undergraduate and the Lonza Prize for the best final year performance in the Biosciences. James spent his placement year at Harvard Medical School in the Hart Lab identifying genetic modifiers of the neurodegenerative disease Spinal Muscular Atrophy. He is currently doing a PhD within the Functional Genomics Unit at the University of Oxford furthering his study of the disease. Throughout his undergraduate degree James developed an interest in many different areas of Biology including parasite resistance, bioinformatics, sexual conflict and neurological disease. James hopes to continue in the field of his PhD, with a view to one day running his own lab.

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