Levamisole receptors: a second awakening

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Abstract

Levamisole and pyrantel are old (1965) but useful anthelmintics that selectively activate nematode acetylcholine ion-channel receptors; they are used to treat roundworm infections in humans and animals. Interest in their actions has surged, giving rise to new knowledge and technical advances, including an ability to reconstitute receptors that reveal more details of modes of action/resistance. We now know that the receptors are plastic and may form diverse species-dependent subtypes of receptor with different sensitivities to individual cholinergic anthelmintics. Understanding the biology of the levamisole receptors is expected to inform other studies on anthelmintics (ivermectin and emodepside) that act on ion-channels.

Keywords

Levamisole; nematode; acetylcholine; ion channel receptor; subtypes; oocyte expression; anthelmintic; resistance

Levamisole and pyrantel: interest and new knowledge

One-third of the human population of the world is at risk of helminth infection, and infection is also very common in animals. Regrettably, there are still no effective vaccines for controlling these infections, so that both treatment and prophylaxis rely on anthelmintic drugs. The continued use of these drugs has given rise to concerns over levels of resistance and promoted the search for new knowledge and understanding that might slow its progress. The cholinergic anthelmintics, which include levamisole and pyrantel, are an important group of anthelmintics and like other major anthelmintics (ivermectin and emodepside) target parasite ion channels. Levamisole selectively opens a restricted subgroup of nematode acetylcholine receptor (AChR) ion channels in nematode nerve and muscle. Opening of AChR channels produces depolarization [1], entry of calcium through the opened channels, and an increase in sarcoplasmic calcium, producing spastic muscle contraction [2]; the

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Conflict of interest
The authors declare no conflict of interest

parasite is then unable to maintain its location (often in the intestine) and is then swept away, effecting the cure. Interest in this class of anthelmintic has increased recently because application of new methods has demonstrated the presence of diverse receptor subtypes and different cholinergic anthelmintic subtype selectivities. It has also allowed better mechanistic explanations of resistance and the development of exciting novel compounds such as monepantel and derquantel. This review describes new knowledge and insights that have increased our understanding of the biology of these receptors.

Levamisole-sensitive AChRs show pharmacological- and species-dependent diversity

The very small currents (1 pA = 1×10^{-12} A) that flow through single receptor channels activated by levamisole or pyrantel have been recorded from muscle of the parasitic nematodes *Ascaris suum* [3], *Oesophagostomum dentatum* [4] and *Brugia malayi* [5] using the patch-clamp technique (Figure 1). In *A. suum* there is the *L*-type (G35 pS) that is preferentially activated by levamisole, the *N*-type (G25 pS) that is preferentially activated by nicotine and the *B*-type (G45 pS) that is preferentially activated by bephenium [6]. At the single-channel level, high concentrations of levamisole activated all three AChR subtypes (Box 1). In the strongyle species, *O. dentatum,* that belongs to the same phylogenetic clade as *Haemonchus contortus* and *Caenorhabditis elegans,* a fourth levamisole-activated AChR subtype with a conductance of 40 pS has also been observed (Figure 1d) [4]. We point out that in the free-living nematode, *C. elegans*, that levamisole activates only one conductance subtype of 30 pS, which is not activated by nicotine and is pharmacologically different from some of the parasite levamisole receptors that can be activated by nicotine [7]. Together, these observations show that levamisole activates a diverse range of receptor subtypes which are separated by their detailed pharmacology, channel conductances and species. This diversity emphasizes a need for molecular and functional characterization of these receptors in different parasitic nematode species as well as in *C. elegans.*

The *C. elegans* model of the levamisole receptor

Although levamisole was developed for treatment of parasitic nematodes, the intensive study of the effects of levamisole in the *C. elegans* model nematode has given us tremendous insight into what may be happening in parasitic nematodes. Starting from the early days of Sydney Brenner [8], levamisole (tetramisole) has been used in genetic studies of *C. elegans,* so that we now have a better understanding of the molecular mechanisms associated with levamisole AChR signaling. Each AChR is composed of five subunits formed in a ring in the membrane with the channel pore in the center (Figure I, Box 2); the subunits are either α subunits with two vicinal cysteines present or β (non- α) subunits that lack these cysteines [9, 10]. There are at least 27 genes for AChR subunits in *C. elegans.* The levamisole receptor channel was found to be composed of five different subunits [11]. Each subunit is about 500 amino acids in length and harbors 4 transmembrane regions (M1, M2, M3, and M4). The M2 region forms the lining of the ion-channel pore. In *C. elegans* (Box 2; Figure 2), three separate genes code for the levamisole α subunits: *unc-63, unc-38, lev-8,* and two genes code for the β subunits, *unc-29* and *lev-1* [12-15]. The canonical agonist binding sites are assumed to be between the positive face of the α subunit and the negative face of the adjacent β subunit; the adjacent pairs of subunits are not identical, so the agonist binding sites are not equivalent (Box 2, Figure I). Evidence from other preparations leads us to believe that at least two agonist molecules need to bind to open the receptor channel effectively. In addition to the canonical sites, anthelmintics may also bind to non-canonical sites formed between the positive face of the β subunit and the negative face of an α subunit and increase opening of the ion-channel [16-18]. Thus the site of action and the potency of a cholinergic anthelmintic will depend upon the channel subunit composition.

In addition to the requirement for the subunits, full function of the levamisole channel is associated with proteins derived from at least seven other genes: *lev-9, lev-10, tpa-1, tax-6, soc-1, nra-1,* and *oig-4* (Figure 2) [19-21]; it is anticipated that other associated proteins will be recognized in the future. Null mutants of these genes can produce a degree of levamisole resistance. The gene *unc-68*, which encodes a ryanodine receptor [22], is associated with amplifying the calcium signal following opening of the levamisole receptor, and two other genes, *unc-22* and *lev-11* [14], are associated with contractile elements of muscle and produce levamisole resistance by reducing the muscle response to the drug. In addition to these genes, other null mutants of genes that can produce levamisole resistance are involved in processing and assembly of the subunits; these genes are *nra-2, nra-4, ric-3, unc-74* and *unc-50* [23-25]. RIC-3 is a small transmembrane protein, which is a chaperone promoting nAChR folding in the endoplasmic reticulum [26]. The gene *unc-74* encodes a thioredoxin protein required for the expression of levamisole AChR subunits [27]. The gene *unc-50* encodes a transmembrane protein in the Golgi apparatus [24]. In *unc-50* mutants, levamisole AChR subunits are directed to lysosomes for degradation. These three ancillary proteins have been widely conserved through evolution from nematodes to humans and have been used for the expression of levamisole receptors in *Xenopus* oocytes. In addition to these proteins, microRNAs (miRNA) are also involved in regulation of receptor subunit expression; miR-1 is one specific miRNA that binds to the mRNA of UNC-63 and UNC-29 but not LEV-1 or LEV-8 [28]. A null mutant of miR-1 leads to reduced sensitivity to levamisole; although the mechanism for this is unclear, it is suggested to involve a change in receptor expression [28].

Molecular diversity: levamisole AChR subunits in parasitic nematodes

Molecular cloning and bioinformatic searches in parasitic nematode genomes and transcriptome databanks have identified homologs of *unc-29, unc-38* and *unc-63* all across the phylum Nematoda [29, 30]. The conservation of these genes in distantly related nematode species may reflect the common and important role they play in the function of the levamisole AChR. It is noteworthy that in the trichostrongylid species, *H. contortus* and *Teladorsagia circumcincta*, that there are four distinct *unc-29* paralogs which have been identified [30]. Unlike the *unc-29, unc-38* and *unc-63* genes, orthologs of the *lev-1* gene have only been identified in Clade V nematode species (*C. elegans* and trichostrongylids). There is currently no evidence for *lev-1* orthologs in nematodes of Clade III species (*A. suum* or *B. malayi*) nor in Clades I, II or IV. Intriguingly, *lev-1* homologs from *H. contortus, T. circumcincta* and *Trichostrongylus colubriformis* lack a signal peptide raising questions about the contribution of LEV-1 to the function of levamisole AChRs [30]. Because AChR subunit oligomerization takes place within the endoplasmic reticulum, the absence of a signal peptide for Hco-LEV-1, Tci-LEV-1 and Tco-LEV-1 suggests that the subunit is processed differently; perhaps it is not involved in levamisole AChR construction, or it could associate with other levamisole AChR subunits using a molecular pathway that remains to be identified. Finally, there is also no evidence to date for a gene orthologous to *lev-8* in the trichostrongylids, despite intensive laboratory and bioinformatic searches [29, 30]. However, homologs of the *acr-8* gene that are closely related to *lev-8* appear to be well conserved in parasitic nematode species [31]. The similarity between *H. contortus, T. circumcincta* and *T. colubriformis* ACR-8 homologs and the *C. elegans* sequence is spread over the length of the primary amino acid sequence. However, the motif, YxxCC, which is part of the ligand binding site is conserved for trichostrongylid ACR-8 and *C. elegans* LEV-8 (YPGCC versus YAGCC for Cel-ACR-8), suggesting that conservation of these residues is associated with specific agonist-binding properties. It has been hypothesized that the *lev-8* and *acr-8* genes arose from a duplication that occurred after the divergence between strongyloidea and rhabditoidea [31]. Alternatively, a common ancestor that had both *lev-8* and *acr-8* may have occurred with the *lev-8* being lost in the trichostrongylids.

Another persuasive observation for ACR-8 being a subunit of levamisole AChR subtypes in parasitic nematodes is that levamisole resistance in *H. contortus* has been associated with the presence of truncated ACR-8 subunits [32] (Box 3).

Functional diversity: *Xenopus* expression of different levamisole AChR subtypes

After cloning *C. elegans* levamisole receptor subunits, it became possible to express the receptor in Xenopus oocytes. Robust expression of the levamisole receptor from *C. elegans* required injection of cRNA of five subunits (UNC-38, UNC-63, LEV-8, UNC-29 and LEV-1) (Figure 3a) and three ancillary proteins (UNC-74, RIC-3 and UNC-50) [31]. The maximum response to acetylcholine is much greater than the response to levamisole with no nicotine response with this expressed *C. elegans* levamisole AChR; the lack of response to nicotine demonstrates that the *C. elegans* receptor is pharmacologically different from the levamisole receptors of some parasitic nematodes. Expression of an acetylcholine and nicotine-sensitive *C. elegans* muscle homomeric receptor was achieved by injecting ACR-16 cRNA (Figure 3b) [12, 33].

Two levamisole-sensitive AChR subtypes from *A. suum* required only two subunits (UNC-38 and UNC-29) and did not require the accessory proteins [34]. Two types of receptor were produced: (i) one type, more sensitive to levamisole and pyrantel than nicotine, was observed when more UNC-29 than UNC-38 cRNA was injected (Figure 3c; Box 1); (ii) the other type that was more sensitive to oxantel and nicotine than levamisole, was observed when more UNC-38 than UNC-29 cRNA was injected (Figure 3d). It is suggested that two types of levamisole receptor are produced by different stoichiometric combinations of UNC-38 and UNC-29: the levamisole/pyrantel receptor by $(UNC-38)_2$:$(UNC-29)_3$ and the nicotine, oxantel receptor by $(UNC-38)_3$:$(UNC-29)_2$. These observations illustrate that the levamisole AChR subunit structure and anthelmintic sensitivity can vary and is plastic.

Expression of the receptor subunits derived from *H. contortus*, which is more phylogenetically related to *C. elegans*, was also different. Expression of a functional receptor highly responsive to levamisole required the presence of 4 subunits (UNC-63, UNC-29, UNC-38, and ACR-8) and the three ancillary proteins (Figure 3e) [31]. This receptor, referred to as Hco-L-AChR-1, is more sensitive to levamisole than acetylcholine and is poorly responsive to pyrantel and nicotine (Figure 3f). When the cRNA for ACR-8 is omitted, the UNC-63, UNC-29, UNC-38 subunit combination gave rise to a second, pharmacologically different, levamisole AChR referred to as Hco-L-AChR-2 (Figure 3f). The Hco-L-AChR-2 was more sensitive to pyrantel and acetylcholine than levamisole. Therefore, two distinct subtypes of recombinant *H. contortus* levamisole AChR could be distinguished, possibly mirroring some of the levamisole AChR diversity revealed with single-channel recording experiments performed on the closely related species *O. dentatum* (Figure 1). The critical role for the ACR-8 subunit in levamisole sensitivity is highlighted by the fact that the Hco-L-AChR-1 and Hco-L-AChR-2 only differ by the presence or absence of Hco-ACR-8. In *C. elegans*, the functional expression of the *C. elegans* levamisole AChR did not require ACR-8 subunits [12]. Of interest in parasitic nematodes is the potential role that ACR-8 plays in levamisole resistance, since absence of ACR-8 in the expressed receptors (Figure 3f) gives rise to the Hco-L-AChR-2 receptor, which is less sensitive to levamisole but highly responsive to pyrantel. Thus, an *acr-8* null mutant parasitic nematode isolate may be sensitive to pyrantel but not levamisole.

Modeling the levamisole receptor

The family of ligand gated ion-channels is present in nearly all animal groups and has also been found in bacteria, predating eukaryotes [35]. The subunit genes of this family may be extremely divergent in sequence but retain a highly conserved structure and specific functional motifs (Box 2). This allows the use of known crystal structures to serve as templates for the prediction of related ion-channel structures and identification of acetylcholine and nicotine within the ligand binding pocket of the AChR [36,37]. The models identify potentially important hydrogen bonds formed upon ligand binding and are able to discriminate between compounds with high and low experimentally determined LD_{50} values. In the future we could use this approach to examine levamisole and pyrantel binding to the various levamisole-sensitive AChRs of identified parasitic nematodes. The next challenge is for *in silico* prediction of ligand receptor interaction for novel compounds. The example of cytisinoid derivatives and their affinity for acetylcholine binding protein demonstrates that it is possible to achieve a good correlation over a wide range of predicted binding energy and IC_{50} values [38].

The neurotransmitter binding pocket lies between two adjacent subunits of the channel pentamer (Box 2), formed from three loops of the positive subunit (known as the A, B and C loops) and three beta-strands of the negative subunit (loops D, E and F). The closed, unbound form of the *Torpedo* fish AChR is an asymmetric pentamer that becomes symmetrical in the presence of acetylcholine [39]. Comparison of a refined version of this structure and that of the channel produced in the open channel configuration shows that the c-loop closes around the bound neurotransmitter, a movement of perhaps 8 Å, accompanied by significant changes in the β-sheet regions of the ligand binding domain and rotation of the transmembrane domain, opening a path for the flow of cations [36, 39].

Modeling interactions of such flexible structures is still being developed and simulation of the ligand-receptor interaction will require either an ensemble of receptors in different physical states, or use of molecular dynamics to produce time simulations of the ligand bound structure. Snapshots can be evaluated to determine the stability of ligand binding and changes in flexibility of residues that interact with the ligand [40]. Although still at the limits of protein modeling, these techniques will help us to understand details of nAChR function and their interaction with levamisole and other anthelmintic compounds.

Resistance: mechanisms of resistance for parasites *in vivo*

In sensitive *O. dentatum* parasites, levamisole readily activates and opens the receptor channels (Figure 1b) frequently. In the levamisole and pyrantel resistant isolates the channels open infrequently and there are fewer receptor channels present in the muscle membrane [4, 43]. A histogram of single-channel conductances from the anthelmintic sensitive isolate of *O. dentatum* [4, 43] shows the presence of four separate channel conductance subtypes: G24 pS, G35 pS, G40 pS, G45 pS (Figure 1c,d). Interestingly, in the levamisole resistant isolate the G35 pS peak is missing, but in the pyrantel resistant isolate this peak is still present [4,43]. These observations suggest that resistance is associated with an overall reduction in the number of receptors, that levamisole resistance may be associated specifically with loss of the G 35 pS subtype but that pyrantel resistance may be different and is not associated with a loss of a specific subtype. We might expect that the changes associated with resistance may lead to subtle changes in motility without compromising the ability of the parasite to complete its life cycle. The changes in motility have been detected by detailed phenotyping [41,42].

Phenotyping sensitive and resistant isolates: microfluidics

We have seen above that there are a range of different levamisole receptor subtypes suggesting some redundancy and that there may be differences between sensitive and resistant isolates. It is now possible to screen and observe effects of drugs such as levamisole affecting motion of L3 larvae of parasitic nematodes with greater resolution using computer tracking and microfluidic technology [41, 42]. These multi-parameter microfluidic bioassays were developed to observe the innate locomotory properties of larval movement along with transient and real time responses to the application of anthelmintics within a single experiment. An electrical field is used in the platform to guide the movement of the larvae (electrotaxis) into and out of the drug wells. Video recording of the experiment along with automatic worm tracking software reveals important information about changes in the locomotion of the worm during entry exposure and exit periods from the drug. The tracking software used can measure subtle locomotion changes in the microfluidic device in the presence or in the absence of drugs. The approach allows quantification of transient drug effects and measurement of locomotory parameters like the sinusoidal properties of the larval movement (velocity, wavelength, frequency and amplitude). The more precise measurements of locomotion can detect levamisole resistance in the absence of drugs from changes in the parameters of locomotion, as well as changes associated with application of anthelmintics [41, 42]. This new method of quantitative phenotyping offers a new way to detect and investigate anthelmintic resistance and anthelmintic interactions that affect the phenotype of locomotion associated with subtle changes in receptor subtypes.

Investigating levamisole resistance at the molecular level in parasitic nematodes

Four sets of proteins have been found to contribute to levamisole resistance in *C. elegans*. The four sets are: (i) Levamisole sensitive AChR subunits, (ii) proteins involved in processing and assembly of levamisole AChR channel subunits; (iii) proteins involved in regulating the levamisole AChR channel; and (iv) proteins involved in the calcium-contraction signaling cascade (Figure 2; Box 3). In parasitic nematodes, searches for expression or sequence polymorphisms associated with levamisole resistance mainly focused on levamisole AChR subunits. In the strongylid parasite, *Ancylostoma caninum* (dog hookworm), genes orthologous to *unc-38, unc-63* and *unc-29* have reduced expression in a highly pyrantel-resistant isolate when compared with a low pyrantel-resistant isolate [44]. By contrast, in the trichostrongylid species *H. contortus, T. circumcincta* and *T. colubriformis*, expression of complete coding mRNAs corresponding to *unc-38, unc-63, unc-29* and *lev-1* homologs were found to be similar in both levamisole susceptible and resistant isolates. However, in addition to full-length coding mRNAs corresponding to *unc-63* orthologs, abbreviated isoforms (*unc-63b*) were found to be specifically expressed in some levamisole resistant isolates from the three nematode species, suggesting a possible role of the truncated UNC-63 subunit in levamisole resistance [30]. Transcript levels may also change with resistance [49]. Recent progress with the functional expression of nematode levamisole AChRs in *Xenopus* oocytes has provided a unique opportunity to test the role of truncated subunits in levamisole resistance [31].

The truncated *Hco-unc-63b* transcript in an *H. contortus* isolate encoded a protein of 343 amino acids that has two transmembrane domains (TM1 and TM2). In *Xenopus* oocytes, expression of the truncated Hco-UNC-63b along with the full length *Hco-unc-63a, Hco-unc-29.1, Hco-unc-38* and *Hco-acr-8* cRNA produced a dose-dependent inhibition of the Hco-L-AChR-1 expression [31]. The results show that the truncated Hco-unc-63b is dominant negative, inhibiting the wild type Hco-UNC-63 subunits and therefore could induce a levamisole-resistant phenotype in parasites expressing this mutant form. Expression

of the truncated UNC–63b is predicted to reduce expression of both the Hco-L-AChR-1 and Hco-L-AChR-2 receptors. Hence, if such a dominant negative effect also occurs *in vivo*, a cross resistance to levamisole and pyrantel could be expected in the *H. contortus* isolates expressing Hco-UNC-63b. The role of truncated ACR-8, which is associated with levamisole resistance [32] remains to be tested in expression experiments.

Concluding remarks

Better molecular and functional platforms have allowed a tremendous increase in our understanding of: (i) the biology of the levamisole AChR channel; (ii) how there are a number of diverse pharmacological subtypes; (iii) how the subunit structure is plastic and affects drug sensitivity; and (iv) how subunit truncation (UNC-63 or ACR-8) or reduced subunit expression may play a role in levamisole resistance. There is optimism that the understanding of the molecular and pharmacological mechanism (or mechanisms) of resistance will allow molecular diagnostic tests for resistance and better pharmacological therapies to be used, including combination drug therapy. The current advances in levamisole receptor molecular biology have bridged the gap between genes and the physiological expression of anthelmintic sensitivity. Further examination of the various receptor subtypes in important parasites such as filaria, hookworm and whipworm, the range of nematode parasite AChR channels in muscle receptors and other tissues, and the changes brought about by resistance and altered subunit composition will provide a deeper understanding of AChR functions and the mechanisms that control the evolution of their diversity. In the future, new approaches that include novel methods to measure small changes in phenotype such as microfluidics, the development of molecular techniques to manipulate parasites *in vivo* and computer simulation of the ligand receptor interaction will usher in a new awakening for receptor research. Over the next ten years we should expect to see a more detailed map of AChR function as well as new anthelmintics that target these receptors.

Acknowledgments

Glossary

Cholinomimetics	drugs which mimic the actions of acetylcholine and activate acetylcholine receptors.
Cholinergic anthelmintic	antinematodal drugs which interact with acetylcholine receptors.
Conductance subtypes	a classification of receptor ion channels based on conductance groupings; G25 (nAChRs with conductances near 25 pS); G35 (nAChRs with conductances near 35 pS); G40 (nAChRs with conductances near 40 pS); G45 (nAChRs with conductances near 45 pS).
IC_{50}	The dose of a drug that inhibits 50% of the response.
LD_{50}	The dose of a drug that kills 50% of the population under test.

Null mutants	defective gene mutants where the genes are non-functional.
Patch-clamp technique	an electrophysiological technique for recording the very small currents that flow through single ion-channels.
Phylogenetic clade	a subgroup within a phylogenetic tree that all trace back to a common point.
Hco-lev-1, Tci-lev -1,Tco-lev-1, Cel- acr-8	*C. elegans* genes are labeled and referred to by a three letter code (e.g. *lev and acr*, followed by an number); to refer homologues of these genes in parasitic nematodes, three letters from the species are added (a gene from *Haemonchus contortus* which is a homologue of *C. elegans lev-1* is referred to as *Hco-lev-1; Tci* is *Teladorsagia circumcincta; Tco* is *Trichostrongylus colubriformis*).

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Box 1. Levamisole AChRs of nematodes *in vivo* are composed of diverse species-dependent subtypes

They have different channel conductances, and the subtypes may be separated pharmacologically.

Table I

Main muscle nAChR channel conductances opened by different anthelmintic in nematodes[a]

Species	Clades	Drugs	Conductances	(pS)[e]			Refs.
A. suum	III	ACh	25-35	-	40-50		[46]
		Lev	23	33	43		[6]
		Nic	26	36	-		[47]
		Pyr	22	-	41		[52]
		Beph	-	36	43		[6]
C. elegans	V						
lev-10 mutant[b]		ACh	30				[7]
		Lev	29.3				[7]
lev-10;lev-1 mutant[c]		Lev	26.9				[7]
lev-10;lev-8 mutant[d]		ACh	27.4				[7]
		Lev	30				[7]
O. dentatum	V	Lev	22	35	40	45	[4]
							[43]

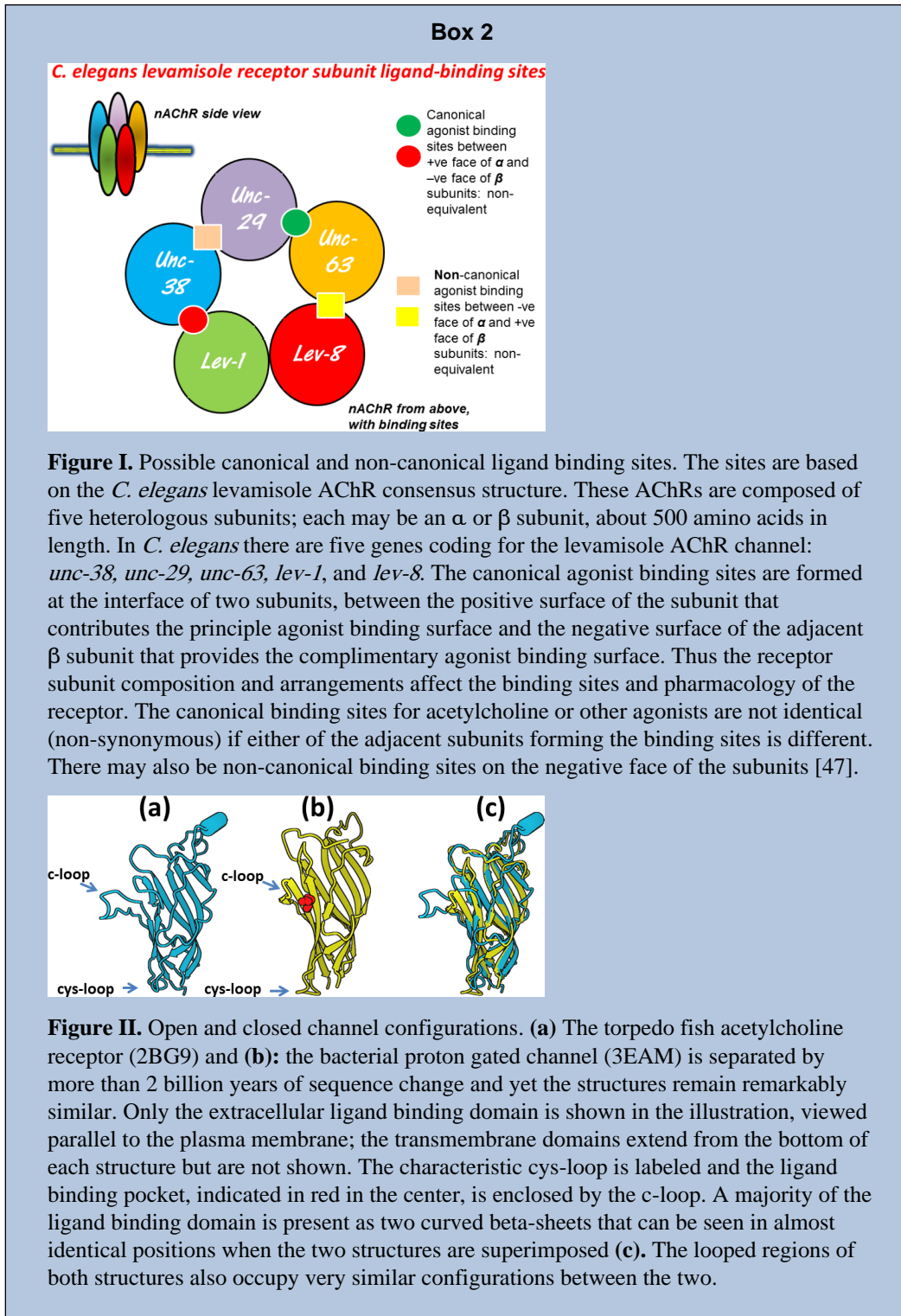Abbreviations: ACh, acetylcholine; Lev, levamisole; Nic, nicotine; Pyr, pyrantel Beph, bephenium

[a]In *C. elegans* only one was separated (approx. 30 pS), in *A. suum* up to three subtypes (approx. G25 pS, G35 pS and G45 pS) were separated, and in *O. dentatum* four were separated (approx. G25 pS, G35 pS, G40 pS and G45 pS).

[b]*C. elegans lev-10* mutant

[c]*lev-1* mutant

[d]*lev-8* mutant

[e]Conductances

Box 2

C. elegans levamisole receptor subunit ligand-binding sites

Figure I. Possible canonical and non-canonical ligand binding sites. The sites are based on the *C. elegans* levamisole AChR consensus structure. These AChRs are composed of five heterologous subunits; each may be an α or β subunit, about 500 amino acids in length. In *C. elegans* there are five genes coding for the levamisole AChR channel: *unc-38*, *unc-29*, *unc-63*, *lev-1*, and *lev-8*. The canonical agonist binding sites are formed at the interface of two subunits, between the positive surface of the subunit that contributes the principle agonist binding surface and the negative surface of the adjacent β subunit that provides the complimentary agonist binding surface. Thus the receptor subunit composition and arrangements affect the binding sites and pharmacology of the receptor. The canonical binding sites for acetylcholine or other agonists are not identical (non-synonymous) if either of the adjacent subunits forming the binding sites is different. There may also be non-canonical binding sites on the negative face of the subunits [47].

Figure II. Open and closed channel configurations. **(a)** The torpedo fish acetylcholine receptor (2BG9) and **(b):** the bacterial proton gated channel (3EAM) is separated by more than 2 billion years of sequence change and yet the structures remain remarkably similar. Only the extracellular ligand binding domain is shown in the illustration, viewed parallel to the plasma membrane; the transmembrane domains extend from the bottom of each structure but are not shown. The characteristic cys-loop is labeled and the ligand binding pocket, indicated in red in the center, is enclosed by the c-loop. A majority of the ligand binding domain is present as two curved beta-sheets that can be seen in almost identical positions when the two structures are superimposed **(c).** The looped regions of both structures also occupy very similar configurations between the two.

Box 2. Levamisole AChR channels and models of subunit structure

Levamisole AChR channels

Levamisole and pyrantel are nematode selective cholinergic (acetylcholine) agonists that open non-selective cation ion-channel receptors (AChRs) present on nematode body muscle. The drugs bind to canonical and/or non-canonical binding sites on the receptors (Figures I and II). Opening of these channels produces depolarization, entry of calcium, and muscle contraction; the nematode parasite becomes paralyzed and is ejected from its location, effecting a cure. The ligand binding sites are between adjacent subunits of the receptor and include canonical and non-canonical binding sites.

Modeling ligand binding and the levamisole AChR channel.

The structure of the nematode levamisole AChR channel and subunits is predictable because the structure of ligand-gated ion-channel subunits is conserved (Figure II).

These structures can predict that of other ion-channels by providing an initial approximate configuration for energy minimization techniques that yield optimal predicted structures [50, 51]. The predictions are used in docking studies where configurations of a ligand are evaluated to find the most likely pose identified as that with the lowest energy. The intrinsic flexibility of ion-channels is essential to their function but creates problems for docking studies *in silico*. This can be seen by the dramatic change is position of the c-loop between the closed channel configuration (Figure IIa): and the open form (Figure IIb). Presence of the ligand stabilizes the c-loop around the ligand. This change is followed by a series of other structural changes that ultimately results in rotation of the M2 transmembrane that opens the channel.

Box 3. Levamisole resistance

C. elegans levamisole resistance

The degree of our understanding of the mechanisms of levamisole resistance depends on species of nematode studied. Levamisole resistance in *C. elegans* has been associated with null mutants of 21 genes [12-15,21-28]. Five of the genes are: *unc-38, unc-29, unc-63, lev-1, and lev-8*, which code for subunits of the levamisole AChR channel; seven of the genes: *oig-4, nra-1, soc-1, tax-6, tpa-1, lev-9* and *lev-10*, are associated with the proper distribution and maintaining the normal opening of the receptor channel. Three genes associated with calcium release or calcium-sensitive contraction are: *unc-68, unc-22* and *lev-11*. Three genes, known as accessory proteins are: *unc-50, ric-3* and *unc-74*. These accessory proteins along with *nra-2* and *nra-4* are associated with the processing, assembly and delivery of the ion-channel receptor to the membrane.

Resistance in nematode parasites

In *O. dentatum* levamisole resistance has been associated with a reduced number of receptor channels at the muscle surface and a loss of the G35 pS conductance subtypes of levamisole AChR channel [4], whereas pyrantel resistance was associated with a reduced number of all conductance subtypes [48]. In *A. caninum*, pyrantel resistance has been associated with reduced expression levels of UNC-38, UNC-63, UNC-29 but not ACR-8. No functional change was detected in the amino acid sequence of either of these proteins nor in the bases coding for the genes (single nucleotide polymorphism: SNPs). In *H. contortus*, levamisole resistance has been associated with truncated forms of UNC-63. With loss of or truncation of ACR-8, the isolate is expected to be less sensitive to levamisole but still sensitive to pyrantel. Thus it is suggested that loss of ACR-8 subunits may lead to a selective loss of sensitivity to levamisole but not pyrantel. Loss of UNC-63, however, is predicted to lead to a loss of sensitivity to both levamisole and pyrantel. The role of the other *C. elegans* genes in resistance to levamisole in parasitic nematodes remains to be investigated.

Transcriptomic approach for identifying resistance genes

In addition to the candidate gene strategy, based on *C. elegans*, a global comparative transcriptomic approach is another useful tool for identification of novel parasitic genes involved in levamisole resistance. This non-hypothesis driven approach using an amplified restriction fragment technique (cDNA-AFLP) has been used to identify genes differentially expressed in levamisole-resistant and susceptible isolates of *H. contortus* [30]. More than 17 000 transcript-derived fragments (TDFs) were amplified and 26 TDFs displayed differential expression including 11 TDFs specifically present in resistant isolates. Among those candidates, HAX was specifically expressed in the two resistant isolates and presented strong homology with the *acr-8* gene. HAX corresponded to a truncated *H. contortus acr-8* mRNA isoform (*Hco-acr-8b*) containing the two first exons and a part of intron 2. The specific expression of *Hco-acr-8b* was confirmed in other *H. contortus* levamisole resistant isolates highlighting its interest as a potent marker for levamisole resistance [32,49].
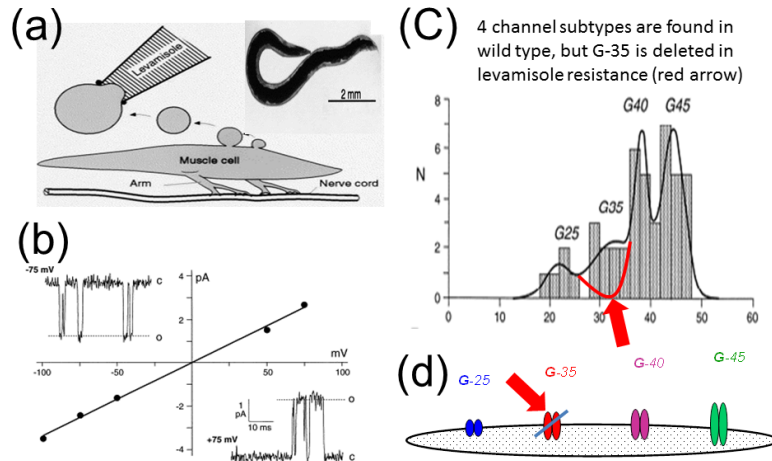
O. dentatum patch preparation

Figure 1.
AChR single-channel currents from *Oesophagostomum dentatum* using the patch-clamp technique [4,43]. **(a)** Diagram of the production of the membrane vesicles and patch-pipette used to record the levamisole activated AChR channel currents; (inset) adult female *O. dentatum*. **(b)** Examples of levamisole activated channel currents recorded at -75mV and +75mV patch potentials and the current voltage plot showing a linear relationship, zero reversal potential, and slope giving a channel conductance of 42 pS. **(c)** Channel conductance distribution for the anthelmintic sensitive isolate (wild type) showing the four peaks (fitted using the sum of 4 normal distributions). The peaks illustrate the presence of four subtypes present: G25 pS, G35 pS, G40 pS and G45 pS. In the levamisole-resistant (LEVR) isolate there were only three subtypes present because the G35 pS subtype was missing. In the pyrantel resistant isolate, the four G subtypes were present and included the G 35 pS subtype, but there were fewer channels present in the muscle compared with the anthelmintic sensitive (wild) isolate. **(d)** Diagram of the different channel subtypes detected and presented by size on a basis of the conductances in *O. dentatum*; G 35 pS is not present in in levamisole resistant isolates, but present in pyrantel isolates.
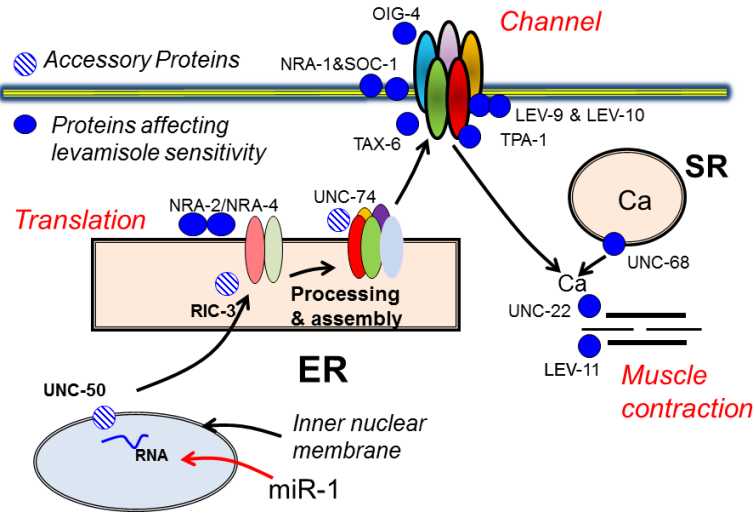
Figure 2.
Assembly and function of levamisole receptors. *C. elegans* levamisole sensitive AChR channels in the muscle membrane are formed by five subunits (UNC-38, blue; UNC-29, purple; UNC-63, yellow; LEV-1, green; and LEV-8, red) with normal function being supported by OIG-1, NRA-1, NRA-2, NRA-4, SOC-1, TAX-6, TPA-1, LEV-9 and LEV-10 proteins. Once the levamisole AChR channel opens, calcium enters and its signal is amplified by the ryanodine receptor (UNC-68); the increased calcium then initiates contraction, requiring the proteins UNC-22 and LEV-11. The expression of the levamisole receptor subunits requires RNAs encoding the five subunits together with three *C. elegans* ancillary proteins involved in AChR assembly (RIC-3), folding (UNC-74) and trafficking (UNC-50) [24, 27, 45]. MicroRNA (miR-1) is also known to regulate and modulate the expression of subunit RNA. Abbreviations: ER, endoplasmic reticulum; SR sarcoplasmic reticulum.
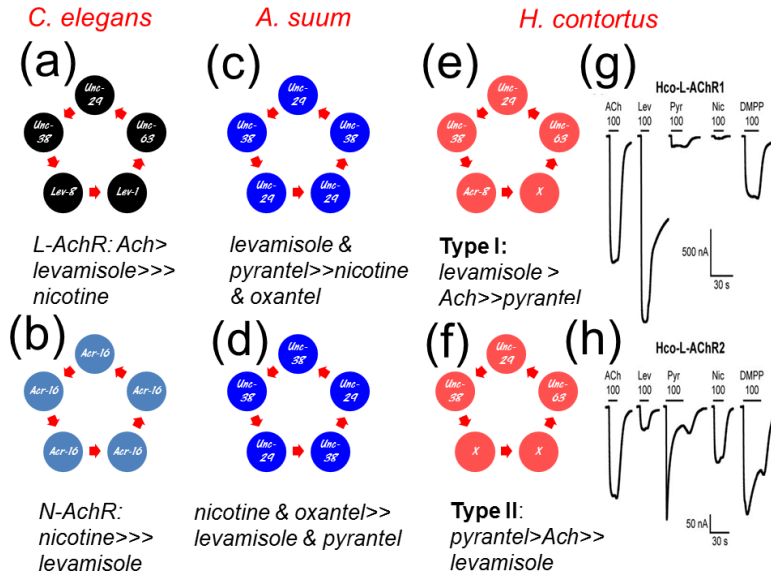
Figure 3.
Diagram of possible stoichiometric arrangements of some different AChRs from *C. elegans*, *A. suum* and *H. contortus* and the relative potencies of anthelmintic agonists. **(a)** The *C. elegans* levamisole AChR. **(b)** The *C. elegans* muscle nicotine AChR. **(c)** The *A. suum* AChR where levamisole is more potent than nicotine when more cRNA for UNC-29 than UNC-38 is injected. **(d)** The *A. suum* AChR where nicotine is more potent than levamisole when more cRNA for UNC-38 than UNC-29 is injected into the *Xenopus* oocytes for expression. **(e)** The *H. contortus* L-AChR 1 receptor, which is most sensitive to levamisole. **(f)** The *H. contortus* L- AChR-2, which is most sensitive to pyrantel [31]. **(g)** Currents recorded from the *Xenopus* oocyte expressing *H. contortus* Hco-L-AChR1 receptor when 100 µM acetylcholine (ACh), 100 µM levamisole (Lev), 100 µM pyrantel (Pyr), 100 µM nicotine (Nic) or 100 µM dimethyphenylpiperazinium (DMPP) are applied. Levamisole is the most potent agonist. **(h)** Currents recorded from the *Xenopus* oocyte expressing *H. contortus* Hco-L-AChR2 receptor when 100 µM acetylcholine (ACh), 100 µM levamisole (Lev), 100 µM pyrantel (Pyr), 100 µM nicotine (Nic) or 100 µM dimethyphenylpiperazinium (DMPP) are applied. Pyrantel produces the biggest peak response.