RESEARCH PAPER

Functional reconstitution of *Haemonchus contortus* acetylcholine receptors in *Xenopus* oocytes provides mechanistic insights into levamisole resistance

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BACKGROUND AND PURPOSE

The cholinergic agonist levamisole is widely used to treat parasitic nematode infestations. This anthelmintic drug paralyses worms by activating a class of levamisole-sensitive acetylcholine receptors (L-AChRs) expressed in nematode muscle cells. However, levamisole efficacy has been compromised by the emergence of drug-resistant parasites, especially in gastrointestinal nematodes such as *Haemonchus contortus*. We report here the first functional reconstitution and pharmacological characterization of *H. contortus* L-AChRs in a heterologous expression system.

EXPERIMENTAL APPROACH

In the free-living nematode *Caenorhabditis elegans*, five AChR subunit and three ancillary protein genes are necessary *in vivo* and *in vitro* to synthesize L-AChRs. We have cloned the *H. contortus* orthologues of these genes and expressed them in *Xenopus* oocytes. We reconstituted two types of *H. contortus* L-AChRs with distinct pharmacologies by combining different receptor subunits.

KEY RESULTS

The Hco-ACR-8 subunit plays a pivotal role in selective sensitivity to levamisole. As observed with *C. elegans* L-AChRs, expression of *H. contortus* receptors requires the ancillary proteins Hco-RIC-3, Hco-UNC-50 and Hco-UNC-74. Using this experimental system, we demonstrated that a truncated Hco-UNC-63 L-AChR subunit, which was specifically detected in a levamisole-resistant *H. contortus* isolate, but not in levamisole-sensitive strains, hampers the normal function of L-AChRs, when co-expressed with its full-length counterpart.

CONCLUSIONS AND IMPLICATIONS

We provide the first functional evidence for a putative molecular mechanism involved in levamisole resistance in any parasitic nematode. This expression system will provide a means to analyse molecular polymorphisms associated with drug resistance at the electrophysiological level.

Abbreviations

AVM, avermectins; BZ, benzimidazoles; DHβE, dihydro-β-erythroidine; DMPP, 1,1-dimethyl-4-phenylpiperazinium; dTC (+)-tubocurarine; Hco-L-AChR1, *H. contortus*-levamisole-sensitive acetylcholine receptor 1; Hco-L-AChR2, *H. contortus*-levamisole-sensitive acetylcholine receptor 2; L-AChR, levamisole-sensitive acetylcholine receptor; N-AChR, nicotine-sensitive acetylcholine receptor

Introduction

Gastrointestinal parasite infections are of major importance for human health and animal welfare. In the absence of effective vaccination strategies, control of parasitic helminths relies mainly on the use of broad spectrum anthelmintics such as levamisole, benzimidazoles (BZ) and avermectins (AVM). Intensive use of these drugs has inevitably led to the selection of resistant parasites. For example, the haematophagous parasite *Haemonchus contortus* (barber pole worm) is one of the most prevalent and pathogenic trichostrongylid species affecting small ruminants and threatening productivity and profitability in sheep and goat farming worldwide (Kaplan, 2004; Waller and Chandrawathani, 2005). *H. contortus* populations resistant to these three classes of anthelminthics and even combinations of drugs have been reported around the world (Kaplan, 2004). However, the rate of resistance selection for levamisole appears to be slower in *H. contortus* in comparison with BZ and AVM. Therefore, levamisole remains a useful tool to control BZ- and AVM-resistant parasites populations (Tyrrell and LeJambre, 2010).

Levamisole and other cholinergic agonists such as pyrantel and oxantel activate acetylcholine receptors (AChRs) expressed in nematode body-wall muscles (Aceves *et al.*, 1970; Aubry *et al.*, 1970; Harrow and Gration, 1985; Colquhoun *et al.*, 1991; Martin *et al.*, 2004). Exposure to these drugs causes spastic paralysis of the worms, which are either killed as with the free-living nematode *Caenorhabditis elegans* or expelled from the host organism in the case of *H. contortus*. The molecular composition of these levamisole-sensitive AChRs (L-AChRs) was first deciphered in *C. elegans* using the powerful genetic tools available in this model organism (Lewis *et al.*, 1980; Lewis *et al.*, 1987). Forward genetic screens for levamisole-resistant mutants in *C. elegans* identified the five genes encoding the five subunits of L-AChRs. They include three α-subunits (UNC-63, UNC-38 and LEV-8) and two non-α-subunits (UNC-29 and LEV-1) (Fleming *et al.*, 1997; Culetto *et al.*, 2004; Towers *et al.*, 2005; Boulin *et al.*, 2008). In addition, mutation of three additional genes, *ric-3*, *unc-74* and *unc-50*, causes a complete loss of L-AChR expression in muscle cells. RIC-3 is a small transmembrane protein thought to act as a chaperone promoting AChR folding in the endoplasmic reticulum (Millar, 2008). It is involved in the assembly or maturation of at least four distinct AChRs in *C. elegans*, including L-AChRs and nicotine-sensitive AChRs (N-AChRs) in muscle (Halevi *et al.*, 2002; 2003). The gene *unc-74* encodes a thioredoxin closely related to the human TMX3 protein (Haugstetter *et al.*, 2005) and this protein is likely to be required for the proper folding of L-AChR subunits, although its function has not been characterized in detail thus far. The gene *unc-50* encodes a transmembrane protein mostly localized to the Golgi apparatus. In *unc-50* mutants, L-AChRs, but no other ionotropic receptors, are targeted to lysosomes for degradation, suggesting a specific role for this protein in the regulation of L-AChR trafficking (Eimer *et al.*, 2007). These three genes have been widely conserved through evolution from nematodes to humans.

In the absence of an efficient expression system for nematode L-AChRs, the biophysical and pharmacological characterization of these receptors has relied for almost four decades on *in vivo* electrophysiological analysis, conducted initially in *Ascaris suum* and *Oesophagostomum dentatum* (Robertson *et al.*, 1999; Qian *et al.*, 2006), and subsequently in *C. elegans* (Richmond and Jorgensen, 1999; Qian *et al.*, 2008). Recently, we have obtained robust expression of *C. elegans* L-AChRs in *Xenopus* oocytes by providing cRNAs not only encoding the five L-AChR subunits, but also the three ancillary proteins RIC-3, UNC-50 and UNC-74 (Boulin *et al.*, 2008). Interestingly, the sole expression of the *A. suum* orthologues of *unc-29* and *unc-38* in *Xenopus* oocytes was sufficient to reconstitute AChRs with varying sensitivity to levamisole and nicotine, depending on the relative expression levels of the two subunits (Williamson *et al.*, 2009). Because *A. suum* is evolutionarily distant from *C. elegans*, these results suggested that the molecular composition of L- and N-AChRs could vary among nematodes, although the potential contribution of additional subunits in the formation of AChRs and the role of ancillary proteins for AChR expression was not explored in this study.

H. contortus and *C. elegans* belong to the same phylogenetic group (clade V), which also includes the human parasites *Ancylostoma ceylanicum* and *Necator americanus* (Mitreva *et al.*, 2004). The precise molecular mechanisms involved in levamisole resistance are still poorly understood in trichostrongylid nematodes, but L-AChR subunit genes are obvious candidates based on results obtained in *C. elegans*. The orthologues of *unc-29*, *unc-63*, *unc-38* and *lev-1* have been cloned in *H. contortus* and other parasitic trichostrongylid species such as *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (Wiley *et al.*, 1996; Hoekstra *et al.*, 1997; Walker *et al.*, 2001; Neveu *et al.*, 2010). No clear orthologue of the *C. elegans* LEV-8 L-AChR subunit has been found (Williamson *et al.*, 2007; Neveu *et al.*, 2010), but a transcriptomic study carried out in *H. contortus* has recently identified another AChR subunit, Hco-ACR-8, as a candidate gene implicated in levamisole resistance (Fauvin *et al.*, 2010). Hco-ACR-8 is most closely related to the *C. elegans* LEV-8 and ACR-8 receptor subunits. A comprehensive analysis performed on the laboratory-selected RHS6 levamisole-resistant isolate identified a truncated isoform of *Hco-unc-63* (*Hco-unc-63b*), which was co-expressed with the full-length *Hco-unc-63a* transcript (Neveu *et al.*, 2010). Truncated *unc-63* transcripts were also identified in levamisole-resistant field isolates of the trichostrongylid species *T. circumcincta* and *T. colubriformis*, suggesting a possible link between variants of the *unc-63* locus and levamisole resistance phenotypes (Neveu *et al.*, 2010). However, this hypothesis could not be tested because of the lack of a heterologous expression system.

In the present study, we have demonstrated that two L-AChRs of *H. contortus* can be functionally reconstituted in *Xenopus* oocytes by co-expressing receptor subunits and conserved ancillary factors. We used this novel expression system to characterize these L-AChRs. Finally, we showed that a truncated AChR subunit found in levamisole-resistant isolates had a dominant-negative effect on the expression of wild-type *H. contortus* receptors in *Xenopus* oocytes. This result suggests a novel mechanism by which levamisole resistance can be obtained in trichostrongylid parasites. It further indicates the capability of this novel expression system to test the functional relevance of polymorphisms associated with resistance to levamisole in wild isolates.

Methods

Animals

All animal care and experimental procedures in this study were in strict accordance with guidelines of good animal practice defined by the Center France-Limousin ethical committee (France). Sheep studies were performed under experimental agreement 6623 approved by the Veterinary Services (Direction des Services vétérinaires) from Indre et Loire (France).

Accession numbers

The accession numbers for protein and cDNA sequences mentioned in this article are: *C. elegans*: ACR-2 NM_076727, ACR-3 NM_076728, ACR-8 – JF416644, ACR-12 NM_077861, LEV-1 NP_502534, LEV-8 NP_509932, RIC-3 NP_501299, UNC-29 NP_492399, UNC-38 NP_491472, UNC-50 NP_499279, UNC-63 NP_491533 and UNC-74 NP_491361; *H. contortus*: *Hco-acr-8* EU006785, *Hco-lev-1* GU060987, *Hco-ric-3.1* HQ116823, *Hco-ric-3.2* HQ116824, *Hco-unc-29.1* GU060980, *Hco-unc-38* GU060984, *Hco-unc-50* HQ116822, *Hco-unc-63a* GU060985, *Hco-unc-63b* GU060986, *Hco-unc-74* HQ116821; *T. circumcincta*: *Tci-acr-8* HQ215517; *T. colubriformis*: *Tco-acr-8* HQ215518.

Nematodes isolates

Experiments with *H. contortus* were performed on the levamisole-susceptible ISE (Roos *et al.*, 2004) and the levamisole-resistant RHS6 isolates (Hoekstra *et al.*, 1997). Adult nematodes (males and females) were collected 30 days after infection from the abomasal mucosa of sheep infected with 10 000 infective larvae (L3s). The *T. circumcincta* and *T. colubriformis* studies were carried out on the levamisole-susceptible TciSO and TcoSO isolates respectively (Neveu *et al.*, 2010). For these species, sheep were experimentally infected with 5000 infective larvae (L3s) and adult nematodes (males) were collected from the abomasum (*T. circumcincta*) or the small intestine (*T. colubriformis*) 30 days after infection. The levamisole susceptibility or resistance status of all isolates used in this study has been confirmed previously (Neveu *et al.*, 2010).

Molecular biology

Total RNA was prepared from 10 adult males from *H. contortus* ISE and RHS6 isolates, and from 50 adult males of *T. circumcincta* (TciSO) or *T. colubriformis* (TcoSO). Frozen worms were homogenized in Trizol reagent (Invitrogen) and total RNA was isolated according to the manufacturer's recommendations. RNA pellets were dissolved in 25 µL of RNA secure resuspension solution (Ambion, Austin, TX, USA) and DNase-treated using the TURBO DNA-free kit (Ambion). RNA concentrations were measured using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). First-strand cDNA synthesis was performed on 3 µg of total RNA using the oligo (dT) RACER primer and superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Full-length cDNA sequences homologous to *Cel-acr-8*, *Cel-ric-3*, *Cel-unc-50* and *Cel-unc-74* were identified in *H. contortus* (ISE) using first-strand cDNA as a template. 3′ cDNA ends were identified by 3′ RACE PCR using the GeneRacer kit (Invitrogen) with primers based on partial genomic sequences available in the *H. contortus*

genome sequence database (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/h_contortus). The 5′ end of each cDNA was amplified by using the nematode spliced-leader sequence SL1 (ggtttaattacccaagtttgag). Amplification products were cloned in pGEMt-easy vector (Promega, Madison, WI, USA) and fully sequenced. Full-length cDNA sequences homologous to *Hco-* and *Cel-acr-8* were identified in *T. circumcincta* (TciSO) *and T. colubriformis* (TcoSO) using similar cloning strategies. 3′-RACE and SL1-PCR experiments were carried out using degenerate primers based on an alignment of *H. contortus* and *C. elegans acr-8* cDNA sequences. The full length cDNAs coding for *Hco-acr-8*, *Hco-lev-1*, *Hco-ric-3.1*, *Hco-ric-3.2*, *Hco-unc-29.1*, *Hco-unc-38*, *Hco-unc-50*, *Hco-unc-63a* and *Hco-unc-74* were amplified by PCR using first-strand cDNA from the *H. contortus* ISE isolate, whereas *Hco-unc-63b* was amplified using cDNA from the RHS6 isolate. Next, amplification products were gel purified, and digested with XhoI and ApaI (HindIII and ApaI for *Hco-unc-29.1*), and cloned into the pTB207 expression vector (Boulin *et al.*, 2008). Each construct was validated by sequencing. Finally, cRNA was synthetized *in vitro* from linearized plasmid DNA templates using the mMessage mMachine T7 transcription kit (Ambion). Lithium chloride-precipitated cRNA was resuspended in RNAse-free water and stored at –80°C. All primer sequences are reported in Supporting Information Tables S1 and S2.

Sequence analysis

Database searches were performed with the BLAST Network Service (NCBI), using the tBLASTn or BLASTX algorithms (Altschul *et al.*, 1997). Signal peptide predictions were carried out using the SignalP 3.0 server (Bendtsen *et al.*, 2004) and membrane-spanning regions were predicted using TMpred. Coiled-coil motifs were predicted using the coiled-coil prediction program (http://www.russell.embl.de/cgi-bin/coils-svr.pl) and conserved protein domains were predicted using SMART (Schultz *et al.*, 1998). Phylogenetic analyses were performed on full-length cDNA sequences as follows: the nucleotide sequences were first translated and amino acid sequences aligned using MUSCLE (Edgar, 2004). The corresponding nucleotide alignment was obtained by concatenating codons using the REVTRANS server. The alignment was analysed using the pipelines available on the phylogeny.fr and the alignment refinement was performed using Gblocks (Castresana, 2000). The Maximum-likelihood (ML) tree was estimated using PhyML taking into account the best-fitting nucleotide substitution model (General Time Reversible) using the Datamonkey server. The statistical support of the inferred tree was evaluated by non-parametric bootstrap analysis with one thousand pseudoreplicates.

Electrophysiological studies in Xenopus laevis oocytes

Xenopus laevis oocytes were prepared, injected, voltage-clamped and superfused as described previously (Paoletti *et al.*, 1995), except that gentamycin was omitted from the conservation medium because prolonged treatments with this antibiotic can inhibit AChRs expressed in *Xenopus* oocytes (Amici *et al.*, 2005). For each oocyte, ~36 nL of a cRNA injection mix containing 50 ng·µL^{-1} of each cRNA species were injected into the animal pole. Oocytes were

recorded 2–3 days after injection. Unless otherwise noted, the standard external solution had the following composition (in mM): 100 NaCl, 2.5 KCl, 1 CaCl$_2$ and 5 HEPES; pH 7.3 (NaOH). In some experiments (dose–response experiments, agonist and antagonist pharmacology) the calcium chelator BAPTA was loaded into oocytes to prevent activation of endogenous calcium-activated chloride conductances. BAPTA-AM (Sigma, St. Louis, MO, USA) was diluted in Barth medium (100 μM final concentration), and oocytes were incubated for ~4 h in 200 μL BAPTA-AM solution at 19°C. Data were collected and analysed using Clampex 9.2 and Clampfit 9.2 (Axon Instruments, Sunnyvale, CA, USA).

Data analysis

Results are shown as means ± SD. Data were fitted using KaleidaGraph 4.0 (Synergy Software). Dose–response curves were established as described previously (Boulin *et al.*, 2008).

Materials

Acetylcholine chloride (ACh), BAPTA-AM, dihydro-β-erythroidine hydrobromide (DHβE), 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (–)-tetramisole hydrochloride (levamisole) (–)-nicotine hydrogen tartrate, pyrantel citrate, (+)-tubocurarine chloride hydrate (dTC) were purchased from Sigma-Aldrich.

Results

The trichostrongylid ACR-8 subunit might be distantly related to C. elegans LEV-8

Based on genetic, biochemical and electrophysiological data (Lewis *et al.*, 1987; Richmond and Jorgensen, 1999; Gottschalk *et al.*, 2005; Boulin *et al.*, 2008), *C. elegans* L-AChR are composed of five receptor subunits (*Cel-lev-1, Cel-lev-8, Cel-unc-29, Cel-unc-38* and *Cel-unc-63*). Clearly identifiable orthologues for four of these subunits have been found in the trichostrongylids *H. contortus, T. circumcincta* and *T. colubriformis*. Trichostrongylid L-AChR subunits are highly similar to their *C. elegans* counterparts, with a few notable differences. First, while *C. elegans* has only a single locus encoding a UNC-29 subunit, *H. contortus* has four paralogues named *Hco-unc-29.1, Hco-unc-29.2, Hco-unc-29.3* and *Hco-unc-29.4* (Neveu *et al.*, 2010). Of these four subunits, Hco-UNC-29.1 is the most similar to UNC-29 (79% identity, 88% amino-acid similarity). Second, no signal peptide is readily identifiable in any trichostrongylid LEV-1 subunit (Neveu *et al.*, 2010). Third, no clear homologue of *Cel-lev-8* has been found so far using bioinformatic or experimental approaches. The closest homolog of *Cel-lev-8* in trichostrongylids is the AChR subunit ACR-8.

Using a PCR-based strategy, we have cloned the full-length *acr-8* cDNA sequences from three levamisole-sensitive trichostrongylid nematodes (*H. contortus* ISE isolate, *T. circumcincta* TciSO isolate, *T. colubriformis* TcoSO isolate). The *acr-8* orthologues identified in *H. contortus, T. circumcincta* and *T. colubriformis*, have been designated *Hco-acr-8, Tci-acr-8* and *Tco-acr-8*, respectively, following recent nomenclature recommendations (Beech *et al.*, 2010). The trichostrongylid ACR-8 sequences contain the typical features of an AChR subunit.

All three subunits possess a YxxCC motif in loop C of the ACh-binding site, defining them as α subunits (Figure 1B). Even though trichostrongylid ACR-8 sequences are mostly similar to Cel-ACR-8 (percentage identities ranging from 68 to 69%), they also share common amino-acid signatures with the Cel-LEV-8 sequence, which are absent in Cel-ACR-8 (residues labelled in red in Figure 1B). Interestingly, these conserved amino-acids are mostly located between the cys-loop and the first transmembrane region, which corresponds to the agonist binding site region of AChR subunits. In *C. elegans*, UNC-29 can interact with the ACR-8 subunit in addition to the L-AChR subunits previously known to contribute to levamisole receptors (Gottschalk *et al.*, 2005). Although *acr-8* mutants are not resistant to levamisole, ACR-8 could replace another subunit in the *C. elegans* L-AChR (Almedom *et al.*, 2009). Taken together, these results suggest that Hco-ACR-8 could be involved in the *H. contortus* L-AChR subunit composition in the absence of a trichostrongylid LEV-8 homologue.

Ancillary factors required for L-AChR expression in C. elegans are conserved in H. contortus

We previously demonstrated that three ancillary proteins encoded by *ric-3, unc-50* and *unc-74* are required for the robust expression of *C. elegans* L-AChRs in *Xenopus* oocytes (Boulin *et al.*, 2008). We hypothesized that these ancillary proteins could also be necessary for heterologous expression of *H. contortus* L-AChRs.

After identifying likely candidates by performing BLAST searches on the *H. contortus* genome, we isolated the corresponding full-length cDNA sequences for *Hco-unc-50, Hco-unc-74* and two *ric-3* orthologues, *Hco-ric-3.1* and *Hco-ric-3.2*. The *H. contortus* and *C. elegans* orthologues share typical sequence features (Table 1 and Supporting Information Figure S1). Hco-UNC-50 harbours a UNC-50 domain and five predicted transmembrane regions that are characteristic of Cel-UNC-50 (Eimer *et al.*, 2007). Hco-UNC-74 contains a predicted signal peptide, a thioredoxin domain and a predicted transmembrane region in its C terminal part. The two *H. contortus* RIC-3 proteins have two transmembrane regions and C-terminal coiled-coil motifs. They only differ by seven amino-acid substitutions that do not affect transmembrane region and coiled-coil motif predictions. The corresponding genes mapped unambiguously to two distinct supercontigs (0006784 and 0043984) suggesting a recent duplication of the gene in *H. contortus*. Interestingly, partial coding sequences sharing significant similarity with each of the *C. elegans* and *H. contortus* ancillary factors can be easily identified in *Brugia malayi* and *A. suum* genomic databanks (Supporting Information Figure S1). This suggests that ancillary proteins are conserved among nematodes distantly related to *C. elegans*.

Reconstitution of H. contortus levamisole-sensitive AChRs in Xenopus laevis oocytes

Our strategy to express *H. contortus* L-AChRs was based on the previous reconstitution of *C. elegans* L-AChRs (Boulin *et al.*, 2008; Jospin *et al.*, 2009). We co-injected *in vitro* transcribed
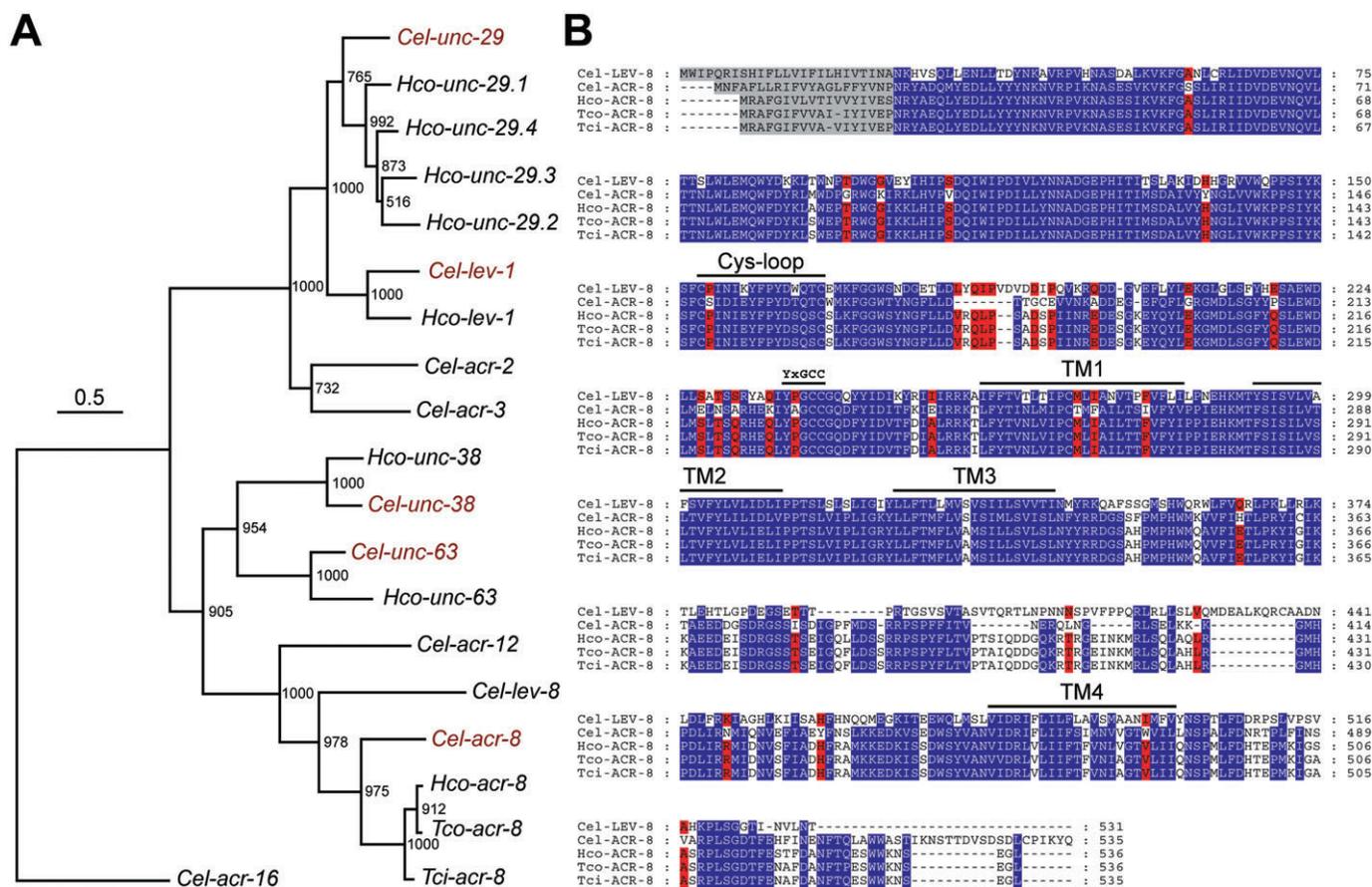
Figure 1

Phylogeny of L-AChR subunits in *C. elegans* and trichostrongylid nematodes. (A) Tree construction was performed on full-length cDNA sequences. Numbers at each node indicate bootstrap values corresponding to 1000 replicates. The scale bar represents nucleotide substitutions per site. The *C. elegans acr-16* N-AChR subunit gene was used as an outgroup. The three letter prefixes in AChR subunit gene names *Cel*, *Hco*, *Tci* and *Tco* refer to *C. elegans*, *H. contortus*, *T. circumcincta* and *T. colubriformis* respectively. The five *C. elegans* AChR subunits required for the functional expression of the levamisole-sensitive AChR in *Xenopus* oocytes are labeled in red. (B) LEV-8 and ACR-8 sequences were aligned using the MUSCLE algorithm (Edgar, 2004) and further processed using GeneDoc. Predicted signal peptide sequences are shaded in grey. Amino acids conserved between Cel-LEV-8 and trichostrongylid ACR-8 sequences – but not *C. elegans* ACR-8 – are highlighted in red. The Cys-loop, the four transmembrane regions (TM1–TM4) and the primary agonist binding site are noted above the sequence.

Table 1

Comparison of *H. contortus* ancillary factors with their *C. elegans* orthologues

Gene name	Accession number	Full-length cDNA size (bp)	Protein sequence length (aa)	*C. elegans* orthologue (e value)	% Amino acid identity / similarity
Hco-ric-3.1	HQ116823	1465	365	Cel-RIC-3 (3e-54)	56/70
Hco-ric-3.2	HQ116824	1468	365	Cel-RIC-3 (5e-54)	56/70
Hco-unc-50	HQ116822	1350	298	Cel-UNC-50 (5e-112)	73/84
Hco-unc-74	HQ116821	1574	445	Cel-UNC-74 (2e-123)	51/70

cRNAs of the four AChR subunits *Hco-acr-8, Hco-unc-29.1, Hco-unc-38* and *Hco-unc-63a* with cRNAs encoding three conserved ancillary factors *Hco-ric-3.1, Hco-unc-50* and *Hco-unc-74* (Figure 2). This combination of cRNAs led to the robust expression of levamisole-sensitive AChRs two to three

days after injection. Application of 100 μM ACh or 100 μM levamisole elicited rapidly increasing currents in the μA range with little to no desensitization (Figure 3A). Addition of the *Hco-lev-1* subunit did not result in any detectable change in expression, suggesting that this subunit is not required for
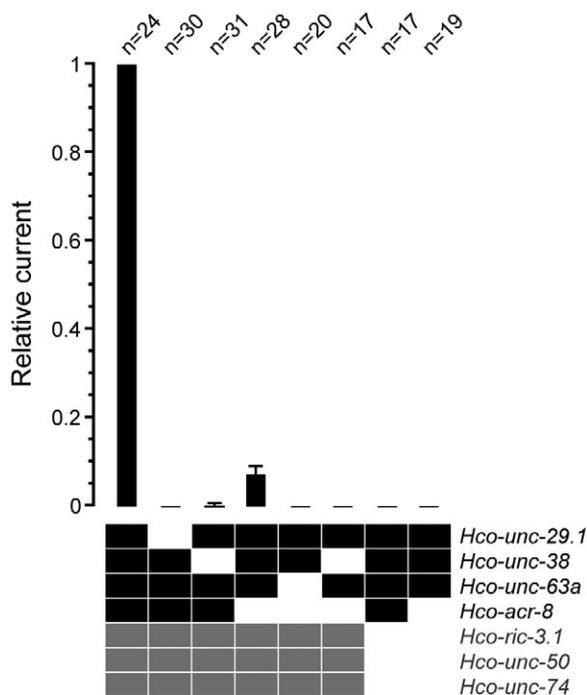
Figure 2

Four receptor subunits and three ancillary factors are required for efficient expression of Hco-L-AChR1. Co-injection of four receptor subunits (black squares) and three ancillary factors (grey squares) yields the strongest currents (average 540 nA (*n* = 24) with 500 μM ACh). Removal of the *Hco-acr-8* subunit from the injection mix reduced average currents by 93 ± 1.6% (*n* = 28). No other combination yielded any measurable currents, except in very rare instances when *Hco-unc-38* was removed. In particular, co-injection of four Hco-L-AChR1 subunits alone is not sufficient for expression, highlighting the crucial role of the three ancillary factors Hco-RIC-3.1, Hco-UNC-50 and Hco-UNC-74. Currents were measured once the steady state was reached. Numbers above bars indicate the number of oocytes recorded for each condition.

the functional expression of the receptor (data not shown). This result is consistent with the absence of predicted signal peptides in any of the trichostrongylid *lev-1* orthologues, suggesting that these subunits cannot be properly inserted in the membrane (Neveu *et al.*, 2010). Hco-LEV-1 was therefore omitted from subsequent expression experiments.

To determine which receptor genes can be combined to produce functional receptors, we removed receptor subunits either individually or in combination. Removal of the *Hco-acr-8* gene reduced expression by 93 ± 1.6% (Figure 2). Despite this strong reduction, expression was reliable and allowed the characterization of a second *H. contortus* receptor (Figure 3B). The combination of *Hco-unc-29.1, –38, –63a* and *Hco-acr-8* resulting in the expression of the first potential *H. contortus* L-AChR will be henceforth referred to as Hco-L-AChR1, whereas the combination of *Hco-unc-29.1, -38* and *-63a* will be referred to as Hco-L-AChR2. Hco-ACR-8 is an α-type AChR subunit and could theoretically assemble as a homopentamer, possibly explaining the observed difference between Hco-L-AChR1 and Hco-L-AChR2 injections. To test

this hypothesis, we injected Hco-ACR-8 with the three ancillary factors. Although parallel expression of Hco-L-AChR1 was robust, we observed no currents for Hco-ACR-8 alone (*n* = 11), demonstrating that Hco-ACR-8 does not assemble into homopentamers in our experimental conditions. Therefore, Hco-L-AChR1 and Hco-L-AChR2 differ most likely by the inclusion of Hco-ACR-8 in Hco-L-AChR1. Next, we removed *Hco-unc-29.1, Hco-unc-38* or *Hco-unc-63a* from the Hco-L-AChR1 injection mix. In the case of *Hco-unc-29.1* or *Hco-unc-63a*, no current was observed, indicating that these two subunits are essential elements of the Hco-L-AChR1 (Figures 2 and 4B). In the case of *Hco-unc-38*, we could find very rare instances where a few oocytes showed very small currents (250 times smaller than control currents on average). Because AChRs were obtained by the co-expression of the two AChR subunits Asu-UNC-29 and Asu-UNC-38 alone (Williamson *et al.*, 2009), we co-injected *Hco-unc-29.1* in combination with *Hco-unc-38* or *Hco-unc-63a* (in addition to the three ancillary factors). These combinations never yielded any detectable current (Figure 2), indicating that results obtained in *Ascaris* are not transposable to *H. contortus*.

Finally, we investigated the requirement for *Hco-ric-3.1, Hco-unc-50* and *Hco-unc-74*. Injection of the Hco-L-AChR1 or Hco-L-AChR2 receptor subunits without the three ancillary factors never yielded any detectable currents (Figure 2). This clearly demonstrates the strict requirement of these factors for expression of *H. contortus* L-AChR in *Xenopus* oocytes, as it was previously reported for *C. elegans* L-AChRs (Boulin *et al.*, 2008).

Pharmacology of H. contortus L-AChRs

Pharmacological profiles of Hco-L-AChR1 and Hco-L-AChR2 were established using cholinergic agonists (ACh, nicotine and DMPP) and anthelminthic agents (levamisole and pyrantel). Both receptors have strikingly different pharmacological profiles. Although Hco-L-AChR1 strongly responds to levamisole (148.8 ± 7.6% of ACh response), Hco-L-AChR2 response was much weaker (32.7 ± 4.2% of ACh response) (Figure 3). In contrast, pyrantel responses were larger in Hco-L-AChR2 (152.1 ± 13.3%) than Hco-L-AChR1 (8.1 ± 1.4%). DMPP activated both receptors (Hco-L-AChR1: 48.5 ± 1.7%; Hco-L-AChR2: 134.6 ± 13.4%). But similarly to pyrantel, response to DMPP differed qualitatively in Hco-L-AChR2. While continuous application of pyrantel and DMPP on Hco-L-AChR1 yielded a rapidly activating response that quickly reached a plateau value (Figure 3A), continuous application of pyrantel and DMPP on Hco-L-AChR2 resulted in a rapidly decaying response (Figure 3B). In addition, a post-wash rebound could be seen in Hco-L-AChR2, but not in Hco-L-AChR1. These differences could be due to reversible channel block by pyrantel and DMPP. Alternatively, pyrantel and DMPP could promote the desensitized state of Hco-L-AChR2. Finally, although Hco-L-AChR1 failed to respond significantly to nicotine (2.0 ± 0.3%), Hco-L-AChR2 responded robustly to nicotine application (62.2 ± 2.5%), suggesting that at least one nicotine-binding site is created when only the three receptor subunits *Hco-unc-29.1, Hco-unc-38* and *Hco-unc-63a* are co-expressed.

Next, we compared the relative sensitivities of Hco-L-AChR1 and Hco-L-AChR2 with ACh and levamisole by establishing full dose–response curves (Figure 3C and D). We find
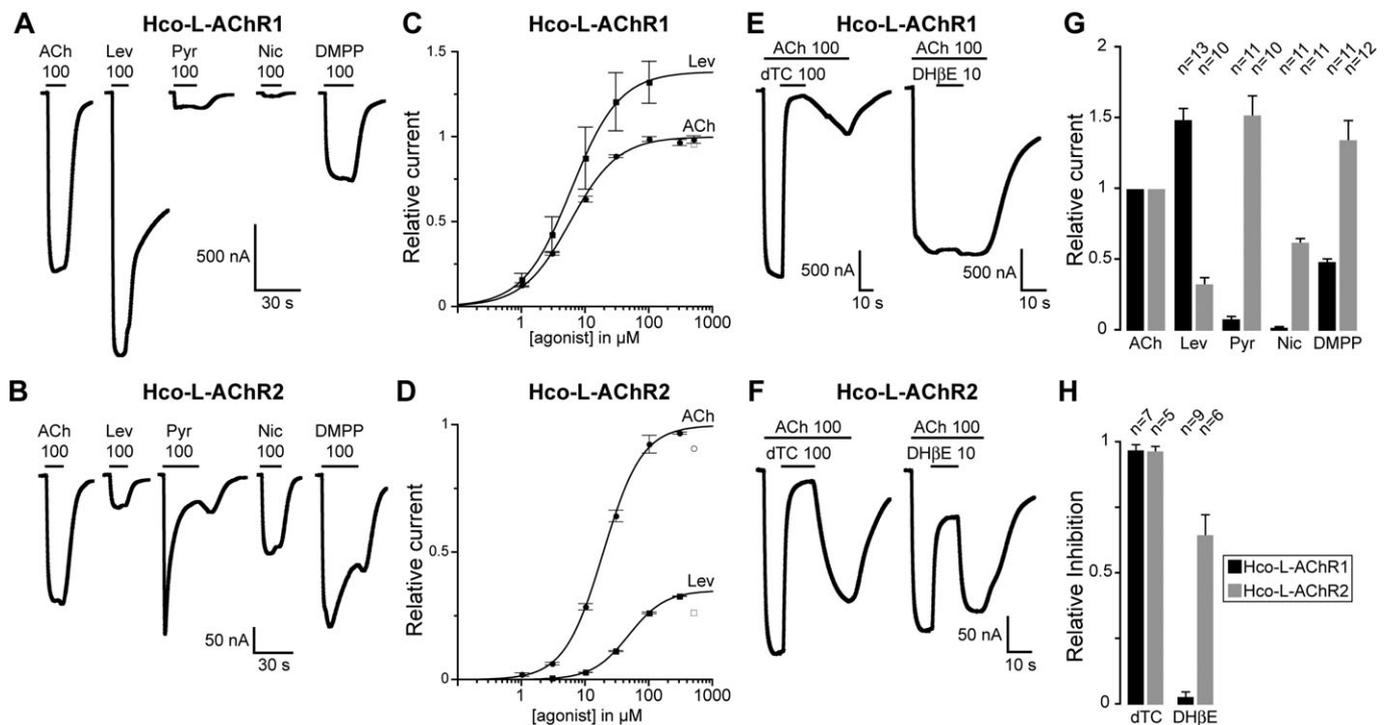
Figure 3

Pharmacology of *H. contortus* levamisole-sensitive AChR. (A) Hco-L-AChR1 and (B) Hco-L-AChR2 were challenged with a series of cholinergic agonists (ACh, DMPP, Nic: nicotine) and anthelmintic agents (Lev: levamisole, Pyr: pyrantel). (C) and (D) ACh and levamisole dose–response curves for Hco-L-AChR1 and Hco-L-AChR2 respectively. The values indicated by open square and open circles were excluded from the fit. (E) and (F) Differential response of Hco-L-AChR to cholinergic antagonists. Currents elicited by 100 μM ACh can be efficiently blocked by 100 μM dTC in Hco-L-AChR1 and Hco-L-AChR2. In contrast, the competitive antagonist DHβE only blocks ACh-induced currents in Hco-L-AChR2 but not in Hco-L-AChR1 consistently with the specific sensitivity of Hco-L-AChR2 to nicotine. (G) Relative efficacy of cholinergic agonists on Hco-L-AChR1 or Hco-L-AChR2. All values are normalized to the current elicited by application of 100 μM ACh. Numbers above bars indicate the number of oocytes recorded for each condition. (H) Relative efficacies of cholinergic antagonists on Hco-L-AChR1 or on Hco-L-AChR2. In (A) (B) (E) and (F) black horizontal bars indicate when agonists and antagonists are applied. All concentrations are indicated in μM. All oocytes were treated with 100 μM BAPTA-AM for 4 h prior to recording. All recordings were made with 1 mM external $CaCl_2$.

that Hco-L-AChR1 has a significantly higher affinity for acetylcholine and levamisole than Hco-L-AChR2. EC_{50} for acetylcholine was 5.8 ± 0.5 μM ($n = 5$) for Hco-L-AChR1 compared with 19.2 ± 0.7 μM ($n = 7$) for Hco-L-AChR2. EC_{50} for levamisole was 6.08 ± 0.3 μM ($n = 5$) for Hco-L-AChR1 compared with 48.4 ± 0.9 μM ($n = 4$) for Hco-L-AChR2. In contrast, cooperativity was more pronounced in the case of Hco-L-AChR2 as measured by the increase in Hill coefficient values (Hco-L-AChR1: ACh nH = 1.16 ± 0.08 and Lev nH = 1.15 ± 0.04; Hco-L-AChR2: ACh nH = 1.42 ± 0.06 and Lev nH: 1.52 ± 0.03).

These results demonstrate that two *H. contortus* L-AChRs with very different pharmacology can be assembled in *Xenopus* oocytes depending on the set of receptor subunits that are provided. Given that no *in vivo* data is available in *H. contortus* regarding the spatial and temporal expression patterns of these receptor subunits, it is not possible at this stage to state whether both of these receptors are expressed *in vivo* and whether they could correspond to the multiple types of L-AChRs that have been described in the muscles of other parasitic nematodes (Robertson *et al.*, 1999).

The competitive antagonist DHβE blocks nicotine-sensitive Hco-L-AChR2 but not nicotine-insensitive Hco-L-AChR1

In the presence of 100 μM ACh, both Hco-L-AChR1 and Hco-L-AChR2 are efficiently blocked by the application of 100 μM of the canonical cholinergic antagonist d-tubocurarine (dTC) ($97.0 \pm 1.9\%$ and $96.6 \pm 1.7\%$, respectively) (Figure 3E, F and H). However, the two receptors respond very differently to the competitive nicotinic antagonist dihydro-β-erythroidine (DHβE). Consistent with their differential response to nicotine, DHβE blocked ACh-activated currents in the nicotine-sensitive Hco-L-AChR2 very efficiently, but did not antagonize ACh-evoked currents in the nicotine-insensitive Hco-L-AChR1 ($64.7 \pm 7.6\%$ and $2.9 \pm 1.7\%$, respectively). This further suggests that a typical nicotine binding site must be formed in *H. contortus* L-AChR when *Hco-acr-8* is absent. This pharmacology is also comparable to that observed in *C. elegans* where L-AChRs are not activated by nicotine and are insensitive to DHβE, while N-AChRs are activated by nicotine and blocked by DHβE, both *in vivo* (Richmond and Jorgensen, 1999) and *in vitro* (Boulin *et al.*, 2008).
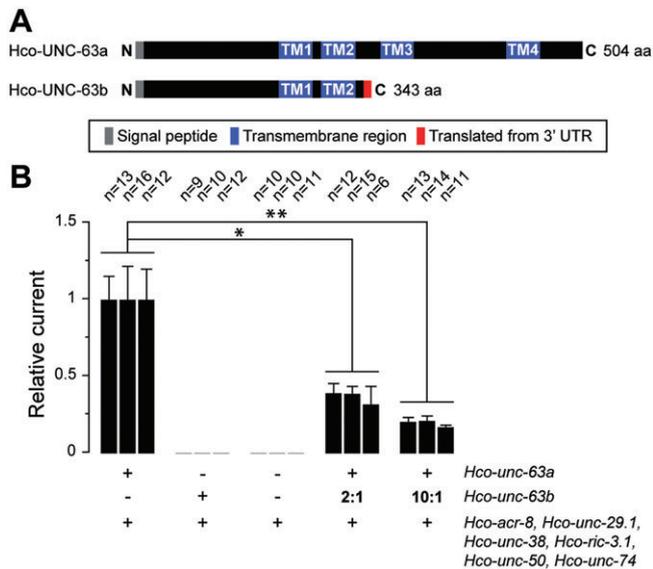
Figure 4

Dominant-negative effect of a truncated form of Hco-UNC-63 on Hco-L-AChR1 currents. (A) Schematic representation of Hco-UNC-63a and Hco-UNC-63b. The Hco-UNC-63b truncated protein retains a signal peptide, the entire N-terminal extracellular domain, two transmembrane domains (TM1 and TM2) and has an additional 26 C-terminal residues resulting from the translation of 3′ UTR sequences. (B) Expression of Hco-UNC-63b in the context of Hco-L-AChR1 decreases receptor expression by $36.5 \pm 4.1\%$ (2:1 ratio) and $19.5 \pm 2.2\%$ (10:1 ratio). Hco-UNC-63a is an essential component of Hco-L-AChR1 and Hco-UNC-63b cannot substitute for wild-type Hco-UNC-63a to form functional receptors. '+' and '−' indicate when a component has been added or not. In each case Hco-acr-8, Hco-unc-29.1, Hco-unc-38, Hco-ric-3.1, Hco-unc-50 and Hco-unc-74 were co-injected with either one or both Hco-unc-63 cRNA variants. '2:1' and '10:1' indicate the relative ratio of truncated to wild-type Hco-unc-63 used. Each wild-type cRNA was injected at 25 ng·μL⁻¹ (vs. 50 ng·μL⁻¹ in all other experiments). Average currents for Hco-L-AChR1 were 161 ± 144 nA. Currents were recorded two days after injection.

A truncated form of Hco-UNC-63 antagonizes *H. contortus* L-AChR expression in *Xenopus* oocytes

The molecular mechanisms and the genes involved in levamisole resistance are still largely unknown in parasitic nematodes. The *unc-63* locus has emerged as a possible candidate as transcripts corresponding to truncated forms of *unc-63* mRNAs (*unc-63b*) were specifically identified in levamisole-resistant isolates of three trichostrongylid species (*H. contortus*, *T. circumcincta* and *T. colubriformis*) (Neveu *et al.*, 2010). Intriguingly, these isolates also expressed full-length *unc-63* transcripts (*unc-63a*) in addition to the truncated mRNAs. The truncated *Hco-unc-63b* transcript identified in the *H. contortus* RHS6 isolate encodes a predicted protein of 343 amino acids including a signal peptide, the entire N-terminal extracellular domain, two transmembrane domains (TM1 and TM2) and 26 residues resulting from translation of the 3′ UTR (Neveu *et al.*, 2010) (Figure 4A). Because co-expression of mammalian AChR subunits trun-

cated after the M2 domain with wild-type subunits resulted in a decrease of surface receptors and associated receptor currents (Verrall and Hall, 1992; Sumikawa and Nishizaki, 1994), the truncated Hco-UNC-63b form might act as a dominant-negative on L-AChR expression.

To test this hypothesis, we injected increasing amounts of *Hco-unc-63b* cRNA with a fixed amount of wild-type L-AChR subunit cRNAs (Figure 4B). In agreement with the dominant-negative hypothesis, we observed a dose-dependent effect of Hco-UNC-63b on L-AChR currents. Injection of *Hco-unc-63b* RNA and Hco-L-AChR1 RNAs at a 2:1 ratio led to a reduction in average current down to $36.5 \pm 4.1\%$ of control (average of three independent experiments). When *Hco-unc-63b* RNA was injected at a 10:1 ratio, average current further decreased to $19.5 \pm 2.2\%$ of control (Figure 4B). This significant reduction in expression was not likely to be due to the saturation of the expression machinery as the total amount of injected RNA in the 10:1 ratio experiment was equivalent to the amount of cRNA injected to express Hco-L-AChR1 in other experiments. We also verified that Hco-UNC-63b does not allow the formation of a functional receptor when *Hco-unc-63a* is omitted.

Taken together, these results suggest that co-expression of the truncated *Hco-unc-63b* form with wild-type *Hco-unc-63* subunits could inhibit the expression levels of the *H. contortus* L-AChR, and therefore could induce a levamisole-resistance phenotype in parasites expressing this mutant form.

Discussion and conclusion

We demonstrate here that two distinct *H. contortus* L-AChRs can be functionally reconstituted in *Xenopus* oocytes by providing different sets of receptor subunits. In addition, robust expression requires the presence of three conserved ancillary factors, as reported in the non-parasitic nematode *C. elegans* (Boulin *et al.*, 2008). This experimental system provides a unique means to describe the pharmacological and biophysical properties of *H. contortus* L-AChRs and to analyse the functional impact of sequence polymorphisms detected in levamisole-resistant nematodes.

Functional conservation of L-AChRs between *C. elegans* and *H. contortus*

One of the striking features of *C. elegans* is the number of AChR subunits encoded in the genome, at least 29 (Jones *et al.*, 2007), as compared with 17 in vertebrates. Despite its small number of neurons, *C. elegans* is the species potentially expressing the most diverse repertoire of AChRs. Genomic studies suggest that many of these subunits are absent in parasitic nematodes including *B. malayi* and *A. suum* (Williamson *et al.*, 2007). Specifically, the *lev-1* and *lev-8* subunits, which are essential for L-AChR expression in *C. elegans*, were not found in these parasites, and L- and N-AChRs of these parasites can be reconstituted by expressing only different ratios of *unc-29* and *unc-38* without the need of accessory proteins (Williamson *et al.*, 2009). Such discrepancies might have reflected divergence caused by different life styles between parasitic and free-living nematodes. Our current results rather suggest that it reflects the phylogenetic distance

between *C. elegans* (clade V) and *B. malayi* and *A. suum* (clade III), as robust expression of L-AChRs from *H. contortus* (clade V) requires four different AChR subunits and three ancillary proteins, as we observed previously in *C. elegans*.

Interestingly, trichostrongylid orthologues of *lev-1* were readily identified but a sequence coding for a signal peptide could never be identified by inspection of genomic sequences or in cloned mRNAs. Consistently, injection of the *Hco-lev-1* cRNA did not change Hco-L-AChR1 expression and levamisole sensitivity, suggesting that it was indeed not incorporated into the receptor. The absence of deleterious variation in the coding sequence suggests, however, that *lev-1* remained under positive selection pressure. Whether complex genetic processing of the mRNA, such as *trans*-splicing between two mRNAs (Fischer *et al.*, 2008), occurs at specific developmental stages or in some specific cells to introduce a signal peptide into Hco-LEV-1 cannot be ruled out.

lev-8 could not be found in *H. contortus*, but functional L-AChR reconstitution indicates that ACR-8, the closest homologue of LEV-8 in *C. elegans*, is assembled in the receptor and plays a pivotal role in drug sensitivity. Two points might be meaningful. First, biochemical data in *C. elegans* suggest that ACR-8 might associate with other subunits found in muscle L-AChR (Gottschalk *et al.*, 2005). Second, the similarity between Hco- and Cel-ACR-8 is widely spread over the length of the primary amino-acid sequence, except for some motifs which are LEV-8 signatures in *C. elegans* (Figure 1B). Most notably, the principal ligand site defined by a YxxCC motif is identical for trichostrongylid ACR-8 and *C. elegans* LEV-8 (YPGCC vs. YAGCC for Cel-ACR-8). It is tempting to hypothesize that the conservation of these particular residues could be associated with their specific agonist-binding properties. It raises the possibility that *lev-8* and *acr-8* arose from a duplication that occurred after the divergence between strongyloidea and rhabditoidea.

Ancillary factors encoded by *ric-3*, *unc-50* and *unc-74* homologues are required for the expression of *H. contortus* L-AChRs in *Xenopus* oocytes, as previously reported for *C. elegans* L-AChRs (Boulin *et al.*, 2008). Although orthologues of these three genes are present in the *Xenopus* genome, they cannot functionally replace their *C. elegans* or *H. contortus* counterparts. They could be expressed at insufficient levels in unfertilized oocytes, or their sequence might have diverged enough that they can not functionally replace their nematode counterparts. Recent work suggests that it is not the case for all parasitic levamisole receptors. *A. suum* UNC-29 and UNC-38 can assemble into functional levamisole- and pyrantel-sensitive receptors in *Xenopus* oocytes in the absence of additional *A. suum* factors (Williamson *et al.*, 2009). However, this required the use of significantly greater amounts of receptor subunit RNA (25 ng of each subunit instead of 1.8 ng in this study) suggesting less efficient assembly or trafficking of the receptor, even though we cannot exclude the possibility that observed differences could reflect specific properties of L-AChRs. A bioinformatic analysis of available genome and EST data from *A. suum* clearly identified partial sequences homologous to the three ancillary factors (Supporting Figure S1). Whether these factors could be used to enhance the efficiency of *A. suum* L-AChR expression remains to be investigated.

Diversity of levamisole-sensitive receptors in nematodes

L-AChRs have been studied at the single channel level in *C. elegans*, *A. suum* and *O. dentatum*. These studies have highlighted some major differences between *C. elegans* and parasitic nematode L-AChR. For instance, three and four conductances ranging from 18 to 53 pS have been described for levamisole-activated channels in *A. suum* and *O. dentatum*, respectively (Robertson *et al.*, 1999; Qian *et al.*, 2006), while only one conductance level (around 30 pS) was found in the *C. elegans* muscle (Rayes *et al.*, 2007; Qian *et al.*, 2008). Consistently, genetic evidence suggests that only a single L-AChR is expressed in *C. elegans* muscle (Richmond and Jorgensen, 1999). In addition, *C. elegans* muscle cells express a second class of AChRs, the N-AChR, with strikingly different biophysical and pharmacological properties. N-AChRs desensitize extremely rapidly upon prolonged exposure to agonists. They are activated by nicotine, blocked by DHβE and insensitive to levamisole. These differences are explained by the molecular composition of N-AChRs, which are homomers of the ACR-16 subunit, a subunit closely related to the α7 subunit in mammals. By contrast, the origin of the diversity of parasitic AChRs is not understood at the genetic level.

Different levamisole-sensitive receptors could either contain different receptor subunits or the same subunits could be combined with different stoichiometries. *In vitro* experiments with *A. suum* levamisole receptors have suggested that different subunit stoichiometries could explain differences in pharmacology (Williamson *et al.*, 2009). We show here for *H. contortus* that two L-AChRs with very different pharmacological properties can be formed when the Hco-ACR-8 receptor subunit is present or absent. Strikingly, Hco-L-AChR1, which contain Hco-ACR-8, are very sensitive to levamisole, weakly responsive to pyrantel, and insensitive to nicotine and DHβE. Hco-L-AChR2, in which ACR-8 was removed, are less responsive to levamisole, more sensitive to pyrantel and respond to nicotine and DHβE. Injecting Hco-ACR-8 with only two other receptor subunits never yielded any currents. We could therefore conclude that Hco-ACR-8 does not entirely replace another receptor subunit but rather that it must be included in the receptor in exchange for one or two other subunits. Indeed, the Hco-L-AChR2 receptor is formed when only Hco-UNC-29.1, Hco-UNC-38 and Hco-UNC-63a are co-injected. This allows for only two stoichiometries since each subunit is essential: either 3:1:1 or 2:2:1. When Hco-ACR-8 is added to form Hco-L-AChR1, the new subunit composition must be either (Hco-ACR-8)$_1$: 2:1:1 or (Hco-ACR-8)$_2$: 1:1:1. Therefore, inclusion of Hco-ACR-8 will modify both the receptor's subunit composition and stoichiometry.

Our oocyte expression system allows for the first time to explore the possible subunit compositions of *H. contortus* AChRs. Ideally, single channel experiments would be required to determine which receptor subtypes correspond to conductances *in vivo*, potentially mirroring the three and four subtypes identified in *A. suum* and *O. dentatum* respectively. In addition, multiple paralogues exist for some of the subunits and it remains possible that specific combinations account for the diversity of conductances observed in parasitic nematode. However, definitive assignment of specific subunit combinations to an *in vivo* conductance would

require genetic experiments, which are not yet feasible in these parasitic nematodes.

Mechanisms of levamisole resistance in trichostrongylid nematodes

In addition to the pharmacological characterization of *H. contortus* L-AChRs, we propose here the first mechanistic model explaining levamisole-resistance in certain isolates of trichostrongylid parasites. We had recently reported that truncated forms of the L-AChR subunit UNC-63 are co-expressed with their full-length counterparts in levamisole-resistant isolates of three trichostrongylid nematodes (*H. contortus* RHS6, *T. circumcincta* TciNZ and *T. colubriformis* TcoGA) (Neveu *et al.*, 2010). We hypothesized that truncated receptor subunits could have a dominant-negative effect on L-AChR function by competing with wild-type subunits during receptor assembly. This hypothesis was consistent with previous data obtained in mammalian muscle AChR where co-expression of truncated and full-length receptor subunits resulted in the alteration of receptor function or expression (Verrall and Hall, 1992; Sumikawa and Nishizaki, 1994). We demonstrate that when co-expressed with Hco-L-AChR1 receptors in *Xenopus* oocytes, the truncated Hco-UNC-63b subunit has a strong dose-dependent dominant-negative effect on receptor expression.

Such a resistance mechanism, involving the down-regulation rather than complete loss of L-AChR expression might be more compatible with the parasitic lifestyle of *H. contortus*. Indeed, complete loss of L-AChRs in *C. elegans* null mutants of the *unc-63* receptor subunit is accompanied by a strong uncoordinated movement phenotype. Because parasites are required to move efficiently to remain infective, the complete loss of L-AChRs might represent a strong selective disadvantage. Accordingly, levamisole-binding experiments performed on wild-type and levamisole-resistant isolates identified changes in the binding characteristics or in the expression level of L-AChR rather than the absence of L-AChRs (Sangster *et al.*, 1988; 1998; Moreno-Guzman *et al.*, 1998). More generally, levamisole-resistant parasites may be less well coordinated than wild-type nematodes, and this loss of general fitness may make selection for levamisole resistance slower to develop and less stable than other types of anthelminthic resistance, in the absence of continuing anthelmintic selection pressure. In the future, our oocyte expression system will provide a means to test the functional relevance of polymorphisms linked to levamisole resistance and to characterize the sensitivity of wild-type and mutated receptors to known and forthcoming anthelminthic compounds.

Acknowledgements

Conflicts of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Conservation of RIC-3, UNC-50 and UNC-74 ancillary factors in *Ascaris suum, Brugia malayi, Caenorhabditis elegans* and *Haemonchus contortus*. Protein alignments were constructed using the MUSCLE algorithm and were further processed using GeneDoc. Consensus regions are shaded in blue. (A) RIC-3 homologues. The seven amino acids which differ between Hco-RIC-3.1 and Hco-RIC-3.2 are labeled in cyan. Domain annotations correspond to *C. elegans* RIC-3. The two transmembrane domains and the three coiled-coil domains are noted above the sequence. The *Brugia malayi* alignment is based on two partial sequences: kb29b08.y1 and XP_001898706.1. The names of the partial RIC-3 sequences from *A. suum* are indicated below the sequence. (B) UNC-50 homologues. Domain annotations correspond to *C. elegans* UNC-50. The five transmembrane domains are noted above the sequence. The *Brugia malayi* alignement is based on XP_1895364.1. The names of the partial sequences from *A. suum* used for the alignment are indicated below the sequence. (C) UNC-74 homologues. Domain annotations correspond to *C. elegans* UNC-74. The C-terminal transmembrane domain and the thioredoxin domain are noted above the sequence. The *Brugia malayi* alignement is based on XP_001901171.1. ED247067 was the only partial sequences from *A. suum* retrieved during our bioinformatic analysis.

Table S1 Primer sequences used for 3′ RACE and SL1-PCR experiments

Table S2 Primers used for cDNA amplification and cloning

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