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Isolation and characterization of a novel member of the ACC ligand-gated chloride channel family, Hco-LCG-46, from the parasitic nematode *Haemonchus contortus*

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Abstract

The ACC-1 family of cys-loop receptors are ligand-gated chloride channels sensitive to acetylcholine (ACh), and are only present in invertebrates. Studies of this family of inhibitory receptors has provided insight into how they bind and respond to ACh in a manner vastly different from nicotinic acetylcholine receptors and appear to be present in tissues that are relevant to anthelmintic action. Here, we have identified two members of the ACC-1 family from the parasitic nematode *Haemonchus contortus*, Hco-LGC-46 and Hco-ACC-4. Hco-LGC-46 is an ACC subunit that has never been previously expressed and pharmacologically characterized. We found that Hco-LGC-46 when expressed in *Xenopus laevis* oocytes forms a functional homomeric channel that is responsive to the cholinergic agonists ACh and methylcholine. *hco-lgc-46* expressed in a *C. elegans lgc-46* null strain (*ok2900*) suppressed hypersensitivity to aldicarb in a manner similar to *cel-lgc-46*. It was also found that Hco-LGC-46 assembles with Hco-ACC-1 and produces a receptor that is over 5-fold more sensitive to ACh and responds to the cholinergic agonists methycholine and carbachol. In contrast, the co-expression of Hco-LGC-46 with Hco-ACC-4 resulted in non-functional channels in oocytes. Hco-ACC-4 also appears to form heteromeric channels with a previously characterized subunit, Hco-ACC-2. Co-expression of Hco-ACC-4 with Hco-ACC-2 resulted in a functional heteromeric channel with an EC_{50} value similar to that of the Hco-ACC-2 homomeric channel. However, the maximum currents generated in the ACC-4/ACC-2 channel were significantly ($p < 0.005$) lower than those from the ACC-2 homomeric channel. Overall, this is the first report confirming that *lgc-46* encodes an acetylcholine-gated chloride channel which when co-expressed with *acc-4* results in reduced receptor function or trafficking in oocytes.

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CRediT authorship contribution statement

Sarah A. Habibi: Conceptualization, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Stephen M. Blazie:** Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Yishi Jin:** Funding acquisition. **Sean G. Forrester:** Conceptualization, Writing - original draft, Writing - review & editing, Funding acquisition.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molbiopara.2020.111276.

Keywords

Haemonchus contortus; Aldicarb sensitivity; Parasitic nematode; Cys-loop receptor; Ion channels

1. Introduction

Ion channels have been extensively studied, due to the role they play in fast synaptic neurotransmission in the nervous system of vertebrate and invertebrate organisms. The cys-loop (cysteine-loop) superfamily of ligand-gated ion channels are a major class of receptor-coupled ion channels that play a significant role in the nervous system of invertebrates, making them prime targets for nematocides [1]. These channels have been shown to be gated by neurotransmitters such as, gamma-aminobutyric acid (GABA) [2,3], glutamate [4], tyramine [5], serotonin [6], and acetylcholine [7].

Cholinergic neurotransmission is mediated by ACh receptors including nicotinic ACh receptors (nAChRs), which are cation-selective channels that result in neuro-excitation in the presence of agonists. However, early studies of the mollusk, *Aplysia*, revealed a population of receptors that resulted in neuro-inhibition in the presence of ACh [8]. Further investigation in the free-living nematode *Caenorhabditis elegans* led to the identification of the inhibitory acetylcholine-gated chloride channel (ACC-1) family of cys-loop receptors [7,9]. The entire family is encoded by eight receptor subunit genes, named *acc-1, −2, −3, and −4,* and *lgc-46, −47, −48,* and *−49* [10]. Of these eight genes, 7 have been identified in the sheep parasite *Haemonchus contortus*, with the absence of *lgc-48* [11]. The ACC-1 family is of interest since they are nematode specific receptors that do not contain homologs in mammalian species [7]. In addition, they are shown to be present in anthelmintic relevant tissues, such as pharyngeal neurons in *C. elegans* [9] and pharyngeal muscle in *H. contortus* [12], making them prime candidates for the development of novel pharmaceuticals.

Recent work by our group identified and pharmacologically characterized two members of the ACC-1 family, ACC-1 and ACC-2, from *H. contortus*. We found that Hco-ACC-2 forms a functional homomeric channel when expressed in *Xenopus laevis* oocytes [13]. Although Hco-ACC-1 does not form a function homomeric receptor on its own, it does associate with Hco-ACC-2 to form a heteromeric receptor which exhibits increased sensitivity to acetylcholine compared to the ACC-2 homomeric channel [12]. Immunolocalization of Hco-ACC-1 revealed specific expression in the anterior part of the pharynx of adult *H. contortus*, revealing a potential role in parasite feeding [12].

Aside from ACC-1 through −4, very little is known about the other members of the ACC-1 family. However, it has been shown that in *C. elegans* LGC-46 and ACC-4 are strongly expressed in cholinergic motor neurons [14,15]. Specifically they are localized to presynaptic terminals on these neurons and are involved in synaptic vesicle release [15]. This suggests the possibility that LGC-46, ACC-4, and other members of the ACC-1 family have the ability to form heteromeric channels. However, to date only ACC-1 through −4 have been expressed and functionally characterized in *Xenopus* oocytes [7,12,13] so it is unknown whether other members, such as LGC-46, can form ACh-gated chloride channels.

Here, we report the first pharmacological characterization of the LGC-46 receptor from the parasitic nematode *H. contortus*. We found that LGC-46 forms a functional homomeric receptor which is sensitive to acetylcholine and other cholinergic ligands. In addition, the co-expression of LGC-46 with ACC-1 results in an acetylcholine receptor that is 5x more sensitive to ACh compared to the LGC-46 homomeric channel. In contrast, the co-expression of ACC-4 with LGC-46 inhibited receptor function in *Xenopus* oocytes. Our data suggests that that LGC-46 interacts with both ACC-2 and ACC-4 in oocytes, but only the LGC-46/ACC-2 channel is functional.

2. Methods

2.1. Isolation of hco-lgc-46 and hco-acc-4

Whole adult *H. contortus* were received from Dr. Prichard (Institute of Parasitology, McGill University). Total RNA was extracted from adult male *H. contortus* worms using Trizol (Invitrogen, Carlsbad, USA). Complementary DNA (cDNA) was synthesized using the Quantitect Reverse Transcriptase kit from Qiagen (Dusseldorf, Germany), using a unique 3' oligo-dT anchor primer sequence (5'CCTCTGAAGGTTCACGGATCCACATCTAGATTTTTTTTTTTTTTTTTVN3'); [where V is either A, C, or G and N is either A, C, G, or T] [16]. Gene specific primers were generated based on a full sequence provided by Dr. Robin Beech (McGill University) as part of the genome sequencing project for *H. contortus* [11]. Amplification of the complete *hco-lgc-46* and *hco-acc-4* genes was carried out using primers specific to the 5' and 3' end of the gene with XBaI and XMaI restrictions sites. *hco-lgc-46* and *hco-acc-4* were subcloned into the *X. laevis* expression vector pGEMHE [17].

2.2. Expression in Xenopus laevis oocytes

All animal procedures followed the Ontario Tech University Animal Care Committee and the Canadian Council on Animal Care guidelines. Channels were expressed in *X. laevis* oocytes according to [18]. Female *X. laevis* frogs were supplied by Nasco (Fort Atkinson, WI, USA). Animals were fed and tanks were cleaned regularly. Frogs were housed in a climate-controlled room (18 °C) with continuous light cycling. Frogs were anesthetized with 0.15 % 3-aminobenzoic acid ethyl ester methanesulphonate salt (MS-222) buffered with $NaHCO_3$ to pH 7 (Sigma-Aldrich, Oakville, ON, CA). Surgical removal of a section of the ovary of the frog was performed, and the lobe was defolliculated with a calcium-free oocyte Ringer's solution (82 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES pH 7.5 (Sigma-Aldrich) (OR-2) containing 2 mg/mL collagenase-II (Sigma-Aldrich). The oocytes in the defolliculation solution were incubated at room temperature for 2 h. Collagenase was washed from the oocytes with ND96 solution (1.8 mM $CaCl_2$, 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES pH 7.5) and allowed one hour to recover at 18 °C in ND96 supplemented with 275 μg/mL pyruvic acid (Sigma-Aldrich) and 100 μg/mL of the antibiotic gentamycin (Sigma-Aldrich) (Supplemented ND96). Stage V and VI oocytes were selected for cytoplasmic injection of cRNA.

The pGEMHE vector containing the *hco-lgc-46, hco-acc-1, hco-acc-2*, and *hco-acc-4* coding sequences were linearized using Sph1 or NheI (New England Biolabs, USA), and used

as templates for an *in vitro* transcription reaction (T7 mMessage mMachine kit, Ambion, Austin, TX, USA) yielding copy RNA corresponding to each gene. *X. laevis* oocytes were injected with 50 nl of each subunit gene (0.5 ng/μL) alone or in combination (0.25 ng/μL each) using the Drummond (Broomall, PA, USA) Nanoject microinjector. Oocytes were also co-injected with the *H. contortus* genes *unc-50, unc-74*, and *ric-3.1*, which encode accessory proteins [19]. The injected oocytes were incubated at 18 °C in supplemented ND96 solution. Electrophysiological recordings of the oocytes were conducted between 48 and 72 h after cRNA injection.

2.3. Electrophysiological recordings

Two-electrode voltage clamp electrophysiology was conducted using the Axoclamp 900A voltage clamp (Molecular Devices, Sunnyvale, CA, USA). Glass electrodes were produced using a P-97 Micropipette Puller (Sutter Instrument Co., Novato, CA, USA). The electrodes were backfilled with 3 M KCl and contained Ag|AgCl wires. The following molecules were first dissolved in ND96; Acetylcholine (ACh), Carbamoylcholine Chloride (Carbachol), Acetyl-β-methylcholine Chloride (Methacholine), Levamisole Hydrochloride (Levamisole), and Pyrantel Citrate Salt (Pyrantel) [Santa Cruz Biotechnology]. These solutions were perfused over oocytes using the RC-1Z recording chamber (Warner Instruments Inc., Hamdan, CT, USA). Data was analyzed using Clampex Software v10.2 (Molecular Devices) and all graphs were generated using Graphpad Prism Software v5.0 (San Diego, CA, USA). EC_{50} values were determined by dose response curves that had been fitted to the following equation:

$$Imax = 1 / \left[1 + \left(\frac{EC50}{[D]}\right)^{h}\right]$$

Where I_{max} is the maximal response, EC_{50} is the concentration of compound required to elicit 50 % of the maximal response, [D] is compound concentration, and h is the Hill coefficient. Both EC_{50} and h are free parameters, and the curves were normalized to the estimated I_{max}. Graphpad prism used the equation to fit a sigmoidal curve of variable slopes to the data. Means were determined from at least 7 oocytes from at least three batches of frogs.

Current-voltage relationships were recorded by changing the holding potential from −60 mV to 40 mV in 20 mV increments. At each step the oocyte was exposed to either a 1 or 10 mM concentration of ACh. For reduced Cl^- trials, NaCl was partially replaced by Na-gluconate (Sigma) in the ND96 buffer solution, for a final Cl − concentration of 62.5 mM. Current-voltage graphs were generated using Graphpad Prism Software v5.0 (San Diego, CA, USA).

2.4. In silico modelling

The protein coding sequences of Hco-LGC-46 and Hco-ACC-1 were aligned with the *Danio rerio* alpha-1 glycine receptor (3JAD). MODELLER v9.21 software [20] was used for the generation of the Hco-LGC-46 homodimer and Hco-ACC-1/Hco-LGC-46 heterodimer. Both homodimer and heterodimers were prepared for agonist docking using AutoDock Tools.

Ligands were obtained from PubChem in their energy-reduced form. AutoDock Vina was used to simulate docking of each ligand to the homo- or hetero-dimers. Pymol was used to visualize the protein models with their associated ligands, and Chimera v1.6.1 [21] was used for the generation of figures.

2.5. Expression of hco-lgc-46 in C. elegans

The *C. elegans* strain (*ok2900*) is a knockout strain for *lgc-46* and the mutant worms are hypersensitive to aldicarb [15], an inhibitor of acetylcholine esterase, compared to WT. To generate P*unc-17β::hco-lgc-46*, we amplified *hco-lgc-46* cDNA from pGEM *hco-lgc-46* using the primers YJ12412 and YJ12414 (Supplementary data T1). We used Gibson Assembly (NEB, Ipswich, MA) to clone the resulting amplicon downstream of the *Punc-17β* promoter sequence within expression vector pCZGY1091 [15], which was amplified using primers YJ12416 and YJ12417. *Punc-17β* is the sequence 498 bp upstream of the annotated (WS220) *C. elegans unc-17* start codon and is active only in *C. elegans* cholinergic motor neurons [22]. Two independent transgenic lines expressing P*unc-17β–hco-lgc-46* from transmitting extrachromosomal arrays were generated by injecting P*unc-17β–hco-lgc-46* (15 ng/μl), P*myo-2::mCherry* (pCFJ90) co-injection marker (2.5 ng/μl), and 100bp DNA ladder (82.5 ng/μl) into the *lgc-46(ok2900)* background using standard *C. elegans* microinjection procedure [23]. The *lgc-46(ok2900)* + empty vector transgenic line included as a control in our aldicarb assay was generated by injecting the empty pCZGY1091 plasmid (15 ng/μl), which contains P*unc-17β* and not the *hco-lgc-46* cDNA, along with P*myo-2::mCherry* (pCFJ90, 2.5 ng/μl), and 100bp DNA ladder (82.5 ng/μl) into the *lgc-46(ok2900)* background. Construction of the extrachromosomal array line expressing P*unc-17β– cel-lgc-46* was reported previously [15]. In transgenic animals with *Pmyo-2* co-injection marker, no toxicity was observed. Aldicarb response was assessed using one-day old young adults on NGM plates seeded with OP50, containing 500 μM aldicarb. Paralysis was defined as absence of movement in response to touch three times with a platinum wire pick and was assessed over a 5 h period. Two independent trials involving n = 15 worms per line were performed on different days. For each trial, the proportion of non-paralyzed worms at each time point was averaged and these results are reported in Fig. 6.

3. Results

3.1. Isolation of hco-lgc-46 and hco-acc-4

The full-length cDNA of the *hco-lgc-46* gene consisted of 1569 nucleotides (GenBank Accession # MN402460). The sequence encodes a protein containing 522 amino acids. The full-length coding sequence of the *hco-acc-4* gene consisted of 1239 nucleotides (GenBank Accession #AHM25235.1) and encodes a protein containing 412 amino acids (KC918365.1). Both protein sequences contain all seven extracellular binding loops, four transmembrane domains, and the Cys-loop motif (Fig. 1). The Hco-LGC-46 protein sequence shares 74 % similarity to the Cel-LGC-46 protein (Fig. 1). The Hco-ACC-4 protein sequence shares 86 % similarity with Cel-ACC-4. The PAR motif present at the beginning of the transmembrane 2 domain is indicative of anion selectivity [24].

3.2. Expression of hco-lgc-46 and hco-acc-1 in Xenopus oocytes

Upon injection of *X. laevis* oocytes with cRNA encoding Hco-LGC-46, along with the accessory proteins Hco-UNC-74, Hco-UNC-50, and Hco-RIC-3.1, a homomeric LGC-46 channel is formed (Fig. 2A). The EC_{50} value for the Hco-LGC-46 homomeric channel in response to ACh was 893 ± 200 μM (Fig. 2C). The Hco-LGC-46 channel also responded to ACh derivative, methacholine, with an EC50 value of 7348 ± 900 μM (Fig. 2A and C). However, the Hco-LGC-46 channel did not respond to 20 μM carbachol. The Hco-ACC-1/Hco-LGC-46 channel was more sensitive to ACh compared to the Hco-LGC-46 homomeric channel (Fig. 2A and B). The EC_{50} value for the Hco-ACC-1/Hco-LGC-46 heteromeric channel in response to ACh was 166 ± 4 μM (Fig. 2C). The heteromeric channel also responded to derivatives, carbachol and methacholine, with EC_{50} values of 1680 ± 70 μM and 3990 ± 600 μM, respectively (Fig. 2C). Carbachol was a partial agonist of the heteromeric channel, whereas for both the Hco-LGC-46 and Hco-LGC-46/ACC-1 channel, methacholine was a weak partial agonist. Neither the Hco-LGC-46 homomeric channel nor the Hco-ACC-1/Hco-LGC-46 heteromeric channel respond to the cholinergic anthelmintics 20 μM levamisole or 20 μM pyrantel.

Current-voltage analysis of the Hco-LGC-46 channel was conducted to confirm anion selectivity (Fig. 3A). A full Cl^- concentration of ND96 (103.6 mM Cl^-) indicated a reverse potential of −17.44 ± 5 mV (n = 5). This is consistent with the calculated Nernst potential for Cl^- of −24.5 mV, when assuming an internal Cl^- concentration of 50 mM [25]. When NaCl was partially replaced with Na-gluconate (final Cl^- concentration of 62.5 mM) the reverse potential shifted to −3.4 ± 2 mV (n = 5), which is also consistent with the assumed Nernst potential of −5.7 mV (Fig. 3A). Current-voltage analysis of the Hco-ACC-1/LGC-46 channel indicated reverse potentials of −27.7 mV and −1.1 mV when recorded in full and partial chloride concentrations respectively (Fig. 3B).

3.3. Homology modelling

To aid in understanding how the agonists sit in the binding pocket, homology models were generated. The Hco-LGC-46 homodimer and a Hco-ACC-1/Hco-LGC-46 heterodimer were produced using the *D. rerio* glycine receptor 3JAD as template. This template was chosen as it contained the highest homology with the ACC-1 family of receptors. The model for the Hco-LGC-46 homodimer is outlined in Fig. 4A. The binding site appears to be composed of several aromatic residues. Docking of channel activators, ACh and methacholine, is outlined in Fig. 4B and C. The quaternary amine on ACh is located 5.6 Å from W259. The aspargine (N182) in loop E is located 3.3 Å away from the carbonyl oxygen in ACh, allowing for potential hydrogen bond interactions to occur. Both ACh and Meth dock in an extended orientation in the LGC-46 binding pocket. However, the quaternary amine of methacholine is shifted away from W259. The model for the Hco-ACC-1/Hco-LGC-46 heteromeric channel is outlined in Fig. 5A. Similar to the homomeric model, the binding site of the heteromeric channel is composed of many aromatic residues. Docking of agonists, ACh, Carbachol, and Methacholine, is shown in Fig. 5B-D. The quaternary amine on ACh and carbachol is located 5.2 Å from W225, whereas this amine in methacholine is located 5.6 Å away from W225. The Y178 reside in loop B is 2.7 Å from N179 allowing for an

increase in potential hydrogen bonding in the binding pocket. When looking at how each molecule docks in the binding pocket, we can see a similar extended orientation for each.

3.4. Expression of hco-lgc-46 in C. elegans

To assess whether *hco-lgc-46* can function *in vivo* we took advantage of a *lgc-46* null strain *ok2900* which is hypersensitive to aldicarb, an acetylcholine esterase inhibitor that causes paralysis over time [15]. Compared to WT, *lgc-46(ok2900)* animals were much more sensitive to 500 μM aldicarb assessed over 5 h (Fig. 6). Expressing *cel-lgc-46* or *hco-lgc-46* specifically in the cholinergic motor neurons (*Punc-17β*) was sufficient to partially suppress aldicarb hypersensitivity. While we did not observe complete suppression, the level is similar to that reported in Takayanagi-Kiya et al. [15] (Fig. 6).

3.5. Expression of Hco-ACC-4 in oocytes

Similar to the *C. elegans* version of *acc-4* [7], injection of *hco-acc-4* cRNA alone in oocytes did not result in a functional ACh-sensitive channel. In addition, the co-injection of cRNA encoding *hco-lgc-46* with *hco-acc-4* did not result in the expression of a sensitive heteromeric channel (Fig. 7). The co-injection of *hco-acc-4* with *hco-acc-2* resulted in a channel that was sensitive to ACh. The resulting ACC-4/ACC-2 receptor had an EC_{50} value of 25 ± 0.3 μM which is similar to the ACC-2 homomeric channel 21 ±0.7 μM [13]. However, the maximum current produced by the channel in the presence of ACC-4/ACC-2 channel was significantly lower ($p < 0.005$) when compared to ACC-2 alone (Fig. 7). This is consistent with results obtained for the *C. elegans* ACC-4/ACC-2 subunits expressed in oocytes [7].

4. Discussion

We have identified and cloned the *Hco-lgc-46* gene from the parasitic nematode *H. contortus*, which encodes a member of the ACC-1 family of cys-loop ligand-gated chloride channels in nematodes. This unique family of inhibitory receptors is only present in nematode species, and thus provides an opportunity for exploration as new potential anthelmintic targets. In addition, phylogenetic analysis has revealed members of the ACC-1 family are present in a vast number of parasitic nematode species [12]. Previously it has been shown that members of this family from *H. contortus* combine to form homomeric and heteromeric channels that are highly sensitive to acetylcholine [12,13]. Here, we report that Hco-LGC-46 expressed in *Xenopus* oocyte can form a homomeric channel and a heteromeric channel with Hco-ACC-1 [12], that responds to cholinergic agonists, albeit at much higher concentrations than previously reported for other members of this family. Hco-LGC-46 when expressed in *C. elegans* cholinergic motor neurons partially suppresses aldicarb hypersensitivity in a similar manner to Cel-LGC-46, confirming that Hco-LGC-46 can function *in vivo*. Whether Hco-LGC-46 functions in a similar manner in *H. contortus* is unknown at this time. We have found previously that ACC-1 is expressed in different tissues in *C. elegans vs H. contortus* [12], so it is possible that there are differences in LGC-46 function between nematodes.

The pharmacology of Hco-LGC-46 and Hco-LGC-46/Hco-ACC-1 receptors, provide insight into the nature of the binding site. The Hco-LGC-46 receptor is minimally sensitive to the partial agonist methacholine (Meth). When referring to the model, Meth can be seen docked in an extended orientation surrounded by aromatic residues in the binding pocket. In mammalian nAChRs a tryptophan residue in loop B has been shown to be a key player in ACh binding where it forms a cationic pi interaction with the quaternary amine of ACh [26]. In nematode ACCs however, this key tryptophan reside is found in loop C [13]. In the Hco-LGC-46 receptor we see Meth docking with its quaternary amine directed away from this tryptophan residue, W259, possibly explaining its lower sensitivity on the receptor. On the other hand, the Hco-LGC-46 receptors appears to be more sensitive to ACh. In the LGC-46 homodimer model ACh docks with its quaternary amine 5.6 Å from the tryptophan residue (W259) in loop C which would allow essential cation pi interactions to occur.

When Hco-LGC-46 was co-expressed with Hco-ACC-1 we generate a receptor that is over 5-fold more sensitive to ACh. Interestingly, when other members of the ACC-1 family are co-expressed with Hco-ACC-1, increased receptor sensitivity is also observed [12]. A pharmacological summary of the ACC-1 family of receptors in response to the cholinergic ligands and anthelmintics can be found in Table 1. Upon analysis of sequence alignments, it is noted that Hco-ACC-1 contains a tyrosine residue (Y178) in binding loop B, whereas the Hco-LGC-46 receptor contains a phenylalanine (F212) in the equivalent position. The presence of a tyrosine residue in loop B has been shown to contribute to hypersensitivity of these receptors when expressed in *Xenopus* oocytes [13]. The addition of the hydroxyl group in the ACC-1/LGC-46 channel places the tyrosine residue (Y178) within 2.7 Å of the amine group found on nearby asparagine (N179), allowing for hydrogen bond interactions to occur and provides a possible reason for increased channel activation. If Hco-ACC-1 is responsible for this increased receptor sensitivity it may indicate that it is playing the role of the principle subunit in pentamer formation (ie contributing this loop B tyrosine). Similarly, the Hco-ACC-1/Hco-LGC-46 receptor was 2-fold more sensitive to Meth, compared to Hco-LGC-46 homomer, and was responsive to ACh derivative carbachol. Both methacholine and carbachol were partial agonists of the ACC-1/LGC-46 receptor. Finally, neither the Hco-LGC-46 nor the Hco-ACC-1/Hco-LGC-46 receptors responded to nAChR anthelmintics pyrantel or levamisole, which have shown to have some activity at the Hco-ACC-2 channel. This could indicate differences in the agonist binding site even within members of the ACC-1 family of receptors.

When Hco-LGC-46 was co-expressed with Hco-ACC-4 we observed no receptor functionality. It is unknown at this time whether the presence of ACC-4 is preventing the expression of LGC-46 in oocytes, or if it is forming a unique heteromeric receptor which is no longer sensitive to cholinergic ligands. Interestingly, nematodes that possess LGC-46 also possess ACC-4, and those lacking one also lack the other [9]. When analyzing the sequence alignments of these two subunits we see that in loop C of the ACC-4 receptor, the key tryptophan residue that has been shown to form essential cation pi interactions in other nematode cys-loop receptors [27] is missing, and instead there is a phenylalanine. Prior research from our group has shown that the substitution of this tryptophan residue for a phenylalanine severely impacts the function of the resulting receptor that is over 200 times less sensitive to ACh compared to wild-type [13]. If LGC-46 and ACC-4 were forming a

heteromeric channel where the binding site is at the interface of the two subunits, this could possibly explain the reason for the inhibited function. In *C. elegans* ACC-4 and LGC-46 have been shown to function together in pre-synaptic cholinergic neurons by regulating synaptic vesicle release. Specifically, *lgc-46* gain-of function mutants showed suppressed activity in the *acc-4* knock-outs, indicating the role of ACC-4 in LGC-46 function [15]. However, while our results provide evidence for an interaction between ACC-4 and LGC-46 in oocytes the interaction appears to negatively affect channel function. Since there are no detectable currents in the ACC-4/LGC-46 receptor, we are unable to conclude whether the result is due to expression of receptor, or changes made to the binding site.

In *C. elegans*, ACC-4 has been shown to negatively impact the function of other ACC-1 family members such as ACC-2, where no currents were detected from the channel [7]. During our investigation we saw a similar observation with ACC-2 and ACC-4, where the presence of ACC-4 significantly ($p < 0.005$) reduced the current flowing through the channel in the presence of ACh with little change in the EC50. This suggests that ACC-4 is negatively impacting expression and not necessarily the agonist binding site in the ACC-4/ ACC-2 channel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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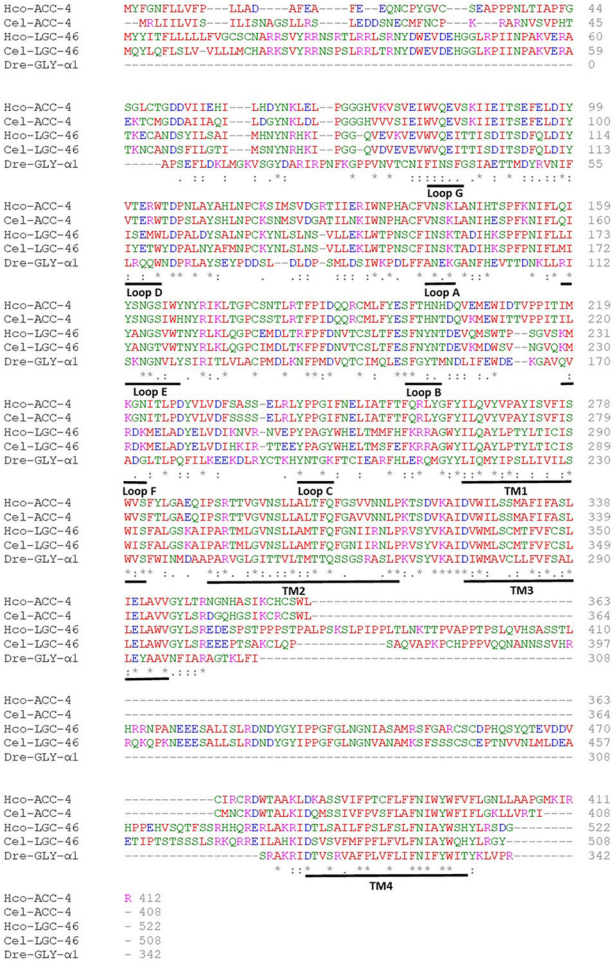

Fig. 1.

Protein sequence alignment of the *H. contortus* Hco-LGC-46 and Hco-ACC-4 receptors with *C. elegans* Cel-LGC-46 and Cel-ACC-4 and *D. rerio* Dre-GLY-α1 receptors. All five binding loops (Loop A-E), the characteristic cysteine residues that form the “cys-loop”, and four transmembrane domains (M1-M4) and highlighted with underlines. (*) indicates identity and (:) indicates similarity.

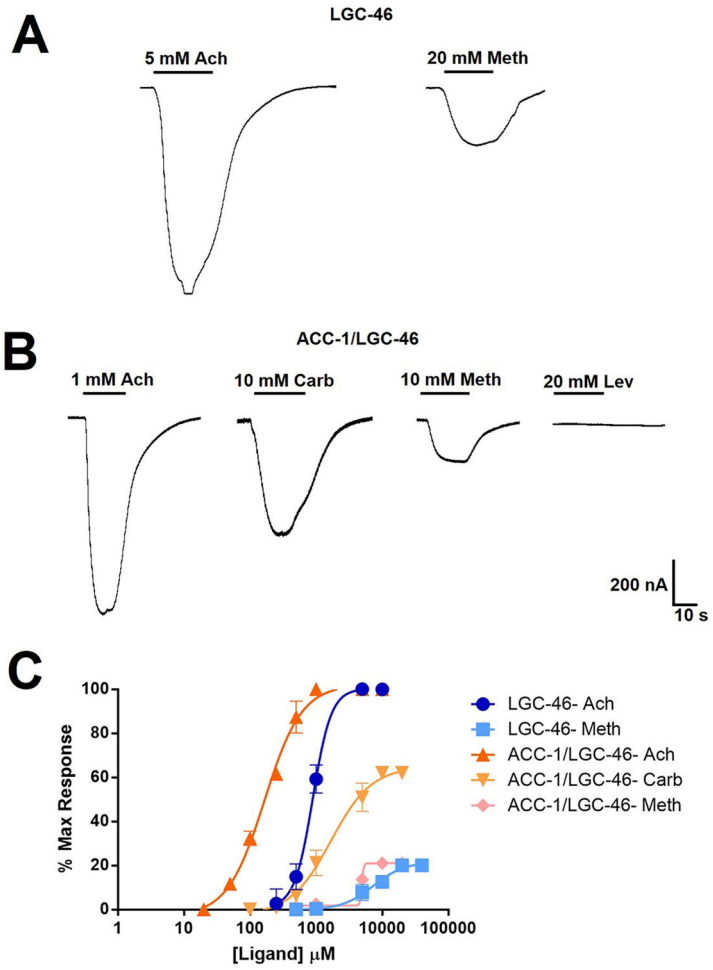

Fig. 2.

(A) Maximal electrophysiological response of the Hco-LGC-46 receptor in response to acetylcholine and methacholine. **(B)** Maximal electrophysiological response of the Hco-ACC-1/Hco-LGC-46 heteromeric receptor in the presence of acetylcholine, carbachol, methacholine, and levamisole. **(C)** Dose-response curves of the Hco-LGC-46 and Hco-ACC-1/Hco-LGC-46 receptors in the presence of ligands shown. Standard errors are shown. $n \geq 7$ oocytes. Each curve is represented as a percent of the maximum acetylcholine response. Partial agonists are those that have a maximum response lower than 100 %.

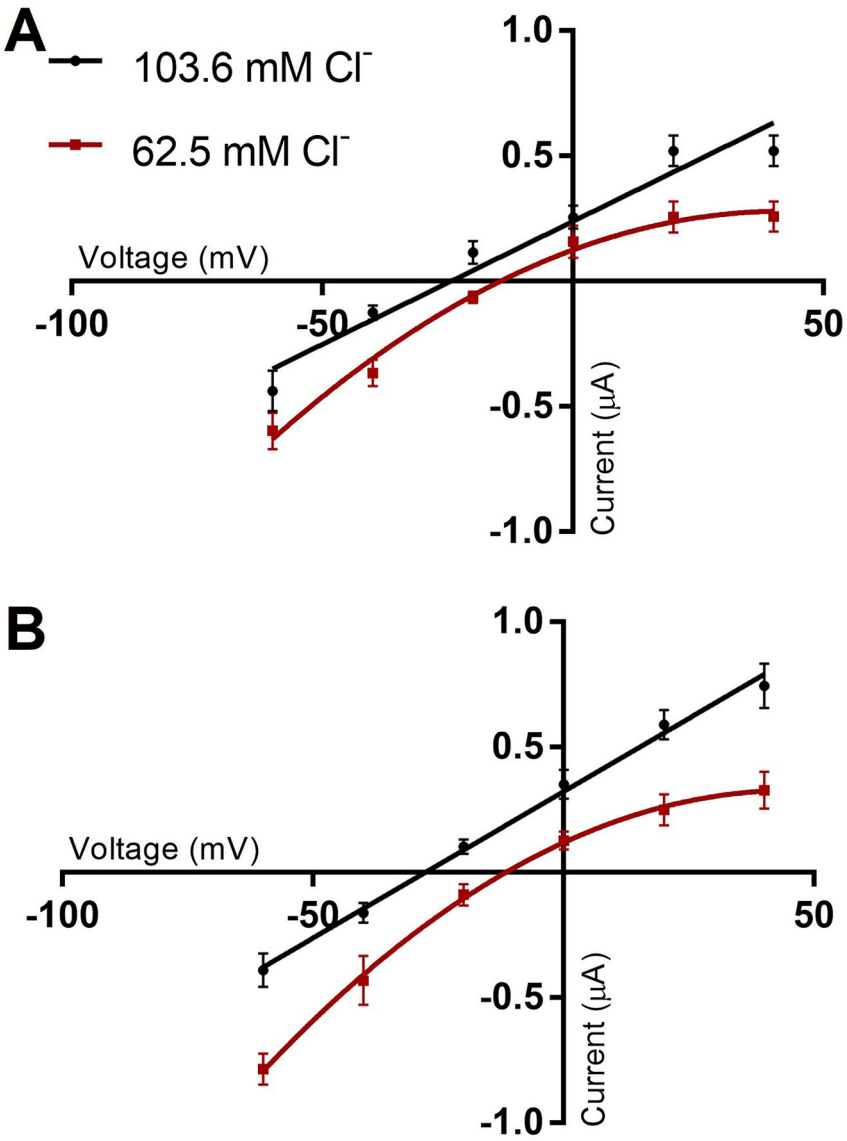

Fig. 3.

(A) Current-voltage analysis of the Hco-LGC-46 channel using 103.6 mM Cl^- and 62.5 mM Cl^- buffer solutions. Acetylcholine response was generated using a maximum concentration. The indicated reverse potentials were −17.44 mV and −3.4 mV when recorded in full and partial chloride concentrations respectively. Standard errors are shown. **(B)** Current-voltage analysis of the Hco-LGC-46/ACC-1 channel using 103.6 mM Cl^- and 62.5 mM Cl^- buffer solutions. Acetylcholine response was generated using a maximum concentration. The indicated reverse potentials were −27.7 mV and −1.1 mV when recorded in full and partial chloride concentrations respectively.

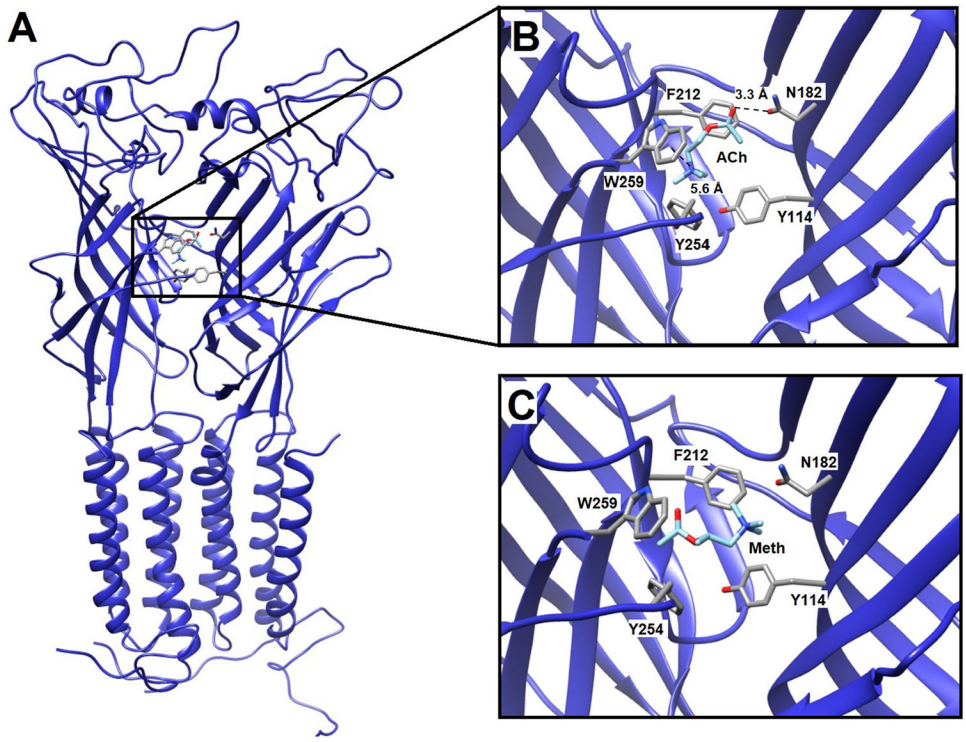

Fig. 4.

(A) Homology model of Hco-LGC-46 homodimer. The principal and complementary subunits are represented by the colour blue. **(B)** View of the Hco-LGC-46 binding pocket with acetylcholine docked. Key aromatic residues in binding pocket are highlighted and distances to tryptophan (W259) and asparagine (N182) are shown. **(C)** View of the Hco-LGC-46 binding pocket with methacholine docked. *A portion of Loop C is removed from the images for clarity.

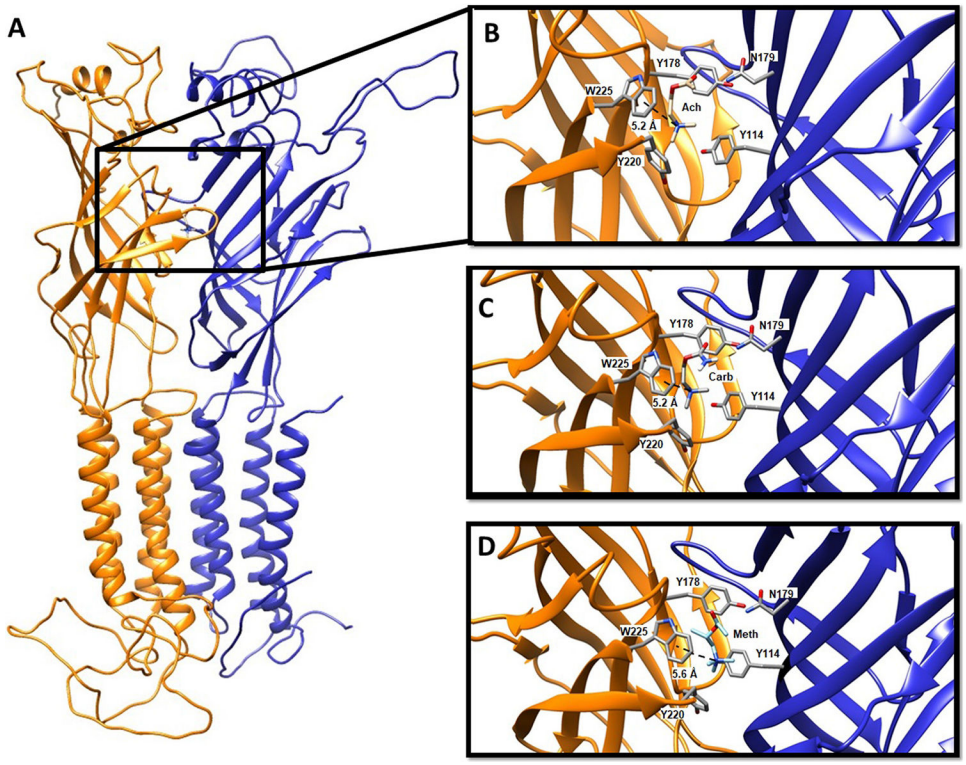

Fig. 5.

(A) Homology model of Hco-ACC-1/ Hco-LGC-46 heterodimer. Hco-ACC-1 and LGC-46 are the principal and complimentary subunits, and are represented by the colours orange and blue, respectively. **(B)** View of the Hco-ACC-1/Hco-LGC-46 binding pocket with acetylcholine docked. **(C)** View of the Hco-ACC-1/Hco-LGC-46 binding pocket with carbachol docked. **(D)** Methacholine docked in binding pocket. Key aromatic residues in binding pocket are highlighted and distance to tryptophan (W225) is shown. *A portion of Loop C is removed from the images for clarity.

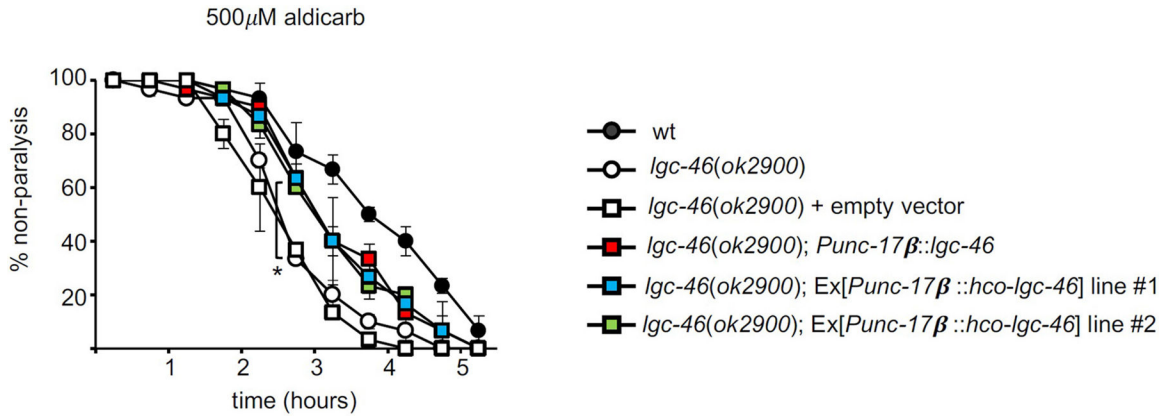

Fig. 6.

Expression of Hco-LGC-46 partially rescues aldicarb hypersensitivity of *C. elegans lgc-46(ok2900)*. Results show the comparison between one extrachromosomal array line expressing *C. elegans* LGC-46 and two independent lines expressing Hco-LGC-46 and under a cholinergic motor neuron specific promoter (P*unc-17β*). When restored to this subset of the nervous system, both *C. elegans* and Hco-LGC-46 partially rescue aldicarb hypersensitivity in the *lgc-46* genetic null (*ok2900*) background. The empty vector control line carries a plasmid containing *Punc-17β* without the *hco-lgc-46* cDNA insert in the *lgc-46(ok2900)* transgenic background. All transgenic lines express co-injection marker *Pmyo-2*::mCherry. Results are from two independent experiments of n = 15 worms per group, performed on different days. Each data point is the mean % non-paralysis ± SEM. *: $p < 0.05$ by two-way ANOVA and Bonferroni post-hoc test.

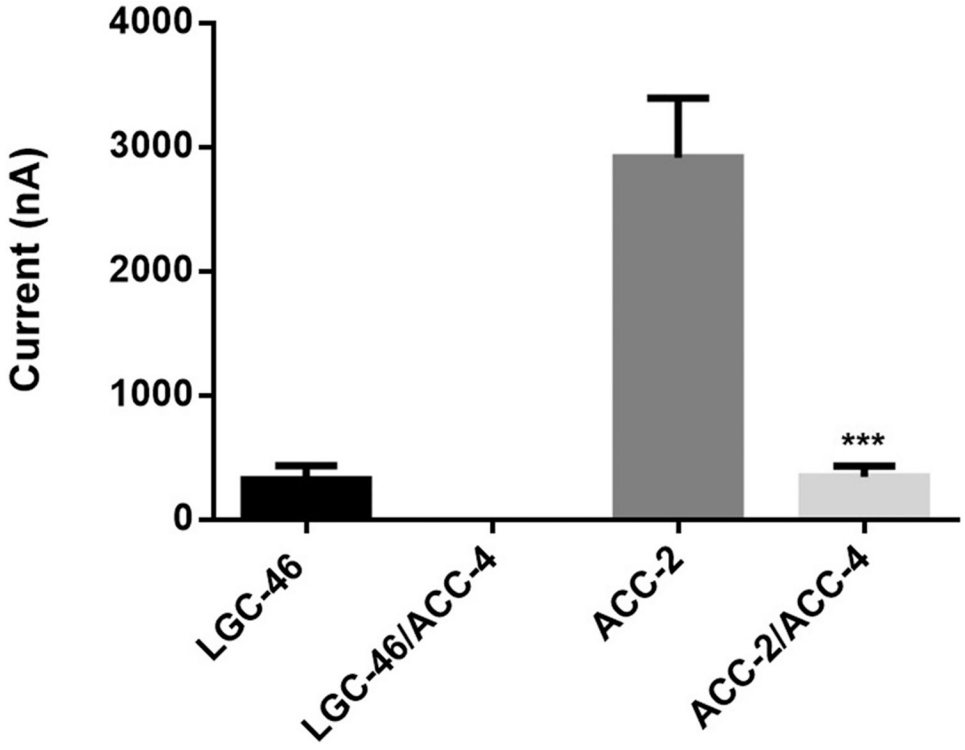

Fig. 7.

Average maximal currents shown for the Hco-LGC-46 and Hco-ACC-2 receptors in comparison to the heteromeric channels with Hco-ACC-4 present. Standard errors are shown, n = 5). *** indicates significant difference between ACC-2 and ACC-2/ACC-4 currents ($P < 0.005$).

Table 1

Pharmacological summary of ACC-1 family of receptors from *H. contortus* in response to various cholinergic ligands and nAChR anthelmintics. Pharmacological responses are represented by EC_{50} ± SE. N.R refers to no observed response of receptor to 20 mM concentration of ligand.

	ACC-2[a]	ACC-1/ACC-2[b]	LGC-46	ACC-1/LGC-46	LGC-46/ACC-4
Acetylcholine	20.86 ± 0.7 μM	5.9 ± 1 μM	893 ± 200 μM	166 ± 4 μM	N.R
Carbachol	43.0 ± 3.6 μM	32.5 ± 3 μM	N.R	1680 ± 70 μM	N.R
Methacholine	100.4 ± 2.1 μM	–	7348 ± 900 μM	3990 ± 600 μM	N.R
Levamisole	98.39 ± 4.0 μM	–	N.R	N.R	N.R
Pyrantel	71.7 ± 3.5 μM	–	N.R	N.R	N.R

[a]Data from Habibi et al. [13].

[b]Data from Callanan et al. [12]).