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Investigating the function and possible biological role of an acetylcholine-gated chloride channel subunit (ACC-1) from the parasitic nematode *Haemonchus contortus*

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ABSTRACT

The cys-loop superfamily of ligand-gated ion channels are well recognized as important drug targets for many invertebrate specific compounds. With the rise in resistance seen worldwide to existing anthelmintics, novel drug targets must be identified so new treatments can be developed. The acetylcholine-gated chloride channel (ACC) family is a unique family of cholinergic receptors that have been shown, using *Caenorhabditis elegans* as a model, to have potential as anti-parasitic drug targets. However, there is little known about the function of these receptors in parasitic nematodes. Here, we have identified an *acc* gene (*hco-acc-1)* from the sheep parasitic nematode *Haemonchus contortus.* While similar in sequence to the previously characterized *C. elegans* ACC-1 receptor, Hco-ACC-1 does not form a functional homomeric channel in *Xenopus* oocytes. Instead, co-expression of Hco-ACC-1 with a previously characterized subunit Hco-ACC-2 produced a functional heteromeric channel which was 3x more sensitive to acetylcholine compared to the Hco-ACC-2 homomeric channel. We have also found that Hco-ACC-1 can be functionally expressed in *C. elegans*. Overexpression of both *cel-acc-1* and *hco-acc-1* in both *C. elegans* N2 and *acc-1* null mutants decreased the time for worms to initiate reversal avoidance to octanol. Moreover, antibodies were generated against the Hco-ACC-1 protein for use in immunolocalization studies. Hco-ACC-1 consistently localized to the anterior half of the pharynx, specifically in pharyngeal muscle tissue in *H. contortus*. On the other hand, expression of Hco-ACC-1 in *C. elegans* was restricted to neuronal tissue. Overall, this research has provided new insight into the potential role of ACC receptors in parasitic nematodes.

1. Introduction

Haemonchus contortus is a pathogenic gastrointestinal parasitic nematode that causes severe livestock damage worldwide, particularly in the sheep industry. The disease, known as haemonchosis, leads to severe symptoms in host ruminants including anemia and death (Besier et al., 2016). Traditionally, *H. contortus* is controlled with broad spectrum anthelmintic chemotherapeutics that target different proteins within the parasite. There are multiple classes of these drugs that target cys-loop ligand-gated ion channels, including macrocyclic lactones which specifically target glutamate-gated chloride channels (GluCls) (Forrester et al., 2003; McCavera et al., 2009; Glendinning et al., 2011) and nicotinic acetylcholine receptor (nAChR) agonists such as pyrantel and levamisole (Boulin et al., 2011; Duguet et al., 2016; Blanchard et al., 2018). Macrocyclic lactones have also been shown to interact with nematode cys-loop GABA receptors (Accardi et al., 2012; Hernando and Bouzat, 2014). There is global concern about the increase in drug resistant populations of *H. contortus* in the field, including documented resistance to more recently developed drugs such as monepantel and derquantel (Raza et al., 2016). This information drives the need for the discovery of novel anthelmintic targets that could be used for the rational design or screening of new and effective anthelmintics.

The cys-loop ligand-gated chloride channel (LGCC) family of receptors is a very attractive group of proteins for drug-target discovery. Information from the *H. contortus* genome suggests that this family of receptors has approximately 35 genes that encode various subunits (Laing et al., 2013). However, approximately half of these potential

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channels have no confirmed ligand. In addition, many of these channels are either not present in mammals or are sufficiently divergent, suggesting the potential to develop highly specific drugs that will not target host receptors (Laing et al., 2013). However, of the 35 possible LGCC targets in the *H. contortus* genome it is likely that only a subset could be developed as targets for broad-spectrum anthelmintics. This is because the genomes of other parasitic nematodes, particularly human pathogens, appear to contain a significantly lower number of *lgcc* genes with some groups of channels being absent (Williamson et al., 2007; Beech et al., 2013). In addition, several LGCCs are likely to have functions that have no real consequence to the parasite if targeted. Therefore, the most attractive LGCCs from an anthelmintic discovery point of view are those present in a broad range of parasitic nematodes, have a function in the parasitic stage that if bound by an anthelmintic would lead to death or expulsion of the parasite and are not similar to host receptors (Wever et al., 2015). The latter point can be achieved by either targeting unique nematode-specific families of receptors or similar receptors that exhibit unique binding sites for potential drugs.

Previous research has suggested that the acetylcholine-gated chloride channels (ACCs) in *Caenorhabditis elegans* (Putrenko et al., 2005) exhibit the characteristics of promising drug targets. The genes that encode the various subunits of this family are present across the nematode phylum and appear to have fairly broad function in the nematode nervous system (Wever et al., 2015). One of the channels, Cel-ACC-1 exhibits a pharmacology distinct from mammalian cys-loop acetylcholine channels and appears in *C. elegans* to be localized to ventral cord and extrapharyngeal neurons (Wever et al., 2015). Due to their broad role in the *C. elegans* nervous system, the ACC family of receptors appear to be promising targets for effective anthelmintic action against parasitic nematodes (Wever et al., 2015). However, directly extrapolating results from *C. elegans* to parasitic nematodes should be done with caution since the expression patterns of individual LGCCs have been shown in some cases to be different (Portillo et al., 2003).

Here we have isolated a member of the ACC family (Hco-ACC-1) from the parasitic nematode *H. contortus*. Electrophysiological examination revealed that while Hco-ACC-1 does not form a functional homomeric channel in *Xenopus* oocytes, co-expression with a previously characterized subunit (Hco-ACC-2) can form a channel highly sensitive to acetylcholine and carbachol. The ACC-1/2 heteromeric channel was 3x more sensitive to acetylcholine compared to the ACC-2 homomeric channel. When expressed in *C. elegans, hco-acc-1,* localizes to pharyngeal neurons and enhances reversal avoidance to octanol demonstrating that *hco-acc-1* can function *in vivo*. In *H. contortus,* ACC-1 may to play an essential role in the pharynx as immunolocalization revealed expression in a specific region of the pharyngeal muscle. Overall, this research has provided some novel insight into the possible role of ACC receptors in parasitic nematodes.

2. Materials and methods

2.1. RNA/cDNA

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, USA) from adult *H. contortus* strain PF23. cDNA was synthesized using the Quantitect Reverse Transcription kit from Qiagen (Dusseldorf, Germany) using a unique 3′ anchor sequence primer (5′CCTCTGAAGGTTCACGGATCCACATCTAGATTTTTTTTTTTTTTTTVN3′); [where V is either A, C, or G and N is either A, C, G, or T] (Weston et al., 1999). The partial *H. contortus* sequence of *acc-1* was initially identified by the Sanger Institute (Cambridge, UK) and used for the creation of gene specific primers. These primers were used in the 5′ and 3′ rapid amplification of cDNA ends (RACE) protocol (Frohman et al., 1988).

2.2. Isolation of hco-acc-1 and sequence analysis

The 5 ‘end of the *hco-acc-1* gene was amplified using two internal *hco-acc-1* specific antisense primers [NESTED PRIMER 5’ GTTGTTCCA AACGCACCTGTGG 3’] and a primer specific for splice leader – 1 sequence (SL1-5′GGTTTAATTACCCAAGTTTGAG3′) (Van Doren and Hirsh, 1988) in a PCR using the PTC-100 Programmable Thermal Controller (MJ Research, Inc, Waltham, MA, USA). For the 3′ end, two *hco-acc-1* gene specific primers [NESTED PRIMER – 5′GTGACTTACTG GAGCTACTAC 3’] and two primers specific for the 3′ oligo-dT anchor sequence were used in a nested PCR reaction. Each amplicon of a predicted size was isolated via gel extraction and subcloned into the pGEMT easy™ vector and sequenced. Amplification of the complete *hco-acc-1* gene was conducted using primers designed targeting 5′ and 3′ untranslated region. This amplicon was subcloned and sequenced to achieve a consensus sequence. Sequence alignments were produced using ClustalW.

For phylogenetic analysis of the nematode ACC subunit family, the most highly conserved regions of the ligand-binding domain and membrane-spanning regions 1–3 (144 amino acids) were aligned using ClustalW and imputed into PhyML 3.0 to create a phylogenetic tree with 100 bootstrap repetitions. The final formatted tree was produced using FigTree v1.4.3.

2.3. Expression in Xenopus oocytes

All animal procedures followed the University of Ontario Institute of Technology Animal Care Committee and the Canadian Council on Animal Care guidelines and according to methods outlined in Abdelmassih et al., (2018). *Xenopus laevis* frogs (all female) were supplied by Nasco (Fort Atkinson, WI, USA). The frogs were housed in a room, which was climate controlled, light cycled, and stored in tanks which were regularly cleaned. 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 1 h to recover at 18 °C in ND96 supplemented with 275 μg mL^{-1} pyruvic acid (Sigma-Aldrich) and 100 μg mL^{-1} of the antibiotic gentamycin (Sigma-Aldrich). Stage V and VI oocytes were selected for cytoplasmic injection of cRNA.

The coding sequence of *hco-acc-1* and *hco-acc-2* (Habibi et al., 2018) was subcloned into the *X. laevis* expression vector pGEMHE (Zhang et al., 2008). The vector was linearized using the restriction enzyme *PstI* (New England Biolabs, USA), and used as template for an *in vitro* transcription reaction (T7 mMessage mMachine kit, Ambion, Austin, TX, USA) yielding *hco-acc-1* and *hco-acc-2* copy RNA. *X. laevis* oocytes were injected with 50 nl of *hco-acc-1* (0.5 ng/nL), *hco-acc-2* (0.5 ng/nL) or *hco-acc-1/2* (0.25 ng/nl each) using the Drummond (Broomall, PA, USA) Nanoject microinjector. Oocytes were also co-injected with the copy RNA encoding three accessory proteins, *hco-unc-50, hco-unc-74, and hco-ric-3.1* (Boulin et al., 2011) which were gifts from Dr. Cédric Neveu (INRA). The injected oocytes were incubated at 18 °C in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES pH 7.5) supplemented with 0.275 μg/mL pyruvate and 50 μg/mL gentamycin. Electrophysiological recordings of the oocytes were conducted between 48 and 72 h after cRNA injection.

2.4. Electrophysiological recordings

The Axoclamp 900A voltage clamp (Molecular Devices, Sunnyvale, CA, USA) was used to conduct two electrode voltage clamp electrophysiology. Glass electrodes were produced using a P-97 Micropipette Puller (Sutter Instrument Co.,Novato, CA, USA). The electrodes were

backfilled with 3M KCl and contained Ag|AgCl wires, and electrodes with resistances of 1–8 MΩ were selected for recordings. All oocytes were clamped at −60 mV for the entirety of the experiments. Acetylcholine and carbamylcholine (Sigma- Aldrich) were first dissolved in ND96. The resultant solutions were perfused over oocytes using the RC-1Z recording chamber (Warner Instruments Inc., Hamdan, CT, USA). Data was subsequently analyzed using Clampex Software v10.2 (Molecular Devices) and all graphs were generated using Graphpad Prism Software v5.0 (San Diego, CA, USA). Acetylcholine and carbamylcholine (carbachol) EC_{50} values were determined by dose response curves which had been fitted to the equation:

$$Imax = \frac{1}{1 + (\frac{EC50}{D})^h}$$

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. Dose-response curves comparing Hco-ACC-2 and Hco-ACC-1/2 were conducted in the same week and with the same batch of eggs. Means were determined from at least 4 oocytes from at least two batches of oocytes.

2.5. In silico modelling

The protein sequences of Hco-ACC-1 and 2 was aligned to the recently crystalized alpha-1 glycine receptor (3jad) from *Danio rerio* for use in MODELLER v9.15 (Sali and Blundell, 1993) for the generation of the hypothetical Hco-ACC-1/2 heteromeric dimer. The associated DOPE score determined the most energetically favorable model. Preparation of the heteromer for agonist docking was carried out using AutoDock Tools (Morris et al., 2009). ACh and carbachol were obtained from the Zinc database in their energy-reduced form (Irwin et al., 2012). AutoDock Vina (Trott and Olson, 2010) was used to simulate docking of each ligand to the Hco-ACC-1/2 heteromer. Pymol was used to visualize the protein heterodimer with its associated ligand docking, and Chimera v1.6.1 (Pettersen et al., 2004) was used to determine the distance between amino acid residues and ligands.

2.6. Immunolocalization in H. contortus

Two peptides specific for a portion of the N-terminal region of the Hco-ACC-1 protein (ACC-1.1-YNKHYIPSHPTQVRVDM and ACC-1.2-YQPVQRSRPERNLLSAIRKW) were synthesized commercially and used to immunize two rabbits (21st Century Biochemicals, Marlboro, MA, USA). Peptides were conjugated to the carrier protein ovalbumin and its sequence was initially BLASTed against the *H. contortus* genome database and the NCBI general database to ensure specificity. Whole sera was collected from the animals and subjected to affinity purification using separate columns for each peptide to isolate and purify each antibody separately. Whole antisera was tested for specificity and titer against the immunogenic peptide by ELISA. For immunolocalization two strains of *H. contortus* were used, PF23 and MOF23. Both strains were derived from the same parental strain. However, the PF23 strain was generated by passage through sheep over 23 generations without anthelmintic treatment whereas MOF23 was generated by passage through sheep over 23 generations with increasing dosage of moxidectin at each generation. Further details of the strains can be found in Urdaneta-Marquez et al., (2014). Adult female *H. contortus* worms (strains PF23 and MOF23) were fixed, permeabilized and subsequently digested as previously described (Rao et al., 2009). Worms were subsequently washed 3 times with PBS, and incubated at 4 °C for 72 h with a 1/150 dilution of primary antibody diluted in 0.1% w/v BSA, 0.5% Triton X-100, and 0.05% sodium azide (Sigma) under slight shaking. The removal of unbound antibodies was conducted by 4 washes of the worms with PBS and a final wash with PBS with 0.1% (v/v) Triton X-100 (PBST). Worms were then incubated at 4 °C with a 1/2000 dilution of Alexa Fluor 448® goat anti-rabbit IgG (H + L) secondary antibody for 24 h. Alexa Fluor 488® was used due its photostability and sensitivity to detect potentially low abundance proteins such as cys-loop LGCCs. Unbound secondary antibody was removed by 4 washes of the worms with PBS, and a final PBST wash. Worms were mounted on slides using Fluoromount™ Aqueous Mounting Medium (Sigma) and examined. Slides were examined using a Zeiss LSM710 confocal microscope (Carl Zeiss Inc., Canada) equipped with the Zeiss Zen 2010 software package. The lasers used for image acquisition were an Argon 488 nm, with the filter sets adjusted to minimize bleed-through due to spectral overlap.

Several controls were conducted including the omission of primary antibody, a pre-immune control (where 1/50 dilutions of pre-immune serum from experimental rabbits was applied instead of the purified primary antibody), and a peptide absorbed control, where an excess of peptide (50μg/ml) was added to the primary antibody dilution and incubated for 24 h at 4 °C before immunolocalization.

2.7. Expression of acc-1 in C. elegans

All transgenic constructs were made by overlap fusion PCR (Hobert, 2002). *H. contortus* and *C. elegans acc-1* cDNAs were fused to *C. elegans acc-1* (5.3 kb) promoter. All transgenes contain sequence encoding a GFP marker (with an *unc-54* 3′-UTR) at the 3′-end of receptor cDNA. PCR products from multiple reactions were pooled and co-injected with coelomocyte-RFP as a screening marker into the appropriate backgrounds (Mello and Fire, 1995). Multiple transgenic lines from each construct were examined. Localization of GFP expression was performed using confocal microscopy.

Well-fed hermaphrodite fourth-stage *C. elegans* larvae carrying a coelomocyte RFP screening marker were picked 24 h prior to assay and incubated overnight at 20 °C. Fresh nematode growth medium (NGM) plates were prepared on the day of assay. Aversive responses were examined off food as described in Chao et al. (2004) and are presented as the time taken to initiate backward locomotion after the presentation of 30% 1-octanol on a hair in front of a forward moving animal.

3. Results

3.1. Hco-ACC-1 isolation and polypeptide analysis

The final consensus nucleotide product obtained through the RACE procedure was sequenced and shown to consist of 1380 nucleotides and assigned the GenBank accession number AHM25233.1. When translated in the appropriate reading frame, the sequence encodes for a protein containing 459 amino acids with the hallmark Cys-loop motif (Fig. 1a). Additionally, 4 hydrophobic transmembrane domains were identified along with a signal peptide cleavage site (Signal P; http://www.cbs.dtu.dk/services/SignalP/). The PAR motif (residues 266–268) was noted in the M2 transmembrane portion of the peptide, indicative of chloride ion selectivity (Jensen et al., 2005). The Hco-ACC-1 peptide shares an 89% similarity with the *C. elegans* ACC-1 subunit. A few key amino acid differences were noted in the major binding loops. Firstly, in ligand binding loop B, a tyrosine (Y178) was identified in the Hco-ACC-1 protein where a phenylalanine (F179) is seen in the analogous position in Cel-ACC-1. Secondly, there are two differences seen in binding loop F. In Hco-ACC-1 two positions P199 and Q201 align to R200 and E201 in Cel-ACC-1 (Fig. 1a). The functional consequences of these differences are not known. In addition, the amino acid sequence of Hco-ACC-1 is identical (except for amino acid 448) to an unnamed protein (CDJ86191.1) deposited to GenBank as part of the *H. contortus* genome sequencing project (Laing et al., 2013).

Using the crystal structure of the *Danio rerio* glycine receptor (PDB 3JAD), a homology model of the ACC receptor (dimer) was generated.

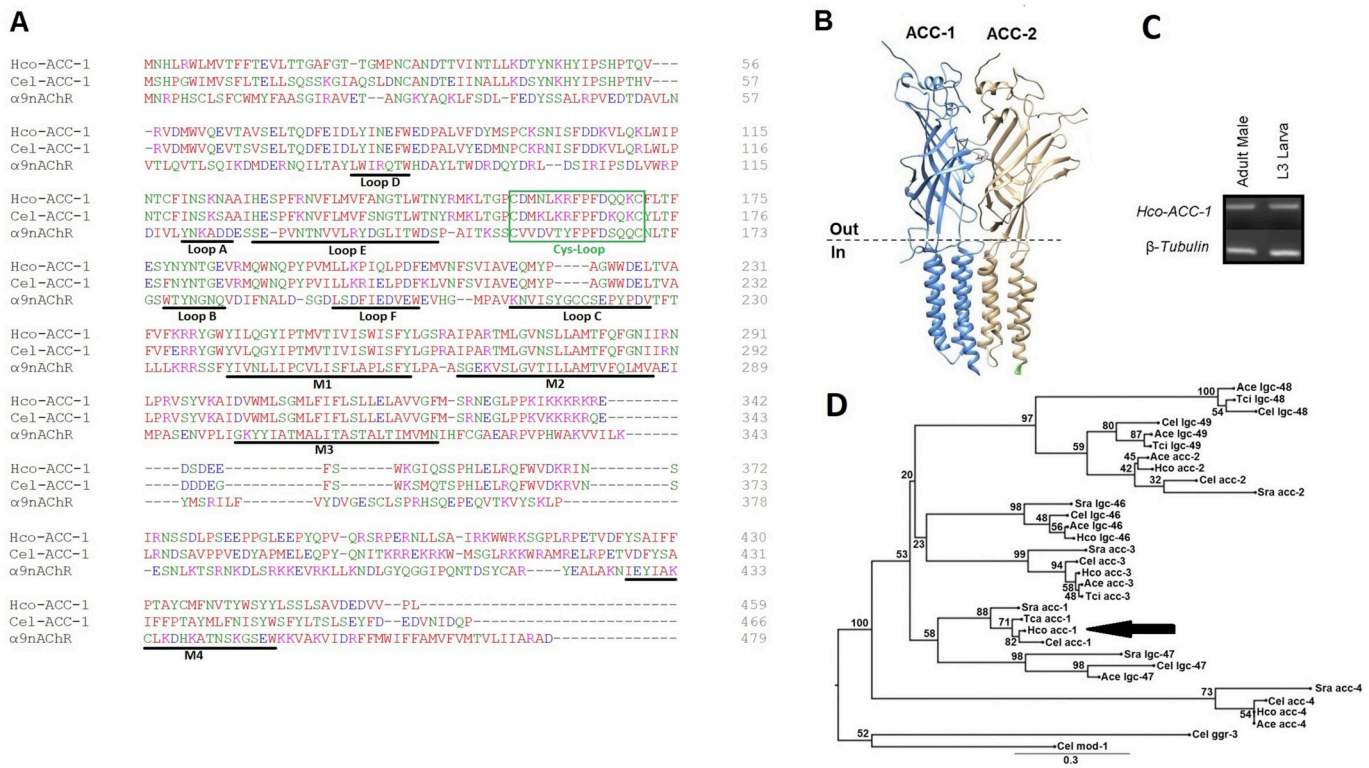

Fig. 1. Isolation of *hco-acc-1* and protein sequence analysis. A. Protein sequence alignment of *H. contortus* and *C. elegans* ACC-1 with the nAChR. Stars indicate regions of amino acid identity. Dashes represent no alignment between sequences while colons indicate similar amino acids. The 6 ligand binding loops (Loops A-F), 4 transmembrane regions (M1-M4) and the cys-loop are indicated. B. Homology model of a hypothetical receptor showing a dimer of Hco-ACC-1 and Hco-ACC-2 that was used for ligand-docking. C. End point PCR analysis of the *hco-acc-1* cDNA in the adult male and L3 larval stages of *H. contortus*. PCR reactions were simultaneously performed on the housekeeping gene β-tubulin. Replicate experiments showed a similar trend. No PCR products were detected in negative controls (including negative RT control). D. Phylogenetic analysis of the ACC family from various nematodes. Cel - *Caenorhabditis elegans* Hco – *Haemonchus contortus*; Tci - *Teladorsagia circumcincta*; Ace - *Ancylostoma ceylanicum;* Sra - *Strongyloides ratti*; Tca - *Toxocara canis.* Hco-ACC-1 is indicated by arrow.

This crystal structure was selected as template as it shares the highest homology with ACC-1 and 2 of the crystal structures currently in the protein database. A hypothetical heteromer was generated where Hco-ACC-1 was designated the primary subunit and Hco-ACC-2 the complementary subunit. Fig. 1B displays the best model (ie. with lowest DOPE and molpdf scores out of 50 possibilities). Acetylcholine was docked in a 30 × 30 × 30 Å box centered between the primary and complementary subunits (between loops A, B and C of the primary and adjacent to loops D, E and F of the complementary subunit).

End-point PCR indicates that *hco-acc-1* is expressed in both the adult male and L3 stage (Fig. 1C) and phylogenetic analysis indicated that Hco-ACC-1 groups with other nematode ACC-1 subunits (Fig. 1D).

3.2. Functional characterization of hco-ACC-1

Oocytes injected with *hco-acc*-1 cRNA alone did not respond to up to 1 mM ACh or carbachol. However, the same batches of oocytes injected with *hco-acc*-2 cRNA responded readily to both ACh and carbachol (Fig. 2a and b) with EC_{50} values similar to those reported in Habibi et al. (2018) of 19 ± 1 μM (n = 5) and 46 ± 2 μM (n = 5), respectively (Fig. 2b). However, co-expression of *hco-acc-1 and 2* produced a channel significantly more sensitive to both ACh and carbachol with EC_{50} values of 5.9 ± 1 μM ($p < 0.001$) (n = 6) and 32.5 ± 3 μM ($p = 0.04$) (n = 4), respectively. Both the ACC-2 and ACC1/2 channels produced currents that were in the microamp range and desensitized. These responses were observed repeatedly in oocytes from different frogs, but was not seen in eggs injected with water. Current-voltage analysis of the Hco-ACC-1/2 heteromeric channel using full Cl^- ND96 (final concentration 103.6 mM Cl^-) indicated a reversal potential of −17.7 ± 5 mV (n = 4) (Fig. 2c) consistent with the calculated Nernst potential for Cl^- of −18.5 mV, assuming 50 mM internal Cl (Kusano et al., 1982). When NaCl was partially replaced with Na-gluconate in the ND96, the reversal potential shifted to −2.1 ± 1 mV (n = 3), consistent with the predicted Nernst potential of −5.7 mV.

3.3. Ligand docking analysis

Docking results clustered all 10 acetylcholine binding poses to the predicted binding location. The dock with the highest affinity (−5.4 kcal) is shown in Fig. 2d. The acetylcholine quaternary amine group was located directly between W225 (loop C) and Y178 (loop B). This quaternary amine was 4.3 Å away from the center of the W225 aromatic ring suggesting the formation of single pi-cation bond with W225. Similarly, carbachol docked at a similar pose and its quaternary amine was 4.6 Å away from W225 (Fig. 2e).

3.4. Immunolocalization

The application of anti-Hco-ACC-1 antibodies was performed on adult female *H. contortus* worms to determine the tissue expression of the ACC-1 protein. Worms from both the PF23 and MOF23 strains were successfully stained, with repeated staining over multiple batches of collected worms. The signal was identified consistently in pharyngeal muscle cells, in the anterior half of the pharynx in both strains (Fig. 3A–D). There were no obvious differences in the signal between *H. contortus* strains. The observed signal was robust and reconstructed stacks of slicing through the Z axis shows the triradiate organization of nematode pharyngeal muscle (supplemental movies 1 and 2). Peptide absorbed controls, pre-immune serum controls and no primary antibody controls displayed no signal beyond background (Fig. 3F and G).

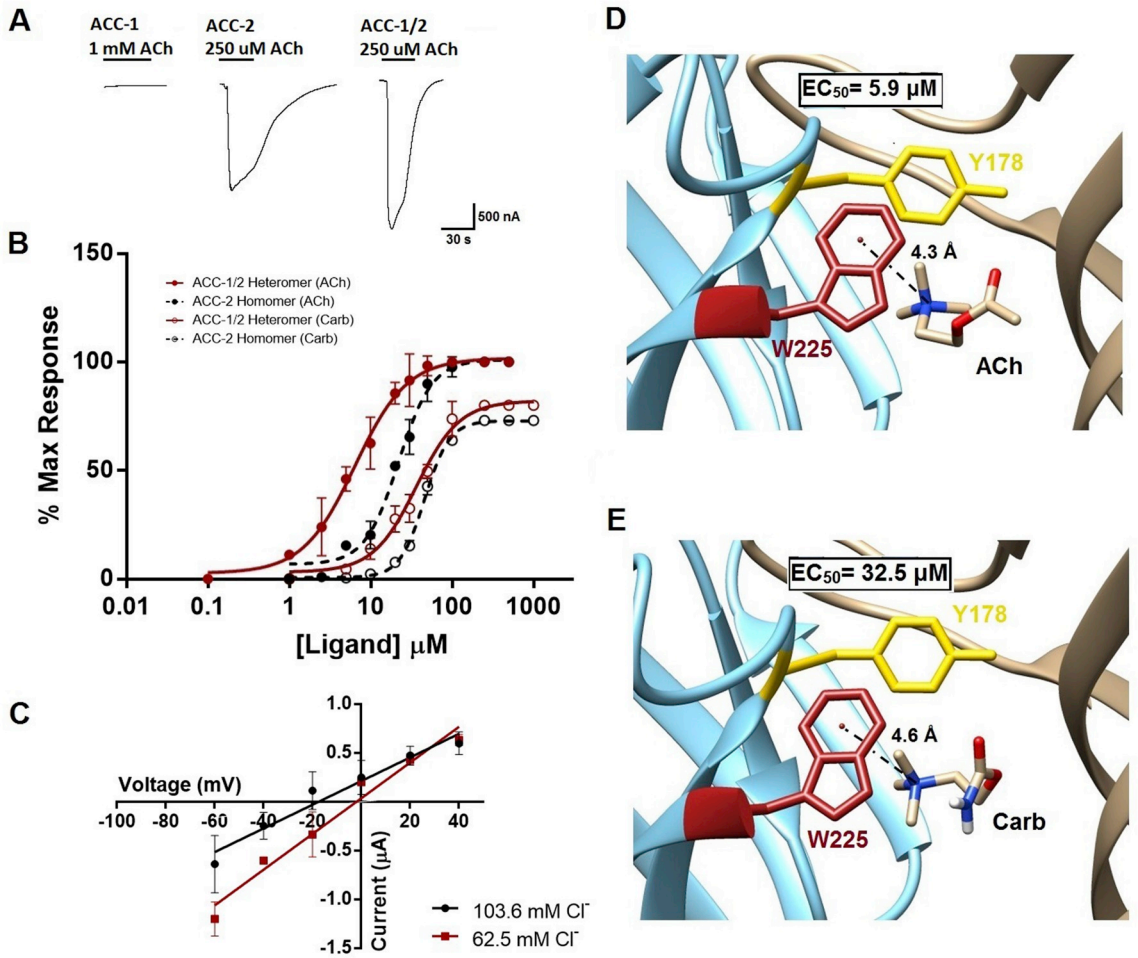

Fig. 2. Hco-ACC-1 and Hco-ACC-2 form a functional heteromeric receptor. A. Representative electrophysiological traces of the acetylcholine responses to oocytes expressing Hco-ACC-1 alone, Hco-ACC-2 alone and Hco-ACC-1 + 2. B. Does response curves comparing the sensitivities of the Hco-ACC-2 channel and the Hco-ACC-1/2 channel to acetylcholine and carbachol. Each data point is a mean ± SEM with $n \geq 4$. C. Current/Voltage relationship of the Hco-ACC-1/2 channel comparing full Cl^- ND96 (103.6 mM) partial Cl^- ND96 (62.5 mM). D. Ligand docking of acetylcholine to the Hco-ACC1/2 receptor shown the distance of the ligand to W225. E. Ligand docking of carbachol to the Hco-ACC-1/2 receptor shown the distance of the ligand to W225.

In addition, no autofluorescence was detected.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.ijpddr.2018.10.010.

3.5. Expression of hco-acc-1 in C. elegans

To determine whether *hco-acc-1* could function *in vivo* we expressed the gene *in C. elegans*. Aversive responses to 1-octanol have been studied extensively in *C. elegans* and are modulated by a complex locomotory circuit involving an array of sensory and interneurons (Komuniecki et al., 2012, 2014). Both *C. elegans* wild-type and *acc-1* null animals initiate aversive responses to 1-octanol in about 10 s, but the overexpression of either the *C. elegans* or *H. contortus acc-1* genes in wild type-animals, driven by 5.2 kb of the predicted *C. elegans acc-1* promoter, significantly decreased the time taken to initiate an aversive response (Fig. 4). Overexpression of the *H. contortus acc-1* in an *acc-1* null background yielded a similar result, confirming the putative orthology of the two proteins. Fluorescence from an *H. contortus acc-1*::*gfp* transgene, driven by the same *C. elegans acc-1* promoter was exclusively neuronal and limited to a small number of neurons in both the anterior (Fig. 5) and the posterior (supplemental Figure 1) ends of the worm, similar to published reports for the *C. elegans acc-1* (Pereira et al., 2015).

4. Discussion

Here we report the isolation and characterization of an acetylcholine-gated chloride channel from a parasitic nematode. Recently, Wever et al. (2015) provided evidence that the ACC family of receptors were potentially good anthelmintic targets in *C. elegans*. Here *avr-15* (encoding a GluCl subunit) under the control of *acc* promoters exhibited high level of sensitivity to ivermectin demonstrating that the ACC family of receptors function in what was referred to as “essential” tissues. In this case one of the essential tissues was extrapharyngeal neurons (Wever et al., 2015). Interestingly, while we did not observe the localization of Hco-ACC-1 in any neurons in *H. contortus* we consistently found this receptor in pharyngeal muscle tissue. It appears therefore that although ACC-1 localizes to different tissues in *H. contortus* compared to *C. elegans* they both appear to function in tissues that would be considered essential from the point of view of anthelmintic action.

The difference in the localization of ACC receptors between *C. elegans* and *H. contortus* resembles the situation observed with the ivermectin target, the GluCl. In *C. elegans,* GluCl subunits have been localized to pharyngeal muscle and various neurons (Dent et al., 1997, 2000; Laughton et al., 1997), but in *H. contortus,* the GluCl receptors localized thus far were found to be exclusively neuronal (Portillo et al., 2003). However, both nematodes are still very sensitive to ivermectin with respect to both locomotion and pharyngeal pumping (Dent et al., 1997; Wolstenholme, 2012). Here, we have found that Hco-ACC-1

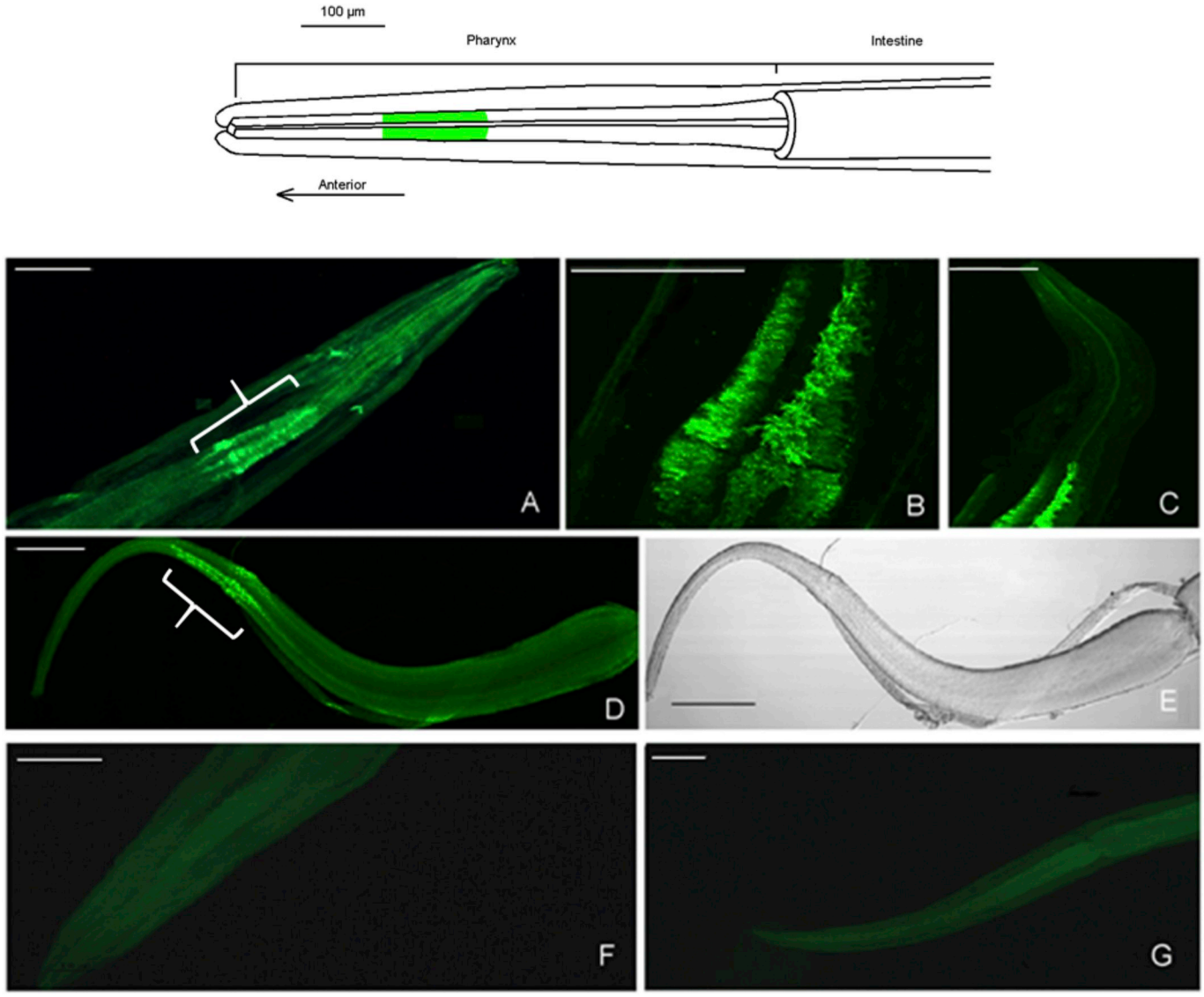

Fig. 3. Immunolocalization of Hco-ACC-1 in adult female *H. contortus* worms. A cartoon schematic of the anterior end of the female *H. contortus* adult nematode is shown at the top. Green denotes detected signal in the anterior half of the pharynx seen during immunlocalization experiments. A. 25x magnification of the anterior end of *H. contortus* PF23 strain. B. 40X magnification stack of 25 of 5 µm confocal slices of *H. contortus* PF23 focused on the anterior half of the pharyngeal muscle tissue. C. 25x magnification of a female adult *H. contortus* MOF23 parasite focusing on the anterior end of the worm. D. 25x magnification of isolated pharynx from a female *H. contortus* MOF23 adult worm. E. 25x magnification light micrograph of D. F. Pre-immune serum negative control of *H. contortus* PF23, 25x magnification focusing on anterior end of the worm. G. Peptide-absorbed control of *H. contortus* MOF23 at 10x magnification focusing on the anterior end of the worm. Lines denote 100 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

localizes to pharyngeal muscle tissue in *H. contortus* but localizes to pharyngeal neurons in *C. elegans*. It is tempting to speculate that like the GluCls, the ACCs family of receptors show different expression patterns but have some overlapping function between *H. contortus* and *C. elegans*. We are in the process of characterizing all additional ACC subunits from *H. contortus* to provide better insight into how conserved the function is between free-living and parasitic nematodes.

An additional difference in ACC-1 between *H. contortus* and *C. elegans* is the results from the oocyte expression experiments. Here we found that unlike Cel-ACC-1 that was reported to form a functional homomeric channel (Putrenko et al., 2005), Hco-ACC-1 does not. However, we have observed that in general the *hco-acc* genes do not express in oocytes as readily as other cys-loop receptors we have examined in our laboratory. However, co-expression of Hco-ACC-1 and 2 yielded a channel 3x more sensitive to ACh (EC_{50} 5.9 µM) compared to the Hco-ACC-2 homomeric channel. Differences in sensitivities between nematode homomeric and heteromeric channels have also been observed in the UNC-49 GABA receptor (Bamber et al., 1999; Siddiqui et al., 2010) and the GluCl (Cully et al., 1994). Interestingly, we have found that changing loop B phenylalanine to a tyrosine in Hco-ACC-2 produced a hypersensitive channel with an EC_{50} of 2.9 µM (Habibi et al., 2018). In Hco-ACC-1 there is a naturally occurring tyrosine (Y178) at the analogous position which may partially explain why the ACC-1/2 heteromeric channel was more sensitive to ACh compared to the ACC-2 homomeric channel. The significance of this naturally occurring tyrosine in Hco-ACC-1 to the *in vivo* function of the receptor is unknown. Moreover, we are still uncertain whether Hco-ACC-1 requires co-expression with additional subunits *in vivo* and while we have found that ACC-1 can co-express with ACC-2 in oocytes we have been unsuccessful, thus far, in determining the tissue location of ACC-2. Thus, we do not know at this time whether Hco-ACC-1 and 2 assemble to form a functional channel *in vivo*. The oocyte experiments merely suggest that the assembly of ACC-1 and ACC-2 is possible.

Examination of the Hco-ACC-1 binding site using *in silico* modelling

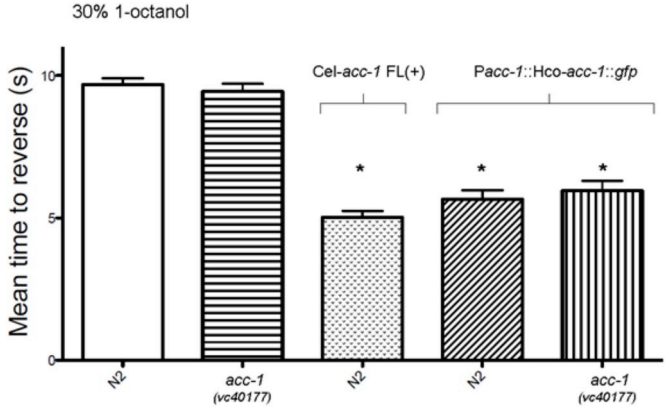

Fig. 4. Mean time to reverse of *C. elegans* in the presence of 30% octanol. N2, wild type; ACC-1 vc40177, Cel-ACC-1 knockout strain. PACC-1::ACC-1 FL(+) – overexpression of Cel-ACC-1 in N2 WT. PACC-1::Hco-ACC-1::GFP expression of Hco-ACC-1 in the wild type N2 *C. elegans* strain and the ACC-1 vc40177 (Cel-ACC-1 knockout) strains. Stars denote significant difference ($P < 0.0005$) compared to N2.

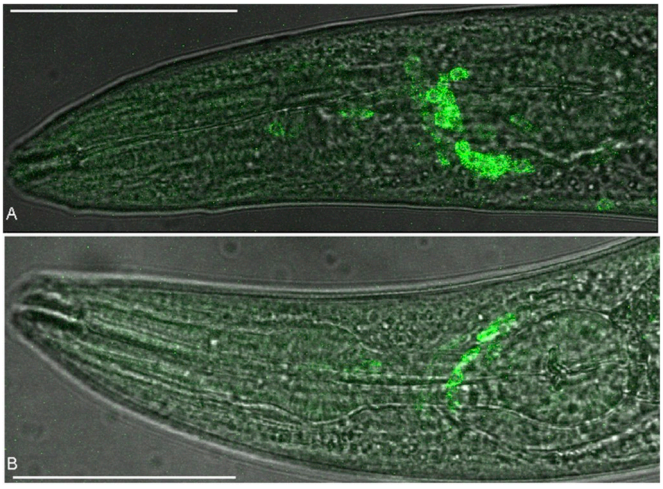

Fig. 5. Confocal image of N2 *Caenorhabditis elegans* worms expressing *hco-acc-1* under control of the *cel-acc-1* wild type promoter. Images A and B both show the expression of the construct in neurons near the posterior (terminal bulb) of the pharynx. Line represents 100 μm.

has also highlighted some unique features of this receptor family. Aside from the fact that ACCs are not present in mammals, they also appear to have a unique agonist binding site. The most striking difference between Hco-ACC-1 and both alpha and non-alpha nAChRs is absence of the signature cys-cys motif in binding loop C (see Fig. 1a). Second, the tryptophan residue that has been shown in nAChRs to contribute to a pi-cationic interaction with the quaternary amine of acetylcholine (and carbachol) is still present in ACC-1, but is located in loop C (W225) rather than in the traditional loop B seen in nicotinic receptors and the AChBP (Beene et al., 2002; Celie et al., 2004). Recently, we have shown that this loop C tryptophan is crucial for receptor function in the Hco-ACC-2 receptor (Habibi et al., 2018). Interestingly, a similar shifting of this key tryptophan residue from loop B to C is also seen when we compare serotonin 5-HT_3 and nematode MOD-1 receptors (Mu et al., 2003). A loop C tryptophan is also present in tyramine and dopamine-gated chloride channels (Beech et al., 2013), suggesting that it may be important for the ability of nematode cys-loop receptors to respond to a diverse array of molecules. However, it is important to note that the binding site for ACh on the Hco-ACC-1/2 channel could also be on the interface of two ACC-1 or ACC-2 subunits (Habibi et al., 2018) or an ACC-2/1 heterodimer. Further research will be important to determine the subunit stoichiometry and variation in binding-sites of the ACC family of receptors.

While traditionally characterized as a muscarinic receptor agonist used primarily for the treatment of glaucoma, we have shown that carbachol (carbamylcholine) is an effective agonist at the nematode ACC-1/2 receptor with an EC_{50} about 2-fold higher than acetylcholine. Carbachol is also an agonist for muscle nicotinic receptors but with an EC_{50} over 10 -fold higher compared to acetycholine (Akk and Auerbach, 1999). Perhaps the structural differences between nAChRs and ACC receptors as outlined above can explain the relatively high sensitivity of carbachol observed in our study. Nevertheless, it is clear that further study of the molecular pharmacology of ACC receptors can provide an excellent opportunity for the study of current AChR agonists and the possible discovery of novel agonists.

In conclusion, this study provides the first investigation of role of ACC receptors in parasitic nematodes. While research using *C. elegans* to evaluate anthelmintic targets is an excellent tool to provide relevant information, research on similar targets in parasitic nematode can provide key information on whether a rational drug design approach might be worthwhile. While there is still much more to be learned about the role of ACC receptors in parasitic nematodes it is clear that comparing the function of the LGCC family in free-living and parasitic nematodes can potentially lead to the discovery of future anthelmintic targets and a better understanding of the evolution of parasitism.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2018.10.010.

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