The Pharmacological Characterization of Hco-UNC-49, a GABA-gated Chloride Channel from the Parasitic Nematode *Haemonchus contortus*

By

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Abstract

Compared to mammals, nematodes appear to exhibit a unique GABAergic nervous system. *Haemonchus controtus* is a parasitic nematode that infects ruminants worldwide. Hco-UNC-49 is a *H. contortus* GABA-gated chloride channel and is an orthologue to the UNC-49 channel from the free-living nematode *Caenorhabditis elegans*. Previous research by our group has shown that while the UNC-49 channels from the two nematodes share similar sequence homology they do not share identical sensitivity to GABA. To further investigate the characteristics of the Hco-UNC-49 channel, this study tested the effects of various modulators, insecticides and anti-parasitic drugs on channel function. Most notably, the molecules penicillin G, propofol and pregnenolone sulfate all had similar effects on Hco-UNC-49 as reported previously for Cel-UNC-49. On the other hand, Hco-UNC-49 appears to be less sensitive to picrotoxin inhibition compared to what has been reported for Cel-UNC-49. Novel effects of a number of anthelmintics were also observed. For example, the anthelmintics ivermectin and moxidectin both enhanced Hco-UNC-49 GABA responses, while piperazine was able to directly activate Hco-UNC-49 at high concentrations. These results suggest that Hco-UNC-49 is likely an *in vivo* target for these anthelmintics.

Keywords

H. contortus, $GABA_A$ pharmacology, UNC-49, anthelmintics, insecticides, channel modulation

Acknowledgements

I would like to dedicate this thesis to the many amazing people who have helped me along during this process. Thank you to my committee, our discussions have brought interesting ideas into this project and really helped shape my education. I would like to thank Dr. Sean Forrester for all of his support throughout the years. You took a chance on me and I'll never forget that it was you who made my dreams of becoming a scientist possible. Through your guidance and strong example I really feel I've begun to make the transition from a questioning student to a questioning scientist. A simple 'thank you' will never do, but I hope you know that your involvement with the students at UOIT has, and will continue to, change lives. I would also like to thank the Forrester Lab in its entirety. Each of you have managed to provoke interesting thoughts, cause laughter and offer a listening ear. You should all be extremely proud of yourselves; I know I am proud to see how our team has evolved over the years. I'd like to thank you again Mike, you've managed to put up with me over this MSc. and have helped me out at every step of the way whenever you could. I know you'll move on and do great things, and I look forward to reading your ground-breaking papers! I'd also of course like to thank my family, without who I could never have achieved so much. You've always supported my dreams and this thesis is a big step in that process, and it's in no small part due to all of you. Lastly I'd like to thank my wife Salma. You are the one person who has helped me 'keep it together' during this time. This Master's has been one of the most profound periods of change in my life, and you are the major reason for these being the best years of my life. I can never thank you for all that you've done, but I won't get too sentimental here…. I'll leave that for my PhD thesis acknowledgements!

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List of Abbreviations

5-HT$_3$ - serotonin

AAD - amino-acetonitrile derivative

ATP - adenosine triphosphate

Cel-UNC-49 - *Caenorhabditis elegans* uncoordinated gene 49 protein product

CNS - central nervous system

[3H]-EBOB – radio-labeled ethynylbicycloorthobenzoate

EC$_{50}$ – concentration of compound which produces half of the maximal response

ECD - extracellular domain

GABA - γ-aminobutyric acid

GABA-R - GABA receptor

GluCl - glutamate gated chloride channel

GRD - GABA and glycine like receptor of *Drosophila*

Hco-UNC-49 - *Haemonchus contortus* uncoordinated gene 49 protein product

IC$_{50}$ – concentration of compound which reduces the response by half

IVM - ivermectin

LCCH3 - ligand gated chloride channel homologue 3

LGCC - Ligand gated chloride channel

M1-M4 - transmembrane domains 1 - 4

MOX - moxidectin

nACh - nicotinic acetylcholine

PenG - penicillin G

PTX - picrotoxin

RDL - resistant to dieldrin (*rdl*) protein product

SE - standard error

unc-49 - uncoordinated gene 49

Chapter 1

Background

Haemonchus contortus

Haemonchus contortus is a blood-feeding gastrointestinal parasitic nematode that infects the abomasum of ruminant hosts such as sheep and goats. Haemonchosis, caused by infection with *H. contortus*, can lead to anemia and in more severe infections death of the host organism and thus, can have a substantial negative impact on livestock-based industries (Nikolaou and Gasser, 2006). *H. contortus* is a major problem because it exists globally and has demonstrated an uncanny ability to develop resistance to many currently administered anthelmintics (Prichard, 1990; Kwa *et al.*, 1994; van Wyk *et al.*, 1997). To complicate issues, *H. contortus* is not very well characterized in terms of its anatomy, development processes and genome. Fortunately, *H. contortus* is a member of the same nematode clade as its closely related free-living cousin *Caenorhabditis elegans* (Blaxter *et al*., 1998). *C. elegans* is a model organism which has been extremely well studied and is a powerful starting point when exploring the biology of *H. contortus*. However, despite the relatedness of these two nematodes, it must be noted that *H. contortus* differs drastically from *C. elegans* as it is a parasitic nematode with a complex life-cycle involving both a free-living and a parasitic phase (Veglia, 1915). On the other hand, the close genetic relatedness, yet drastic lifestyle difference suggests that studies comparing the biology of *C. elegans* with *H. contortus* may provide

important information that will ultimately better define the biology of parasitic nematodes.

H. contortus Life Cycle

Free Living Phase

Originally described by Veglia (1915), the life cycle of *H. contortus* exists in two major phases, a free living phase, followed by a parasitic phase. Adult female worms lay their eggs within the abomasum of their infected host at around the 4 cell stage. By the time the eggs develop to the 11-26 cell stage they are deposited into the environment within fecal matter expelled from the host. Oxygen is required at this point to continue the development process and must therefore continue outside of the host. The parasite egg will continue to develop over the next 14-17 hours at which point it will hatch, releasing the L1 larval stage. Each larval stage is characterized by two periods of development; a period of activity which can include feeding and growth and a second period known as “lethargis” where prominent morphological changes take place. The larva will continue to develop through the L1, L2 and L3 larval stages, at which point the worm waits to be consumed by its future host before sexually differentiating and entering its parasitic life phase (Veglia, 1915; Nikolaou and Gasser, 2006).

Parasitic Phase

Once consumed by the host through the grazing of contaminated grass, the parasite will enter the abomasum and continue to develop through the L4 larval stage. At this point it will develop a buccal capsule to facilitate blood feeding, sexually differentiate, and later develop into a full-fledged adult parasitic worm. Adult *H. contortus* display interesting anterior modifications compared to their free-living *C. elegans* counterparts. *C. elegans* possesses an anterior "mouth-like" structure (www.wormatlas.org) whereas adult *H. contortus* possess a "needle-like" structure known as the buccal lancet which aids in penetrating the host's abomasum lining to allow for blood-feeding (Veglia, 1915). The parasites will remain in the abomasum of the host and will feed off of the host's blood while mating (Nikolaou and Gasser, 2006). Adult females can lay upwards of 4500 eggs per day (Coyne and Smith, 1992). It is notable that L4 larvae can undergo a process known as hypobiosis, or arrested development, within the abomasum wall of the host (Blitz and Gibbs, 1971a,b) in response to seasonal changes and host immune factors, and wait for more optimal conditions to differentiate into the adult stage (Michel, 1975.; Gibbs, 1986; Eysker, 1997).

Anthelmintic Control

Anthelmintics are currently employed to treat *H. contortus* infections (Nikolaou and Gasser, 2006). Many of these drugs target the nematode nervous system to

incapacitate the parasite so it can be expelled from the host. There are three classes of anthelmintics currently in widespread use. The first class is the benzimidazoles which disrupt β-tubulin (Lubega and Prichard, 1990, 1991). The other two classes of widely used anthelmintics are the macrocyclic lactones and the nicotinic agonists which both target members of the cys-loop ligand gated ion channel family (Martin *et al.*, 2005; Wolstenholme and Rogers, 2005). A popular and potent macrocyclic lactone anthelmintic, ivermectin, activates glutamate-gated chloride channels (GluCls) in an essentially irreversible manner and can potentiate the inhibitory effect of the endogenous ligand (Cully *et al.*, 1994; Forrester *et al.*, 2002, 2003). Ivermectin has been shown to also target GABA-gated chloride channels and enhance the effects of GABA (Kass *et al.*, 1980; Boisvenue *et al.*, 1983; Holden-Dye *et al.*, 1988; Holden-Dye and Walker, 1990). These drugs all affect the parasite's ability to function normally which ultimately results in the elimination of the parasite from the host.

Despite the effectiveness of some of these anthelmintics, such as ivermectin, there are growing trends of drug resistance developing in *H. contortus* (Prichard, 1994). Therefore, there is an ongoing need to develop new classes of anthelmintics. Regardless of the fact that resistance has occurred to every known class of anthelmintic, including those that target ion channels, the cys-loop ligand-gated ion channel family remains a viable target for future drug discovery research. This is illustrated by the recent discovery of a new class of anthelmintics, the amino-acetonitrile derivatives

(AADs), which are very effective against parasitic nematodes and target the nicotinic acetylcholine receptor ACR-23 (Kaminsky *et al*., 2008). However, the potential for AAD-resistant strains of *H. contortus* (Kaminsky *et al*., 2008) highlights the need for continued research into the development of new anthelmintics. To accomplish this, a greater understanding of the biology of parasitic nematodes, such as *H. contortus,* and potential protein targets is required before a focused effort can be placed on developing effective treatments.

Ligand Gated Chloride Channels

Ligand gated chloride channels (LGCCs) are members of the cys-loop superfamily of ligand-gated ion channels. Receptors of this type are transmembrane complexes that become activated by specific chemical ligands which cause the receptor channels to open, allowing chloride ions across the cellular membrane to induce cellular inhibition. All cys-loop ligand gated ion channels are thought to be pentamers (hence contain five subunits) and play prominent roles in the function of invertebrate nervous (Harrison *et al.*, 1996) and muscle (Bamber *et al.*, 2005) tissue as well as the CNS (central nervous system) of vertebrate organisms. LGCCs are responsible for mediating fast inhibitory neurotransmission (Raymond and Sattelle, 2002).

The LGCC pentamer arrangement of subunits forms a central pore in the membrane (Ramond and Sattelle, 2002) (see Figure 1). Different combinations of subunits give rise to channels with unique ligand binding kinetics and pharmacological

properties (Bamber *et al.*, 2003). As members of the cys-loop superfamily, each subunit possesses a pair of disulphide bonded cysteine residues which are separated by 13 amino acids in the extracellular N-terminal domain which forms a characteristic "cys-loop" (Figure 2) (Karlin 2002). In addition to the cys-loop, subunits of this family possess a long extracellular N-terminal domain, four transmembrane spanning regions, termed M1-M4, and an intracellular loop that occurs between M3 and M4 (Raymond and Sattelle, 2002).

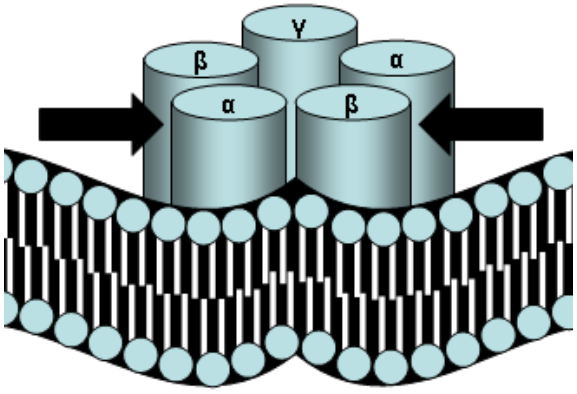

Figure 1: Pentameric assembly of a LGCC (Ligand-Gated Chloride Channel) with indicated ligand binding domains at the interface of subunits. Binding domains are indicated by black arrows at the interfaces of two subunits. (Modified from Kash *et al*., 2004)

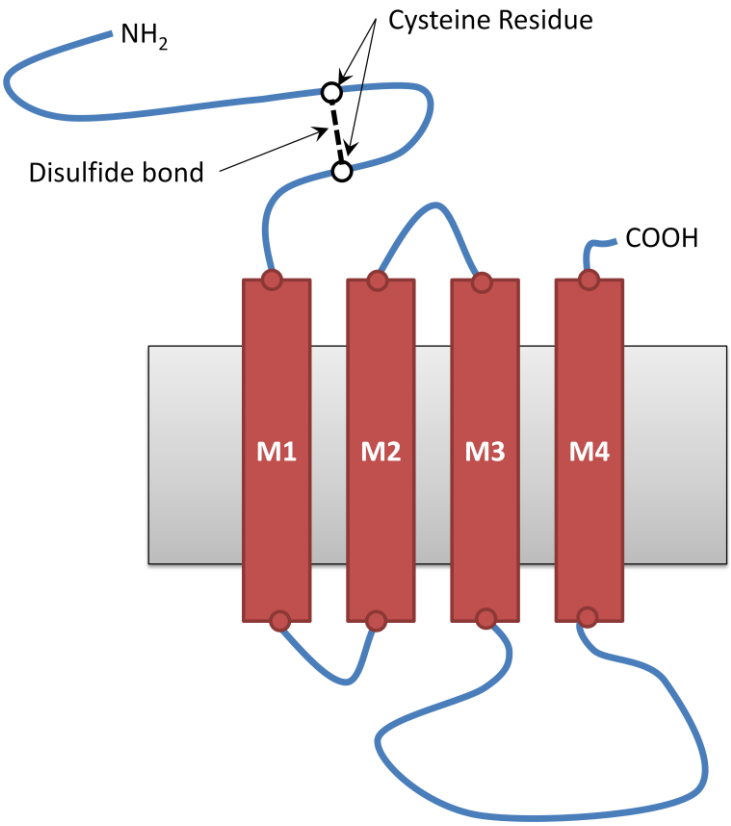

Figure 2: Graphical depiction of a cys-loop ligand-gated ion channel subunit. Shown here is a generic cys-loop LGCC subunit complete with the characteristic cys-loop, extracellular N-terminal domain, four transmembrane (M1-M4) spanning regions and a large intracellular loop between M3 and M4 (Adapted from Raymond and Sattelle, 2002).

Once bound by ligand, LGCCs will undergo a conformational change into an "open" state where it will conduct chloride ions across a cellular membrane, reducing the probability of an action potential. Ligand binding is thought to occur at the interface between different subunits (Kash *et al*., 2004) (Figure 1) which allows for ligand binding kinetics to be modulated by different subunit composition and arrangements.

The M2 transmembrane spanning region is believed to line the channel pore (Olsen and Tobin, 1990). Residues in this region play a role in ion selectivity and in modulating the kinetic properties of the channel itself (Raymond and Sattelle, 2002; Hosie *et al.*, 1995). The intracellular loop between the M3 and M4 transmembrane regions contains regulatory phosphorylation sites and motifs responsible for synaptic localization (Moss and Smart, 1996; Bamber *et al.*, 2005).

LGCCs Subtypes

The two types of LGCCs found in vertebrates are GABA and glycine receptors (Ortells and Lunt, 1995). However, invertebrates such as nematodes and insects contain a more diverse and unique array of LGCC subtypes, including those gated by acetylcholine, serotonin, GABA, histamine and glutamate (Dent, 2006). Interestingly, in the free-living nematode *C. elegans*, it was found that glutamate-gated chloride channels (GluCls) differ significantly from vertebrate excitatory glutamate-gated cation channels and are in fact more similar to vertebrate $GABA_A$ and glycine receptors (Cully *et al.*, 1994). In addition, GluCl subunits have been found to co-assemble with an invertebrate $GABA_A$ receptor-like subunit known as RDL (resistant to dieldrin), which was the first evidence for co-assembly of subunits from different classes (Buckingham *et al.*, 2005). GluCls are believed to be a target of the anthelmintic ivermectin, resulting in paralysis of the pharyngeal muscles in parasites (Arena et al., 1992). In addition, it has

been suggested that ivermectin may target GABA receptor subunits which in turn lead to the paralysis of somatic muscle (Kass *et al.*, 1980; Boisvenue *et al.*, 1983).

GABA-gated chloride channels

GABA (γ-aminobutyric acid) receptors are widespread in both vertebrates as well as invertebrates and are primarily responsible for fast inhibitory neurotransmission (Sattelle, 1990). In vertebrates, two classes of ionotropic GABA receptors have been found, $GABA_A$ and $GABA_C$ (Hosie *et al.*, 1997). $GABA_A$ receptors are found throughout the vertebrate CNS, are sensitive to bicuculline antagonism, and can be regulated by allosteric modulators (Hosie *et al.*, 1997). $GABA_C$ receptors on the other hand are insensitive to bicuculline and a great majority of allosteric modulators (Hosie *et al.*, 1997). Both classes of ionotropic GABA receptors are blocked by the plant toxin known as picrotoxin which is a classical chloride channel blocker (Hosie *et al.*, 1997). Insect GABA receptors, on the other hand, exhibit differences in their pharmacological profile compared to their vertebrate counterparts. For example, most insect receptors are insensitive to bicuculline, but differ from vertebrate $GABA_C$ receptors in their sensitivity to modulators, such as benzodiazapines, barbituates and GABA analogues (Satelle, 1990; Hosie *et al.*, 1997).

There have been many different subtypes of $GABA_A$ receptors found in both vertebrates and invertebrates. The vertebrate GABA receptor subunit subtypes appear more extensive with a total of eight that have been discovered (α, β, γ, δ, ε, θ, π, ρ), all of which possess multiple isoforms, with the exception of δ, ε, θ, and π (McKernan and Whiting, 1996). However, the majority of vertebrate $GABA_A$ receptors are thought to be composed of α, β, γ or δ subunits while only ρ subunits are found in the majority of $GABA_C$ receptors (Cutting *et al.*, 1991). However, heteromultimeric channels containing ρ, α, and γ subunits have also been observed in brainstem neurons (Milligan *et al.*, 2004). In *D. melanogaster*, there appears to be three types of GABA receptor subunits, RDL (resistance to dieldrin), GRD (GABA and glycine like receptor of *Drosophila*) (Harvey *et al.*, 1994) and LCCH3 (ligand gated chloride channel homologue 3) (Henderson *et al.*, 1993), and these classes do not fit into the vertebrate classes observed to date (Hosie *et al.*, 1997). In *C. elegans*, there are at least four genes that encode $GABA_A$ receptor (chloride channel) subunits, *unc49*, *lgc-37*, *lgc-38* and *gab-1* (Jones and Satelle 2008).

In addition to differences in the number of encoded subunits, vertebrate and invertebrate $GABA_A$ receptors differ in overall *in vivo* function. For example, the insect GABA receptor, known as RDL (ffrench-Constant *et al*., 1991), has been observed throughout the *Drosophila melanogaster* nervous system in all developmental stages (Buchner *et al.*, 1988) and has been implicated in olfactory learning (Liu and Davis, 2008) and motility (Leal and Neckameyer, 2002; ffrench-Constant *et al*., 1993). *C. elegans*

GABA receptors have been found primarily at the neuromuscular junctions and play key roles in locomotion (Bamber et al, 1999). With respect to vertebrate function, it is well established that GABA receptors function mostly in the CNS and are responsible for several human psychiatric disorders such as anxiety, insomnia and epilepsy (Landolt and Gillin 2000; Meldrum 1989; Miczek *et al*., 1995).

Anatomy of a GABA-gated chloride channel

The 5' Extracellular Domain

The 5' extracellular domain (ECD) of $GABA_A$ receptor subunits contains a GABA binding site or pocket and the cys-loop. These two sites in the ECD of $GABA_A$ receptor subunits are highly important for channel function (Padgett *et al.*, 2007; Schofield *et al.*, 2003). The cys-loop is a 13 amino-acid structure which is flanked by two cysteine residues which are highly conserved across all members of the cys-loop ligand-gated ion channel super-family of receptor subunits. The cys-loop appears to be essential for channel function and studies which have mutated this loop have observed defects in channel gating responses (Schofield *et al.*, 2003). In light of this evidence and in conjunction with *in silico* modeling of the GABA-R, the cys-loop appears ideally located to translate the effects of GABA binding into channel opening (Unwin, 2005). The putative $GABA_A$ binding site is also in the ECD and is believed to exist at the interface of adjoining subunits (Sigel *et al.*, 1992; Amin and Weiss, 1993). Modeling of the GABA-R suggests that on the primary subunit there are 3 loop structures (A-C) comprising one

half of the binding site and on the secondary subunit there are three more loop structures (D-F) comprising the other half of the binding site (Corringer *et al.*, 2000). The mechanism behind the GABA binding site is still largely unknown but there is a growing body of evidence to suggest the importance of several residues within these loops. Loop A has been proposed to be the primary site of GABA docking on the $GABA_A$ channel (Padgett *et al.*, 2007), at which point loop C can constrict and cause a "capping" action which has been suggested to result in channel opening (Hansen *et al.*, 2005).

The Channel Pore and Gate

The pentameric structure of the GABA-R forms a pore in the cellular membrane so that chloride ions can be conducted across the membrane and into the cell to induce cellular inhibition. To keep the effects of these channels controlled, the opening and closing of the pore needs to be tightly regulated. A primary structure that ensures this regulation is known as the channel gate and is thought to be formed by a few key residues in the M2 transmembrane domain of channel subunits that form a "kink" (Unwin, 2005). This kink in the M2 domain is thought to result in a "closed" channel conformation which restricts the passage of chloride ions into the cell (Miyazawa *et al.*, 2003). Mutational analysis of the gate residues resulted in a channel that is constitutively open (Pan *et al.*, 1997). The channel pore of a GABA-R serves two purposes; it allows ions to flow into the cell, and it also selects for which ions may pass through the pore. This is accomplished primarily via charged residues lining the pore

which form concentric rings that are able to attract ions of opposing charge and repel ions of similar charge. Mutating residues in these rings have resulted in channels that conduct ions of the opposite charge (Galzi *et al.*, 1992). This demonstrates that a few residues in key locations can vary a channel's function enormously. In addition, the size of the pore plays a role in ion selectivity, which restricts the passage of ions based on size. In combination, these properties create highly regulated channels capable of conducting ions across a cell membrane without the need for ATP or other energy reserves.

$GABA_A$ receptor pharmacology

Picrotoxin

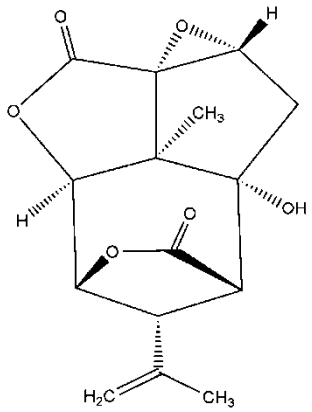

Figure 3: Chemical structure of Picrotoxin.

Picrotoxin (structure shown in Figure 3) is a well studied $GABA_A$ channel antagonist derived from the moonseed family of plants known as *Menispermaceae* as well as its close relative *Coriaria arborea* from New Zealand. *Coriaria arborea*, known as a "loco weed", was found to be the cause of poisoning in cattle and humans (Olsen, 2006). The active ingredient in picrotoxin is known as picrotoxinin which is a polycyclic compound that contains no nitrogen and acts as a non competitive antagonist in $GABA_A$ channels (Olsen, 2006). The effects of picrotoxin are able to reverse the effects of barbiturates and benzodiazepines (Olsen and Gordey, 2000; Takeuchi and Takeuchi, 1969). Mutational studies have identified T246 in the M2 domain of the β_2 $GABA_A$ subunit as well as analogous positions on the α2 and γ2 subunits as important for

picrotoxin's antagonistic effects (Gurley *et al.*, 1995). Several additional residues lining the M2 pore (V257-T261, L301, A252) have also been identified as important for picrotoxin blockage of $GABA_A$ channels (Xu *et al.* 1995; Zhang *et al.* 1995; Chang and Weiss 1998, 2000; Buhr *et al.* 2001). Picrotoxin is also potent against RDL-like GABA-Rs in invertebrate species such as *Drosophila*, *C. elegans* and *H. contortus* (Buckingham *et al.*, 1994; Bamber *et al.*, 2003; Siddiqui *et al.*, 2010). With respect to *C. elegans* and *H. contortus* $GABA_A$ receptors, the homomeric UNC-49 channel (channel with only UNC-49B subunits) are highly sensitive to the effects of picrotoxin, while the heteromeric channels (channel with both UNC-49B and C subunits) are quite resistant to the plant toxin's effects (Bamber *et al.*, 2003; Siddiqui *et al.*, 2010). Picrotoxin resistance in UNC-49 appears to be the result of a methionine residue at position 6' in the M2 transmembrane region of the UNC-49C subunit (Siddiqui *et al.*, 2010; Zhang *et al.*, 1995; Bamber *et al.*, 2003).

Propofol

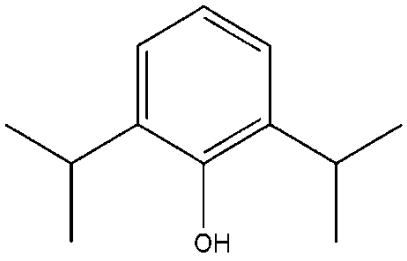

Figure 4: Chemical structure of Propofol.

Propofol (structure is shown in Figure 4) is an intravenous anesthetic which has been shown to target $GABA_A$ channels and enhance GABAergic currents in mammalian systems (Hales and Lambert, 1991). Studies have elucidated several residues which appear to be important for propofol's action at the $GABA_A$ receptor. One study identified β_2 M286 near the extracellular end of the M3 transmembrane domain of the β_2 subunit as important for propofol sensitivity (Bali and Akabas, 2004). Propofol was found to enhance recombinant human GABA-Rs (Pistis *et al.,* 1997) which is in contrast to the effects of propofol on the *C. elegans* UNC-49 GABA receptors where propofol was found to enhance the homomeric channel but inhibit the heteromeric channel (Bamber *et al.*, 2003). This inhibition of the Cel-UNC-49 heteromeric channel is believed to occur due to a 15' methionine residue in the M2 domain of the Cel-UNC-49C subunit (Bamber

et al., 2003). This residue has been shown to affect propofol sensitivity in other GABA channels (Pistis *et al*., 1999).

Penicillin G

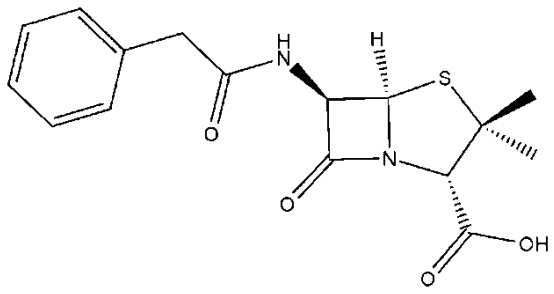

Figure 5: Chemical structure of Penicillin G.

Penicillin G (structure shown in Figure 5) is a β-lactam antibiotic, part of a known class of pro-convulsants, which acts as an open channel blocker in $GABA_A$ receptors (Fujimoto *et al.*, 1995). When penicillin G was tested against Cel-UNC-49 receptors it was found that at a lower concentration (1 mM) the heteromeric and homomeric channels were slightly inhibited to near equal extents whereas at a higher concentration (100 mM) the heteromeric channel was inhibited nearly twice as much as the homomeric channel (Bamber *et al.*, 2003). The reason for this change in channel sensitivity to penicillin G block is not yet known. Penicillin G is thought to bind along the M2 region of the channel pore to exert its effects and may bind to a common site with picrotoxin (Kalueff, 2007). The suggestion that it shares a common binding site with

picrotoxin arises from the fact that the binding sensitivities of both compounds are reduced by a phenylalanine residue at position 6' in the M2 domain of murine $GABA_A$ receptors (Sugimoto *et al.*, 2002). However, this similarity does not appear to hold true in *C. elegans* where the presence of the UNC-49C subunit confers resistance to picrotoxin, but increased sensitivity to penicillin G (Bamber *et al.*, 2003). Whether or not the binding sites simply overlap, or some other structural features of the UNC-49C subunit are contributing to penicillin G sensitivity remains unclear.

Pregnenolone Sulfate

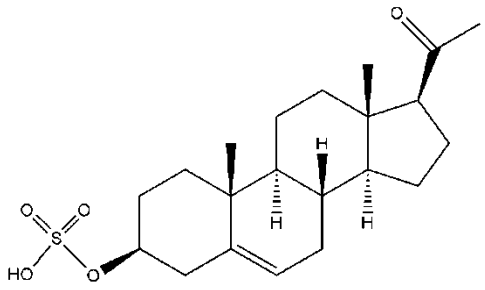

Figure 6: Chemical structure of Pregnenolone Sulfate.

Pregnenolone is a pregnane steroid which is produced endogenously in humans as a cholesterol derivative. Several steroidal compounds have been found to modulate $GABA_A$ channels as agonists, enhancers and even inhibitors (Hosie *et al*., 2006; Stell *et al*., 2003; Majewska *et al*., 1988). Previous research has identified two putative binding sites for agonistic and enhancing neurosteroids. The first, at position T236, at the β/α interface (Hosie *et al*., 2006) and the second at position Q241 which is at the base of an

aqueous pocket formed by the M1-M4 domains of subunits (Hosie *et al*., 2007). Currently the site of action for inhibitory neurosteroids is unknown but several key residues in the M1 domain (L258, Q259, F262, S265) and one residue in the M2-M3 linker domain (R306) have been shown to be important for pregnenolone sulfate (structure shown in Figure 6) sensitivity in Cel-UNC-49 (Wardell *et al*., 2006). In addition, pregnenolone sulfate has been shown to reduce GABA responses in rat cerebral cortical neurons (Majewska *et al*., 1988).

Dieldrin

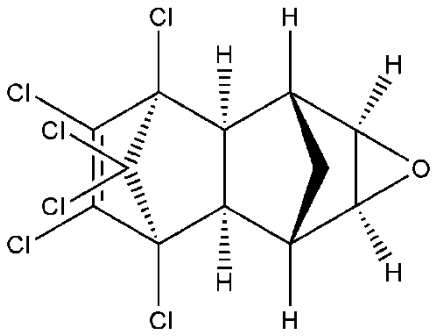

Figure 7: Chemical structure of Dieldrin.

Dieldrin (structure shown in Figure 7) is a cyclodiene insecticide developed in the 1940's which was used heavily during the 1950's. Dieldrin has been linked to various health problems and has subsequently been banned worldwide. Dieldrin was found to inhibit $GABA_A$ chloride fluxes in the rat brain (Gant *et al.*, 1987). Resistance to dieldrin in *Drosophila melanogaster* led to the discovery of RDL, the very first invertebrate $GABA_A$ subunit homologue ever isolated (ffrench-Constant *et al.*, 1991). Furthermore, it was

found that a single point mutation (A302S) endows this channel with resistance to dieldrin as well as picrotoxin and fipronil (ffrench-Constant *et al.*, 1993; Buckingham *et al.*, 1996; Hosie *et al.*, 1995). However, studies on the RDL-like UNC-49 channel from *C. elegans* have revealed that these channels are highly resistant to the inhibitory effects of dieldrin (Bamber *et al.*, 2003). Interestingly, these channels do not possess the classic A302S resistance mutation but it is of note that Cel-UNC-49B does have an A302G mutation which has been associated with cyclodiene resistance in various insect species (Bamber *et al*., 1999; ffrench-Constant *et al.*, 2000).

Fipronil

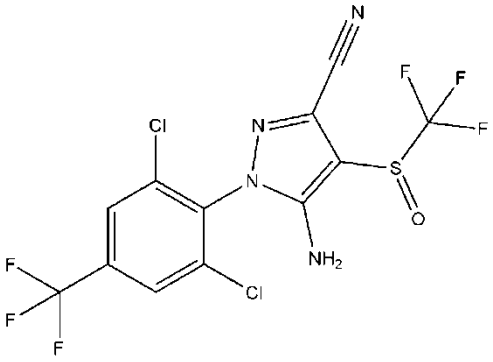

Figure 8: Chemical structure of Fipronil.

Firponil (structure shown in Figure 8) is a phenyl pyrazole insecticide which has been shown to block both GABA-gated and glutamate gated chloride channels (Hosie *et al.*, 1995; Horoszok *et al.*, 2001). The binding site for fipronil has not yet been completely defined and characterized but it has been suggested to bind to the same site

as dieldin and picrotoxin (Hosie *et al.*, 1995). Despite this suggestion, research has shown that highly dieldrin resistant insects show low levels of fipronil resistance (Le Goff *et al*., 2005). In addition, binding studies revealed that picrotoxin and dieldrin both show competitive displacement of [^{3}H]-EBOB while fipronil displaces this compound in a non-competitive and more complex manner (Deng *et al.*, 1991, 1993). Whether this suggests that fipronil has more than one binding site on the GABA-R or simply exhibits unique binding kinetics remains unclear.

Ivermectin

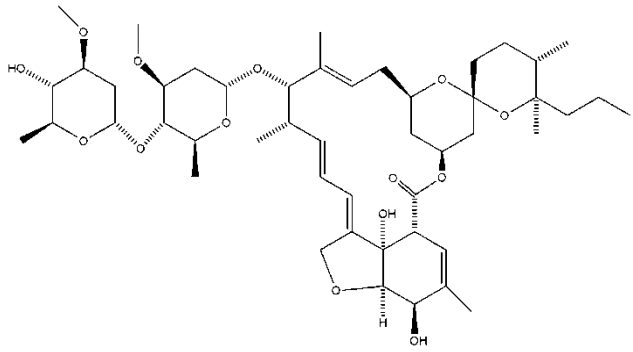

Figure 9: Chemical structure of Ivermectin.

Ivermectin (structure shown in Figure 9) is an antiparasitic macrocyclic lactone that has been employed to treat infections of gastrointestinal helminths such as *H. contortus*. It has been shown to exert its paralytic effects by activating glutamate-gated chloride channels, which results in paralysis of the pharyngeal muscle tissue (Arena *et*

al., 1992; Cully *et al.*, 1994; Dent *et al.*, 1997; Dent *et al.*, 2000; Geary *et al.*, 1993, Martin, 1997), and on GABA-gated chloride channels, which is believed to cause somatic muscle paralysis (Kass *et al.*, 1980; Boisvenue *et al.*, 1983; Holden-Dye *et al.*, 1988; Holden-Dye and Walker, 1990). Ivermectin has been shown to bind irreversibly to glutamate-gated chloride channels in *H. contortus* which stabilizes the channel in an open state resulting in uncontrolled chloride currents (Arena *et al.*, 1992; Cully *et al.*, 1994; Forrester *et al.*, 2003). In addition, ivermectin has been shown to modulate both glutamate-gated chloride channel and GABA receptor function (Arena *et al.*, 1992; Holden-Dye and Walker, 1990; Feng *et al.*, 2002).

Moxidectin

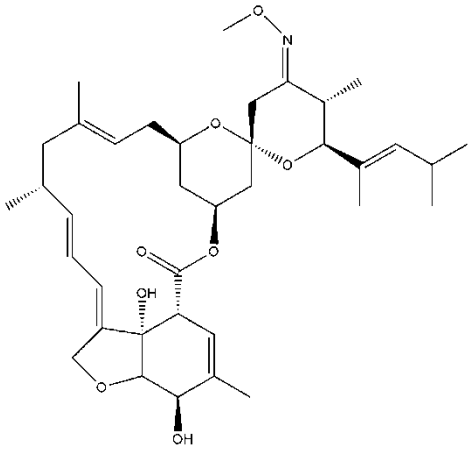

Figure 10: Chemical structure of Moxidectin.

Moxidectin (structure shown in Figure 10) is a milbemycin anti-parasitic compound that is structurally related to the avermectins such as ivermectin. Like

ivermectin, moxidectin has been shown to affect glutamate-gated chloride channels (Forrester *et al.*, 2002, 2003) and GABA-gated chloride channels (Huang and Casida, 1997). Both anthelmintic structures share a 16-membered macrocyclic unit. However, the avermectins possess a disaccharide substituent at C-13 that is not present in the milbemycins, and moxidectin itself is substituted at C-23 and C-25 compared to ivermectin (Shoop *et al*., 1995). Although ivermectin and moxidectin do share similarities in their structures, some reports suggest that moxidectin is effective in treating nematode infections when ivermectin fails (Craig *et al.,* 1992), suggesting that the two compounds show some differences in their action. However, other reports have shown that common alleles are linked to both ivermectin and moxidectin resistant strains of *H. contortus* (Blackhall *et al.*, 1998; 2003) indicating that both compounds act at similar targets and share similar mechanisms of resistance.

Piperazine

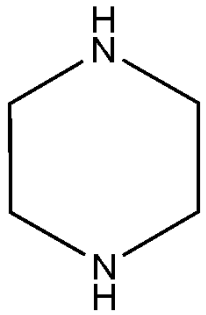

Figure 11: Chemical structure of Piperazine.

Piperazine (structure shown in Figure 11) is an anthelmintic compound which was originally used to treat infections from *Ascaris lumbricoides* and *Enterobius vermicularis* (Abdi, 1982). Treatment with piperazine results in flaccid paralysis of the parasite and ultimately expulsion from the host (Abdi, 1982). The effectiveness of piperazine against ascariasis ranged between 70%-95% based on dosages between 3g daily to 75mg/kg over 2 days (Brown and Sterman 1954; Brown *et al*., 1956). Piperazine has been shown to produce its paralytic effects by activating GABAergic-like chloride currents on muscle tissue in the parasitic nematode *Ascaris suum* (Martin, 1982). However, despite this decades-old demonstration, it has yet to be definitively shown that piperazine produces its effects directly through GABA-gated chloride channels, and whether this targeting is due to a specific receptor, or has broad effects across the GABA-gated chloride channels.

The nematode GABA receptor, UNC-49

Cel-UNC-49

Cel-unc-49 is a *C. elegans* gene that encodes a GABA-gated chloride channel and is of potential importance in terms of anthelmintic drug development because this receptor has been found to be expressed at the neuromuscular junction in the worm (Bamber *et al.*, 1999) and thus may be a prime target for novel anti-parasitic drugs. Through alternative splicing, the *unc-49* gene encodes three distinct GABA-gated chloride channel subunit transcripts (*Cel-unc-49a*, *Cel-unc-49b*, *Cel-unc-49c*) of which

two, *Cel-unc-49b* and *Cel-unc-49c*, have been detected in the worm at significant levels (Bamber *et al.*, 1999) and encode two different channel subunits. Cel-UNC-49B forms a functional homomeric channel and Cel-UNC-49C does not form a functional channel on its own, but can associate with Cel-UNC-49B to form a functional heteromeric channel in *Xenopus laevis* oocytes (Bamber *et al.*, 1999).

Cel-UNC-49B and Cel-UNC-49C are co-expressed together at the neuromuscular junction in *C. elegans* (Bamber *et al.*, 2005). Bamber and colleagues went on to demonstrate that the pharmacology of *C. elegans* muscle tissue closely matched that of the Cel-UNC-49 heteromeric channel due to an observed resistance to picrotoxin and increased sensitivity to pregnenolone sulfate (Bamber *et al.*, 2005). Despite the presence of heteromeric channels at the neuromuscular junction, it has been demonstrated that Cel-UNC-49C is not required for the correct expression or function of Cel-UNC-49B (Bamber *et al.*, 2005). The UNC-49C subunit likely plays an important role physiologically because it is shown to be conserved in other nematode species such as *H. contortus* (Siddiqui *et al.*, 2010). It is likely that UNC-49C plays an important role in modulating the sensitivity and kinetics of the channel.

Hco-UNC-49

Recently an orthologue of Cel-UNC-49 has been isolated and initially characterized in the parasitic nematode *H. contortus* (Hco-UNC-49) (Siddiqui *et al.*, 2010). Two LGCC subunit sequences have been isolated from *H. contortus* mRNA, *Hco-*

unc-49b and *Hco-unc-49c*, which encode two different subunits that share a common 5' N-terminus but have different 3' C-termini (Siddiqui *et al.*, 2010). Hco-UNC-49B forms a functional homomeric GABA-R and can also combine with Hco-UNC-49C to form a functional heteromeric GABA-R in *X. laevis* oocytes (Siddiqui *et al.*, 2010). Hco-UNC-49 demonstrates some similarities with its *C. elegans* counterpart in that the UNC-49B subunit can form a functional homomer that is highly sensitive to picrotoxin, but when associated with UNC-49C produces a channel that is highly resistant to picrotoxin (Siddiqui *et al*., 2010; Bamber *et al.*, 2003). However, there are some striking differences between Hco-UNC-49 and Cel-UNC-49, as the heteromeric channel in *H. contortus* is more sensitive to GABA than is the homomeric channel (Siddiqui *et al*., 2010). This trend is reversed in *C. elegans*, where the heteromeric channel is less sensitive to GABA compared to the homomeric channel (Bamber *et al*., 1999). Differences such as these may extend to an overall functional difference in the GABAergic nervous system in *H. contortus* versus that of *C. elegans*. To date, Hco-UNC-49 expression has not been localized in *H. contortus*. However, if these receptors are expressed at the neuromuscular junction, as is the case in *C. elegans* (Bamber et al., 2005), they could prove to be an extremely important drug target in the treatment of *H. contortus* infection.

Objective

The overall objective of this research is to evaluate the pharmacological profile of the Hco-UNC-49 channel using classical $GABA_A$ receptor modulators, insecticides and currently used anthelmintics. This approach will determine how pharmacologically unique Hco-UNC-49 is compared to GABA receptors from other invertebrates as well as mammals. This may provide information on the overall potential of GABA receptors from parasitic nematodes as targets for novel anti-parasitic drugs.

Chapter 2

Introduction

GABA (γ-aminobutyric acid) is a major inhibitory neurotransmitter in both vertebrate and invertebrate nervous systems. GABA exerts its inhibitory effects through ionotropic GABA receptors. These receptors are members of the cys-loop ligand-gated ion channel superfamily which also contains the nACh, 5-HT_3, glutamate and glycine-gated ion channels. GABA receptor channels have been a focus of much study over the years since they play important roles in several human disorders such as anxiety and insomnia and are targets for several insecticides and anti-parasitic drugs (Concas *et al*., 1990; ffrench-Constant *et al*., 1991; Martin, 1982). Vertebrate GABA-Rs differ from invertebrate GABA-Rs in both pharmacology and overall function (Rauh *et al*., 1993; Sattelle, 1990). Therefore, these differences may be exploited in the development of pest-control and anthelmintic compounds which can be formulated to preferentially target invertebrate receptors while at the same time exhibit minimal toxicity to mammals.

Haemonchus contortus is a parasitic nematode that infects ruminants worldwide (Nikolaou and Gasser, 2006). Currently used anti-parasitic drugs that treat *H. contortus* infections include macrocyclic lactone anthelmintics such as ivermectin and moxidectin, which target glutamate-gated chloride channels, and levamisole which affect nAChRs. Indeed, most of the effective treatments against *H. contortus* target receptors found in the parasite nervous system which leads to paralysis of the worm and expulsion from

the ruminant host. Thus, the nervous system of parasitic nematodes remains an attractive target for drug discovery. Recently, two GABA receptor subunits (Hco-UNC-49B and Hco-UNC-49C) were isolated and characterized from *H. contortus* which are orthologues to well characterized neuromuscular GABA receptor subunits from the free-living nematode *Caenorhabditis elegans* (Cel-UNC-49) (Siddiqui *et al*., 2010; Bamber *et al*., 1999, 2003, 2005). While the UNC-49 receptors from the two nematodes share several similar characteristics, they exhibit a notable difference; specifically, for the *H. contortus* receptor, the UNC-49C subunit appears to be a positive modulator of GABA sensitivity, whereas in the *C. elegans* receptor, UNC-49C is a negative modulator. Differences such as these warrant further investigations into the functional characteristics of parasitic nematode receptors as this may have important implications for the development of new anthelmintics.

In this study, we have further characterized the *H. contortus* UNC-49 channel and tested the effects of various known GABA modulating drugs, some insecticides, and several anti-parasitic drugs. Results from this study indicate that the Hco-UNC-49 channel has binding sites for several molecules, is positively modulated by ivermectin and moxidectin, and is activated by high concentrations (mM) of piperazine. These results suggest that Hco-UNC-49 is an *in vivo* target for both macrocyclic lactone anthelmitics and piperazine.

Materials and Methods

Expression of unc-49b and unc-49c in Xenopus laevis oocytes

In order to optimize the expression of parasite protein in *X. laevis* oocytes, *Hco-unc-49b* (Genbank Accession #: EU939734.1) and *Hco-unc-49c* (Genbank Accession #: EU049602.1) were sub-cloned into the *Xenopus* expression vector pT7Ts. These constructs were then linearized and used as template to produce *Hco-unc-49b* and *Hco-unc-49c* cRNA via an *in vitro* transcription reaction using the T7 mMessage mMachine kit (Ambion, Austin, TX, USA). The cRNA was then precipitated with lithium chloride and resuspended in RNAse free water. *X. laevis* oocytes were injected with 50nL of *Hco-unc-49b* and/or *Hco-unc-49c* (0.5ng/nL) using a Drummond nanoject microinjector and incubated at 20^{o}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 100 µg/mL gentamycin (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Control oocytes were injected with 50nL of sterile water. For expression of the heteromeric channel, *Hco-unc-49b* and *Hco-unc-49c* cRNA was mixed in equal proportions prior to injection. Recordings were performed 2-5 days post injection.

GABA and Modulator Solutions

All compounds used were purchased from Sigma Aldrich, (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) except for moxidectin which was provided by Dr. Roger Prichard, McGill University. GABA, piperazine hexahydrate and penicillin G were

dissolved in ND96 to stock concentrations of 1M, 1M and 268mM, respectively whereas pregnenolone sulfate (119mM), propofol (50mM), dieldrin (83mM), fipronil (228mM), moxidectin and ivermectin (5mM) were dissolved in DMSO to stock concentrations indicated. Stock solutions were diluted with ND96 to desired concentrations for use in recordings.

Electrophysiological Recordings

To test the effect of the compounds against the Hco-UNC-49 channel, two electrode voltage clamp was performed using an Axoclamp 900A voltage clamp (Molecular Devices, Sunnyvale, CA, USA). Glass electrodes with Ag|AgCl wire were filled with 3M KCl and possessed resistances between 1-5 MΩ. Oocytes were then pierced with the electrodes and clamped at -60 mV for the duration of each recording. Solutions were perfused over clamped oocytes using an RC-1Z perfusion chamber (Warner Instrument Inc., Hamdan, CT, USA). Data was obtained and analyzed using Clampex software (Molecular Devices). To determine the effect that each compound has on the GABA response, oocytes were perfused with GABA in the presence of the compound and normalized to the response of the same concentration of GABA without compound. An average normalized effect and its corresponding standard error of the mean was calculated from the pool of oocytes (from different batches of frogs). Each oocyte represents a replicate experiment. A paired Student's *t*-test or the two-tailed Wilcoxon signed rank test was performed against the raw output data (current) to determine if

the effect of each compound was statistically significant ($P < 0.05$). IC_{50} values for picrotoxin inhibition were produced by generating dose response curves fitted to the equation: $I_{PTX+}/I_{PTX-} = 1/\{[PTX]/IC_{50})^n + 1\}$; I_{PTX+}/I_{PTX-} is the current generated by GABA when picrotoxin is present compared to GABA without picrotoxin, IC_{50} is the concentration of picrotoxin required to reduce the GABA response by 50% and n is the Hill coefficient. All graphs as well as dose response curves and analysis were produced using Graphpad Prism Software v5.0 (San Diego, CA, USA).

Results

Classical GABA modulators affect Hco-UNC-49

Known GABA modulators (penicillin G, pregnenolone sulfate and propofol) were tested against Hco-UNC-49 homomeric and heteromeric channels. None of these modulators produced a response on their own when applied to the Hco-UNC-49 channels. When co-applied with 50µM GABA, 10mM penicillin G inhibited the heteromeric channel by 29% ($P < 0.001$; n=10) and the homomeric channel by 11% ($P = 0.065$; n=10). Propofol (50µM) inhibited the heteromeric channel by 32% ($P = 0.009$; n=4) and enhanced the homomeric channel by 58% ($P = 0.031$; n=6). Pregnenolone sulfate inhibited the heteromeric channel by 11% ($P= 0.021$; n=13) and the homomeric channel by 17% ($P = 0.011$; n=14) (Figure 12a and b). These results demonstrate that Hco-UNC-49 is sensitive to modulation by several known GABA modulators.

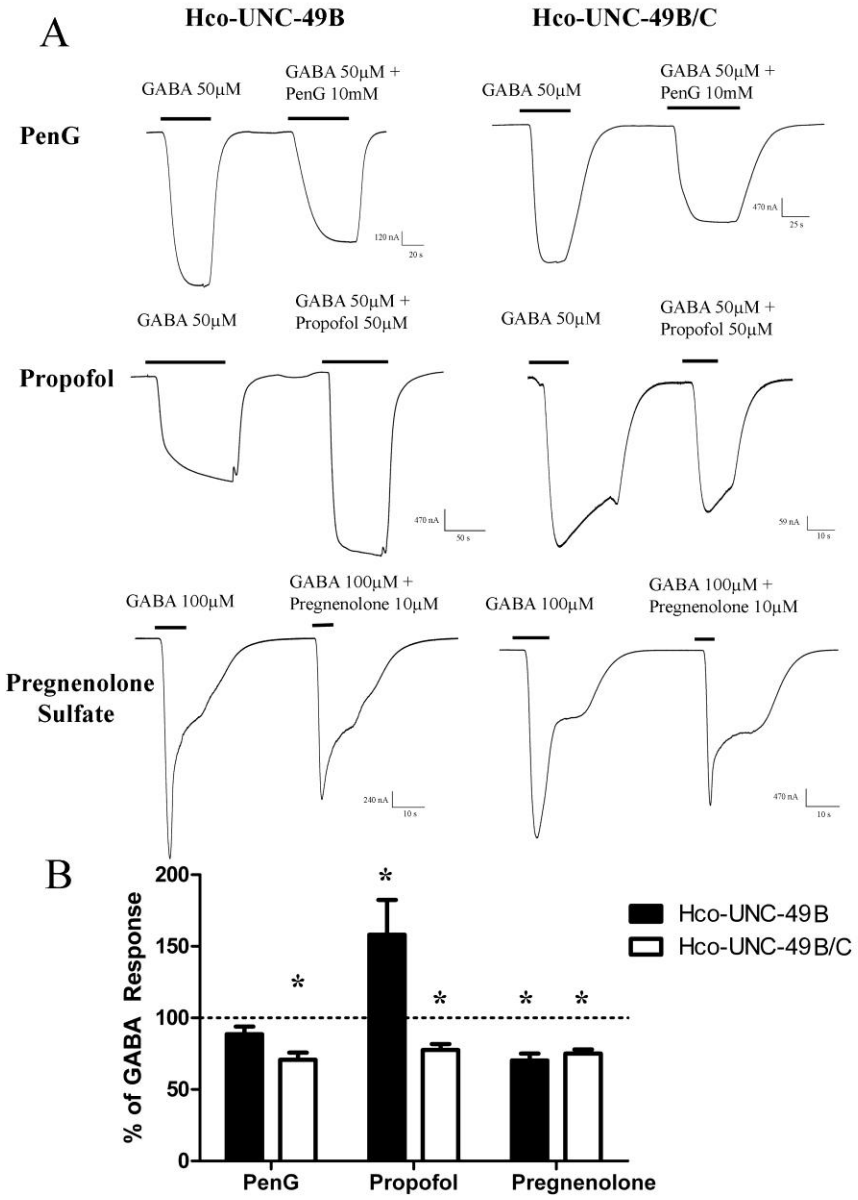

Figure 12: Hco-UNC-49 is modulated by known $GABA_A$ channel modulators. A) Responses of Hco-UNC-49 homomeric (left) and heteromeric (right) channels in response to the application of GABA and GABA in combination with penicillin G, propofol or pregnenolone sulfate. B) Overall modulatory effect of GABA modulators on Hco-UNC-49. Concentrations of modulators used are as follows: penicillin G (10mM), propofol (50µM), pregnenolone sulfate (10µM). GABA concentration used in penicillin G and propofol trials was 50µM and 100µM for the pregnenolone sulfate trials. Error bars represent SE of the mean. Bars marked with (*) denote modulator co-application effect was significantly different than the effect produced by GABA alone ($P \leq 0.05$).

The presence of Hco-UNC-49C causes both picrotoxin and fipronil resistance

Picrotoxin, dieldrin and fipronil, all known $GABA_A$ channel blockers, were applied to Hco-UNC-49 to determine the homomeric and heteromeric channel sensitivities to the blocking effects of these compounds. The insecticide dieldrin (10μM) exhibited very little effect causing a 3% enhancement in the homomeric and a 4% reduction in the heteromeric channels. Another insecticide, fipronil, caused a 49% reduction in current for the homomeric channel ($P < 0.001$; n=4). However, the heteromeric channel was resistant to fipronil and was inhibited by only 2% ($P = 0.051$; n=7) (Figure 13b). Picrotoxin had a similar effect to fipronil where it inhibited the homomeric channel to a greater degree compared to the heteromeric channel. To further characterize picrotoxin's effect on homomeric and heteromeric channels, inhibitory dose response trials using increasing concentrations of picrotoxin were performed. The IC_{50} of picrotoxin against the Hco-UNC-49 homomeric channel was 3.65 ± 0.64 μM (n=16) and for the heteromeric channel was 134.56 ± 44.12 μM (n=16) (Figure 13c).

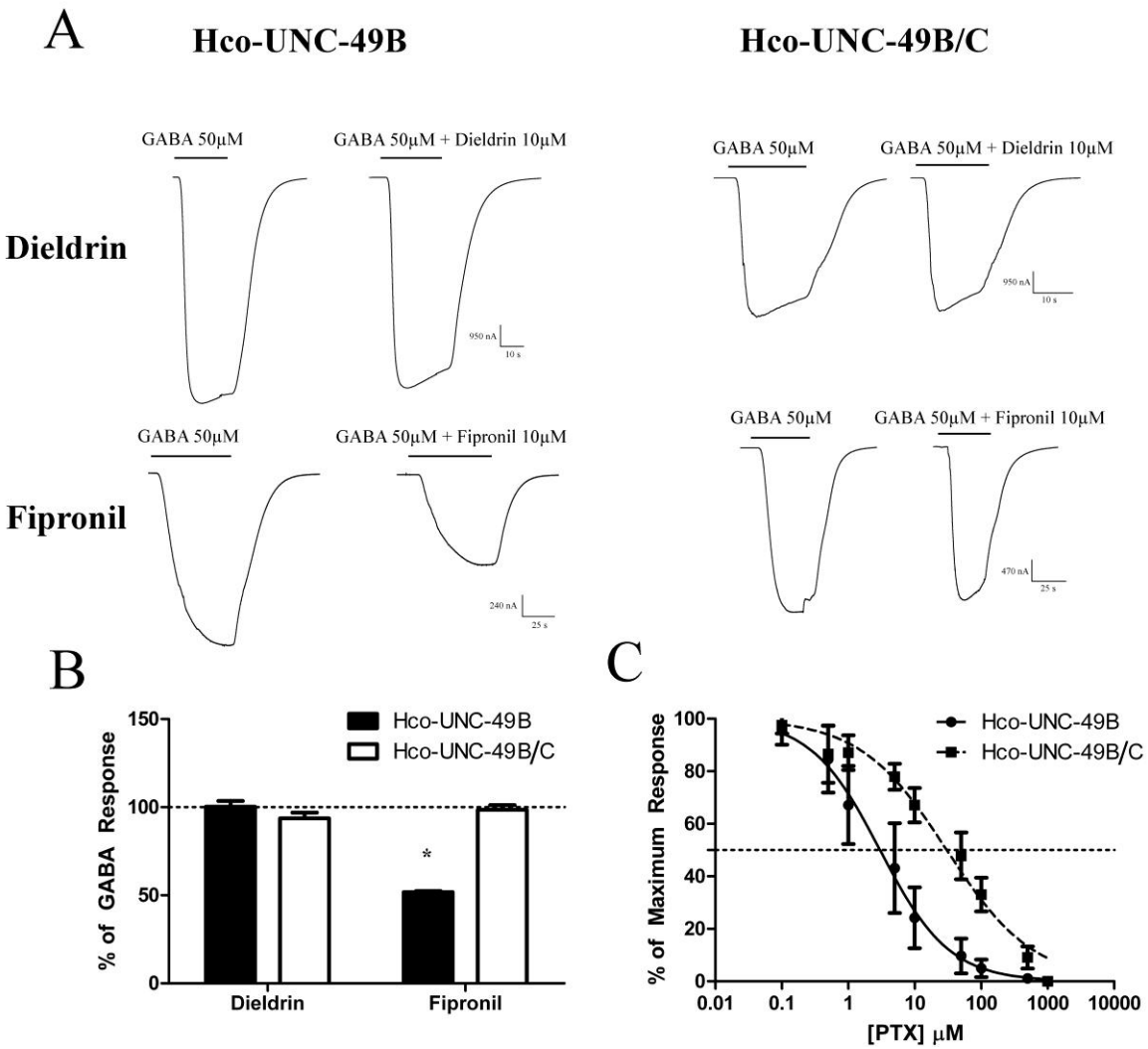

Figure 13: Hco-UNC-49C confers resistance to known GABA channel blockers. A) Responses of Hco-UNC-49 channels to GABA alone and the co-application of GABA and known channel blockers dieldrin or fipronil. B) Overall blocking effects of the insecticides dieldrin and fipronil (at 10 µM) on Hco-UNC-49 homomeric and heteromeric channels. The concentration of GABA used was 50µM. Error bars represent the SE of the mean. C) Inhibitory dose response of the Hco-UNC-49 homomeric and heteromeric channel with picrotoxin. The concentration of GABA used corresponded to the EC_{50} for the channel (40µM for Hco-UNC-49B/C and 64µM for Hco-UNC-49B). Error bars represent the SE of the mean. Bars marked with (*) denote modulator co-application effect was significantly different than the effect produced by GABA alone ($P \leq 0.05$).

Hco-UNC-49 is modulated by the anthelmintics ivermectin and moxidectin

Modulation of Hco-UNC-49 by several anthelmintic drugs (ivermectin, moxidectin and piperazine) was tested to determine if this channel could be a target for any of these drugs in the parasite (Figure 14). During co-application of ivermectin (10µM) with 50µM of GABA, both the homomeric and heteromeric Hco-UNC-49 channels displayed a large increase in response (homomeric channel 72.83 %, n=8 ($P < 0.001$); heteromeric channel 49.78 %, n=8 ($P < 0.001$)) compared to 50µM GABA alone (Figure 14b and d). A similar pattern was observed for the co-application of moxidectin (10µM) with 50µM GABA where the homomeric channel displayed a 50 % (n=4, $P = 0.05$) enhancement and the heteromeric channel showed a 40 % (n=6, $P = 0.007$) enhancement compared to 50µM GABA applied alone (Figure 14a and d). When piperazine (50µM) was co-applied with GABA (50µM), no modulatory effects were observed (Figure 14c and d).

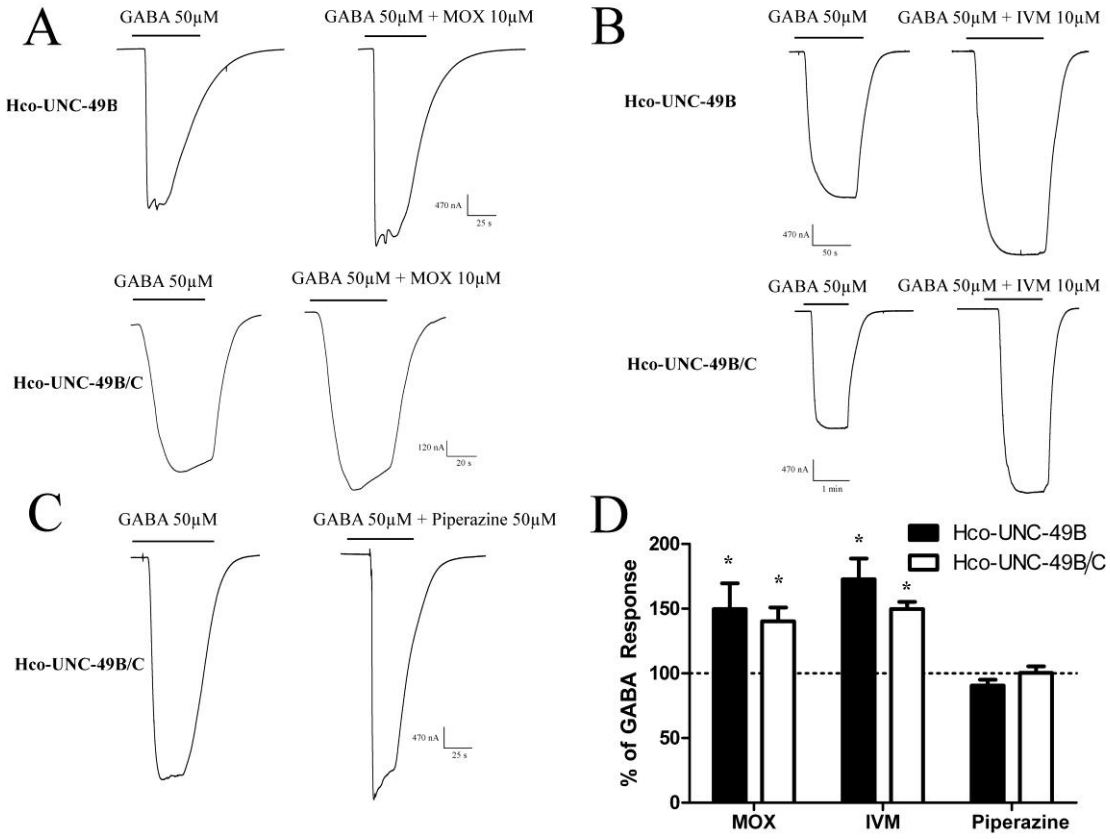

Figure 14: Anthelmintic modulation of Hco-UNC-49. Responses of Hco-UNC-49B homomeric and Hco-UNC-49B/C heteromeric channels to the co-application of GABA and the anthelmintics A) MOX B) IVM and C) Piperazine. D) Overall Hco-UNC-49 modulation by 10 µM MOX, 10 µM IVM and 50 µM piperazine. 50µM GABA was used in all trials. Error bars represent the SE of the mean. Bars marked with (*) denote modulator co-application effect was significantly different than the effect produced by GABA alone ($P \leq 0.05$).

The anthelmintic piperazine activates Hco-UNC-49 at high concentrations

Previous studies on parasite muscle tissue have strongly suggested that piperazine is capable of activating invertebrate GABA-gated chloride channels. Here, we have tested high concentrations of piperazine to see if this compound is capable of activating Hco-UNC-49. Piperazine concentrations between 2-4mM activated both Hco-UNC-49 homomeric and heteromeric channels (Figure 15a). In general, piperazine activated the channel at a rate comparable to GABA, and at 6 mM produced currents of -1394 ± 327 nA that were completely reversible after wash. Oocytes injected with water did not generate currents in response to the same concentrations of piperazine that activated Hco-UNC-49 (n= 4) (Figure 15b). Dose response studies indicate that piperazine activates Hco-UNC-49 homomeric channels with an EC_{50} value of 6.23 ± 0.45mM (n=8) and Hco-UNC-49 heteromeric channels with an EC_{50} value of 5.09 ± 0.32mM (n=7) (Figure 15c).

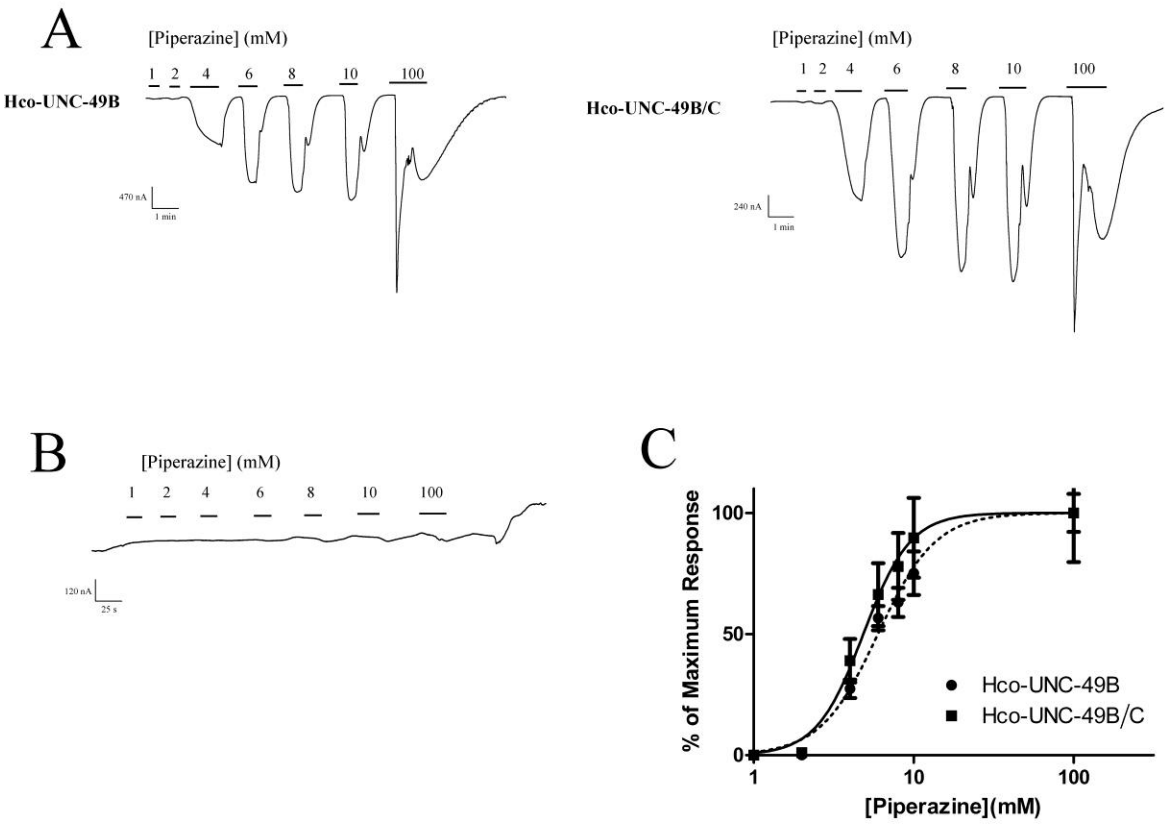

Figure 15: The anthelmintic piperazine activates Hco-UNC-49 at high concentrations. A) Piperazine dose response electrophysiological traces on the Hco-UNC-49 homomeric (left) and heteromeric channels (right). B) The effect of piperazine on water-injected oocytes. C) Piperazine dose response curves of Hco-UNC-49 channels. Error bars represent the SE of the mean.

Discussion

Hco-UNC-49 is the *H. contortus* orthologue of the well studied GABA-gated chloride channel Cel-UNC-49 from the free living nematode *C. elegans.* To further characterize this channel, the pharmacological profile of Hco-UNC-49 using several known GABA channel modulators (penicillin G, propofol, pregnenolone sulfate) was examined. The Hco-UNC-49 response to these three modulators demonstrated similar patterns as observed in Cel-UNC-49. Specifically, penicillin G inhibited both the homomeric and heteromeric channels, propofol enhanced the homomeric channel and inhibited the heteromeric channel, and pregnenolone sulfate inhibited both the homomeric and heteromeric channels (Bamber *et al*., 2003; current study). Previous research, however, has indicated that the UNC-49 channels from the two species do differ in their sensitivity to GABA (Siddiqui *et al*., 2010; Bamber *et al*., 1999). These differences are likely due to differences in the GABA binding site between the two species (Siddiqui *et al*., 2010). On the other hand, the similarity in the response of the modulators observed here suggests that the binding sites for these modulators in the UNC-49 channel from the two organisms may be similar. Indeed, the *H. contortus* and *C. elegans* UNC-49 channels share a high degree of amino acid sequence homology (>80%), so it is not surprising that the pharmacological profile examined here is similar to what has been observed previously.

To further characterize the pharmacological profile of Hco-UNC-49, the effects of several known GABA channel blockers (dieldrin, fipronil, picrotoxin) were tested. When comparing our results to what has been found for the *C. elegans* UNC-49 channel (Bamber *et al*., 2003), it appears that the Cel-UNC-49 homomeric channel is more sensitive to picrotoxin block compared to the Hco-UNC-49 homomeric channel with IC_{50} values of 0.9 ± 0.2µM (Bamber *et al*., 2003) and 3.65 ± 0.64µM (present study) respectively. Since the M2 region (thought to be the binding site for picrotoxin) in UNC-49B from both nematodes is identical, the differences in IC_{50} values may either be the result of differences in our experimental set up or it is possible that structural differences outside the M2 region contribute to overall picrotoxin sensitivity. Further, investigation will be required to determine whether there are indeed other structural elements on the UNC-49 receptor that may account for the different picrotoxin sensitivities between the *C. elegans* and *H. contortus* channel.

Similar to the *C. elegans* UNC-49 channel, Hco-UNC-49 was found to be highly resistant to dieldrin (Bamber *et al*., 2003) which is interesting because there is evidence that both picrotoxin and dieldrin inhibit GABA-Rs through the same mechanism (Kadous *et al*., 1983; Yarbrough *et al*., 1986; ffrench-Constant *et al*., 1993). However, based on our study and that of Bamber *et al*. (2003) it appears that picrotoxin and dieldrin inhibit GABA-gated chloride channels through different mechanisms. It is possible that dieldrin binds to the UNC-49 channel at a lower affinity compared to picrotoxin. While we have

not examined this in our study, one experiment (using either a binding assay or 2-electrode voltage clamp) could evaluate whether dieldrin can competitively displace or reduce picrotoxins effect. This would answer some of the questions regarding the binding capability of dieldrin on the UNC-49 channel.

Hco-UNC-49 responded to fipronil in a similar pattern as picrotoxin, where the Hco-UNC-49 homomeric channel was sensitive to fipronil block, and the heteromeric channel was highly resistant. Previous research has implicated two mutations in the M2 and M3 transmembrane domains (A302G, T350M) that contribute to fipronil resistance in *Drosophila simulans* RDL GABA receptor (Le Goff *et al*., 2005). It is interesting to note that Hco-UNC-49B naturally possesses the resistant-associated glycine residue at the position analogous to 302, and a valine residue at the position analogous to 350. Therefore, one could suggest that Hco-UNC49B should be somewhat resistant to fipronil. However, without conducting mutational analysis and detailed fipronil dose-response experiments on Hco-UNC49B, it is not known what effect these two positions have on the degree of fipronil sensitivity. Interestingly, Hco-UNC-49C possesses an isoleucine and cysteine residue, at the positions analogous to 302 and 350, respectively and when assembled with Hco-UNC-49B causes the channel to be fipronil resistant. However, whether these two residues within Hco-UNC-49C is the direct cause of the fipronil resistance in the heteromeric channels is unknown at this time.

Various anthelmintic compounds (ivermectin, moxidectin, piperazine) were screened for potential modulatory effects on Hco-UNC-49 to determine if this receptor could potentially play a role in the anti-parasitic effects produced by these compounds. Ivermectin and moxidectin were found to enhance both homomeric and heteromeric Hco-UNC-49 channels. This enhancement of GABA response could indicate a partial role of Hco-UNC-49 in larval and possibly adult paralysis by ivermectin and possibly moxidectin. It has been speculated that while glutamate-gated chloride channels are the primary targets for these compounds, GABA receptors may be a secondary target resulting in somatic muscle paralysis of *H. contortus* (Beech *et al*., 2010). Indeed, various studies, in addition to ours, have shown that macrocyclic lactones can enhance GABA-induced currents (Crichlow *et al*., 1986; Kruosek and Zemkova 1994; Feng *et al*., 2002). This study provides further mounting evidence that GABA receptors are an *in vivo* target for these macrocyclic lactone anthelmintics.

When piperazine is applied to the muscle tissue of *Ascaris suum* it was observed to evoke a GABA-like chloride current (Martin, 1982). This result suggested that piperazine is able to activate GABA-gated chloride channels. However, the identity of the receptor responsible for this effect has not been identified. In this study, it was found that piperazine activates Hco-UNC-49 at high concentrations in the mM range. This is consistent with the low potency of piperazine on *Ascaris* muscle tissue which has been shown to be roughly 100 times less potent than GABA (Martin, 1980, 1982). This is

consistent with our EC_{50} data for the Hco-UNC-49 channel which clearly shows piperazine to be about 100-130 fold less potent compared to GABA. Furthermore, it has been noted that the GABA receptor characterized in the piperazine-sensitive *A. suum* muscle tissue shares a similar pharmacological profile with UNC-49 (Bamber *et al*., 2003; Martin, 1993; Siddiqui *et al*., 2010). Additionally, there is evidence for the presence of an *unc-49*-like coding sequence in the *A. suum* genome (GenBank Accession Number: BM319703). All of these findings suggest that UNC-49 may play a key role in piperazine sensitivity in *A. suum* (Martin, 1982) and possibly *H. contortus*.

This research has resulted in several additional questions that could be explored. For instance, it still remains to be seen where Hco-UNC-49 is expressed in *H. contortus* and in what form (homomeric versus heteromeric), as this knowledge will aid in understanding the activity of several antiparasitic drugs against the nematode. Furthermore, mutational analysis may be useful in revealing which residues of Hco-UNC-49C are responsible for resistance against inhibition by picrotoxin and fipronil and would contribute to a deeper understanding of some of the functional elements of UNC-49 channels in comparison to GABA receptors from other invertebrates and mammals. Finally, a closer examination of where piperazine binds and how it activates UNC-49 relative to GABA would be important for an enhanced understanding of the activation kinetics and possibly the overall function of nematode GABA receptor channels.

Conclusion

This study has characterized the pharmacological profile of Hco-UNC-49 through the use of known GABA channel modulators, various insecticides and several anthelmintic compounds. Analysis of the modulation of Hco-UNC-49 by the known GABA modulators, penicillin G, propofol, and pregnenolone sulfate has revealed that Hco-UNC-49 shares a similar profile with that of Cel-UNC-49. It therefore appears that nematodes in this clade may share similar properties in regards to the effects of these compounds on UNC-49 GABA receptors. The similarity observed between the two species may aid in the identification and characterization of the binding sites of these compounds and the modulatory mechanisms that they initiate. In addition, the channel blocking effects of the insecticides dieldrin, fipronil and picrotoxin have revealed that Hco-UNC-49C bestows the heteromeric channel a high degree of resistance to these blockers and provides further evidence that dieldrin exhibits differences in its mode of action compared fipronil and picrotoxin (Bamber *et al*., 2003). Furthermore, Hco-UNC-49 was enhanced by the anthelmintics ivermectin and moxidectin. Though much emphasis has been placed on the GluCls as the physiological targets of these macrocyclic lactones, this study, and several others provide strong evidence that GABA-Rs may also be affected by these anthelmintics *in vivo*. Finally, this study has advanced our understanding of the GABA-like effect of the anthelmintic piperazine which, before this study, had only been characterized on the muscle tissue of *A. suum* (Martin, 1982).

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Appendicies

Appendix I: Mean GABA current (nA) values in the absence and presence of modulating/blocking compounds.

Compound	**Hco-UNC-49B**		**Hco-UNC-49B/C**	
	GABA Only	*GABA + Compound*	*GABA Only*	*GABA + Compound*
Penicillin G	-933 ± 124*	-819 ± 107	-2006 ± 220	-1491 ± 267
Propofol	-691 ± 280	-1096 ± 498	-631 ± 174	-511 ± 158
Pregnenolone Sulfate	-1995 ± 272	-1483 ± 166	-2837 ± 217	-2428 ± 148
Ivermectin	-1499 ± 200	-2421 ± 203	-1755 ± 208	-2581 ± 242
Moxidectin	-2413 ± 200	-3496 ± 166	-783 ± 192	-1037 ± 241
Piperazine	-2867 ± 278	-2798 ± 345	-3439 ± 561	-3600 ± 635
Dieldrin	-4280 ± 597	-4118 ± 545	-2998 ± 438	-2760 ± 371
Fipronil	-1292 ± 72	-671 ± 44	-2401 ± 170	-2349 ± 133

* Values are presented as number of nA ± SEM