

**Molecular characterization of GABA receptor subunits from the
parasitic nematode *Haemonchus contortus***

By

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ABSTRACT

Haemonchus contortus is a parasitic nematode that is controlled by several nematocides which target ion channels. We have identified two *H. contortus* ion channel genes, *Hco-unc-49B* and *C* that encode two GABA-gated chloride channel subunits. Electrophysiological analysis shows that the Hco-UNC-49B subunit forms a functional homomeric channel in *Xenopus laevis* oocytes that produces a robust response to GABA and is highly sensitive to picrotoxin. In contrast, Hco-UNC-49C alone does not respond to GABA but can assemble with Hco-UNC-49B to form a heteromeric channel with an increased sensitivity to GABA and a lower sensitivity to picrotoxin. To investigate the subunit requirements for high agonist sensitivity, we generated cross-assembled channels by co-expressing the *H. contortus* subunits with UNC-49 subunits from the nematode *Caenorhabditis elegans* (Cel-UNC-49). Co-expressing the Cel-UNC-49B with Hco-UNC-49C produced a heteromeric channel with a low sensitivity to GABA. In contrast, co-expressing Hco-UNC-49B with Cel-UNC-49C produced a heteromeric channel that was highly sensitive to GABA. These results suggest that the Hco-UNC-49B subunit is the key determinant for the high agonist sensitivity of heteromeric channels.

KEYWORDS

GABA, ligand-gated chloride channel, *H. contortus*, electrophysiology, homomeric channel, heteromeric channel

DEDICATION

This thesis is dedicated to my parents and my husband, who offered their immeasurable support and love throughout the duration of my masters and taught me the importance of pursuing my dreams. Without them, this thesis would not have been complete.

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LIST OF ABBREVIATIONS

5-HT ₃	serotonin-gated cation channel
AAD	amino acetonitrile derivatives
BD	binding domain
cDNA	copy deoxyribonucleic acid
cRNA	copy ribonucleic acid
cys-loop	cysteine-loop
Cys-loop LGIC	cysteine-loop ligand-gated ion channel
DMSO	dimethylsulfoxide
EC ₅₀	half maximal effective concentration
GABA	γ-amino butyric acid
GluCl	glutamate-gated chloride channel
LCCH3	ligand-gated chloride channel homolog 3
LGCC	ligand-gated chloride channel
LGIC	ligand-gated ion channel
MS-222	ethyl 3-amino benzoate methane sulfonate
nAChR	nicotinic acetylcholine receptor
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RT-PCR	reverse transcription polymerase chain reaction
RDL	resistance to dieldrin
cRNA	copy ribonucleic acid
rRNA	ribosomal ribonucleic acid

SL1	splice leader sequence 1
TBPS	t-butylbicyclophosphorothionate
UTR	untranslated region

CHAPTER 1
COMPREHENSIVE LITERATURE REVIEW

Haemonchus contortus

Nematodes are one of the most diverse groups of organisms known (Lorenzen and Platt, 1994). *H. contortus* is a gastrointestinal parasitic nematode, of the order Strongylida, which infects small ruminants such as cattle, sheep and goats (Nikolaou and Gasser, 2006). Infection with *H. contortus* is referred to as Haemonchosis (Mehlhorn, 2008). The parasite lives in the abomasum (one of the four stomach chambers) of its host where it latches onto the abomasum wall and feeds on blood. Infection of the host with *H. contortus* can result in changes in weight (partly due to impaired digestion), reduction in the quality and quantity of wool in sheep, decrease in milk production in cattle, lowered reproductive abilities (Parkins and Holmes, 1989), anemia and finally hypoproteinemia (Mehlhorn, 2008). The latter two complications are due to the blood-sucking nature of the parasite. The degree of which these symptoms may occur depends on host nutritional and immunological status, the level of infection and host age (Parkins and Holmes, 1989). In severe cases, *H. contortus* infection may lead to death (Nikolaou and Gasser, 2006). Due to these effects, these gastrointestinal parasites are the leading cause in productivity losses in the sheep meat and wool industries around the world (Newton and Meeusen, 2003).

Haemonchus contortus – Life cycle

The life cycle of *H. contortus* was initially described in detail by Veglia (1915). The life cycle begins when adult females lay their eggs in the abomasum of small ruminants. The eggs are laid in the four cell stage and are approximately 70 x 46 µm in size. The eggs are then released into the outside environment via the host's feces around

the 11 to 26 cell stage. Development beyond this stage requires the presence of oxygen, and must therefore occur outside the host. After excretion from the host, the eggs hatch in feces at approximately 14 to 17 hours, releasing L1 stage larvae (Figure 1.1). Development continues through stages L2 to L4 before reaching the adult stage. It is during the L3 stage of development that the parasite is infective. Sheep become infected by accidentally ingesting L3 larvae while grazing. Once inside the host, the parasite develops into the L4 stage and later will sexually differentiate and develop into male or female adult worms. L4's can also undergo hypobiosis, where their development ceases as a result of various seasonal, host-immune and parasite factors. Adult female worms lay their eggs after about 18 days following the host's oral infection (Stoll, 1929). Female adults lay around 4500 eggs daily (Coyne and Smith, 1992) and can grow to 25-30 mm x 170-380 μ m, whereas males can grow to 15-18 mm x 160-270 μ m (Nikolaou and Gasser, 2006).

Each stage of the *H. contortus* life-cycle occurs in two phases of development. The first being a period of activity and the second a period of inactivity, frequently termed "lethargis", where structural changes take place within the larvae (Nikolaou and Gasser, 2006). Following lethargis, the larvae are fully developed and can continue on to the next stage of development. The degree of growth in each life-cycle stage differs depending on the *H. contortus* strain as well as both environmental and host factors (Rossanigo and Gruner, 1996).

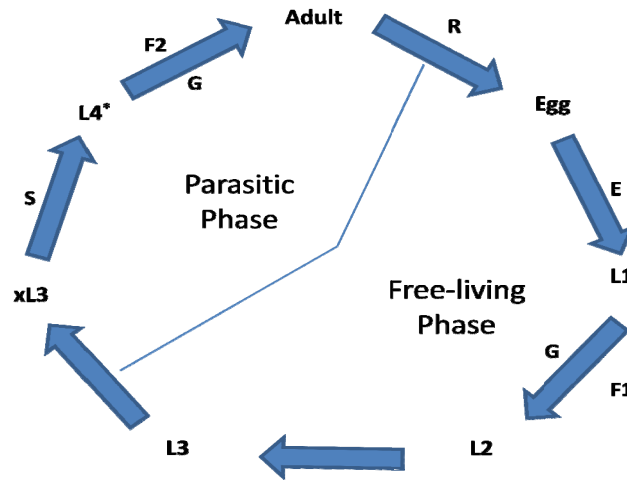


Figure 1.1: The developmental cycle of *H. contortus*. Specific features of development: E, embryogenesis; S, sexual differentiation; R, reproduction; F1, bacterial-feeding phase; F2, blood-feeding phase; G, rapid growth phases; *, indicates potential to undergo hypobiosis. L1, L2, L3, exsheathed L3 and L4 larval stages. (Adapted from Nikolaou and Gasser, 2006)

Anthelmintic Drugs

There currently are no vaccines available for the control of gastrointestinal nematodes. Therefore, the control of these infections relies primarily on chemotherapy, or specifically anthelmintic drugs (Kaminsky *et al.*, 2008).

Since the 1960's, there have been three major classes of anthelmintics used in veterinary medicine, these include benzimidazoles, imidazthiazoles and macrocyclic lactones. Benzimidazoles include thiabendazole, mebendazole and fenbendazole, which all have broad spectrum action against gastrointestinal nematodes (Martin *et al.*, 1997). The benzimidazole class of anthelmintics binds to a parasite protein called β -tubulin, which prevents the formation of microtubules (Sangster *et al.*, 1985) and are used for the control of nematode infections in ruminants, swine, horses, dogs, cats and humans (Harder *et al.*, 2003). The imidazthiazoles class of anthelmintics, which includes levamisole, act as agonists of the nematode nicotinic acetylcholine receptor (nAChR) (Martin *et al.*, 1997) and indirectly target acetylcholineesterase (Harder *et al.*, 2003).

Macrocyclic lactones are composed of two subclasses of anthelmintics, the avermectins and milbemycins. The avermectins and milbemycins were both isolated in the 1970s from a soil living *Streptomyces* species (Yates *et al.*, 2003). These drugs are active against both nematodes (endoparasites) as well as ectoparasites, but do not have a significant effect on tapeworms or flukes. The avermectins are comprised of ivermectin, abamectin, and doramectin (Martin *et al.*, 1997). The avermectin primarily used in livestock is ivermectin (Dourmishev *et al.*, 2005), whereas the milbemycin, moxidectin, is used in livestock as well as a preventative therapy in dogs and cats (Harder *et al.*, 2003). Both drugs are thought to primarily act on a specific class of invertebrate-specific receptors called the glutamate-gated chloride channels (GluCl_s) (Cully *et al.*, 1994, 1996; Dent *et al.*, 1997, 2000; Laughton *et al.*, 1997; Jagannathan *et al.*, 1999; Forrester *et al.*, 1999, 2002; Cheeseman *et al.*, 2001; Horoszok *et al.*, 2001). The avermectins may act on GluCl_s that are associated with the nematode pharynx and thus inhibits pharyngeal pumping required for feeding. An inhibition in motility may also be associated with the action of macrocyclic lactones (Geary *et al.*, 1993).

Several other smaller classes of anthelmintics that target other ion channels have been discovered. Piperazine acts as a γ -amino butyric acid (GABA)-gated chloride channel agonist in nematode somatic muscle. It acts to increase the chloride conductance which leads to hyperpolarization and relaxation of the somatic muscle, resulting in paralysis (Martin, 1982, 1985). Praziquantel is thought to act on calcium channels, increasing the permeability of the trematode tegument (covering) to calcium, causing muscle contractions (Martin *et al.*, 1997). This specific drug is used to control the trematode *Schistosoma mansoni*, which is the causative agent of schistosomiasis

(Redman *et al.*, 1996). Lastly, a new class of anthelmintics has been recently discovered called, amino-acetonitrile derivatives (AADs). AADs are thought to act on a nematode specific group of acetylcholine receptor subunits, and exhibit broad spectrum activity on sheep and cattle nematodes (Kaminsky *et al.*, 2008).

Due to the reliance and poorly managed use of anthelmintics, resistance to each new anthelmintic class is becoming increasingly widespread (Newton and Meeusen, 2003). To date, *H. contortus* has become resistant to all known anthelmintics, with the exception of the newly discovered AADs. The first case of resistance to the avermectins was reported in 1988 in South Africa and is becoming increasingly common in Australasia, Africa and South America (Newton and Meeusen, 2003). In some areas resistance is becoming so prevalent that the profitability of the entire sheep industry has been threatened (Besier and Love, 2003). Resistance is a serious obstacle to agriculturally dependant incomes in many parts of the world. Therefore, ongoing research into new anthelmintics is essential in order to maintain a level of parasite control appropriate for modern agriculture.

Ligand-gated Ion Channels

Ligand-gated ion channels (LGICs) play key roles in rapid synaptic transmission when bound to a ligand, such as a neurotransmitter. The binding of a ligand causes a conformational change in the channel, allowing for channel opening (For review see Unwin, 1993). This open channel conformation permits the flow of ions down their electrochemical gradients. The resulting pathway, a few atoms in diameter, is formed for only a matter of milliseconds. However, thousands of ions of the correct charge are able

to pass through the channel (Unwin, 1993). The function of these channels is to respond to a stimulus such as a neurotransmitter and quickly modify the potential of the post-synaptic membrane.

The cysteine-loop ligand-gated ion channels (cys-loop LGICs) are neurotransmitter receptors with a characteristic protein structure. Each channel is composed of five LGIC subunits embedded in the cell membrane in such a way that they create a central pore (Figure 1.2A) (Hille, 1992). Each subunit is characterized by a large extracellular N-terminal ligand binding domain (Figure 1.2B). This region contains the cysteine-loop (cys-loop), which is formed by two cysteine residues bonded together by a disulphide bond (Cockcroft *et al.*, 1990). In addition, each subunit contains four trans-membrane spanning regions, M1-M4, with the M2 lining the pore of the ion channel and determining the charge of the ions allowed to pass (Chebib and Johnston, 2000). An intracellular loop of variable sequence and length is located between the M3 and M4 regions and is generally thought to contribute to subtype specificity and intracellular regulatory processes (Olsen and Tobin, 1990).

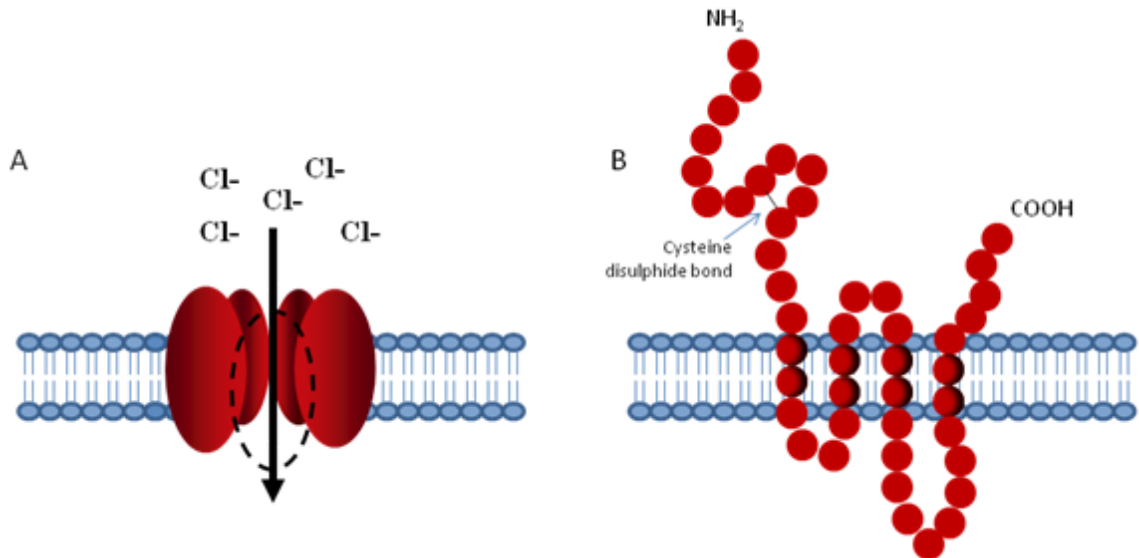


Figure 1.2: Structure of LGICs from the cys-loop family A. The channel is formed by 5 subunits arranged in a ring-like configuration (the fifth subunit denoted by the dashed shape). B. Schematic representation of the topology of one subunit of a LGIC. The subunit topology of the four transmembrane spanning loops (TM1-TM4) and large N-terminal domain containing the cys-loop is shown. (Adapted from Raymond and Sattelle, 2002)

The subunit combination can change the properties of the resulting channel, such as neurotransmitter affinity, localization and pharmacology (Bamber *et al.*, 1999, 2003, 2005).

Four primary types of LGICs have been recognized in vertebrates, which are organized based on their associated ligand. The excitatory or cationic channels include the nAChRs and serotonin-gated cation channels (5-HT₃) (Ortells and Lunt, 1995). The inhibitory or anionic channels include the GABA_A receptors and glycine receptors (Ortells and Lunt, 1995).

Invertebrate LGICs not only include the excitatory nAChRs and inhibitory GABA receptors, but a broad array of unusual LGICs. For example, there have been several novel ligand-gated chloride channels (LGCCs) identified that have been shown to be activated by neurotransmitters such as histamine (Gisselmann *et al.*, 2001; Zheng *et al.*, 2002), glutamate (Cully *et al.*, 1994), acetylcholine (Putrenko *et al.*, 2005) serotonin

(Ranganathan *et al.*, 2000), dopamine (Rao *et al.*, 2009) and tyramine (Pirri *et al.*, 2009). In addition, excitatory GABA receptors have been identified in both *C. elegans* and *Drosophila melanogaster* (Beg and Jorgensen, 2003; Gisselmann *et al.*, 2004).

Mammalian GABA-gated chloride channels

GABA is the most widely distributed inhibitory neurotransmitter found throughout the vertebrate central nervous system (Sivilotti and Nistri, 1991). In mammals, GABA is able to activate three different classes of receptors, GABA_A, GABA_B and GABA_C receptors. GABA_A and GABA_C receptors are LGCCs, whereas GABA_B receptors are part of the G-protein coupled receptor superfamily (McKernan and Whiting, 1996).

When activated, the GABA_A receptors allow for the influx of Cl⁻, causing hyperpolarization of the associated neuron. Hence, the GABA_A receptors are a means of decreasing neuronal excitability by providing inhibitory input. In mammals, these receptors are expressed in the peripheral and central nervous system and have modulatory binding sites for benzodiazepines, barbiturates, and neurosteroids (Figure 1.3) (Bormann, 1988; MacDonald and Olsen, 1994). The convulsant bicuculline acts as a competitive antagonist for the GABA_A receptor and picrotoxin acts as non-competitive channel blocker (Enna and Mohler, 2007).

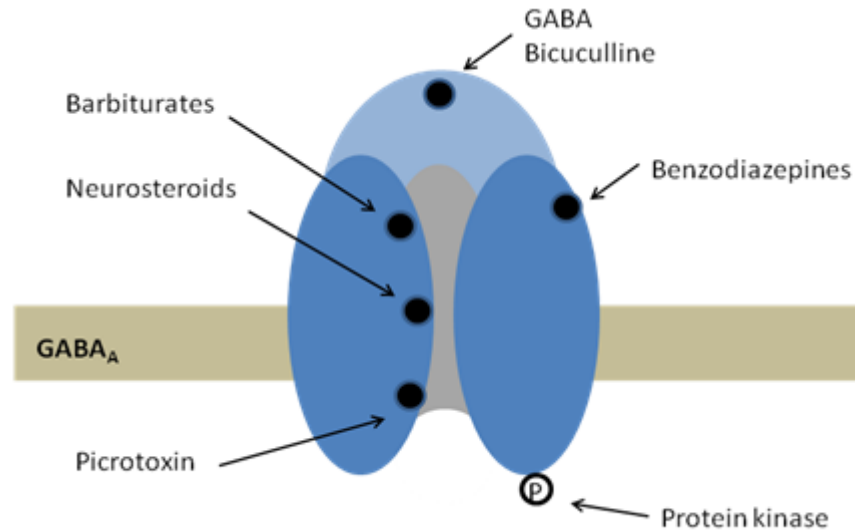


Figure 1.3. The GABA_A receptor which is a LGCC containing modulatory sites for benzodiazepines, barbiturates and neurosteroids. Channel activity is blocked by picrotoxin and bicuculline. Intracellular modulation is achieved by protein kinases (Adapted from Bormann, 2000).

The protein sequences of the GABA_A receptor subunits show 20-30% sequence similarity with other LGICs such as the nAChR, the glycine receptor and the 5-HT₃ receptor (Mehta and Ticku, 1999). Vertebrate GABA_A subunits can be organized into several subtypes, including α (6 isoforms), β (3 isoforms), γ (3 isoforms), δ , ϵ , θ , π (1 isoform each) and ρ (3 isoforms) (Mehta and Ticku, 1999). There is approximately 70% amino acid identity between isoforms within the same class and approximately 30% amino acid identity between subtypes (Mehta and Ticku, 1999). Studies have shown that the GABA_C receptors may actually be composed solely of ρ subunits that primarily localizes to the mammalian retina (Cutting *et al.*, 1991; Enz and Cutting, 1998; Feigenspan and Bormann, 1998). Their classification as a separate group of GABA receptors is no longer supported by the International Union of Pharmacology which recommends they be classified as a minor subtype of the GABA_A receptor family (Barnard *et al.*, 1998).

While a large variety of subunit types have been identified, the precise combination of the native GABA_A receptor remains unknown. The actual number of subtypes and isoforms identified allows for the assembly of an extremely large number of pentameric channel combinations. However, not all combinations of subunits are able to respond to GABA. It is estimated that the actual number of native vertebrate GABA_A receptor subunit combinations occurring in the nervous system may be less than 20 (McKernan and Whiting, 1996). However, the majority of vertebrate GABA_A receptors are likely composed of α , β and γ subunits (Pritchett *et al.*, 1989; Pritchett and Seeburg, 1991; Malherbe *et al.*, 1990). Although the α and β subunits are able to form functional GABA responsive channels *in vitro*, they lack several GABA_A receptor characteristics (Pritchett *et al.*, 1988). For example, the inclusion of the γ subunit is necessary for receptor sensitivity to benzodiazepines (Pritchett *et al.*, 1989). In addition to channel pharmacology, the GABA_A receptor subunit combinations determine receptor trafficking and localization. It was found that the γ_2 subunit is important for bringing receptors to synaptic regions, whereas the δ subunit is involved in targeting receptors to extra-synaptic regions (Nusser *et al.*, 1998; Wei *et al.*, 2003).

The major GABA binding site was identified by radiolabeling purified GABA_A receptors with [³H]muscimol and [³H]flunitrazepam. This method identified binding sites on both the α and β subunits (Deng *et al.*, 1986; Bureau and Olsen, 1988), indicating that the GABA binding site may be located at the interface of the two subunits.

GABA neurotransmission in C. elegans

In the model free living nematode, *Caenorhabditis elegans*, GABA containing neurons are mostly associated with neuromuscular junctions and comprises less than 10% of the nervous system (Docherty *et al.*, 1985). In contrast, mammalian GABA receptors act in the central nervous system and are involved in 30% to 40% of synapses (Docherty *et al.*, 1985). Analysis of the *C. elegans* genome suggests that nematodes possess both GABA_A and GABA_B-like receptors (Jorgensen, 2005). However, the vertebrate GABA_A receptor subtype and isoform classification is not conserved, making comparisons between nematodes and mammals more challenging.

GABA acts mainly as an inhibitory neurotransmitter in *C. elegans* (Jorgensen, 2005). GABA synthesis occurs in the neuron by glutamic acid decarboxylase, which converts the amino acid glutamate to GABA (Figure 1.4). As GABA must be packaged for its subsequent release from the pre-synaptic neuron, its transport is mediated through a synaptic vesicle by the vesicular GABA transporter (Schuske *et al.*, 2004). Once released from the pre-synaptic neuron, it can act on inhibitory GABA receptors (on the post-synaptic neuron), causing the influx of anions into the neuron (Schuske *et al.*, 2004).

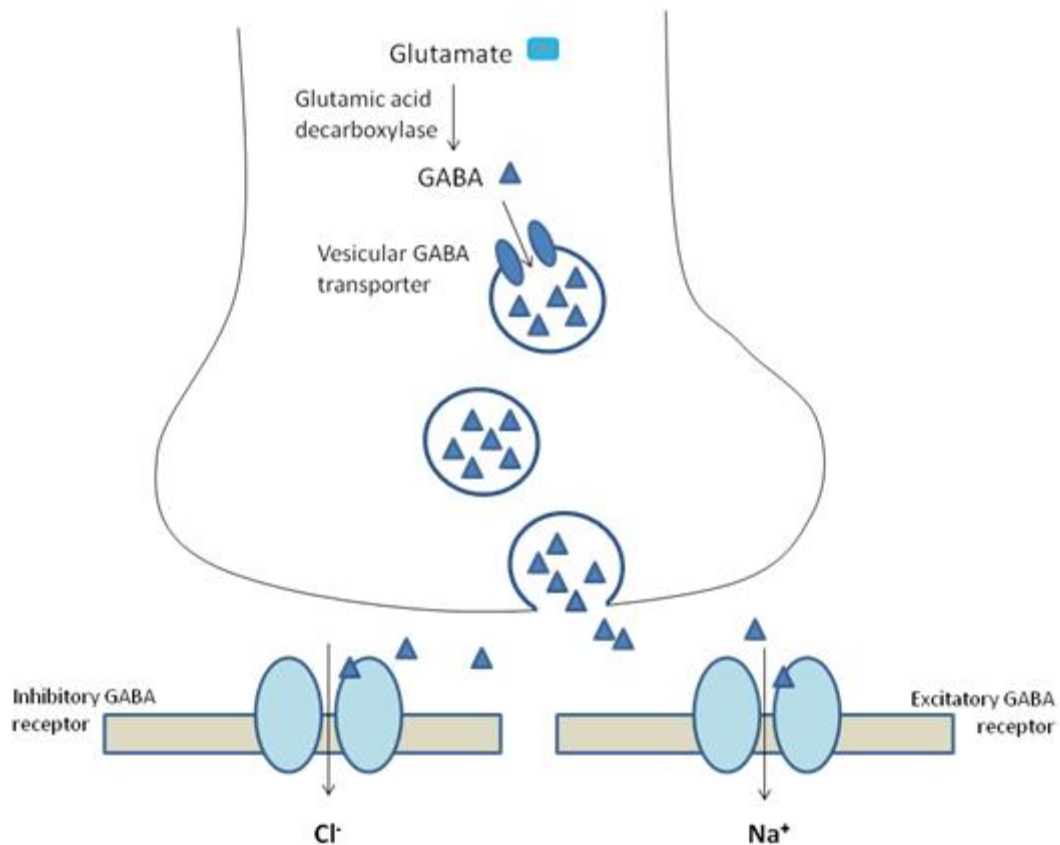


Figure 1.4. GABA synthesis and transport. GABA is synthesized in the neuron from glutamate using the biosynthetic enzyme glutamic acid decarboxylase. GABA is then transported into a vesicle with the aid of the vesicular GABA transporter and subsequently transported to the plasma membrane, for release. GABA released from the presynaptic neuron is free to act on inhibitory or excitatory GABA receptors (Adapted from Schuske *et al.*, 2004)

In order to identify which neurons are GABAergic in *C. elegans*, McIntire (*et al.*, 1993a) stained the nematode with antibodies against GABA. It was found that of the 302 neurons present (White *et al.*, 1986) 26 contained GABA. Specifically, they identified 19 motor neurons that innervate the dorsal and ventral body muscles, 4 motor neurons that innervate the head muscles, 2 motor neurons that innervate the enteric muscles and finally 1 interneuron. The parasitic nematode, *Ascaris suum* appears to have a similar pattern of GABA localization compared to *C. elegans* (Johnson and Stretton, 1987; Guastella *et al.*, 1991).

Laser ablation studies of *C. elegans* GABAergic neurons were performed in order to identify GABA's actual role in the nematode (McIntire *et al.*, 1993b). Motor neurons innervating the dorsal and ventral body wall muscles are responsible for worm movement. Movement is characterized by a sinusoidal wave in the nematodes' body. Essentially a bend in the body is made by contracting muscles on one side of the body by excitatory input, while at the same time relaxing muscles on the opposite side of the body by inhibitory input (McIntire *et al.*, 1993b). When the neurons innervating the dorsal and ventral body wall muscles are laser ablated, the worm is not able to move in the sinusoidal pattern as efficiently and when touched, it hyper-contracts muscles on both sides of the body (McIntire *et al.*, 1993b), indicating a loss of muscle inhibition. Therefore, it was suggested that GABA neurons are responsible for the inhibitory input used in locomotion (McIntire *et al.*, 1993b).

The motor neurons innervating the head region regulate foraging, where the tip of the nose moves side to side (Jorgensen, 2005). Ablating the GABA containing neurons in the head region results in worms with an exaggerated, side to side, foraging movement. It is thus thought that these GABA containing neurons in the head are involved in relaxing the muscles on the opposite site of the bend to restore the head to its forward position (Jorgensen, 2005). Therefore GABA is again playing a role in neuromuscular inhibition.

The GABA motor neurons identified in the enteric muscles are responsible for nematode defecation (McIntire *et al.*, 1993b). The worm undergoes a series of muscular contractions that eventually results in the excretion of intestinal matter. These muscular contractions begin with the posterior body muscles, followed by the anterior body

muscles and end with the contraction of the enteric muscles (Thomas, 1990). When the GABA containing motor neurons associated with the enteric muscles are laser ablated, the worm is no longer able to contract the enteric muscles and expel its intestinal contents and as a result the worm swells with food and waste (McIntire *et al.*, 1993b). This effect suggests an excitatory role of GABA in enteric muscle contractions, rather than the characteristic inhibitory role seen in vertebrates.

GABA Receptors in C. elegans

Observations from the above laser ablation studies have been complemented by the identification of specific GABA receptors in *C. elegans*. *exp-1* encodes a GABA-gated cation channel and was originally identified by mutants with defects in their defecation cycle (Thomas, 1990). Mutants of *exp-1* were able to move and forage normally, however they were unable to undergo enteric muscle contractions required for release of their intestinal contents (McIntire *et al.*, 1993b). *exp-1* was first isolated and characterized by Beg and Jorgensen (2003) and identified as the only gene known to be specifically required for excitatory GABA functions. *exp-1* encodes a protein closely related to the LGIC subunits and shares 22% identity to another *C. elegans* GABA receptor subunit, UNC-49B, and 21% identity to the human $\beta 2$ GABA receptor subunit (Beg and Jorgensen, 2003). The primary variation observed when compared to other GABA receptor subunits is in the M2 domain (Beg and Jorgensen, 2003) where ion selectivity occurs (Chebib and Johnston, 2000). Electrophysiological analysis showed EXP-1 as able to form homomeric channels (ion channels composed of a single population of subunits) that are highly sensitive to GABA and to a lesser degree

responsive to the GABA agonist, muscimol. In contrast to all other GABA-gated anion channels, EXP-1 is selective for monovalent cations and is not sensitive to the classic GABA_A receptor channel blocker, picrotoxin (Beg and Jorgensen, 2003).

The GABA_A receptors responsible for inhibiting body muscle contraction during movement and abnormal foraging are encoded by the *unc-49* gene (Schuske *et al.*, 2004). Studies by Bamber (*et al.*, 1999) demonstrated that the *unc-49* gene, under the control of one promoter, encodes three different GABA_A receptor subunits. This unusual gene structure is conserved with *Caenorhabditis briggsae*, a related nematode (Schuske *et al.*, 2004). Through alternative splicing, the subunits share a common N-terminal domain but different GABA-binding and trans-membrane domains, generating UNC-49A, UNC-49B and UNC-49C. However, only UNC-49B and UNC-49C are expressed at high levels, while UNC-49A has a low expression in adult worms (Bamber *et al.*, 1999). Electrophysiological studies show that when expressed in *Xenopus* oocytes, UNC-49B alone is able to form functional homomers, whereas UNC-49C is not. In addition, green fluorescent protein tagging in *C. elegans* shows UNC-49B and UNC-49C both localize to neuromuscular junctions and when both are expressed in oocytes, they co-assemble to form functional GABA receptors (Bamber *et al.*, 1999). However, all *unc-49* mutants (or worms with abnormal locomotion) lack functional UNC-49B but none of these mutants lack UNC-49C, suggesting that only UNC-49B is required for receptor function (Bamber *et al.*, 2005). It is currently predicted that UNC-49B is responsible for channel activation, and interacts with the cytoskeleton at the neuromuscular synapse, enabling synaptic localization (Figure 1.5) (Bamber *et al.*, 2005). UNC-49C is thought to negatively

regulate receptor function by reducing sensitivity to GABA and increasing its desensitization (Bamber *et al.*, 2005).

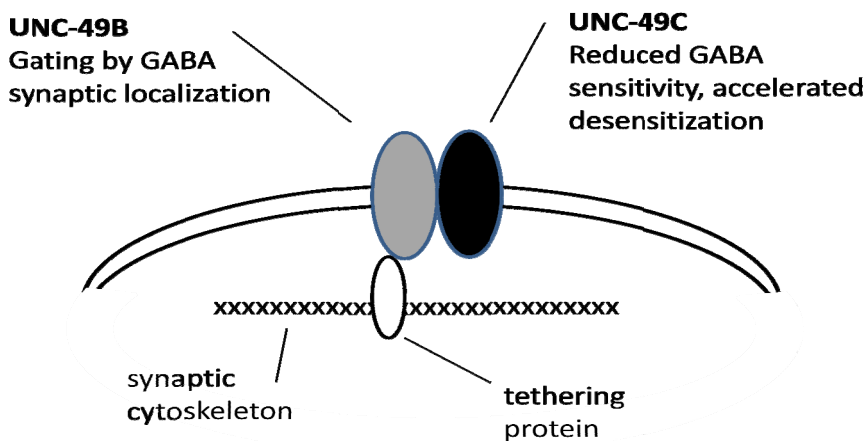


Figure 1.5. The UNC-49 heteromeric channel at the synapse. UNC-49B predicted to play a role in channel activation and interaction with the synaptic cytoskeleton. UNC-49C decreases GABA sensitivity and increases desensitization. UNC-49C associates with UNC-49B to localize to the synapse. (Adapted from Bamber *et al.*, 2005)

Although UNC-49C is not thought to be required for receptor function, it may offer an evolutionary advantage that has yet to be investigated. The evidence presented above does not preclude the possibility that another subunit can co-assemble with the UNC-49B/C heteromer and alter the receptor's pharmacological properties.

GABA Receptors in H. contortus

Although several GABA receptors have been identified in *C. elegans*, the identification of GABA receptors in *H. contortus* remains limited. In fact, only one GABA-gated chloride channel subunit has been identified in *H. contortus*. This inhibitory amino acid receptor subunit was first identified by Laughton *et al.*, (1994) called *HG1*. Initial electrophysiological analysis showed that *HG1* was unable to form a homomeric GABA responsive channel (Laughton *et al.*, 1994). When compared to

vertebrate GABA receptors, HG1 has the closest similarity to the α/γ subunit sequences of GABA_A receptor subunits (Feng *et al.*, 2002). Vertebrate GABA_A α and γ subunits generally need to be expressed in combination with a β subunit to form functional channels (Mehta and Ticku, 1999). Thus, a β -like GABA_A receptor subunit sequence from *C. elegans*, GAB-1, was isolated and co-expressed with HG1 for further electrophysiological characterization (Feng *et al.*, 2002). It was demonstrated that the HG1 GABA receptor subunit was able to co-assemble with the *C. elegans* GAB-1 to produce a GABA-sensitive channel, suggesting that HG1 is in fact from the GABA receptor subunit family. However, since HG1 requires the GAB-1 subunit for channel assembly, it is not known how relevant this subunit is for the biology of *H. contortus*.

Other Evidence for the Role of GABA in parasitic nematodes

In another closely related parasitic nematode, *Ascaris suum*, the presence of extra-synaptic inhibitory GABA receptors were identified in muscle bag cells due to their inhibitory response to locally applied GABA (Martin, 1980; Holden-Dye *et al.*, 1989). These receptors were initially believed to have close similarity to the vertebrate GABA_A receptors, although typical vertebrate GABA_A receptor agonists, including bicuculline, dieldrin and t-butylbicyclophosphorothionate (TBPS) had weak actions on the *Ascaris suum* GABA receptors (Martin, 1980; Holden-Dye *et al.*, 1989; Martin *et al.*, 1991). However, the anti-parasitic drug, piperazine, thought to act on GABA receptors, was found to have a hyperpolarizing effect on the *Ascaris* muscle (Martin, 1985). Immunolocalization studies using antibodies raised against the *H. contortus* HG1 subunit

was able to detect possible HG1-like subunits in *Ascaris* neuromuscular junctions, which is consistent with the localization observed in *H. contortus* (Skinner *et al.*, 1998).

GABA receptors in other invertebrates

GABA receptors in insects are the targets of several different classes of insecticides as well as convulsants. Studies into the mechanism of resistance to the insecticide dieldrin lead to the isolation of a GABA-gated chloride channel gene from *Drosophila* called *rdl* (resistance to dieldrin) (Ffrench-constant *et al.*, 1991). A mutation in this gene appears to confer cyclodiene resistance, which was caused by an amino acid change from alanine to serine at position 302 located in the M2 channel pore lining domain (Ffrench-constant *et al.*, 1991). Electrophysiological characterization of the wild type RDL channel found that it is equally responsive to GABA and the GABA receptor agonist muscimol. In addition, the GABA-gated chloride channel blocker, picrotoxin, and the cyclodiene insecticide dieldrin, were able to inhibit the GABA response. The mutant RDL homomeric channel appears to have a similar response to GABA and muscimol as the wild type, but was no longer sensitive to the inhibitory effects of picrotoxin and dieldrin. The mutation in the M2 domain appears to provide the resulting channel with approximately 100 fold less sensitivity to picrotoxin compared to the wild type (Ffrench-constant *et al.*, 1993). The resistant nature of the mutant RDL has been suggested to be caused by the mutation in the M2 region located near the picrotoxin/cyclodiene binding site, causing a direct structural change in the antagonist binding site, and as a result weakening the bound drug conformation (Zhang *et al.*, 1994). RDL subunits have been localized to the adult and embryonic nervous system of *D.*

melanogaster and appear to play a role in processing olfactory, visual and mechanosensory information (Harrison *et al.*, 1996; Aronstein *et al.*, 1996).

A LGCC subunit from *D. melanogaster* with high similarity to vertebrate GABA_A β subunits was discovered, and called ligand-gated chloride channel homolog 3 (LCCH3) (Henderson *et al.*, 1993). This subunit alone is unable to form functional homomeric channels (Zhang *et al.*, 1995; Gisselmann *et al.*, 2004). When simultaneously expressed with RDL, the resulting RDL/LCCH3 heteromeric channel (ion channels composed of more than one population of subunits) had different properties than the RDL subunit on its own. The heteromeric channel is insensitive to picrotoxin as well as bicuculline and has a reduced sensitivity to GABA (Zhang *et al.*, 1995). However, this channel is not thought to function as an *in vivo* channel since RDL and LCCH3 localize to different tissues (Aronstein and French-constant, 1995; Aronstein *et al.*, 1996) and expression of each gene occurs in different stages in *D. melanogaster* (Aronstein *et al.*, 1996; Harrison *et al.*, 1996).

A third *D. melanogaster* subunit named GRD has been cloned, and has a high degree of similarity with vertebrate ionotropic GABA_A β receptor subunits (Harvey *et al.*, 1994). Electrophysiological analysis revealed that GRD does not form functional homomeric channels (Gisselmann *et al.*, 2004). However when expressed simultaneously with LCCH3, the GRD/LCCH3 channel responds to GABA and muscimol. Interestingly, the heteromeric channel was not sensitive to the antagonistic effects of bicuculline. When attempting to identify the ions that flow through this GRD/LCCH3 channel, results indicated channel permeability to both sodium and potassium, with no significant contribution of chloride ions. This suggested that the

GRD/LCCH3 channel is forming a cation selective channel (Gisselmann *et al.*, 2004). This channel however has little homology to the *C. elegans* cation channel EXP-1 (described above). Since the localization of GRD has not yet been studied, it is not known if GRD and LCCH3 are expressed in the same tissues and life-stages. Therefore, whether these subunits would assemble *in vivo* is currently unknown.

Conclusion and rationale for current study

It is clear from the available literature that most of our current understanding of the role of GABA in the nervous system of invertebrates has emerged mostly from two model organisms, the nematode *C. elegans* and the fruit-fly *Drosophila melanogaster*. While information from these two organisms provides a very important basis for our understanding of GABAergic neurotransmission in lower organisms, it may not entirely relate to other invertebrate organisms. Parasitic nematodes have an entirely different lifestyle and may therefore exhibit differences in their specific GABAergic nervous system when compared to *C. elegans*. Therefore, a more comprehensive understanding of these channels in parasitic nematodes will allow us to understand the differences in GABA neurotransmission between parasitic and non-parasitic nematodes. This may lead to a better understanding of a nervous system designed specifically for parasitism and may in turn lead to the discovery of new targets for future anti-parasitic drugs. This thesis will focus on one aspect of the GABA nervous system of a parasitic nematode and describe the isolation and function of two GABA receptor subunits, Hco-UNC-49B and C, from the parasitic nematode *H. contortus*, which are orthologues of the UNC-49 subunits from the free-living nematode, *C. elegans*.

REFERENCES

- Aronstein,K. and Ffrench-Constant,R. 1995. Immunocytochemistry of a novel GABA receptor subunit Rdl in *Drosophila melanogaster*. *Invertebr.Neurosci.* (1) 25-31.
- Aronstein,K., Auld,V. and Ffrench-Constant,R. 1996. Distribution of two GABA receptor-like subunits in the Drosophila CNS. *Invertebr.Neurosci.* (2) 115-120.
- Bamber,B.A., Beg,A.A., Twyman,R.E. and Jorgensen,E.M. 1999. The *Caenorhabditis elegans unc-49* Locus Encodes Multiple Subunits of a Heteromultimeric GABA Receptor. *The Journal of Neuroscience* (19) 5348-5359.
- Bamber, B. A., Twyman, R. E., & Jorgensen, E. M. (2003). Pharmacological characterization of the homomeric and heteromeric UNC-49 GABA receptors in *C. elegans*. *British Journal of Pharmacology*, 138, 883-893.
- Bamber,B.A., Richmond,J.E., Otto,J.F. and Jorgensen,E.M. 2005. The composition of the GABA receptor at the *Caenorhabditis elegans* neuromuscular junction. *Br.J.Pharmacol.* (144) 502-509.
- Barnard,E.A., Skolnick,P., Olsen,R.W., Mohler,H., Sieghart,W., Biggio,G., Braestrup,C., Bateson,A.N. and Langer,S.Z. 1998. International Union of Pharmacology. XV. Subtypes of γ -aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. *Pharmacol.Rev.* (50) 291-314.
- Beg,A.A. and Jorgensen,E.M. 2003. EXP-1 is an excitatory GABA-gated cation channel. *Nat.Neurosci.* (6) 1145-1152.
- Besier, R.B, Love, S.C.J. 2003. Anthelmintic resistance in sheep nematodes in Australia: the need for new approaches. *Aust.J.Exp.Agric.* (43) 1383-1391.
- Bormann, J. 1988. Electrophysiology of GABA_A and GABA_B receptor subtypes. *Trends Neurosci.* (11) 112-116.
- Bormann, J. 2000. The "ABC" of GABA receptors. *Trends Pharmacol. Sci.* (21) 16-19.
- Bureau,M. and Olsen,R.W. 1988. GABA/benzodiazepine receptor protein carries binding sites for both ligands on two major peptide subunits. *Biochem. Biophys. Res. Commun.* (153) 1006-1011.
- Chebib,M. and Johnston,G. 2000. GABA-activated ligand gated ion channels: medicinal chemistry and molecular biology. *J.Med.Chem.* (43) 1427-1447.
- Cheeseman,C.L., Delany,N.S., Woods,D.J. and Wolstenholme,A.J. 2001. High-affinity ivermectin binding to recombinant subunits of the *Haemonchus contortus* glutamate-gated chloride channel. *Molecular & Biochemical Parasitology* (114) 161-168.

- Cockcroft, V.B., Osguthorpe, D.J., Barnard, E.A. and Lunt, G.G. 1990. Modeling of agonist binding to the ligand-gated ion channel superfamily of receptors. *Proteins: Structure, Function, and Genetics* (8) 386-397.
- Coyne, M.J. and Smith, G. 1992. The mortality and fecundity of *Haemonchus contortus* in parasite-naive and parasite-exposed sheep following single experimental infections. *Int.J.Parasitol.* (22) 315-325.
- Cully, D.F., Vassilatis, D.K., Liu, K.K., Paress, P.S., Van der Ploeg, L.H.T., Schaeffer, J.M. and Arena, J.P. 1994. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. *Nature* (371) 707-711.
- Cully, D.F., Paress, P.S., Liu, K.K., Schaeffer, J.M. and Arena, J.P. 1996. Identification of a *Drosophila melanogaster* glutamate-gated chloride channel sensitive to the antiparasitic agent avermectin. *J.Biol.Chem.* (271) 20187-20191.
- Cutting, G.R., Lu, L., O'hara, B.F., Kasch, L.M., Montrose-Rafizadeh, C., Donovan, D.M., Shimada, S., Antonarakis, S.E., Guggino, W.B. and Uhl, G.R. 1991. Cloning of the gamma-aminobutyric acid (GABA) rho 1 cDNA: a GABA receptor subunit highly expressed in the retina. *Proc.Natl.Acad.Sci.U.S.A.* (88) 2673-2677.
- Deng, L., Ransom, R.W., Olsen, R.W. 1986. [3H] muscimol photolabels the γ -aminobutyric acid receptor binding site on a peptide subunit distinct from that labeled with benzodiazepines. *Biochemical and biophysical research communications* (Print) (138) 1308-1314.
- Dent, J.A., Smith, M.H.M., Vassilatis, D.K. and Avery, L. 2000. The genetics of ivermectin resistance in *Caenorhabditis elegans*. *Proc.Natl.Acad.Sci.* (97) 2674-2679.
- Dent, J., Davis, M. and Avery, L. 1997. avr-15 encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in *Caenorhabditis elegans*. *EMBO J.* (16) 5867-5879.
- Docherty, M., Bradford, H.F., Wu, J.Y., Joh, T.H. and Reis, D.J. 1985. Evidence for specific immunolysis of nerve terminals using antisera against choline acetyltransferase, glutamate decarboxylase and tyrosine hydroxylase. *Brain Res.* (339) 105-113.
- Dourmishchev, A.L., Dourmishchev, L.A. and Schwartz, R.A. 2005. Ivermectin: pharmacology and application in dermatology. *Int.J.Dermatol.* (44) 981-988.
- Enna, S. J., & Mohler, H. (Eds.). 2007. *The GABA receptors* (3rd ed.). Totowa, NJ: Humana Press. doi:10.1007/978-1-59745-465-0
- Enz, R. and Cutting, G.R. 1998. Molecular composition of GABA_C receptors. *Vision Res.* (38) 1431-1441.

- Feigenspan,A. and Bormann,J. 1998. GABA-gated Cl⁻ channels in the rat retina. *Prog.Retin.Eye Res.* (17) 99-126.
- Feng,X.P., Hayashi,J., Beech,R.N. and Prichard,R.K. 2002. Study of the nematode putative GABA type-A receptor subunits: evidence for modulation by ivermectin. *J.Neurochem.* (83) 870-878.
- Ffrench-Constant,R.H., Mortlock,D.P., Shaffer,C.D., MacIntyre,R.J. and Roush,R.T. 1991. Molecular cloning and transformation of cyclodiene resistance in *Drosophila*: an invertebrate gamma-aminobutyric acid subtype A receptor locus. *Proc.Natl.Acad.Sci.U.S.A.* (88) 7209-7213.
- Ffrench-Constant,R.H., Rocheleau,T.A., Steichen,J.C. and Chalmers,A.E. 1993. A point mutation in a *Drosophila* GABA receptor confers insecticide resistance. *Nature* (363) 449-451.
- Forrester,S.G., Hamdan,F.F., Prichard,R.K. and Beech,R.N. 1999. Cloning, Sequencing, and Developmental Expression Levels of a Novel Glutamate-Gated Chloride Channel Homologue in the Parasitic Nematode *Haemonchus contortus*. *Biochem.Biophys.Res.Commun.* (254) 529-534.
- Forrester,S.G., Prichard,R.K. and Beech,R.N. 2002. A glutamate-gated chloride channel subunit from *Haemonchus contortus*: expression in a mammalian cell line, ligand binding, and modulation of anthelmintic binding by glutamate. *Biochem.Pharmacol.* (63) 1061-1068.
- Geary,T.G., Sims,S.M., Thomas,E.M., Vanover,L., Davis,J.P., Winterrowd,C.A., Klein,R.D., Ho,N.F. and Thompson,D.P. 1993. *Haemonchus contortus*: ivermectin-induced paralysis of the pharynx. *Exp.Parasitol.* (77) 88-96.
- Gisselmann,G., Plonka,J., Pusch,H. and Hatt,H. 2004. *Drosophila melanogaster* GRD and LCCH3 subunits form heteromultimeric GABA-gated cation channels. *Br.J.Pharmacol.* (142) 409-413.
- Gisselmann,G., Pusch,H., Hovemann,B.T. and Hatt,H. 2001. Two cDNAs coding for histamine-gated ion channels in *D. melanogaster*. *Nat.Neurosci.* (5) 11-12.
- Guastella,J., Johnson,C.D. and Stretton,A.O.W. 1991. GABA-immunoreactive neurons in the nematode *Ascaris*. *J.Comp.Neurol.* (307) 584-597.
- Harder,A., Schmitt-Wrede,H.P., Krücken,J., Marinovski,P., Wunderlich,F., Willson,J., Amliwala,K., Holden-Dye,L. and Walker,R. 2003. Cyclooctadepsipeptides—an anthelmintically active class of compounds exhibiting a novel mode of action. *Int.J.Antimicrob.Agents* (22) 318-331.

- Harrison,J.B., Chen,H.H., Sattelle,E., Barker,P.J., Huskisson,N.S., Rauh,J.J., Bai,D. and Sattelle,D.B. 1996. Immunocytochemical mapping of a C-terminus anti-peptide antibody to the GABA receptor subunit, RDL in the nervous system of *Drosophila melanogaster*. *Cell Tissue Res.* (284) 269-278.
- Harvey,R.J., Schmitt,B., Hermans-Borgmeyer,I., Gundelfinger,E.D., Betz,H. and Darlison,M.G. 1994. Sequence of a *Drosophila* ligand-gated ion-channel polypeptide with an unusual amino-terminal extracellular domain. *J.Neurochem.* (62) 2480-2483.
- Henderson,J.E., Soderlund,D.M. and Knipple,D.C. 1993. Characterization of a putative gamma-aminobutyric acid (GABA) receptor beta subunit gene from *Drosophila melanogaster*. *Biochem.Biophys.Res.Commun.* (193) 474-482.
- Hille, B. Ionic channels of excitable membranes 1992. *Sunderland, MA: Sinauer Associates*, 4-19.
- Holden-Dye,L., Krogsgaard-Larsen,P., Nielsen,L. and Walker,R.J. 1989. GABA receptors on the somatic muscle cells of the parasitic nematode, *Ascaris suum*: stereoselectivity indicates similarity to a GABA_A-type agonist. *Br.J.Pharmacol.* (98) 841-850.
- Horoszok,L., Raymond,V., Sattelle,D.B. and Wolstenholme,A.J. 2001. GLC-3: a novel fipronil and BIDN-sensitive, but picrotoxinin-insensitive, L-glutamate-gated chloride channel subunit from *Caenorhabditis elegans*. *Br.J.Pharmacol.* (132) 1247-1254.
- Jagannathan,S., Laughton,D.L., Critten,C.L., Skinner,T.M., Horoszok,L. and Wolstenholme,A.J. 1999. Ligand-gated chloride channel subunits encoded by the *Haemonchus contortus* and *Ascaris suum* orthologues of the *Caenorhabditis elegans* gbr-2 (avr-14) gene. *Molecular & Biochemical Parasitology* (103) 129-140.
- Johnson,C.D. and Stretton,A.O. 1987. GABA-immunoreactivity in inhibitory motor neurons of the nematode *Ascaris*. *Journal of Neuroscience* (7) 223-235.
- Jorgensen, E.M. GABA (August 31, 2005), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.14.1, <http://www.wormbook.org>
- Kaminsky,R., Ducray,P., Jung,M., Clover,R., Rufener,L., Bouvier,J., Weber,S.S., Wenger,A., Wieland-Berghausen,S. and Goebel,T. 2008. A new class of anthelmintics effective against drug-resistant nematodes. *Nature* (452) 176-180.
- Laughton,D.L., Amar,M., Thomas,P., Towner,P., Harris,P., Lunt,G.G. and Wolstenholme,A.J. 1994. Cloning of a putative inhibitory amino acid receptor subunit from the parasitic nematode *Haemonchus contortus*. *Recept.Channels* (2) 155-163.

- Laughton,D.L., Lunt,G.G. and Wolstenholme,A.J. 1997. Reporter gene constructs suggest that the *Caenorhabditis elegans* avermectin receptor beta-subunit is expressed solely in the pharynx. *J.Exp.Biol.* (200) 1509-1514.
- Lorenzen,S. and Platt,H. 1994. 1994. The phylogenetic systematics of free living nematodes. Ray Society, London.
- Macdonald, R.L., Olsen, R.W. 1994. GABA_A receptor channels. *Annu.Rev.Neurosci.* (17) 569-602.
- Malherbe,P., Sigel,E., Baur,R., Persohn,E., Richards,J.G. and Mohler,H. 1990. Functional characteristics and sites of gene expression of the alpha 1, beta 1, gamma 2-isoform of the rat GABA_A receptor. *Journal of Neuroscience* (10) 2330-2337.
- Martin,R.J. 1980. The effect of γ -aminobutyric acid on the input conductance and membrane potential of *Ascaris* muscle. *Br.J.Pharmacol.* (71) 99-106.
- Martin,R.J. 1982. Electrophysiological effects of piperazine and diethylcarbamazine on *Ascaris suum* somatic muscle. *Br.J.Pharmacol.* (77) 255-265.
- Martin,R.J. 1985. γ -Aminobutyric acid and Piperazine activated single channel currents from *Ascaris suum* body muscle. *Br.J.Pharmacol.* (84) 445-461.
- Martin,R.J., Pennington,A.J., Duittoz,A.H., Robertson,S. and Kusel,J.R. 1991. The physiology and pharmacology of neuromuscular transmission in the nematode parasite, *Ascaris suum*. *Parasitology* (102 Suppl) S41-58.
- Martin,R.J., Robertson,A.P. and Bjorn,H. 1997. Target sites of anthelmintics. *Parasitology* (114) 111-124.
- McIntire,S.L., Jorgensen,E. and Horvitz,H.R. 1993a. Genes required for GABA function in *Caenorhabditis elegans*. *Nature* (364) 334-337.
- McIntire,S.L., Jorgensen,E., Kaplan,J. and Horvitz,H.R. 1993b. The GABAergic nervous system of *Caenorhabditis elegans*. *Nature* (364) 337-341.
- McKernan,R.M. and Whiting,P.J. 1996. Which GABA_A-receptor subtypes really occur in the brain? *Trends Neurosci.* (19) 139-143.
- Mehlhorn, H. (Ed.). (2008). *Encyclopedia of parasitology* (3rd ed.) Springer Berlin Heidelberg.
- Mehta,A.K. and Ticku,M.K. 1999. An update on GABA_A receptors. *Brain Res.Rev.* (29) 196-217.

- Newton,S.E. and Meeusen,E.N.T. 2003. Progress and new technologies for developing vaccines against gastrointestinal nematode parasites of sheep. *Parasite Immunol.* (25) 283-296.
- Nikolaou,S. and Gasser,R.B. 2006. Prospects for exploring molecular developmental processes in *Haemonchus contortus*. *Int.J.Parasitol.* (36) 859-868.
- Nusser,Z., Sieghart,W. and Somogyi,P. 1998. Segregation of different GABA_A receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *Journal of Neuroscience* (18) 1693-1703.
- Olsen, RW, Tobin, AJ. 1990. Molecular biology of GABA_A receptors. *The FASEB Journal* (4) 1469-1480.
- Ortells,M.O. and Lunt,G.G. 1995. Evolutionary history of the ligand-gated ion-channel superfamily of receptors. *Trends Neurosci.* (18) 121-127.
- Parkins,J.J. and Holmes,P.H. 1989. Effects of gastrointestinal helminth parasites on ruminant nutrition. *Nutrition Research Reviews* (2) 227-246.
- Pirri,J.K., McPherson,A.D., Donnelly,J.L., Francis,M.M. and Alkema,M.J. 2009. A Tyramine-gated chloride channel coordinates distinct motor programs of a *Caenorhabditis elegans* escape response. *Neuron* (62) 526-538.
- Pritchett,D.B. and Seeburg,P.H. 1991. Gamma-Aminobutyric acid type A receptor point mutation increases the affinity of compounds for the benzodiazepine site. *Proc.Natl.Acad.Sci.U.S.A.* (88) 1421.
- Pritchett,D.B., Sontheimer,H., Gorman,C.M., Kettenmann,H., Seeburg,P.H. and Schofield,P.R. 1988. Transient expression shows ligand gating and allosteric potentiation of GABA_A receptor subunits. *Science* (242) 1306-1308.
- Pritchett,D.B., Sontheimer,H., Shivers,B.D., Ymer,S., Kettenmann,H., Schofield,P.R. and Seeburg,P.H. 1989. Importance of a novel GABA_A receptor subunit for benzodiazepine pharmacology. *Nature* (338) 582-585.
- Putrenko,I., Zakikhani,M. and Dent,J.A. 2005. A family of acetylcholine-gated chloride channel subunits in *Caenorhabditis elegans*. *J.Biol.Chem.* (280) 6392-6398.
- Ranganathan,R., Cannon,S.C. and Horvitz,H.R. 2000. MOD-1 is a serotonin-gated chloride channel that modulates locomotory behaviour in *C. elegans*. *Nature* (408) 470-475.

- Rao,V.T.S., Siddiqui,S.Z., Prichard,R.K. and Forrester,S.G. 2009. A dopamine-gated ion channel (HcGGR3*) from *Haemonchus contortus* is expressed in the cervical papillae and is associated with macrocyclic lactone resistance. *Molecular & Biochemical Parasitology* (166) 54-61.
- Raymond,V. and Sattelle,D.B. 2002. Novel animal-health drug targets from ligand-gated chloride channels. *Nat.Rev.Drug Discov.* (1) 427-436.
- Redman,C., Robertson,A., Fallon,P., Modha,J., Kusel,J., Doenhoff,M. and Martin,R. 1996. Praziquantel: an urgent and exciting challenge. *Parasitology Today* (12) 14-20.
- Rossanigo,C.E. and Gruner,L. 1996. The length of strongylid nematode infective larvae as a reflection of developmental conditions in faeces and consequences on their viability. *Parasitol.Res.* (82) 304-311.
- Sangster,N.C., Prichard,R.K. and Lacey,E. 1985. Tubulin and benzimidazole-resistance in *Trichostrongylus colubriformis* (Nematoda). *J.Parasitol.* (71) 645-651.
- Schuske,K., Beg,A.A. and Jorgensen,E.M. 2004. The GABA nervous system in *C. elegans*. *Trends Neurosci.* (27) 407-414.
- Sivilotti,L. and Nistri,A. 1991. GABA receptor mechanisms in the central nervous system. *Prog.Neurobiol.* (36) 35-92.
- Skinner,T.M., Bascal,Z.A., Holden-Dye,L., Lunt,G.G. and Wolstenholme,A.J. 1998. Immunocytochemical localization of a putative inhibitory amino acid receptor subunit in the parasitic nematodes *Haemonchus contortus* and *Ascaris suum*. *Parasitology* (117) 89-96.
- Stoll,N.R. 1929. Studies with the Strongyloid nematode, *Haemonchus contortus*: I. Acquired resistance of hosts under natural re-infection conditions out-of-doors. *Am.J.Epidemiol.* (10) 384-418.
- Thomas,J.H. 1990. Genetic analysis of defecation in *Caenorhabditis elegans*. *Genetics* (124) 855-872.
- Unwin,N. 1993. Neurotransmitter action: opening of ligand-gated ion channels. *Neuron-Cambridge MA-* (10) 31-41.
- Veglia,F. 1915. The anatomy and life-history of the *Haemonchus contortus* (Rud). Third and fourth annual reports of the Director of Veterinary Research. Pretoria, Union of South Africa, 349–500.
- Wei,W., Zhang,N., Peng,Z., Houser,C.R. and Mody,I. 2003. Perisynaptic localization of δ subunit-containing GABA_A receptors and their activation by GABA spillover in the mouse dentate gyrus. *Journal of Neuroscience* (23) 10650-10661.

White,J.G., Southgate,E., Thomson,J.N. and Brenner,S. 1986. The structure of the nervous system of the nematode *Caenorhabditis elegans*. Philosophical Transactions of the Royal Society of London. Series B, *Biological Sciences* (314) 1-340.

Yates,D.M., Portillo,V. and Wolstenholme,A.J. 2003. The avermectin receptors of *Haemonchus contortus* and *Caenorhabditis elegans*. *Int.J.Parasitol.* (33) 1183-1193.

Zhang,H.G. 1994. A unique amino acid of the *Drosophila* GABA receptor with influence on drug sensitivity by two mechanisms. *J.Physiol.(Lond.)* (479) 65-75.

Zhang,H.G., Lee,H.J., Rocheleau,T., Ffrench-Constant,R.H. and Jackson,M.B. 1995. Subunit composition determines picrotoxin and bicuculline sensitivity of *Drosophila* gamma-aminobutyric acid receptors. *Mol.Pharmacol.* (48) 835-840.

Zheng,Y., Hirschberg,B., Yuan,J., Wang,A.P., Hunt,D.C., Ludmerer,S.W., Schmatz,D.M. and Cully,D.F. 2002. Identification of two novel *Drosophila melanogaster* histamine-gated chloride channel subunits expressed in the eye. *J.Biol.Chem.* (277) 2000-2005.

CHAPTER 2

**MOLECULAR CHARACTERIZATION OF NOVEL GABA-GATED
CHLORIDE CHANNEL SUBUNITS FROM *HAEMONCHUS*
*CONTORTUS***

*The initial cloning of *Hco-unc-49C* was conducted as an undergraduate research project
(Salma Siddiqui, UOIT Honours Thesis, 2007)

INTRODUCTION

Livestock infected with parasitic nematodes can cause great losses in productivity in the agricultural sector. One gastrointestinal nematode responsible for great economic hardship is the blood-feeding parasite, *Haemonchus contortus*, which resides in the abomasum of sheep and goats where it causes anemia and other complications (Mehlhorn, 2008). Infection with *H. contortus* is commonly controlled through different classes of anthelmintics that target various proteins including β -tubulin (targets for benzimidazoles) as well as receptors called ligand-gated chloride channels (LGCCs). Currently used drugs that target LGCCs include the macrocyclic lactones which activate glutamate-gated chloride channels (GluCl_s) and piperazine which is thought to target γ -amino butyric acid (GABA)-gated chloride channels (Kohler, 2001). However, increased drug resistance has rendered several currently available anthelmintics, including the macrocyclic lactones, less effective (Prichard, 1994) and thus, there is an ongoing need to discover novel protein targets for future anti-parasitic drugs.

Mammalian GABA_A-gated chloride channels play an important role in the function of the inhibitory nervous system and are mainly found in the brain. Several classes of GABA_A- receptor subunits have been identified in mammals which include α , β , γ , δ , ϵ , π , θ and ρ subunits (for review see Mehta and Ticku, 1999; Whiting, 2003). The individual subunits are part of the cysteine-loop ligand-gated ion channel (cys-loop LGIC) superfamily. Each subunit exhibits a large extracellular N-terminal region containing a cysteine-loop (cys-loop) (Cockcroft *et al.*, 1990), and four highly conserved membrane spanning domains (M1- M4) with the second membrane spanning domain lining the channel pore (Unwin, 1993). The various types of subunits assemble as

pentamers and the different combinations of these subunits can result in channels with different properties and pharmacology (Mehta and Ticku, 1999; Sieghart, 1995).

Invertebrates, such as nematodes and arthropods, are also known to utilize GABA_A receptors for inhibitory neurotransmission. One particular class of GABA_A receptors, the RDLs (Resistance to Dieldrin), appear to be in many ways divergent from mammalian GABA_A receptors and thus may be unique to invertebrates (Dent, 2006). In insects, RDL receptors have been shown to be associated with cyclodiene resistance (Ffrench-constant *et al.*, 1991) and appear to play a role in GABA-mediated processing of olfactory, visual and mechanosensory information (Harrison *et al.*, 1996; Aronstein *et al.*, 1996). In the model free-living nematode, *Caenorhabditis elegans*, three RDL-like subunits (UNC-49A, UNC-49B and UNC-49C) have been characterized and are encoded by a single gene that is differentially spliced (Bamber *et al.*, 1999). When expressed in *Xenopus laevis* oocytes, the Cel-UNC-49B subunit alone is able to form a functional homomeric channel and can assemble with Cel-UNC-49C to form a functional heteromeric channel that exhibits a reduced sensitivity to GABA (Bamber *et al.*, 1999) and differing responses to various channel modulators and inhibitors (Bamber *et al.*, 2003). The Cel-UNC-49B/C heteromeric channel appears to be the native channel which is expressed at neuromuscular junctions and plays a key role in GABA-mediated control of locomotion (Bamber *et al.*, 1999, 2005).

In contrast to *C. elegans*, the role of GABA in parasitic nematodes is less understood. Evidence for GABA neurotransmission has been demonstrated in the parasitic nematodes *Ascaris suum* (Guastella *et al.*, 1991; Martin, 1980, 1985; Martin *et al.*, 1991), *H. contortus* (Laughton *et al.*, 1994; Portillo *et al.*, 2003) and *Trichinella*

spiralis (Ros-Moreno *et al.*, 1999). In *A. suum*, GABA receptors associated with somatic muscle have been shown to be neither bicuculline- nor picrotoxin-sensitive (Holden-Dye *et al.*, 1988), which is a pharmacological profile distinct from mammalian GABA_A receptors. The *A. suum* receptor is also partially sensitive to the GABA_A receptor agonist, muscimol (Holden-Dye *et al.*, 1989). To date, however, the only putative GABA receptor subunit gene isolated and characterized from a parasitic nematode is the *H. contortus* gene *Hco-HG1*, which encodes a protein orthologous to the uncharacterized *C. elegans* subunit, LGC-37 (Laughton *et al.*, 1994) and is expressed in ring motor- and inter-neurons (Skinner *et al.*, 1998). Although *Hco-HG1* does not appear to form a functional GABA receptor channel in *Xenopus* oocytes, it can co-assemble with a *C. elegans* subunit, GAB-1, to form a functional GABA-sensitive channel (Feng *et al.*, 2002). While these reports provide evidence that GABA is utilized by parasitic nematodes, we still do not know the full extent of the repertoire of GABA receptors in these organisms, nor do we fully understand the potential differences in GABA neurotransmission between free-living and parasitic nematodes.

To better understand inhibitory GABA neurotransmission in parasitic nematodes, we have isolated two GABA receptor subunit genes from *H. contortus*, *Hco-unc-49B* and *C*, which are orthologous to the *unc-49* genes from the free-living nematode *C. elegans* (*Cel-unc-49*). Similar to what has been reported for *C. elegans*, we found that the *Hco-UNC-49B* and *C* subunits form a functional heteromeric channel that is picrotoxin resistant. However, in contrast to the *C. elegans* channel, this *Hco-UNC-49B/C* heteromeric channel is more GABA sensitive than the *Hco-UNC-49B* homomeric channel. Furthermore, the *Hco-UNC-49B/C* channel is almost 3x more sensitive to

GABA compared to the previously reported *C. elegans* B/C heteromeric channel. By co-expressing *H. contortus* UNC-49 subunits with *C. elegans* UNC-49 subunits we found that only heteromeric channels containing the Hco-UNC-49B subunit exhibited high sensitivity to GABA. These results demonstrate that *H. contortus* channels may have different properties than those found in *C. elegans* and these differences may be the result of the unique properties of the Hco-UNC-49B subunit when it is assembled in a heteromeric channel.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of Hco-unc-49B and Hco-unc-49C. RNA was isolated using Trizol (Invitrogen) from adult *H. contortus* worms supplied by Dr. Roger Prichard (Institute of Parasitology, McGill University). Copy DNA (cDNA) was synthesized using the RevertAid™ minus first strand cDNA synthesis kit (Fermentas) using the manufacturer's recommendations. A modified oligo-dT anchor primer (5'CCTCTGAAGGTTACGGATCCACATCTAGATTTTTTTTTTTTTTTTTTTVN3'); [where V is either A, C, or G and N is either A, C, G, or T (Weston *et al.*, 1999)] was used to create the cDNA, which was subsequently used as template for the isolation of *Hco-unc-49C* and *Hco-unc-49B*.

Partial *H. contortus* gene sequences were identified by searching a *H. contortus* specific online sequence database (Sanger Institute, Cambridge, UK). Partial sequences predicted to encode GABA_A-receptor subunits were chosen for further molecular cloning and functional analysis. These sequences were used to design gene specific primers for

the 5' and 3' rapid amplification of cDNA ends (RACE) procedure (Frohman *et al.*, 1988).

Isolation of Hco-unc-49C. The 5' region of *Hco-unc-49C* was isolated using a sense primer specific to the splice leader 1 (SL1) sequence (SL1-5' GGTTTAATTACCCAAGTTTGAG 3') [a sequence commonly found spliced onto the 5' end of nematode mRNA (Van Doren and Hirsh, 1988)] along with two antisense gene specific primers in a nested polymerase chain reaction (PCR) (nested primer 5' ATAGCAGAAGCCCAGATAGAC 3') using a PTC-100 Programmable Thermal Controller (MJ Research, Inc.). PCR cycling conditions were as follows: 20 s 94°C, 30 s 55°C, and 60 s 72°C; 35 cycles. Resultant amplicons of the predicted size were sub-cloned into pGEM-T easy (Promega) and sequenced (Genome Quebec). The 3' sequence was amplified using two sense gene specific primers in a nested PCR (nested primer 5' TCACAGAGACGCATTTGATG 3') with two antisense primers specific to the modified oligo-dT anchor added to the cDNA. Amplicons of the expected size were sub-cloned and sequenced (Genome Quebec), yielding the predicted full length *Hco-unc-49C* gene. The full length sequence was verified (Genbank Accession Number 1003415) using primers (named *Hco-unc-49 5'* and *Hco-unc-49C 3'*), which flank the 5' and 3' coding sequence, in a nested PCR.

Isolation of Hco-unc-49B. To isolate the 3' region of *Hco-unc-49B* a nested PCR was performed using two sense gene specific primers (nested primer 5' CGATTCCGCTTATTCAACATCCTC 3') along with primers specific to the modified oligo-dT anchor at the 3' end as mentioned above. PCR amplicons were cloned and sequenced as above. Since it is predicted that, like the *unc-49B* transcripts in *C. elegans*,

the *Hco-unc-49B* transcript shares the same 5' end with *Hco-unc-49C*, the full length *Hco-unc-49B* coding sequence (Genbank Accession Number 1119896) was amplified using the same sense *Hco-unc-49* 5' primer as above along with an antisense primer specific to the 3' coding sequence of *Hco-unc-49B*.

Sequence analysis. The predicted full-length genes of *Hco-unc-49B* and *Hco-unc-49C* were translated and the resulting amino acid sequences were aligned with their *C. elegans* counterparts using the program, MacVector (MacVector Inc., North Carolina, USA). Phylogenetic analysis was performed using the neighbour joining algorithm in MacVector.

Reverse-transcription PCR. Transcription levels of *Hco-unc-49B* and *Hco-unc-49C* in different life stages of *H. contortus* were examined using reverse transcription PCR (RT-PCR). Total RNA was isolated from *H. contortus* eggs, third stage larvae (L3), adult male and adult female using RNStat (TelTest Inc). The resultant RNA (1-2 µg) was then DNase (Ambion) treated, to remove any genomic DNA contamination, and used in a reverse transcription reaction (Quantitect RT Kit, Qiagen) as per the manufacturer's recommendations. Expression levels of the corresponding cDNA were determined by PCR. *Hco-unc-49B* amplicons were generated using the sense primer 5'TGACCACTCTCATCA-CCGCAAC3' and antisense primer 5'CGACCGCATATTCGACCAATG3'. *Hco-unc-49C* was amplified using the sense primer 5'CCTGGGTTTCTGCTATCTACTGGTC3' and antisense primer 5'TTTGTGCTCGCTTTTCTTCTCC3'. The resultant amplicons for both genes were approximately 100 bp in size. A control gene, 18s ribosomal RNA (rRNA) (Genbank Accession Number L04153.1), was amplified using the sense primer

5'AATGGTTAAGAGGGACAATTCG3' and antisense primer 5'CTTGGCAAATGCTTTCGC3'. A second control gene, *Hco-leg*, encoding an adult-specific asparaginyl proteinase (Genbank Accession Number AM177177), was amplified to verify there was no cross contamination of adult RNA with other life-stages. The *Hco-leg* sense primer used was 5'GAAAAGATAAGTTCGGACACCTGG3' and the antisense primer was 5'TCAGAATCATCCATCCAAGTCACC3'. At least three replicates were performed for each gene.

Expression of unc-49B and unc-49C in Xenopus laevis oocytes. The coding sequence of *Hco-unc-49B* and *Hco-unc-49C* were sub-cloned into the oocyte expression vector, pT7Ts. The vector was then linearized and used as template in an *in vitro* transcription reaction (T7 mMessage mMachine kit, Ambion) using the manufacturer's recommendations, producing the corresponding copy RNA (cRNA). The cRNA was subsequently precipitated using lithium chloride and diluted in H₂O. *Cel-unc-49B* and *Cel-unc-49C* full length cDNA clones (provided by Dr. B. Bamber, University of Toledo) were linearized using Asp-718I (Roche) and used as template in an *in vitro* cRNA reaction (T3 mMessage mMachine kit, Ambion) according to the manufacturer's recommendations.

X. laevis oocytes were surgically removed from females anesthetized with 0.15% (w/v) ethyl 3-amino benzoate methane sulfonate (MS-222) (Sigma). Defolliculation of the oocytes was achieved using 2 mg/mL collagenase (Sigma) in OR2 buffer (82 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM Hepes pH 7.5) for 2 hours while rocking at room temperature. The oocytes were then stored in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM Hepes pH 7.5) supplemented with 0.275 µg/mL

pyruvate and 100 µg/mL gentamycin (Sigma). Each oocyte was injected with 50 nL of *Hco-unc-49B* and/or *Hco-unc-49C* cRNA (0.3 – 0.5 ng/nL) using a Drummond Nanoject microinjector. Oocytes were incubated at 20°C in supplemented ND96, which was replaced at least every 24 hours. Recordings were routinely made 2-5 days post cRNA injection.

Electrophysiological Recordings. Two-electrode voltage clamp electrophysiology was performed using the Axoclamp 900A voltage clamp (Molecular Devices). Glass electrodes containing Ag|AgCl wire were filled with 3 M KCl and had a resistance between 1 and 5 MΩ. Oocytes were clamped at a -60 mV for the duration of the experiments. The drugs GABA and muscimol (both from Sigma) were dissolved in ND96. Picrotoxin (Sigma) and moxidectin (Fort Dodge Animal Health) were dissolved in dimethylsulfoxide (DMSO). Drugs were washed over the oocytes using an RC-1Z recording chamber (Warner Instrument Inc.). Data was obtained and analyzed using the Clampex software (Molecular Devices) and graphs were produced using Graphpad. GABA and muscimol EC₅₀ values were determined by generating dose response curves fitted to the equation:

$$I_{\max} = \frac{1}{\{1 + (EC_{50}/[D])^h\}}$$

where I_{\max} is the maximal response, $[D]$ is the concentration of drug, EC_{50} is the concentration of drug that is required to produce half-maximal current, and h is the Hill coefficient. I_{\max} , EC_{50} and h were free parameters. The curves were then normalized to the estimated I_{\max} . The equation generating the dose response curves was used to fit a sigmoidal curve of variable slope to the normalized data (GraphPad). The significance of

the EC₅₀ values were verified using a student's t-test where a $p \leq 0.01$ was considered significant.

Current voltage relationships were performed by changing the holding potential in 20 mV steps from -60 mV to +40 mV, and at each step the oocytes was exposed to 1 mM GABA. For reduced chloride trials, NaCl was partially substituted with Na-gluconate (Sigma) in the ND96 for a final Cl⁻ concentration of 62.5 mM.

RESULTS

Cloning of the Hco-unc-49 genes. PCR isolation of the *Hco-unc-49C* gene yielded a 1767 bp cDNA sequence. This full-length sequence included a 276 bp 5' untranslated region (UTR). We observed that some of our clones were missing this 5' UTR and portions of 5' coding sequence (Figure 2.1A). However, the SL1 sequence was found attached to the extreme 5' ends of all 5' RACE PCR amplicons, suggesting the occurrence of trans-splicing. These putative 5' truncated products had lost their signal peptide cleavage site and are therefore predicted to be non-functional. The 3' end contained 171 bp UTR as well as a poly-A tail. When translated in the appropriate reading frame, the sequence encodes for a 440 amino acid polypeptide, containing the signature cys-loop. In addition, four hydrophobic trans-membrane domains were identified as well as a signal peptide cleavage site using the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>). The Hco-UNC-49C protein sequence shares a 66% homology with its predicted *C. elegans* orthologue, Cel-UNC-49C, a GABA_A receptor subunit. An amino acid alignment of Hco-UNC-49C and Cel-UNC-49C

revealed a high degree of sequence homology, especially in the membrane spanning domains (Figure 2.2).

The *Hco-unc-49B* gene was found to have 1489 bp of coding sequence and 699 bp of unique 3' UTR. Several of our clones differed in the length of the 3'UTR (data not shown) and one clone contained only the first 560 bp of 5' coding region ending with the last 280 bp of the 3' UTR (Figure 2.1B).

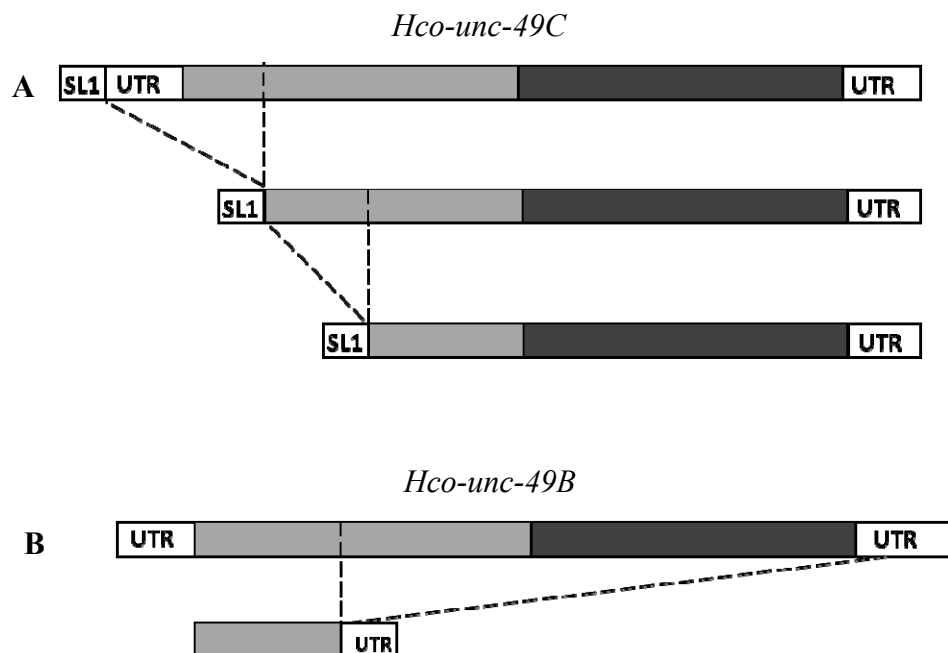


Figure 2.1. Splicing pattern of *Hco-unc-49C* and *Hco-unc-49B*. A) Trans-splicing identified, yielding 3 putative transcripts that were missing different portions of the 5' end of *Hco-unc-49C*. B) Alternative splicing identified at the 3' end of *Hco-unc-49B* yielding a transcript containing only a portion of the N-terminal domain (top diagram is the full predicted *Hco-unc-49B* and below is the spliced version identified). Light grey regions indicate the N-terminal coding polypeptide sequence and dark grey regions indicate the membrane spanning domains

The full predicted Hco-UNC-49B polypeptide consists of 496 amino acids and shares a 68% homology with the *C. elegans* subunit Cel-UNC-49B.1. However, the *H. contortus* polypeptide has a longer M3-M4 intracellular domain and is therefore more

similar to Cel-UNC-49B.3 (Bamber *et al.*, 1999). The Hco-UNC-49B and Hco-UNC-49C polypeptides share a common N-terminal domain and are identical until just after the cys-loop; at this point the sequences diverge and thus exhibit two different C-terminal ends (Figure 2.2).

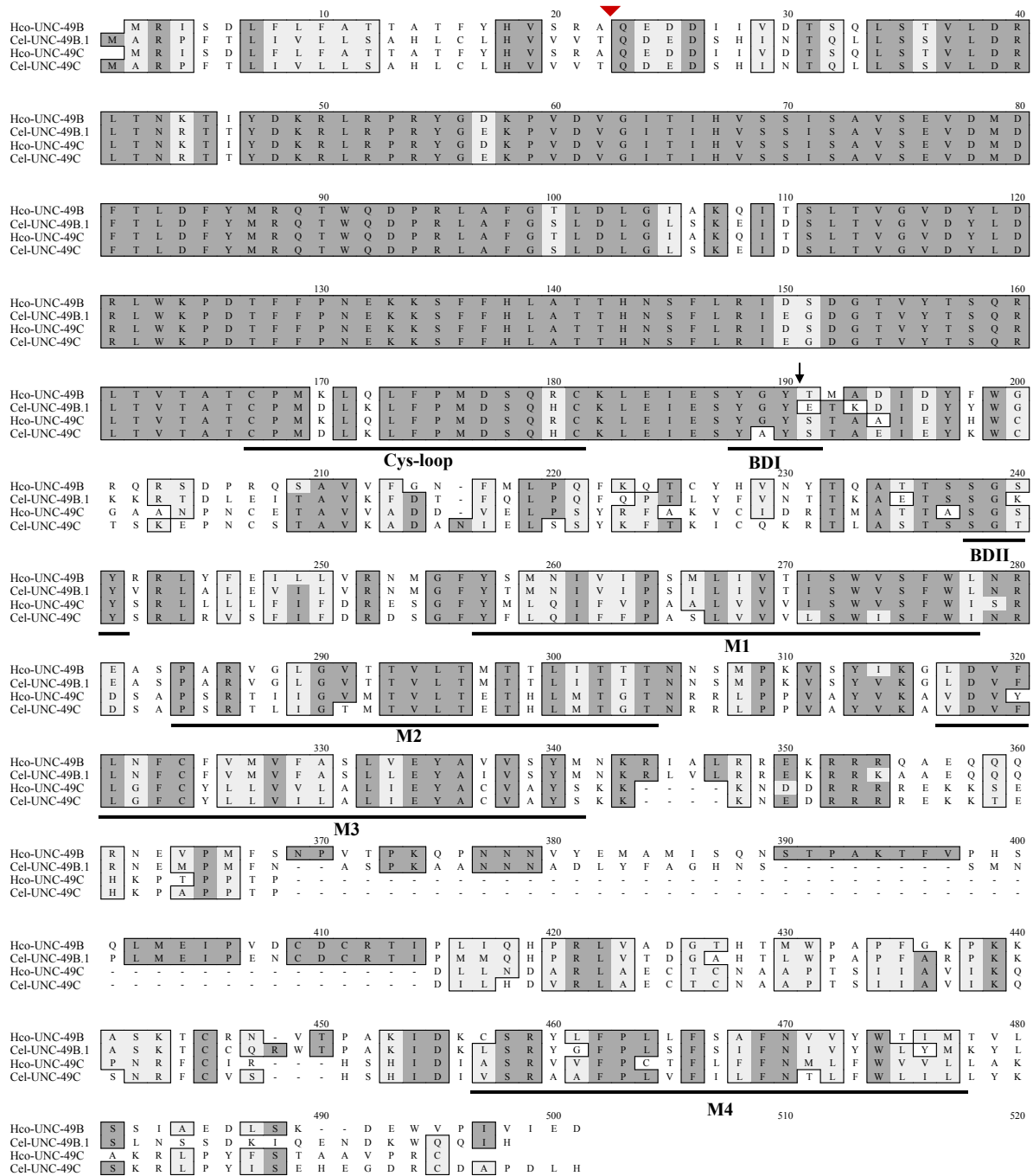


Figure 2.2. Protein sequence alignment *H. contortus* and *C. elegans* UNC-49B and C. Dark shaded areas indicate regions of amino acid identity or no alignment between the two sequences, lightly shaded areas indicate similar amino acids and no shading indicates no similarity. Signal peptide cleavage site is identified by an arrowhead. The downward facing arrow indicates the region where Hco-UNC-49 is spliced, generating Hco-UNC-49B and C. The putative binding domains, BDI and BDII (Bamber *et al.*, 1999) are indicated. The cys-loop and four membrane spanning domains are identified by the appropriate bars.

Phylogenetic analysis revealed that Hco-UNC-49B and Hco-UNC-49C group closely together with their *C. elegans* counterparts and with the *C. elegans* LGC-38 RDL-like protein (GenBank Accession Number AAA81158) (Figure 2.3A). The UNC-49 subunit sequences appear to form a separate grouping from vertebrate GABA-gated chloride channel subunits, suggesting the UNC-49s are invertebrate specific.

Hco-unc-49B and Hco-unc-49C are differentially expressed in various life stages. RT-PCR analysis of *Hco-unc-49B* and *Hco-unc-49C* detected the presence of transcripts in four life-stages of *H. contortus* (eggs, L3 larvae, adult female and adult male) (Figure 2.3B). However, PCR amplicons of *Hco-unc-49B* and *Hco-unc-49C* were least intense in the egg stage compared to the other life stages. No PCR products were visible in the negative controls lacking reverse transcriptase, verifying that there was no genomic DNA contamination. As well, only *Hco-leg* amplicons were detected in the adult male and female stages confirming that there was no cross contamination of RNA between the adult and juvenile stages.

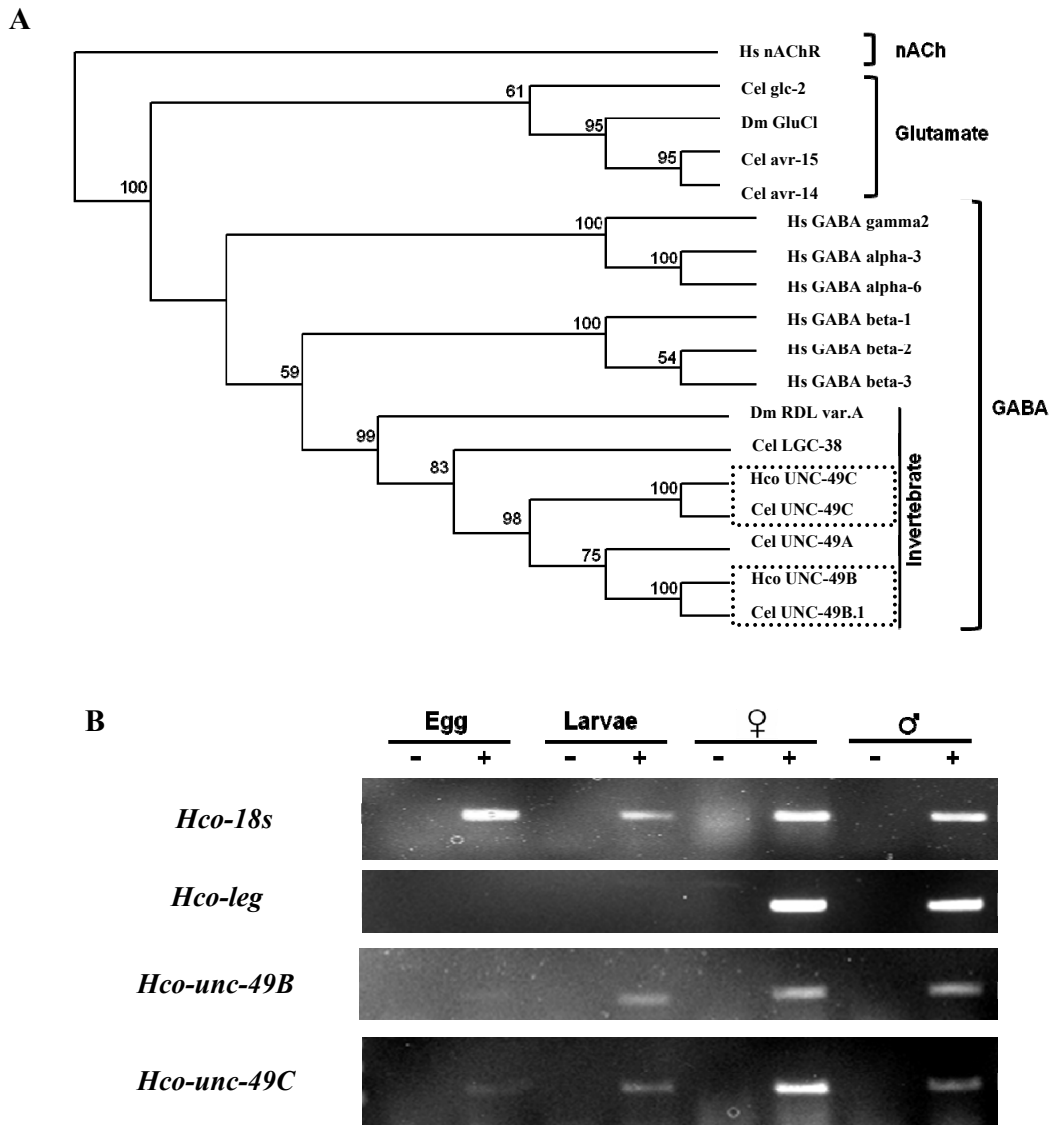


Figure 2.3. A) Phylogenetic analysis of Hco-UNC-49B and Hco-UNC-49C. Polypeptide sequences of various GABA-gated chloride channel subunits, as well as GluCl subunits sequences are included. A nACh gated cation channel was included as an out group. B) Life stage expression of *Hco-unc-49B* and *Hco-unc-49C* in egg, third stage larvae and adult female and male worms. *Hco-18s* and *Hco-leg* are used as control genes to determine cDNA quality and if cross-contamination occurred between life stages, respectively.

Pharmacological characterization of Hco-UNC-49B and Hco-UNC-49C. When expressed in *X. laevis* oocytes, Hco-UNC-49B alone was able to form a homomeric channel and produce a robust response (current) to 100 μ M GABA (Figure 2.4A) which was consistently observed. In contrast, Hco-UNC-49C alone or oocytes injected with

water did not respond to GABA (Figure 2.4B). When both Hco-UNC-49B and Hco-UNC-49C (Hco-UNC-49B/C) were injected in equal amounts a strong response to 100 μ M GABA was also observed (Figure 2.4C). Neither Hco-UNC-49B, Hco-UNC-49C, nor Hco-UNC-49B/C responded to 1 mM glutamate or glycine.

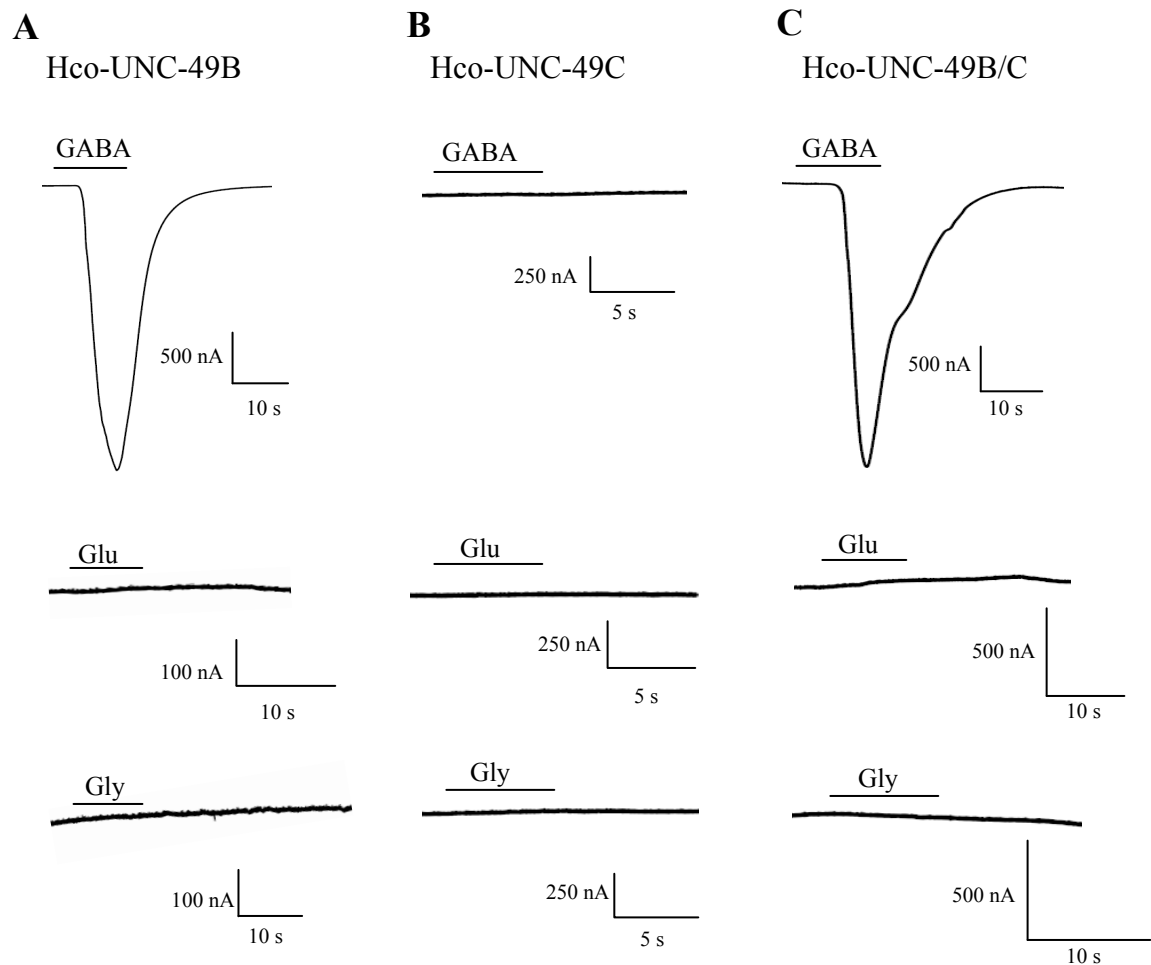


Figure 2.4. Hco-UNC-49B and B/C channels expressed in *Xenopus laevis* oocytes respond to GABA, but not to glutamate (Glu) or glycine (Gly). A) Hco-UNC-49B responds to 100 μ M GABA but not to 1 mM glutamate or 1 mM glycine (below traces). B) *Hco-unc-49C* injected oocytes do not respond to 100 μ M GABA, 1 mM glutamate or 1 mM glycine. C) Hco-UNC-49B/C responds to 100 μ M GABA but not to 1 mM glutamate or 1 mM glycine.

The GABA response for both Hco-UNC-49B and Hco-UNC-49B/C was dose dependent when tested with concentrations of GABA ranging from 1 μ M to 5 mM (Figure 2.5A).

Oocytes expressing Hco-UNC-49B exhibited a slightly, but significantly, lower sensitivity to GABA compared to oocytes expressing both Hco-UNC-49B and C subunits (Figure 2.5B). The GABA EC_{50} value for Hco-UNC-49B was $64.0 \pm 4.4 \mu\text{M}$ (n=6) with a Hill coefficient of 1.9 ± 0.2 , suggesting that at least two GABA molecules are required to open the channel. Hco-UNC-49B/C exhibited an EC_{50} of $39.9 \pm 5.7 \mu\text{M}$ (n=10) with a Hill coefficient of 2.2 ± 0.4 , similar to that of the homomeric channel.

Current-voltage analysis of the Hco-UNC-49B/C channel using full Cl^- ND96 (103.6 mM Cl^-) indicated a reversal potential of $-17.2 \pm 4.0 \text{ mV}$ (n=5) (Figure 2.5C) consistent with the calculated Nernst potential for Cl^- (-18.5 mV), assuming 50 mM internal Cl^- (Kusano *et al.*, 1982). When NaCl was partially replaced with Na-gluconate in the ND96 (final concentration 62.5 mM Cl^-), the reversal potential shifted to $-6.9 \pm 4.2 \text{ mV}$ (n=4), consistent with the predicted Nernst potential of -5.7 mV.

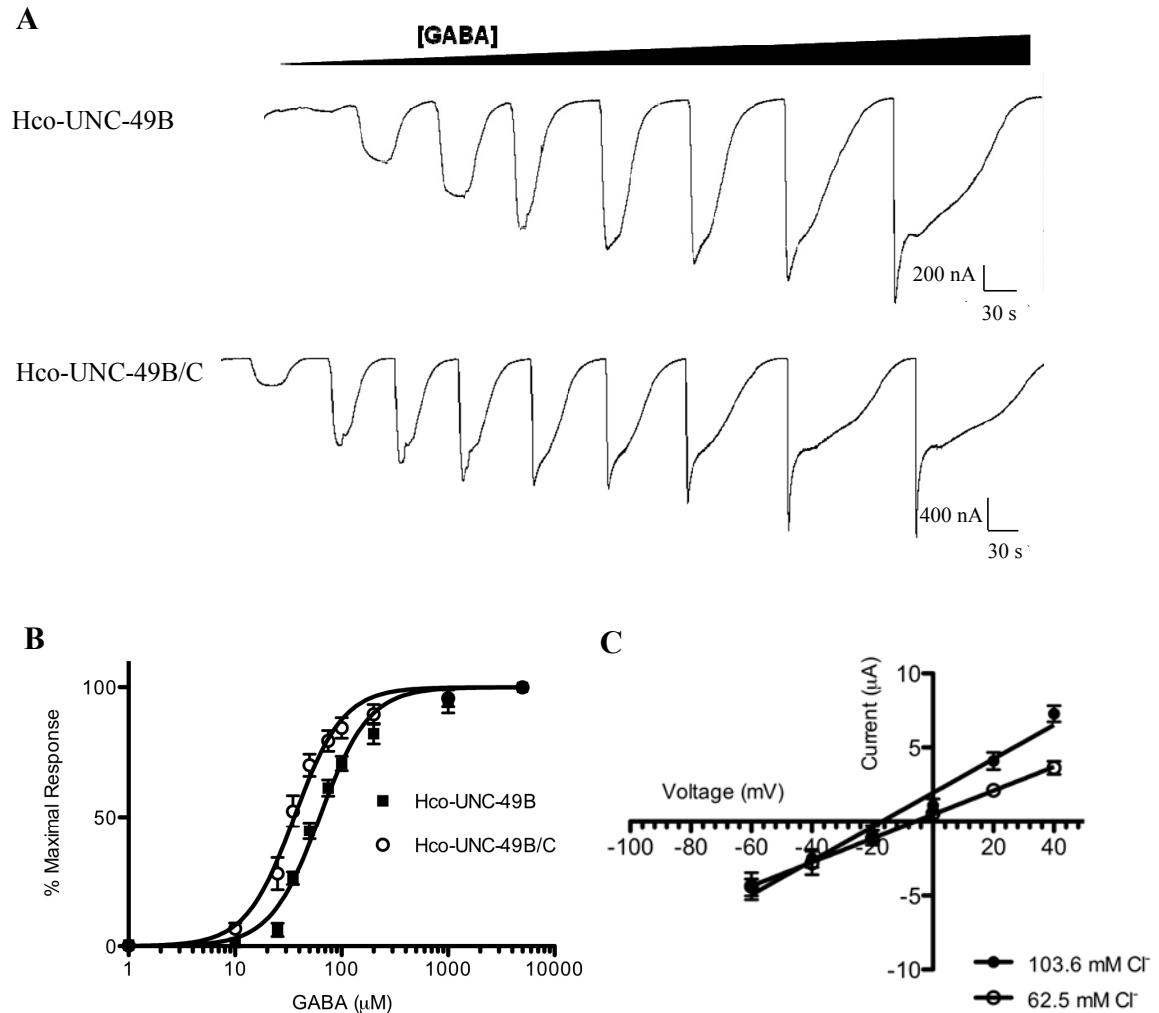


Figure 2.5. GABA activates the Hco-UNC-49 channels and allows for the influx of Cl^- . A) Dose response trial of Hco-UNC-49B and Hco-UNC-49B/C with concentrations of GABA ranging from 1 μM to 5 mM. B) Dose response curve of Hco-UNC-49B and Hco-UNC-49B/C with the current normalized to the percentage of maximal response. C) Current voltage analysis of Hco-UNC-49B/C using 103.6 mM Cl^- in ND96 buffer solution and reduced chloride concentration of 62.5 mM in ND96 buffer solution. GABA responses were generated with 1 mM GABA.

The Hco-UNC-49B homomeric channel was much more sensitive to the GABA-gated chloride channel blocker picrotoxin, compared to the Hco-UNC-49B/C heteromeric channel. In the presence of picrotoxin, the response of Hco-UNC-49B to GABA was

reduced by $94.0 \pm 0.9\%$ ($n=4$) compared to a $46.0 \pm 5.9\%$ ($n=4$) reduction in the response of Hco-UNC-49B/C to GABA (Figure 2.6).

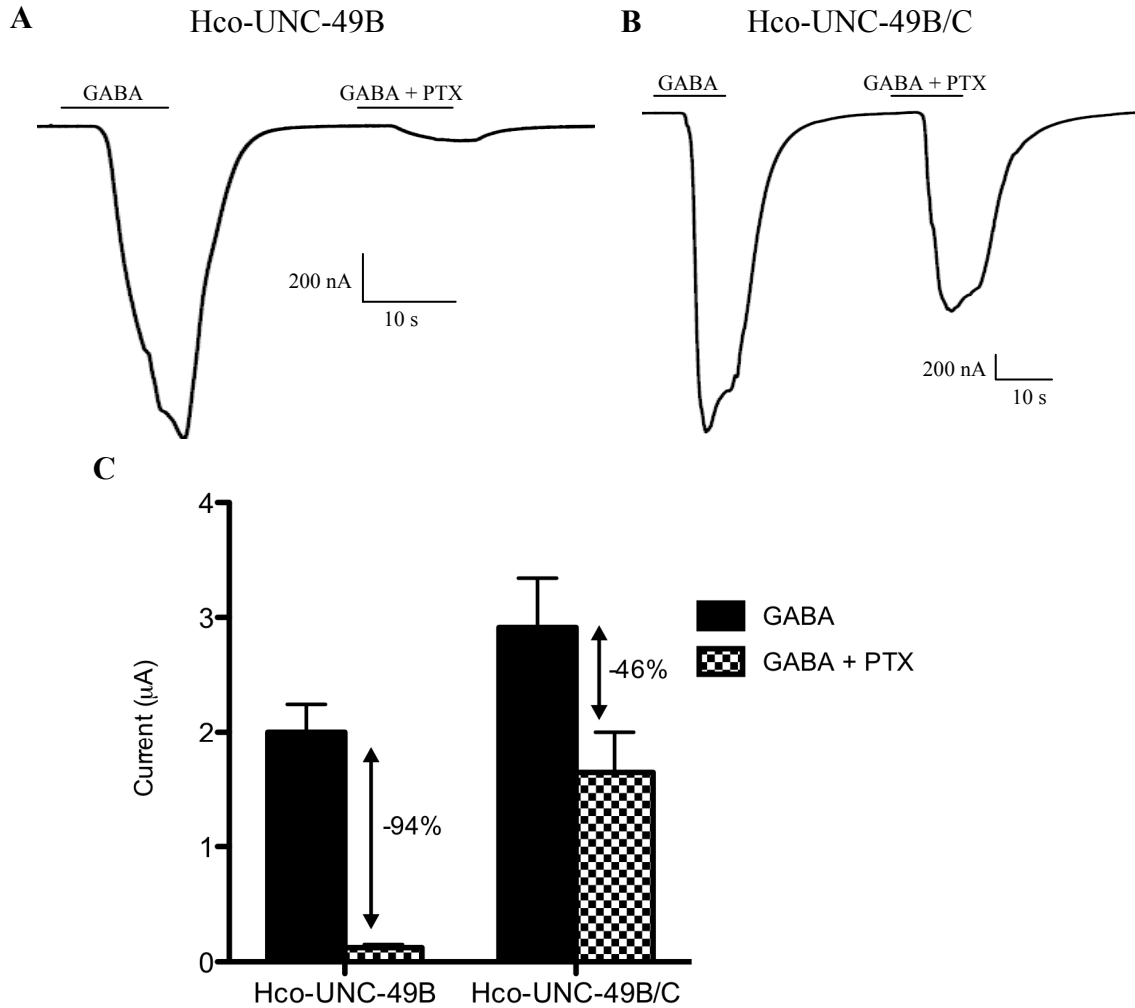


Figure 2.6. Hco-UNC-49B/C is more resistant to the inhibiting effects of picrotoxin compared to Hco-UNC-49B. A) Hco-UNC-49B and B) Hco-UNC-49B/C channel response to $50 \mu\text{M}$ GABA alone followed by $50 \mu\text{M}$ GABA combined with $100 \mu\text{M}$ picrotoxin. C) Graph indicating the percent picrotoxin dependant inhibition of the GABA response for each Hco-UNC-49 channel.

The Hco-UNC-49B/C channel has a higher sensitivity to muscimol compared to the Hco-UNC-49B channel. Both Hco-UNC-49B and Hco-UNC-49B/C responded to $100 \mu\text{M}$ muscimol (Figure 2.7A, 2.7B). However, the Hco-UNC-49B current generated by muscimol was only $23.5 \pm 3.5\%$ ($n=3$) of the current generated by the same

concentration of GABA (Figure 2.7C). In contrast, the response of Hco-UNC-49B/C to muscimol was $48.1 \pm 15.1\%$ (n=3) of the GABA response. The muscimol EC_{50} values for the Hco-UNC-49B and Hco-UNC-49B/C channels were $157.5 \pm 13.2 \mu\text{M}$ (n=8) (Hill coefficient of 2.8 ± 0.3), and $62.2 \pm 4.0 \mu\text{M}$ (n=8) (Hill coefficient of 2.0 ± 0.2), respectively (Figure 2.7D).

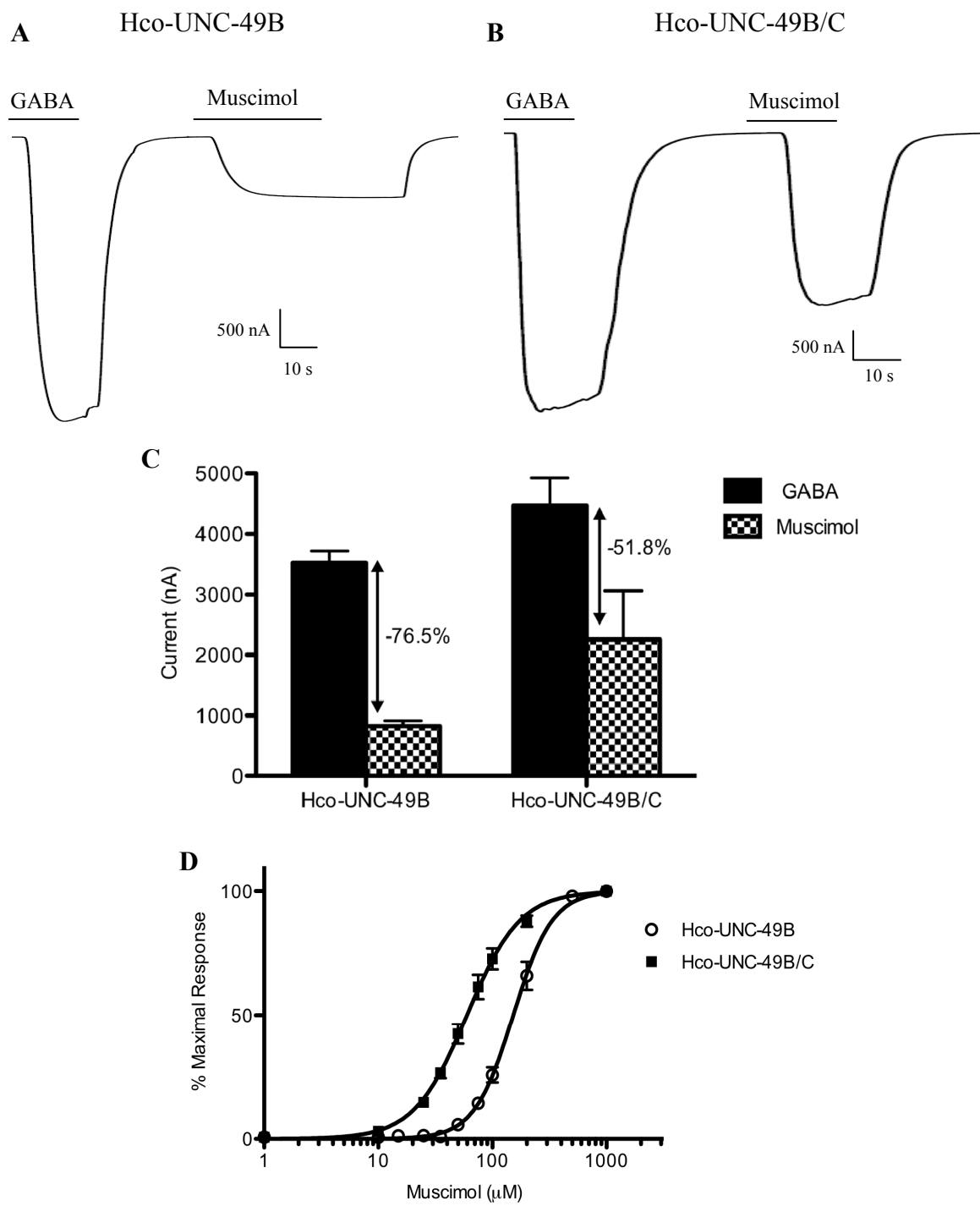


Figure 2.7. Muscimol activates the Hco-UNC-49 channels. A) Muscimol response for the Hco-UNC-49B and B) Hco-UNC-49B/C channels. Oocytes were first washed in 100 μ M GABA and then in 100 μ M muscimol. C) Bar graph representing the channel response of muscimol in comparison to GABA. Percentage indicates the difference in channel response between GABA and muscimol. D) Dose response analysis of muscimol for the Hco-UNC-49B and Hco-UNC-49B/C channels.

Anti-parasitic drug effects. The antiparasitic drug moxidectin, which is thought to selectively target GluCl_s (Forrester *et al.*, 2002), potentiates the GABA response of Hco-UNC-49B/C by $40.2 \pm 10.7\%$ (n=6) when oocytes are washed with 50 μ M GABA followed by 50 μ M GABA with 10 μ M moxidectin (Figure 2.8).

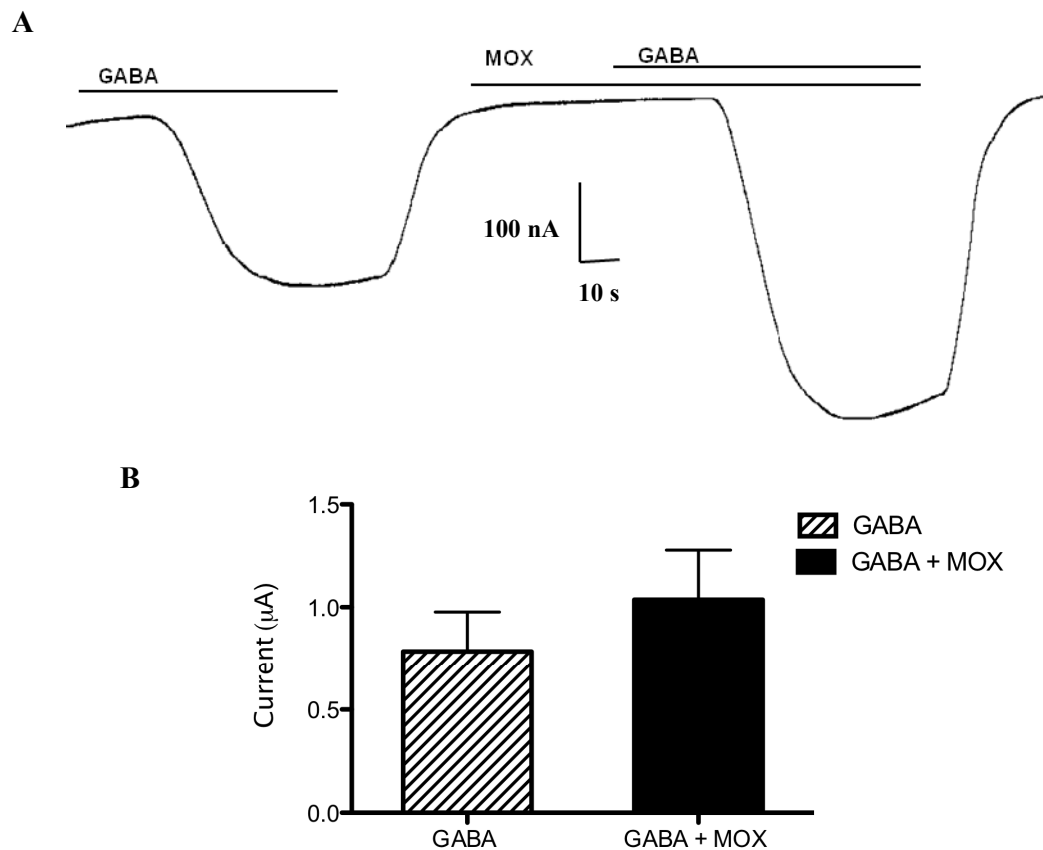
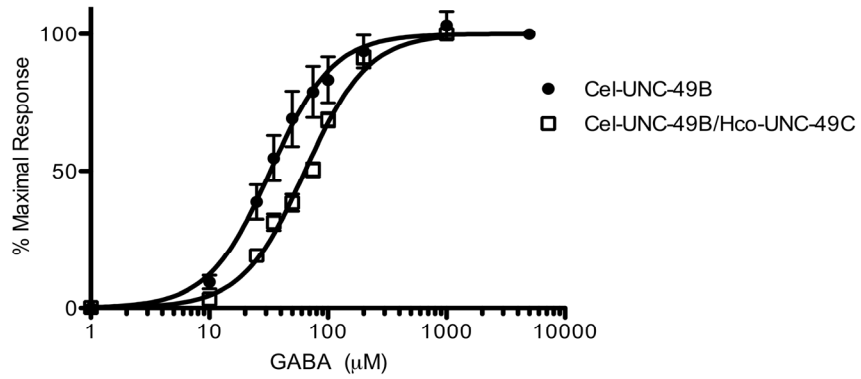


Figure 2.8. Moxidectin (MOX) potentiates the response of Hco-UNC-49B/C to GABA. A) The response of the Hco-UNC-49B/C channel to 50 μ M GABA. The oocyte was then washed with 10 μ M MOX followed by 50 μ M GABA/10 μ M MOX combined. B) Bar graph representing the GABA response in the presence and absence of MOX.

The Hco-UNC-49B subunit is associated with high GABA sensitivity in nematode heteromeric channels. Previous reports on the UNC-49 channel in *C. elegans* have found that the assembly of Cel-UNC-49C with Cel-UNC-49B produces a heteromeric channel

with a lower sensitivity to GABA compared to the Cel-UNC-49B homomeric channel (Bamber *et al.*, 1999). Our results from the *H. contortus* UNC-49 subunits demonstrate the opposite trend; the heteromeric channel has higher sensitivity to GABA compared to the homomeric channel. To determine which specific subunit (either B or C) is associated with high GABA sensitivity in the *H. contortus* heteromeric channels, we produced cross-assembled channels that contain both *C. elegans* and *H. contortus* UNC-49 subunits. Dose response curves were generated and the associated EC₅₀ values for GABA were calculated for each resulting channel. The calculated EC₅₀ for Cel-UNC-49B was 41.7 ± 8.7 μM (n=10), which is similar to that previously reported (Bamber *et al.*, 1999). When the Cel-UNC-49B subunit was co-expressed with Hco-UNC-49C, a significant decrease in GABA sensitivity was observed with an EC₅₀ of 97.2 ± 10.2 μM (n=10) (Figure 2.9A). In contrast, when Hco-UNC-49B was co-expressed with Cel-UNC-49C the resulting channel was significantly more sensitive to GABA with an EC₅₀ of 24.5 ± 2.1 μM (n=8), compared to the Hco-UNC-49B channel (Figure 2.9B). A summary of EC₅₀ values and hill coefficients is shown in Table 1.

A



B

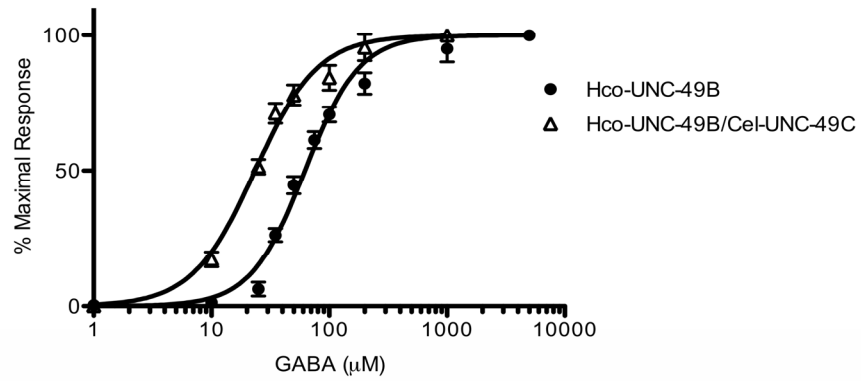


Figure 2.9. Hco-UNC-49 and Cel-UNC-49 cross-assembled heteromeric channels have differing GABA sensitivities. A) Dose response curves generated by Cel-UNC-49B and Cel-UNC-49B/Hco-UNC-49C showing a decrease in GABA sensitivity due to the introduction of Hco-UNC-49C. B) Dose response curves showing the response of Hco-UNC-49B (from Figure 2.5) and Hco-UNC-49B/Cel-UNC-49C to GABA.

Table 1. Comparison of EC₅₀ and Hill Coefficient values for *H. contortus* and *C. elegans* UNC-49 homomeric, heteromeric and cross-assembled heteromeric channels

Drug	Channel	EC ₅₀ (μM)	Hill Coefficient
GABA	Hco-UNC-49B	64.0 ± 4.4	1.9 ± 0.2
GABA	Hco-UNC-49B/C*	39.9 ± 5.7	2.2 ± 0.4
GABA	Hco-UNC-49B/ Cel-UNC-49C***	24.5 ± 2.1	1.6 ± 0.1
GABA	Cel-UNC-49B	41.7 ± 8.7	1.9 ± 0.3
GABA	Cel-UNC-49B/Hco-UNC-49C**	97.2 ± 10.2	1.1 ± 0.1
Muscimol	Hco-UNC-49B	157.5 ± 13.2	2.8 ± 0.3
Muscimol	Hco-UNC-49B/C***	62.2 ± 4.0	2.0 ± 0.2

p-values comparing the UNC-49B/C heteromeric channel with the corresponding UNC-49B homomeric channel
 * *p*<0.01, ***p*<0.001, ****p*<0.0001

DISCUSSION

This thesis describes the isolation and pharmacological characterization of two Hco-UNC-49 subunits, Hco-UNC-49B and Hco-UNC-49C, from the sheep parasitic nematode, *H. contortus*. Like their *C. elegans* orthologues, Hco-UNC-49B and C share the same N-terminal sequence but differ in their C-terminal sequence, which includes the membrane spanning regions. It is assumed, therefore, that similar to the situation in *C. elegans*, these *unc-49B* and *C* transcripts are generated by alternative splicing of the same *unc-49* gene. A similar phenomenon has been reported for a GluCl gene called *avr-14* which has been shown, in several nematode species, to be alternatively spliced to form two different transcripts (Laughton *et al.*, 1997; Jagannathan *et al.*, 1999; Yates and Wolstenholme, 2004). It appears, therefore, that this type of transcript generation is conserved in the nematode phyla.

Analysis of the *unc-49* transcripts has revealed some interesting characteristics. Firstly, we found the length of the 5' UTR between *H. contortus* and *C. elegans unc-49B* and *C* transcripts is similar. However, the 3' UTR of *Cel-unc-49B* appears to be a few hundred base pairs longer than its *H. contortus* counterpart. Since it is generally accepted that the 3' UTR plays an important role in transcript regulation, it is possible that *Cel-unc-49B* may be under a slightly different control mechanism compared to the equivalent gene in *H. contortus*. It has also been suggested the increased length of the 3' UTR may infer a decreased evolutionary age or an increase in the complexity of the organism (Mazumder *et al.*, 2003). Secondly, we have found higher levels of both *Hco-unc-49B* and *C* expression in adult and L3 larvae compared to eggs. This suggests that both transcripts are important in processes that occur specifically in more mature stages of life such as those associated with motility.

Functional analysis of the Hco-UNC-49 channels revealed properties that were similar to previously reported *C. elegans* UNC-49 channels. Firstly, the *H. contortus* UNC-49B subunit, like its *C. elegans* counterpart, forms a functional homomeric channel with a similar EC₅₀ for GABA and is highly sensitive to the open channel blocker, picrotoxin. Secondly, similar to studies in *C. elegans*, Hco-UNC-49C does not form a functional channel alone but will associate with Hco-UNC-49B to produce a picrotoxin resistant heteromeric channel. The resistance of the *H. contortus* and *C. elegans* channels to picrotoxin can be attributed to the fact that the M2 region of the UNC-49C subunit of both species exhibits a methionine at a key position that is associated with picrotoxin resistance (Bamber *et al.*, 2003; Zhang *et al.*, 1995) (Figure 2.2). Thus, incorporation of the nematode UNC-49C subunit causes heteromeric channels to become resistant to

microtoxin. It is interesting to note that the GABA_A receptor characterized from *Ascaris* muscle is also microtoxin resistant (Holden-Dye *et al.*, 1988) suggesting that this receptor shares similar properties to the Hco-UNC49B/C heteromeric channel.

Both *H. contortus* homomeric and heteromeric channels responded to the restricted structural analogue of GABA, muscimol. However, like with GABA, the heteromeric channel had a higher sensitivity to muscimol compared to the homomeric channel. Although, compared to GABA, muscimol was a less potent agonist for these channels. This is different than what has been observed for mammalian, particularly rat, GABA_A receptors, where muscimol appears to be a more potent agonist compared to GABA (Amin and Weiss, 1993; Vien *et al.*, 2002). A similar trend was observed for GABA_A receptors from the tobacco budworm (Wolff and Wingate, 1998). Our results appear more similar to the *Ascaris* GABA_A receptor where muscimol was ~60% less potent compared to GABA (Holden-Dye *et al.*, 1989), which further supports the notion that the previously characterized *Ascaris* muscle receptor was an Hco-UNC-49B/C-like channel.

Further comparison of the *H. contortus* and *C. elegans* UNC-49 channels revealed an important difference; the parasite UNC-49B/C channel appears to have a higher sensitivity to GABA. In an attempt shed some light on the subunit determinants for either high or low GABA sensitivity of heteromeric channels, we co-expressed *C. elegans* UNC-49 subunits with *H. contortus* UNC-49 subunits. First, we observed the same sensitivity of the Cel-UNC-49B homomeric channel to GABA as reported by Bamber *et al.*, 1999 (EC₅₀ of 42 μM). However, co-expression of Cel-UNC-49B and Hco-UNC-49C resulted in a heteromeric channel with a decreased sensitivity to GABA

(EC₅₀ of 97 μM). This was, in fact, the same trend as observed for the Cel-UNC-49B/C channel, where incorporation of the C subunit decreased the sensitivity of the channel to GABA (Bamber *et al.*, 1999). Interestingly, co-expression of Hco-UNC-49B with Cel-UNC-49C produced a channel with a higher sensitivity to GABA than both the Hco-UNC-49B homomeric channel and the Hco-UNC-49B/C heteromeric channel. Therefore, it appears that when Hco-UNC-49B assembles with an UNC-49C subunit, the sensitivity of the channel to GABA increases, regardless of whether the co-injected *UNC-49C* RNA is from *H. contortus* or *C. elegans*.

The cause for the increased GABA sensitivity in Hco-UNC-49B-associated heteromeric channels is unknown. It should be noted that the only UNC-49B subunit that we detected from *H. contortus* exhibited a longer M3-M4 intracellular loop compared to the *C. elegans* UNC-49B subunit that we used in this study. Whether this difference is associated with the results of the current study is not known at this time. The M3-M4 intracellular loop is generally thought to contribute to subtype specificity as well as intracellular regulatory processes (Olsen and Tobin, 1990) and not necessarily binding affinities. We believe that the more likely cause may be found in the amino acid differences in the BDs of Hco-UNC-49B and Cel-UNC-49B. In mammalian GABA_A receptors, a mutation of a highly conserved threonine residue in BDI of the β subunit caused a decrease in GABA sensitivity to the resulting α₁β₂γ₂ channel (Amin and Weiss, 1993). In the Hco-UNC49B subunit, a threonine is present in the equivalent position while in the *C. elegans* UNC-49B subunit, a glutamic acid is present. Interestingly, however, when comparing the EC₅₀ values of the UNC-49B homomeric channels from both species, there is only a slight difference (64 vs 42 μM). Thus, if this unique

threonine residue found on Hco-UNC-49B causes an enhancement of GABA binding, it may only become involved when Hco-UNC-49B becomes associated with an UNC-49C subunit suggesting that, like mammalian GABA_A receptors, the GABA binding site is on the interface of adjacent subunits (Deng *et al.*, 1986; Bureau and Olsen, 1988) (in our case in the interface of UNC-49B and UNC-49C). Another amino acid variation between the UNC-49B subunits from both species resides in the BDII where a serine is found in the Hco-UNC-49B subunit and a lysine is found in the equivalent position in Cel-UNC-49B. The significance of this variation is currently not known. However, we are currently investigating residues in both BDs to verify their role in GABA binding.

CONCLUSIONS

This thesis describes the isolation and pharmacological characterization of two novel GABA receptor subunits from the parasitic nematode *H. contortus*. In addition, a novel method was used to understand the binding characteristics of a parasite ion channel and its subunits by creating cross-species heteromeric channels and comparing their channel properties. Future research will aim to further investigate how Hco-UNC-49B is able to confer increased GABA sensitivity to Hco-UNC-49B/C heteromeric channels.

The goal here was to better understand the role of GABA receptors in a parasite such as *H. contortus* and how they compare to homologous receptors found in a non-parasitic nematode. Clear differences in channel properties were observed between the *H. contortus* homomeric and heteromeric channels when compared to the free-living nematode *C. elegans*. How these different channel properties affect overall GABA neurotransmission in parasitic nematodes will be an important area of future research.

REFERENCES

- Amin, J. and Weiss, D.S. 1993. GABA_A receptor needs two homologous domains of the α and β -subunit for activation by GABA but not by pentobarbital. *Nature* (366) 565-569.
- Aronstein, K., Auld, V. and French-Constant, R. 1996. Distribution of two GABA receptor-like subunits in the *Drosophila* CNS. *Invertebr. Neurosci.* (2) 115-120.
- Bamber, B.A., Beg, A.A., Twyman, R.E. and Jorgensen, E.M. 1999. The *Caenorhabditis elegans unc-49* Locus Encodes Multiple Subunits of a Heteromultimeric GABA Receptor. *The Journal of Neuroscience* (19) 5348-5359.
- Bamber, B.A., Twyman, R.E. and Jorgensen, E.M. 2003. Pharmacological characterization of the homomeric and heteromeric UNC-49 GABA receptors in *C. elegans*. *Br.J.Pharmacol.* (138) 883-893.
- Bamber, B.A., Richmond, J.E., Otto, J.F. and Jorgensen, E.M. 2005. The composition of the GABA receptor at the *Caenorhabditis elegans* neuromuscular junction. *Br.J.Pharmacol.* (144) 502-509.
- Bureau, M. and Olsen, R.W. 1988. GABA/benzodiazepine receptor protein carries binding sites for both ligands on two major peptide subunits. *Biochem. Biophys. Res. Commun.* (153) 1006-1011.
- Cockcroft, V.B., Osguthorpe, D.J., Barnard, E.A. and Lunt, G.G. 1990. Modeling of agonist binding to the ligand-gated ion channel superfamily of receptors. *Proteins: Structure, Function, and Genetics* (8) 386-397.
- Deng, L., Ransom, R.W. and Olsen, R.W. 1986. [³H]Muscimol photolabels the GABA receptor site on a peptide subunit distinct from that labeled with benzodiazepine. *Biochem. Biophys. Res. Commun.* (138) 1308-1314.
- Dent, J.A. 2006. Evidence for a diverse Cys-loop ligand-gated ion channel superfamily in early bilateria. *J.Mol.Evol.* (62) 523-535.
- Feng, X.P., Hayashi, J., Beech, R.N. and Prichard, R.K. 2002. Study of the nematode putative GABA type-A receptor subunits: evidence for modulation by ivermectin. *J.Neurochem.* (83) 870-878.
- French-Constant, R.H., Mortlock, D.P., Shaffer, C.D., MacIntyre, R.J. and Roush, R.T. 1991. Molecular cloning and transformation of cyclodiene resistance in *Drosophila*: an invertebrate gamma-aminobutyric acid subtype A receptor locus. *Proc.Natl.Acad.Sci.U.S.A.* (88) 7209-7213.
- Forrester, S.G., Prichard, R.K. and Beech, R.N. 2002. A glutamate-gated chloride channel subunit from *Haemonchus contortus*: expression in a mammalian cell line, ligand

- binding, and modulation of anthelmintic binding by glutamate. *Biochem.Pharmacol.* (63) 1061-1068.
- Frohman,M.A., Dush,M.K. and Martin,G.R. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proceedings of the National Academy of Sciences* (85) 8998-9002.
- Guastella,J., Johnson,C.D. and Stretton,A.O.W. 1991. GABA-immunoreactive neurons in the nematode *Ascaris*. *J.Comp.Neurol.* (307) 584-597.
- Harrison,J.B., Chen,H.H., Sattelle,E., Barker,P.J., Huskisson,N.S., Rauh,J.J., Bai,D. and Sattelle,D.B. 1996. Immunocytochemical mapping of a C-terminus anti-peptide antibody to the GABA receptor subunit, RDL in the nervous system of *Drosophila melanogaster*. *Cell Tissue Res.* (284) 269-278.
- Holden-Dye,L. 1989. GABA receptors on the somatic muscle cells of the parasitic nematode, *Ascaris suum*: stereoselectivity indicates similarity to a GABA_A-type agonist. *Br.J.Pharmacol.* (98) 841-850.
- Holden-Dye,L., Hewitt,G.M., Wann,K.T., Krogsgaard-Larsen,P. and Walker,R.J. 1988. Studies involving avermectin and the 4-aminobutyric acid(GABA) receptor of *Ascaris suum* muscle. *Pestic.Sci.* (24) 231-245.
- Jagannathan,S., Laughton,D.L., Critten,C.L., Skinner,T.M., Horoszok,L. and Wolstenholme,A.J. 1999. Ligand-gated chloride channel subunits encoded by the *Haemonchus contortus* and *Ascaris suum* orthologues of the *Caenorhabditis elegans gbr-2 (avr-14)* gene. *Molecular & Biochemical Parasitology* (103) 129-140.
- Köhler,P. 2001. The biochemical basis of anthelmintic action and resistance. *Int.J.Parasitol.* (31) 336-345.
- Kusano,K., Miledi,R. and Stinnakre,J. 1982. Cholinergic and catecholaminergic receptors in the *Xenopus* oocyte membrane. *J.Physiol.(Lond.)* (328) 143-170.
- Laughton,D.L., Amar,M., Thomas,P., Towner,P., Harris,P., Lunt,G.G. and Wolstenholme,A.J. 1994. Cloning of a putative inhibitory amino acid receptor subunit from the parasitic nematode *Haemonchus contortus*. *Recept.Channels* (2) 155-163.
- Laughton,D.L., Lunt,G.G. and Wolstenholme,A.J. 1997. Alternative splicing of a *Caenorhabditis elegans* gene produces two novel inhibitory amino acid receptor subunits with identical ligand binding domains but different ion channels. *Gene* (201) 119-125.
- Martin,R.J. 1985. Y-Aminobutyric acid and Piperazine activated single channel currents from *Ascaris suum* body muscle. *Br.J.Pharmacol.* (84) 445-461.

- Martin,R.J. 1980. The effect of γ -aminobutyric acid on the input conductance and membrane potential of *Ascaris muscle*. *Br.J.Pharmacol.* (71) 99-106.
- Martin,R.J., Pennington,A.J., Duittoz,A.H., Robertson,S. and Kusel,J.R. 1991. The physiology and pharmacology of neuromuscular transmission in the nematode parasite, *Ascaris suum*. *Parasitology* (102 Suppl) S41-58.
- Mazumder,B., Seshadri,V. and Fox,P.L. 2003. Translational control by the 3'-UTR: the ends specify the means. *Trends Biochem.Sci.* (28) 91-98.
- Mehlhorn, H. (Ed.). (2008). *Encyclopedia of parasitology* (3rd ed.) Springer Berlin Heidelberg.
- Mehta,A.K. and Ticku,M.K. 1999. An update on GABA_A receptors. *Brain Res.Rev.* (29) 196-217.
- Olsen, RW, Tobin, AJ. 1990. Molecular biology of GABA_A receptors. *The FASEB Journal* (4) 1469-1480.
- Portillo,V., Jagannathan,S. and Wolstenholme,A.J. 2003. Distribution of Glutamate-Gated Chloride Channel Subunits in the Parasitic Nematode *Haemonchus contortus*. *J.Comp.Neurol.* (462) 213-222.
- Prichard,R. 1994. Anthelmintic resistance. *Vet.Parasitol.* (54) 259-268.
- Ros-Moreno,R.M., Moreno-Guzman,M.J., Jimenez-Gonzalez,A. and Rodriguez-Caabeiro,F. 1999. Interaction of ivermectin with γ -aminobutyric acid receptors in *Trichinella spiralis* muscle larvae. *Parasitol.Res.* (85) 320-323.
- Sieghart,W. 1995. Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. *Pharmacol.Rev.* (47) 181-234.
- Skinner,T.M., Bascal,Z.A., Holden-Dye,L., Lunt,G.G. and Wolstenholme,A.J. 1998. Immunocytochemical localization of a putative inhibitory amino acid receptor subunit in the parasitic nematodes *Haemonchus contortus* and *Ascaris suum*. *Parasitology* (117) 89-96.
- Unwin,N. 1993. Neurotransmitter action: opening of ligand-gated ion channels. *Neuron-Cambridge MA-* (10) 31-41.
- Van Doren,K. and Hirsh,D. 1988. Trans-spliced leader RNA exists as small nuclear ribonucleoprotein particles in *Caenorhabditis elegans*. *Nature* (335) 556-559.

- Vien,J., Duke,R.K., Mewett,K.N., Johnston,G.A.R., Shingai,R. and Chebib,M. 2002. trans-4-Amino-2-methylbut-2-enoic acid (2-MeTACA) and (±)-trans-2-aminomethylcyclopropanecarboxylic acid ((±)-TAMP) can differentiate rat ρ 3 from human ρ 1 and ρ 2 recombinant GABA_C receptors. *Br.J.Pharmacol.* (135) 883-890.
- Weston,D., Patel,B. and Van Voorhis,W.C. 1999. Virulence in *Trypanosoma cruzi* infection correlates with the expression of a distinct family of sialidase superfamily genes. *Molecular & Biochemical Parasitology* (98) 105-116.
- Whiting,P.J. 2003. GABA-A receptor subtypes in the brain: a paradigm for CNS drug discovery? *Drug Discov.Today* (8) 445-450.
- Wolff,M.A. and Wingate,V.P.M. 1998. Characterization and comparative pharmacological studies of a functional γ -aminobutyric acid (GABA) receptor cloned from the tobacco budworm, *Heliothis virescens* (Noctuidae: Lepidoptera). *Invertebr.Neurosci.* (3) 305-315.
- Yates,D.M. and Wolstenholme,A.J. 2004. An ivermectin-sensitive glutamate-gated chloride channel subunit from *Diriofilaria immitis*. *Int.J.Parasitol.* (34) 1075-1081.
- Zhang,D., Pan,Z.H., Zhang,X., Brideau,A.D. and Lipton,S.A. 1995. Cloning of γ -aminobutyric acid type C receptor subunit in rat retina with methionine residue critical for picrotoxinin channel block. *Proc.Natl.Acad.Sci.USA* (92) 11756-11760.