Molecular Characterization of the Binding Site of Nematode GABA$_A$

Receptors

By

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Certification of Approval
Abstract

*Haemonchus contortus* is a parasitic nematode that is controlled in large part by nematocidal drugs that target receptors of the parasitic nervous system. Hco-UNC-49 is a nematode GABA receptor that has a relatively low overall sequence homology to mammalian GABA receptors but is very similar to the UNC-49 receptor found in the free living nematode *Caenorhabditis elegans*. However, the nematode receptors do exhibit different sensitivities to GABA which may be linked to differences in the putative GABA binding domains. Mutational analysis conducted in this study identified at least one amino acid, positioned near the GABA binding domain, which may partially account for differences in nematode GABA sensitivity. In addition, positions reported to be crucial for GABA sensitivity in mammalian receptors also affect GABA sensitivity in Hco-UNC-49 suggesting that the GABA binding domains of the mammalian and nematode GABA receptors share some pharmacological similarities. However, there were some differences observed. For example, in mammalian GABA$_\alpha$ receptors amino acids from both $\beta$ and $\alpha$ subunits appear to be important for GABA sensitivity. For residues examined in this study, only those on the UNC-49B subunit, and not UNC-49C, appear important for GABA sensitivity.

**KEYWORDS**

*Haemonchus contortus*, GABA, ligand binding, mutational analysis, homology modelling
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List of Abbreviations

- 5-HT<sub>3</sub> Serotonin-gated cation channels
- AChBP Acetylcholine binding protein
- Ala Alanine
- Arg Arginine
- *ben-1* Benzimidazole resistant mutant
- BDI Binding domain I
- BDII Binding domain II
- cDNA Complementary DNA
- Cel-UNC-49 *Caenorhabditis elegans* uncoordinated gene 49
- CNS Central nervous system
- cRNA copy ribonucleic acid
- Cys-loop Cysteine-loop
- DALY Disability adjusted life year
- DD Dorsal D-type motor neuron
- DNA Deoxyribonucleic acid
- EC<sub>50</sub> Half-maximal channel activity
- GABA γ-aminobutyric acid
- *Grd* *Glycine-like receptor* of Drosophila
- Hco-LGC-38 *Haemonchus contortus* RDL homologue
- Hco-UNC-49 *Haemonchus contortus* uncoordinated gene 49
- IVM Ivermectin
- KCL Potassium chloride
- L1-L4 Larval growth stage 1 - 4
- *Lcch3* *Ligand-gated chloride channel homologue 3*
- Leu Leucine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LGCC</td>
<td>Ligand-gated chloride channels</td>
</tr>
<tr>
<td>LGIC</td>
<td>Ligand-gated ion channels</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>MS-222</td>
<td>Ethyl 3-amino benzoate methane sulfonate</td>
</tr>
<tr>
<td>nAChRs</td>
<td>Nicotinic acetylcholine receptors</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>PTX</td>
<td>Picrotoxin</td>
</tr>
<tr>
<td>Rdrl</td>
<td>Resistance to dieldrin-like</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>Ser</td>
<td>Serine</td>
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<tr>
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<td>Small subunit ribosomal RNA</td>
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<tr>
<td><em>tub</em>-1</td>
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<tr>
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<td>Tyrosine</td>
</tr>
<tr>
<td><em>unc</em></td>
<td>Uncoordinated genes</td>
</tr>
<tr>
<td>VD</td>
<td>Ventral D-type motor neuron</td>
</tr>
<tr>
<td>VGAT</td>
<td>Vesicular GABA transporter</td>
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Chapter 1 –
Introduction
1.1 The Phylum Nematoda

The phylum Nematoda (roundworms) represents one of the most diverse groups of organisms found on the planet (Gilleard, 2004; Nikolaou and Gasser, 2006). Estimates suggest that nematodes encompasses roughly 100,000 to 1 million extant species exploiting a wide variety of niches include free living, terrestrial and marine microbivores, meiofaunal predators, herbivores, and plant and animal parasites (Parkinson et al. 2004). Small subunit ribosomal ribonucleic acid (SSU rRNA) phylogenetics suggest that nematode phylogeny consists of five major clades (I to V) which include Dorylaimia (clade I), Enoplia (clade II) and Chromadorea (which includes Rhabditida; see Figure 1) (Gilleard, 2004; Parkinson et al. 2004). Rhabditida, a paraphyletic taxon, can further be divided into several other taxa including Spirurina (clade III), Tylenchina (clade IV) and Rhabditina (clade V) (Gilleard, 2004; Parkinson et al. 2004). Most clades are comprised of a combination of free-living nematodes as well as animal and plant parasites (Blaxter, 2002; Gilleard, 2004). Consequently, it is believed there have been multiple independent events of gain of parasitism during nematode evolution (Blaxter, 2002). Interestingly, clade III contains only animal parasites suggesting that parasitism is the ancestral state of this taxon (Blaxter, 2002).
Figure 1. Phylogenetic tree showing evolutionary relationships within the phylum Nematoda. Species indicated are of great importance within this thesis. The evolutionary relationships are based on SSU rRNA sequences. The species underlined represents a parasitic nematode. The species shown in italics represent the related free-living nematode. Adapted from Gillett (2004) and Parkinson et al. (2004).

One of the best known characteristics of the Nematoda is the vast number of parasitic species, many of which infect humans and domesticated animals (Blaxter, 2002). Thus, nematode infections represent a significant source of economical and health-related crises worldwide. Presently, it is estimated that approximately 2.9 billion people are inflicted with nematode infections resulting in a myriad of debilitating illnesses such as African river blindness and elephantiasis (Parkinson et al. 2004). The highest incidence rates are most commonly associated with tropical regions, especially that of Africa, Asia and the Americas (Parkinson et al. 2004). Consequently, it has been estimated that nematode-associated human morbidity rivals that of diabetes and lung cancer in worldwide disability adjusted life year (DALY) measurements (Parkinson et al. 2004). Fortunately mortality rates are low with respect to the incidence of infection; however, death rates may still exceed 100,000 annually. (Parkinson et al. 2004).
Another significant consequence to nematode infections is the substantial losses in livestock and companion animals. Major losses have been observed in the productivity within sheep wool and meat industries (Newton and Meeusen, 2003). In addition, nematode infections are responsible for approximately $80 billion in annual crop damage worldwide (Parkinson et al. 2004). Although nematode parasites infect an array of species, a vast majority of parasitic nematodes parasitize vertebrates, specifically mammals. The evolution of vertebrate parasitism has been quite successful, arising in at least four independent taxonomic groups: Trichocephalida (clade I), the three orders in clade III, the Strongyloidae (clade IV) and the Strongylida (clade V) (Dorris et al. 2002). Clade V is of high economical importance as this clade represents the strongylid nematodes; many of which are important veterinary pathogens (Gilleard, 2004). Most notable are the Trichostrongyloidea which include important parasitic nematode genera infecting domestic livestock such as Haemonchus, Ostertagia, and Trichostrongylus, (Gilleard, 2004).

1.1.1 Caenorhabditis elegans

The majority of our current understanding about the molecular and developmental biology of nematodes can be attributed to the nematode Caenorhabditis elegans. C. elegans is a free living soil nematode approximately 1 mm in length (Strange, 2006). This nematode is well adapted for survival in soil environments where it can outcompete its competitors by producing large numbers of offspring which in turn rapidly deplete local food sources (Strange, 2006). The sex of adult C. elegans is largely hermaphroditic with males comprising less than 0.1% of the wild-type populations (Strange, 2006).
As the first multi-cellular organism whose genome was completely assembled, *C. elegans* has been fundamental in medical research especially within the fields of cancer, ageing, neurobiology and parasitology (Parkinson *et al.* 2004). The scientific value of *C. elegans* is attributed to its simplistic life-cycle and ease of maintenance. Parasitic nematodes, unlike *C. elegans*, lack *in vitro* culture systems and require the propagation of life cycles *in vivo* (Gilleard, 2004). Alternatively, at 25°C, *C. elegans’* embryogenesis occurs in approximately 14 hours and post embryonic development (i.e. L1-L4 larval development) lasts a total of 35 hours (Strange, 2006). Furthermore, the typical life span of *C. elegans* is about 2-3 weeks. Thus, life-cycle experiments can be completed very rapidly. As a result, there is an increasing interest in the use of *C. elegans* as a standard tool for molecular helminthology (Gilleard, 2004).

For the wealth of information available regarding *C. elegans* there is comparatively little known about other members within this phylum. To date there are few studies directly comparing aspects of *C. elegans* biology with analogous processes in parasitic nematode species (Gilleard, 2004). One explanation for this lack of comparison is the extensive diversity of the phylum Nematoda (Gilleard, 2004). Thus, the value of *C. elegans* in helminthology will differ depending on its phylogenetic distance from a particular parasitic nematode species (Gilleard, 2004). For instance, *C. elegans* are located within clade V (Figure 1; Gilleard, 2004). Clade V also consists of strongylid nematodes, which as indicated previously, contain important parasitic nematode species infecting domestic livestock which include the *Haemonchus* family (Gilleard, 2004). As a result, *C. elegans*, a rhabditine nematode, is closely related to the vertebrate-parasite *Haemonchus contortus* (Parkinson *et al.* 2004). Thus it is reasonable to assume that *C.*
elegans biology will, in general, have more relevance to *H. contortus* than the more distantly related parasites within other clades.

### 1.1.2 Haemonchus contortus

One of the most economically important parasitic nematodes is *Haemonchus contortus* (Nikolaou and Gasser, 2006). *H. contortus* is a gastrointestinal parasitic nematode commonly infecting ruminants, such as sheep and cattle. The pathogenesis of a *Haemonchus* infection results from the bloodsucking activity of the parasite within the abomasum, or fourth stomach, of its host (Nikolaou and Gasser, 2006). *Haemonchus* infections may lead to serious health complications such as acute Haemonchosis or anaemia, which if left untreated, often leads to the animal’s death (Nikolaou and Gasser, 2006).

The life cycle of *H. contortus* is complex consisting of two major phases: a free living and parasitic phase (Nikolaou and Gasser, 2006). Briefly, an adult hermaphrodite releases approximately 4,500 eggs within the abomasum of an infected host (Nikolaou and Gasser, 2006). These eggs develop to the 11-26 cell stage at which point oxygen becomes essential to proper development (Nikolaou and Gasser, 2006). Consequently, the eggs are released into the environment within the fecal matter and remain there until they develop into a developmentally arrested L3 infective larva (Nikolaou and Gasser, 2006). At this stage, the juvenile nematode waits on blades of grass to be consumed by a host. Upon being consumed, temperature, pH and chemical conditions within the host’s gut stimulate the L3 larvae to attach to the mucosa of the abomasum initiating its parasitic life phase (Nikolaou and Gasser, 2006). The development of a buccal capsule
during the L4 larval stage is required to facilitate blood feeding (Nikolaou and Gasser, 2006). Finally, sexual differentiation of the juvenile parasite signals developmental completion (Nikolaou and Gasser, 2006).

At the moment, the most common treatment for *Haemonchus* infections is through the use of anthelmintics. There are several drugs used to treat *Haemonchus* infections and most target specific receptors of the parasite nervous system called ligand-gated ion channels (LGICs). Presently, there are three major classes of anthelmintics currently in use. The first class, the benzimidazoles, disrupt parasite tubulin polymerization. The levamisole/morantel family disrupt sodium transport through cholinergic receptor channels. Finally the avermectins, consisting of macrocyclic lactones (i.e. Ivermectin, IVM), activate glutamate-gated chloride channels that are present on nematode neuromuscular systems (Mes, 2004). As a result, parasitic nematodes are effectively paralyzed.

Unfortunately the development of genetic resistance (i.e. anthelmintic resistance) to several of these drugs in *H. contortus* is an unwanted consequence of the evolutionary process. Genetic resistance within a population is perpetuated to the next generation via the survival of a parental line under selective pressures such as anthelmintic treatment (Njue and Prichard, 2004). Ultimately, the frequency of resistant individuals within the population will increase resulting in overall treatment failure (Njue and Prichard, 2004; Blackhall *et al.* 2008).
1.1.3 C. elegans as a model for H. contortus

As the rise of resistance to several anthelmintics in *H. contortus* continues to reduce treatment efficacy, the need for novel treatment development has increased. As a result, there is growing interest in the use of the free-living nematode *C. elegans* as a standard tool for molecular helminthology (Gilleard, 2004). However, there currently is an ongoing debate regarding the relevance of *C. elegans* biology in parasitology, especially with respect to *H. contortus* (Gilleard, 2004). Therefore, in order to use this understanding to develop novel drug treatments for nematode-induced diseases, the relationship between *C. elegans* and *H. contortus* must be understood. Previous studies have demonstrated that *C. elegans* can be used to study *H. contortus* biology at the molecular level. For instance, the *H. contortus* isotype 1 β-tubulin gene (*tub-1*), involved in benzimidazole resistance, was the first parasite gene to be functionally expressed under the control of its own cis regulatory elements in *C. elegans* (Kwa et al. 1995). This approach provided a functional assay to test putative benzimidazole resistance-conferring alleles from the parasitic nematode *H. contortus* (Kwa et al. 1995; Gilleard, 2004). Moreover, transforming *C. elegans ben-1*, benzimidazole resistant, mutants with susceptible alleles of the *H. contortus* *tub-1* genes increased sensitivity of the strain to benzimidazoles (Kwa et al. 1995). This work exemplifies how an interspecies mutant rescue experiment allows for a parasite gene to be functionally examined in some detail (Kwa et al. 1995; Gilleard, 2004).

Studies involving GABA receptor gene co-expression between *C. elegans* and *H. contortus* have also been conducted. A LGIC receptor subunit known as Hco-LGC-37 has been identified in *H. contortus* which can only form GABA sensitive channels in
Xenopus laevis oocytes in the presence of a GABA_\text{A} \beta\text{-like receptor subunit (Feng et al. 2002). As a result, when the Hco-LGC-37 subunit was co-expressed with a C. elegans \beta\text{-like receptor subunit, known as GAB-1, a fully functional heteromeric GABA-sensitive channel was produced (Feng et al. 2002). Thus, this investigation demonstrated the potential for interspecies gene co-expression studies for exploring the function of parasite LGIC receptor subunits.

1.2 Ligand-gated Ion Channels

Fast synaptic neurotransmission, both excitatory and inhibitory, for invertebrates and vertebrates is mediated in part by the nicotinic class of LGIC receptors (Johnston, 2005). LGICs, also called cysteine-loop (Cys-loop) receptors (Mes, 2004), can be subdivided into cation- and anion-selective channels (Johnston, 2005). Excitatory receptors are generally activated by molecules such as acetylcholine or serotonin whereas inhibitory receptors are generally activated by \(\gamma\)-aminobutyric acid (GABA), glutamate (only in invertebrates) or glycine (Yates et al. 2003; Johnston, 2005). Both cationic and anionic receptors can form homomeric receptors in which five copies of a single type of subunit assemble together (Johnston, 2005). However, LGICs more commonly assemble from several different types of subunits forming hetero-pentamers (Johnston, 2005). These pentamers are composed of five subunits which are arranged in the post-synaptic membrane to form a central ion channel (Johnston, 2005). Each subunit consists of an N-terminal extracellular domain wherein the ligand binding sites reside, four transmembrane domains (TM1-TM4) - the second of which form the channel-lining, and an extracellular C-terminus (Johnston, 2005; see Figure 2). Due to numerous subunit
types, there can be a large variety of possible heteromeric channels which in turn offers a myriad of functional diversity able to meet a wide range of synaptic needs (Johnston, 2005).

**Figure 2. Schematic structure of LGIC receptors.** Left: Monomeric subunit located within a cellular membrane. The cellular membrane is represented by the lipid bi-layer. The transmembrane regions are located within the cellular membrane depicted by shaded rectangles. TM1 is located on the far left (I) and TM4 is located on the far right (IV). TM2 and 3 are positioned accordingly. The characteristic disulfide bond of the Cys-loop family of receptors is located in the N-terminal extracellular domain depicted as a black line. Right: A representative illustration of five subunits symmetrically arranged around the central pore. The ions which pass through the central pore are dependent upon the receptor class. For clarity, chloride ions were chosen to indicate passage through the channel pore. Adapted from Lester *et al.* (2004).

Due to the high degree of functional diversity within the LGIC superfamily, a variety of LGICs have been described in both vertebrates and invertebrates. The function of each receptor has evolved to meet a specific synaptic role. For instance, nicotinic acetylcholine receptors (nAChRs) and serotonin-gated cation channels (5-HT₃) are both expressed in nerve cells while the former is additionally expressed in muscle cells (Connolly and Wafford, 2004). Consequently, both receptors play an essential role in fast synaptic cationic neurotransmission (Connolly and Wafford, 2004). Conversely, the glycine and GABA receptor are part of the inhibitory nervous system and as such are expressed within the spinal cord and central nervous system, respectively (Connolly and
Additionally, invertebrate systems have been shown to express ligand-gated chloride channels (LGCC) which are activated by non-traditional neurotransmitters such as histamine (Gisselmann et al. 2001), glutamate (Cully et al. 1994), dopamine (Rao et al. 2009) and tyramine (Pirri et al. 2009).

The activation of LGICs has been linked to six discontinuous extracellular loops within the extracellular N-terminal (Kash et al. 2004, Lummis, 2009). This N-terminal loop model appears to be well conserved across a wide variety of LGICs and contains the ligand (neurotransmitter) binding pocket (Kash et al. 2004). Channel opening is initiated by neurotransmitter binding which causes constriction of the binding pocket (Kash et al. 2004). This constriction causes a ‘conformational wave’ of several loops within the extracellular structure resulting in interactions between the TM2–3 linker and pre-TM1 segments (see Figure 2; Kash et al. 2004). Subsequently, both a rotation and tilting of the TM helices allows the channel pore to open (Kash et al. 2004).

1.3 GABA-gated Chloride Channels

The most abundant inhibitory neurotransmitter in vertebrate nervous systems is GABA (Schuske et al. 2004), which is estimated to be present in approx 20-50% of cerebral cortex synapses (Hevers and Lüddens, 1998). Thus, it is of no surprise that GABA has been implicated in numerous psychiatric disorders such as anxiety, insomnia and epilepsy (Hevers and Lüddens, 1998). GABA invokes neuronal inhibition by acting on three classes of membrane-bound receptors encoded within the vertebrate genome (Johnston, 2005). These receptors can be divided into two major types: ionotropic receptors (GABA\textsubscript{A} and GABA\textsubscript{C} receptors) and metabotropic receptors that are G-protein
coupled receptors (GABA$_B$ receptors; Hevers and Lüddens, 1998; Johnston, 2005); the latter acts via second messengers (Johnston, 2005). However, it is the fast inhibitory synaptic neurotransmission, mediated by ionotropic GABA receptors, which play the most extensive roles in regulating mechanisms within the nervous system of both vertebrates and invertebrates (Lee et al. 2003).

The ionotropic GABA receptors are members of the nicotinic-like LGIC superfamily, which form pentameric complexes arranged around a central ion conducting pore (Johnston, 2005). Similar to all members of the LGIC, these GABA receptors are composed of a large extracellular N-terminal domain and four transmembrane domains (Johnston, 2005). The cytoplasmic loop, between the third and fourth transmembrane domains of the receptor is thought to be the target for protein kinases, required for subcellular targeting and membrane clustering (Johnston, 2005). GABA$_A$ and GABA$_C$ receptors assemble into chloride-selective transmembrane channels ubiquitously distributed within the central nervous system (CNS) of vertebrates where their main physiological role is the mediation of inhibitory neurotransmission (Hevers and Lüddens, 1998; Johnston, 2005).

1.3.1 The Vertebrate GABA$_A$ Receptor

Of all the receptors which constitute the LGIC superfamily, the GABA$_A$ receptor represents one of the most thoroughly characterized receptors in the vertebrate nervous system. GABA$_A$ receptors embody a sophisticated and complex receptor family comprised of 16 different subunits: $\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_{1-3}$, $\delta$, $\varepsilon$, $\pi$ and $\theta$ (Johnston, 2005). As a result GABA$_A$ receptors have the ability to form either homomeric or, more commonly,
heteromeric channels. Many of the subunits also have possible splice variants which increase the overall complexity and receptor variability (Johnston, 1996). Consequently, GABA<sub>A</sub> receptors have the potential to form thousands of functional pentameric receptors with diverse subunit arrangements (e.g. α<sub>1</sub>, β<sub>2</sub> and γ<sub>2</sub> subunits). Over 2000 different pentameric GABA<sub>A</sub> receptors could exist even if combinations were restricted to those containing at least two α and two β subunits (Johnston, 2005). However, different neuron classes appear to express specific combinations of subunit genes with roughly a dozen major GABA<sub>A</sub> receptor subtypes identified experimentally (McKernan and Whiting, 1996). Thus, it appears that the number of GABA<sub>A</sub> receptor subtypes is constrained by subunit expression patterns indicating that the formation of these receptors is a highly regulated process (Bamber et al. 1999).

GABA<sub>A</sub> receptors are one of the most complicated receptors found in the LGIC superfamily not only in terms of the large number of receptor subtypes but also the large variety of ligands which interact with specific sites on the receptor (Johnston, 1996). It is estimated that approximately 11 distinct ligand binding sites are located on the GABA<sub>A</sub> receptor which associate with more than 100 different known agents such as GABA, picrotoxin (PTX), muscimol, bicuculline and propofol (Johnston, 1996). Although the number of specific sites may vary, depending upon the subunit arrangement, all receptors must contain at least one β-subunit to form a properly functioning receptor (Amin and Weiss, 1993; Johnston, 1996). This is a result of two separate and homologous domains on the β-subunit which are essential for activation by GABA (Amin and Weiss, 1993). These homologous domains, known as binding domains I and II (BDI and BDII, respectively), are both four amino acids in length and each contribute a tyrosine and
threonine essential in the binding of a GABA molecule (Amin and Weiss, 1993). These amino acids are highly conserved across a wide spectrum of species due to their essential roles in the activation of GABA\textsubscript{A} receptors (see Table 1). Both the tyrosine and threonine are said to form hydrogen-bonds between the main chain amino and the carboxyl groups (Amin and Weiss, 1993). Additionally, a conserved glycine is said to assist in the formation of hairpin turns aligning the tyrosine and threonine in close proximity to the agonist (Amin and Weiss, 1993).
**Table 1. Comparative analysis of GABA receptor binding loop sequences from several species.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Common Name</th>
<th>Vertebrate</th>
<th>Receptor/Subunit</th>
<th>Loop B(^1) BDI Sequence</th>
<th>Loop C(^1) BDII Sequence</th>
<th>Loop D Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Homo sapiens</em></td>
<td>Human</td>
<td>Yes</td>
<td>GABA(_A/\beta)-subunit</td>
<td>ESYGYTT</td>
<td>VVFSTGSYP</td>
<td>LTMYFQQQA</td>
<td>Schofield <em>et al.</em> 1987</td>
</tr>
<tr>
<td><em>Rattus norvegicus</em></td>
<td>Rat</td>
<td>Yes</td>
<td>GABA(_A/\beta)-subunit</td>
<td>ESYGYTT</td>
<td>VVFSTGSYP</td>
<td>LTMYFQQY</td>
<td>Lolait <em>et al.</em> 1989</td>
</tr>
<tr>
<td><em>Bos Taurus</em></td>
<td>Bovine</td>
<td>Yes</td>
<td>GABA(_A/\beta)-subunit</td>
<td>ESYGYTT</td>
<td>VVFSTGSYP</td>
<td>LTMYFQQA</td>
<td>Ymer <em>et al.</em> 1989</td>
</tr>
<tr>
<td><em>Gallus gallus</em></td>
<td>Chicken</td>
<td>Yes</td>
<td>GABA(_A/\beta)-subunit</td>
<td>ESYGYTT</td>
<td>VVFATGAYP</td>
<td>LTMYFQQY</td>
<td>Bateson <em>et al.</em> 1990</td>
</tr>
<tr>
<td><em>Caenorhabditis elegans</em></td>
<td>Roundworm</td>
<td>No</td>
<td>UNC-49/B-subunit</td>
<td>ESYGYET</td>
<td>AETSSGKYV</td>
<td>LDFYMRQT</td>
<td>Bamber <em>et al.</em> 1999</td>
</tr>
<tr>
<td><em>Haemonchus contortus</em></td>
<td>Barber Pole</td>
<td>No</td>
<td>UNC-49/B-subunit</td>
<td>ESYGYTM</td>
<td>ATTSSGYSR</td>
<td>LDFYMRQT</td>
<td>Siddiqui <em>et al.</em> 2010</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td>House Fly</td>
<td>No</td>
<td>RDL/isoform B</td>
<td>ESFGYTMs</td>
<td>INLTTGNYK</td>
<td>LDFYFRQF</td>
<td>ffrench-Constant <em>et al.</em> 1991</td>
</tr>
<tr>
<td><em>Lymnea stagnalis</em></td>
<td>Pond Snail</td>
<td>No</td>
<td>GABA(_A/\beta)-like subunit</td>
<td>ESYGYTM</td>
<td>EELSTGDYQ</td>
<td>ITMYLNNQY</td>
<td>Harvey <em>et al.</em> 1991</td>
</tr>
</tbody>
</table>

1 The bolded amino acids, within each respective loop sequence, denote the putative binding domains.
The complexity of GABA binding in GABA\textsubscript{A} receptors is attributed to the fact that the GABA binding site is located at subunit interfaces (Lummis, 2009). Based on structural modelling of the related acetylcholine binding protein (AChBP) it has been demonstrated that, like other Cys-loop receptors, the entire binding pocket of the GABA\textsubscript{A} receptor is constructed from six regions, known as Loops A-F (Lummis, 2009; see Figure 3A). In fact, the formation of the ligand-binding site appears to be composed of residues contributed from Loops A-C of the principle subunit and residues from Loops D-F of the adjacent subunit (Lummis, 2009). For instance, the most common GABA\textsubscript{A} receptor observed in the vertebrate nervous system is composed of $\alpha_1$, $\beta_2$ and $\gamma_2$ subunits (McKernan and Whiting, 1996). From this composition it has been shown through mutagenesis that residues critical for binding can be found in Loop B ($\beta_2$Tyr\textsuperscript{157} and $\beta_2$Thr\textsuperscript{160}; BDI residues) and C ($\beta_2$Thr\textsuperscript{202} and $\beta_2$Tyr\textsuperscript{205}; BDII residues) of the principle subunit and Loop D ($\alpha_1$Phe\textsuperscript{64}) of the adjacent subunit (Sigel \textit{et al.} 1992; Amin and Weiss, 1993; Lummis, 2009; see Figure 3B). Additionally, other loops have been shown to be involved in GABA sensitivity within the $\beta_2$-$\alpha_1$ interface such as Loop A ($\beta_2$Tyr\textsuperscript{97}; Boileau \textit{et al.} 2002), E ($\alpha_1$Arg\textsuperscript{120}; Westh-Hansen \textit{et al.} 1999), and F ($\alpha_1$Asp\textsuperscript{181}; Newell and Czajkowski, 2003).
Figure 3. Model of the GABAβα subunit interface. A) Docking of GABA into the β2-α1 subunit interface. The β2 subunit is located on the left. The α1 subunit is located on the right. The six discontinuous loops involved in the binding of GABA are highlighted in black and identified with their corresponding letter designation. The grey box represents the areas enlarged in Figure 3B. B) Amino acids (represented in their stick form) shown to be essential for GABA binding in the GABAβα receptor. Amino acid loop designations are identified as follows: Loop A (magenta), Loop B (purple), Loop C (cyan), Loop D (dark gray). The blue colour on the GABA molecule represents the nitrogen atom. The red colours on the GABA molecule represent oxygen atoms.

1.3.2 The Invertebrate GABA Receptors

Similar to their vertebrate counterparts, invertebrate ionotropic GABA receptors can be found dispersed throughout the nervous system of many insects and worms (Hosie et al. 1997). Invertebrate ionotropic GABA receptors form anion-selective ion channels
which perform rapid synaptic inhibitory neurotransmission (Hosie et al. 1997). Although both vertebrate and invertebrate ionotropic GABA receptors share similar functional and structural characteristics, their pharmacological profiles are quite different (Hosie et al. 1997). For instance, vertebrate GABA_A receptors are antagonized by both bicuculline and PTX (Hosie et al. 1997). Although some invertebrate ionotropic GABA receptors are also antagonized by PTX, they are typically insensitive to bicuculline (Hosie et al. 1997). In fact, invertebrate ionotropic GABA receptors differ from both vertebrate GABA_A and GABA_C receptors in their sensitivity to many GABA analogues and allosteric modulators (Hosie et al. 1997).

In contrast to what is known regarding vertebrate GABA receptors, there have been only a limited number of GABA receptor subunits cloned and characterized from invertebrate organisms. Thus far there have been three Cys-loop receptor subunit classes identified in Drosophila melanogaster (common fruit fly). The three classes are encoded by three genes: the glycine-like receptor of Drosophila (Grd), the ligand-gated chloride channel homologue 3 (Lcch3), and the resistance to dielddrin like (Rdl) (Hosie et al. 1997). However, the most extensively characterized non-vertebrate GABA receptor is the major product of the Rdl gene, the RDL receptor (Hosie et al. 1997).

The Rdl gene has the ability to produce four possible gene products all possessing features characteristic of ionotropic GABA receptors (Hosie et al. 1997). In fact, the subunits encoded within the Rdl gene display approximately 30-38% sequence identity to that of vertebrate GABA_A β-subunits and GABA_C ρ-subunits – which is typical sequence identity for vertebrate verses invertebrate GABA receptors (Hosie et al. 1997). Moreover, the RDL receptor has the ability to form homomeric channels, a feature characteristic of
GABA$_A$ β-subunits and GABA$_C$ ρ-subunits (Hosie et al. 1997). Immunocytochemical studies indicated that the Rdl gene products are in fact distributed throughout the Drosophila CNS and concentrated in regions of neuropil (Hosie et al. 1997). Furthermore, it appears that the distribution of RDL antibody staining correlates closely with that of immunoreactivity for GABA, as well as for the enzyme glutamic acid decarboxylase and the synaptic vesicle protein synaptotagmin (Hosie et al. 1997). Thus, the evidence convincingly implements the Rdl gene products, specifically the RDL protein, as synaptic neuronal GABA receptors (Hosie et al. 1997). Partial and full-length complementary deoxyribonucleic acids (cDNAs) encoding homologues of the Drosophila RDL subunits have further been identified in species within three orders of insects including: the yellow fever mosquito Aedes aegypti (Diptera), the German cockroach Blattella germanica (Dictyoptera), and the beetle Tribolium castaneum (Coleoptera) (Hosie et al. 1997).

With respect to nematodes there have been several GABA receptors characterized. Previous studies on H. contortus have identified a GABA receptor subunit gene called Hco-lgc-37 (Laughton et al. 1994) which is expressed in ring motor- and inter-neurons (Skinner et al. 1998). Functional analysis indicates that while Hco-LGC-37 does not form a functional homomeric channel, it can co-assemble with the C. elegans subunit Cel-GAB-1 to form a functional GABA-sensitive channel (Feng et al. 2002). Recently, a RDL homologue was isolated from H. contortus (Hco-LGC-38; Siddiqui et al., unpublished). However, to date, it is unknown in which tissues these receptor subunits are expressed (Siddiqui et al., unpublished). Other nematode GABA receptors
include the UNC-49 GABA receptors which are discussed below.

1.4 The Uncoordinated Genes

The importance of the GABAergic nervous system in nematodes was first determined through investigations in which laser ablation was conducted on 26 of the 302 neurons present in C. elegans which express the neurotransmitter GABA (McIntire et al. 1993a). When the dorsal and ventral D-type motor neurons (DD or VD, respectively) were ablated, C. elegans exhibited abnormal locomotion (McIntire et al. 1993a). Typically when a C. elegans travels, muscle contractions on one side of the body cause the body to bend whilst simultaneous muscle relaxation, via GABA innervations, occurs on the opposite side (Schuske et al. 2004). As a result, movement is driven through a sinusoidal body wave. However, if laser ablation is used to kill the VD and DD neurons required for relaxation of body wall muscles, the operated worm has only excitatory input into its muscles (Schuske et al. 2004). Hence locomotion becomes abnormal. The resultant phenotype, known as a ‘shrinker’ phenotype, occurs as the worm pulls its head in and its body shortens owing to hypercontraction of the body wall muscles on both sides of the body (Schuske et al. 2004).

In nematode locomotion, GABA release from the VD neurons is essential for muscle relaxation to reset posture when changing directions (Schuske et al. 2004). If GABA is not properly utilized, locomotion becomes ‘uncoordinated’ and the shrinker phenotype develops. It is from this understanding that five uncoordinated (unc) genes, required for GABA function, were identified in C. elegans mutants which resembled the shrinker phenotype after laser ablation (Schuske et al. 2004). Three C. elegans mutants,
unc-25, unc-46 and unc-47, appear to shrink and lack enteric muscle contractions (Schuske et al. 2004). This suggests the mutated genes encode proteins that are required for both inhibitory and excitatory GABA functions (Schuske et al. 2004). Additionally, two other mutants, unc-30 and unc-49, solely display the ‘shrinker’ phenotype signifying that the associated proteins are required only for inhibitory GABA function (Schuske et al. 2004).

McIntire et al. (1993b) used the GABA receptor agonist muscimol to determine whether the unc gene products were pre- or post-synaptic. If the unc gene encoded a protein required in the presynaptic neuron, muscles in the C. elegans mutants would respond normally to muscimol (McIntire et al. 1993b; Schuske et al. 2004). Accordingly, muscimol will activate postsynaptic GABA receptors constitutively (Schuske et al. 2004). When McIntire et al. (1993b) bathed wild-type worms in muscimol, contractions ceased in the body muscles causing the animal to become flaccid. This effect was seen with the muscimol sensitive mutants: unc-25, unc-30, unc-46 and unc-47 (McIntire et al. 1993b; Schuske et al. 2004). It was later discovered that these genes encoded the biosynthetic enzyme for GABA - glutamic acid decarboxylase, a homeodomain transcriptional factor required for GABA neuronal specification in the D-type neurons, a gene which regulates the transport of GABA into vesicles and the vesicular GABA transporter (VGAT), respectively (McIntire et al. 1993b; Jin et al. 1994; Jin et al. 1999; Schuske et al. 2004; see Figure 4). However, the unc-49 mutant was resistant to the effects of muscimol on the body suggesting this gene encodes postsynaptic GABA receptors (McIntire et al. 1993b). Since the unc-49 mutants were defective only for
locomotion (an inhibitory GABA function), this gene was predicted to encode an inhibitory GABA receptor (Schuske et al. 2004).

Figure 4. Uncoordinated proteins required for GABA synthesis and transport in Caenorhabditis elegans. GABA is synthesized from glutamate by the unc-25 gene product - glutamic acid decarboxylase. GABA is then transported into synaptic vesicles via the vesicular GABA transporter (VGAT), a protein encoded by the unc-47 gene. It is proposed UNC-46 modulates vesicular GABA loading. UNC-30, a homeodomain transcription factor, is necessary for UNC-25 and UNC-47 expression as denoted by the asterisk. Finally, GABA is released from the neuron activating the inhibitory GABA receptor UNC-49 increasing Cl\textsuperscript{−} uptake and thus relaxing body muscles. Adapted from Schuske et al. (2004).
1.4.1 C. elegans UNC-49 GABA Receptor

*C. elegans* mediate body muscle inhibition during locomotion by means of the GABA receptor encoded by the *unc-49* gene (Schuske *et al.* 2004). The *unc-49* gene locus possesses a gene structure which encodes three distinct GABA receptor subunits. Through alternative splicing, a common N-terminal ligand binding domain is spliced to one of three potential C-terminal domains (Bamber *et al.* 1999). As a result, three different *C. elegans* GABA receptor subunits (Cel-UNC-49A, Cel-UNC-49B and Cel-UNC-49C) are produced (Bamber *et al.* 1999). This unusual gene structure is also conserved in the related nematode *Caenorhabditis briggsae* (Schuske *et al.* 2004).

Of the three aforementioned Cel-UNC-49 subunits, Cel-UNC-49A is barely detectable *in vivo* (Bamber *et al.* 1999). Additionally, it has been shown that Cel-UNC-49A does not co-assemble with Cel-UNC-49B or Cel-UNC-49C to form a functional heteromeric receptor *in vitro* (Bamber *et al.* 1999). Alternatively, strong expression of the Cel-UNC-49B and Cel-UNC-49C subunits has been identified at neuromuscular junctions from the D-type GABA motor neurons (Bamber *et al.* 1999; Schuske *et al.* 2004). Electrophysiological studies have demonstrated that Cel-UNC-49B subunits can form functional homomeric receptors, whereas Cel-UNC-49C cannot (Bamber *et al.* 1999). However, the two subunits can associate to form functional heteromeric channels when co-expressed in *Xenopus laevis* oocytes indicating that a UNC-49B/C heteromer may be the native receptor *in vivo* (Bamber *et al.* 1999). This was later confirmed through *in vivo* studies which detected the heteromeric channel at the neuromuscular junction (Bamber *et al.* 2005).
To better understand the pharmacological properties of UNC-49 GABA receptors, it is essential to understand their relationship to other LGICs. Phylogenetic analysis indicates that UNC-49 subunits are closely related to the RDL receptors of *Drosophila melanogaster* (Bamber *et al.* 2003). Although vertebrate GABA<sub>A</sub> receptors and UNC-49 receptors are related, the UNC-49 subunits are not orthologues to any class of GABA<sub>A</sub> receptor subunits (Bamber *et al.* 2003). However, like RDL receptors, UNC-49 subunits do share some sequence similarity with specific GABA<sub>A</sub> receptor subunits. UNC-49B and UNC-49C share roughly 35-45% sequence similarity with that of mammalian α, β, γ and ρ subunits: UNC-49B shares 42.5% similarity with the GABA<sub>A</sub> β subunit (Bamber *et al.* 2003).

When comparing the pharmacology of the UNC-49 GABA receptors with that of mammalian GABA receptors there are key differences observed that appear to be linked to structural differences between the channels. For instance, Bamber *et al.* (2003) demonstrated that Cel-UNC-49B homomers are sensitive to PTX, whereas Cel-UNC-49B/C heteromers were resistant. This change in PTX sensitivity has been attributed to a single residue within the M2 domain (Gurley *et al.* 1995; Bamber *et al.* 2003; Schuske *et al.* 2004). Gurley *et al.* (1995) showed that the conserved threonine at the 6' position in the M2 domain is essential in determining PTX sensitivity in the rat ρ1 GABA<sub>C</sub> receptor subunit. A mutation replacing the threonine with a methionine in the rat ρ1 GABA<sub>C</sub> receptor subunit resulted in PTX resistance (Gurley *et al.* 1995). In fact, the Cel-UNC-49C subunit contains a methionine in this position which confers PTX resistance to the Cel-UNC-49B/C heteromer (Bamber *et al.* 2003). Conversely, the Cel-UNC-49B subunit possesses the aforementioned conserved threonine at the 6' position resulting in a PTX
sensitive channel when it is assembled as a homomeric channel (Bamber et al. 2003).

1.4.2 H. contortus UNC-49 GABA Receptor

Recently, two orthologous Cel-UNC-49 subunit genes were isolated in H. contortus known as Hco-UNC-49B and C. Structure-function analysis has shown that the Hco-UNC-49 subunits share similar characteristics with those observed in C. elegans. For instance, the Hco-UNC-49 subunits have been shown to possess similar N-terminal amino acid sequences whereas their C-terminal sequences exhibit some variability. Thus, it is believed that the Hco-UNC-49 gene transcripts are under the same alternative splicing mechanisms as their Cel-UNC-49 counterparts. Furthermore, like their C. elegans orthologues, the Hco-UNC-49C subunit is unable to form a functional homomeric channel, yet possesses the ability to form a functional PTX resistant heteromeric channel when co-expressed with Hco-UNC-49B (Siddiqui et al. 2010).

The most interesting characteristic of the Hco-UNC-49 receptor is its relative sensitivity to GABA compared to the Cel-UNC-49 receptor. Specifically, the Hco-UNC-49B/C heteromeric channel exhibits an increased GABA sensitivity compared to that of the Hco-UNC-49B homomeric channel (EC$_{50}$ of 39.9 ± 5.7 µM to 64.0 ± 4.4 µM, respectively; Siddiqui et al. 2010) – a trend which was opposite in C. elegans. Additionally, the Hco-UNC-49B/C heteromer is approximately 2.5 times more sensitive to GABA than the Cel-UNC-49B/C heteromer; a feature which may be indicative of a requirement for a more sensitive receptor by the parasite in vivo. In addition, through experiments which combined Cel-UNC-49 and Hco-UNC-49 receptor subunits, it was demonstrated that Hco-UNC-49B was essential for this increased sensitivity. However,
the understanding as to why there is an increase in GABA sensitivity with Hco-UNC-49B-associated heteromeric channels remains unclear (Siddiqui et al. 2010). One potential cause may be the amino acid differences within the BDs of the C. elegans and H. contortus receptors.

Overall, there are current gaps in our understanding of the relative importance of the putative binding loops and binding domains of the UNC-49 receptor for the activation of the channel by GABA. In addition, it is not known whether these BDs can explain the observed differences in GABA sensitivity between H. contortus and C. elegans UNC-49 receptors. This represents a substantial gap in scientific knowledge which warrants further research. Any information obtained in this investigation will present a novel understanding of the elements required in the activation of nematode GABA receptors.

### 1.5 Objectives

The aim of this study was to investigate which amino acids are essential for GABA sensitivity in the H. contortus UNC-49 GABA receptor. The focus here was on Loops B, C and D which are three of the six loops that have been identified in other organisms as important for GABA sensitivity. A variety of amino acids, identified as essential for GABA binding to mammalian GABAA receptors, were mutated via site-directed mutagenesis. In addition, a parallel study examined whether observed differences in GABA sensitivity between the H. contortus and C. elegans UNC-49 receptors is the result of amino acid differences in the putative BDs found in Loops B and C. Specifically, mutations were introduced into the BDs of the H. contortus unc-49 gene in order to change the amino acid to what is present in the C. elegans’ UNC-49 receptor.
In all cases, the effect of each mutation was examined by two-electrode voltage clamp electrophysiology. Those mutations that caused a substantial affect on GABA sensitivity were also analyzed via homology modelling.

The goal of this research is to better understand how key binding residues in nematode GABA receptors determine function. In doing so, this investigation will also provide valuable information of the evolution of these primitive channels and may reveal clues on the relationship between the *H. contortus* UNC-49 GABA binding pocket and that of the *C. elegans* UNC-49 and vertebrate GABA\textsubscript{A} receptors.
Chapter 2 –
Molecular Characterization of the Binding Site of Nematode GABA$_A$
Receptors
2.1 Introduction

Fast synaptic neurotransmission in both vertebrate and invertebrate systems is mediated, in part, by a large superfamily of ligand-gated ion channels known as Cys-loop receptors. In mammals, the Cys-loop superfamily can be broadly categorized as excitatory receptors (nicotinic acetylcholine, 5-HT) and inhibitory receptors (glycine, γ-aminobutyric acid or GABA; Miller and Smart, 2010). In nematodes, such as the model organism Caenorhabditis elegans, inhibitory neurotransmission is predominately mediated by ionotropic GABA receptors (referred to as UNC-49 receptors) which are concentrated at neuromuscular junctions wherein their physiological role is the GABA-mediated control of locomotion (Bamber et al. 1999). These nematode GABA<sub>A</sub> receptors differ considerably from mammalian GABA<sub>A</sub> receptors in sequence homology, pharmacology and overall function. The unc-49 gene encodes for three subunits (UNC-49A, UNC-49B and UNC-49C) which are involved in C. elegans locomotion (Bamber et al. 1999). However, only UNC-49B and UNC-49C are expressed at physiologically relevant levels and co-assemble to form the native receptor in vivo (Bamber et al. 1999; Bamber et al. 2005).

Recently, two orthologues of the Cel-unc-49 genes (Hco-unc-49B and Hco-unc-49C) were identified in the related parasitic nematode Haemonchus contortus (Siddiqui et al. 2010). Although there is a high degree of amino acid sequence homology between the receptors in the two species, there are pharmacological differences. Notably, the H. contortus heteromeric channel has approximately a 2.5-fold higher sensitivity to GABA compared to the C. elegans channel (Siddiqui et al. 2010). In addition, UNC-49C appears to be a positive modulator of GABA sensitivity in the H. contortus heteromeric
channel, but is a negative modulator of GABA sensitivity in the *C. elegans* heteromeric channel (Bamber *et al.* 1999; Siddiqui *et al.* 2010). These differences may be attributed to differences within the putative binding pocket of each receptor. However, the exact identity of the essential elements involved in GABA sensitivity in nematode GABA$_A$ receptors is largely unknown.

Knowledge regarding the molecular elements required for mammalian GABA$_A$ receptor activation has stemmed from mutational analysis, photoaffinity labelling, radioligand-binding assays and *in silico* homology modelling studies (Sigel *et al.* 1992; Amin and Weiss, 1993; Smith and Olsen, 1994; Cromer *et al.* 2002; Boileau *et al.* 2002). From these studies it is generally accepted that the GABA binding site is comprised of the interactions between six discontinuous loops (Loops A-F) found within the extracellular domains of interacting subunits; Loops A-C of the principle subunit and Loops D-F of the adjacent subunit (Lummis, 2009). These loops can be readily observed in homology models that have been generated using the low resolution extracellular structure of the related acetylcholine binding protein (AChBP; Boileau *et al.* 2002; Sixma and Smit, 2003; Padgett *et al.* 2007; Lummis, 2009). The validity of these homology models have been demonstrated in parallel mutagenesis and radioligand-binding assay studies which have identified key residues within these loops that are essential for ligand binding (Sigel *et al.* 1992; Amin and Weiss, 1993; Smith and Olsen, 1994; Cromer *et al.* 2002; Boileau *et al.* 2002).

Homology modelling of invertebrate Cys-loop receptors has been limited even though these receptors have been shown to be important targets for both pesticides and anthelmintics (Casida, 1993; Casida, 2009; McGonigle and Lummis, 2010). Very
recently, a 3-dimensional homology model of the most thoroughly studied insect GABA receptor, the *Drosophila melanogaster* RDL receptor, was constructed and used to study the binding of several GABA analogues (McGonigle and Lummis, 2010). This study revealed key binding structures within the RDL receptor, several of which are analogous to vertebrate GABA<sub>A</sub> receptors (McGonigle and Lummis, 2010). Invertebrate receptor studies such as these are key to providing insight into the similarities and differences between the invertebrate and mammalian GABA binding pocket. This will be important for an enhanced understanding of the evolution of GABA neurotransmission and possibly the discovery of novel pesticides and anthelmintics.

Analysis of the extracellular domain of the nematode UNC-49 GABA<sub>A</sub> receptor has revealed the presence of sequences that are homologous to the six binding loops known to be crucial for vertebrate GABA<sub>A</sub> receptor activation. Thus, the aim of this study is evaluate the importance of these loops for UNC-49 channel activation. Through site-directed mutagenesis and homology modelling, this study has uncovered several amino acid residues that are important for GABA sensitivity and appear to have analogous functions to what has been reported for mammalian receptors. Intriguingly however, several mutations have unveiled potential differences in the binding pocket between nematode and mammalian GABA<sub>A</sub> receptors. Finally, there is at least one amino acid that may partially account for the observed differences in GABA sensitivity between *H. contortus* and *C. elegans* UNC-49 channels.
Chapter 3 –
Materials and Methods
3.1 Mutation introduction and in vitro transcription of Hco-UNC-49

The coding sequences of Hco-UNC-49B and Hco-UNC-49C were sub-cloned into a pT7TS transcription vector. This transcription vector incorporates *Xenopus laevis* β-globin untranslated DNA to the 5’ and 3’ end of the *unc-49* gene (Dent et al. 1997). Mutagenic primers, that introduce single nucleotide changes in the *Hco-unc-49B* and *Hco-unc-49C* coding sequence, were designed using Stratagene’s web-based QuikChange® Primer Design Program (http://www.stratagene.com/sdmdesigner/default.aspx). These nucleotide changes will subsequently change several amino acids in order to determine their contribution to GABA sensitivity within the *H. contortus* GABA receptor (see Appendix A, Table A1-A4). Introduction of the mutations in the Hco-UNC-49 coding sequence was performed using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and verified using DNA sequencing (Genome Quebec). A linearized version of the mutated construct (100-600 ng) was then used as a template in the mMessage mMachine *in vitro* transcription reaction using T7 RNA polymerase provided in a capped RNA transcription kit (Ambion, Austin, TX, USA). Capped *Hco-unc-49* copy RNA (cRNA) was then precipitated using lithium chloride and subsequently resuspended in H₂O at a final concentration of 0.5 ng/ml. Approximately 10–25 µg of cRNA was generated per *in vitro* transcription reaction.
3.2 Expression of Hco-UNC-49 in Xenopus laevis oocytes

Oocytes were surgically removed from *X. laevis* females (Nasco) while anesthetised using 0.15% (w/v) 3-aminobenzoic acid ethyl ester, methane sulphonate salt (MS-222, pH 7; Sigma, Oakville, Ont.), buffered using sodium bicarbonate at pH 7. Harvested oocytes were partitioned into small clusters of less than 20 oocytes and defolliculated using type II collagenase solution (2 mg/mL; Sigma) in OR2 buffer (82 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM Hepes pH 7.5) for 2 hours under light shaking at room temperature. Oocytes were then transferred into ND96 frog saline (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES) supplemented with 275 µg/ml pyruvate (a carbon source), and gentamycin (100 µg/ml; Sigma). Oocytes were cytoplasmically injected with 50 nl of capped *Hco-unc-49* cRNA (0.5 ng/ml; wild-type and mutants) using the Drummond Nanoject microinjector (Broomhall, PA, USA). Incubation and maintenance of the injected oocytes occurred over a 1-4 day period at 20°C while submerged in supplemented ND96. Continual replacement of supplemented ND96 occurred approximately every 12 hours. Electrophysiological recordings were performed 2-5 days, post cRNA injections.

3.3 Electrophysiological trials

Two-electrode voltage clamp using microelectrodes was conducted ~ 48 hours, post injection, by means of an Axoclamp 900A voltage clamp (Axon Instruments, Foster City, CA, USA) at 20°C. Standard bath solution and subsequent dose-response recordings were conducted in non-supplemented ND96 frog saline for the duration of the
experiments. Microelectrodes containing Ag|AgCl wires were filled with 3M KCl, uphold ing a resistance of between 1 – 5 MΩ. The *X. laevis* oocytes were clamped and held at a membrane potential of -60 mV and a subsequent 5 s application of neurotransmitter (at various concentrations, in ND96) was proceeded by a 1-2 min wash with ND96 frog saline. Drugs were washed over the oocytes using an RC-1Z recording chamber (Warner Instrument Inc., Holliston, MA, USA). Data was obtained using Axograph and Clampex 10.0 software (Axon Instruments). Dose–response curves were produced using Graphpad Prism Software 5.0 (San Diego, CA, USA).

### 3.4 Statistical analysis

Statistical analysis between samples was conducted using a student’s t-test where a p ≤ 0.01 was considered significant (Graphpad Prism Software 5.0). Equations used to generate dose–response curves were fitted to a sigmoidal curve with variable slope. Generation of dose–response curves occurred via fitting obtained data to the following equation:

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

Where \(I_{\text{max}}\) is the maximal response, \([D]\) is the concentration of drug, \(EC_{50}\) is the concentration of drug producing a half-maximal effect, and \(h\) is the Hill coefficient (Forrester *et al.* 2003). The previous equation allows for a sigmoid curve of variable slope to be fitted for normalized data (Forrester *et al.* 2003). Data gathered consisted of at least four independent oocyte recordings for each dose of neurotransmitter. To ensure consistency, mutants were tested over at least two independent batches of oocytes.
3.5 Homology modelling and ligand docking

The protein coding sequence of Hco-UNC-49B (GenBank accession number ACL14329) and Hco-UNC-49C (ABW22635) were aligned with the coding sequence of the AChBP (P58154) using the align2d command in MODELLER 9v7 (Sali and Blundell, 1993) as well as with MacVector v 8.0 (Accelrys, San Diego, USA) and ClustalX v 2.0.12. The crystal structure of the AChBP in the HEPES-bound state at 2.7 Å resolution (Protein Data Bank ID 1I9B) was selected as the template for homology modelling of the Hco-UNC-49 receptor as many other GABA<sub>A</sub> receptors, including the related Drosophila RDL receptor, have used this template. A three-dimensional model of the extracellular region of the Hco-UNC-49 receptor was constructed using the default parameters in MODELLER through multi-subunit modelling in which one to five subunits were modelled in a single run by repeatedly aligning the template sequence to the desired number of Hco-UNC-49 subunit sequences. A total of 50 models were generated to compensate for low sequence homology. The most energetically favourable models were selected from the MODELLER output file using MODELLIST (http://mordred.bioc.cam.ac.uk/~ricardo/Servers/modellist_s.html) and were subsequently evaluated for violations in stereochemical, volume, and surface properties using PROCHECK (Laskowski et al. 1996) and Ramachandran plot analysis (RAMPAGE, http://mordred.bioc.cam.ac.uk/~rapper/rampage.php; Lovell et al. 2003). All models were viewed and images generated using UCSF Chimera v 1.4.1 (Pettersen et al. 2004). Protonated structural models of GABA were generated using ChemBio3D Ultra 12.0 (CambridgeSoft, Cambridge, U.K.) and were energy minimized using the MM2 force field. Docking of GABA into the Hco-UNC-49 receptor homology model was conducted
using DOCK v 6.4. The binding site of the receptor models were defined using the
default parameters in sphgen, grid and dock6 - all accessory programs of DOCK.
Chapter 4 – Results
4.1 Template selection for homology modelling

The coding sequence for both Hco-UNC-49B and Hco-UNC-49C were aligned to the coding sequence of the AChBP template (1I9B) to ensure minimal thresholds were met for homology modelling (Figure 5B). ClustalX alignment of Hco-UNC-49B to the template sequence indicated that there was approximately 24% identity and 64% similarity between the sequences. Additionally, Hco-UNC-49C aligned to the template sequence with approximately 28% identity and 66% similarity. To verify 1I9B as a suitable template, the profile.build() command in MODELLER was used. The profile.build() command uses local dynamic algorithms and the target query to identify related sequences from a library of sequences of known protein structures from the Protein Data Bank (Eswar et al. 2005). From the resulting output file, the 1I9BA (chain A) template was found to be the top hit for strictly extracellular domain protein templates with an identity score for both Hco-UNC-49B and C of 26% and an E value of 0.1 x 10^{-2}.

Additionally, the extracellular domains of both Hco-UNC-49 subunits were aligned to the α and β2 of the GABA_A receptor (Figure 5B). The Hco-UNC-49B had approximately 37% and 42% identity and 69% and 73% similarity to the α1 and β2 subunits, respectively. The Hco-UNC-49C had approximately 36% and 40% identity and 70% and 71% similarity to the α1 and β2 subunits, respectively. Most of the key amino acids composing the six discontinuous loops associated with ligand binding are conserved across the aligned sequences (Figure 5B). The major sequence differences between the AChBP and the Hco-UNC-49 receptors, with respect to agonist binding structures, occur within loop A and F.
Figure 5. Extracellular domains of the Hco-UNC-49 receptor. A) Model of the Hco-UNC-49B homodimer interface. The binding pocket is formed through the interactions of the six discontinuous loops indicated: Loops A-C (in grey) on the principle subunit and Loops D-F (in black) on the secondary subunit. The GABA molecule can be seen docked near the center of the loops. B) Sequence alignment of the nematode UNC-49 receptor subunits with other receptors from of the Cys-loop superfamily. Identities and similarities between sequences are both shown in dark grey for clarity. Key amino acids which form the loops that are essential to agonist binding are indicated in boxes. A dotted box indicates the sequence of a loop is continued on the next row. Sequences are numbered according to the numbers associated with the Hco-UNC-49 receptor sequences.
4.2 Model selection for homology modelling

The most energetically favourable models, with the lowest structural violations in stereochemical, volume, and surface properties were chosen using MODELLIST and ramachandran plot analysis. Models with the lowest DOPE and molpdf scores were selected and screened on ramachandran plots. DOPE and molpdf scores are values assigned in the MODELLER output file which, when taken together, access the energy of an overall protein in the form of an ‘energy score’ which sum all the restraint functions. Final models were chosen based on their similarity to the template’s associated ramachandran plot, DOPE and molpdf scores. All generated models had no more than 10% of their residues in unfavourable positions on their respective ramachandran plot; a percentage used by PROCHECK which is acceptable for homology models (Hooft et al. 1997).

The final extracellular Hco-UNC-49B homodimer structure (Figure 5A) shows a very similar structure to previously modelled vertebrate and invertebrate ionotropic GABA receptors. In addition, the modelling of the UNC-49 and other vertebrate and invertebrate GABA_A subunit monomers revealed similarities in structure and binding loop arrangements (Figure 6). The structure of the homodimer (see Figure 5A) consists of two energetically favourable interacting asymmetric Hco-UNC-49B monomers each with an N-terminal α-helix and 11 β-strands ranging in size from three to 14 amino acids. Due to the interactions of the subunits, the primary subunit has lost a short, three amino acid long 3_{10} helix structure near the N-terminus where each amino acid corresponds to a 120° turn in the right-handed helical structure. This structure is visible on the adjacent subunit.
Additionally, the TM1 would attach to each monomer via the C-terminus located towards the bottom of the each structure immediately after the 11th β-strand.

Figure 6. Homology models of various ionotropic GABA receptor monomers. Hco-UNC-49B, Cel-UNC-49B and Drosophila RDL represent invertebrate GABA_A receptor subunits. The Cel-UNC-49B monomer was modelled on the AChBP and has not been previously modelled in the literature. The Human GABA_A – β2 represents the sole vertebrate GABA_A receptor subunit. For orientation, the N and C termini are located at the ‘top’ and ‘bottom’ of each structure, respectively.

The docking of GABA into the binding pocket of the Hco-UNC-49B homodimer interface placed the molecule consistently into the crevice surrounded by Loops B-D (Figure 7); a trend similar to the RDL receptor model (McGonigle and Lummis, 2010). In its most favoured orientation, the carboxyl group of the GABA molecule was positioned adjacent to Arg^{66} of Loop D and to a lesser extent Tyr^{64} of Loop D (Figure 7). The orientation of GABA also placed its ammonium group in close proximity to Tyr^{166} of Loop B as well as Tyr^{218} of Loop C, potentially allowing for ligand-protein cationic-π interactions (Figure 7). Phe^{106} (not a primary focus within this investigation) is an amino acid also located in close proximity to the GABA molecule. A predicted hydrogen-bond between Tyr^{166} and Arg^{66} may align and secure Tyr^{166} in proper orientation for cationic-π bonds with GABA (Figure 7). Thr^{169} is also shown due to its relevance in the current study.
Figure 7. GABA docked within the putative binding pocket of an Hco-UNC-49B homodimer. All amino acids (shown in stick form) predicted to be important in GABA activation are indicated. The ribbon structure of amino acids found in loop C of the primary subunit, that proceed Tyr\textsuperscript{218}, have been removed for clarity. Amino acids Tyr\textsuperscript{64}, Phe\textsuperscript{106}, Tyr\textsuperscript{166} and Tyr\textsuperscript{218} make up the conserved aromatic box found in other GABA\textsubscript{A} receptors. The GABA molecule is shown in space-filling form for ease of viewing. The blue colours represent nitrogen atoms. The red colours represent oxygen atoms.

4.3 Pharmacological characterizations of the Hco-UNC-49 receptors

Heterologous expression of the majority of the mutant Hco-unc-49B and Hco-unc-49C cRNA in X. laevis oocytes formed fully functional GABA-gated channels. Wild-type heteromeric channels produced robust responses (≥3 µA) at 100 µM GABA (Figure 8). Channels with mutations in the Hco-UNC-49B sequence that we found to affect GABA sensitivity also typically produced robust responses (≥3 µA), albeit at higher concentrations compared to the wild-type (Figure 8).
Figure 8. Representative GABA-activated dose-response currents from oocytes expressing wild-type or mutant Hco-UNC-49B channels. GABA concentrations that were applied to the oocytes are indicated at the top.

In general, the wild-type Hco-UNC-49B homomeric channel was slightly less sensitive to GABA (EC$_{50}$: 63.1 ± 4.2 µM, n = 6; Hill coefficient: 1.7 ± 0.2) compared to its wild-type Hco-UNC-49B/C heteromeric counterpart (EC$_{50}$: 41.6 ± 4.5 µM, n = 9; Hill coefficient: 1.2 ± 0.1). In most cases, channels with only a single mutated UNC-49B subunit type (i.e. homomeric channels) had a lower sensitivity to GABA compared to channels with a combination of the same mutated UNC-49B subunit with wild-type UNC-49C subunits (i.e. heteromeric channels; see Table 2, 3, and 4).

4.4 Characterization of Loop B in UNC-49B and C subunits

The potential functional differences in Loop B between UNC-49 subunits from *H. contortus* and *C. elegans* were assessed by changing amino acids in the *H. contortus*
Loop B to reflect what is naturally present in Loop B from *C. elegans*. With respect to the UNC-49C subunit, there is only one amino acid difference at position 167 between the two nematodes within BDI of Loop B (see Figure 9). However, mutating Gly^{167} \rightarrow \text{Ala}^{167} in Hco-UNC-49C had no effect on GABA sensitivity (Table 2). For the UNC-49B sequences between the two nematode species, there is a difference immediately following BDI (position 170; Figure 9). In addition, there is a difference immediately outside the loop at position 171 (see Figure 9). Of these differences, only the Met^{170} \rightarrow \text{Thr}^{170} mutant in the Hco-UNC-49B subunit produced a slight, but statistically significant, decrease in GABA sensitivity in both homo- and heteromeric UNC-49 channels; most notably a two-fold decrease in the UNC-49B/C heteromeric channel (EC_{50}: 88.3 ± 3.3 \mu M, n = 7; Table 2; Figure 10A-C). To further investigate the importance of position 170 in GABA sensitivity, an additional mutation (M170S) was generated. This mutation had no affect on GABA sensitivity in heteromeric channels. However, the homomeric channel showed an approximate 2.5-fold decrease in GABA sensitivity (EC_{50}: 171.5 ± 12.3 \mu M, n = 6; Table 2; Figure 10A-C).
To better understand the role of Loop B in the sensitivity of GABA in nematode UNC-49 receptors, mutations were introduced into the Hco-UNC-49 subunits that were shown to dramatically decrease GABA sensitivity in human GABA<sub>A</sub> receptors when the mutations were introduced into the β<sub>2</sub>-subunit. Specifically, when Y166F or T169S were introduced into the Hco-UNC-49B subunit, the sensitivity of the heteromeric channels (i.e. mutant Hco-UNC-49B/ wild-type Hco-UNC-49C) to GABA decreased substantially and shifted the EC<sub>50</sub> to 384.3 ± 49.3 µM (n = 5) and 728.4 ± 75.8 µM (n = 4), respectively (Table 2; Figure 10A-C). The most dramatic effects were seen in the Hco-UNC-49B homomeric channel where the mutations Y166F and T169S decreased GABA sensitivity approximately 9- and 29-fold, respectively (Table 2; Figure 10A-C). An
additional mutation was incorporated at position 166 (Y166S) in Hco-UNC-49B which resulted in heteromeric channels which produced only small responses (≤ 300 nA) to 25 mM GABA and homomeric channels that were unresponsive to GABA at 50 mM (Table 2).

Interestingly, when Y166F was introduced into Hco-UNC-49C, no change in GABA sensitivity was observed (Table 2). It should be noted that a serine is already present at position 169 in Hco-UNC-49C and thus this position was not mutated. All remaining Hco-UNC-49C mutants had no effect on GABA sensitivity (Table 2).

Figure 10. Several Loop B associated mutations affect GABA sensitivity. A) Dose-response curves of wild-type and mutated Hco-UNC-49B receptors. Each data point represents the mean current value (n ≥ 4 oocytes) normalized to the mean maximum current observed. **Left:** Heteromeric wild-type and mutated Hco-UNC-49B receptors. **Right:** Homomeric wild-type and mutated Hco-UNC-49B receptors. B) Fold increase in EC$_{50}$ of Hco-UNC-49B mutants. C) Comparisons of EC$_{50}$ between heteromeric and homomeric Hco-UNC-49B mutant channels. All error-bars represent standard error of mean (SEM).
Table 2. Comparisons of EC$_{50}$ and Hill coefficient values for wild-type and mutated Loop B Hco-UNC-49 receptors

<table>
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<tr>
<th>Mutation$^2$</th>
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<th>Number of oocytes</th>
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<td>EC$_{50}$ ± S.E. (µM)</td>
<td>Number of oocytes</td>
<td>EC$_{50}$ ± S.E. (µM)</td>
<td>Number of oocytes</td>
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<tr>
<td></td>
<td>(Hill Coefficient ± S.E.)</td>
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<td>(Hill Coefficient ± S.E.)</td>
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<tr>
<td>Wild-type</td>
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<td>63.1 ± 4.2</td>
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<td>(1.2 ± 0.1)</td>
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<td>(1.7 ± 0.2)</td>
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<tr>
<td>UNC-49B</td>
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<tr>
<td>Y166S</td>
<td>Small responses (≤ 300 nA) at 25 mM</td>
<td>6</td>
<td>No response at 50 mM</td>
<td>6</td>
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<tr>
<td>Y166F</td>
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<td>709.7 ± 69.8*</td>
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<td>(1.6 ± 0.1)</td>
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<td>(2.2 ± 0.3)</td>
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<tr>
<td>T169S</td>
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<td>1838 ± 102.8*</td>
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<td>(1.6 ± 0.2)</td>
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<td>(2.1 ± 0.01)</td>
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<tr>
<td>M170T</td>
<td>88.3 ± 3.3*</td>
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<td>97.0 ± 7.4*</td>
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<td></td>
<td>(2.3 ± 0.2)</td>
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<td>(2.8 ± 0.02)</td>
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<tr>
<td>M170S</td>
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<td>171.5 ± 12.3*</td>
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<td>(1.8 ± 0.1)</td>
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<td>(2.8 ± 0.2)</td>
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<tr>
<td>A171K</td>
<td>42.7 ± 4.6</td>
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<td>51.7 ± 4.4</td>
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<td>(2.3 ± 0.2)</td>
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<td>UNC-49C</td>
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</tr>
<tr>
<td>Y166F</td>
<td>49.0 ± 3.8</td>
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<td></td>
<td>(1.2 ± 0.1)</td>
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<tr>
<td>Y167A</td>
<td>32.6 ± 1.8</td>
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</tr>
<tr>
<td></td>
<td>(1.7 ± 0.2)</td>
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</table>

$^1$Heteromeric channels are composed of an aforementioned mutant and its wild-type counterpart

$^2$Mutants are classified by the amino acid that was changed

All EC$_{50}$ values significantly different from their corresponding wild-type counterpart are denoted by an asterisk (*)
4.5 Homology modelling of the major Loop B mutations

Homology models of the mutants with the largest effect on GABA sensitivity, that remained functional, were generated to visualize the possible affects they have on the structural conformation of the Hco-UNC-49 channels (Figure 11). The most prominent changes occurred within the portions of the loops not found in either α-helices or β-sheets (Figure 11A). Within the Y166F mutant, the orientation of the introduced phenylalanine (shown in blue) is shifted away from the binding pocket and from Try\textsuperscript{218} (shown in orange). In addition, as a result of the switch from Try\textsuperscript{166} to Phe\textsuperscript{166} there is now no hydrogen-bond connecting position 166 to Arg\textsuperscript{66} (Loop D; shown in purple).

Furthermore, there is a slight shift of Loop B as well as the introduction of two new hydrogen-bonds which interact with Thr\textsuperscript{169} (shown in red), one of which connects Thr\textsuperscript{169} with Loop C (Figure 11B). In the homology model of mutant T169S (shown in red within Figure 11B), there is a small four amino acid long helical structure (shown in yellow) at the beginning of Loop C that is not present in the wild-type (Figure 11A). In addition, there are some shifts in the binding pocket. For instance, Arg\textsuperscript{66} (shown in purple) is now shifted away from the binding pocket. Additionally, there is also a loss of the predicted hydrogen-bond between Try\textsuperscript{166} (shown in blue) and Arg\textsuperscript{66}.
Figure 11. Homology models of the effects of Loop B mutations. A) Hco-UNC-49B wild-type and mutant Loop B homodimer models. The binding pocket is located at the interface of the primary subunit (left) and adjacent subunit (right). B) Close up view of the Hco-UNC-49B wild-type and mutant homodimer binding pocket. Amino acids are identified as follows: position 166 (Loop B; blue), position 169 (Loop B; red), position 218 (Loop C; orange), position 66 (Loop D; purple). Predictive hydrogen-bonds connecting interacting amino acids are indicated as black lines. The four amino acid long helical structure associated with the T169S mutation is shown in yellow.
4.6 Characterization of Loop C in UNC-49B and C subunits

Between the *H. contortus* and *C. elegans* UNC-49C sequences, there are three amino acid differences in Loop C (Figure 9). However, only one difference is located within BDII (Figure 9). Mutational analysis demonstrated that neither A214S nor S217T had any effect on GABA sensitivity when introduced in Hco-UNC-49C (Table 3). The UNC-49B sequences between the two nematode species have only two differences within Loop C, one of which is located within BDII at position 217 (Figure 9). The mutagenesis of Ser$^{217} \rightarrow$Lys$^{217}$ in the Hco-UNC-49B subunit resulted in no change in GABA sensitivity in either the homo- or heteromeric receptor forms (Table 3).

Two mutations were introduced into Loop C of both Hco-UNC-49B and Hco-UNC-49C subunits at positions that were previously shown to disrupt GABA sensitivity in human GABA$_\alpha$ receptors (T202S and Y205F). However, in Hco-UNC-49B, a serine is already present at the 202 equivalent (Ser$^{215}$). Therefore, this position was mutated back to a threonine (i.e. Ser$^{215} \rightarrow$ Thr$^{215}$). However, no affect on GABA sensitivity was observed in either the homomeric or heteromeric channels. Only Y218F (205 equivalent) in the Hco-UNC-49B subunit significantly decreased GABA sensitivity (Figure 12A). Within the mutant Hco-UNC-49B heteromeric channel (i.e. mutant Hco-UNC-49B/ wild-type Hco-UNC-49C), the Y218F mutation resulted in a shift in the channels EC$_{50}$ to $697.6 \pm 193.8$ µM ($n = 4$); a decrease in GABA sensitivity of approximately 17-fold (Table 3; Figure 12A-C). As a homomeric channel, the Y218F mutation also resulted in a decreased GABA sensitivity lowering the channels EC$_{50}$ to $654.3 \pm 41.3$ µM ($n = 4$) (Table 3; Figure 12A-C). Due to the slight difference in EC$_{50}$ between the homo- and heteromeric channel, there is a possibility that the mutated UNC-49B subunit cannot...
assemble with UNC-49C. To confirm the formation of heteromeric channels, the mutant Y218F channels were tested with the GABA receptor antagonist PTX (Figure 12D). Similar to previous reports (Siddiqui et al. 2010), there was almost complete inhibition within the mutant homomeric channel, but when mutated UNC-49B was co-expressed with wild-type UNC-49C, the resulting channel became PTX resistant (Figure 12D) indicating that the UNC-49B/C subunits were in fact co-assembled. Interestingly, when the Y218F mutation was introduced into Hco-UNC-49C, it had no affect on GABA sensitivity (Table 3).
Figure 12. One Loop C associated mutation lowers GABA sensitivity. A) Dose-response curves of wild-type and mutated Hco-UNC-49B receptors. Each data point represents the mean current value (n ≥ 4 oocytes) normalized to the mean maximum current. Left: Heteromeric wild-type and mutated Hco-UNC-49B receptors. Right: Homomeric wild-type and mutated Hco-UNC-49B receptors. B) Fold increase in EC₅₀ of Hco-UNC-49B mutants. C) Comparisons of EC₅₀ between heteromeric and homomeric Hco-UNC-49B mutant channels. D) Hco-UNC-49B mutant Y218F heteromeric channels are more resistant to PTX inhibition compared to Y218F mutant homomeric channels. Receptors were initially hit with 700 µM GABA proceeded by 700 µM GABA combined with 100 µM PTX. E) The percent PTX-dependent inhibition of the GABA responses for each channel. All error-bars represent standard error of mean (SEM).
Table 3. Comparisons of EC$_{50}$ and Hill coefficient values for wild-type and mutated Loop C Hco-UNC-49 receptors

<table>
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<th>Mutation$^2$</th>
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<td>EC$_{50}$ ± S.E. (µM)</td>
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</tr>
<tr>
<td></td>
<td>(Hill Coefficient ± S.E.)</td>
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<tr>
<td>Wild-type</td>
<td>41.6 ± 4.5 (1.2 ± 0.1)</td>
<td>9</td>
</tr>
<tr>
<td>UNC-49B</td>
<td>34.3 ± 4.2 (1.3 ± 0.2)</td>
<td>6</td>
</tr>
<tr>
<td>S215T</td>
<td>42.5 ± 7.6 (1.9 ± 0.2)</td>
<td>13</td>
</tr>
<tr>
<td>S217K</td>
<td>697.6 ± 193.8* (1.6 ± 0.1)</td>
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</tr>
<tr>
<td>Y218F</td>
<td>31.6 ± 1.9 (1.3 ± 0.1)</td>
<td>13</td>
</tr>
<tr>
<td>UNC-49C</td>
<td>35.6 ± 2.1 (1.9 ± 0.1)</td>
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<tr>
<td>S215T</td>
<td>34.6 ± 3.1 (1.4 ± 0.1)</td>
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<tr>
<td>Y218F</td>
<td>53.9 ± 3.0 (1.9 ± 0.01)</td>
<td>4</td>
</tr>
</tbody>
</table>

$^1$Heteromeric channels are composed of an aforementioned mutant and its wild-type counterpart  
$^2$Mutants are classified by the amino acid that was changed  
All EC$_{50}$ values significantly different from their corresponding wild-type counterpart are denoted by an asterisk (*)

4.7 Homology modelling of the major Loop C mutations

Similar to the Loop B homology models, the most disruptive changes to structure were observed in non-secondary structure configurations (Figure 13A). In the model for Y218F (shown in orange within Figure 13B), Ser$^{217}$ (shown in green) now has no predicted hydrogen-bonds associated with it, whereas Ser$^{217}$ in the wild-type homodimer
has one associated hydrogen-bond (Figure 13B). In addition, Loop C in the Y218F homology model forms a more relaxed structure, in relation to the wild-type homodimer (Figure 13A and B). Furthermore, there are subtle differences in protein structure throughout the mutant model with respect to the wild-type model. Additionally, Arg$^{66}$ (in Loop D; shown in purple) is shifted away from the binding pocket and the predicted hydrogen-bond between Try$^{166}$ (in Loop B; shown in blue) and Arg$^{66}$ is not present which may possibly destabilize the architecture of the binding pocket (Figure 13B).

![Figure 13. The effect of Loop C mutation Y218F. A) Hεo-UNC-49B wild-type and mutant Loop C homodimer models. The binding pocket is located at the interface of the primary subunit (left) and adjacent subunit (right) B) Close up view of the Hεo-UNC-49B homodimer binding pocket in the wild-type and Y218F mutant receptor. Amino acids are identified as follows: position 166 (Loop B; blue), position 217 (Loop C; green), position 218 (Loop C; orange) and position 66 (Loop D; purple). Predictive hydrogen-bonds connecting interacting amino acids are indicated as black lines.](image-url)
4.8 The effect of Loop D mutations on Hco-UNC-49 subunits

Based on the model of the UNC-49 channel binding pocket, as well as other research on mammalian GABA<sub>A</sub> receptors, Loop D is found on the adjacent subunit. In the mammalian GABA<sub>A</sub> receptor binding pocket, the α-subunit donates Loops D-F to GABA binding. However, when aligned with the α-subunit, the Hco-UNC-49 subunits do not have a high degree of sequence similarity (Figure 9). Nonetheless, Try<sup>64</sup> in both Hco-UNC-49 sequences appear to be equivalent to Phe<sup>64</sup> in the α-subunit (Figure 9); an amino acid shown to be essential to GABA sensitivity in mammalian GABA<sub>A</sub> receptors when mutated to a leucine (Sigel et al. 1992). When the Try<sup>64</sup>→Leu<sup>64</sup> mutant was introduced into Hco-UNC-49B, it had a profound effect on GABA sensitivity, decreasing the sensitivity of the heteromeric channel to GABA by roughly 19-fold (EC<sub>50</sub> to 804.3 ± 104.9 µM, n = 7) and the homomeric channel by roughly 194-fold (EC<sub>50</sub> to 12 425 ± 849.7 µM, n = 8; Table 4; Figure 14A-C). Leucine substitutions were also conducted on amino acids at the adjacent positions, 63 and 65, in the Hco-UNC-49B subunit. These mutants produced small differences in GABA sensitivity in the homomeric channel (Table 4; Figure 14A-C). Consistent with previous loops analyzed in this study, the same mutations introduced into Hco-UNC-49C did not affect GABA sensitivity (Table 4).
Figure 14. One Loop D associated mutation lowers GABA sensitivity. A) Dose-response curves of wild-type and mutated Hco-UNC-49B receptors. Each data point represents the mean current value (n ≥ 4 oocytes) normalized to the mean maximum current. **Left:** Heteromeric wild-type and mutated Hco-UNC-49B receptors. **Right:** Homomeric wild-type and mutated Hco-UNC-49B receptors. B) Fold increase in EC$_{50}$ of Hco-UNC-49B mutants. C) Comparisons of EC$_{50}$ between heteromeric and homomeric Hco-UNC-49B mutant channels. All error-bars represent standard error of mean (SEM).
### Table 4. Comparisons of EC$_{50}$ and Hill coefficient values for wild-type and mutated Loop D Hco-UNC-49 receptors

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<td>EC$_{50}$ ± S.E. (µM)</td>
<td>Number of oocytes</td>
<td>EC$_{50}$ ± S.E. (µM)</td>
<td>Number of oocytes</td>
</tr>
<tr>
<td>Wild-type</td>
<td>(Hill Coefficient ± S.E.)</td>
<td></td>
<td>(Hill Coefficient ± S.E.)</td>
<td></td>
</tr>
<tr>
<td>F63L</td>
<td>37.9 ± 5.6</td>
<td>7</td>
<td>93.2 ± 10.3*</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>(1.9 ± 0.2)</td>
<td></td>
<td>(2.2 ± 0.4)</td>
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</tr>
<tr>
<td>Y64L</td>
<td>804.3 ± 104.9*</td>
<td>7</td>
<td>12,425 ± 849.7*</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>(2.0 ± 0.3)</td>
<td></td>
<td>(5.5 ± 0.3)</td>
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<tr>
<td>M65L</td>
<td>67.7 ± 8.4</td>
<td>6</td>
<td>94.7 ± 10.0*</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>(2.1 ± 0.06)</td>
<td></td>
<td>(2.7 ± 0.4)</td>
<td></td>
</tr>
</tbody>
</table>

UNC-49C

| F63L         | 30.6 ± 0.8      | 5          | -----| -----|
|   | (1.7 ± 0.1)    |            | -----| -----|
| Y64L         | 61.3 ± 8.2      | 9          | -----| -----|
| M65L         | 51.3 ± 8.8      | 6          | -----| -----|
|   | (1.3 ± 0.2)    |            | -----| -----|

$^1$Heteromeric channels are composed of an aforementioned mutant and its wild-type counterpart
$^2$Mutants are classified by the amino acid that was changed
All EC$_{50}$ values significantly different from their corresponding wild-type counterpart are denoted by an asterisk (*)

### 4.9 Homology modelling of the major Loop D mutations

Similar to the Loop B and C homology models, the most considerable changes to structure are seen within non-secondary structural arrangements (Figure 15A). In the Y64L homology model, the mutation of Try$^{64}$→Leu$^{64}$ (shown in magenta) removes the aromatic tyrosine residue from the binding pocket (Figure 15B). Additionally, Arg$^{66}$ (shown in purple) now forms two intra-loop hydrogen-bonds with Thr$^{68}$ (shown in cyan;
Furthermore, Arg$^{66}$ is completely removed from the binding pocket and does not form the predicted hydrogen-bond with Tyr$^{166}$ (shown in blue; Figure 15B).

**Figure 15.** The effect of Hco-UNC-49B Loop D mutation Y64L. A) Hco-UNC-49B wild-type and mutant Loop D homodimer models. The binding pocket is located at the interface of the primary subunit (left) and adjacent subunit (right) B) Close up view of the Hco-UNC-49B homodimer binding pocket. Amino acids are identified as follows: position 166 (Loop B; blue), position 64 (Loop D; magenta), position 66 (Loop D; purple), position 68 (Loop D; cyan). Predictive hydrogen-bonds connecting interacting amino acids are indicated as black lines. Tyr$^{218}$ is shown in white to indicate the location of Loop C.

### 4.10 Homomeric channels typically have higher Hill coefficients compared to heteromeric channels

Homomeric channels were shown to typically possess higher Hill coefficients than heteromeric channels (Figure 16). Although several mutations caused the UNC-49 channels to require more GABA for channel activation, there were no significant changes
in desensitization kinetics as all mutants recovered from GABA activation similarly to their wild-type counterparts.

Figure 16. Hill coefficients for mutant Hco-UNC-49B channels. All the bars represent either a homomeric or heteromeric mutated Hco-UNC-49B receptor. Each bar represents the mean Hill coefficient (n ≥ 4 oocytes). Error-bars represent standard error of mean (SEM).
Chapter 5 – Discussion
5.1 Equivalent amino acid positions play analogous roles in invertebrate and vertebrate GABA<sub>A</sub> receptors

Most of our understanding of the Cys-loop receptor agonist binding has been developed through work with the nAChR (Cromer et al. 2002). Nonetheless, methods such as amino acid mutagenesis and photo-affinity labelling have been fundamental in advancing our understanding of the essential elements involved in GABA<sub>A</sub> receptor activation (Sigel et al. 1992; Amin and Weiss, 1993; Smith and Olsen, 1994; Boileau et al. 2002; Padgett et al. 2007). Unfortunately, there is currently a gap in our understanding regarding the molecular characterization of the determinants important for agonist activation of invertebrate Cys-loop receptors which have been demonstrated to be important targets for both insecticides and anthelmintics (Casida, 1993; Casida, 2009). This thesis has attempted to bridge this gap and represents, to our knowledge, the first investigation of molecular determinants important for the activation of an invertebrate GABA<sub>A</sub> receptor by its natural agonist.

Cys-loop receptor subunits appear to have a high degree of structural similarity particularly within the architecture of their extracellular or ‘ligand-binding’ domain (Ernst et al. 2005). This is especially true when one observes the architectural conservation within the binding loops of these receptors. The homology models generated for the Hco-UNC-49 subunits produced the characteristic six discontinuous loops (Loops A-F) associated with binding in roughly the same orientation and location as other Cys-loop receptors such as the nAChR, 5-HT<sub>3</sub>, GABA<sub>A</sub>, GABA<sub>C</sub> and the Drosophila RDL receptor (Boileau et al. 2002; Karlin, 2002; Ernst et al. 2005, Harrison and Lummis, 2006; Thompson and Lummis, 2007; McGonigle and Lummis, 2010). This is not surprising as even proteins with low amino acid sequence homology may still form
similar structural and functional domains (Murzin, 1998). However, in our case there is a relatively high degree of amino acid similarity between the Loops A-E of the Hco-UNC-49 subunits and the α- and β-subunits of the vertebrate GABA_A receptor. This suggests that the structural determinates important for GABA activation in invertebrates and vertebrates are similar.

The role of these loops, particularly B, C and D (the focus of this study), in the function of the Hco-UNC-49 channel were investigated through site directed mutagenesis and homology modelling. Overall, results from this study determined that the majority of amino acids shown to be essential for GABA sensitivity within the vertebrate GABA_A receptor also had analogous roles in the Hco-UNC-49 receptor. For instance, previous data has implicated β₂Thr₁⁶⁰ (in Loop B) as essential for vertebrate GABA receptor activation (Amin and Weiss, 1993). Similarly, when the Hco-UNC-49 equivalent position (Thr₁⁶⁹) was mutated to a serine it also caused a dramatic decrease in GABA sensitivity suggesting that both vertebrate and invertebrate GABA_A receptors require this conserved threonine within Loop B. While homology modelling suggests that Thr₁⁶⁹ may not be in direct contact with GABA (Figure 7), its change to a serine appears to result in significant structural changes in the binding site which may explain the altered sensitivity of the channel to GABA.

In the vertebrate GABA_A receptor, it has been shown that four aromatic residues are essential for binding; forming what is commonly referred to as an ‘aromatic box’ located on the inter-subunit interface between Loops A-D of two dissimilar subunits (Padgett et al. 2007; Pless et al. 2008). In fact, many Cys-loop receptors (the nAChR, the 5-HT₃ receptor, and the C. elegans 5-HT-gated Cl⁻ channel, MOD-1) possess a conserved
‘aromatic box’ indicating its importance in agonist binding (Lester et al. 2004; Pless et al. 2008). Homology modelling of the Hco-UNC-49 receptor has also shown the presence of a putative ‘aromatic box’ (Figure 7) suggesting that this region is also important for GABA binding in invertebrate receptors.

Within the vertebrate GABA\textsubscript{A} receptor, four amino acids (\(\alpha_1\text{Phe}^{64}\), Loop D; \(\beta_2\text{Try}^{97}\), Loop A; \(\beta_2\text{Try}^{157}\), Loop B; \(\beta_2\text{Try}^{205}\), Loop C) form the aromatic box with \(\beta_2\text{Try}^{97}\) participating in cationic-\(\pi\) interactions with the ammonium of the GABA molecule and \(\beta_2\text{Try}^{157}\) and \(\beta_2\text{Try}^{205}\) interacting with the carboxyl group of GABA (Padgett et al. 2007). The roles of \(\text{Try}^{157}\) and \(\text{Try}^{205}\) have been thoroughly investigated in the GABA\textsubscript{A} receptor and both have been shown, when mutated to phenylalanine, to dramatically decrease GABA sensitivity (Amin and Weiss, 1993). In this study, similar effects were observed when the same mutations were introduced into the equivalent \(\text{Try}^{166}\) and \(\text{Try}^{218}\) positions of the Hco-UNC-49B subunit indicating that these amino acids play analogous roles in the UNC-49 receptor. Indeed, both amino acids are positioned in close proximity to GABA when docked (Figure 7) and likely contribute to an aromatic box in the Hco-UNC-49 receptor. In addition, it has been speculated that residue \(\text{Try}^{157}\) (\(\text{Try}^{166}\) equivalent) plays a role in the hydrogen-bond network within the binding pocket of the vertebrate GABA\textsubscript{A} receptor forming a hydrogen-bond with the -NH of the \(\alpha_1\text{Thr}^{130}\) backbone (Padgett et al. 2007). This bond is thought to stabilize the binding pocket allowing \(\beta_2\text{Tyr}^{97}\) to form a cation–\(\pi\) interaction with GABA (Padgett et al. 2007). This arrangement is consistent with a high majority of Cys-loop receptors which have aromatic residues located on either Loop B (nACh, 5-HT\textsubscript{3} and GABA\textsubscript{C} receptors) or Loop C (MOD-1 receptors) which form cation-\(\pi\) interactions with the ligand (Pless et al. 2008).
Homology modelling of the Hco-UNC-49 receptor indicates a similar function of Try\textsuperscript{166} which appears to form a stabilizing hydrogen-bond with Arg\textsuperscript{66} (Figure 7) which may be important for overall structure of the binding site and the positioning of key amino acid residues that interact with GABA. It is interesting to note that in the homology models of all the mutations that had a substantial affect on GABA sensitivity, the hydrogen-bond between Try\textsuperscript{166} and Arg\textsuperscript{66} was lost. Finally, the importance of the Try\textsuperscript{166} is further illustrated by the finding that when the mammalian equivalent was mutated into a serine, there was a near 1000-fold reduction in GABA sensitivity (Amin and Weiss, 1993). Consistent with this, when we mutated position 166 to a serine in Hco-UNC-49B, there was only a small response (≤ 300 nA) to very high concentrations of GABA (>25 mM) in the heteromeric channel and no current was observed using up to 50 mM GABA in the homomeric channel. This further suggests a conserved role for this position in nematode GABA receptors.

A notable difference between Hco-UNC-49 and vertebrate GABA\textsubscript{A} receptors is the role of Thr\textsuperscript{202} (vertebrate) and Ser\textsuperscript{215} (Hco-UNC-49 equivalent). Previous data suggests that when Thr\textsuperscript{202} in the vertebrate GABA\textsubscript{A} receptor is mutated to a serine, GABA sensitivity was decreased approximately 21-fold (Amin and Weiss, 1993). However, both Hco-UNC-49 subunits (B and C) possess a serine in the equivalent position. To examine this position further, Ser\textsuperscript{215} was mutated to Thr\textsuperscript{215} which resulted in no change in GABA sensitivity. Interestingly, in the Hco-UNC-49 homology model between receptors with either a Ser\textsuperscript{215} or Thr\textsuperscript{215} there were only minute differences (data not shown). On the other hand, the Thr\textsuperscript{202} → Ser\textsuperscript{202} vertebrate GABA\textsubscript{A} receptor mutant model (i.e. β2-α1 dimer) produced drastic changes in both shape and orientation of Loop
A. These changes resulted in the upward ‘kinking’ of Loop A allowing the formation of new predicted hydrogen-bonds between Loop A of the β2-subunit and the amino acids just prior to Loop E on the α1-subunit (data not shown). Thus it appears that position 202 in the vertebrate GABA_A receptor may be critical for the overall structural conformation of the binding pocket rather than directly interacting with the GABA molecule itself. In fact, research by other groups has revealed that Thr^{202} may not make direct contact with GABA in the binding pocket (Wagner and Czajowski, 2001). It is clear, however, that this position is important for channel function in vertebrate GABA receptors, but possibly not as much in Hco-UNC-49.

Another interesting observation was that while positions 166 and 218 in the UNC-49 receptor appear to have analogous roles to the mammalian GABA_A receptor, the mutations in the UNC-49 channel (specifically the heteromeric channel) had a less drastic affect on GABA sensitivity compared to what was previously observed for the mammalian channel (Amin and Weiss, 1993). These differences may be the result of our particular perfusion system and experimental setup. However, it is more likely that there are in fact inherent differences in the structure of the binding pocket between nematode and mammalian GABA_A receptors. Further research would be required to determine the exact structural differences important for GABA binding and activation in nematode verses mammalian receptors.

5.2 *One amino acid may partially account for differences in GABA sensitivity between nematode UNC-49 receptors*

Previous research by our group demonstrated that the Hco-UNC-49B/C heteromeric channel was approximately 2.5-fold more sensitive to GABA than that of the
Cel-UNC-49B/C receptor (Siddiqui et al. 2010). The apparent increased sensitivity appears to be linked to the Hco-UNC-49B subunit (Siddiqui et al. 2010). Examination of Loop B and C identified several key amino acids that were variable between the *H. contortus* and *C. elegans* UNC-49B subunits. However, of all the positions that were different between the two nematodes, the only mutations that had any noticeable affect were those introduced at position 170 of Loop B. In *H. contortus* a Met$_{170}$ is present while *C. elegans* exhibits a threonine at the equivalent position. The mutation M170T resulted in an approximate 2-fold decrease in GABA sensitivity in the heteromeric channel. The importance of position 170 was also verified by introducing an additional mutation (M170S) which caused a 2.5-fold decrease in GABA sensitivity in the homomeric channel. Interestingly, in the GABA$_A$ receptor $\beta_2$-subunit, a mutation from threonine to arginine in the equivalent position (Thr$^{161}$) altered GABA sensitivity by almost 3-fold (Amin and Weiss, 1993). Therefore, it appears that this position may play a small role in GABA activation in both vertebrate and UNC-49 receptors and may partially account for the difference in GABA sensitivity between *H. contortus* and *C. elegans* channels.

5.3 *The binding site of Hco-UNC-49 is located between similar subunits*

UNC-49 receptors have been identified in many free-living nematodes such as *C. elegans* (Bamber et al. 1999), *C. briggsae, C. brenneri, C. japonica* and *C. remanei* (Wormbase) as well as in the parasitic nematode *H. contortus* (Siddiqui et al. 2010). Their presence in a wide variety of nematode species may be indicative of their importance to nematode GABAergic systems. In *C. elegans*, the UNC-49 receptors are
expressed at the neuromuscular junction where the co-assembly of two subunits (UNC-49B and UNC-49C) results in the formation of a heteromeric GABA receptor which has been demonstrated to act as the neuromuscular GABA\textsubscript{A} receptor \textit{in vivo} (Bamber \textit{et al.} 1999; Bamber \textit{et al.} 2003; Bamber \textit{et al.} 2005).

The significance of Cel-UNC-49C in heteromeric channels is quite evident as its co-assembly with Cel-UNC-49B confers PTX resistance, a decrease in GABA sensitivity and changes in desensitization kinetics (Bamber \textit{et al.} 1999; Bamber \textit{et al.} 2003; Bamber \textit{et al.} 2005). In \textit{H. contortus}, Hco-UNC-49C exerts its influence by increasing GABA sensitivity in heteromeric channels while simultaneously causing the channels to become PTX resistant (Siddiqui \textit{et al.} 2010). The characterization of native GABA receptors in the muscle cells of the parasitic nematode \textit{Ascaris suum} have shown these receptors possess similar pharmacological properties to those observed for the UNC-49B/C heteromeric channel (Holden-Dye \textit{et al.} 1989; Walker \textit{et al.} 1992; Martin, 1993, Bamber \textit{et al.} 2003). Thus, it appears as though the formation of heteromeric UNC-49-like channels \textit{in vivo} is conserved and functionally important in nematodes (Bamber \textit{et al.} 2003). However, what is not known is whether UNC-49C exerts a direct or indirect effect on GABA binding and channel activation.

Within vertebrate GABA\textsubscript{A} receptors, the GABA binding site is located at the interface of two dissimilar subunits: the primary \textbeta–subunit and the secondary, or adjacent, \textalpha–subunit (Lummis, 2009). However, it is not known whether the UNC-49C subunit contributes to the binding-site interface in UNC-49 receptors. To begin to address this question, mutations in Loops B and C were introduced into the Hco-UNC-49C subunit which included mutations at positions shown to dramatically affect GABA
sensitivity when introduced in Hco-UNC-49B (positions 166 and 218). However, none of the Hco-UNC-49C mutations produced a noticeable effect on GABA sensitivity in the heteromeric channels. Furthermore, in vertebrate GABA\textsubscript{A} receptors, the mutation of $\alpha_1$Phe\textsuperscript{64} (found in Loop D on the adjacent subunit) to Leu\textsuperscript{64} caused a dramatic decrease in GABA sensitivity by roughly 200-fold (Sigel \textit{et al.} 1992). Intriguingly, when the same mutation was introduced into the Hco-UNC-49C equivalent, Try\textsuperscript{64}, no change in GABA sensitivity was observed. However, when the same mutation was introduced into Hco-UNC-49B, a significant reduction in GABA sensitivity was observed.

Taken together, these results suggest two possibilities about the nature of the UNC-49 binding site. First, there is a binding site on the UNC-49B-C interface, but other residues on the UNC-49C subunit (not examined in this study) contribute directly to GABA binding and channel activation. Second, the binding site is on the UNC-49B-B interface and UNC-49C plays an indirect role in the sensitivity of the channel to GABA. The latter scenario seems more likely since UNC-49B homomeric channels with functional agonist binding sites are readily formed in oocytes. In addition, in \textit{C. elegans} only UNC-49B is essential for receptor function (Bamber \textit{et al.} 1999). Interestingly, in most cases, the Hill slopes for homomeric channels are higher than the heteromeric channels, suggesting that a channel with five UNC-49B subunits requires more molecules of GABA to activate the channel compared to a channel with a combination of UNC-49B and C subunits (Weiss, 1997).

If Hco-UNC-49C does not play a direct role in GABA binding, then what is the function of this subunit? As stated previously, UNC-49C likely indirectly modulates the sensitivity and the overall kinetics of the channel. Indeed, the introduction of Hco-UNC-
49C increases the sensitivity of the majority of the channels examined in this study. Therefore, the assembly of the Hco-UNC-49 with Hco-UNC-49B subunits may influence the conformation of the channel which in turn alters either the binding site so that GABA binds at a higher affinity or produces a more sensitive channel. However, further research is required to determine the exact changes that occur in the channel when assembled as a homomer or heteromer. For illustrative purposes, a model of an Hco-UNC-49B homomeric channel and a hypothetical Hco-UNC-49B/C heteromeric channel with indicated binding site regions can be found below (see Figure 17).

**Figure 17. Homomeric and heteromeric Hco-UNC-49 pentameric receptors.** A) Ariel view of homomeric and heteromeric Hco-UNC-49 pentameric receptors. Each UNC-49 subunit is denoted by a corresponding number (1-5). Potential UNC-49B-B interface binding sites are denoted by an asterisk. Hco-UNC-49B subunits are highlighted in light grey. Hco-UNC-49C subunits are highlighted in dark grey B) Side view of homomeric and heteromeric Hco-UNC-49 pentameric receptors.
5.4 Future directions

Within this investigation, individual residues which play a key role in GABA sensitivity were identified via mutational analysis and two-electrode voltage clamp electrophysiology. This approach determined the effect of the various mutations on the macroscopic characteristics of the channel, such as the channel’s EC$_{50}$ (Wagner et al. 2004). Further analysis of the microscopic processes such as ligand binding-unbinding, channel opening-closing and channel desensitization-resensitization which may have contributed the changes in GABA sensitivity observed in this study (Colquhoun, 1998; Wagner et al. 2004) can be investigated using single-channel recordings and rapid ligand application (Wagner et al. 2004). However, based on previous research it appears as though the results associated with this study are caused by changes in GABA binding and not gating or channel activation (Sigel et al. 1992; Amin and Weiss, 1993; Boileau et al. 2002).

Homology modelling of both mutant and wild-type Hco-UNC-49 receptors indicate that it is highly plausible that Try$^{166}$ (Loop B), Try$^{218}$ (Loop C) or both interact with GABA via cationic-$\pi$ interactions. One method to identify cation–$\pi$ interactions is through the incorporation of fluorinated aromatics at prospective cation–$\pi$ sites (Padgett et al. 2007). This method exploits the electrostatic properties of the cation–$\pi$ binding affinity of aromatics by using the highly electronegative fluorine atom (Padgett et al. 2007). Fluorine’s placement around the aromatic ring results in the diminished negative electrostatic potential on the face of the ring which in turn reduces the cation–$\pi$ binding affinity of the residue (Padgett et al. 2007).

The exact role and function of Hco-UNC-49C in GABA binding in nematode
GABA<sub>A</sub> receptors has remained elusive. Despite its apparent contributions to channel pharmacology and desensitization kinetics, it appears that Hco-UNC-49C does not contribute directly to GABA binding. However, additional experiments would be required to confirm this hypothesis. Additionally, the receptor composition and potential arrangement of subunits within Hco-UNC-49 receptors has yet to be identified. Baur <i>et al.</i> (2006) has shown that mammalian GABA<sub>A</sub> receptors formed from a concatenated five subunit construct possessed comparable pharmacological properties to a corresponding non-concatenated GABA<sub>A</sub> receptor. Thus, a similar methodology with Hco-UNC-49 channels will allow the creation of perfectly defined receptor subunit composition and arrangement which may be useful in elucidating the function of Hco-UNC-49C.

The polymorphism Y166S is associated with a complete loss of GABA-activated responses in the homomeric channel. Interestingly, there are small responses within oocytes expressing Y166S mutant heteromeric channels at 25 mM GABA. Additionally, the equivalent mutation in mammalian GABA<sub>A</sub> receptors (Y157S) was also shown to respond to GABA albeit at very high concentrations of GABA (Amin and Weiss, 1993). Therefore, it is unclear whether the lack of GABA-evoked responses with the homomeric mutant is a result of defective channel assembly or trafficking of the channel to the membrane surface. To address these issues both radioligand-binding assays as well as Western blot analysis could be implemented to quantify the binding, if any, of GABA to the mutant channel and to identify whether the channel is expressed on the membrane surface, respectively.
5.5 Conclusion

The goal of this investigation was to identify which amino acids are essential for GABA sensitivity in the *H. contortus* UNC-49 GABA receptor. As a result, this thesis represents the first investigation of the molecular determinants important for the activation of an invertebrate GABA<sub>A</sub> receptor by its natural agonist. Therefore, this study has contributed an important step forward towards an increased understanding of the evolution of GABA<sub>A</sub> receptors.
Chapter 6 – References


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Chapter 7 – Appendices
Appendix A –
Mutation Primers
Table A1: Designed primers for Hco-UNC-49-C. elegans associated mutation introduction

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<th>Mutation</th>
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*All mutation positions are located in the coding sequence of the Hco-UNC-49B protein

** All Mutation positions are located in the coding sequence of the Hco-UNC-49C protein
Table A2: Designed primers for Hco-UNC-49B- GABA\textsubscript{A} \(\beta_2\) subunit associated mutation introduction

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<th>Reverse Primer (3' - 5') Antisense</th>
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<td><strong>Y166F</strong></td>
<td>CGATGCAAGCTGAAAATTGAAAGC</td>
<td>CCATTGTATAGCCAAAGCTTTCAAT</td>
</tr>
<tr>
<td>(40 base pairs)</td>
<td>TTTGGCTATACATGGCTGATATCG</td>
<td>TTCCAGCTTGATCG</td>
</tr>
<tr>
<td>79.55 °C Tm</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T169S</strong></td>
<td>GAAATTTGAAAGCTATGGCTATTCA</td>
<td>AGTAGTCGATATCCGCAATTTGAAT</td>
</tr>
<tr>
<td>(43 base pairs)</td>
<td>ATGGCTGATATCGACTACT</td>
<td>AGCCATAGCTTTCAATTTTC</td>
</tr>
<tr>
<td>78.73 °C Tm</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S215T</strong></td>
<td>ATTACACACAAACACATCAA</td>
<td>CGCCTATACGACCCCCGTGTATGTG</td>
</tr>
<tr>
<td>(40 base pairs)</td>
<td>CGGGGTCGTATAGGCG</td>
<td>GTTGCTTGATGTGTAAAT</td>
</tr>
<tr>
<td>79.10 °C Tm</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Y218F</strong></td>
<td>CCACATCATCAGGGTCTTCTGAGGC</td>
<td>TCAAAGTATAAACGCCTAAACGAC</td>
</tr>
<tr>
<td>(37 base pairs)</td>
<td>GTTTCTAATTTTG</td>
<td>CCTGATGATGTGG</td>
</tr>
<tr>
<td>78.28 °C Tm</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*All mutation positions are located in the coding sequence of the Hco-UNC-49B protein
### Table A3: Designed primers for Hco-UNC-49C- GABA<sub>A</sub> β<sub>2</sub> subunit associated mutation introduction

<table>
<thead>
<tr>
<th>Mutation*</th>
<th>Forward Primer (5’ - 3’)</th>
<th>Reverse Primer (3’ - 5’) Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Y166F</strong> (36 base pairs) 78.19 °C Tm</td>
<td>GCAAGCTCGAAATTGAAAGCTTTTATT</td>
<td>CCGTGGAAATAACCAAGCTTTCAATT</td>
</tr>
<tr>
<td></td>
<td>GGTTATCCACGG</td>
<td>TCGAGCTTGC</td>
</tr>
<tr>
<td><strong>S215T</strong> (33 base pairs) 78.59 °C Tm</td>
<td>CAATGGCCACTACCCGGCTACGGGC</td>
<td>GGAATAGGAGCCCGTAGCGGTAGT</td>
</tr>
<tr>
<td></td>
<td>TCCTATTCCC</td>
<td>GGCCATTG</td>
</tr>
<tr>
<td><strong>Y218F</strong> (29 base pairs) 80.22 °C Tm</td>
<td>CGCTTCAGGCTCCTTTTCCGACT</td>
<td>GCAGGAGTCGGGAAGGAGCCTGA</td>
</tr>
<tr>
<td></td>
<td>CCTGC</td>
<td>AGCG</td>
</tr>
</tbody>
</table>

*All mutation positions are located in the coding sequence of the Hco-UNC-49C protein*
Table A4: Designed primers for Hco-UNC-49- GABA<sub>A</sub> α<sub>1</sub> subunit associated mutation introduction

<table>
<thead>
<tr>
<th>Mutation*</th>
<th>Forward Primer (5’ - 3’)**</th>
<th>Reverse Primer (3’ - 5’) Antisense**</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>F63L</strong></td>
<td>ATATGGACTTTACGCTAGATTTATA</td>
<td>CTGCCATGTTTTGTCGATATATAAA</td>
</tr>
<tr>
<td></td>
<td>TATGCGACAAACATGGCAG</td>
<td>TCTAGCGTAAAGTCCATAT</td>
</tr>
<tr>
<td>(44 base pairs)</td>
<td>78.80 °C Tm</td>
<td></td>
</tr>
<tr>
<td><strong>Y64L</strong></td>
<td>GGATATGGACTTTACGCTAGATTTTT</td>
<td>CCTGCCATGTTTTGTCGATTAAGAA</td>
</tr>
<tr>
<td></td>
<td>CTTAATGCGACAAACATGGCAG</td>
<td>ATCTAGCGTAAAGTCCATATCC</td>
</tr>
<tr>
<td>(47 base pairs)</td>
<td>80.33 °C Tm</td>
<td></td>
</tr>
<tr>
<td><strong>M65L</strong></td>
<td>GATATGGACTTTACGCTAGATTTTC</td>
<td>CCATGTTTGTCAATAGAAAATCT</td>
</tr>
<tr>
<td></td>
<td>TATTTGCGACAAACATGG</td>
<td>AGCGTAAAGTCCATATC</td>
</tr>
<tr>
<td>(42 base pairs)</td>
<td>78.67 °C Tm</td>
<td></td>
</tr>
</tbody>
</table>

*All mutation positions are located in the coding sequence of the Hco-UNC-49 subunit proteins

** Primers were designed for complementation to both Hco-UNC-49 subunit nucleotide sequences