Isolation and partial characterization of three members of the GGR-1 family in the parasitic nematode *Haemonchus contortus*

By

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An oral defense of this thesis took place on August 13, 2019 in front of the following examining committee:

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The above committee determined that the thesis is acceptable in form and content and that a satisfactory knowledge of the field covered by the thesis was demonstrated by the candidate during an oral examination. A signed copy of the Certificate of Approval is available from the School of Graduate and Postdoctoral Studies.

ABSTRACT

Haemonchus contortus is a blood sucking parasitic nematode that infects ruminant animals. These parasites are of significant economic importance as they are estimated to cost the agricultural industry billions of dollars each year in management. Reliance and overuse of drugs has resulted in resistance, and there is an urgent need for novel anti-parasitic drugs. Cys-loop ligand gated ion channels have been recognized as important invertebrate specific drug targets. However, specific targets must be identified and characterized prior to drug development. This thesis describes the identification and partial characterization of three ion channel subunits from the GGR-1 family – *Hco-lgc-39, Hco-lgc-40,* and *Hco-ggr-2.* The phylogenetic analysis conducted in this study revealed that these genes are widely conserved across several different parasitic nematodes. Two of these genes, *lgc-39* and *ggr-2,* have not been previously characterized in another organism. Although *lgc-40* has been previously investigated in *C. elegans*, the channel found in *H. contortus* was found to be similarly sensitive to choline and 18-fold more sensitive to acetylcholine. This increased sensitivity to acetylcholine could potentially indicate a different function in *H. contortus*. Pharmaceutical characterization of Hco-LGC-39, revealed that this protein subunit forms a homomeric channel that is responsive to acetylcholine, and to a higher degree, methacholine. A ligand for the Hco-GGR-2 homomeric channel has not yet been identified. Additionally, homology modelling of Hco-LGC-39 and Hco-LGC-40 provided further insight into receptor ligand interactions, and helped to identify several residues that may be important for ligand binding. This partial characterization of unique receptor subunits provides the beginning step for the production of novel anthelminthic drugs to combat the increasingly problematic parasite, *H. contortus.*

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_____ Kristen Nazareth

STATEMENT OF CONTRIBUTIONS

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication. I have used standard referencing practices to acknowledge ideas, research techniques, or other materials that belong to others. Furthermore, I hereby certify that I am the sole source of the creative works and/or inventive knowledge described in this thesis.

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ACC	(Nematode) Acetylcholine-Gated Chloride Channel
Ach	Acetylcholine
Asp (D)	Aspartic acid
C. elegans (Cel)	*Caenorhabditis elegans*
cDNA	Complementary DNA
Cys-loop	Cysteine loop
EC50	50% of maximal response
ECD	Extracellular domain
GABA	γ-Aminobutyric acid
GGR	GABA/Glycine Receptor family
h	Hill slope
H. contortus (Hco)	Haemonchus contortus
ICD	Intracellular domain
Lgc	Ligand gated channel
LGIC	Ligand gated ion channel
M (#)	Transmembrane domain (1-4)
RACE	Rapid Amplification of cDNA ends
Ser (S)	Serine
TEVC	Two-electrode Voltage-Clamp
TMD	Transmembrane domain
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
UNC	Uncoordinated
X. laevis	Xenopus laevis (African clawed frog)

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Haemonchus contortus is a blood sucking parasitic nematode that lives in the abomasum of ruminant animals. Currently, infection with *H. contortus* is controlled through the use of broad spectrum anthelminthic drugs. However, overuse and reliance on these treatments has resulted in resistance and there is an urgent need for new methods of management before these parasites become completely unmanageable. The ideal anthelminthic target should meet four criteria: 1) the target is not in the host organism, 2) the target is not a target of any existing anthelminthic, 3) the target belongs to a multigene family and therefore multiple targets may be activated with the use of one drug, and 4) the target plays a vital role in the parasite life cycle (Wever et al., 2015). This study provides the beginning stages in the evaluation of the GGR-1 family of cys-loop receptors as potential future anthelmintic targets.

1.2 The Parasitic Nematode Haemonchus contortus

H. contortus, also referred to as the barber pole worm, is a parasitic nematode that is of significant economic importance due to its detrimental effects on the agricultural industry. *H. contortus* is typically found in tropical and subtropical areas, with its evolutionary origin being in Africa. However, the parasite is now a global concern. In its infective stage, these parasites live in the abomasum of ruminants where they feed on the blood of these animals. An infected animal loses 0.05 mL of blood per worm per day, which, given the number of worms an animal is infected with, amounts to a total blood loss volume of about 250 mL daily (Rodríguez *et al.*, 2015). This significant blood loss can lead to problems including anemia and hemorrhagic gastritis, often resulting in animal death

(Miller *et al*., 1998). Infection reduces the quality and quantity of wool and milk, and decreases reproduction, which affects the herd size and decreases meat production. This problem has been magnified by the parasite's increasing resistance to many groups of anthelmintics (Van Wyk, Malan, & Randles, 1997). *H. contortus* has great genetic diversity, which, in combination with its large population size, allows it to quickly select for alleles that give the parasite resistance to a particular anthelminthic (Prichard, 2001). This growing resistance creates an urgent need for new methods of management.

1.3 H. contortus Life Cycle

In 1915, the complex life cycle of *H. contortus* was described in detail by Veglia (Figure 1.1). This life cycle beings when adults, living in the abomasum of their host, mate and the females produce thousands of eggs that are released into the external environment with the host feces. These eggs hatch into first stage larvae (L1), which then undergo two consecutive molts becoming second stage larvae (L2) with the first molt and third stage larvae (L3) with the second molt. Although there is no significant growth in this larval stage, the L3 larvae begin to develop a needle-like structure that is later used to pierce the stomach mucosa of its host, enabling it to feed on host blood (Veglia, 1915). The L3 larvae move into the pasture where they are consumed by grazing ruminants (Laing et al., 2013). Upon being ingested, specific molecules in the ruminant's body, particularly carbon dioxide, signal the L3 to transition into the parasitic L4 stage (Schwarz *et al*., 2013). They shed their protective cuticle, retained from the L2 stage, and migrate into the abomasum where they become parasitic L4 larvae. This transition from L3 to L4 larvae has been studied extensively, and appears to be the result of two separate pathways. Both pathways are stimulated by carbon dioxide and facilitated by carbonic anhydrase, an enzyme which

assists in the conversion of carbon dioxide into carbonic acid. In this process, noradrenaline is released, which then activates various genes required for further development and molting (Davey et al., 1982). L4 larvae develop into adults within two to three weeks (Laing *et al*., 2013). Although the development into the adult phase is quick in optimal conditions, it is possible for the L4 larval stage to pause their development in less than optimal conditions in order to survive (Laing et al., 2013). This feature has greatly contributed to the success of the species. Both the L4 and adult stage feed on the host's blood, contributing to blood loss.

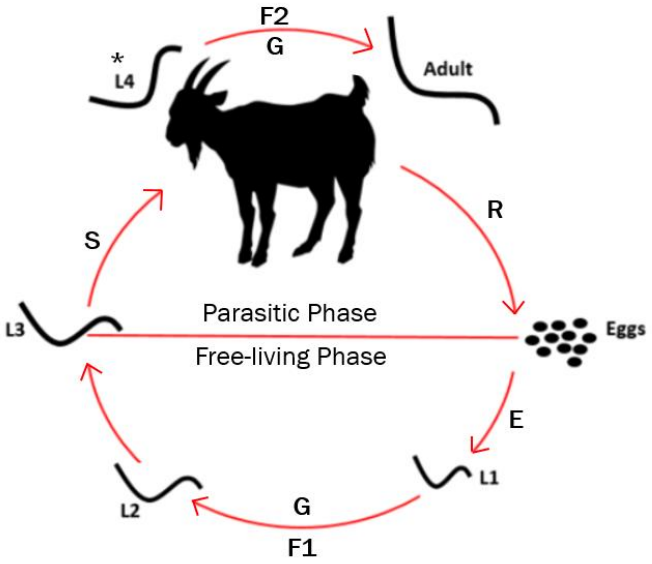


Figure 1.1: The life cycle of *H. contortus*. Important developmental features include: E – embryogenesis, S – sexual differentiation, R – reproduction, F1 – bacterial feeding phase, F2 – blood feeding phase, G – rapid growth phases. The * indicates the ability to undergo arrested development if required (Adapted from (Nikolaou & Gasser, 2006).

1.4 Current Anthelminthics Used for H. contortus Management and Resistance

Historically, there have been three classes of drugs used to treat *H. contortus* infection: nicotinic agonists, avermectins, and benzimidazoles. Nicotinic agonists, including levamisole, are strong anthelmintic drugs which act on nematode nicotinic acetylcholine receptors (Hoekstra *et al*., 1997). Upon binding to the receptor, these drugs

activate acetylcholine-gated cation channels found on muscle membranes, resulting in a depolarization, and therefore prolonged muscle contraction (Martin & Robertson, 2010). This causes spastic paralysis and allows for the parasite to be eliminated from the host (Martin, 1997). Despite initial success in *H. contortus* management with nicotinic agonists, the parasites have since become resistant to the drugs, greatly reducing their effect. It has been determined that resistance to levamisole occurs due to down-regulation of the nicotinic acetylcholine receptor subunit after treatment with the drug for several generations (Sarai *et al*., 2015).

Avermectins, including ivermectin, are a group of anthelminthic drugs that act to increase muscle permeability to chloride ions, which selectively paralyzes and eliminates the parasite (Martin, 1997). It is believed that multiple channels are activated by avermectins, but all are believed to cause long-lasting hyperpolarization in muscle cells through the binding and opening of chloride channels, primarily glutamate-gated chloride channels (Martin, 1997). *H. contortus* resistance to this class of drugs has been studied extensively and multiple mechanisms of resistance have been found. One mechanism of resistance involves P-glycoprotein, a membrane protein that binds to and removes cytotoxins from the cell (Blackhall *et al*, 1998).

Benzimidazoles, the most commonly used drugs to treat livestock, bind to β-tubulin and act in inhibiting microtubule formation (Martin, 1997). Since microtubules are essential for intracellular support, including transportation, movement, and cell division, treatment with benzimidazoles allow for parasite elimination (Saunders *et al*., 2013). Resistance to these drugs appears to be due to signal nucleotide polymorphisms present in the genes coding for β-tubulin (Zhang *et al*., 2016).

In the past 25 years, no new treatment for *H. contortus* has been developed with the exception of cyclodepsipeptides, which were not developed for use in livestock (Kaminsky *et al*., 2008). Recently, a new drug class, amino-acetonitrile derivatives that overcomes resistance to historical treatments, has been developed (Kaminsky *et al*., 2008). Monepantel, an amino-acetonitrile derivative, binds irreversibly to MPTL-1, a receptor unique to nematodes, causing long-lasting depolarization of muscle cells, and therefore nematode paralysis (Baur *et al.*, 2015). Another novel class used to treat livestock infected with *H. contortus* is the spiroindole class (Roeber et al., 2013). Derquantel, a common spiroindole, is an agonist of nematode muscle nicotinic acetylcholine receptors, resulting in parasite paralysis (Buxton *et al*., 2014).

Through the process of evolution, *H. contortus* has become resistant to the many anthelmintics used to treat livestock. The historic anthelminthic resistance observed in *H. contortus* is evidence for urgent need for new targets for anthelminthic drugs.

1.5 Ligand-Gated Ion Channels-Potential Targets for New Anthelmintics

Ligand-gated ion channels (LGICs) are a group of integral membrane proteins that contain a pore to selectively allow the passage of specific ions across the plasma membrane (Araud *et al.*, 2010). The movement of ions, including Na^+, K^+, Ca^{2+}, and Cl^-, through these channels is passive and driven by the electrochemical gradient (Alexander *et al*, 2008). Upon binding of a ligand, such as a neurotransmitter, these channels undergo a conformational change, opening the channel pore and creating an aqueous pathway for ion transfer across the membrane (Unwin, 1993). Opening of LGICs allow for a rapid ion flow across the membrane, changing the membrane potential (Unwin, 1993). This change in

membrane potential can either be excitatory, due to cations depolarizing the membrane, or inhibitory, due to anions hyperpolarizing the membrane (Connolly & Wafford, 2004).

1.6 Cys-Loop Ligand-Gated Ion Channels

An important family of LGICs are the cys-loop receptors, named due to the presence of a disulfide bridge in a loop of 13 amino acids (Absalom *et al.*, 2009). In mammals, these receptors allow for fast excitatory and inhibitory neurotransmission in the central and peripheral nervous system, and include the nicotinic acetylcholine receptor and serotonin receptors, both selective for cations, and the glycine receptor and the γ-aminobutyric acid (GABA) receptor, both selective for anions (Absalom *et al.*, 2009). The cys-loop receptors have a structure consisting of five subunits surrounding an ion pore (Connolly & Wafford, 2004) (Figure 1.2B). Most receptors of this family are composed of different subunits that can combine differently to form a variety of heteromeric receptors with different properties (Thompson *et al.*, 2010). Each subunit contains regions of one of three domains: the extracellular domain (ECD), the transmembrane domain (TMD), and the intracellular domain (ICD), each contributing to the overall structure of the receptor (Thompson *et al.*, 2010) (Figure 1.2A, Figure 1.3).

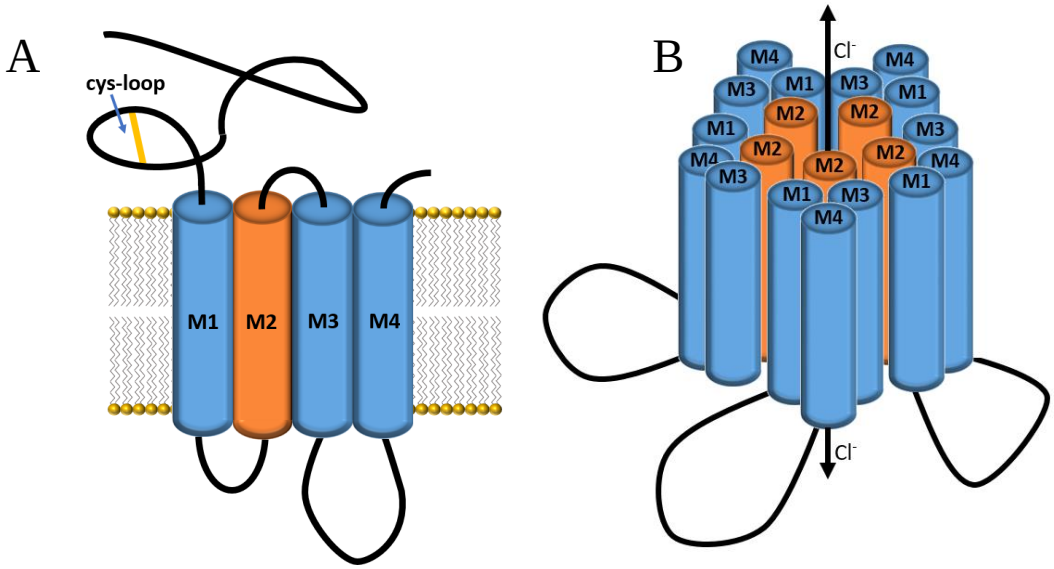


Figure 1.2: (A) Structure of an individual cys-loop LGIC subunit. Each subunit is composed of four transmembrane domains (M1-M4) and the cys-loop is found in the extracellular domain. (B) Structure of a pentameric cys-loop LGIC. The central pore is lined by transmembrane domain 2 (M2)

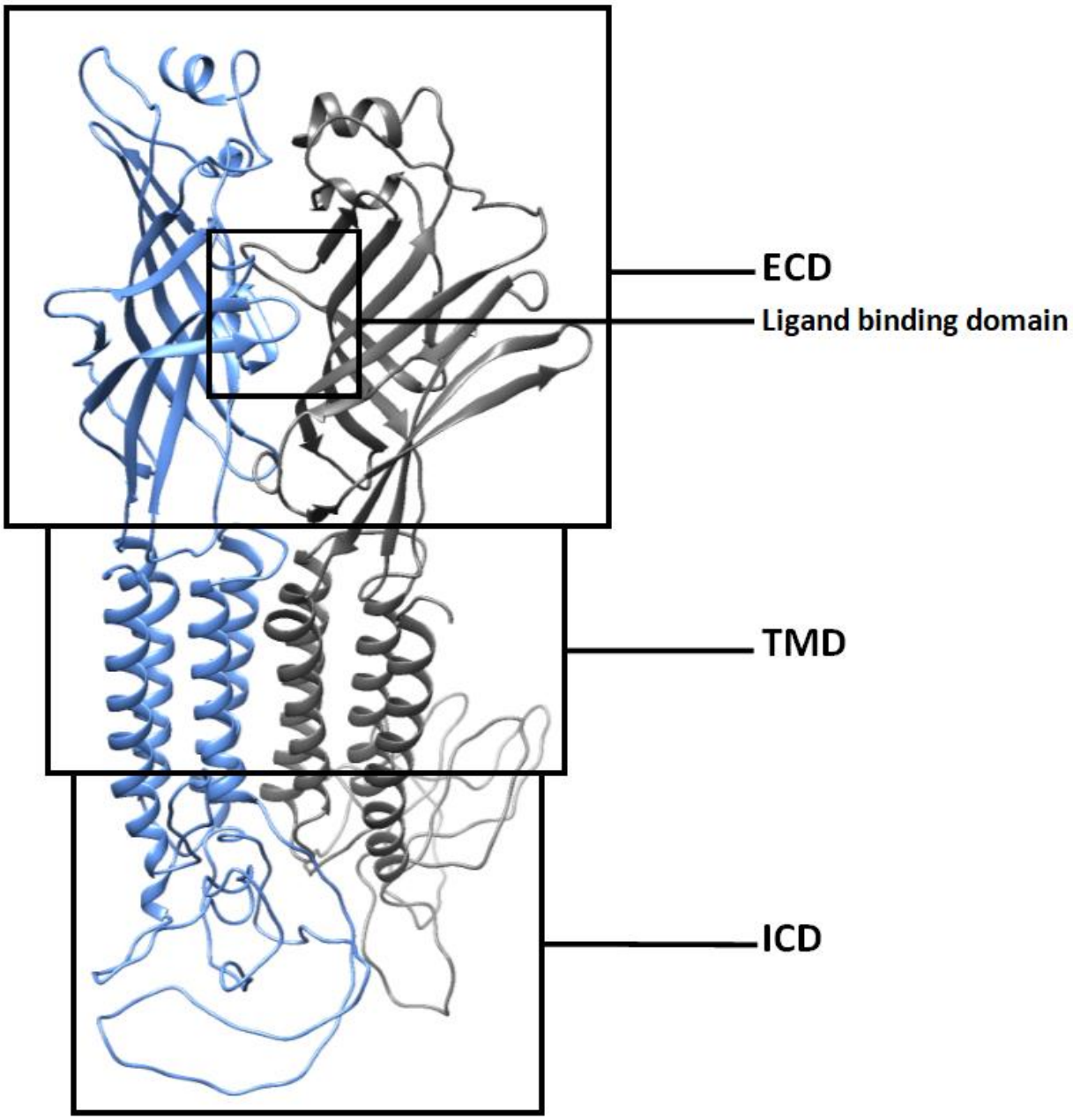


Figure 1.3: Structure of a Hco-LGC-40 dimer. The ligand binding site is located in the extracellular domain. Each subunit contributes four membrane spanning regions in the transmembrane domain. A small portion of the protein is found intracellularly.

The ligand-binding site is located in the ECD at the interface between two adjacent subunits (Brejc et al., 2001). The ligand-binding site is formed due to the junction of seven peptide 'loops' (Figure 1.4). The key regions of the ligand-binding domain are formed by

the α-subunit, which contribute to loops A, B, and C, and the remainder of the binding site is formed by the complementary subunit, which contributes three β-strands and one loop ('loops' D-G) (Hibbs & Gouaux, 2011). Although the complementary subunit loops D-F do not contribute actual peptide loop structure, the terminology was introduced prior to the discovery of the complementary subunit's β-sheet contributions (Thompson *et al.*, 2010). These 'loops' contain aromatic residues, which interact with the natural ligand, forming cation-π interactions (Pless et al., 2008). From these loops forming the binding region, only one to a few of the amino acids within each loop face into the binding site, and the remaining likely aid in conserving the structure of the binding site or contributing to the conformational changes associated with the opening and closing of the channel pore (Schulte et al., 2006; Thompson et al., 2006). Different agonist and antagonists may interact with different residues on the binding pocket, and agonists can interact with different residues to open the channel pore (Bower et al., 2008). Most of the cys-loop LGICs require ligand binding at two binding sites for channel activation.

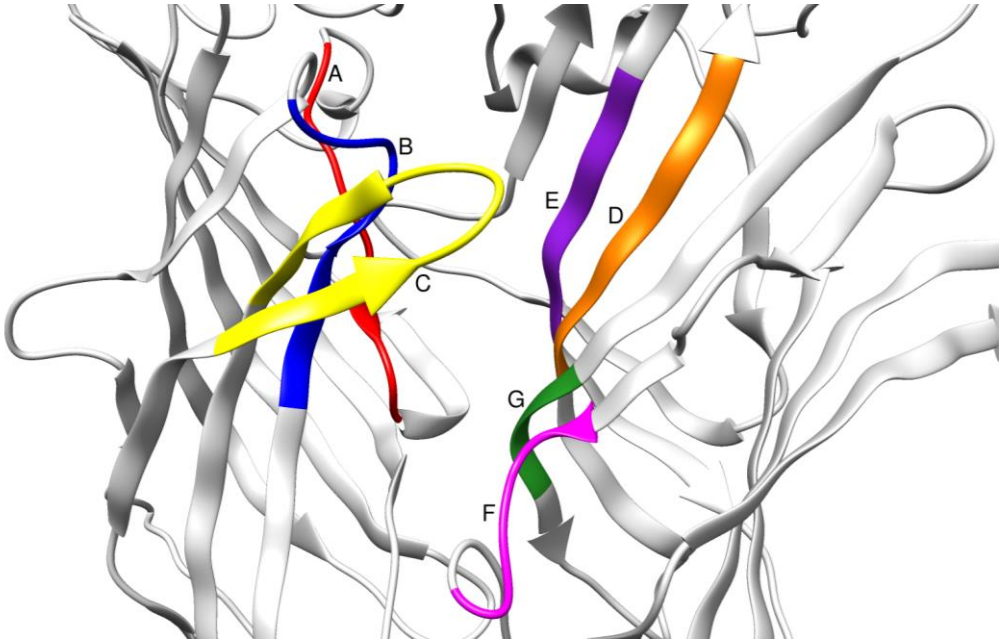


Figure 1.4: The ligand-binding domain is located between two adjacent subunits. The α subunit contributes loops A-C and the β subunit contributes loops E-F.

The TMD is composed of four transmembrane α-helices (M1-M4) from each subunit, including a total of 20 membrane-crossing α-helices per receptor. The receptor pore is formed by the M2 helices from each subunit, and undergoes a conformational change upon agonist binding resulting in the opening of the receptor channel (Cederholm *et al.*, 2009). Although M2 is the only transmembrane α-helix that is directly involved in the formation of the channel, M1, M3, and M4 helices have been found to play important roles in the structure and function of the channel (Tsetlin *et al.*, 2011). M1 forms a part of the outer ring, which interacts with the lipid environment, and may also have an influence on M2, as mutations in M1 have previously been linked to changes in receptor response (Bianchi et al., 2001; Dang et al., 2000; Engblom et al., 2002; Greenfield et al., 2002). The M2-M3 loop has repeatedly been show to play an important role in transmitting the energy associated with binding of a ligand into channel opening (Campos-Caro et al., 1996; Grosman et al., 2000; Kusama et al., 1994; O'Shea & Harrison, 2000). Structural analysis has revealed that M3 and M4 function in shielding M2 from the lipid bilayer (Miyazawa et al., 2003).

The ICD contains a loop located between M3 and M4 that consists of an amphipathic α-helix, called the membrane-associated (MA) stretch, which is important for channel conductance (Cederholm et al., 2009).

Cys-loop receptor activation and channel opening follow a series of specific steps. The binding of an agonist to the receptor causes movements in the ECD that begin with rotation of the α-subunits and are mediated by the M2-M3 linker (Cymes & Grosman, 2008). These movements affect the stability of the hydrophobic 'girdle', a hydrophobic

region blocking the channel pore. The hydrophobic girdle moves away from the channel pore, therefore opening the channel (Law et al., 2000).

1.7 The Binding Site of Cys-Loop LGICs

There are a number of aromatic residues located in the binding pocket of cys-loop LGICs. These are believed to facilitate neurotransmitter binding by providing a hydrophobic region, thereby creating a water-free environment where a ligand can dock, and the receptor can form cation-π interactions with positively charged ligands (Dougherty and Stauffer, 1990). In particular, four aromatic amino acids, known as the aromatic box, surround a bound ligand (Figure 1.5). The aromatic amino acids - tryptophan, phenylalanine, and tyrosine - contribute to cation-π interactions in different cys-loop receptors. Even if receptors are activated by the same ligand, different amino acids may contribute to the stability of the ligand. In the 5-HT_3R, for example, serotonin is stabilized through a cation-π interaction with a tryptophan residue in loop B (Beene et al., 2002); whereas in the MOD-1 receptor, serotonin forms a cation-π interaction with a tryptophan residue located in loop C (Mu, Lester, & Dougherty, 2003).

The strength of the cation-π interaction depends on two factors: the aromatic residue contributing to the interaction, and the positive charge of the ligand. Tryptophan is believed to form the strongest cation-π interaction, due to the presence of the six-membered ring of the indole (Campo-Cacharrón et al, 2014) followed by tyrosine, and finally phenylalanine. Ligands such as acetylcholine and serotonin tend to form cation-π interactions with tryptophan, whereas GABA and glycine tend to form interactions with tyrosine and phenylalanine.

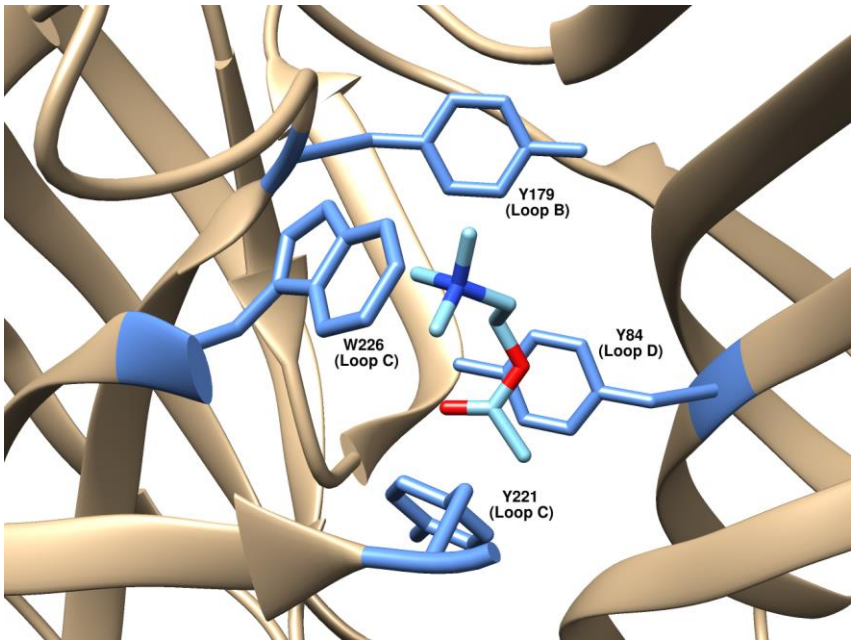


Figure 1.5: Computer generated structure of the predicted aromatic box for GGR-2 with acetylcholine bound. Aromatic amino acids forming the aromatic box are shown in blue.

1.8 Cys-Loop LGIC Subgroups in nematodes

C. elegans is powerful model organism in nematode studies and is often used for genetic comparison to other organisms. This is for two reasons: First, the complete genome of *C.* elegans was sequenced in 1998, and has shown conservation of many genes with other organisms. Second, *C. elegans* are ideal organisms to study in a lab as they have a very short life cycle, with the overall life cycle lasting 2-3 weeks. In 2008, Jones and Sattelle analyzed cys-loop LGICs in *C. elegans* through phylogenetic analysis and found that this species possesses the most extensive known superfamily of cys-loop LGICs, totaling 102 subunit-encoding genes. These genes show great diversity coding cation and anion permeable channels gated by acetylcholine (Ach), γ-aminobutryic acid (GABA), glutamate, and serotonin. In addition this study found that several subunits form distinct

groups. These groups included the EXP-1, AVR-14, UNC-49, GGR-1, LGC-45, ACC-1, MOD-1, and GGR-3 (Figure 1.6).

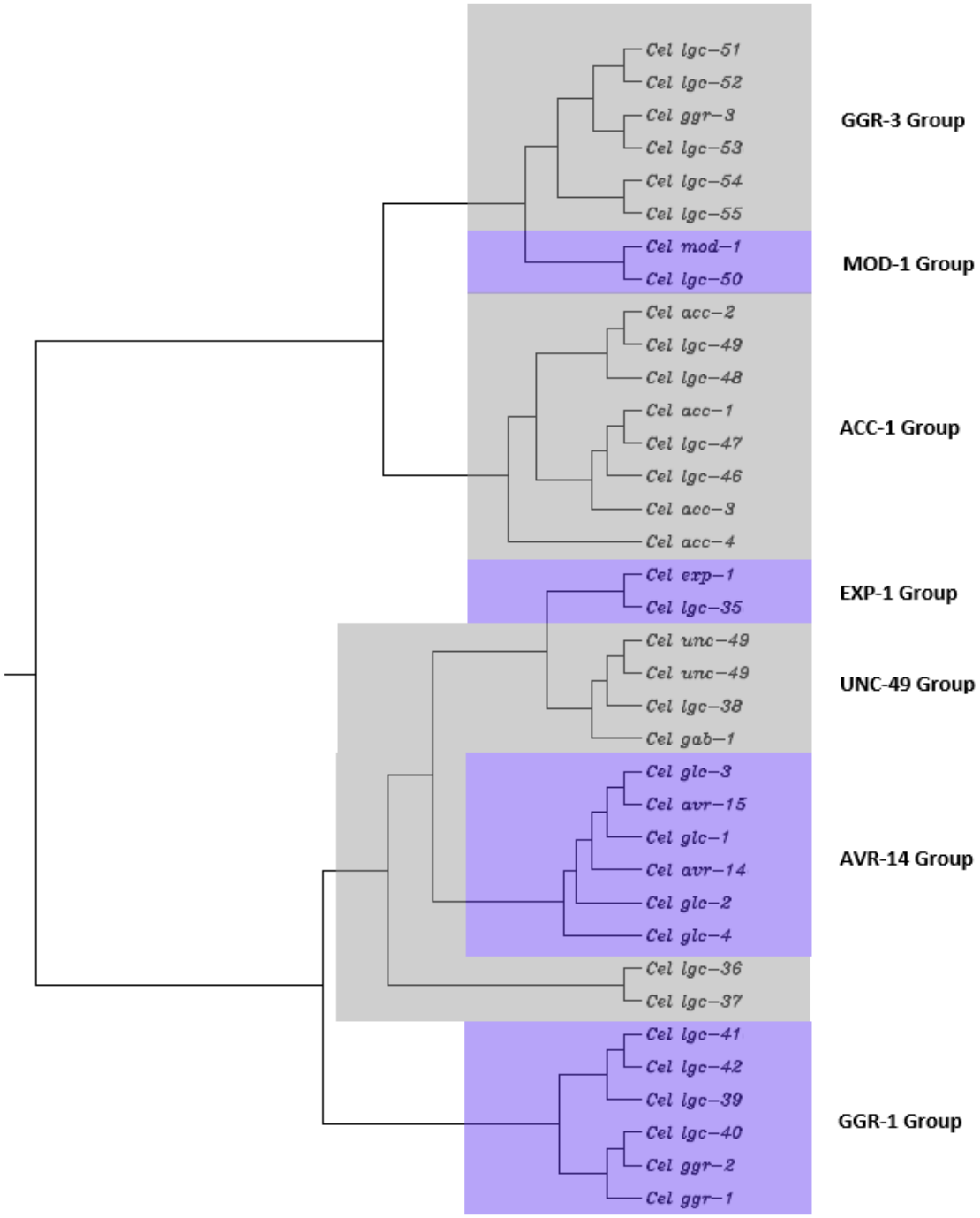


Figure 1.6: Phylogenetic tree showing the cys-loop LGIC subgroups in *C. elegans.* (Adapted from Jones & Sattelle, 2008)

Studies conducted on these cys-loop LGIC subtypes have highlighted their diversity in structure and function. For example, the EXP-1 group is a unique group of GABA-gated channels, which are cation permeable. They have been found to play a role in the mediation of muscle contraction (Beg & Jorgensen, 2003). The AVR-14 group is comprised of six subunits that are glutamate-gated chloride channels. Channels within this group are involved in several functions including the inhibition of pharyngeal pumping (Dent *et al.,* 1997; Laughton *et al.,* 1997; Pemberton *et al.*, 2001), regulating locomotion (Cook *et al*., 2006), and olfactory behavior (Chalasani *et al.*, 2007). These channels are targeted by avermectin and milbemycin anthelmintics (Wolstenholme & Rogers, 2005). The UNC-49 group includes receptor subunits which form a GABA-gated anion channel that controls muscle relaxation during locomotion (Bamber *et al*., 1999; Richmond & Jorgensen, 1999). This group of receptors is closely related to mammalian and insect GABA-gated anion channels (Dent, 2006). The ACC-1 group includes eight subunits that encode of ACh-gated anion channels (Putrenko *et al.*, 2005). The MOD-1 group consists of serotonin-gated chloride channels that modulate locomotory behavior. The GGR-3 group is composed of channels that respond to biogenic amines including dopamine, and tyramine (Rao et al., 2009; Ringstad et al., 2009). LGC-55, a member of this family, has been localized in neck muscle of *C. elegans* where it plays a role in head oscillations in response to anterior touch (Pirri et al., 2009).The functional characterization of the GGR-1 group and LGC-45 has not yet been published, and the ligands or neurotransmitters that these receptors respond to cannot easily be deciphered through phylogenetic analysis.

Both *H. contortus* and *C. elegans* are clade V nematodes; due to this close relationship, Laing *et al* (2013) were able to identify many LGICs in *H. contortus* through

sequence homology with *C. elegans*. These genes may be involved in many parasite functions, such as blood feeding, egg laying, and movement.

1.9 The GGR-1 Receptor Family

There are six genes in the GGR-1 family in both *C. elegans* and *H. contortus* (Jones & Sattelle, 2008; Laing *et al*., 2013). These genes include *ggr-1* and *ggr-2* as well as *lgc-39-42* (Jones & Sattelle, 2008). It is likely that all genes in this family are anion selective (Jones & Sattelle, 2008) as they have the amino acids proline, alanine, and arginine, known as the PAR motif, preceding TM2. This has been shown to be important for anion selectivity (Jensen *et al.*, 2005). GGR-1 is the only receptor subunit that does not contain this PAR motif, having the amino acids PGR instead (Jones & Sattelle, 2008). Thus far, there is very little known about the functional characterization of this family of genes, with only one publication investigating GGR-1 Family subunits in *C. elegans* (Ringstad *et al.*, 2009).

In 2009, Ringstad *et al* studied LGC-40 in *C. elegans* and found that it is capable of forming a homomeric channel, which is activated in response to choline, acetylcholine, and serotonin. The EC_{50} values of these compounds are 3.4 µM, 87 µM, and 905 µM, respectively. It was also found that the LGC-40 channel was blocked by low concentrations of *d*-tubocurarine, known to block nicotinic acetylcholine receptors, and inhibited by hemicholinium-3, a potent inhibitor for high affinity choline receptors. This provides further evidence of this receptor being cholinergic in nature. The function that this channel plays in *C. elegans* is currently unknown, as *lgc-40* deletion mutants appeared to be normal in locomotion, egg laying, defecation, and pharyngeal pumping (Ringstad *et al.*, 2009).

Other members of this family, *lgc-39*, *lgc-42*, *ggr-1*, and *ggr-2*, were also investigated by this group (Ringstad et al., 2009). These channels were screened for responsiveness to 100 µM of GABA, glycine, glutamate, histamine, dopamine, octopamine, and tyramine, and 1 mM of serotonin. The channels were not activated in response to the above ligands. To test for formation of a functional channel, subunits were exposed to 10 µM of ivermectin, an anti-parasitic drug that is known to activate many ligand-gated channels. A response to ivermectin was observed in GGR-2 injected oocytes, suggesting the formation of a functional homomeric channel. However, the ligand for this channel has not yet been determined.

Despite the research presented by Ringstad *et al.*, (2009), very little is known about the function and importance of the GGR-1 family receptors in parasitic nematodes such as *H. contortus.* Studying this receptor family further in *H. contortus* may reveal more information regarding binding and receptor activation, which will aid in drug design and provide knowledge about receptor evolution.

1.10 The Function of Cholinergic Compounds and Serotonin in *C. elegans*

As determined by Ringstad et al. (2009), Cel-LGC-40 was found to be responsive to choline, acetylcholine, and serotonin. Although the exact function of this channel is unknown, the activating neurotransmitters are vital to a variety of nematode behaviours.

Expression of choline acetyltransferase is often used to determine whether a neuron is cholinergic. By this criteria, there are approximately 120 cholinergic neurons found in the *C. elegans* hermaphrodite (Jinmahn Kim et al., 2015). There are a variety of behaviours believed to be mediated by acetylcholine and other cholinergic compounds. These include locomotion, egg laying, pharyngeal pumping, defecation cycling, and male mating.

Locomotion in *C. elegans*, and other nematodes, occurs via the worm bending its body in the shape of a sinusoidal curve, in waves known as muscular waves (Niebur & Erdös, 1991). This is accomplished by an alternating contraction and relaxation of the dorsal and ventral striated muscle cells (Hwang et al., 2016). A large number of cholinergic neurons in C. elegans are motor neurons. These neurons appear to play a role in both neuron-muscle communication as well as neuron-neuron communication. Egg laying in *C. elegans* involves several different neurotransmitters including acetylcholine, which appears to inhibit egg laying. This was observed in a study by Bany et al. (2003) in which mutant worms defective in acetylcholine synthesis were hyperactive egg layers. Similarly, these mutations were observed to cause longer and more variable defaecation cycles (Thomas, 1990) and were linked with disruption of male mating behavior (Garcia et al., 2001). It also appears that other acetylcholine sensitive receptors, particularly those that are responsive to the anti-parasitic drug, levamisole, can stimulate egg laying (Jinah Kim et al., 2001). In addition, several acetylcholine sensitive receptors appear to be located on the pharynx, where they mediate different aspects of pharynx function (Steger & Avery, 2004). Moreover, the MC neuron, a neuron associated with the stimulation of the pharynx muscle, is also cholinergic (McKay et al., 2004).

In addition to the above functions, cholinergic compounds in the body of *C. elegans* may play more complex roles. For example, the four motor neurons linked to the function of egg-laying muscles, VC4, VC5, HSNL, and HSNR, have been found to release both acetylcholine and serotonin, indicating that the function of these neurons may be linked to the co-expression of both neurotransmitters (Duerr et al., 2001). Cholinergic compounds have also been associated with negative regulation of starvation-dependent proteolysis in

C. elegans. Mutated worms deficient in acetylcholine release or reception show an increase in starvation-induced protein turnover (Szewczyk et al., 2000). Finally, cholinergic compounds have been linked to the regulation of developmental timing. In 2006, Ruaud & Bessereau showed that application of DMPP, a nicotinic receptor agonist, slowed the larval development during the L2 stage. However, the molt timing was unaffected, and the lack of synchronicity between the L2 development and the molt timing resulted in death of the worm.

Unlike the large number of neurons believed to be associated with cholinergic compounds, serotonin is produced in eight types of neurons in *C. elegans*. To understand the roles that serotonin plays within the worms, numerous studies have analyzed mutant worms lacking in the genes encoding for serotonin biosynthetic enzymes, or have studied the function through the use of drugs which altered serotonin signaling pathways. From these studies, serotonin is believed to play a role in locomotion, defecation, egg laying and pharyngeal pumping.

When food-deprived worms are exposed to a bacterial lawn, their rate of locomotion dramatically slows. This behavior has been linked to serotonin, as mutants lacking this neurotransmitter are defective in this "enhanced slowing response" (Sawin et al., 2000). This finding is quite significant as the serotonin induced "enhanced slowing response" almost completely reduces locomotion, thereby ensuring that the food-deprived worms have access to a food source. In addition, serotonin is synthesized in neurons which have sensory nerve endings in the pharynx and extend to the outside of the pharynx and the pseudocoelom. Mutants of serotonin biosynthetic enzymes were shown to have a decrease in both egg laying and pharyngeal pumping. Taken together this may indicate that

serotonin communicates information about a food source to the rest of the body, thereby controlling egg laying and pharyngeal pumping (Sze et al., 2000).

1.11 Rational and Objectives

There is an urgent need for the identification and characterization of drug targets for novel anti-parasitic drugs. Ion channels have long been exploited as targets for anthelminthic drugs, and it is likely that members of the cys-loop LGIC family of receptors can be exploited as drug targets. However, prior to being used as a drug target, the function and structure of the target must be elucidated. The majority of the GGR-1 family of receptors have not been studied in any organism. The overall goal of this study is to identify and partially characterize the GGR-1 family subunit genes in the parasitic nematode *H. contortus.* This goal is broken down into five main objectives. The first objective is to conduct a phylogenetic analysis of the GGR-1 family genes. The purpose of this objective is to determine whether these genes are present in a wide range of parasitic nematodes. The second objective is to determine the relative expression of GGR-1 family receptors in adult and L2 stage larvae *H. contortus.* Subunits found to be present in the adult stage will be selected for further analysis. The third objective is to clone the GGR-1 family genes into pGEMHE, a vector designed for optimal expression in *X. laevis* oocytes. The fourth objective is to characterize the cloned genes using two-electrode voltage-clamp electrophysiology. Finally, the fifth objective is to further understand the GGR-1 family receptors through homology modeling and ligand docking. This will begin the process of determining whether these genes are sufficient targets for a novel anti-parasitic drug.

2.1 Phylogenic Analysis

C. elegans GGR-1 family protein sequences were used to search the NCBI database (http://blast.ncbi.nlm.nih.gov/) and sequences from other nematodes that had a high degree of similarity were selected for further analysis. The sequences were aligned using ClustalW and a phylogenetic tree was created using PhyML 3.0 with 100 bootstrap repetitions. Only highly conserved regions of the sequences were used to create the phylogenetic tree. The sequences were named based on the resulting tree.

2.2 RNA Isolation and cDNA Synthesis

Adult male and L2 stage larvae *H. contortus* were received from Dr. Prichard (Institute of Parasitology, McGill University). Total RNA was isolated from *H. contortus*, in both life stages using Trizol (Invitrogen, Carlsbad, USA). Complementary DNA (cDNA) was synthesized for both life stages using the Quantitect Reverse Transcriptase kit from Qiagen (Dusseldorf, Germany) with a unique 3' oligo-dT anchor primer sequence: (5′CCTCTGAAGGTTCACGGATCCACATCTAGATTTTTTTTTTTTTTTTVN3′); [where V is either A, C, or G and N is either A, C, G, or T] (Weston *et al.*, 1999). Partial sequences of GGR-1 family receptor subunit genes were provided by Dr. Robin Beech (McGill University) and used to design gene specific primers for the end-point PCR expression tests and rapid amplification of cDNA ends (RACE).

2.3 Semi-Quantitative PCR

Standard end-point PCR (2 min 95 ^{o}C, [15 s 95 ^{o}C, 15 s 54 ^{o}C, 15 s 72 ^{o}C] × 35, 3 min 72 ^{o}C) was performed using Taq polymerase and primers specific for each subunit in the GGR-1 family. Primers designed for each subunit targeted a 200-300 base pair region.

Each reaction was conducted using both adult and L2 larval cDNA as a template. The housekeeping gene, α-tubulin was used as a control in these reactions. In addition, a water control and a –RT control was conducted for each primer set in both adult and L2 stage larvae. Following the PCR, products were run on an agarose gel and gene presence and relative quantity were analyzed in the adult stage as compared to the L2 larval stage.

2.4 Isolation of GGR-1 Family Genes

Gene sequences provided by Dr. Robin Beech (McGill University) were analyzed for completion. The genes *lgc-40*, *lgc-41*, *ggr-1*, and *ggr-2* appeared to be complete sequences, while *lgc-39*, and *lgc-42* appeared to contain the full 3' end of the gene, but were lacking the sequence encoding the signal peptide cleavage site on the 5' end. The incomplete genes were cloned using RACE procedure (Frohman *et al.*, 1988). The 5' end was amplified using two internal gene specific primers and one of two primers specific for a splice leader (SL) sequence:

SL1 – 5'-GGTTTAATTACCCAAGTTTGAG or

SL2 – 5'-GGTTTTAACCCAGTTACTCAAG-3' (Van Doren & Hirsh, 1988).

The resulting amplicon was then isolated using the QIAquick Gel Extraction Kit (Qiagen, Dusseldorf, Germany), sub-cloned into the pGEMT easy™ sequencing vector, and sequenced at Genome Quebec. The predicted protein sequence was analyzed using SignalP-4.0 to confirm presence of a signal peptide.

2.5 Expression of GGR-1 Family Genes in *Xenopus laevis* Oocytes

Amplification of the GGR-1 family subunit complete sequences was conducted using two sets of primers targeting the full length coding sequence of each gene. Following amplification, the amplicon was cloned into the pGEMHE *Xenopus laevis* expression

vector. This vector was then linearized and used as a template for *in vitro* cRNA synthesis, conducted using the mMESSAGE mMACHINE T7 Transcription Kit (Ambion, Austin, TX, United States).

All animal procedures followed the University of Ontario Institute of Technology Animal Care Committee and the Canadian Council on Animal Care guidelines. *Xenopus laevis* female frogs were supplied by Nasco (Fort Atkinson, WI, USA). The frogs were kept in climate controlled, light cycled room, and stored in regularly cleaned tanks.

X. laevis were anesthetized with 0.15% 3-aminobenzoic acid ethyl ester methanesulphonate salt (MS-222) buffered with $NaHCO_3$ to pH 7 (Sigma-Aldrich, Oakville, ON, CA). During surgery, a section of the frog ovary was removed and the lobe was defolliculated with OR-2, calcium-free oocyte Ringer-s solution [82 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES pH 7.5 (Sigma-Aldrich)] and 2 mg/mL collagenase-II (Sigma-Aldrich). The oocytes were incubated in the defolliculation solution for 2 hours at room temperature under agitation. Collagenase was washed from the oocytes with ND-96 solution (96 mM NaCl, 2mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) and oocytes were incubated in ND96 supplemented with 0.275 µg/mL pyruvate and 50 µg/ mL gentamycin for one hour at 18^{o}C prior to injection.

X. laevis oocytes were injected with 50 nL cRNA encoding either a single subunit, to test the presence of a homomeric channel, or two subunits, to test the presence of a heteromeric channel. The injected oocytes were incubated at 18^{o}C in a supplemented ND96 solution. The oocytes were incubated for a minimum of 48 hours and a maximum of 72 hours following injection and supplemented ND96 solution was changed every 24 hours until electrophysiology was performed.

2.6 Electrophysiological Recordings

Two-electrode voltage clamp (TEVC) electrophysiology was performed using the Axoclamp 900A voltage clamp (Molecular Devices, Sunnyvale, CA, USA). Glass electrodes were made using a P-97 Micropipette Puller (Sutter Instrument Co. Novato, CA, USA). The electrodes were backfilled with 3M KCl and contained Ag|AgCl wires, which connected the electrodes to their respective Axon Instrument Headstage (Molecular Devices). Oocytes were clamped at a holding potential of -60 mV and a second electrode was used to measure current changes during channel activation.

2.7 Determination of Receptor Ligands

In order to determine potential channel activators of homomeric and heteromeric channels, solutions containing various cholinergic, aminergic, and GABAergic compounds, dissolved in non-supplemented ND96, were washed over injected oocytes. Channel activation was observed as the presence of a current. Before testing of each compound, oocytes were washed with non-supplemented ND96 until the stable resting membrane potential was reached. Washing of solution over the oocytes was accomplished using the RC-IZ perfusion chamber (Warner Instruments, Holliston, MA, United States) and a Fisherbrand Variable-Flow Peristaltic Pump (Fisher Scientific, Hampton, NH, United States) to remove liquid waste. Responses to each ligand at increasing concentrations were recorded in individual oocytes, and dose-response curves were generated using GraphPad Prism (GraphPad Software, San Diego, CA, United States) with data fit to the following equation:

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

In this equation, I_{max} is the maximal response, EC_{50} is the concentration of the agonist that produces 50% of the maximal response, [D] is the agonist concentration, and *h* is the hill coefficient. The hill coefficient provides a measure of cooperative binding. A hill coefficient of 1 indicates independent binding, less than one indicates negative cooperativity, and greater than one indicates positive cooperativity.

2.8 Current-Voltage Relationships

To confirm that the receptors studied were indeed chloride channels, current-voltage relationships were recorded. This was done by changing the holding potential from -40 mV to +40 mV in 20 mV increments. At each 20 mV increase, the oocyte was exposed to the EC_{50} concentration of the responding ligand. These tests were done using ND96 as well as reduced Cl^- ND96, where NaCl was partially replaced with Na-gluconate (Sigma), creating a Cl^- concentration of 62.5 mM. Current-voltage graphs were generated using Graphpad Prism Software v5.0 (San Diego, CA, USA).

2.9 In Silico Homology Protein Modelling

To further analyze the structure of the GGR-1 Family subunits, a homodimer model was generated for each receptor subunit. The template used to model the homodimers was the *Danio rerio* alpha-1 glycine receptor (3JAD), as it was the template with the highest identity. The chosen template and the specific GGR family sequence was used to generate a homodimer model in MODELLER v9.20 using automated scripts. The most energetically favoured model was chosen based on DOPE score, and selected for computational agonist docking.

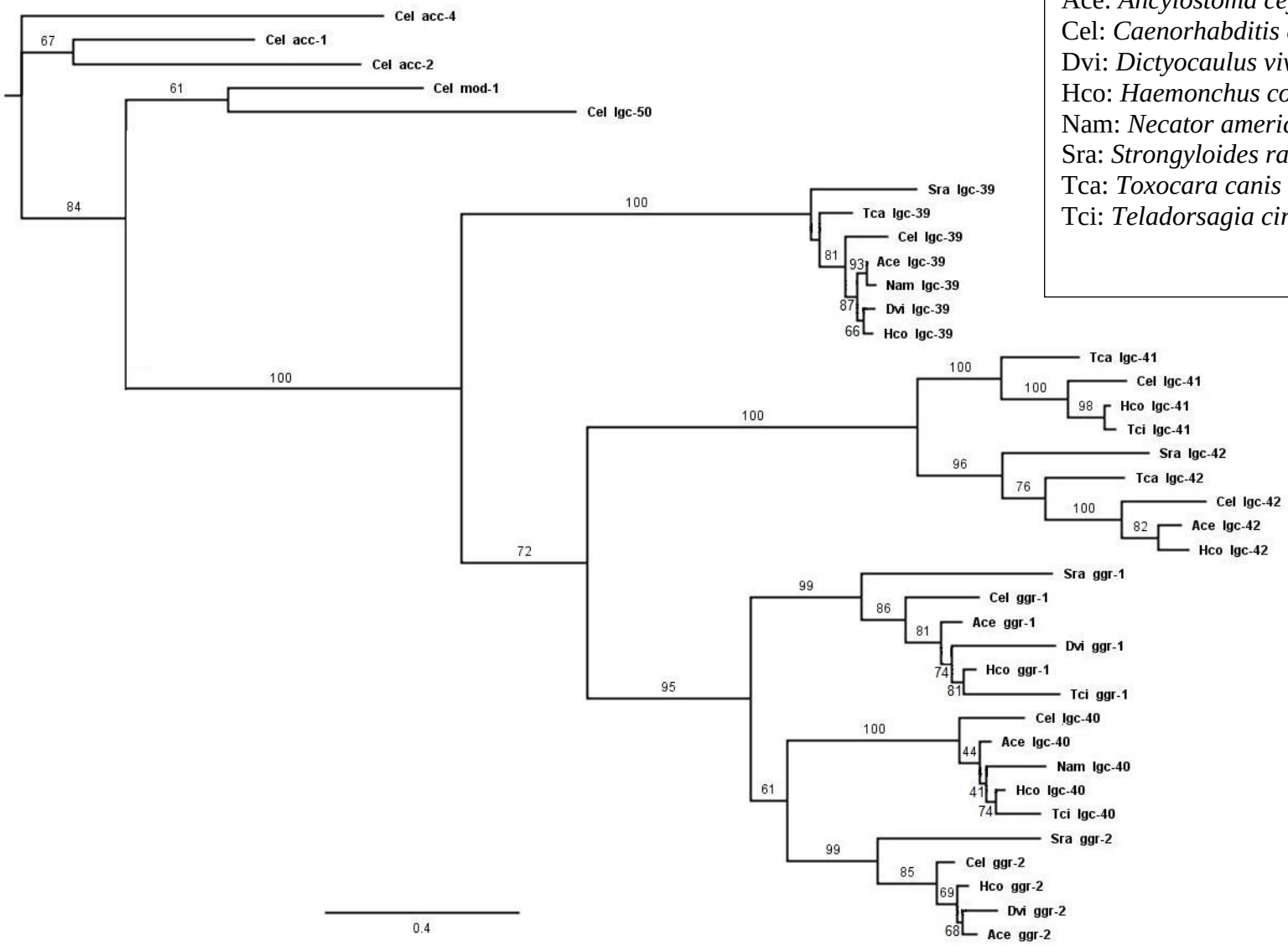
2.10 Computational Agonist Docking

Ligands, in their energy reduced form, were obtained from the Zinc database http://zinc.docking.org/ (Irwin *et al.*, 2012). Homodimer models, previously created using MODELLER, were prepared for ligand docking using AutoDock tools (Morris *et al*., 2009). This preparation included the addition of polar hydrogens, which are lacking in both the model generated by MODELLER as well as the energy reduced form from the Zinc database. This was necessary as it allows for the possibility of hydrogen bonds between the receptor binding site and the ligand. AutoDock Vina was used to simulate docking of a ligand in the binding site of the homodimers (Trott & Olson, 2010). Pymol was used to visualize the docked ligand on its corresponding homodimer, and Chimera v1.6.1 (Pettersen et al., 2004) was used to determine the distance between the amino acid residues and the ligand.

CHAPTER 3: RESULTS

3.1 Phylogenetic Analysis

The phylogenetic analysis conducted on the six GGR-1 family subunits indicated that members of this family are present in a wide range of parasitic nematodes, including many nematodes from different clades (Figure 3.1). The subunits are highly conserved across different clades, with a high degree of similarity found between the protein sequences of different parasites and *C. elegans*.



Legend
Ace: *Ancylostoma ceylanicum*
Cel: *Caenorhabditis elegans*
Dvi: *Dictyocaulus viviparus*
Hco: *Haemonchus contortus*
Nam: *Necator americanus*
Sra: *Strongyloides ratti*
Tca: *Toxocara canis*
Tci: *Teladorsagia circumcincta*

Figure 3.1: Maximum likelihood phylogeny of GGR-1 Family genes from a variety of parasitic nematodes. Members of the ACC-1 and MOD-1 families were used as outgroups. The distance to the outgroup has been shortened for image clarity.

3.2 Semi-Quantitative PCR

Semi-Quantitative PCR was done on all six subunits of the GGR-1 family to determine the relative expression of the gene subunits in adult and L2 stage larvae *H. contortus* (Figure 3.2). *Lgc-39, lgc-40, lgc-41,* and *ggr-2* were detected in both the adult and the L2 larval stage, with *lgc-39* and *lgc-40* being expressed to a high degree in the adult stage. No PCR product was obtained for *lgc-42* in the adult stage, although it appeared to be present in the L2 larval stage. No PCR product was obtained for *ggr-1* in either the adult or L2 stage. No amplification was observed in the negative reverse transcriptase controls, verifying that there was no genomic DNA contamination. The genes that were detected in the adult stage were selected for further investigation.

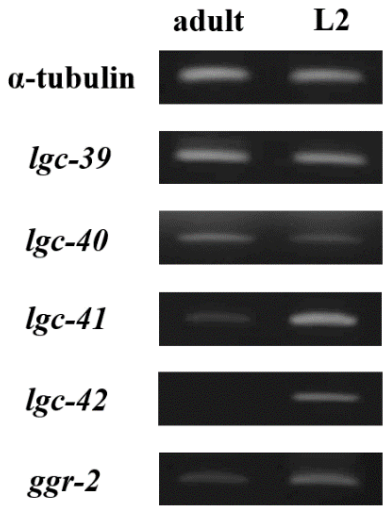


Figure 3.2: End point PCR analysis of GGR-1 family genes with the housekeeping gene, α-tubulin, used as a control. No amplification was obtained for *ggr-1* and it was therefore excluded. No amplification was obtained for –RT controls.

3.3 Isolation and Characterization of lgc-39, lgc-40, and ggr-2

PCR amplification of the *Hco-lgc-39* gene yielded a 1845 base pair cDNA sequence. This sequence was submitted to GenBank (accession number QCU71394.1). When translated, this sequence encoded for a protein 610 amino acids in length and

included the cys-loop, characteristic of this type of receptors. A homodimer of Hco-LGC-39 was generated using *in silco* protein modelling with the structure 3JAD used as a template, as this template was most similar to the protein sequence. Appropriate ligands were docked in the interface between two adjacent subunits (Figure 3.4).

In addition to the characteristic cys-loop, the seven peptide 'loops' were located in the extracellular domain, and the four hydrophobic transmembrane domains were found. The PAR motif, indicative of Cl^- selectivity (Jensen et al., 2005), was noted preceding the M2 domain (Figure 3.3). The sequence obtained using the RACE procedure revealed a signal peptide at the 5' end, with a cleavage site between the 17^{th} and 18^{th} amino acid, which was identified using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP-4.0/). The Hco-LGC-39 protein sequence shares approximately a 66% similarity with the Cel-LGC-39 protein, with highly conserved regions observed in the binding sites (Figure 3.5).

Figure 3.3: ClustalW alignment between Hco-LGC-39, Hco-LGC-40, and Hco-GGR-2 amino acid sequences. Important regions including the signal peptide and cleavage site, binding loops (A-G), and transmembrane domains are highlighted.

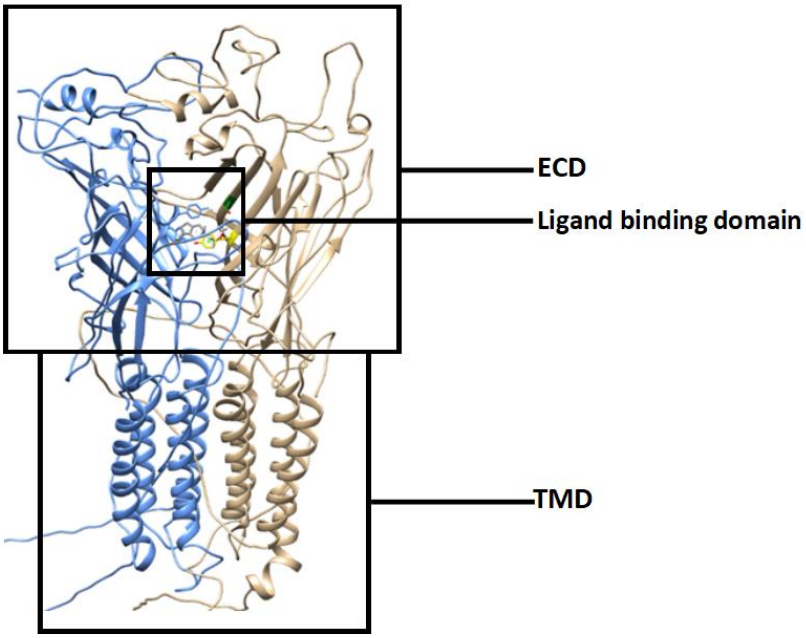


Figure 3.4: Homology model of Hco-LGC-39. The primary subunit (blue) is located on the left, and the complimentary subunit (tan) is located on the right. Methacholine is docked in the binding site located at the interface between the two subunits. The intracellular domain is not shown.

```
Cel-LGC-39      SGDRLLKVIGNGVVEDGYNMFMAPGQSQGKRTDVHVAVYIESMSSFKAQSMDFEVDMYLA
Hco-LGC-39      PGDRLLKVIGNGVVKDGYNMFLAPGQAQGRRTDVHVAVYIESMSSFKAQTMDFEVDMYLA
                .**************:******:****:**:**********************:**********
                                                          Loop G          Loop D
Cel-LGC-39      MGWFDRRLAHNCTHPILVTSKLIADRIWYPDLYFVNSKYAYLQEVTTPNLMVIVYPDGLI
Hco-LGC-39      MGWFDRRLAHNCTHPILVTSKLIADRMWHPDLYFVNSKFAYLQEVTTPNLMVIVYPDGLI
                ***************************:*:*********:**********************
                                                Loop A
Cel-LGC-39      FKTMRLDVTLSCMMDLKLFPLDYQECPLTIQSFAYIEQIVNLTWRDDPPNFPIGFNPDIK
Hco-LGC-39      FKSMRLDVTLSCMMDLKLFPIDHQECPLKIQSYAYIEQIVNLTWHVAPPYFPVGSNEEIK
                **:**********************:*:******.***:************:  ** **:* * :**
                Loop E          cys-loop          Loop B                Loop F
Cel-LGC-39      LNDMQITNKRFVKCAGPYPMFRGEAAWSCIQGYIVMKRLVLFHIIQTYIPTGMLVSISWM
Hco-LGC-39      LNDMAITRTRYEKCAGPYPMFRGSAYWSCIRAFIVMKRLVLFHIIQTYIPTGMLVSISWM
                **** **..*: **********.* ****:.:**************************
                                Loop C
```

Figure 3.5: ClustalW protein alignment of Cel-LGC-39 and Hco-LGC-39. Binding loops (A-G) are highlighted in red and the cys-loop is highlighted in green.

Amplification of the complete *Hco-lgc-40* sequence yielded a 1422 base pair sequence, submitted to GenBank (accession number QCU71395.1), which encodes for a protein 473 amino acids in length. The protein subunit was analyzed for key features and

a dimer was generated using 3JAD as a template (Figure 3.6). Ligands of interested were docked in the interface between the two subunits.

This protein has a 73% similarity with the Cel-LGC-40 protein (Figure 3.7), and contains the characteristic cys-loop and seven binding loops in the extracellular domain as well as four transmembrane domains (Figure 3.3). In addition, the PAR motif was observed preceding M2, which is indicative of this channel being Cl^- conductive. The signal peptide cleavage site occurs between the 15^{th} and 16^{th} amino acid, as determined using SignalP 4.0.

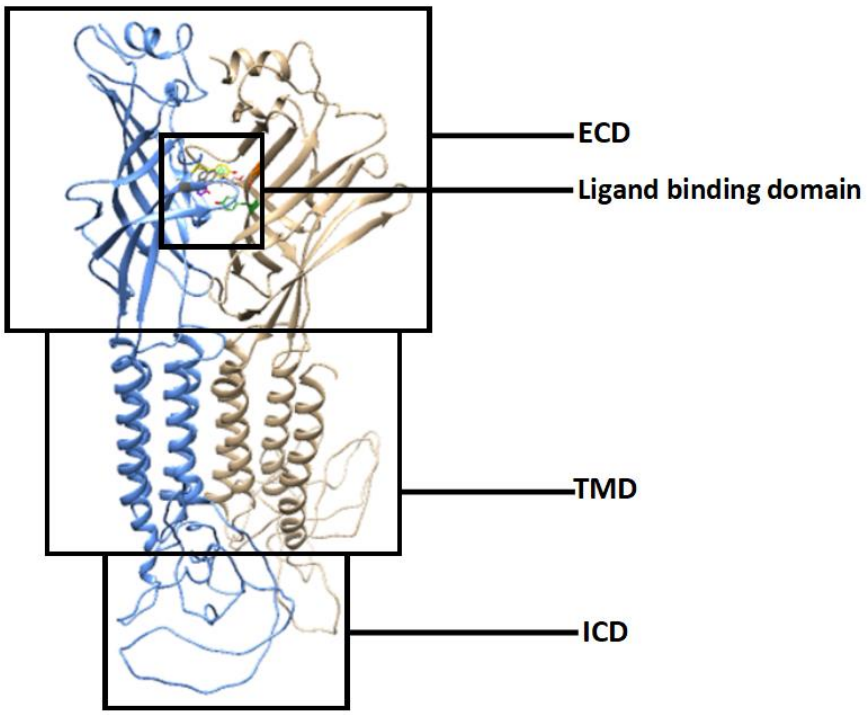


Figure 3.6: Homology model of Hco-LGC-40. The primary subunit (blue) is located on the left, and the complimentary subunit (tan) is located on the right. Choline is docked in the binding site located at the interface between the two subunits.

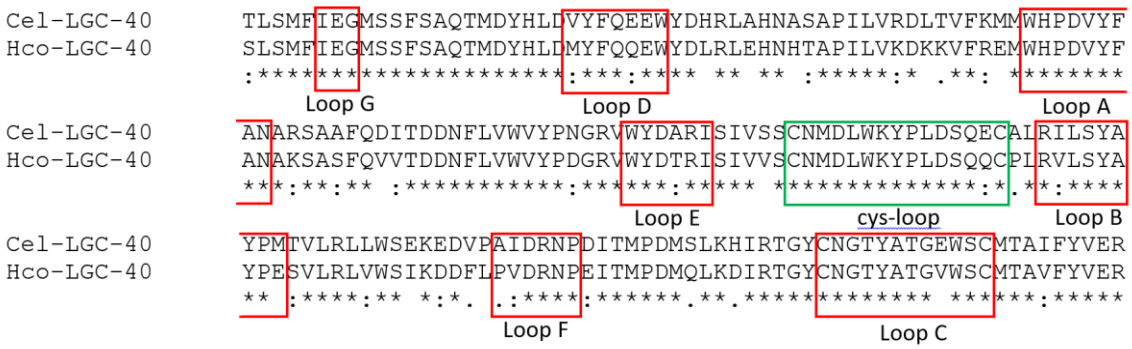


Figure 3.7: ClustalW protein alignment of Cel-LGC-40 and Hco-LGC-40. Binding loops (A-G) are highlighted in red and the cys-loop is highlighted in green.

Amplification of the complete *Hco-ggr-2* sequence yielded a 1551 base pair sequence, submitted to GenBank (accession number QCU71396.1), which encodes for a protein 516 amino acids in length. The protein subunit was analyzed for key features and a dimer was generated using 3JAD as a template (Figure 3.8). Ligands of interested were docked in the interface between the two subunits.

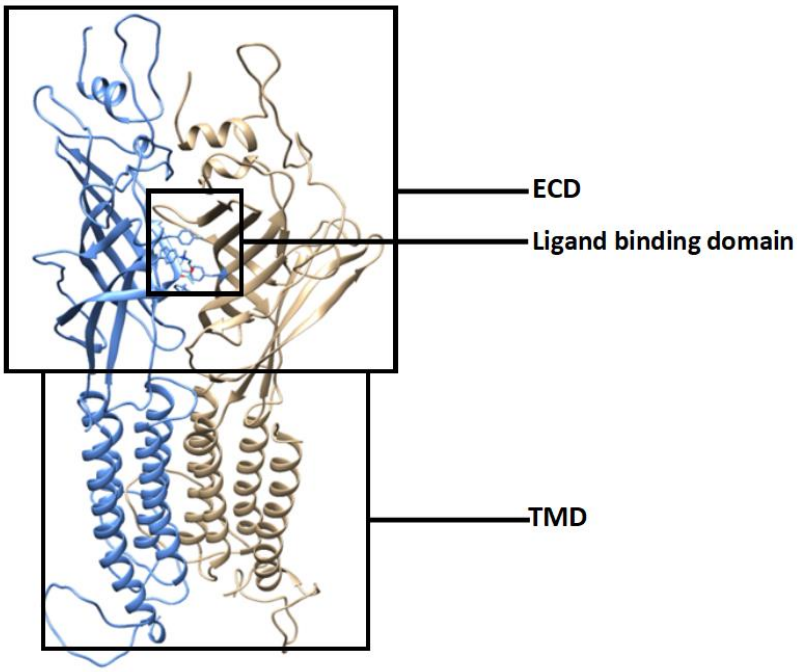


Figure 3.8: Homology model of Hco-GGR-2. The primary subunit (blue) is located on the left, and the complimentary subunit (tan) is located on the right. Acetylcholine is docked in the binding site located at the interface between the two subunits.

This protein has approximately a 65% homology with the Cel-GGR-2 protein (Figure 3.9). The signal peptide cleavage site for Hco-GGR-2 occurs between the 23rd and 24th amino acid, and the protein contained all the distinguishing features of a cys-loop LGIC (Figure 3.3).

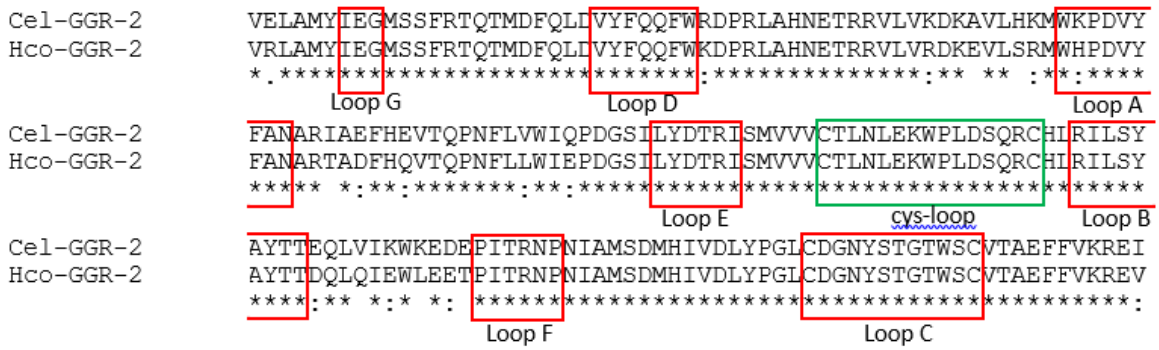


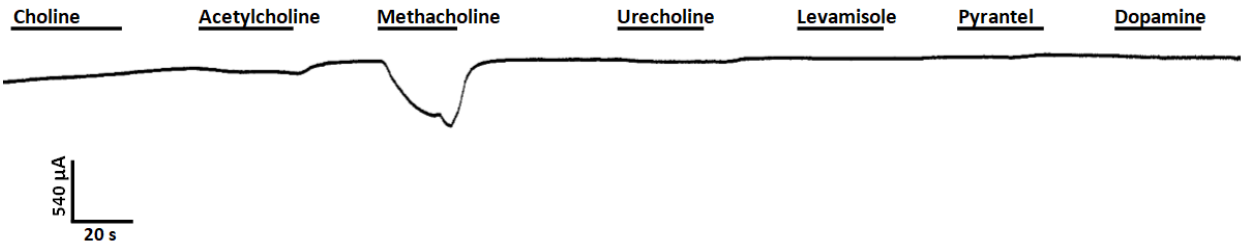
Figure 3.9: ClustalW protein alignment of Cel-GGR-2 and Hco-GGR-2. Binding loops (A-G) are highlighted in red and the cys-loop is highlighted in green.

3.4 Pharmacological Characterization of LGC-39, LGC-40 and GGR-2

When expressed in *X. laevis* oocytes, Hco-LGC-39 and Hco-LGC-40 were both able to form homomeric channels when injected alone. Hco-LGC-39 showed a strong response to methacholine and a weaker response to acetylcholine at 100 µM concentration (Figure 3.10A) and Hco-LGC-40 showed smaller amplitude responses to both choline and acetylcholine at 100 µM concentration (Figure 3.10B). Neither channel was activated at a 100 µM concentration of urecholine, levamisole, pyrantel, GABA, glycine, tyramine, dopamine, and octopamine. It is unclear whether Hco-GGR-2 is capable of forming a homomeric channel as it showed no response to any of the tested ligands.

A

Hco-LGC-39



B

Hco-LGC-40

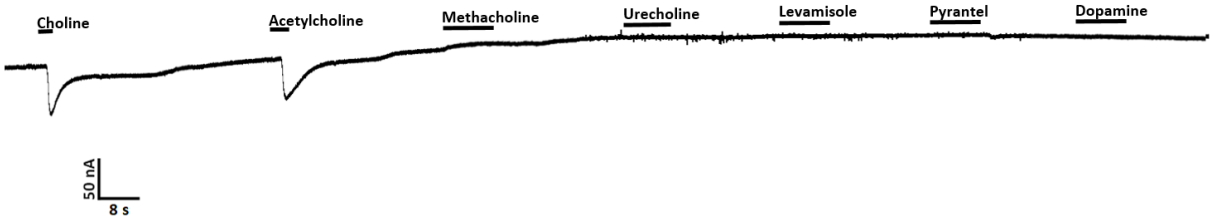


Figure 3.10: (A) Hco-LGC-39 homermeric channel activation in response to 100 µM concentrations of the labelled ligands. (B) Hco-LGC-40 homomeric channel activation in response to 100 µM concentrations of the labelled ligands. Additional ligands (GABA, glycine, tyramine, dopamine, and octopamine) were tested. However, they elicited no response in all tested homomeric channels and are therefore excluded from the figure.

The response of Hco-LGC-39 was dose dependent when exposed to increasing concentrations of their activating ligands (Figure 3.11 A). The methacholine EC_{50} value for Hco-LGC-39 was 447.5 ± 32.4 µM (n=5) with a Hill coefficient of 1.604 ± 0.18, which suggests that more than one methacholine molecule is required to open the channel. The acetylcholine EC_{50} value for Hco-LGC-39 is 1049 ± 92 µM (n=5) with a Hill coefficient of 1.796 ± 0.279, suggesting the binding of at least two acetylcholine molecules for channel activation (Figure 3.11 B).

Current voltage analysis of the Hco-LGC-39 channel using full chloride (103.6 mM Cl^-) indicated a reversal potential of -18.58 ± 2.91 mV (n=6), which is consistent with the calculated Nernst potential for Cl^- (-18.5 mV), assuming 50 mM internal Cl^- (Kusano et al., 1982). When the chloride concentration was reduced to 62.5 mM the reversal potential shifted to 0.3640 ± 2.713, which is consistent with the predicted Nernst potential of -5.7 mV (Figure 3.11 C).

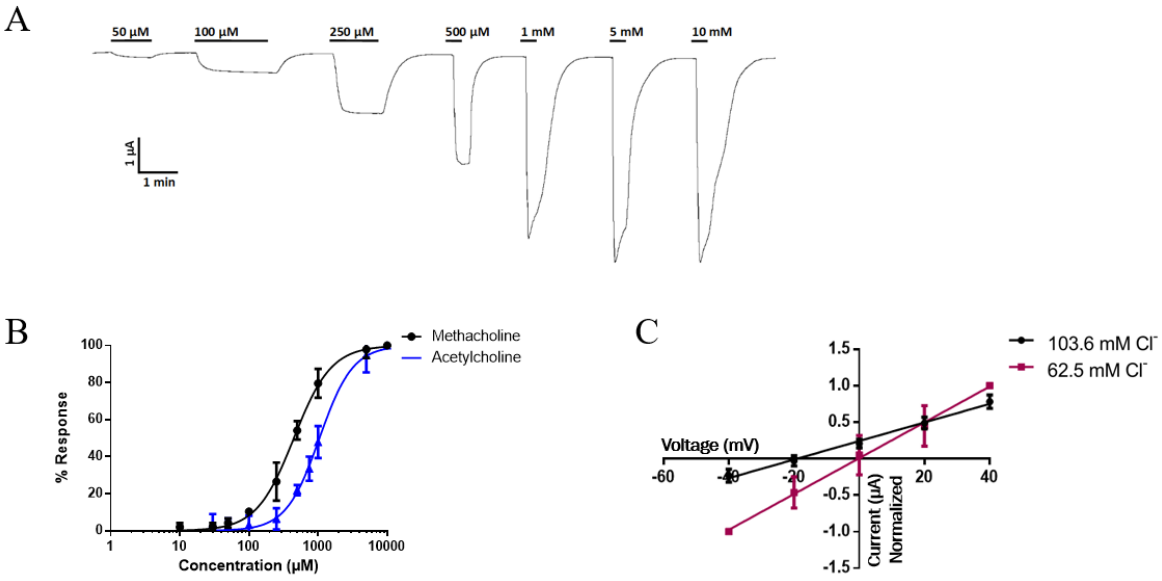


Figure 3.11: (A) Hco-LGC-39 homomeric channel responses to increasing concentrations of methacholine. (B) Hco-LGC-39 dose response curves of methacholine and acetylcholine. (C) Current-voltage analysis conducted using 500 µM concentration of methacholine.

The Hco-LGC-40 homomeric channel responds in a dose dependent manner to both choline and acetylcholine (Figure 3.12 A). The choline EC_{50} value for Hco-LGC-40 was 2.323 ± 0.369 µM (n=6) with a Hill coefficient of 2.794 ± 0.117, which suggests that more than one choline molecule is required to open the channel. The acetylcholine EC_{50} value for Hco-LGC-40 is 4.828 ± 0.239 µM (n=4) with a Hill coefficient of 3.992 ± 0.882,

suggesting the binding of at least two acetylcholine molecules for channel activation (Figure 3.12 B).

Similarly, current voltage analysis of the Hco-LGC-40 channel using full chloride (103.6 mM Cl^-) indicated a reversal potential of -25.01 ± 11.15 mV (n=3), which is consistent with the calculated Nernst potential for Cl^- (-18.5 mV), assuming 50 mM internal Cl^- (Kusano et al., 1982). When the chloride concentration was reduced to 62.5 mM the reversal potential shifted to -1.738 ± 4.768, which is consistent with the predicted Nernst potential of -5.7 mV (Figure 3.12 C).

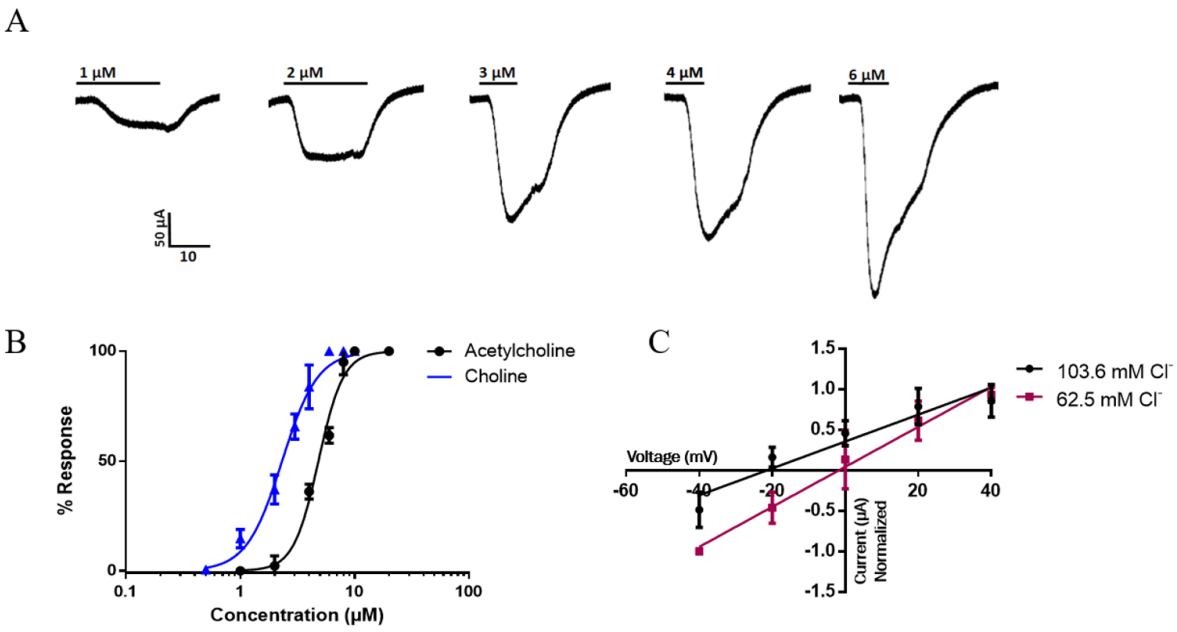


Figure 3.12: (A) Hco-LGC-40 homomeric channel responses to increasing concentrations of choline. (B) Hco-LGC-40 dose response curves of choline and acetylcholine. (C) Current-voltage analysis conducted using 3 µM concentration of methacholine.

The Hco-GGR-2 channel was not activated in response to any of the tested ligands at a 100 µM concentration.

3.5 *In Silico* Homology Protein Modeling

In silico homology protein modeling was done for Hco-LGC-39 and Hco-LGC-40 using the *D. rerio* α-1-glycine receptor 3JAD crystal template. This template showed a high degree of similarily to both protein subunits. Acetylcholine and methacholine were docked on the Hco-LGC-39 model, and choline and acetylcholine were docked on the Hco-LGC-40 model. All docked ligands appear to be in close proximity to the tryptophan residue found in loop C, W327, in Hco-LGC-39, and W222, in Hco-LGC-40. The close proximity of the positively charged quaternary amine group to the center of the tryptophan aromatic ring, gives reason to suggest stability of the ligand through a strong π-cationic interaction with this residue.

In Hco-LGC-39 (Figure 3.13), it appears as though the tryptophan residue from loop C, W327, is important in stabilizing the docked acetylcholine, potentially through π-cationic interactions with the quaternary amine. The positively charged quaternary amine group of the acetylcholine ligand is located 4.466 Å away from the center of the W327 aromatic ring, allowing for this type of interaction (Figure 3.7, Table 1).

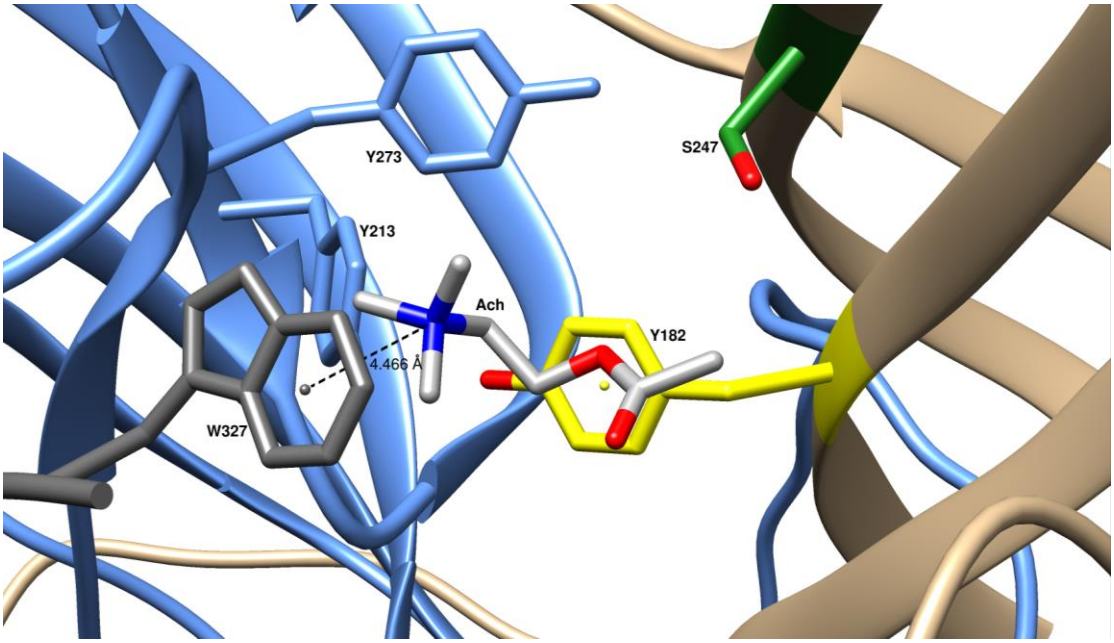


Figure 3.13: Acetylcholine docked on the Hco-LGC-39 binding site. A portion of loop C has been removed for clarity.

When methacholine was docked on the Hco-LGC-39 binding site (Figure 3.14), the positively charged quaternary amine group was located 4.587 Å away from W327, tryptophan in loop C, suggesting a strong π-cation interactions with this residue. Additional stability may be provided by the tyrosine residue on loop D, Y182, which is 5.416 Å away from the positively charged quaternary amine group. The serine residue on loop E (S247) may be contributing to stability through interactions between the hydroxyl group of the serine and the carbonyl group of the methacholine.

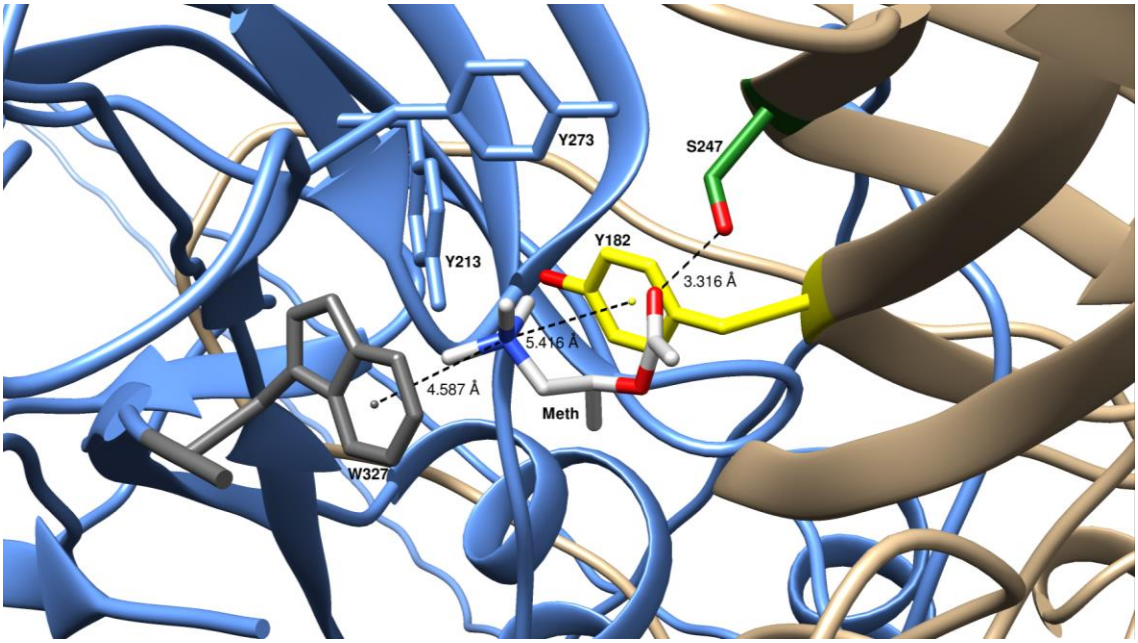


Figure 3.14: Methacholine docked on the Hco-LGC-39 binding site. A portion of loop C has been removed for clarity.

Table 3.1: Calculated affinities and distances from the quaternary amine group to the stabilizing tryptophan residue for the activating ligands of Hco-LGC-39.

Ligand	*Affinity to Hco-LGC-39 (kcal/mol)*	*Distance to W327 (Å)*
Acetylcholine	**-4.2**	**4.466**
Methacholine	**-4.4**	**4.587**

When choline was docked on the Hco-LGC-40 binding site (Figure 3.15), the positively charged quaternary amine group was 4.587 Å away from the tryptophan residue on loop C (W222), which likely stabilizes it through π-cationic interactions (Figure 3.15, Table 3.2). In addition, stability of this amine group may be improved through interactions with the tyrosine residue (Y172), which is predicted to be 5.087Å away. Stability may also be improved through hydrogen bonding occurring between the hydroxyl group of the choline molecule and the aspartic acid residue on loop E (D142). The distance between the hydroxyl group and the carboxylic acid group is predicted to be 3.059 Å (Figure 3.9).

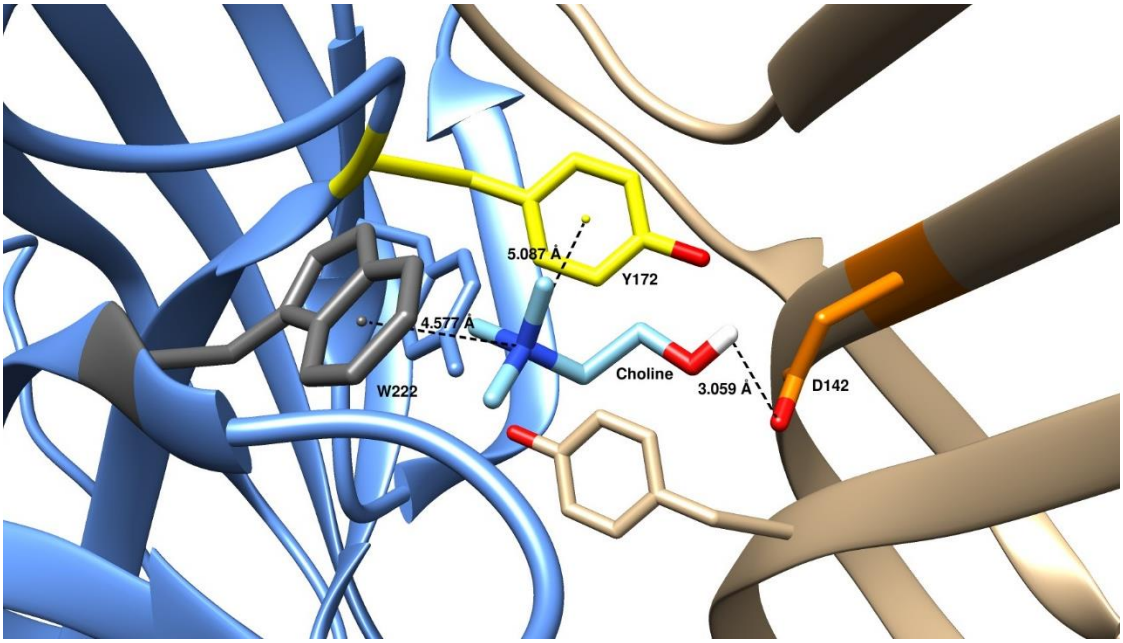


Figure 3.15: Choline docked on the Hco-LGC-40 binding site. A portion of loop C has been removed for clarity.

The quaternary amine group of acetylcholine also appears to be stabilized by the tryptophan residue (W222), predicted to be situated 4.706 Å away from the center of the aromatic ring (Figure 3.16, Table 3.2). In addition, the polar carbonyl group of the ester in the acetylcholine molecule appears to be in close proximity (3.270 Å) to the hydroxyl group of a tyrosine residue, contributing to stability of the molecule.

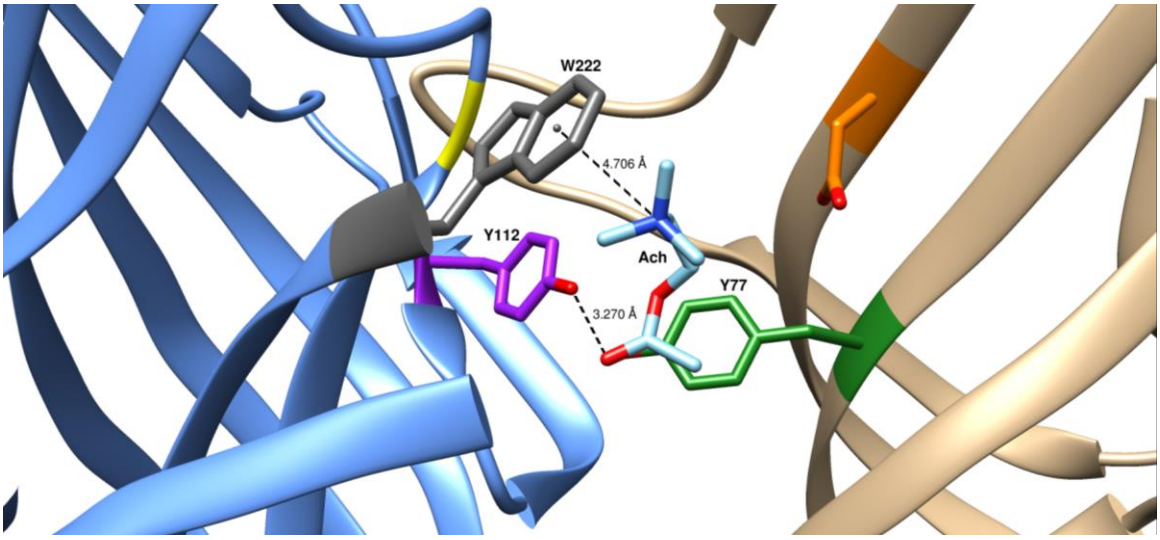


Figure 3.16: Acetylcholine docked on the Hco-LGC-40 binding site. A portion of loop C has been removed for clarity.

Table 3.2: Calculated affinities and distances from the quaternary amine group to the stabilizing tryptophan residue for the activating ligands of Hco-LGC-40.

Ligand	*Affinity to Hco-LGC-40 (kcal/mol)*	*Distance to W222 (Å)*
Acetylcholine	**-4.6**	**4.706**
Choline	**-3.6**	**4.577**

CHAPTER 4: DISCUSSION AND CONCLUSION

The purpose of this thesis was to isolate and characterize the nematode specific receptor subunits genes *hco-lgc-39, hco-lgc-40*, and *hco-ggr-2*. These three genes are the first from the GGR-1 family to be isolated from a parasitic nematode.

Although the functions of these receptors have yet to be determined, genes from this family appear to be highly conserved among a variety of parasitic nematodes. In addition, the expression tests conducted revealed that *lgc-39, lgc-40, lgc-41*, and *ggr-2* are present in both the adult and L2 larval stage, indicating that these receptors may play an important role in both the free-living and parasitic stages of these nematodes.

4.1 Characterization of Hco-LGC-39

The sequencing and modelling completed on Hco-LGC-39 has helped to further understand the structure and identify residues that may be important in Hco-LGC-39 channel function. The work done in this study revealed that Hco-LGC-39 forms a homomeric channel that is activated in response to acetylcholine and methacholine.

Although methacholine is a synthetic ligand, and therefore is not the natural ligand of Hco-LGC-39, the receptor was approximately two-fold more sensitive to it. Structurally, the only difference between acetylcholine and methacholine is the presence of a β-methyl group in the backbone. The presence of this methyl group appears to dock the molecule in a more structurally favorable manner, where the polar ester group is in close proximity to the polar serine, potentially contributing to the stability, and therefore receptor sensitivity to the molecule. A difference in sensitivity to methacholine compared to acetylcholine was also observed in the *H. contortus* acetylcholine receptor ACC-2 (Habibi et al., 2018). Although Hco-ACC-2 was found to be sensitive to many cholinergic compounds,

methacholine was the only compound found to be a full agonist of the receptor (Habibi et al., 2018). Taken together, these findings appear to indicate that the addition of a β-methyl group in the backbone of acetylcholine increases the responsiveness of some acetylcholine sensitive cys-loop LGICs.

The PAR motif preceding TM2, as well as the information obtained from the current-voltage tests, suggest that Hco-LGC-39 conducts chloride ions. This is interesting as vertebrates do not have anion channels gated by acetylcholine. However, other anionic channels gated by acetylcholine are present in invertebrates, including, but not limited to, the ACC-1 family receptors (Jones & Sattelle, 2008).

When acetylcholine activates a LGIC, it is typically stabilized through π-cationic interactions with a tryptophan residue. However, in nicotinic acetylcholine receptors, this tryptophan residue is traditionally found in loop B (Beene et al., 2002), unlike the loop C tryptophan predicted to stabilize the acetylcholine and methacholine molecules docked in this study. This phenomenon was also observed in Hco-ACC-2, where the tryptophan found in loop C was deemed essential for channel function, as mutating this residue to a tyrosine or a phenylalanine greatly reduced channel function (Habibi et al., 2018). This is interesting as tyrosine and phenylalanine are aromatic amino acids capable of forming π-cationic interactions with positively charged compounds. However, despite having electron dense regions due to the presence of an aromatic group, the interactions provided by these residues are much weaker than that provided by tryptophan. This is due to the presence of the indole nitrogen group found in the tryptophan residue, which contributes to the electron density of this side chain (Campo-Cacharrón et al., 2014). This finding of tryptophan residue being found in loop C has also been observed in MOD-1 receptors (Ranganathan,

Cannon, & Horvitz, 2000), where the tryptophan residue has been shown to be essential for receptor function through π-cationic interactions with serotonin (Mu et al., 2003). In addition, this loop C tryptophan has been observed in a wide range of cys-loop LGICs including the dopamine receptor LGC-53 (Beech et al., 2013), and the tyramine receptor LGC-55 (Rao et al., 2010). This loop C tryptophan residue appears to be a necessary component in the nematode cys-loop receptor family, and contributes to the diverse array of molecules these receptors are activated by.

4.2 Characterization of Hco-LGC-40

Similar to Hco-LGC-39, the sequencing and modelling helped to further understand the structure, and identify residues that may be important in Hco-LGC-40 channel function. This channel also contains a PAR motif preceding TM2, which, in combination with the current-voltage relationships, indicates that this channel conducts chloride ions. The electrophysiological analysis completed on this receptor revealed that Hco-LGC-40 forms a homomeric channel that is sensitive to both choline and acetylcholine. When compared to the homologous protein in *C. elegans* the *H. contortus* homomeric channel was similarly sensitive to choline and 18-fold more sensitive to acetylcholine (Ringstad et al, 2009). Differences in sensitivity between homologous receptors in *H. contortus* and *C. elegans* have been observed in other members of the cys-loop LGICs, including the ACC-1 family and the UNC-49 family (Siddiqui et al., 2010). The difference in sensitivity between the *C. elegans* and *H. contortus* UNC-49 receptors was attributed to the difference of at least one residue found in the binding site (Accardi & Forrester, 2011). Although it is unclear why these differences in sensitivity of homologous receptors occur, differences among

different nematodes are likely to occur based on the specific requirements that the nematode has for the receptor function.

Similar to *Hco-LGC-39*, a tryptophan residue is present in loop C that appears to be stabilizing the docked choline and acetylcholine molecules through π-cationic interactions with the charged quaternary amine. In addition, choline is likely stabilized in the binding site through hydrogen bonding between the hydroxyl group of the choline and the aspartic acid residue approximately 3 Å away. In addition to the π-cationic interactions with the tryptophan residue, acetylcholine is likely stabilized through interactions between the polar carbonyl group and the polar hydroxyl group of a nearby tyrosine residue.

4.3 Characterization of Hco-GGR-2

In this study, GGR-2 was revealed to be present in both and adult and L2 stage larvae, and therefore may play an important role in multiple life stages. Cloning and sequencing of Hco-GGR-2 aided in further understanding the structure of the receptor subunit. The cloned adult Hco-GGR-2 subunit was unresponsive as a homomeric channel to all of the tested ligands (choline, acetylcholine, methacholine, urecholine, levamisole, pyrantel, GABA, glycine, tyramine, dopamine, and octopamine) at a 100 µM concentration. This is similar to the result observed by Ringstad et al. (2009), who observed no response in the Cel-GGR-2 homomeric channel to 100 µM GABA, glycine, glutamate, histamine, dopamine, octopamine, and tyramine, and 1 mM of serotonin. However, Ringstad et al. (2009) observed a response to ivermectin, confirming that Cel-GGR-2 is capable of forming a homomeric channel. It would be interesting to determine if a similar response would be found in the Hco-GGR-2 channel. To this date, an activating ligand is yet to be determined for this channel.

4.6 Future Work

In this study end-point PCR was used to determine the presence and absence of genes in the adult and L2 stage larvae. Although this test aided in screening for genes that were present in the adult stage, it did not give a reliable estimate of the exact gene expression. For this reason, it would be of interest to analyze the gene expression of all six GGR-1 family members using qPCR. This would allow for a more accurate measure of expression, giving insight to the importance of these genes in different life stages, and allowing to select for the genes that are highly expressed in the adult stage, as these genes would be ideal drug targets.

The homology modelling completed in this study aided in visualizing the binding site of the receptors and identifying potential residues that may contribute to the stability of ligands. These models were generated using 3JAD, the *Danio rerio* alpha-1 glycine receptor. Although this template had the highest similarity to Hco-LGC-39 and Hco-LGC-40, there was only a 30-35% identity between the sequences of interest and the template. Therefore, the models generated were treated as predictions of the binding site. Information gained from homology modeling needs to be verified using site-directed mutagenesis.

A loop C tryptophan residue was identified in both Hco-LGC-39 and Hco-LGC-40. These residues were W327 and W222 from Hco-LGC-39 and Hco-LGC-40, respectively. In both models the quaternary amine of the ligand docked within 5 Å of the tryptophan residue, indicating potential cation π-interactions. To confirm if these residues are vital to channel function, mutagenesis needs to be conducted. Since tryptophan is known to form the strongest cation-π interactions (Campo-Cacharrón et al., 2014), mutating these residues to the other aromatic amino acids, tyrosine and phenylalanine,

would be of interest. Tyrosine and phenylalanine are capable of forming weaker cation-π interactions than that of tryptophan, and a decrease in channel sensitivity would provide support for the tryptophan residues being vital for the function of the two channels.

In addition, other residues were identified as important through homology modelling. In the Hco-LGC-39 channel the serine residue, S247, appeared to be important in the stability of methacholine through a potential interaction with the carbonyl group of methacholine. To investigate the importance of this residue in the binding site, S247 could be mutated to a non-polar amino acid. This interaction would be supported if a decrease in sensitivity to methacholine is observed. In the Hco-LGC-40 channel, an aspartic acid residue, D142, was identified as important as it appeared to be interacting with the hydroxyl group of choline, thereby contributing to the stability. To investigate the importance of this residue, D142 could be mutated to a non-polar group, as a non-polar amino acid would not interact in the same manner with choline. If D142 does in fact contribute to ligand stability, mutation to a non-polar amino acid would be detrimental to the channel function.

In this study, it was found that Hco-LGC-39 was activated in response to acetylcholine and methacholine, with EC_{50} values of 1049 ± 92 µM and 447.5 ± 32.4 µM respectively. Although these values are too large to conclude that acetylcholine is the natural ligand of this receptor, the natural ligand may be cholinergic in nature, allowing the receptor to be activated in response to other cholinergic compounds. In addition, Hco-LGC-39 may be a member of a heteromeric channel that has an increased sensitivity to acetylcholine and other compounds. It would be of interest to investigate this potential heteromer by co-injecting Hco-LGC-39 with other GGR-2 family members.

4.5 Conclusion

This thesis is the first to characterize GGR-1 family receptor subunits in a parasitic nematode. Although the function of these receptors has yet to be determined, and the receptor subunits have not been localized in the parasite, several findings indicate a potential for these receptors to be used as drug targets. First, a phylogenetic analysis of receptors from this family indicate that the family is well conserved with many members of the six subunits found in a variety of parasitic nematodes. Therefore, if a drug targeting these receptors was developed, it would have the potential to act on a wide variety of parasitic nematodes. Second, many of the receptor subunits studied appear to be present in multiple life stages of the parasite, which may indicate importance in function across various life stages in *H. contortus*. Third, these receptors are members of the cys-loop LGIC family, which are highly diverse and unique in nematodes. Therefore, drugs targeting these receptors are unlikely to have an adverse effect on the host. Finally, the work done in this study gives insight to a novel agonist binding site, which could potentially be exploited as a drug target for a novel drug.

Despite the work done in this thesis, the natural ligand of the receptor subunits was likely not found, especially in the case of Hco-LGC-39 and Hco-GGR-2. However, due to the responses elicited in response to cholinergic compounds in both Hco-LGC-39 and Hco-LGC-40, there is reason to believe that the natural ligands of this receptor family may be cholinergic in nature. To further explore the possibility of this family as targets for novel anthelminthic drugs, further testing is required to determine the natural ligand and the function that this family of receptors plays in the worms.

In conclusion, this thesis is the first to examine GGR-1 family receptors in parasitic nematodes. The work done in this study also builds on the investigation of Cel-LGC-40 by Ringstad et al., (2009). Although studying ion channels in *C. elegans* is an effective introductory step in characterizing ion channels, additional research needs to be done in parasites to ensure that the channels may indeed be exploited for use as drug targets for treatment of parasitic infection. This study serves as a starting step for characterization of GGR-1 family subunits in a parasitic nematode.

References

Absalom, N. L., Schofield, P. R., & Lewis, T. M. (2009). Pore structure of the Cys-loop ligand-gated ion channels. *Neurochemical Research*, *34*(10), 1805–1815.

Accardi, M. V, & Forrester, S. G. (2011). The Haemonchus contortus UNC-49B subunit possesses the residues required for GABA sensitivity in homomeric and heteromeric channels. *Molecular and Biochemical Parasitology*, *178*(1–2), 15–22.

AJ, W., & Rogers, A. (2005). Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. *Parasitology*, *131(Suppl)*, S85-95.

Alexander, S. P. H., Mathie, A., & Peters, J. A. (2008). Guide to receptors and channels (GRAC). *British Journal of Pharmacology*, *153*(S2), S1–S1.

Araud, T., Wonnacott, S., & Bertrand, D. (2010). Associated proteins: The universal toolbox controlling ligand gated ion channel function. *Biochemical Pharmacology*, *80*(2), 160–169.

Bamber BA, Beg AA, Twyman RE, J. E. (1999). The Caenorhabditis elegans unc-49 locus encodes multiple subunits of a heteromultimeric GABA receptor. *Journal of Neuroscience*, *19*, 5348–5359.

Bany, I. A., Dong, M.-Q., & Koelle, M. R. (2003). Genetic and cellular basis for acetylcholine inhibition of Caenorhabditis elegans egg-laying behavior. *Journal of Neuroscience*, *23*(22), 8060–8069.

Baur, R., Beech, R., Sigel, E., & Rufener, L. (2015). Monepantel irreversibly binds to and opens Haemonchus contortus MPTL-1 and Caenorhabditis elegans ACR-20 receptors. *Molecular Pharmacology*, *87*(1), 96–102.

Beech, R. N., Callanan, M. K., Rao, V. T. S., Dawe, G. B., & Forrester, S. G. (2013).

Characterization of cys-loop receptor genes involved in inhibitory amine neurotransmission in parasitic and free living nematodes. *Parasitology International*, *62*(6), 599–605.

Beene, D. L., Brandt, G. S., Zhong, W., Zacharias, N. M., Lester, H. A., & Dougherty, D. A. (2002). Cation− π interactions in ligand recognition by serotonergic (5-HT3A) and nicotinic acetylcholine receptors: the anomalous binding properties of nicotine. *Biochemistry*, *41*(32), 10262–10269.

Beg, A. A., & Jorgensen, E. M. (2003). EXP-1 is an excitatory GABA-gated cation channel. *Nature Neuroscience*, *6*(11), 1145.

Bianchi, M. T., Haas, K. F., & Macdonald, R. L. (2001). Structural Determinants of Fast Desensitization and Desensitization–Deactivation Coupling in GABAAReceptors. *Journal of Neuroscience*, *21*(4), 1127–1136.

Blackhall, W. J., Liu, H. Y., Xu, M., Prichard, R. K., & Beech, R. N. (1998). Selection at a P-glycoprotein gene in ivermectin-and moxidectin-selected strains of Haemonchus contortus. *Molecular and Biochemical Parasitology*, *95*(2), 193–201.

Bower, K. S., Price, K. L., Sturdee, L. E. C., Dayrell, M., Dougherty, D. A., & Lummis, S. C. R. (2008). 5-Fluorotryptamine is a partial agonist at 5-HT3 receptors, and reveals that size and electronegativity at the 5 position of tryptamine are critical for efficient receptor function. *European Journal of Pharmacology*, *580*(3), 291–297.

Brejc, K., van Dijk, W. J., Klaassen, R. V, Schuurmans, M., van der Oost, J., Smit, A. B., & Sixma, T. K. (2001). Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature*, *411*(6835), 269.

Buxton, S. K., Charvet, C. L., Neveu, C., Cabaret, J., Cortet, J., Peineau, N., … Martin,

R. J. (2014). Investigation of acetylcholine receptor diversity in a nematode parasite leads to characterization of tribendimidine-and derquantel-sensitive nAChRs. *PLoS Pathogens*, *10*(1), e1003870.

Campo-Cacharrón, A., Cabaleiro-Lago, E. M., Carrazana-García, J. A., & Rodríguez-Otero, J. (2014). Interaction of aromatic units of amino acids with guanidinium cation: The interplay of π··· π, X□ H··· π, and M+··· π contacts. *Journal of Computational Chemistry*, *35*(17), 1290–1301.

Campos-Caro, A., Sala, S., Ballesta, J. J., Vicente-Agulló, F., Criado, M., & Sala, F. (1996). A single residue in the M2-M3 loop is a major determinant of coupling between binding and gating in neuronal nicotinic receptors. *Proceedings of the National Academy of Sciences*, *93*(12), 6118–6123.

Cederholm, J. M. E., Schofield, P. R., & Lewis, T. M. (2009). Gating mechanisms in Cys-loop receptors. *European Biophysics Journal*, *39*(1), 37.

Chalasani SH, Chronis N, Tsunozaki M, Gray JM, Ramot D, G., & MB, B. C. (2007). Dissecting a circuit for olfactory behaviour in Caenorhabditis elegans. *Nature*, *450*, 63–70.

Connolly, C. N., & Wafford, K. A. (2004). The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function. Portland Press Limited.

Cook A, Aptel N, Portillo V, Siney E, Sihota R, H.-D. L., & A, W. (2006). Caenorhabditis elegans ivermectin receptors regulate locomotor behaviour and are functional orthologues of Haemonchus contortus receptors. *Molecular and Biochemical Parasitology*, *147*(118–125).

Cymes, G. D., & Grosman, C. (2008). Pore-opening mechanism of the nicotinic

acetylcholine receptor evinced by proton transfer. *Nature Structural & Molecular Biology*, *15*(4), 389.

Dang, H., England, P. M., Farivar, S. S., Dougherty, D. A., & Lester, H. A. (2000). Probing the role of a conserved M1 proline residue in 5-hydroxytryptamine3 receptor gating. *Molecular Pharmacology*, *57*(6), 1114–1122.

Davey, K. G., Sommerville, R. I., & Rogers, W. P. (1982). The effect of ethoxyzolamide, an analogue of insect juvenile hormone, nor-adrenaline and iodine on changes in the optical path difference in the excretory cells and oesophagus during exsheathment in Haemonchus contortus. *International Journal for Parasitology*, *12*(6), 509–513.

Dent, J. A. (2006). Evidence for a diverse cys-loop ligand-gated ion channel superfamily in early bilateria. *Journal of Molecular Evolution*, *62*, 523–535.

Dent, J. A., Davis, M. W., & Avery, L. (1997). avr-15 encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in Caenorhabditis elegans. *The EMBO Journal*, *16*(19), 5867–5879.

Duerr, J. S., Gaskin, J., & Rand, J. B. (2001). Identified neurons in C. elegans coexpress vesicular transporters for acetylcholine and monoamines. *American Journal of Physiology-Cell Physiology*, *280*(6), C1616–C1622.

Engblom, A. C., Carlson, B. X., Olsen, R. W., Schousboe, A., & Kristiansen, U. (2002). Point mutation in the first transmembrane region of the b2 subunit of the GABAA receptor alters desensitization kinetics of GABA and anesthetic-induced channel gating. *Journal of Biological Chemistry*, *277*, 17438–17447.

Frohman, M. A., Dush, M. K., & Martin, G. R. (1988). Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific

oligonucleotide primer. *Proc. Natl. Acad. Sci. Unit. States Am.*, *85*, 8998–9002.

Garcia, L. R., Mehta, P., & Sternberg, P. W. (2001). Regulation of distinct muscle behaviors controls the C. elegans male's copulatory spicules during mating. *Cell*, *107*(6), 777–788.

Greenfield, L. J., Zaman, S. H., Sutherland, M. L., Lummis, S. C. R., Niemeyer, M.-I., Barnard, E. A., & Macdonald, R. L. (2002). Mutation of the GABAA receptor M1 transmembrane proline increases GABA affinity and reduces barbiturate enhancement. *Neuropharmacology*, *42*(4), 502–521.

Grosman, C., Salamone, F. N., Sine, S. M., & Auerbach, A. (2000). The extracellular linker of muscle acetylcholine receptor channels is a gating control element. *The Journal of General Physiology*, *116*(3), 327–340.

Habibi, S. A., Callanan, M., & Forrester, S. G. (2018). Molecular and pharmacological characterization of an acetylcholine-gated chloride channel (ACC-2) from the parasitic nematode Haemonchus contortus. *International Journal for Parasitology: Drugs and Drug Resistance*, *8*(3), 518–525.

Hibbs, R. E., & Gouaux, E. (2011). Principles of activation and permeation in an anion-selective Cys-loop receptor. *Nature*, *474*(7349), 54.

Hoekstra, R., Visser, A., Wiley, L. J., Weiss, A. S., Sangster, N. C., & Roos, M. H. (1997). Characterization of an acetylcholine receptor gene of haemonchus contortus in relation to levamisole resistance1. *Molecular and Biochemical Parasitology*, *84*(2), 179–187.

Hwang, H., Barnes, D. E., Matsunaga, Y., Benian, G. M., Ono, S., & Lu, H. (2016). Muscle contraction phenotypic analysis enabled by optogenetics reveals functional

relationships of sarcomere components in Caenorhabditis elegans. *Scientific Reports, 6*, 19900.

Irwin, J. J., Sterling, T., Mysinger, M. M., Bolstad, E. S., & Coleman, R. G. (2012). ZINC: a free tool to discover chemistry for biology. *Journal of Chemical Information and Modeling*, *52*(7), 1757–1768.

Jensen, M. L., Pedersen, L. N., Timmermann, D. B., Schousboe, A., & Ahring, P. K. (2005). Mutational studies using a cation-conducting GABAA receptor reveal the selectivity determinants of the Cys-loop family of ligand-gated ion channels. *Journal of Neurochemistry*, *92*(4), 962–972.

Jensen, M., Schousboe, A., & Ahring, P. (2005). Charge selectivity of the Cys-loop family of ligand-gated ion channels. *Journal of Neurochemistry, 92*, 217–225.

Jones, A. K., & Sattelle, D. B. (2008). The cys-loop ligand-gated ion channel gene superfamily of the nematode, Caenorhabditis elegans. *Invertebrate Neuroscience*, *8*(1), 41–47.

Kaminsky, R., Ducray, P., Jung, M., Clover, R., Rufener, L., Bouvier, J., … Goebel, T. (2008). A new class of anthelmintics effective against drug-resistant nematodes. *Nature*, *452*(7184), 176.

Kim, J., Poole, D. S., Waggoner, L. E., Kempf, A., Ramirez, D. S., Treschow, P. A., & Schafer, W. R. (2001). Genes affecting the activity of nicotinic receptors involved in Caenorhabditis elegans egg-laying behavior. *Genetics*, *157*(4), 1599–1610.

Kim, J., Yeon, J., Choi, S.-K., Huh, Y. H., Fang, Z., Park, S. J., … Kweon, H.-S. (2015). The evolutionarily conserved LIM homeodomain protein LIM-4/LHX6 specifies the terminal identity of a cholinergic and peptidergic C. elegans sensory/inter/motor

neuron-type. *PLoS Genetics*, *11*(8), e1005480.

Kusama, T., Wang, J.-B., Spivak, C. E., & Uhl, G. R. (1994). Mutagenesis of the GABA rho 1 receptor alters agonist affinity and channel gating. *Neuroreport*, *5*(10), 1209–1212.

Kusano, K., Miledi, R., & Stinnakre, J. (1982). Cholinergic and catecholaminergic receptors in the Xenopus oocyte membrane. *The Journal of Physiology*, *328*(1), 143–170.

Laing, R., Kikuchi, T., Martinelli, A., Tsai, I. J., Beech, R. N., Redman, E., … Britton, C. (2013). The genome and transcriptome of Haemonchus contortus, a key model parasite for drug and vaccine discovery. *Genome Biology*, *14*(8), R88.

Laughton, D. L., Lunt, G. G., & Wolstenholme, A. J. (1997). Reporter gene constructs suggest that the Caenorhabditis elegans avermectin receptor beta-subunit is expressed solely in the pharynx. *Journal of Experimental Biology*, *200*(10), 1509–1514.

Law, R. J., Forrest, L. R., Ranatunga, K. M., La Rocca, P., Tieleman, D. P., & Sansom, M. S. P. (2000). Structure and dynamics of the pore-lining helix of the nicotinic receptor: MD simulations in water, lipid bilayers, and transbilayer bundles. *Proteins: Structure, Function, and Bioinformatics*, *39*(1), 47–55.

Martin, R. J. (1997). Modes of action of anthelmintic drugs. *The Veterinary Journal*, *154*(1), 11–34.

Martin, R. J., & Robertson, A. P. (2010). Control of nematode parasites with agents acting on neuro-musculature systems: lessons for neuropeptide ligand discovery. In *Neuropeptide Systems as Targets for Parasite and Pest Control* (pp. 138–154).

Springer.

McKay, J. P., Raizen, D. M., Gottschalk, A., Schafer, W. R., & Avery, L. (2004). eat-2 and eat-18 are required for nicotinic neurotransmission in the Caenorhabditis elegans pharynx. *Genetics*, *166*(1), 161–169.

Miller, J. E., Bahirathan, M., Lemarie, S. L., Hembry, F. G., Kearney, M. T., & Barras, S. R. (1998). Epidemiology of gastrointestinal nematode parasitism in Suffolk and Gulf Coast Native sheep with special emphasis on relative susceptibility to Haemonchus contortus infection. *Veterinary Parasitology*, *74*(1), 55–74.

Miyazawa, A., Fujiyoshi, Y., & Unwin, N. (2003). Structure and gating mechanism of the acetylcholine receptor pore. *Nature*, *423*(6943), 949.

Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., & Olson, A. J. (2009). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of Computational Chemistry*, *30*(16), 2785–2791.

Mu, T.-W., Lester, H. A., & Dougherty, D. A. (2003). Different binding orientations for the same agonist at homologous receptors: a lock and key or a simple wedge? *Journal of the American Chemical Society*, *125*(23), 6850–6851.

Niebur, E., & Erdös, P. (1991). Theory of the locomotion of nematodes: dynamics of undulatory progression on a surface. *Biophysical Journal*, *60*(5), 1132–1146.

Nikolaou, S., & Gasser, R. B. (2006). Prospects for exploring molecular developmental processes in Haemonchus contortus. *International Journal for Parasitology*, *36*(8), 859–868.

O'Shea, S. M., & Harrison, N. L. (2000). Arg-274 and Leu-277 of the γ-aminobutyric

acid type A receptor α2 subunit define agonist efficacy and potency. *Journal of Biological Chemistry*, *275*(30), 22764–22768.

Pemberton DJ, Franks CJ, Walker RJ, H.-D. L. (2001). Characterization of glutamate-gated chloride channels in the pharynx of wild-type and mutant Caenorhabditis elegans delineates the role of the subunit GluCl-alpha2 in the function of the native receptor. *Molecular Pharmacology*, *59*, 1037–1043.

Pettersen, E., Goddard, T., Huang, C., Couch, G., Greenblatt, D., Meng, E., & Ferrin, T. (2004). UCSF Chimera--a visualization system for exploratory research and analysis. *Journal of Computational Chemistry*, *25*(13), 1605–1612.

Pirri, J. K., McPherson, A. D., Donnelly, J. L., Francis, M. M., & Alkema, M. J. (2009). A tyramine-gated chloride channel coordinates distinct motor programs of a Caenorhabditis elegans escape response. *Neuron*, *62*(4), 526–538.

Pless, S. A., Millen, K. S., Hanek, A. P., Lynch, J. W., Lester, H. A., Lummis, S. C. R., & Dougherty, D. A. (2008). A cation-π interaction in the binding site of the glycine receptor is mediated by a phenylalanine residue. *Journal of Neuroscience*, *28*(43), 10937–10942.

Prichard, R. (2001). Genetic variability following selection of Haemonchus contortus with anthelmintics. *Trends in Parasitology*, *17*(9), 445–453.

Putrenko, I., Zakikhani, M., & Dent, J. (2005). A family of acetylcholinegated chloride channel subunits in Caenorhabditis elegans. *The Journal of Biological Chemistry*, *280*, 6392–6398.

Ranganathan, R., Cannon, S., & Horvitz, H. (2000). MOD-1 is a serotonin-gated chloride channel that modulates locomotory behaviour in C. elegans. *Nature*, *408*, 470–475.

Rao, V. T. S., Accardi, M. V, Siddiqui, S. Z., Beech, R. N., Prichard, R. K., & Forrester, S. G. (2010). Characterization of a novel tyramine-gated chloride channel from Haemonchus contortus. *Molecular and Biochemical Parasitology*, *173*(2), 64–68.

Richmond JE, J. E. (1999). One GABA and two acetylcholine receptors function at the C. elegans neuromuscular junction. *Nature Neuroscience*, *2*, 791–797.

Ringstad, N., Abe, N., & Horvitz, H. (2009). Ligand-gated chloride channels are receptors for biogenic amines in C. elegans. *Science*, *325*(5936), 96–100.

Rodríguez, A. V, Goldberg, V., Viotti, H., & Ciappesoni, G. (2015). Early detection of Haemonchus contortus infection in sheep using three different faecal occult blood tests. *Open Veterinary Journal*, *5*(2), 90–97.

Roeber, F., Jex, A. R., & Gasser, R. B. (2013). Impact of gastrointestinal parasitic nematodes of sheep, and the role of advanced molecular tools for exploring epidemiology and drug resistance-an Australian perspective. *Parasites & Vectors*, *6*(1), 153.

Ruaud, A.-F., & Bessereau, J.-L. (2006). Activation of nicotinic receptors uncouples a developmental timer from the molting timer in C. elegans. *Development*, *133*(11), 2211–2222.

Sarai, R. S., Kopp, S. R., Knox, M. R., Coleman, G. T., & Kotze, A. C. (2015). In vitro levamisole selection pressure on larval stages of Haemonchus contortus over nine generations gives rise to drug resistance and target site gene expression changes specific to the early larval stages only. *Veterinary Parasitology*, *211*(1–2), 45–53.

Saunders, G. I., Wasmuth, J. D., Beech, R., Laing, R., Hunt, M., Naghra, H., … Gilleard, J. S. (2013). Characterization and comparative analysis of the complete

Haemonchus contortus β-tubulin gene family and implications for benzimidazole resistance in strongylid nematodes. *International Journal for Parasitology*, *43*(6), 465–475.

Sawin, E. R., Ranganathan, R., & Horvitz, H. R. (2000). C. elegans locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. *Neuron*, *26*(3), 619–631.

Schulte, M. K., Hill, R. A., Bikádi, Z., Maksay, G., Parihar, H. S., Joshi, P., & Suryanarayanan, A. (2006). The structural basis of ligand interactions in the 5-HT3 receptor binding site. *Biological and Biophysical Aspects of Ligand-Gated Ion Channel Receptor Superfamilies*, 127–153.

Schwarz, E. M., Korhonen, P. K., Campbell, B. E., Young, N. D., Jex, A. R., Jabbar, A., … Pell, J. (2013). The genome and developmental transcriptome of the strongylid nematode Haemonchus contortus. *Genome Biology*, *14*(8), R89.

Siddiqui, S. Z., Brown, D. D. R., Rao, V. T. S., & Forrester, S. G. (2010). An UNC-49 GABA receptor subunit from the parasitic nematode Haemonchus contortus is associated with enhanced GABA sensitivity in nematode heteromeric channels. *Journal of Neurochemistry*, *113*(5), 1113–1122.

Steger, K. A., & Avery, L. (2004). The GAR-3 muscarinic receptor cooperates with calcium signals to regulate muscle contraction in the Caenorhabditis elegans pharynx. *Genetics*, *167*(2), 633–643.

Sze, J. Y., Victor, M., Loer, C., Shi, Y., & Ruvkun, G. (2000). Food and metabolic signalling defects in a Caenorhabditis elegans serotonin-synthesis mutant. *Nature*, *403*(6769), 560.

Szewczyk, N. J., Hartman, J. J., Barmada, S. J., & Jacobson, L. A. (2000). Genetic defects in acetylcholine signalling promote protein degradation in muscle cells of Caenorhabditis elegans. *J Cell Sci*, *113*(11), 2003–2010.

Thomas, J. H. (1990). Genetic analysis of defecation in Caenorhabditis elegans. *Genetics*, *124*(4), 855–872.

Thompson, A. J., Chau, P.-L., Chan, S. L., & Lummis, S. C. R. (2006). Unbinding pathways of an agonist and an antagonist from the 5-HT3 receptor. *Biophysical Journal*, *90*(6), 1979–1991.

Thompson, A. J., Lester, H. A., & Lummis, S. C. R. (2010). The structural basis of function in Cys-loop receptors. *Quarterly Reviews of Biophysics*, *43*(4), 449–499.

Trott, O., & Olson, A. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, *31*(2), 455–461.

Tsetlin, V., Kuzmin, D., & Kasheverov, I. (2011). Assembly of nicotinic and other Cys-loop receptors. *Journal of Neurochemistry*, *116*(5), 734–741.

Van Doren, K., & Hirsh, D. (1988). Trans-spliced leader RNA exists as small nuclear ribonucleoprotein particles in Caenorhabditis elegans. *Nature*, *335*, 556–559.

Van Wyk, J. A., Malan, F. S., & Randles, J. L. (1997). How long before resistance makes it impossible to control some field strains of Haemonchus contortus in South Africa with any of the modern anthelmintics? *Veterinary Parasitology*, *70*(1–3), 111–122.

Veglia, F. (1915). *The anatomy and life-history of Haemonchus contortus (Rud.)*. Pretoria: Government Printer and Stationery Office.

Weston, D., Patel, B., & Van Voorhis., W. C. (1999). Virulence in Trypanosoma cruzi

infection correlates with the expression of a distinct family of sialidase superfamily genes. *Molecular and Biochemical Parasitology, 98*(1), 105–116.

Wever, C. M., Farrington, D., & Dent, J. A. (2015). The validation of nematode-specific acetylcholine-gated chloride channels as potential anthelmintic drug targets. *PloS One, 10*(9), e0138804.

Zhang, Z., Gasser, R. B., Yang, X., Yin, F., Zhao, G., Bao, M., … Zou, F. (2016). Two benzimidazole resistance-associated SNPs in the isotype-1 β-tubulin gene predominate in Haemonchus contortus populations from eight regions in China. *International Journal for Parasitology: Drugs and Drug Resistance, 6*(3), 199–206.