Searching for an Exclusion Mechanism in the Evolution of Neurotransmitter Receptor Subunits

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Contribution of Authors

All parts of this manuscript were written by Eyre Nomi. Revisions and corrections were provided by supervisor Dr. Robin Beech.

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Abbreviations

AAD = Amino-acetonitrile derivative

AChR = Acetylcholine receptors

cDNA = Complementary DNA

EC_{50} = Half maximal concentration

HGI = Helminth Genome Initiative

LGIC = Ligand-gated ion channels

MR = Mineralocorticoid receptor

MS-222 = 0.15% tricaine methanesulfonate solution

PAML = Phylogenetic analysis by maximum likelihood

TEVC = Two-electrode voltage clamp

TM = Transmembrane

UTR = Untranslated region

mAChR = Muscarinic acetylcholine receptors

nAChR = Nicotinic acetylcholine receptors

pLGIC =Pentameric ligand-gated ion-channel

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Abstract

Pentameric ligand-gated ion-channels (pLGIC) are neurotransmitter receptors that mediate fast synaptic transmission and muscle contraction in animals. At the neuromuscular junction, ligand binding allows either cations or anions to pass through the post-synaptic cell membrane and induce or inhibit muscle contraction, respectively. The pLGIC subunit family arose before eukaryotes diverged from prokaryotes, then expanded to a family that binds a diverse repertoire of ligands. This expansion occurred through gene duplication followed by functional divergence, a process constrained by the mechanisms of subunit assembly and receptor processing. Therefore, details of this evolutionary process will reveal features of these underlying mechanisms.

Subunit gene duplication initially creates identical gene copies, whose respective protein products may co-assemble into one receptor. Eventually, the genes may evolve to produce two distinct subunits that do not interact, producing separate, distinct homomeric receptors. If this has occurred recently, then minimal sequence change should allow identification of the mechanisms responsible.

This study investigates two paralogous subunits, Cel-ACR-20 and Cel-ACR-23, from the free-living nematode, *Caenorhabditis elegans*. Both are the products of gene duplications within the *Caenorhabitis* group, some 100 MYA, from their common ancestor, MPTL-1. These subunits form homomeric betaine receptors, providing a model to examine the possible evolution of mutual exclusion. This may be through physical exclusion, or spatial or temporal separation of gene expression. The first of these is addressed in this thesis.

Codon substitution rate analysis provided evidence that selection pressure was different for MPTL-1 and its two descendants, consistent with a change in function. Reconstitution of receptors in *Xenopus* oocytes and two-electrode voltage clamp electrophysiology found no evidence for the formation of a heteromer between Cel-ACR-20 and Cel-ACR-23. A more sensitive test was implemented, using the ability of truncated, non-functional versions of each subunit to incorporate into and inhibit the formation of functional receptors. Inhibition of the cognate receptor and failure to inhibit a receptor formed from the other subunit suggests that the protein sequence from the signal peptide to the end of the second transmembrane region has adapted to prevent binding of the two subunits to form a heteromer.

This work establishes a model system with which the specific mechanism that excludes two subunits from co-assembling may be determined in future. Since pLGICs are an important anthelmintic drug target, understanding the regulation of subunit oligomerization can strengthen our current understanding of how anthelminthic targets may change in parasitic nematodes and model organisms.

Abrégé

Récepteurs ionotropes pentamériques (pLGIC) sont des récepteurs de neurotransmetteurs qui assurent une transmission synaptique rapide et une contraction musculaire chez les animaux. A la jonction neuromusculaire, la liaison des ligands permet aux cations ou aux anions de passer à travers la membrane cellulaire post-synaptique et d'induire ou d'inhiber la contraction musculaire. D'un point de vu évolutif, la famille des sous-unités pLGIC est apparue avant que les eucaryotes ne divergent des procaryotes, puis s'est étendue à une famille qui lie un répertoire varié de ligands. Cette expansion s'est produite par la duplication de gènes suivie d'une divergence fonctionnelle, un processus contraint par des mécanismes d'assemblage des sous-unités et de traitement des récepteurs. Par conséquent, les détails de ce processus évolutif révéleront les caractéristiques de ces mécanismes sous-jacents.

La duplication du gène de la sous-unité crée initialement des copies de gènes identiques, dont les produits protéiques respectifs peuvent s'assembler en un seul récepteur. Finalement, les gènes peuvent évoluer pour produire deux sous-unités distinctes qui n'interagissent pas, produisant des récepteurs homomériques séparés et distincts. Si ce phénomène s'est produit récemment, une modification minimale de la séquence devrait permettre d'identifier les mécanismes responsables.Cette étude porte sur deux sous-unités paralogues, Cel-ACR-20 et Cel-ACR-23, du nématode vivant de façon autonome, *Caenorhabditis elegans*. Toutes deux sont les produits de duplications de gènes au sein du groupe *Caenorhabditis*, qui se sont produit il y a environ 100 millions d'années, les séparant de leur ancêtre commun, MPTL-1. Ces sous-unités forment des récepteurs homomériques activés par la bétaïne, fournissant un modèle pour examiner l'évolution possible de l'exclusion mutuelle. Cela peut se faire par l'exclusion physique, ou par la séparation spatiale ou temporelle de l'expression des gènes. La première de ces sous-unités est abordée dans cette thèse.

L'analyse du taux de substitution des codons a fourni la preuve que la pression de la sélection était différente pour la MPTL-1 et ses deux descendants, ce qui correspond à un changement de fonction. La reconstitution des récepteurs dans les ovocytes de *Xenopus* et l'étude électrophysiologique en potentiel imposé deux électrodes n'ont pas réussi à fournir la preuve de la formation d'un hétéromère entre Cel-ACR-20 et Cel-ACR-23. Un test plus sensible a été mis en place, utilisant la capacité des versions tronquées et non fonctionnelles de chaque sous-unité à s'incorporer dans les récepteurs fonctionnels et à en inhiber la formation. L'inhibition du récepteur apparenté et l'échec de l'inhibition d'un récepteur formé à partir de l'autre sous-unité suggèrent que la séquence protéique du peptide signal à l'extrémité de la seconde région transmembranaire s'est adaptée pour empêcher la liaison des deux sous-unités pour former un hétéromère.

Ce travail a établit un modèle de système avec lequel le mécanisme spécifique qui exclut deux sous-unités du co-assemblage peut être déterminé. Puisque les pLGICs sont une cible importante des médicaments anthelminthiques, la compréhension de la régulation de l'oligomérisation des sous-unités aide à renforcer notre compréhension actuelle de la façon dont les cibles anthelminthiques peuvent changer chez les nématodes parasitaires et l'organisme modèle de laboratoire vivant en autonomie.

1- Introduction

1.1 Rational

Pentameric ligand-gated ion channels (pLGICs) are ubiquitous metazoan structures that play a critical role in muscle contraction and motility, and are common anthelmintic drug targets (Berridge, 2014). Knowledge of their structure and function has led to a detailed understanding of the action of a variety of pharmacologically active drug classes, including antidepressants, anesthetics and anthelmintics (Dwyer et al., 2014).

While the structural organization and coordinated motion of pLGIC subunits during channel gating is well characterized, the mechanisms that regulate which subunits co-assemble and produce functional receptors remains unclear. Different configurations of pLGIC subunits can alter the biochemical activity of the overall ion channel, which can be useful for organisms that need to make rapid, nuanced adaptations in response to changing environmental conditions or somatic structures at different life cycle stages. In fact, receptor composition has been found to differ between even closely related nematodes (Boulin et al., 2008, 2011, Buxton et al., 2014).

Novel subunit types arise by gene duplication followed by adaptation. How this occurs can reveal details of the mechanisms regulating receptor assembly and function. Investigation of recent duplication of the *unc-29* gene in the trichostrongylid nematode *Haemonchus contortus* revealed that ligand binding and sensitivity to a variety of ligands changed little, while a major change occurred in the interaction between UNC-29 and other subunits in the levamisole sensitive acetylcholine receptor (L-AChR) (Duguet et al., 2016).

Ideally, a simpler system could provide a model to investigate details of such subunit interaction, such as the scenario where a single gene that encodes a homomeric receptor has duplicated to produce two subunits that each encode a separate homomeric receptor. One possible example can be found in the homomeric MPTL-1 betaine receptor that confers sensitivity to monepantel in parasitic nematodes. This gene has duplicated within the *Caenorhaditis* group of nematodes to produce the homomeric receptors ACR-20 and ACR-23 (Peden et al., 2013, Rufener et al., 2013, Baur et al., 2015). The correspondence between resistance to monepantel and loss of each gene suggests the target receptor is not heteromeric. One possibility, that is investigated in this thesis, is that an intrinsic incompatibility has evolved between ACR-20 and ACR-23 to prevent formation of a heteromer.

The theory of how gene duplication leads to novel functions is relatively well understood (Ohno, 1970, Taylor & Raes, 2004). Typically, the functional adaptation of new genes occurs against a

relatively stable background of other cellular functions. However, in the case of pLGIC proteins that co-assemble, evolution acts additionally on the interactions between duplicate gene copies. This aspect of gene duplicate evolution is remains understudied. The goal for characterization of subunit interaction between ACR-20 and ACR-23 is to establish a model system that could later be used to investigate details of this evolutionary process.

1.2 Background

1.2.1 Neurotransmission

Neurotransmission is fundamental for animal life, mediating both central nervous system activity and muscular movement. Neurotransmitters act as ligands at chemical synapses for specific post-synaptic receptors that are activated upon ligand binding. When a presynaptic neuron is excited, it fuses vesicles containing neurotransmitter with the surface membrane and delivers them into the synaptic cleft. The neurotransmitter then binds to receptors on the postsynaptic cell that can be a neuron or muscle cell.

There are two main classes of receptors responsible for neurotransmission, metabotropic receptors and ionotropic receptors (Voglis & Tavernarakis, 2006). Metabotropic receptors, also known as G-protein-coupled receptors, result in slow chemical neurotransmisison via signal transduction cascades, whereby neutotransmitter binding amplifies chemical signals in a series of indirect, downstream, sequential reactions (Voglis & Tavernarakis, 2006). An example of this is found in serotonin receptors that mediate both excitatory and inhibitory neurotransmission on other neurons.

Pentameric ligand-gated ion channnels (pLGIC) are ionotropic receptors responsible for fast acting neurotransmission (Voglis & Tavernarakis, 2006). This involves a ligand binding directly to its binding site on the extracellular side of its specific receptor, causing a conformational change in the receptor that allows ion flow across the cell membrane. This changes the membrane potential of the cell, and can result in polarization, depolarization, or hyperpolariation of the post-synaptic cell, propagating or blocking an electrical signal for a variety of downstream events. Motile organisms manage patterns of excitatory and inhibitory membrane potential changes to control muscular motion in a rapid and coordinated manner (Wolstenholme, 2011). Every animal that moves does so because of fast synaptic transmission. This makes ligand-gated ion channels both ubiquitous and highly conserved structures.

Random drug screens of anthelmintic compounds show that the most common targets are pLGIC neurotransmitter receptors belonging to the nicotinic acetylcholine superfamily (Greenberg, 2014). These nicotinic acetylcholine receptors (nAChR) mediate fast synaptic transmission and muscle

contraction in animals, and even some prokaryotes (Smart & Paoletti, 2012, Courtot et al., 2015). Ligand binding allows either cations or anions to pass through the post-synaptic cell membrane and induce, or inhibit, muscle contraction, respectively. For example, in nematodes, acetylcholine receptors are permeable to cations and cause neuronal excitation, while GABA and glutamate receptors are permeable to anions and cause inhibition to mediate cell excitability (Smart & Paoletti, 2012).

1.2.2 The Origin of pLGIC

The specific origins of pLGIC are unknown but likely predate the split between eukaryotes and prokaryotes (Tasneem et al., 2004). Homologs of metazoan pLGICs have been found in prokaryotic species, suggesting that rudimentary pLGIC structures were initially acquired through lateral gene transfer from prokaryotic species. Since then, a wide diversity of pLGIC have evolved as the result of gene duplication events followed by functional divergence.

More than 550 million years ago, the Cambrian Explosion gave rise to the enormous complexity of the metazoans (Erwin, 2015). As animals began to inhabit different and more complex environments, demands for new body structures and complex, coordinated movement guided their evolution. To accommodate the increasing complexity of somatic structures involved in motility, pLGIC evolved to form many kinds of receptors that respond to different activating ligands (Pless & Sivilotti, 2018). While pLGIC are highly conserved structures, any change in their binding affinity for other subunits or ligand specificity can cause dramatic changes in neurotransmission (Jaiteh et al., 2016, Pless & Sivilotti, 2018). Understanding more about the mechanisms involved in functional divergence following a gene duplication event can tell us more about how subunits interactions evolve and what determines receptor composition.

1.2.3 Nematodes

Nematodes are well suited for use as lab models, as they have many complete genome sequences published, transparent bodies allowing internal structures to be visualized, a diversity of pLGICs, and even have complete connectomes mapped (White et al., 1986, Blaxter, 2011). This also means the *acr-20*, and *acr-23* receptors used as a model for this study have been characterized (Peden et al., 2013, Rufener et al., 2013, Baur et al., 2015).

The phylum Nematoda has a high degree of genetic diversity as well, and a large repertoire of duplicated genes on which dynamic and ongoing evolution is acting. Nematoda includes both free-living and parasitic species, although both the high rate of gene duplication events and the adaptability

the phylum represents lends towards parasitic adaptations and the acquisition of new hosts or niches (Blaxter et al., 1998).

The Helminth Genome Initiative (HGI) is a global community of researchers working to provide high quality, annotated genome data for parasitic nematodes causing infection (Howe et al., 2016, International Helminth Genomes Consortium, 2019). Through this project, genomes for more than 80 different nematode species from all five major nematode clades are publicly available in repositories such as WormBase ParaSite, which seeks to compile all available genomic data on parasitic worm species (Howe et al., 2016, International Helminth Genomes Consortium, 2019). Since many sequences for *mptl-1*, *acr-20* and *acr-23* were required to do the bioinformatic analysis for this project, this database provided the data required for the bioinformatics analysis in this project.

1.3 Hypothesis

A recently duplicated homomeric subunit gene will be identical to the original copy, unless there is a mutation associated with the duplication event. Both genes will therefore have the potential to combine into a heteromeric receptor. Over time, the subunits will diverge in sequence and may acquire different functional characteristics. Paralogous subunits that ultimately produce homomeric receptors may have acquired some mechanism to inhibit the formation of heteromers. Since exclusively homomeric receptors are observed in nature, such as ACR-16 in *C. elegans* (Charvet et al., 2018), some mechanism of exclusion must have evolved to prevent the formation of heteromers. Therefore, this project hypothesizes there exists a mechanism that evolved to prevent certain homomeric subunits from co-assembling into a heteromeric receptor in vivo. This could be an intrinsic incompatibility, a separation in space or in time, or any combination of these. In the case of the ACR-20 and ACR-23 subunits from *C. elegans*, I hypothesize that they have evolved some physical incompatibility to reduce the formation of a heteromeric receptor.

1.4 Specific Aims

This project implemented both *in silico* and wet lab work. First, evidence for functional change in *acr-20* and *acr-23* was explored by detecting differences in evolutionary pressure using analysis of codon substitution rates. Reconstitution of ACR-20 and ACR-23 functional homomers and a baseline functional profile for these receptors was established and concentration-response curves for betaine were determined to verify the maximum response and EC_{50} values for each homomer.

Evidence for any functional heteromer between ACR-20 and ACR-23 was evaluated from the concentration-response curve for betaine when co-injecting their respective RNA into the same oocyte.

Non-functional, truncated subunits were designed that interrupt normal receptor function, presumably by integration into the receptor. Interaction between the truncated and full-length versions of each subunit was demonstrated, but not so for each subunit against the other, consistent with an evolved exclusion mechanism.

2- Literature Review

The goal of this thesis is to investigate a specific feature of a proposed model of the evolution of homomeric pentameric ligand-gated ion-channels, specifically that homomeric receptors that derive from a homomeric ancestor should display some mechanism that prevents formation of heteromeric receptors. The following literature review provides background information to understand the process and consequences of gene duplication, the general structure and function of the receptors used in the study, and the rationale of choosing the *acr-20*, and *acr-23* subunits specifically as the experimental model.

2.1 Gene Duplication

This study aims to understand the events immediately following a recent gene duplication event in ion channel subunits, and how new functions that arise in duplicate genes affect subunit interaction. Therefore, it is useful to understand what happens during a gene duplication event and how new functions emerge.

2.1.1 What is Gene Duplication?

Gene duplication is a random event that can happen during DNA replication, with vertical transmission leading to two identical copies of a gene within a genome where previously there was only a single copy (Lipinski et al., 2011). Duplications may range in size from parts of a single gene, for example *avr-14* and *unc-49* in the nematode *C. elegans,* up to many hundreds of genes or even an entire genome (Jones & Sattelle, 2008, Gout & Lynch, 2015).

Gene duplication is a common occurrence. According to observations on genomic datasets from eukaryotic species, gene duplicates arise at an average rate of roughly 1% per gene per million years (Lynch & Conery, 2000). In some organisms, such as *C. elegans*, gene duplication is common enough to make gene copy-number polymorphisms the dominant kind of mutation over nucleotide substitutions or deletions (Lipinski et al., 2011). The gene duplication rate in *C. elegans* is two orders of magnitude greater than its rate of spontaneous point mutations per nucleotide site, at 10^{-7} duplications per gene per generation (Lipinski et al., 2011). The frequency of gene copy-number polymorphisms is affected by the rate of spontaneous duplication as well as the probability of evolutionary pressures, such as natural selection, genetic drift, and various mutations, acting to preserve or eliminate the duplicated gene in the genome (Otto & Yong, 2002, Zhang, 2003, Lipinski et al., 2011).

Within a population, genomes carrying newly duplicate genes can increase and decrease in frequency at random unless the mutation is harmful, in which case it will be eventually lost (Kimura & Ohta, 1974, Lynch & Conery, 2000). A duplication is said to become fixed when all individuals within the population carry the new variant.

This study focuses on the fate of individual genes that have undergone a recent gene duplication event and are fixed, or are in the process of becoming fixed, in *C. elegans* as a distinct and measurable target. This allows for the analysis of an acquired mechanism to prevent interaction between the two paralogous genes.

2.1.2 The Gene Duplication Event

Before a gene duplication event, a gene first exists in an individual organism, typically as one copy. During the replication process, the gene can be randomly duplicated. There are now two copies of that gene in the genome of the individual. What happens to this identical copy?

Most commonly, the duplicated gene is inactive and accumulates mutations that do not experience selective pressure (Lynch & Conery, 2000, Dittmar & Liberles, 2010). If one of the copies acquires an inactivating mutation it will ultimately be lost from the population with no consequence for the organism (Lynch, 2002).

In cases where both gene copies are retained there are different possible outcomes for the two genes. These include increased expression of the gene product from multiple genes, altered regulation

of one copy so they are expressed in different locations or at different times, division of existing functions between the two copies and the acquisition of new functionality.

Finally, and only very rarely, the duplicated gene can be retained in the organism's genome and accumulate mutations over time through its progeny (Hurles, 2004). Random genetic drift then causes each gene copy to drift apart in sequence similarity. Selection pressure acts on each gene copy individually to retain genes that confer neutral or positive traits, while removing organisms with deleterious mutations from the population (Lynch et al., 2001). Where there is a lack of purifying selection pressure, the duplicated gene is spread through the population so that most individuals have this gene, at which point the gene is determined to be fixed (Innan & Kondrashov, 2010).

2.1.3 Gene Duplication and Gene Dosage

Gene duplication can be understood as a recurring process that reaches an equilibrium, defined by the ratio between the rate of gene duplicate formation and deletion (Zhao et al., 2015). This dosage balance promotes the retention of interaction networks, as changes in gene product stoichiometry could alter downstream protein expression and assembly, potentially altering the fitness of the organism.

Scientists generally agree that an increase in genetic redundancy relaxes the constraints of selective pressure (Lynch & Conery, 2000, Keane et al., 2014, Rodrigo & Poyatos, 2016). Under conditions of neutral selection pressure, duplicated genes can be thought of as the medium evolution uses to give rise to novel structures and functions. Since shortcomings in gene expression for one duplicate can be compensated for by the other, far more neutral mutations can be tolerated and silently propagate at higher rates than any other type of mutation (Rodrigo & Poyatos, 2016). This allows the duplicated gene to silently spread through the population at random simply because the consequences of carrying an extra gene copy have no significant effect on survival. In this way, gene duplication events can be considered the medium evolution acts upon to give rise to novel structures and functions, the majority of which are nonsense mutations and are cleared from the population unnoticed (Katju, 2012). It is specifically because these duplicated genes are expendable that an organism can accommodate neutral mutations without killing off the population. This is where mutations leading to a change in protein function becomes possible.

2.1.4 Purifying Selection

Purifying selection remains present at all stages of a gene duplication event (Innan & Kondrashov, 2010). While genetic redundancy can shield duplicate genes from purifying selection, any change in sequence that has an adverse effect on existing structures and functions, or that takes too great a toll on the cellular resources, tend to be negatively selected against and eliminated from the population (Innan & Kondrashov, 2010).

2.1.5 Positive Selection

Positive selection favors the survival of organisms with mutations in their duplicate gene copies that allow for better adaptation to the environment. Positive selection favors mutations that improve the organism's ability to respond to its environment, such as a change in substrate specificity, acquisition of new somatic structures such as limbs, or resistance to drugs (Conant & Wolfe, 2008). Positive selection can also act on increased gene dosage, especially where the speed of a biological process is affected by levels of gene expression. An example is the *mdr1* gene, which encodes the drug efflux pump P-glycoprotein in the malaria parasite *Plasmodium falciparum* (Conant & Wolfe, 2008).

2.1.6 Pseudogenization

The most common outcome for duplicate genes is pseudogenization (Rastogi & Liberles, 2005). In this process, duplicate genes accumulate mutations that disrupt regular gene function, but are not yet been deleted from the genome (Rastogi & Liberles, 2005). These tend to be found in the larger genomes of more complex eukaryotic organisms which contain sections of non-coding regions of DNA, such an introns, that may not directly experience the same level of selection as exons, where genes are actively transcribed (Lynch & Marinov, 2015). The genome of the nematode *L. loa,* for example, contains genes that are in the process of being lost. An example is the *glc-2* gene, a glutamate-gated chloride channel that exists as a functional copy in its close relative, *B. malayi* (Beech & Neveu, 2015). Some would argue that pseudogenes are an interim stage of genes that are in the process of being deleted from the genome by stochastic processes (Lynch & Marinov, 2015).

2.1.7 Subfunctionalization

Subfunctionalization is a process similar to pseudogenization, but is achieved through the distribution of ancestral gene functions between its two duplicates (Rodrigo & Fares, 2018). Typically, each duplicate gene loses different subsets of the original function of the ancestral gene, thus dividing and distributing any number of the functions of the ancestral gene between them (Gout & Lynch, 2015). The more restricted but complementary set of sub-tasks allocated to each gene copy means both copies are required to maintain the functionality of the original gene (Lynch, 2002). This makes duplicate genes that have undergone subfunctionalization more likely to be maintained in the population (Lynch, 2002).

2.1.8 Co-option

Proteins tend to have additional, minor functions in addition to the primary function they evolved to perform, especially proteins with high levels of interactions such as enzymes and receptors (Conant & Wolfe, 2008). In the context of gene duplication, co-option allows for a seemingly new function of one gene copy, which was always present, to be appropriated to a primary role after the duplication event, while the other gene remains identical to the ancestral copy.

The most obvious example of co-option is the feather, which originally functioned in temperature homeostasis, then was later co-opted for flight (Conant & Wolfe, 2008). This is also true of various kinds of receptors that switch their substrate specificity or binding affinity, as seen in the diversity of the steroid hormone receptor family (Conant & Wolfe, 2008). Finally, co-option is also observed in cellular signaling systems (Chute et al., 2019). In *C. elegans*, for example, machinery from inter-cellular signaling was co-opted for inter-organismal signaling, allowing a nicotinic acetylcholine receptor to sense choline in order to function in inter-organismal communication pathways (Chute et al., 2019).

2.1.9 Neofunctionalization

The final and least likely outcome for a duplicated gene is the accumulation of mutations that lead to a new function (Ohno, 1970). Since the majority of mutations are deleterious or neutral, neofunctionalization is relatively uncommon (Ohno, 1970). Rarely, a duplicated gene can undergo mutations that bestow a novel and advantageous function and become fixed in a population (Lynch,

2002). This usually happens when the new function contributes an immediate adaptive value to the organism in which it occurs, or when the organism is only subject to neutral selection and is able accommodate an extra gene copy (Rodrigo & Fares, 2018). In comparison to the retention rate of subfunctionalized genes, genes encoding new functions are more likely to be fixed in the population as the population size increases (Lynch et al., 2001).

2.1.10 Fixation

Fixation occurs when the majority of organisms in the population carry the gene duplicate (Lynch et al., 2001). The fixation of a gene duplication event in a population often depends on the selective conditions provided by the environment. Many duplicated genes become fixed in the population by random genetic drift under neutral selection (Lynch and Conery, 2003). Positive selection tends to favor the fixation of duplicated genes where an increase in gene dosage is advantageous, or where a new function confers an adaptation that increases fitness (Rodrigo & Fares, 2018).

2.1.11 What Kinds of Genes Tend to Undergo Duplication Events?

While gene duplication events are ubiquitous, certain genes whose products have a high degree of molecular interactions, are evolving slowly, and are not considered a key component of the organism's suite of housekeeping genes seem to be favored for gene duplication events (Taylor & Raes, 2004, Conant & Wolfe, 2008). There are several postulated reasons for this. First, it is thought the rate of evolution affects the likelihood of a gene duplication event (Conant & Wolfe, 2008). Genes that are evolving slowly have been found to undergo gene duplications more frequently (Taylor & Raes, 2004, Conant & Wolfe, 2008). This does not seem to apply to essential housekeeping genes, however, which tend to be under high selection pressure and are quickly purged should a deleterious mutation arise (Conant & Wolfe, 2008). *C. elegans* genes have been found to have below average rates of evolution, which would relate to the numerous and rapid rates of gene duplication observed in this species, and in nematodes at large (Blaxter et al., 1998, Davis & Petrov, 2004, Conant & Wolfe, 2008). Genes whose products engage in a high degree of biochemical interactions with other molecules, such as enzymes, transcription factors, and ion channels, can more easily adapt novel functions, (Conant & Wolfe, 2008). This is reflected in the observed rates of gene duplication and functional classes of genes, which did not

seem to have a pattern, implying that the interactions between gene products was a more important feature (Conant & Wolfe, 2008).

2.1.12 Gene Duplication in pLGIC

Gene duplication events are fairly common in pLGIC, giving rise to the wide diversity of receptors we observe today (Han et al. 2019). This is, in part, due to the features of pLGIC that make them conducive to gene duplication events, particularly their slower rates of evolution and their complex networks of protein-protein interactions, which includes other subunits (Jaiteh et al., 2016, Pless & Sivilotti, 2018).

The majority of our understanding of the consequences and outcomes of gene duplication are drawn from examples where proteins interact individually with their biochemical environment, which is relatively unchanging. However, subunits that assemble into a pLGIC receptor can be understood as having the function of interacting with other subunits in their environment, resulting in different patterns of receptor oligomerization. A receptor can be homomeric if a single gene produces all five, identical subunits that associate to form a homomeric channel, or heteromeric if receptor subunits come from different genes that share a common ancestor (Bamber et al., 2003, Jaiteh et al., 2016). For example, *acr-16* encodes a homomeric receptor in *C. elegans*, whereas *acc-1* encodes an obligate heteromer in *H. contortus* (Touroutine et al., 2005, Callanan et al., 2018).

Changes in oligomerization can occur immediately following a gene duplication event. For example, in *C. elegans*, the subunits DEG-3 and DES-2 form a heteromer (Yassin et al., 2001). The MPTL-1 receptor is descended from DES-2, but forms a homomer (Rufener et al., 2013). Different organizations of subunits in the receptor affect the receptor's biochemical behavior. For example, UNC-49B and UNC-49C are GABA receptor subunits in *C. elegans* (Bamber et al., 2003). UNC-49B forms a homomeric GABA receptor, but can also co-assemble with UNC-49C to form a heteromeric receptor (Bamber et al., 2003). UNC-49B homomers were shown to be more sensitive to picrotoxin than UNC-49B/C heteromers (Bamber et al., 2003). The switch between self-interaction and non-self interaction is an important distinction with implications for receptor pharmacology, and could reflect an ability of proteins to control self-interactions separately from interactions with different proteins.

There are different ways evolutionary pressure can act on duplicate pLGIC subunit genes to produce a diversity of ion channel receptors. One source of diversity is the stoichiometric arrangement

of subunits in the receptors they form, with the downstream effect of altering substrate specificity (Jaiteh et al., 2016). Take, for example, *Ode-unc-38*, *Ode-unc-29, Ode-unc-63* and *Ode-acr-8,* which are found in the nematode parasite *Oesophagostomum dentatum* and are homologues of the *C. elegans* levamisole muscle receptor genes (Buxton et al. 2014). The heteromeric channel *Ode(29-63-38)*, is most sensitive to acetylcholine, but upon incorporating ACR-8 into this receptor, the *Ode(29–63–38–8)* heteromer responds primarily to levamisole instead (Buxton et al. 2014). These subunits act differently in *C. elegans,* where they bind a fifth subunit, Cel-LEV-1 and result in a heteromeric receptor with no affinity for nicotine (Buxton et al. 2014). Instead, *C. elegans* seems to have evolved a separate homopentamer of ACR-16 subunits to form a nicotine-sensitive muscle receptor (Buxton et al. 2014). Clearly, the evolution of subunits affects receptors in terms of both their formation and their ligand-binding behaviors.

Strong purifying selection is pervasive for pLGICs, leading to their conserved sequence (Beech et al 2013, Duguet et al. 2016). Loss of orthologous pLGIC genes following gene duplication events is common in nematode species (Beech & Neveu, 2015). For example, the *glc-5* and *glc-6* genes, which are involved in glutamate signaling and sensitivity to ivermectin in *H. contortus,* share a common ancestor that predates the divergence between *H. contortus* and *C. elegans*, but these genes have been lost in the *C. elegans* lineage (Beech & Neveu, 2015). There is some speculation that divergence and purifying selection in pLGIC duplicates in nematodes could be driven, in part, by different evolutionary pressures acting on parasitic and free-living lifestyles (Boulin at al., 2011, Li et al., 2015). For example, *acr-13* and *acr-8* are thought to have arisen from a gene duplication event occurring after the divergence between strongyloidea and rhabditoidea (Boulin at al., 2011). ACR-13 is essential for the formation of the L-AChR in *C. elegans*, but is markedly absent from many related parasites, including *H. contortus*, *B. malayi* and *A. suum* (Boulin at al., 2011). These parasites instead form L-AChRs expressing different ratios of the *unc-29* and *unc-38* subunits (Boulin at al., 2011).

Positive selection in pLGIC is rare, but has been observed in selection for α9 and α10 cholinergic nicotinic receptor subunits, which assemble to form the receptor that mediates sensitive hearing in mammals (Lipovsek et al., 2012). However, there is growing evidence that functional divergence of acetylcholine receptors followed by positive selection, which is common in clade V parasitic nematodes, may be mediated by alteration of receptor assembly (Duguet et al., 2016). For example, multiple independent gene duplications were identified in the *unc-29* acetylcholine receptor subunit, and codon substitution rate analysis identified positive, directional selection acting on amino acid positions associated with subunit assembly (Duguet et al., 2016).

There is also evidence to suggest differences in the timing and location of expression of subunits could be driving the evolution of pLGIC subunits (Li et al., 2015). AChRs in *C. elegans* generally seem to be enriched during the embryonic stage and are suspected to play a role in larval development (Von Stetina et al., 2007, Li et al., 2015). In parasitic nematodes, AChRs seem to be developmentally regulated (Li et al., 2015). These observations are further validated by the observation that levamisole has a strong paralytic effect on numerous filarial nematode species at the microfilariae stage compared to the adult stage (Li et al., 2015). An example of spatial separation can be observed in the *acr-26* receptor, which was found to be expressed in *A. suum* in muscle cells in the head but not the body wall (Bennett et al., 2012, Li et al., 2015). *Bm1_48815*, an orthologue *acr-26* found in *B. malayi*, was found in body muscles of only male worms (Li et al., 2015). This may explain the observed trend of higher motility rates among male *B. malayi* worms compared to females (Li et al., 2015). Another example can be found in *unc-63* and *acr-16* subunits, which both contribute to levamisole-insensitive receptors for neuromuscular transmission in *C. elegans* (Touroutine et al., 2005). However, ACR-16 is enriched in body wall muscle cells and is a requirement for levamisole-insensitive nicotinic responses (Touroutine et al., 2005). This suggests ACR-16 subunits evolved specifically for locomotion and are differentially expressed in body wall muscle. The observed spatial and temporal separation of subunit expression could indicate that some receptors have evolved specific purposes for specific life stages.

2.2 nAChR Subunits

2.2.1 DEG-3 Subfamily

The multitude of studies on *C. elegans* have identified at least 29 nAChR subunit genes that can be divided into five groups based on sequence homology: DEG-3, ACR-16, ACR-8, UNC-38 and UNC-29 (Brown et al., 2006, Jones et al., 2007, Li et al., 2015). The subunits used in this study are all members of the DEG-3 subfamily. Genes in the DEG-3 subfamily appear to be nematode specific and are not found in mammals, making them ideal targets for anthelmintic compounds (Brown et al., 2006, Rufener et al., 2009, 2010). They are also characterized by their ability to respond to amino-acetonitrile derivatives (AADs), a new class of anthelmintic compounds found to be effective against drug-resistant nematodes, making this clade important to ongoing studies of anthelmintic resistance (Brown et al., 2006, Rufener et al., 2009, Rufener et al., 2010). *C. elegans* and *H. contortus* share many DEG-3 homologues in common, implying the mode of activation of nAChR signaling pathways may be conserved between these two species (Kaminsky et al., 2008, Rufener et al., 2009).

2.2.2 Subunit Structure

Each nAChR subunit is made up of an extracellular hydrophilic amino-terminal N-terminal domain of around 200 amino acids that is required for receptor assembly and ligand binding, four hydrophobic α-helical transmembrane (TM) domains of around 20 amino acids that form the ion-conducting pore, an intracellular loop between TM3 and TM4 in the cytoplasmic domain involved in modulating channel activity and ion conductance, and a small extracellular carboxyl-terminal (Corringer et al., 2000, Brown et al., 2006, Choudhary et al., 2020). TM1-TM3 are separated by short loops, and TM3 is connected by a large, variable intracellular domain to TM4 (Corringer et al., 2000). The TM2 of each subunit lines the pore of the channel, comprising a barrel through which ions are conducted (Corringer et al., 2000). The intracellular loop is typically between 100 and 270 amino acids, and plays an important role in receptor modulation, sorting, and trafficking (Corringer et al., 2000).

The majority of subunits are classified as either α or non-α (Corringer et al., 2000, Pless & Sivilotti, 2018, Choudhary et al. 2020). The α-subunits have two adjacent cysteines in the ligand binding C-loop of the extracellular N-terminal and are considered the site of action for binding receptor agonists and antagonists (Corringer et al., 2000). The non-α-subunits β, γ, δ, and ε are missing these cysteine residues (Corringer et al., 2000, Choudhary et al. 2020). Non-α-subunits form the complementary face of the binding site, and are not known to bind ligands on their own (Corringer et al., 2000, Changeux, 2012). All the cation-selective nAChRs studied to date have been found to contain at least two α subunits (Choudhary et al. 2020).

A receptor can be homomeric if a single gene produces all five, identical subunits. If the receptor subunits come from different, homologous genes that share a common ancestor, a heteromeric receptor is formed. In homomeric receptors, all subunits are α-subunits and occupy both the principal and complementary faces of the ligand binding site, such as in the α9α10 nAChR receptor (Boffi et al. 2017). In heteromeric receptors, nonequivalent subunits create a diversity of binding sites composed of different subunit interfaces with different properties (Corringer et al., 2000).

We know from solved structures of heteromeric receptors that different subunits have evolved specifically defined positions within the receptor. For example, the main *C. elegans* L-AChR is composed of five different subunits encoded by the *unc-38*, *unc-63*, *lev-8*, *unc-29* and *lev-1*, all of which inhabit a specific position in the receptor (Charvet et al., 2018). By contrast, the main *C. elegans* N-AChR is homomeric, composed of five identical subunits encoded by the *acr-16* gene (Charvet et al., 2018). Some subunits are also known to exclusively form heteromers. For example, Hco-ACC-1 subunit does not form a functional homomeric channel, but requires Hco-ACC-2 to form a heteromer that is highly sensitive to acetylcholine and carbachol (Callanan et al., 2018). While a diversity of

oligomeric states contributes to the wide diversity of pLGIC, what remains unknown are the mechanisms that prevent certain subunits from assuming a place in the receptor.

2.2.3 nAChR Binding Sites

The nAChR binding site is formed by the interface between the extracellular domains of adjacent subunits, with a total of six noncontiguous regions labeled loops A–F (Boffi et al., 2017). The α-subunit contributes three, highly conserved loops (A–C) at the principal face, and the non-α-subunit contributes three loops (D–F) that have more sequence variability between subunits (Boffi et al., 2017). The α-subunit side of each binding site features four core aromatic residues that line the binding pocket, a critical component in the binding domain that interacts with the ligand and makes the α-subunit a requirement in producing an active binding site (Hansen & Taylor, 2007). Comparatively, interfaces between non-α-subunits are thought to be inactive or form an important site to accommodate modulatory ligands for allosteric modulation of the receptor (Hansen & Taylor, 2007, Taly et al., 2014). This means only sites containing the α-subunit principal face will actively bind agonist. The nAChR in *T. marmorata*, for example, only have two distinct binding sites composed of the α-δ and α-γ subunit interfaces (Boffi et al, 2017).

Ligand binding causes a conformational change in the receptor that propagates into the transmembrane domain, affecting steric changes in TM2 helices that cause the ion pore to open (Cheng et al. 2006). The most drastic change occurs in the C-loop of the α-subunit in the principal face of the binding site, which moves around 4 Å to close over the ligand-binding pocket and act as lid that effectively closes the ligand-binding pocket (Cheng et al. 2006, Hansen & Taylor, 2007). Steric changes in the complementary face also contribute to opening the ion pore, making the identity of the subunit contributing the complementary face important to the functioning and pharmacology of the ion channel (Cheng et al. 2006).

A considerable amount of receptor diversity comes from the arrangement and composition of its subunits (Corringer et al., 2000, Hansen &Taylor, 2007). For example, in *A. suum,* different combinations of UNC-29 and UNC-38 create heteromers with different pharmacological profiles depending on the stochiometry of their subunits (Williamson et al., 2009, Holden-Dye et al., 2013). When there are more UNC-29 than UNC-38, the receptor is considered L-type with levamisole and pyrantel as full agonists (Williamson et al., 2009, Holden-Dye et al., 2013). The converse ratio results in a receptor considered N-type with nicotine and oxantel as full agonists (Williamson et al., 2009, Holden-Dye et al., 2013). The variation between subunit composition and placement in the receptor affects its overall substrate affinity and binding kinetics, and therefore also its ability to propagate

neurotransmission signals (Corringer et al., 2000). This is the premise which allows this study to use differences between the pharmacological profiles of each receptor to detect the formation of a heteromer.

2.3 Experimental Model

We know from solved structures of heteromeric receptors that the different subunits have evolved specifically defined positions within the receptor (Unwin, 1995, Morales-Perez et al., 2016, Phulera et al., 2018). To explain the diversity we observe in ion channel receptors and the stochiometry of their subunits, there must be some mechanism that regulates the ability of subunits to interact with other subunits. Three different outcomes can result from gene duplication. First, gene duplication of a homomeric ancestral receptor may lead to the formation of two different homomeric receptors. This requires some mechanism to prevent association between the two new subunits, which could be separation in time, in space, or some biochemical exclusion of the two subunits. Second, gene duplication of a homomeric ancestor may give rise to a heteromeric receptor, and this would require some mechanism to specify subunit position while retaining their ability to interact. Third, gene duplication could lead to a change in the composition of a heteromeric receptor. In this case, the specific interactions that determine subunit position would have to change. The most simple experimental system would involve analysis of homomeric receptors and so this project will focus on the first scenario.

Nematodes are good model organisms for the study of gene duplication events, as the phylum Nematoda is one of the largest most diverse animal phyla due to a high rate of gene duplications (Mitreva et al., 2005, Blaxter, 2011, International Helminth Genomes Consortium, 2019). Nematodes have undergone fast expansion and functional divergence, giving an abundance of examples of recent gene duplication events (Mitreva et al., 2005, Duguet et al., 2016). Furthermore, a large amount of data sequenced, annotated, and verified Clade V Nematode genes are available through repositories such as NCBI thanks to the Helminth Genome Initiative (Howe et al., 2016, International Helminth Genomes Consortium, 2019).

C. elegans is a well-studied Clade V Nematode and laboratory model organism that has one of the largest nAChR gene families known for any organism (Brown et al., 2006, Howe et al., 2016). Following complete sequencing of its genome, most of its genes have been annotated and assigned functions that have been verified in the lab (Brown et al., 2006). Furthermore, many aspects of synaptic transmission and neural mapping in *C. elegans* have been characterized, allowing for this organism to be used as a model in understanding components of anthelmintic drug targets (Blaxter, 2011, Dwyer et

al., 2014). Finally, *C. elegans* also possesses one of the largest repertoires of AChR gene families of any organism that can be easily expressed in *Xenopus* oocytes (Brown et al., 2006, Blaxter, 2011, Duguet et al., 2016, Howe et al., 2016).

An excellent model to investigate what happens after a recent gene duplication event can be found in members of the DEG-3 subfamily that are targeted by monepantel (AAD-2225) (Baur et al., 2015). *Haemonchus contortus*, a species of parasitic nematodes closely related to *C. elegans*, possess *mptl-1*, a gene that encodes a homomeric betaine receptor regulating movement and whose loss results in monepantel resistance (Rufener et al., 2013, Peden et al., 2013, Baur et al., 2015). Within the *Caenorhabditis* clade, duplication of *mptl-1* has led to the paralogs, *acr-20* and *acr-23* (Peden et al., 2013, Baur et al., 2015). The major advantages of this model system are that MPTL-1, ACR-20 and ACR-23 receptors have been well characterized and have all been shown to function as homomeric betaine receptors (Rufener et al., 2013, Peden et al., 2013, Baur et al., 2015).

ACR-23 was discovered during random drug screening trials of *C. elegans* to identify AAD targets (Rufener et al., 2013). This screen led to the identification of 44 monepantel resistance mutant alleles, of which 27 alleles fell into one complementation class that caused disruptions in the *acr-23* gene (Rufener et al., 2013). These receptors have been localized in the body wall muscle and in mechanosensory neurons, and are believed to be important to worm locomotion (Rufener et al., 2013, Peden et al., 2013). ACR-20 is paralogous to ACR-23, but is more closely related in sequence to their shared ancestor, *mptl-1* (Baur et al., 2015). In *C. elegans*, MPTL resistance maps to mutations in *acr-23*, while mutations in *acr-20* do not lead to significant MPTL resistance (Baur et al., 2015). Interestingly, both MPTL-1 and ACR-20 channels are constitutively opened by monepantel (Baur et al., 2015). This could be explained by a decreased affinity of ACR-20 for monepantel compared to ACR-23, or the receptor may play a decreased physiological role that does not affect viability of the nematodes (Baur et al., 2015). Importantly for the work presented here, the fact that MPTL resistance is not affected equally by mutations of *acr-20* and *acr-23* means that the drug target receptor is not an obligate heteromer.

These channels respond primarily to betaine, a ubiquitous noncanonical amino acid which serves as an organic osmolyte to protect cells against osmotic stress (Baur et al., 2015). The mode of action of betaine toxicity is largely unknown, but is known to result in nematode death and is important in agricultural settings to help control parasite infection (Peden et al., 2013, Baur et al., 2015). A main focus of this thesis is to determine whether ACR-20 and ACR-23 can form a heteromeric receptor. It is important to note that the half maximal concentration, or EC_{50} value, for betaine on ACR-23 is 56 times

higher, at 1.4 mM (Penen et al., 2013), than ACR-20, at 25 μM (Baur et al., 2015). Since EC_{50} can be diagnostic for some receptors, this difference increases the possibility of detecting any distinct EC_{50} that might indicate formation of a heteromer.

2.4 Detecting Differences in Evolutionary Selection Pressure

Any differences in selection pressure due to a change in functional requirements may be revealed by a change in the non-synonymous/synonymous codon substitution rate. The branch length for *acr-23* appears greater than *acr*-20 in a phylogenetic tree (Baur et al., 2015) suggesting that these genes may have experienced differential selection pressure in their history. Phylogenetic analysis by maximum likelihood (PAML) compares maximum likelihood models of sequence evolution in a way that allows testing of specific model components. These include site specific substitution rates as well as evidence for varying substitution rates among specific branches of a phylogenetic tree (Yang, 2007). This method seems particularly appropriate to estimate any change in selection pressure and therefore possible changes in function for these genes.

The program CODEML considers codon triplets as the unit of evolution and compares the nonsynonymous/synonymous substitution rate ratio between each codon site. This ratio, or ω, measures the direction and magnitude of selection acting on different codon sites. $\omega < 1$ indicates negative or purifying selection, whereas $\omega > 1$ indicates positive selection changing the function of the protein, and $\omega = 1$ indicates neutral evolution (Yang, 2007). The smaller the value of ω, the stronger the purifying selection, and the larger, the stronger the positive selection.

3- Methods

3.1 Cloning

Gene sequences for *C. elegans acr-20* (R06A4.10) and *acr-23* (F59B1.9) were obtained from GenBank to design forward and reverse primers for each gene using the Primer3 plugin (Rozen and Skaletsky, 2000) of Geneious 10.2.6 (http://www.geneious.com) on Linux Ubuntu 18.04.5 LTS. The forward primer contained a terminal *Not*-I site and the reverse a terminal *Apa*-I site, shown in bold below. The *acr-20* and *acr-23* genes were amplified from *C. elegans* Bristol N2 cDNA (gift from J. D.

Noonan) with Q5® High-Fidelity DNA Polymerase (New England Biolabs) or DreamTaq (Thermofisher) respectively as described in their instructions.

Primers for *acr-20* were:

5'-**GCGGCCGC**GATATAGGAATTGATGTCGAGTGG-3'

5'-**GGGCCC**CCGCTCCGTAGACTATACTCTTAC-3'

Primers for *acr-23* were:

5'-**GCGGCCGC**ATGCACAGGATCTACACATTTTTGA-3'

5'-**GGGCCC**TCACATCAGAGCAGATCAATCGA-3'

All PCR amplicons were verified for amplification and size by electrophoresis through a 0.8% agarose/TBE gel and visualized with GreenGlo (Denville Inc.).

PCR amplicons were purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research). Fragments were ligated into the pGEM-T vector and transformed into DH5-α competent *E. coli* cells using the heatshock method according to the manufacturer's instructions (Promega Technical Manual, 2018). Purified plasmid DNA was prepared using the miniprep procedure (Bio Basic) and the cloned genes were confirmed by Sanger sequencing (Nanuq, Genome Quebec). A total of 88 clones of *acr-20* and 24 clones of *acr-23* were screened. Clones identical to the reference sequence were digested with both *Not*-I and *Apa*-I, and ligated into pTD2 (Duguet, 2017), a plasmid expression vector specialized for protein expression in *Xenopus* oocytes modified from pTB207 (Boulin et al., 2008). The pTD2 plasmid contains both the 5' and 3' UTR from *Xenopus* β-globin, which flank the cloning site, to increase stability of the cRNA *in vivo*.

Truncated subunits were made by designing a reverse primer with a terminal *Apa*-I site to bind immediately following the second transmembrane domain of both *acr-20* and *acr-23:*

5'-**GGGCCC**TCAAGATGACGTGGATGGCATTTTGTC-3'

This reverse primer also contains a stop codon terminating the protein immediately after the second transmembrane domain. Truncated subunits were cloned as described for the full-length genes.

3.2 cRNA Synthesis

pTD2 clones were linearized using *Nhe*-I for 12 hours and used as template for *in vitro* transcription using the the mMESSAGE mMACHINE T7 Transcription Kit (Ambion), according to the

manufacturer's instructions. *In vitro* transcription reactions ran for 6 – 12 hours, then the synthesized cRNA was DNase treated and precipitated with lithium chloride and dissolved in RNAse-free distilled water. The concentration and purity of the cRNA was established using a Nanodrop spectrophotometer (ThermoFisher Scientific) and the amplification and quality was verified by electrophoresis on a denaturing 0.8% agarose MOPS gel. Small aliquots of RNA were made from concentrated RNA stocks, diluted with nuclease-free water to 225 ng/µL for single injections, 500 ng/µL for double injections, and a range from 225 ng/µl to 2000 ng/µL for the different concentrations of co-injected truncated subunits.

3.3 Animals

All work involving *Xenopus leavis* frogs was carried out according to the approved Animal Use Protocols (AUP #2015-7758).

Oocytes were surgically removed from adult female *Xenopus* frogs under anesthesia by submerging in 0.15% tricaine methanesulfonate solution (MS-222) for 10 - 15 minutes, pH corrected to 7 using $NaHCO_3$. For terminal surgeries, 0.45% MS-222 was used for 2 hours. A small incision was made near the posterior end of the abdomen, and 1 – 2 mL of eggs were extracted using tweezers and stored in sterile calcium-free OR2 solution (80 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH to 7.3 with NaOH). Eggs were mechanically separated into groups of 8-10 and defolliculated using Collagenase Type II (Thermofischer) at a concentration of 0.2% (w/v) in calcium-free OR-2 and left to shake gently for 1 – 2 hours. Oocytes were then washed and stored at 18 °C in ND-96-penstrep (95 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM Na-HEPES, supplemented with 1M penicillin-streptomycin solution and 0.275 g/L pyruvate as an energy source) for at least 30 minutes before cRNA injections. All solutions were sterilized via vacuum filtration with a 0.2 µm PES filter.

3.4 Oocyte Injections

A total volume of 54 nL cRNA mixture was injected into each oocyte and remained constant. The number of ng of cRNA was controlled by preparing different dilutions of concentrated cRNA stock in RNAase-nuclease-free water. Stage V or VI oocytes were placed in a petri dish immersed in ND96 and injected with the Nanoject II system by Drummond Scientific Company. Needles were pulled from borosilicate glass capillaries (World Precision Instruments) and backfilled with light mineral oil to

create a plunger system to use with the cRNA mixtures. Two subunits were co-expressed by injecting 13 ng cRNA for each subunit, totaling 26 ng. Truncated subunit RNA was co-injected in addition to 13ng of whole subunit RNA at a 1:1 and 1:5 ratio of whole-subunit to truncated-subunit for both ACR-20 and ACR-23 homomers, and for ACR-20 ACR-23 co-injections, creating a total of eight different admixtures. This means a total of 26 ng RNA was injected for the 1:1 ratio, and 78 ng for the 1:5 ratio. Maximum betaine responses were then measured for 1.5 mM betaine on *acr-20* and 10 mM betaine for *acr-23*. After injection, oocytes were left to incubate at 18 °C for roughly 48 hours in ND-96-penstrep, and medium was changed every 24 hours.

3.5 Electrophysiology

Two-electrode voltage clamp electrophysiology was conducted using the Axoclamp 900A computer-controlled microelectrode amplifier and Axon Digidata 1550B from Axon Instruments. Electrodes were pulled from B150F-4 borosilicate glass capillaries (World Precision Instruments). The tip of each electrode was clipped with a fine pair of tweezers and filled with 3 M KCl before being positioned onto Ag|AgCl wires connected to headstages connected to the amplifier. The electrodes were checked for a resistance between 0.5 and 5 MΩ.

A RC3Z oocyte housing chamber (Harvard Apparatus) was connected to a gravity-fed perfusion system to deliver both the recording buffer and the ligand solutions. Recording buffer was Ringer's solution (100 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, HEPES 5 mM, pH to 7.3 with NaOH). The perfusion chamber was connected to a reference electrode through a 3 M KCl 1% agar bridge. Individual oocytes were placed into the inlet of a RC3Z chamber (Harvard Apparatus) filled with Ringer's solution and pierced by both electrodes. Voltage was clamped at -60 mV, and only oocytes that held a clamping current less than 1000 nA were used.

Ligand solutions were made using Ringer's solution as the solvent. A stock of 100 mM betaine in Ringer's solution was prepared and used to make concentrations of 15 mM, 10 mM, 1.5 mM, 1 mM, 100 μM, 10 μM 1 μM. Additional dilutions of betaine were made at 67.5 μM, 45 μM, and 22.5 μM for *acr-20*, and 675 μM, 450 μM, and 225 μM for *acr-23*. A stock of 10 mM acetylcholine and 10 mM nicotine were prepared and used to make a concentration of 100 μM for each ligand solution. Drug perfusion with different concentrations of drugs was performed for the duration of time it took for each oocyte response to plateau. In between recording responses to different drug doses, drugs were washed out of the perfusion chamber with the recording buffer, allowing the oocyte to return to their basal

current before the next treatment was administered. At least three oocytes from five different batches were examined for each condition.

3.6 Bioinformatics

A multiple sequence alignment was performed using sequences retrieved from the WBPS13 version of the WormBase ParaSite database (http://parasite.wormbase.org/). The sequences collected were *mptl-1* from all Clade III, IV, and V nematodes where this gene is found, and *acr-20* and *acr-23* from *C. elegans*. Sequences were then imported into Geneious v 10.2.6 for Ubuntu (https://www.geneious.com). Sequences from the signal peptide, the intracellular loop between TM3 and TM4 and the C-terminal region beyond TM4 were removed from the alignment. Any positions with ambiguity or gaps were also removed using the clean data option. A multiple sequence alignment based on codons was obtained using the MAFFT plugin (Katoh & Standley, 2013).

Phylogenetic trees were produced using the PhyML plugin for Geneious (Guindon et al., 2010). Codon substitution models were examined using CODEML from the PAML v4.6 software package (Yang, 2007) implemented on the Beluga Compute Canada cluster though Compute Canada (www.computecanada.ca). The likelihood of three nested models, with either one (M0), two (M3k2) or three (M3k3) classes of codon substitution rates were maximized (M0: run model = 0, NSSites = 0, ncatG = 0; M3k2: model = 0, NSSites = 3, ncatG = 2; M3k3: model = 0, NSSites = 3, ncatG = 3) were used to evaluate if each additional rate class explained the data significantly better. Branch models and clade models (MD) allowing one of the three rate classes to differ on specified branches of the phylogeny were optimized to evaluate evidence of different selection pressures acting on the three genes (MD: run model = 3, NSsites = 3, ncatG = 3). Since the likelihood distribution of model MD often has local maxima, the most likely model was determined starting with twelve initial substitution rates ranging from 0.001 to 10 (Yang, 2007). For these nested models, probability was determined from the likelihood ratio test using twice the difference in log likelihood values, which follows the χ^2 distribution (Yang, 2007).

The CODEML program tests branch models with different ω ratio parameters at different branches in the phylogenic tree to detect where natural selection is creating different rates of evolutionary change. This program first takes a set of user trees as input and then calculates their log likelihood values under a variety of branch models. The first model, M0, uses a constant value for ω at all points in the tree. The second, a two-ratio model, assumes specific branches have different ω values

from the rest of the tree, specifically the branches leading up *acr-20* and *acr-23*. The third, a free-ratio model, where ω is independent for each branch in the tree. In this study, the M0 model was used, as well as the M3K2 model, which assumes 2 substitution rate classes, and M3K3 model, which assumes 3 substitution rate classes. Model D uses a variable number of site classes, which is set by the user. In this study, Model D implements a 4^{th} and 5^{th} rate class. Where codon substitution rates differ, this is evidence of natural selection acting differently at different points in the phylogeny.

3.7 Data Analysis

Electrophysiology recordings were taken with the Clampex 9.2 software (Axon Instruments) and analyzed with Clampfit 9.2. All final recordings were filtered at 10 Hz before the amplitude of currents corresponding to drug responses were measured and recorded in Excel spreadsheets (Microscoft). GraphPad Prism 8.0 (GraphPad Software) was used to calculate concentration-response curves for betaine for each type of receptor by applying the Hill equation as described by Boulin et al. (2008). GraphPad Prism 8.0 was also used to perform one-way ANOVA.

4- Results and Discussion

The monepantel sensitive homomeric betaine receptors ACR-20 and ACR-23 in *C. elegans* are paralogous duplications of the ancestral homomeric MPTL-1 receptor (Peden et al., 2013, Rufener et al., 2013, Baur et al., 2015). Adaptation to new, independent functions as homomers would be disrupted if ACR-20 and ACR-23 could form a heteromeric receptor. It could therefore be expected that there would be increased selection pressure to prevent formation of a heteromeric receptor and evolution of some mechanism that causes *acr-20* and *acr-23* to exclusively form homomers. The work presented here aimed to verify if such a mechanism exists.

In silico work was done to provide evidence that a suitable model was chosen to investigate the hypothesis of this study. Functional change is associated with changes in selection pressure that may leave evidence in the protein sequence (Yang, 2007, Duguet et al., 2016). The sequences of *mptl-1*, *acr-20* and *acr-23* from a variety of species were examined to determine if codon substitution rates revealed evidence of functional change specific to *acr-20* and *acr-23*.

Homomeric ACR-20 and ACR-23 receptors were reconstituted in Xenopus oocytes to verify that their characteristics corresponded to those published previously (Peden et al., 2013, Rufener et al., 2013, Baur et al., 2015). The betaine dose-response curve was examined for evidence of a distinct EC_{50} that might indicate that a heteromeric receptor was formed when both subunits were co-expressed.

A more sensitive method for detecting subunit interaction through concentration dependent poisoning of receptor expression using non-functional, truncated versions of ACR-20 and ACR-23 was developed. Initial characterization experiments revealed a high degree of variability in oocyte response. An internal control based on an unrelated homomeric receptor, *Dme-acr-16*, was developed to minimize this variability. In addition, it was possible to document that expression of ACR-20 led to increased oocyte membrane permeability, possibly due to spontaneous opening of the ACR-20 receptor.

Ultimately, it was possible to show that truncated ACR-20 poisoned the ACR-20 receptor in a concentration dependent manner that was not observed with truncated ACR-23, and vice versa. The lack of evidence that the N-terminal domains of ACR-20 and ACR-23 interact suggests that these paralogous genes have evolved an exclusion mechanism.

4.1 Substitution Rate Analysis

The two genes *acr-20* and *acr-23* represent a duplication within the *Caenorhabditis* group of the *mptl-1* gene found in other nematodes. Comparison of the codon substitution rates between *acr-20*, *acr-23*, and *mptl-1* provides a way to compare how selection pressure might be acting differently on each of the genes. Evidence for different evolutionary rates may indicate a change in function of the protein.

The maximum likelihood models produced by PAML analysis are summarized in Table 1, based on the phylogeny shown in Figure 1. The nested model tests based on these data are shown in Table 2. Models with three different codon substitution rate classes explained the data significantly better than those with either one or two different classes. Models that included an additional rate class only for the branches immediately following the duplication event (branch A, B and both) or for all branches after the duplication (clade A, B and both) did not make significant improvements (Table 2).

Model	Rate Classes	lnL	np
M0	One codon rate class on all branches	-34309	107
M3K2	Two codon rate classes on all branches	-33278	109
M3K3	Three codon rate classes on all branches	-33061	111
MD 20-branch	Three codon rates plus Branch A	-33059	112
MD 23-branch	Three codon rates plus Branch B	-33059	112
MD 20&23-branch	Three codon rates plus Branches A and B	-33059	113
MD 20-clade	Three codon rates plus Clade 20	-33053	112
MD 23-clade	Three codon rates plus Clade 23	-33052	112
MD 20 & 23-clade	Three codon rates plus Clades 20 and 23	-33054	113

Table 1. PAML analysis results. Each model is listed in the first column (M0: run model = 0, NSSites = 0, ncatG = 0; M3k2: model = 0, NSSites = 3, ncatG = 2; M3k3: model = 0, NSSites = 3, ncatG = 3; MD: run model = 3, NSsites = 3, ncatG = 3). The relevant rate classes are given in the second column. The lnL column represents the log likelihood for each model, and the np column represents the number of parameters (np) for each model. M0 assumes one class of codon substitution rates, M3K2 assumes two classes, and M3K3 assumes three classes. MD models assume three classes of codon substitution rates and allow one of the three rate classes to differ on specified branches. Trees 01- 03 are branch models; trees 04 – 06 are clade models. 20-branch has a different rate class on the branch leading to *acr-20*, 23-branch has a different rate class on the branch leading to *acr-23*, and 20 & 23-branch includes a different rate class for each of these two branches. Trees 04 –06 were produced by the same logic, but each rate class extends to include all the branches for each gene's group of descendants together as a clade.

Six tree models were constructed with PAML. The model MD 20 & 23-clade was chosen as an example model because it exemplifies how both clade and branch models were constructed.

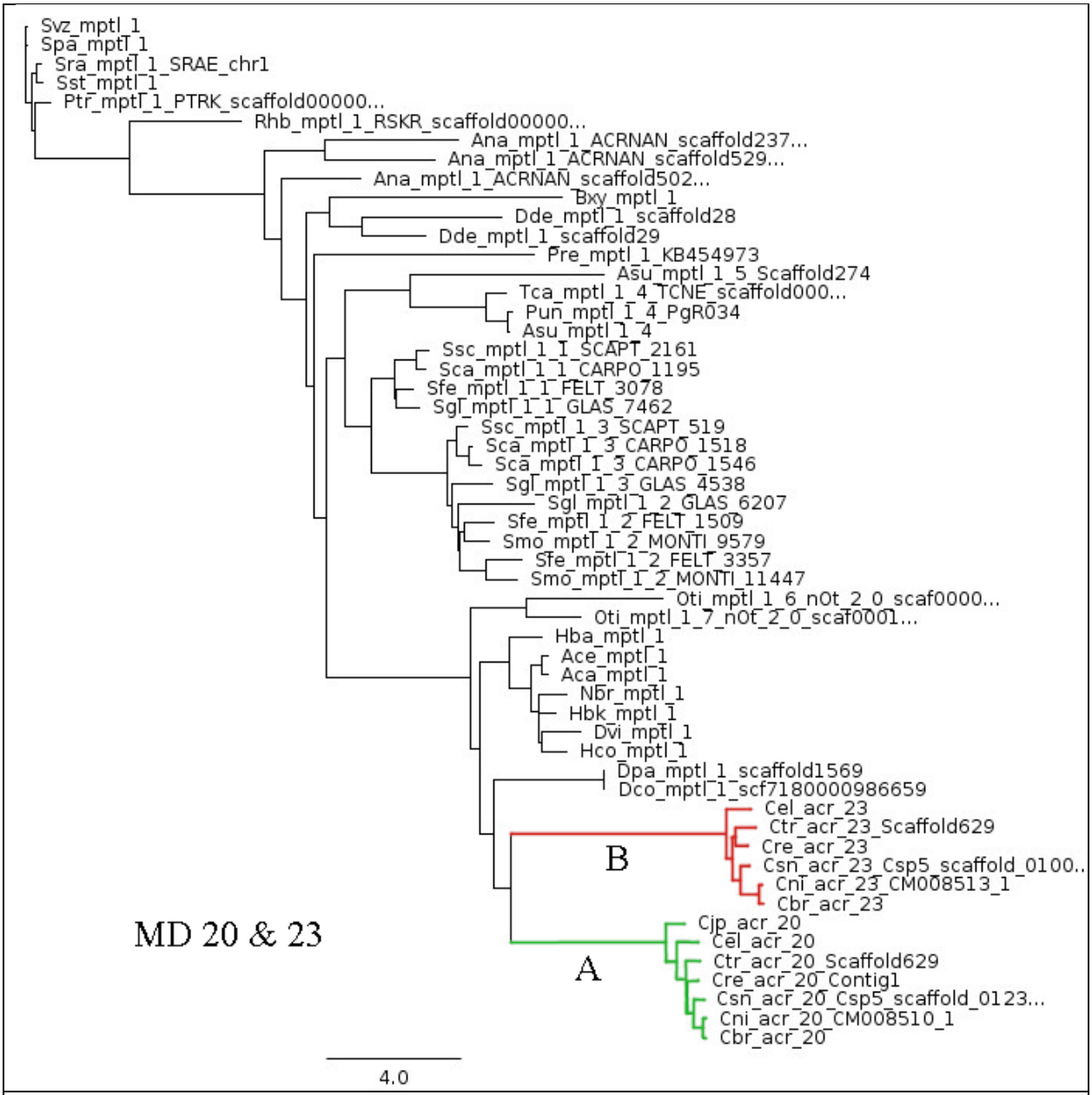

Figure 1. Phylogenetic tree showing both branch and clade models. Tree model MD 20 & 23-clade was chosen as a representative example for all models tested. The branch leading to *acr-20* is labeled A, the branch leading to *acr-23* is labeled B. *acr-20* and its descendants are labeled as a clade in green, and *acr-23* and its descendants are labeled as a clade in red. Black branches represent the background rate class. A scale bar is shown for reference, indicating branch lengths which represent the number of nucleotide substitutions per codon.

When a DNA sequence change alters an amino acid, a non-synonymous change, then that change is exposed to selection pressure. If this has little effect on survival of the organisms, the site should evolve at the same rate as synonymous changes that do not affect the protein sequence at all. If the change leads to a faulty protein that doesn't work well, selection pressure will remove those

changes, in which case we'd expect to observe a conserved sequence and the rate of non-synonymous change will be lower than the rate of synonymous change.

The rate classes depicted in Table 2 describe codon substitution rates (ω), and the proportion of amino acids in the protein (p) that are subject to that substitution rate. $\omega < 1$ indicates purifying selection, $\omega > 1$ indicates positive selection changing the function of the protein, and $\omega = 1$ indicates neutral evolution (Yang, 2007). The first column represents the largest proportion of amino acids in the protein and their corresponding ω value, and the last column represents the smallest proportion. A majority of the subunit experiences very strong purifying selection that agrees with the strong conservation of subunit structure (Lipovsek et al. 2012, Beech et al 2013, Duguet et al. 2016).

Model		Rate Classes		
M0 (One Class)	p	1.0		
	ω	0.02786		
M3k2 (Two Classes)	p	0.65041	0.34959	
	ω	0.00904	0.06989	
M3k3 (Three Classes)	p	0.40811	0.39576	0.19613
	ω	0.02587	0.00303	0.09844
MD 20-branch (branch model)	p	0.40335	0.40291	0.19408
	ω	0.00325	0.02627	0.09885
	ω_A	0.00065	0.02627	0.09885
MD 23-branch (branch model)	p	0.40786	0.39452	0.19762
	ω	0.02551	0.00298	0.09865
	ω_B	0.02551	0.00298	0.06231
MD 20 & 23-branch (branch model)	p	0.40335	0.40275	0.1939
	ω	0.00329	0.02628	0.09886
	ω_A	0.00065	0.02628	0.09886
	ω_B	0.00264	0.02628	0.09886
MD 20-clade (clade model)	p	0.40851	0.39651	0.19498
	ω	0.02701	0.00317	0.1066
	$\omega_{20clade}$	0.02701	0.00317	0.05622
MD 23-clade (clade model)	p	0.40689	0.39118	0.20193
	ω	0.02613	0.00304	0.10435
	$\omega_{23clade}$	0.02613	0.00304	0.05544
MD 20 & 23-clade (clade model)	p	0.41675	0.39271	0.19054
	ω	0.00377	0.02734	0.10066
	$\omega_{20clade}$	0.00032	0.02734	0.10066
	$\omega_{23clade}$	0.00264	0.02734	0.10066

Table 2. PAML tree model rate classes. The value p represents proportion of codons falling under the same class of codon substitution rate, ω represents the ratio of non-synonymous to synonymous substitutions (dN/dS), ω_A represents dN/dS specifically for the branch leading to *acr-20* (branch A), and ω_B represents dN/dS specifically for the branch leading to *acr-23* (branch B). Clade models are represented by $\omega_{20clade}$ and $\omega_{23clade}$ where the same dN/dS is

present on the branch leading to *acr-20* and all its descendants, and to *acr-23* and all its descendants, respectively.

Since the nested models created by PAML assume a chi-square distribution, the chi-squared test can be used to determine the best model (Yang, 2007). Significant improvements between models were determined by calculating the difference in their log likelihood values, multiplying the resultant by two, and looking up this value in a chi-squared distribution table using the corresponding degrees of freedom, which were calculated as the difference in np from Table 1 for each model (Yang, 2007). This allows a comparison of p-values to determine which of the models best fits the data.

Chi-Squared Test

Comparison	Outer	Nested	2x difference in lnL	df	p-value
M3k2 - M0	-33278	- 34309	2062	2	< 0.001
M3k3 - M3k2	-33061	- 33278	434	2	< 0.001
MD 20-branch - M3k3	-33059	- 33061	4	1	< 0.05
MD 23-branch - M3k3	-33059	- 33061	4	1	ns
MD 20 & 23-branch – MD 20-branch	-33059	- 33059	0	1	ns
MD 20 & 23-branch - MD 23-branch	-33059	- 33059	0	1	ns
MD 20 & 23-branch - M3k3	-33059	- 33061	4	2	ns
MD 20-clade - M3k3	-33053	-33061	16	1	< 0.001
MD 23 clade - M3k3	-33052	-33061	18	1	< 0.001
MD20 & 23-clade – MD 20-clade	-33054	-33053	-2	1	ns
MD 20 & 23-clade – MD 23-clade	-33054	-33052	-4	1	ns
MD 20 & 23-clade - M3k3	-33054	-33061	14	2	< 0.001

Table 3. Chi-Squared Test on tree model comparisons. Each comparison between models was made by taking the twice the difference between the log likelihood values of the models being compared and taking the difference in degrees of freedom (df) between the nested model and the outer model. Degrees of freedom represent the number of parameters for each model, which includes the lengths of each branch on the phylogenetic tree, the transition to transversion ratio, nucleotide frequencies, codon frequencies, and different rate classes of codon change. P-values were calculated by looking up the resulting values on the $\chi 2$ distribution table. A level of $p < 0.005$ was considered significant.

The first three comparisons between nested models M0, M3K2, and M3K3 showed that the two codon rate class model was significantly better than the one rate model, and three rate classes were better than two.

Comparisons between branch models showed that having a different rate on the branch leading to *acr-20* compared to the rest of the tree provided a significant improvement to the model. This was not true when only the branch leading to *acr-23* had a different rate. Comparing the 20-branch model to the 20 & 23-branch model showed no evidence that the branch leading to *acr-23* had a different rate when *acr-20* already had a different rate. The same held true when comparing the 23-branch model to the 20 & 23-branch model. Interestingly, having different rates for both *acr-20* and *acr-23* was not an improvement over the three-rate model (M3K3). The results for these branch models imply that only *acr-20* has a significantly different rate of codon substitution compared to the rest of the tree. This suggests a functional change occurred in *acr-20* after the gene duplication event that led to *acr-20* and *acr-23*.

Comparisons between clade models showed the same pattern but included both *acr-20* and *acr-23*, suggesting both the *acr-20* and *acr-23* clades have different rates of codon substitution from the rest of the tree, and to each other. This is consistent with a functional switch at the base of each clade, after which strongly purifying selection pressure resumed to maintain this new function.

An interesting result was found when comparing the model with different rates leading to both *acr-20* and *acr-23* clades to models with a different rate to only *acr-20* or only *acr-23*. A negative value was returned for these model comparisons, suggesting a model where functional change occurred for both *acr-20* and *acr-23* is worse. However, since a different rate for the *acr-20* and *acr-23* clades were separately an improvement over the three-rate model, we'd expect a model that includes different rates for each of these genes to also be an improvement. A possible explanation for this observation is the PAML algorithm found a local and not a global maximum for the 20 & 23-clade model. PAML utilizes different ω values at the starting point of the search specifically to avoid this problem. The results of this analysis, then, could mean the likelihood surface for this model is more complex than this analysis was able to capture.

Overall, this bioinformatics analysis suggests there may have been a functional change associated with the duplication of *mptl-1* to form *acr-20* and *acr-23*. The change in function could be affecting each subunit's binding behavior, pointing towards evidence for the evolution of an exclusion mechanism so that neither subunit can bind to the other. Furthermore, rates of codon substitution are most likely different between *acr-20* and *acr-23*, which suggests evolutionary divergence between these gene duplication products.

4.2. Electrophysiology

Cel-acr-20, *Cel-acr-23*, and *mptl-1* have been characterized in the literature as homomeric betaine receptors (Peden et al., 2013, Rufener et al., 2013, Baur et al. 2015). Resistance to monepantel is caused by knockout mutations in *acr-23* (Peden et al., 2013, Rufener et al., 2013). There is no evidence so far that loss of *acr-20* leads to resistance (Baur et al. 2015). This observation suggests *acr-20* and *acr-23* do not form a heteromeric receptor *in vivo*. In the following electrophysiology experiments, these receptors were reconstituted to confirm homomeric assembly in *Xenopus* oocytes. The concentration-response curve of the homomeric receptors compared to the curve obtained when both are co-injected failed to confirm or reject formation of a heteromer. Co-expression with a non-functional, truncated version of each subunit demonstrated a physical interaction between each subunit and its truncated version, but not with the other subunit.

4.2.1 Receptor Expression and EC_{50} Calculations

Concentration-response curves for each injection scheme are shown in Figure 2. Homomeric channels were successfully reconstituted and were fully responsive to betaine. The EC_{50} value for betaine with *acr-20* was 44.21 +/- 22.79 μM, with a Hill coefficient of 0.86, and for *acr-23* was 1.16 +/- 0.6 mM, with a Hill coefficient of 1.16. These EC_{50} values are consistent with the published values of 25 +/- 7 μM betaine for *acr-20* with a Hill coefficient of 1.3 (Baur et al. 2015) and 1.4 +/- 0.1 mM betaine for *acr-23* with a Hill coefficient of 1.2 (Peden et al., 2013). The concentration-response curves also revealed the concentration of betaine producing a maximum response. For *acr-20* this was 1.5 mM betaine, and for *acr-23* this was 10 mM betaine. This confirms the subunits cloned here correspond to those published previously.

The human alpha4/beta2 receptor is found in two different stoichiometries with a difference in EC_{50} values of about 100-fold (Zhou et al., 2003). When both receptors are present, the dose response curve shows evidence for two distinct EC_{50} values (Zhou et al., 2003). The response to betaine for oocytes co-injected with both *acr-20* and *acr-23* did not show evidence for a biphasic curve, with an EC_{50} of 224.4 +/- 84.9 μM, and Hill coefficient of 0.86. This value falls between the EC_{50} values for the two homomers. This could reflect that a single heteromeric receptor was produced, or that this method was not sufficiently sensitive to detect one.

A data set to represent a hypothetical mixture of homomers was created by combining the data for homomeric *acr-20* and *acr-23* and plotting these as a single dataset in Figure 2. This yields a curve with an EC_{50} value of 253.4 +/- 6.2 mM, with a Hill coefficient of 0.54. These values are not significantly different from the response when *acr-20* and *acr-23* were co-injected into oocytes. Although this does not rule out the possibility that heteromeric receptors were formed, the data provides no evidence for this.

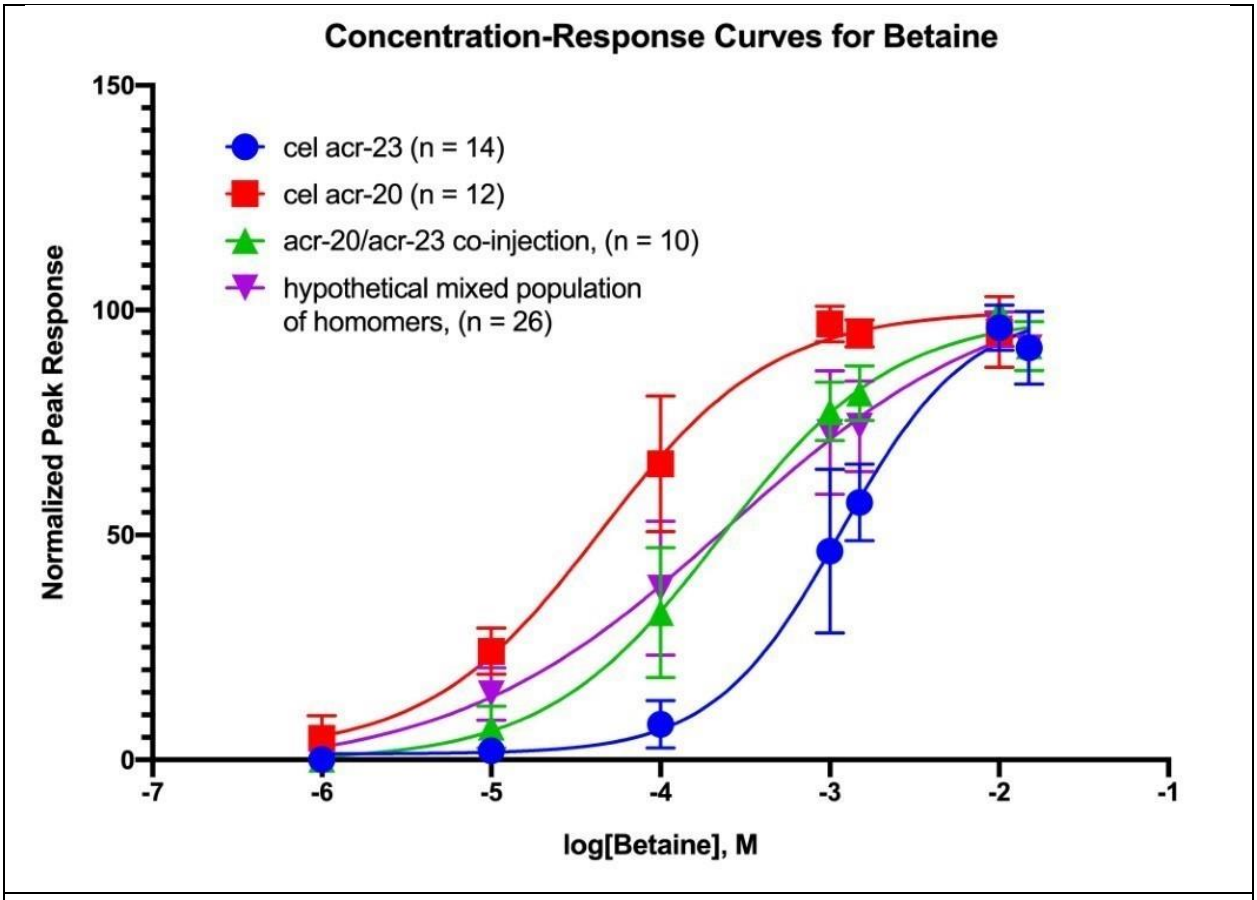

Figure 2. Concentration-response curves for betaine. EC50 curves for three different conditions: *Cel-acr-20* alone, *Cel-acr-23* alone, both combined and a fourth, artificial dataset combining the data from homomers. Error bars represent mean ± 95% confidence interval. 15 mM, 10 mM, 1.5 mM, 1 mM, 100 µM, 10 µM, and 1 µM of betaine were tested per oocyte. Three applications per concentration of betaine were tested per oocyte, and their average was calculated to represent that oocyte's response.

These EC_{50} calculations served to confirm proper assembly of homomeric channels. While evidence of a functional heteromer was inconclusive, this experiment was still useful in proving the experimental system was working properly.

4.2.2 Poisoning Receptor Function with Truncated Subunits

The use of concentration-response curves to detect the presence of heteromers was not sufficiently sensitive to detect heteromer formation. Therefore, a more sensitive test was devised to utilize protein-protein interactions between subunits to look for evidence of heteromer formation. The truncated version of each subunit inactivates the receptor it assumes a place in as a dominant effect. Since subunits are synthesized and assembled into receptors in the ER, every receptor is equally likely to incorporate these truncated subunits. If heteromers are being formed by co-injecting full length and truncated subunit RNA of opposite genes, we would expect to see a reduction in the total number of receptors produced, and therefore a reduction of maximal current, that is directly proportional to the amount of truncated cRNA injected (Boulin et al., 2011).

Oocytes injected with the truncated subunits alone did not respond, confirming they could not produce a functional receptor, as shown in Figure 3. Truncated subunit RNA was co-injected into *Xenopus* oocytes at ratios of 1:1 and 1:5 of full length to truncated subunits. Maximum betaine responses were then measured for 1.5 mM betaine on *acr-20* and 10 mM betaine for *acr-23.* A poisoning effect of receptor function was confirmed for truncated ACR-20 on full length ACR-20 and truncated ACR-23 on full length ACR-23. The maximal betaine response decreased with increasing truncated cRNA injected. This confirmed that the truncated subunits were able to poison receptors from full length subunits, presumably through physical interaction and co-assembly into a non-functional receptor.

The addition of truncated subunits to the full-length version of the same subunit showed a dose-dependent decrease in the maximal current for both ACR-20 and ACR-23 homomers. This provides evidence for the proper expression and assembly of truncated subunits into the receptor, and in doing so, their ability to sequester functional, full length subunit proteins in non-functional receptor complexes.

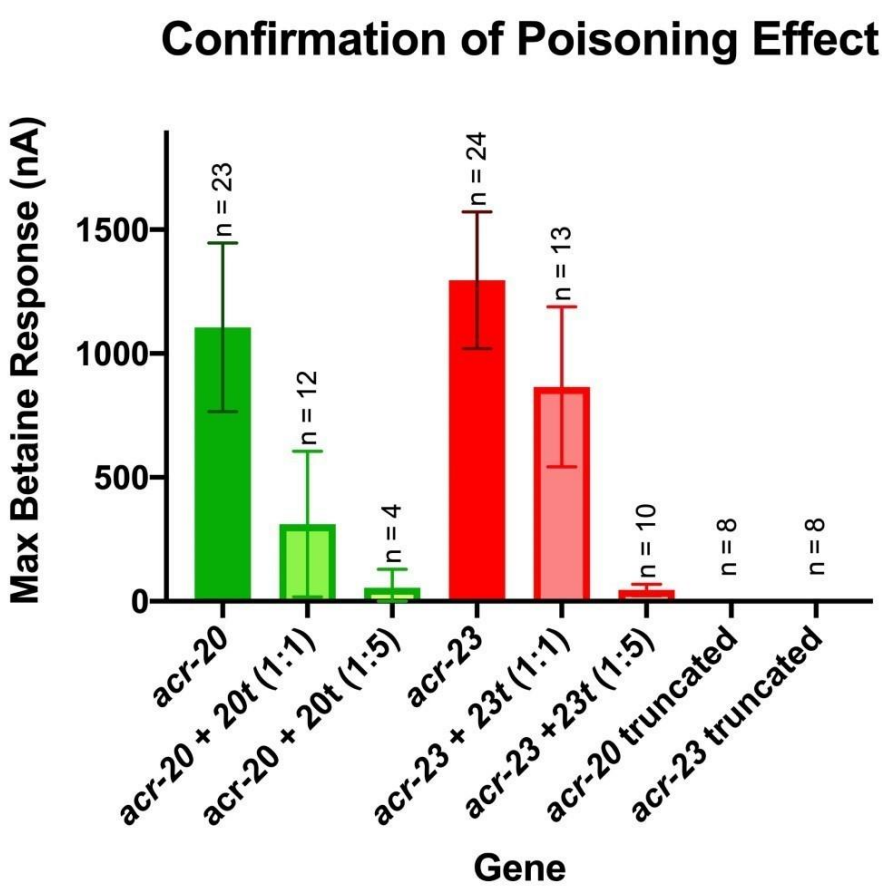

Figure 3. Confirmation of poisoning effect. Reduction in maximal response to betaine is shown with increasing co-injection of truncated subunits for homomeric receptors. Error bars represent mean ± 95% confidence interval. Error bars have considerable overlap, but a dose dependent poisoning effect is observed in both *acr-20* and *acr-23* homomers with increasing concentration of truncated subunit in the co-injection admixture. Truncated subunits elicit no response when injected into oocytes alone, indicating that the truncated subunits are inactive on their own.

A high degree of variability was observed in maximal responses to betaine between oocytes, resulting in large error bars for each injection scheme in Figure 3. For example, maximum responses for *acr-20* range from 400 nA to 2,000 nA. The electrophysiology recordings that produced EC_{50} curves utilized a method of standardization, whereby every oocyte's response to betaine was expressed as a proportion to its maximal response. The truncated subunit experiments had no such internal control. Nonetheless, there appears to be a trend of overall reduction in betaine responses produced by homomers as the concentration of co-injected truncated subunit increases. Responses for both *acr-20*

and *acr-23* homomers were greatly reduced for the 1:5 injection of full length to truncated subunit, indicating the truncated subunit was assembled into the receptor and reduced function as predicted.

The high degree of variability between oocyte maximum betaine responses meant that to determine statistical significance for the results would require sample sizes over 200 oocytes per experimental group (https://www.calculator.net/sample-size-calculator). Given that oocytes are injected one at a time, as well as the limitations imposed by the COVID-19 pandemic, it became important to find an alternative method of measuring functional poisoning by truncated subunits.

4.2.3 Oocyte Variability

Multiple factors introduced variability in electrophysiology responses. First and foremost was the integrity of the oocytes. The oocytes were separated by size, not their stage in the maturation cycle, which may have introduced differences in the cellular machinery available to each oocyte for synthesizing proteins. This could also explain differences observed in each oocyte's ability to heal after receiving an injection wound. Proper expression and assembly of receptors from the injected cRNA may have also varied. While the mechanics of RNA injections are kept constant, there is no practical way to control the volume of the injection that leaks back out of the oocyte, so the precise amount of RNA each oocyte receives can never truly be constant. Ideally, there should be some way to standardize between oocytes to correct for these factors. In order to develop a method of standardizing electrophysiology responses, the inclusion of ACR-16 as an internal control was evaluated.

4.2.4 Determining an Internal Control for the Poisoning Experiment

Variability between oocytes was high in terms of maximum response to betaine although each individual oocyte did produce consistent responses to multiple doses of the same concentration of betaine. This indicated that each oocyte expressed receptors that were stable and functional, so that between-oocyte variability might be mitigated by introducing an internal control. Expressing oocyte responses relative to such an internal standard would reduce inter-oocyte variability.

A suitable internal control would consist of a receptor that responds to a ligand neither ACR-20 nor ACR-23 respond to, and that would not oligomerize with the other subunits to form heteromers. Therefore, the *acr-16* subunit from *Dracunculus medinensis*, *Dme-acr-16,* was selected, with the additional feature that this ACR-16 subunit does not require accessory proteins to be expressed, as is

the case for *cel-acr-16* (Abongwa et al., 2016, J. Noonan, pers. comm.). This receptor has acetylcholine as its principal agonist, which does not illicit a response in ACR-20 or ACR-23 (Rufener et al., 2013, Peden et al., 2013, Baur et al., 2015). Dme-ACR-16 was shown not to respond to betaine, see below. This internal control allows the betaine response of each oocyte to be normalized to its acetylcholine response, and for these normalized responses to provide the basis of comparison between treatment groups.

4.2.5. Confirming Internal Controls

Plasmid containing *Dme-acr-16* (gift from J. Noonan) was used to produce RNA as described in Methods. Oocytes were injected with 13 ng *Dme-acr-16* alone and tested for their response to 100 μM ACh and 10 mM betaine. While *acr-20* and *acr-23* were shown in the literature to have no response to acetylcholine (Rufener et al., 2013, Peden et al., 2013, Baur et al., 2015), *acr-20* and *acr-23* homomers were also tested with 100 μM ACh to verify no response to acetylcholine. Doses were administered in random order. 100 μM ACh was used for all receptors, 1.5 mM betaine was used for ACR-20, and 10 mM betaine was used for ACR-23 and ACR-16.

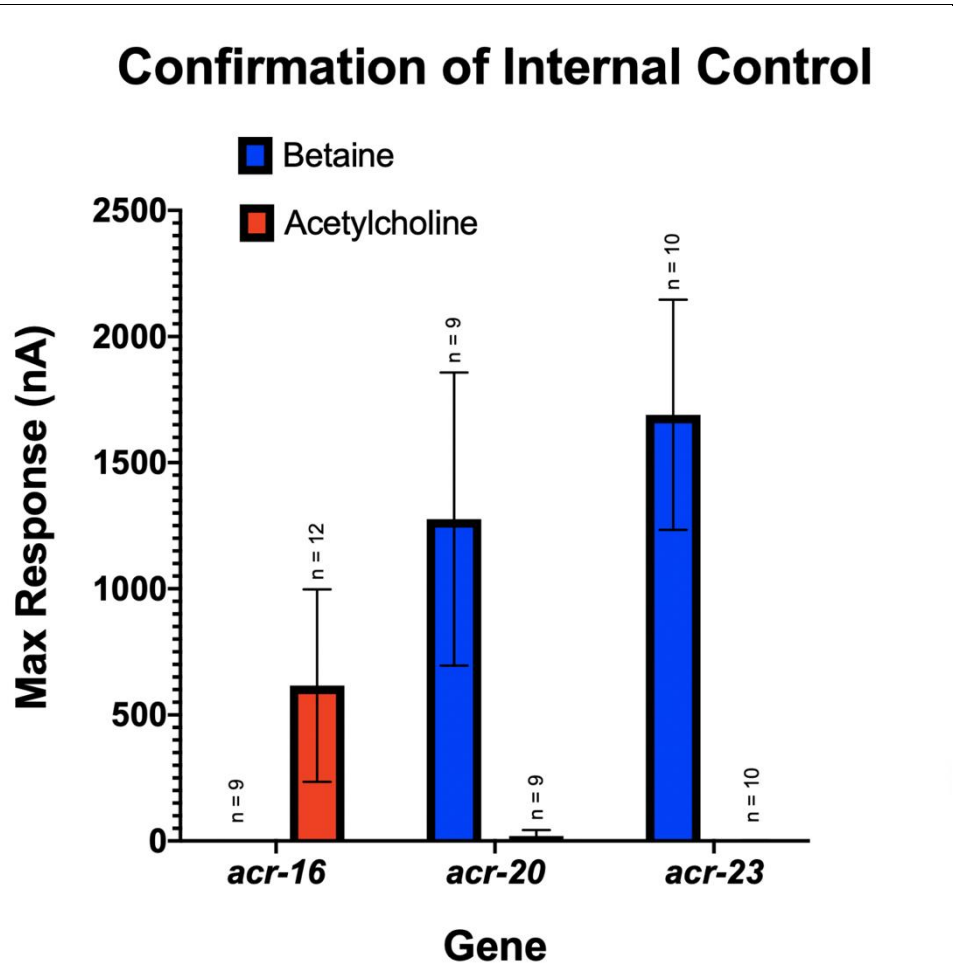

Figure 4. Confirmation of internal control. This graph shows the maximal response of ACR-16 and ACR-23 to either 1.5 mM betaine and ACR-20 to 10 mM betaine and all three to 100 μM ACh. There are 2-3 responses to each drug for each biological replicate, and 3 biological replicates per group. Error bars represent mean ± 95% confidence interval. No response to betaine was observed for ACR-16, and no response to ACh for ACR-23 and only a negligible response for ACR-20.

Variation in the ability of oocytes to express receptors should affect *acr-16* and *acr-20*/*acr-23* in the same way. A robust internal standard must therefore show a linear correlation between the betaine and ACh response for each oocyte.

The next test to validate the internal control determined whether or not a linear relationship exists between ACh and betaine responses. If the oocyte has properties that increase or decrease its ability to produce protein receptors, then those properties would be expected to affect *acr-16* and *acr-20*/*acr-23* in the same way. Oocytes were co-injected with 13ng *acr-16* and 13 ng *acr-20* or *acr-23*, and

exposed to acetylcholine and betaine, separately as before. The correlations between their responses are shown in Figure 5.

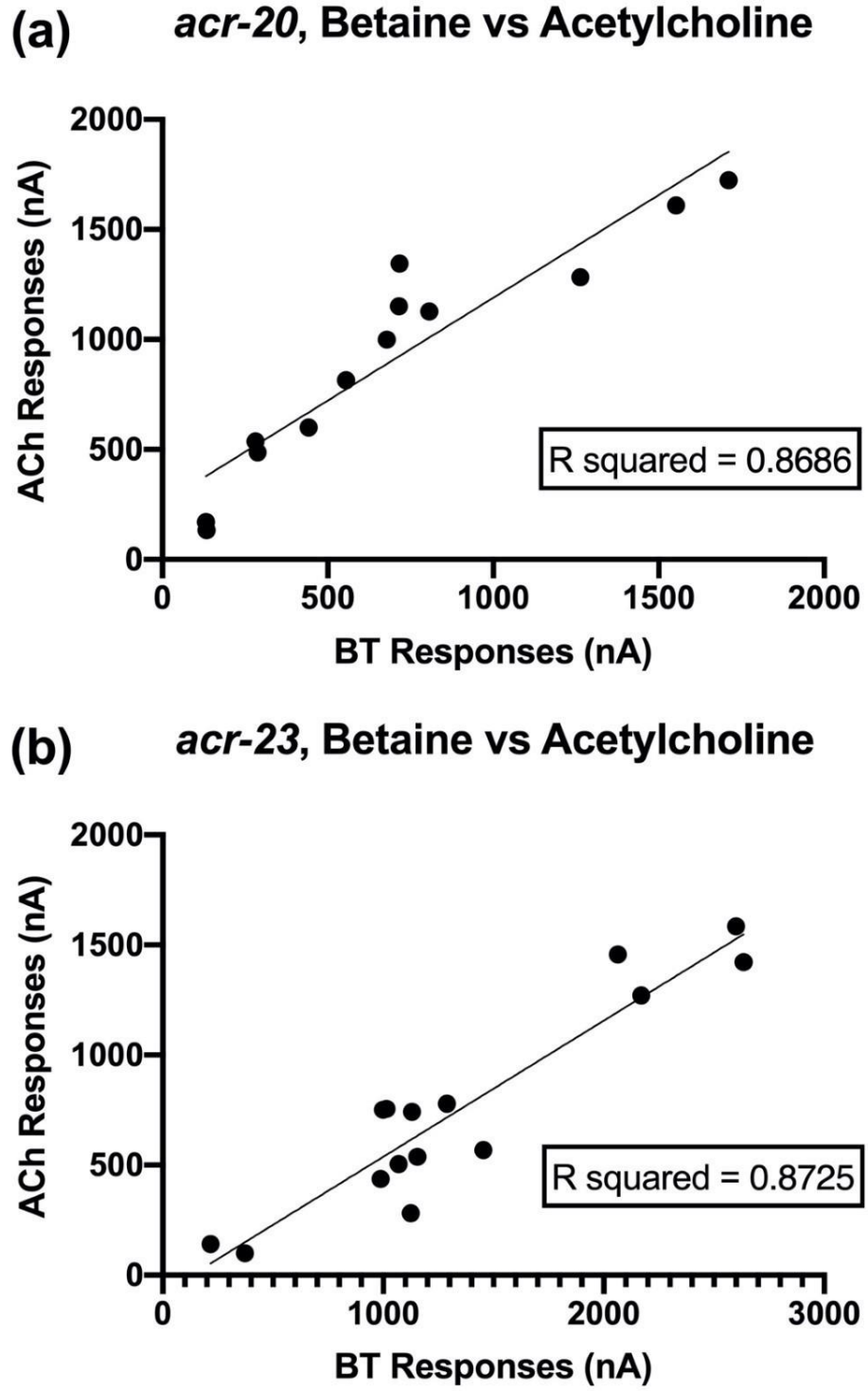

Figure 5. Confirmation of linear relationship between acetylcholine and betaine for oocytes co-injected with *dme-acr-16*. **(a)** A linear relationship exists between betaine and acetylcholine

responses for *acr-20*, with a R^2 value of 0.8686. **(b)** A linear relationship exists between betaine and ACh response for *acr-23*, with an R^2 value of 0.8725. Dme-ACR-16 is therefore an appropriate internal control for expression for both ACR-20 and ACR-23

A linear correlation was found between betaine and acetylcholine response for both ACR-20 and ACR-23, indicating their responses share a dependency on the quality of the oocyte in which they are expressed. ACR-16 was therefore used as an internal reference going forward.

4.2.6 Poisoning Experiment Using Internal Controls

The poisoning experiment was repeated, co-injecting every oocyte with 13ng *dme-acr-16* RNA in addition to the previous RNA injection scheme. Each oocyte was then treated with 100 μM ACh, as well as 1.5 mM betaine for *acr*-20 and 10 mM betaine for *acr*-23. Each oocyte was treated three times with ACh and three times with betaine. The average of these responses was used to represent each biological replicate in Figure 6.

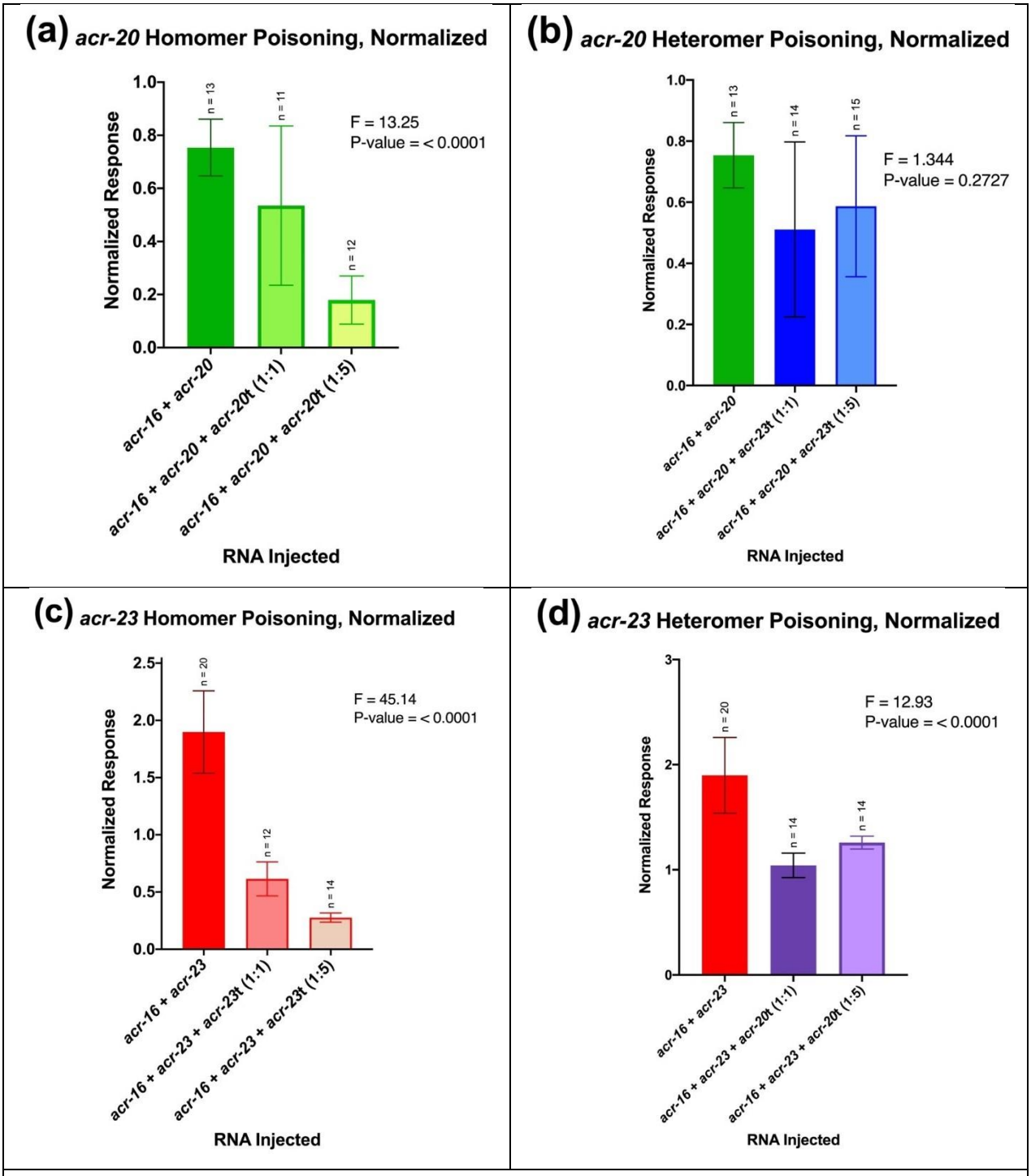

Figure 6. Normalized Poisoning Experiment. All values are normalized by diving betaine responses by acetylcholine responses on a per-oocyte basis. Error bars represent mean ± 95% confidence interval. Injections with truncated subunits were compared to homomers of full-length subunits to test for homomer and heteromers formation separately. F - and P -values show results of one-way ANOVA. **(a)** Poisoning effect on *acr-20* homomers. Here, a statistically significant poisoning effect is observed for homomeric channels. **(b)** Poisoning effect of truncated *acr-23* on full-length *acr-20*. Here, the p-value shows no statistical significance. Overlap between error bars provides inconclusive evidence of a poisoning effect,

indicating too much variability between oocytes. Therefore, evidence for a poisoning effect is inconclusive for heteromers. **(c)** Poisoning effect on *acr-23* homomers. Here, a statistically significant poisoning effect is observed for homomeric channels. **(d)** Poisoning effect of truncated *acr-20* on full-length *acr-23*. Here, there is statistically significant evidence for no poisoning effect. This is apparent in the 1:5 full-length to truncated subunit injection, which did not result in smaller maximal responses compared to the 1:1 ratio. This suggests heteromers were not formed with truncated subunits.

Both *acr-20* and *acr*-23 homomers showed an observable poisoning effect by co-injecting an increasing concentration of their own truncated subunits. This established that the poisoning experiment worked as expected in the presence of *Dme-acr-16*. Considerable variability remains for oocytes injected with full-length *acr-20*.

The profile for ACR-20 co-injected with increasing concentrations of truncated-*acr-23* subunits was clearly different than when co-injected with truncated ACR-20. There did not appear to be evidence for a dose dependent poisoning effect, although the decrease in response upon the addition of more truncated subunit was not significant in either case due to the variability associated with the ACR-20 response which resulted in large, overlapping error bars (see below).

Taking error bars into consideration, the average normalized responses for *acr-20*/truncated-*acr-23* heteromers are not significantly different from the full-length *acr-20* homomer. Furthermore, if a poisoning effect were present, there should be a clear difference between the *acr-20*/truncated-*acr-23* injected oocytes at a 1:1 and a 1:5 ratio. No such difference can be observed.

The response of *acr-23* co-injected with truncated-*acr-20* showed no evidence for a poisoning effect. While responses for the 1:1 and 1:5 ratios were both smaller than *acr-23* injected on its own, the betaine response for the 1:5 ratio was not significantly lower than the 1:1 ratio. Interestingly, the least oocyte variability was observed for the *acr-23*/truncated-*acr-20* injected oocytes. This points towards a specific problem with expression of the ACR-20 subunit, examined in the next section.

4.2.7 Poisoning Receptor Function With Truncated Subunits

The truncated subunits used in this study have an intact amino-terminal and interface at the ligand binding domain, but are missing the last two transmembrane domains, intracellular loop and carboxyl terminal. This modified version of each subunit produced no current through the oocyte

membrane on their own. When present along with the full-length version of the same subunit, there was a dose dependent poisoning of the betaine response.

The poisoning experiment provides evidence that there is reduced interaction between the ACR-20 and ACR-23 subunits, based on the extra-cellular domain and first two transmembrane regions. Further confirmation would be required before concluding that the two subunits do not form a heteromeric receptor, but it appears that there has been an evolutionary adaptation to favor formation of the homopentameric receptors.

4.3 Cytotoxic Effect of ACR-20

Throughout the course of this study, oocytes injected with *acr-20* were shown an increased variability in terms of their ability to clamp at –60 mV or to detectably respond to betaine. The TEVC electrophysiology used in this study requires the voltage across the oocyte membrane be clamped at -60 mV. The basal current, or clamping current, required to achieve this membrane potential reveals the oocyte's ability to allow ions to pass across its membrane in the absence of exogenous activating ligand (Goldin, 2006). If this current was below -1000 nA, the oocyte was discarded in the study presented here. A value of -200 nA is more typically used as a threshold value. However, there were inherent problems when expressing ACR-20 that lead to accepting a more lenient threshold current. More oocytes injected with ACR-20 required a current below -1000 nA to clamp, suggesting an issue with membrane integrity. Qualitatively, a general increase in discoloration of the oocytes was also observed for *acr-20* injected oocytes. The brightness of unfertilized oocytes is generally considered a measure of their overall health, as they tend to dim and take on a mottled appearance as they die (Goldin, 2006). Interestingly, the same was not observed in oocytes injected with other subunits nor the truncated version of *acr-20* alone. Since the truncated subunit does not encode a functional subunit, this implies the effect could be caused by the oocyte expressing a population of ACR-20 homomeric receptors that are leaky or spontaneously open. To investigate this further and to determine if there was a correlation with ACR-20 expression, the ability of oocytes to clamp at –60 mV was determined.

Figure 7 shows the proportion of oocytes that required a clamping current in excess of -1000 nA to maintain –60 mV for the poisoning experiment shown in Figure 6. This proportion was consistently higher with oocytes injected with *acr-20* compared to those injected with *acr-23*.

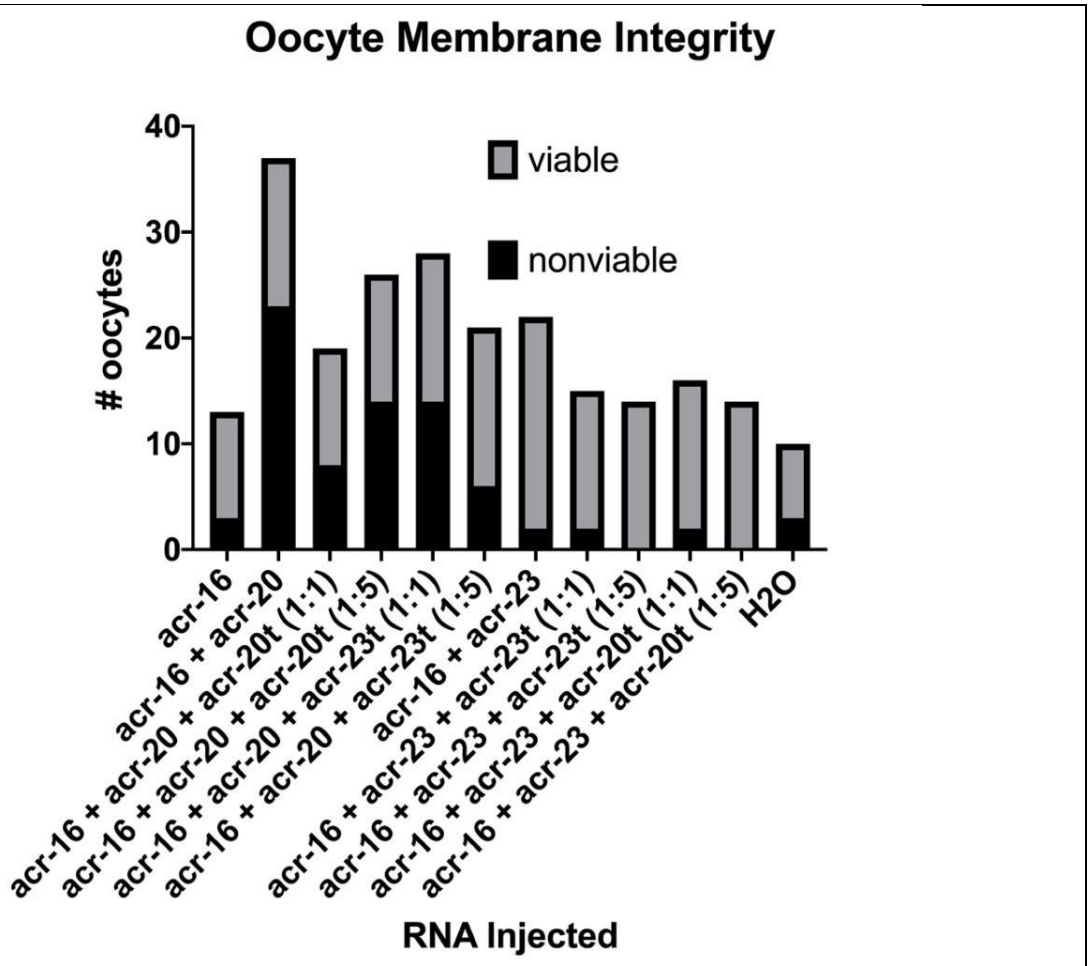

Figure 7. Oocyte membrane integrity assay for oocytes used in the poisoning experiment. Proportions of viable versus non-viable oocytes are shown for each injection scheme, including negative controls of water injections. Oocytes were binned into two categories: clamping above –1000 nA, in which case they were considered viable and used in the poisoning experiment, and clamping below –1000 nA, in which case they were considered nonviable and not used in the study. Oocytes injected with full-length *acr*-20 show a larger proportion of non-viable oocytes, indicating a cytotoxic effect for oocytes injected with *acr-20*.

To provide a viability assay, a series of oocytes injected with *acr-20* alone at concentrations of 500 ng/µL, 250 ng/µL, 125 ng/µL and 62 ng/µL were tested for their ability to clamp at –60 mV. Each oocyte was then pierced by electrodes and the current required to clamp at –60 mV was recorded. During this process, each oocyte was also tested for their ability to respond to betaine. As long as at least one oocyte in a given batch was able to respond to betaine, indicating the integrity of the RNA injected, the measurement of membrane potential for every oocyte in that batch were included in the assay. The results are shown in Figure 8.

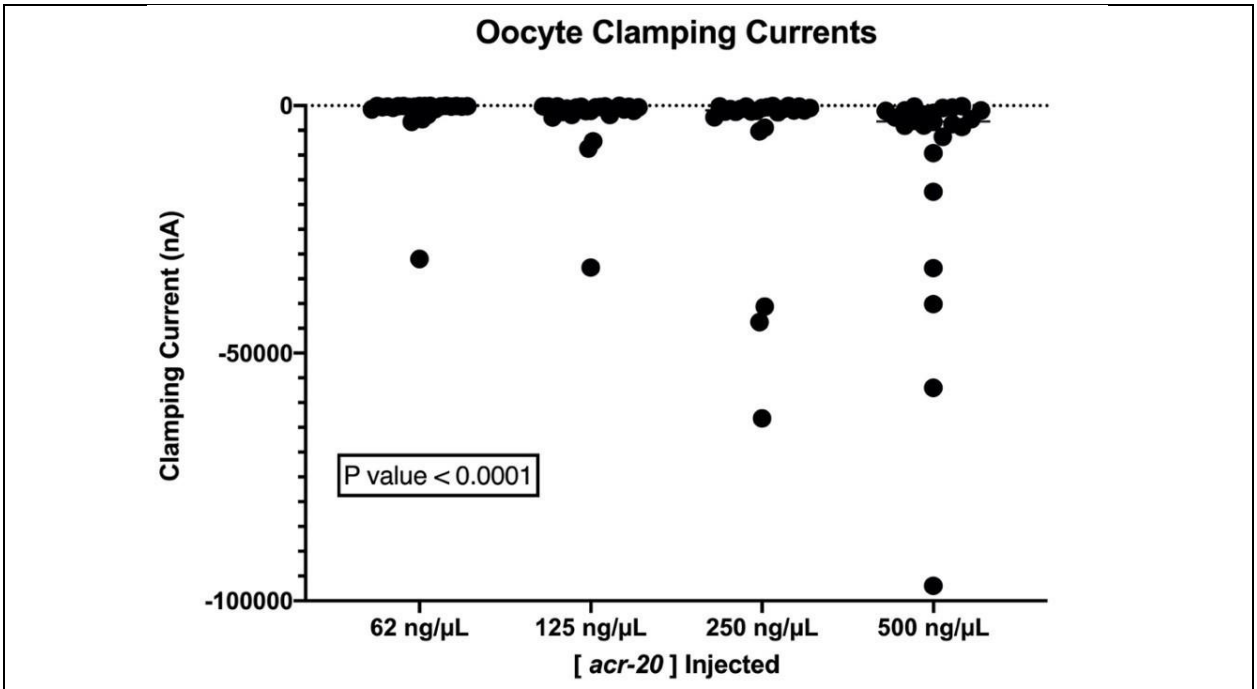

Figure 8. Viability assay comparing oocyte clamping values. n = 24 for each experimental group. Dot plots indicate biological replicates for each concentration of *acr-20* injected. As the concentration of *acr-20* injected increases, the current passing through oocytes to clamp them at –60 mV increases, indicating a decrease in membrane integrity. This oocyte viability assay did not pass the normality test (alpha = 0.05). Therefore, the Kruskal-Wallis test was used to test for statistical significance.

As the concentration of *acr-20* injected increases, the proportion of oocytes requiring a clamping current above –1000 nA increases. Not all oocytes behaved the same way, and some were able to clamp with low current despite the maximal concentration of cRNA injected. As can be seen from Fig. 7, oocytes that clamp successfully responded to betaine.

In a previous study, the expression of ACR-23 was found to be toxic to *Xenopus* oocytes (Peden et al., 2013). In the present study, the viability of *acr-20*-injected oocytes showed a much greater toxic effect on oocytes. Given that *acr-20* and *acr-23* arose from the same gene duplication event, this may be a feature they share. Furthermore, monepantel has shown to irreversibly open ACR-20 homomers (Baur et al., 2015). While an irreversible response was not observed for betaine, the recovery time between doses for betaine on *acr-20* receptors was observed to be longer than that for *acr-23*, suggesting *acr-20* receptors are slower to close their channels after exposure to ligand.

These viability assays suggest that the functional homomeric ACR-20 receptor, when expressed in *Xenopus* oocytes, may spend a significant fraction of its time in an open configuration and therefore produce a cytotoxic effect.

5- Conclusion

Gene duplication is understood to be essential to adaptation, and precedes the emergence of new functions (Ohno, 1970). The duplication of *mptl-1* occurred in the *Caenorhabditis* lineage after the speciation event that led to the divergence of *H. contortus* and *C. elegans* as separate species. Since *C. elegans* is not a parasitic species, *C. elegans* presumably posed a different set of evolutionary pressures on these duplicate genes, creating conditions conducive to a change in function. This change in function is reflected in the identification of different evolutionary rates acting on *acr-20* and *acr-23* using PAML, and the exclusion mechanism suggested by the results of the electrophysiology experiments.

PAML analysis identified evidence for the relatively rapid evolution of an exclusion mechanism in *acr-20* and *acr-23* since their split from their ancestor, *mptl-1*. Rapid evolution involves increased amounts of sequence change as well as a relaxed rate of evolutionary pressure acting on those changes. This rapid evolution can be linked to neofunctionalization, which would lead to new protein-protein interactions, or to subfunctionalization, which would reduce the set of proteins the subunit is able to interact with. Either scenario could explain the emergence of an exclusion mechanism. Presumably, once this change in function has occurred, evolutionary pressure becomes more strongly purifying and prevents further sequence change. Small ω values, indicating strong purifying selection pressure, were identified in *acr-20* and *acr-23*, which could indicate that this functional change has occurred and is becoming fixed in the population. Furthermore, *acr-20* and *acr-23* were identified to likely have different evolutionary rates from each other, implying evolutionary divergence occurred since the original split from their ancestral *mptl-1*.

There was some evidence that the *acr-20* clade could be evolving differently from the *acr-23* clade, since clade models were an improvement when differing the rate class for only *acr-20* and only *acr-23*, respectively. The clade model with different rate classes for both *acr-20* and *acr-23* scored lower than models with a different rate class for only *acr-23* or only *acr-20.* This could be an

observational artifact of a complex likelihood surface which prevented the algorithm from finding global optima, or it could be evidence that suggests *acr-20* is evolving at a different rate from *acr-23*, in which case *acr-20* could be undergoing a functional change on its own. It is also possible *acr-23* did not change function immediately after the duplication event, but after the gene was established in the population.

Evidence for an exclusion mechanism was identified from co-expression with the truncated subunit forms. A clear reduction in function was observed when truncated subunits were incorporated into *acr-23* homomers, but not for *acr-23* heteromers. The same pattern was true for *acr-20,* but the increased variability due to the apparent cytotoxicity for the oocytes meant that the 1:5 ratio showed a significant reduction for the truncated *acr-20* but the 1:1 ratio did not. This study lays out an important feature of *acr-20* in terms of increased rates of cytotoxicity in *Xenopus* oocytes. The observation that *acr-20* has a more cytotoxic effect than *acr-23* might be explained by functional divergence. If the *acr-20* homomers cause ions to leak through the channel, this could serve a purpose if *acr-20* is expressed in tissues where the ion concentration gradient between cells would not cause excessive leakage, or if *acr-20* serves a purpose as a leaky channel for functions such as nociceptive transduction (Chatzigeorgiou et al., 2010).

This study represents an important piece of the evolutionary story of ion channel diversity, structure, and evolution in terms of how homomeric pLGIC subunits might be maintained in a population. The evolution of an exclusion mechanism could explain how subunits maintain their subunit binding specificity. Conversely, identifying where an exclusion mechanism has not evolved could indicate which subunits are able to form heteromers.

5.1 Future Work

A complete understanding of *acr-20* and *acr-23* as homomeric pLGIC is still underway. Further study of their binding behaviors could prove useful to understand the hypothesized exclusion mechanism laid out by this study.

It is worth noting that *acr-20, acr-23,* and *mptl-1* are not known to require accessory proteins to be expressed in *Xenopus* oocytes (Rufener et al., 2013, Baur et al., 2015). However, it could still be worthwhile to test whether co-injections with *ric-3*, an accessory protein required by many pLGIC for proper assembly, effects their functional expression (Ben-Ami et al., 2005). It could also be interesting

to determine whether the toxic effect of ACR-20 expression could be mitigated by RIC-3, which may improve the stability of subunits to ensure they are properly assembled.

Patch clamp electrophysiology could be used to detect ionic currents through a single receptor at a time, rather than TEVC, which looks at the summation of responses for an entire population of receptors (Sakmann & Neher, 1984). This means the patch clamp method could identify heteromers based solely on their EC_{50} values.

The poisoning experiment could be repeated with truncated subunits that constitute the second half of each subunit, spanning only the third and fourth transmembrane domain as well as the carboxyl terminal, to test if the poisoning effect persists. This could tell us whether the receptor behaves differently when it incorporates truncated subunits lacking a ligand-binding domain. If a poisoning effect isn't observed with these alternatively truncated subunits, this would narrow down the area of the subunit where the hypothesized exclusion mechanism evolved for easier investigation in future projects.

In this project, the exclusion mechanism was hypothesized to have physical, temporal, and spatial components, but only the physical components were investigated. The temporal and spatial components could be explored with an additional project to build and inject fluorescently labeled plasmids into *C. elegans.* Fluorescent tags of opposing colors could be fused to protein immediately after the signal peptide of each subunit. Confocal microscopy would then be used detect fluorescence where these subunits are expressed somatically, and at different points in the *C. elegans* life cycle.

5.2 Impact of the Covid Pandemic

This project was interrupted by the COVID-19 pandemic, hindering access to the laboratory, and reducing the total number of hours spent in the wet lab. While work continued, data collection for electrophysiology slowed significantly. This methodology is generally time consuming, which means sample sizes were smaller than originally planned. During the course of the experiments, it became clear that due to the nature of these particular receptors, variability was a constant complicating factor. The normal response would have been to increase sample size, but due to circumstances this was not possible. Instead, a method to provide an internal control in the form of ACR-16 was developed.

5.3 Implications of Findings

This study represents a small piece of the puzzle of our understanding of how ion receptors have evolved, and what mechanisms influence receptor oligomeric state. Since anthelmintic resistance in nematodes is a rapidly growing problem, there is clear motive to continue investigating the structure and evolution of these important drug targets. Furthermore, because pLGIC are involved in neurotransmission responsible for motility in all animals, and *C. elegans* is a common laboratory model for understanding receptors other vertebrates, this work can be extended to fields outside the domain of parasitology to understand the evolution of muscle control at large. Wherever pLGIC are acting to mediate synaptic transmission at the neuromuscular junction, the evolution of their subunits is an important feature to understand their binding behavior, stoichiometry, and subsequent effect on the receptors they form.

How homomeric subunits maintain their binding exclusivity remains to be fully understood. This study shows a promising method of studying this binding behavior, using subunits that are products of recent gene duplication events. Groundwork has been laid out for the continuation of this study, particularly to look for further evidence of an exclusion mechanism that has spatial or temporal components.

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